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IOP Conference Series: Earth and Environmental Science

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To cite this article: A Adelina *et al* 2019 *IOP Conf. Ser.: Earth Environ. Sci.* **348** 012008

IOP Conf. Series: Earth and Environmental Science **348** (2019) 012008 doi:10.1088/1755-1315/348/1/012008

Fermented chicken feathers using *Bacillus subtilis* to improve the quality of nutrition as a fish feed material

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Abstract. Chicken feather meals constitutes 80-85% protein, the main component being beta-keratin, a fibrous and insoluble structural protein extensively cross linked by disulfide bonds. The keratins can not be absorbed directly in the digestive system, therefore a processing technique is required to make it more absorbable. Processing technique can be fermentation by microorganism *Bacillus subtilis* to degrade keratin by secretion of keratinase. The aim of this study was to examine fermentation of chicken feather meal with *B. subtilis* to improve the quality of fish feed ingredients. The treatments were: no-fermented; fermentation of chicken feather meals: 6, 8, 10, 12 and 14 ml inoculum fermentation using *B. subtilis,* respectively for chicken feather meal as much as 2 g. The results showed that the processing fermentation with 10 ml inoculum *B. subtilis* gives the best results in the highest keratinase activity (273.33 U/ml), increased the protein content of chicken feather meals (74.16 to 85.20%), but decreased of lipid content (2.44 to 1.42%) and carbohydrate content (7.86 to 2.05%) with a change in the physical properties of white -yellow (color), soft (texture), and less typical sting (smell).

1. Introduction

Intensive fish cultivation requires the availability of artificial feed continuously with quality and quantity to meet the needs of fish, so that it can accelerate fish growth. The supply of fish feed is still relying on fish meal as the main component. Source of animal protein is needed up to 40-50% of the total feed ingredients. To meet national fish meal needs, 75% are still met from imports. Continuity of fluctuating supply of local materials to lower product quality has made the domestic feed processing industry prefer imported products. To produce good quality fish food that is relatively cheap, the supply of imported fish meal can be substituted with local materials that are also of good quality, guaranteed supply continuity and cheaper prices, such as chicken feather meal.

Chicken feathers have a high protein content (80-85%), higher than soybean meal protein (42.5%) and fish meal (66.5%). Chicken feathers contain 0.19% calcium minerals, 0.04% phosphorus, potassium 0.15% and sodium 0.15% [1,2]. However, chicken feather protein is a type of protein that is difficult to digest, because 90% of its protein is composed of beta-keratin and fibrous fiber components [3].

Keratin is a product of hardening of the epidermal tissue of the body that is often found in feathers, hair, nails, horns and other epidermal tissues that are hardened [4]. Keratin consists of cystine disulfide bond components, hydrogen bonds and hydrophobic interactions of keratin molecules. Keratin is a protein that is rich in sulfur amino acids, cystine. Disulfide bonds formed between the cystine amino acids cause these proteins to be difficult to digest properly by proteolytic enzymes such as trypsin, pepsin and papain in the digestive organs [5]. To eliminate keratin can be done with fermentation technology using microbes [4].

Keratin degrading microorganisms including *Bacillus subtillis*. These bacteria produce the enzyme keratinase which functions to degrade keratin found in chicken feathers. The keratinase enzyme produced by Bacillus bacteria is able to hydrolyze various soluble proteins and insoluble proteins such as keratin proteins [6,7]. The results of the research by Adelina et al [8] showed that the protein digestibility of feed containing fermented chicken feather meal *B. subtilis* increased from 39.09% to 48.75%. In this study, fermented chicken feather meal was carried out using *Bacillus subtilis* bacteria which was isolated from the digestive system of tiger shrimp [9]. The great potential possessed by chicken feathers makes us interested in analyzing the ability of *Bacillus subtilis* bacteria to improve and increase digestibility for fish diet.

2. Materials and Methods

This research was conducted from May to November 2018. The study was conducted in several places: the multiplication of *B. subtilis* bacteria and fermented chicken feather meal was carried out in the Laboratory of Parasites and Fish Diseases and making feed the test was carried out at the Fish Nutrition Laboratory, Fisheries and Marine Sciences Faculty, University of Riau.

2.1. Research Materials

Materials used in the study: chicken feathers are still good, washed thoroughly, dried and mashed into flour; bacterium *B. subtilis* strain, access code JX188065.1, resulting from isolation from the digestive tract of tiger shrimp [9].

2.2. Experimental design

The research was conducted by experimental methods. The completely randomized design (CRD) with one factor and 6 levels of experiment were used in this study. The number of different *B. subtilis* bacteria for the fermentation process of chicken feather meal was treated as a reference to Mulia et.al [10] and Adelina et.al [8]. The tested treatments were; 2 g chicken feather meal without *B. subtilis* (P0), 6 ml *B. subtilis*/ 2 g chicken feather meal (P1), 8 ml *B. subtilis*/ 2 g chicken feather meal (P2), 10 ml *B. subtilis*/ 2 g chicken feather meal (P3), 12 ml *B. subtilis*/ 2 g chicken feather meal (P4) and 14 ml *B. subtilis*/ 2 g chicken feather meal (P5). To minimize errors, each treatment was repeated three times.

2.3. B. subtilis Bacteria Production

Isolate *B. subtilis* was vortexed for inoculum, then transferred by dropping as much as 50 μ into NA medium (Nutrient Agar), the bacteria was flattened using a sprider, then incubated for 24 hours. The bacteria were then purified 4 times on the NA medium to obtain pure *B. subtilis* colonies. Pure *B. subtilis* is transferred to NB (Nutrient Broth) liquid media for propagation. Propagation is done in stages: 1). Grow B. *subtilis* on 10 ml NB media, incubated for 24 hours, 2). The growing *B. subtilis* was transferred to 90 ml NB media, incubated for 24 hours, 3). *B. subtilis* was transferred to 900 ml NB media and incubated for 24 hours. *B. subtilis* can then be used as a fermentor for fermented chicken feathers.

2.4. Fermentation of Chicken Feathers Using B. subtilis

The stages of chicken feather fermentation as follows: (1). chicken feathers was sterilized for 15 minutes then cooled, (2). Sterile petridisk dishes were prepared as many as 15 pieces, then into each petridisk inserted 2 g of chicken feathers, (3). Pure *B. subtilis* was taken as much as 6 ml from 900 ml NB media, then dropped over 2 g of chicken feathers and repeated 3 times; the same thing was done on pure *B. subtilis* taken 8, 10, 12 and 14 ml, then each drop was over 2 g of chicken feathers and repeated 3 times. (4). All of chicken feathers samples that have been extracted with *B. subtilis* were then incubated in the incubator at 50ºC, pH 8 for 72 hours [11]. The results of the fermentation process obtained chicken feather hydrolyzate (5). Fermented of chicken feathers was then analyzed for protein, fat and carbohydrate levels by the Takeuchi [12] method.

2.5. Calculation of the Number of Colonies of B. subtilis

Calculation of *B. subtilis* using a spectrophotometer, namely: looking at cell density by approaching the measurement of optical density (optical density) at a wavelength of 625 nm. Furthermore, the absorbance value is entered into the equation Y = a + bx which is converted to obtain the value of bacterial cell density in culture [13].

2.6. Keratinase B. subtilis activity

The procedure for measuring keratinase activity from *B. subtilis* used was following the modified method from Desi [11], Ali et al. [14] and Marzuki [15].

2.6.1 Bacillus subtilis inoculum preparation. The making of the inoculum begins with purification of *B. subtilis* on NA media 3 times, to obtain pure colonies. Furthermore, *B. subtilis* is transferred to liquid media (NB).

2.6.2 Production of keratinase in pepton media. The liquid peptone media containing 1% glucose as much as 100 ml was adjusted pH to 8.5. After that, add 1 g of chicken feather meal, then sterilize with autoclave. Then into the enzyme production medium, 1 ml of *B. subtilis* inoculum solution was added, then incubated for 3 days. After 3 days, 400 μL NaN3 20% was added to stop the production of the enzyme, then centrifuged at a speed of 3500 rpm for 20 minutes. Centrifuged filtrate is a keratinase enzyme solution.

2.6.3 Keratinase B. subtilis activity test. The test for keratinase *B. subtilis* was carried out using a spectrophotometer. The enzyme obtained from centrifugation added chicken feather meal or chicken feather hydrolysate for each treatment as much as 20 mg which was dissolved in a buffer solution with a ratio of enzyme and buffer solution of 1: 4 (200 μl enzyme: 800 μl buffer solution), then incubated for 15 minutes. After that, 2 ml of 10% TCA was added. Then centrifuged at a speed of 3500 rpm for 15 minutes. The filtrate from the centrifuge then measured its absorbance with a spectrophotometer with a wavelength of 571 nm. Keratinase activity was measured by the formula Ali et al. [14].

$$\text{Keratinase activity (Unit / ml)} = \frac{4 \times n \times A}{0{,}01 \times T}$$

Notation: 4 = volume of final solution (ml); n = dilution factor; A = absorbance value (unit); T = incubation time (minutes)

2.7. Changes in the physical appearance of chicken feather meal

Unfermented and fermented chicken feather meal is considered a change in physical appearance such as: color, texture and smell.

2.8. Data analysis

Data on *B. subtilis* colonies, keratinase *B. subtilis* activity, and nutien content (protein, fat and carbohydrate) in each treatment were analyzed using variance analysis. If there is a difference between treatments followed by Duncan's test at a 95% confidence interval (Steel and Torrie, 1993)[16]. Changes in the physical appearance of chicken feather meal before and after fermentation were analyzed descriptively.

3. Results and Discussion

3.1. Colony Amount and Keratinase B. subtilis Activity in Fermentation of Chicken Feathers Meals

Fermented chicken feather meal using *B. subtilis* with a number of 6, 8, 10, 12 and 14 ml *B. subtilis*/ 2 g chicken feather meal. After 72 hours of incubation, the number of *B. subtilis* colonies produced during fermentation and keratinase activity was shown in Table 1. The number of colonies *B. subtilis* used for fermentation of chicken feather meal in all treatments was the same ie 9.63x108 CFU/ ml. After incubation for 72 hours, there was an increase in the number of colonies. Increasing the number of *B. subtilis* colonies due to the presence of nutrients derived from chicken feather meal used by bacteria for their growth, these nutrients are carbon, nitrogen and energy [17]. In the exponential or logarithmic growth phase, microbes divide rapidly and constantly, in this phase the speed of microbial growth is strongly influenced by the medium of growth such as pH and nutrient content, as well as environmental conditions including temperature and air humidity [18].

Table 1. Number of *B. subtilis* colonies and keratinase activity in fermented chicken feathers

Treatment Number of *B. subtilis* (ml / 2 g chicken feather meal)	Number of colonies *B. subtilis* (x 10^9 CFU/ml)	Keratinase activity (Unit/ml)
P0 (0)	9.63 x 10^{8a*}	3.11 ± 0.40^{a*}
P1 (6)	4.76 ± 0.04^{b}	205.60 ± 2.97^{b}
P2 (8)	4.83 ± 0.02^{c}	237.60 ± 2.44^{c}
P3 (10)	4.94 ± 0.03^{d}	273.33 ± 2.44^{e}
P4 (12)	5.11 ± 0.01^{e}	266.40 ± 1.92^{d}
P5 (14)	5.17 ± 0.00^{f}	237.33 ± 2.11^{c}

Information: *different letters in the same column show there are significant differences between treatments (P<0,05); CFU = Colony Forming Unit

The number of *B. subtilis* colonies in this study was higher than Setyahadi and Rahayu [19] who also fermented chicken feather using *B. subtilis*, incubated at pH 7.5; the temperature of 37oC for 16 hours resulted in the highest number of *B. subtilis* cells 53.7x107 cells/ml, Supriati et.al [20] produced a number of *B. licheniformes* grown on peptone media, incubated at pH 8.0; the temperature of 45°C for 5 days is 1.0-1.2 x107 cells/ml.

The production of keratinase produced by microbes influenced by temperature and pH. Brandelli et.al [4] state that keratinase is most produced in alkaline or neutral conditions, pH 7.5-9. Imtiaz and Rehman [23] obtained the highest keratinase *B. subtilis* BML5 activity at pH 8 and temperature of 37°C. The highest keratinase *B. subtilis* activity in the study of Mousavi et.al [24] found at optimum conditions of 40°C and pH 11. Anitha and Eswari [21] reported that *B. megaterium* (A1), *B. licheniformis* 511 and *B. subtilis* 1-1 which were isolated from the Pasumalai Indian feather plume soil had keratinase activity of 72,875 U/mg, 242 U/mg and 198 U/mg, after 96 hours, 48 hours and 48 hours incubation period at 35°C and pH 7.5. Furthermore Mazotto et.al [22] stated *B. subtilis* and *B. licheniformis* had 319 unit/ml keratinase enzyme activity and 412 units/ml in medium 10 g feaher meal at 37°C and 2 days incubation time. The high level of keratinase activity produced shows that bacteria have varying potential in utilizing nutrients from the substrate and their metabolic abilities such as the amount of enzymes and proteins that bacteria have different, so that the bacteria will adapt to the conditions that best suit their metabolic needs.

3.2. Changes in chemical composition of chicken feathers before and after fermentation

3.2.1. Content of chicken feather meals protein. Fermented chicken feather using B. subtilis meal results in changes in content of chicken feather protein as shown in Figure 1. The use of B. subtilis 10 ml/ 2 g chicken feather meal was able to produce the highest increase in protein (11.04%), but not different (P> 0.05) with the use of B. subtilis 8 ml/ 2 g chicken feather meals (protein increased 8.56%) and 12 ml (protein increases 8.06%). The same thing was found by Sari et.al [25] that a significant increase in the number of B. lichenoformis microbial cells in the fermentation process could increase the protein content of shrimp waste. B. subtilis has a fairly good proteolytic power so that the proteolytic properties possessed by microbes are able to break down substrate proteins into cell biomass products called single cell proteins. The presence of single cell proteins and protease

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enzymes produced by B. subtilis during fermentation can increase substrate proteins, because single cell proteins and these enzymes are proteins [26].

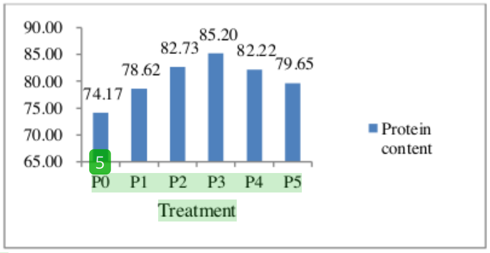

Figure 1. Histogram of meal protein content of chicken feathers before and after fermentation at various concentrations of *B. subtilis*

3.2.2. Content of chicken feather meals lipid. Fermentation of chicken feather meal using several doses of B. subtilis inoculum resulted in a decrease in lipid content of chicken feather meal as shown in Figure 2.

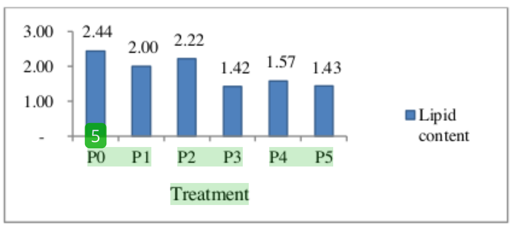

Figure 2. Histogram of chicken feather lipid content before and after fermentation at various concentrations of *B. subtilis*

Fermentation of chicken feather meal using *B. subtilis* can reduce the lipid content of chicken feather meal. Figure 2 shows that the initial lipid content of chicken feather meal was 2.44%, then after fermentation it was reduced to 1.42-2.22% or a decrease of 1.02–0.22%. The use of *B. subtilis* as much as 10 ml in fermented chicken feather can reduce the largest lipid content (1.02%), but not different from the use of *B. subtilis* 12 and 14 ml (P> 0.05). Pamungkas and Khasani [27] stated that fermentation using *B. subtilis* was able to reduce crude lipid content of oil palm cake, as well as Setyahadi and Rahayu [19] stated that the use of protease enzyme from *B. subtilis* in fermented chicken feathers reduced lipid content from 13.9 % to 4.6-6.1%.

3.2.3. Content of chicken feather meals carbohydrates. Fermentation of chicken feather meal using several B. subtilis inoculum concentrations resulted in a decrease and increase in carbohydrate starch meal as shown in Figure 3.

Pérez et.al [28] stated that B. subtilis produces extracellular enzymes such as cellulase which serves to hydrolyze carbohydrates of cellulose and hemicellulose which can be used as an energy source to increase dissolved protein levels. The results of this study were supported by Wizna et.al [29] who stated that organic materials which experienced a decrease during fermentation were starch and fat because they were used to meet energy needs for the growth of fermenters.

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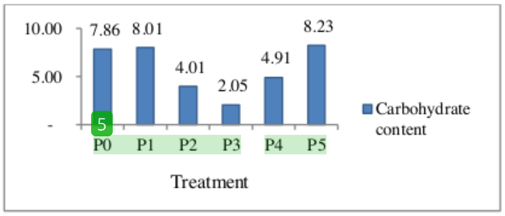

Figure 3. Histogram of carbohydrate content of chicken feather meal before and after fermentation at various concentrations of *B. subtilis*

3.3. Physical changes of chicken feather meals before and after fermentation
Fermentation of chicken feather meals using *B. subtilis* causes changes in color, texture and smell. The physical appearance of chicken feather meal before and after fermentation using *B. subtilis* can be seen in Figure 4. The enzymatic process results in changes in the color and flavor of a material. Furthermore, Blackwell [30], explained that enzymatic browning occurs in materials containing phenolic substrates, one of which is the amino acid tyrosine. The phenolic compound is a good substrate for the browning process. Phenolic compounds will produce phenolase enzymes which play a role in the browning process of a material. NRC [31], states that chicken feather meal contains 14 types of amino acids and one of them is the amino acid tyrosine of 2.48%. Browning reactions in this study occur because of the presence of tyrosine in chicken feather meal which can produce phenolase enzyme which works in the browning process. Muthmainna et.al [32] states that smell arising during fermentation are caused by the activity of proteolytic enzymes that break down proteins into anaerobic peptides or amino acids which produce H_2S, ammonia, methyl sulfid, amines and other compounds that cause unpleasant odors.

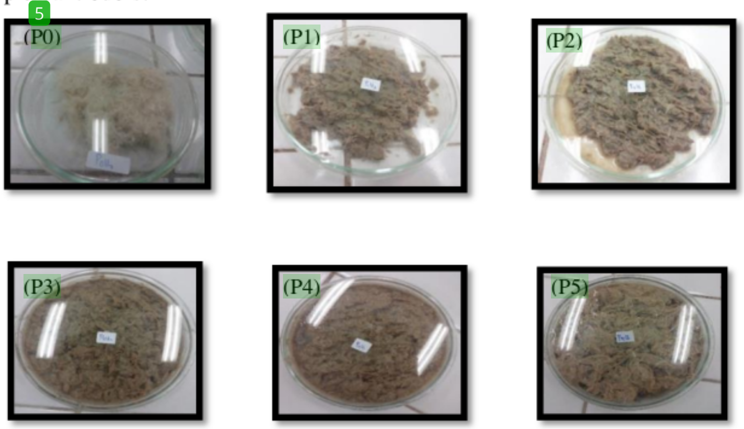

Figure 4. Changes in the physical appearance of chicken feather meals before and after fermentation

4. Conclusion
Fermentation technology using *B. subtilis* microbes can improve the quality of chicken feather meal. The fermentation process at pH 8 and a temperature of 50°C for 72 hours resulted in discoloration of flour of chicken feathers to brown, the texture changed to soft and the aroma more stinging. The use of *B. subtilis* 10 ml/ 2 g chicken feather meal, at the end of the fermentation process produced the highest keratinase enzyme, 273.33 units/ml and was able to degrade keratin chicken feather protein indicated by an increase in chicken feather protein 11.04%, lipid loss 1.02% and a decrease in carbohydrates 5.81%.

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