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Immobilization of *Bacillus* sp. SLII-1 on Chitosan-Alginate Hybrid Material for Promising Feedstock Supplement

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Abstract. The increase in poultry meat production led to an increase in feed demand. On the other hand, poultry industry produces feather waste which composes 5-7 wt% of the total body weight. Feed diversification by utilizing feather waste can be an alternative to overcome the problem of the high price of feed and to reduce environmental waste. The use of feather as a feed has a disadvantage because of the low digestibility of feather waste. The strategy to increase the digestibility of feather waste is to deliver keratinolytic bacteria to poultry intestine using immobilization technique. The purpose of this study was to determine the sodium alginate and chitosan concentration of immobilant with the highest number of released cell in poultry digestive system simulation. *Bacillus* sp. SLII-I as keratinolytic bacteria was able to increase soluble protein in feather meal liquid medium by 117.69% (wt/v). The composition of sodium alginate (2% w/v) and chitosan (0,8% w/v) was the most effective in delivering bacteria to poultry intestine as much as $2.54x10^7$ CFU per gram bead in digestive system simulation.

INTRODUCTION

The increase of poultry industry led to an increase in feed demand. On the other hand, poultry industry produces environmental waste since feather composes 5-7 wt% of the total body weight [1]. Feed diversification by utilizing feather waste can be an alternative to increase the economic value of feather and to reduce environmental waste. Currently, feather waste is processed by the mechanical, chemical and heating method. The mechanical method is not able to hydrolyze complex structure of keratin and still produce low digestible feed. Heating and chemical methods require high costs and damage the essential amino acids of keratin which are susceptible to high temperatures [2].

Utilization of keratinase [EC 3.4.21] is a beneficial alternative in processing feather waste because keratinase can hydrolyze keratin by breaking disulfide bonds and peptide bonds to produce amino acids that can be digested by poultry [3]. Application of keratinase from *Bacillus* sp. SLII-1 for hydrolysis of feather meal as a feed is reported by [1]. Keratinase-treatment feather meal can substitute soybean meal as a protein source for broiler chickens.

Probiotics are feed supplements in the form of live microbes that have a positive effect on the health and growth of poultry through a mechanism increasing feed digestibility [4]. *Bacillus* sp. SLII-1 has the potential as a probiotic because of the ability to produce keratinase which is beneficial for poultry in the digestion of keratin in the feed. Probiotic effects can be obtained if probiotics can reach the poultry intestine. Probiotic delivery to the intestine through feed directly is constrained by very low pH conditions in poultry ventricular organs that damage probiotics [5].

Cell immobilization is defined as the loading of cells in material to maintain viability and control the release of probiotic cells [6]. Many researchers used chitosan-coated calcium alginate to deliver probiotics to human intestine [7–10]. Calcium alginate is used widely for cell immobilization because of its easy handling, non-toxic, cheap, and

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safe as a feed additive [7]. Chitosan is used widely as a coating for alginate capsules because it can form complexes with alginate and increasing cell stability under acidic conditions [11]. The strategy to increase the digestibility of feather as feed is immobilization of *Bacillus* sp. SLII-1 in chitosan-coated sodium alginate. *In vitro* model of the digestive system of poultry is used to determine the most effective immobilization formulation.

MATERIALS AND METHODS

Reconfirmation of *Bacillus* sp. SLII-1 as Keratinolytic Bacteria

Keratinolytic potential of *Bacillus* sp. SLII-1 was evaluated by comparing total soluble protein content between two treatments shown in Table 1.

TABLE 1. Treatment for Evaluating Keratinolytic Potential of *Bacillus* sp. SLII-1

No.	Treatment
I	Medium FMB (Feather Meal Broth) without *Bacillus* sp. SLII-1
II	Medium FMB (Feather Meal Broth) with *Bacillus* sp. SLII-1

Medium FMB (100 ml) contains 0.05 g NaCl, 0.03 g K_2HPO_4, 0.04 g KH_2PO_4, and 1 g feather meal. A loopful of *Bacillus* sp. SLII-1 culture was inoculated to 100 ml of Feather Meal Broth (Treatment II). The treatment I and II were incubated at 120 rpm (room temperature) for 48 hours. After the incubation period, FMB was filtered by Whatman filter paper No.1. Filtrate as soluble protein was measured its total protein using the Bradford method. Bradford reagent (100 ml) contains 0.001 g Coomassie Brilliant Blue G-250, 5 ml ethanol 95%, and 10 mL phosphoric acid. Filtrate (0.1 ml) and Bradford reagent (5 ml) were homogenized and incubated for 10 minutes. Absorbance value at a wavelength of 595 nm of the mixture was measured by spectrophotometer. The absorbance value was converted to total soluble protein content using a standard BSA (Bovine Serum Albumin) curve equation below.

$$\text{Total Protein (mg/ml)} = \frac{\text{Absorbance value}+0.0017}{0.0049}$$

Growth Profile of *Bacillus* sp. SLII-1

Growth profile was made to determine the incubation time that produces the highest number of cell. A loopful of *Bacillus* sp. SLII-1 culture was inoculated to 100 ml of FMB. The culture was incubated at 120 rpm (room temperature) for 48 hours. Culture (0.1 ml) was taken every 2 hours from 0^{th} to 48^{th}-hour and transferred to haemacytometer. The cell density was calculated using a haemacytometer under a binocular microscope with a magnification of 1000X.

Cultivation was conducted by inoculating a loopful of *Bacillus* sp. SLII-1 to 100 ml FMB. The culture was incubated at 120 rpm (room temperature) for 42 hours.

Immobilization of *Bacillus* sp. SLII-1

Immobilization of *Bacillus* sp. SLII-1 was performed using the extrusion method according to (12). 42^{nd}-hour culture of *Bacillus* sp. SLII-1 was filtered in order to separate the culture from the remaining undigested feather waste. Sodium alginate (with variable concentration 1% (w/v), 2% (w/v), and 3% (w/v)) was mixed with 5.5 ml glycerol and 100 mL *Bacillus* sp. SLII-1 culture. Cell suspension in sodium alginate was injected into 50 ml of $CaCl_2$ 0.45 M using a sterile syringe. The formed beads were incubated in $CaCl_2$ solution for 30 minutes for the hardening process. Beads were washed twice with sterile water and were incubated in 50 ml of chitosan solution 0.8% (w/v) in 1%(v/v) acetic acid with stirring for 15 minutes. Then, the beads were washed twice with sterile water.

Poultry Digestive System Simulation

In vitro model of poultry digestive system was made to test the viability of immobilized *Bacillus* sp. SLII-1 in poultry digestive conditions. *In vitro* model of poultry digestive system used in this study was a model from [13]. The first step was simulating digestion in the crop phase by adding 0.1 g of bead to 6 ml of phosphate buffer pH 6.0 in the test tube, then incubated without agitation in a water bath at 40℃ for 30 minutes. Simulation of the gizzard was carried out by adjusting the pH to 3.0 by adding 1M HCl solution gradually. The solution was homogenized and incubated for 45 minutes at 40° C. Finally, the $NaHCO_3$ solution was dropped gradually with constant stirring until the pH was 6.5. The tube was homogenized and incubated for 60 minutes. This step simulated digestion in the small intestine in the poultry. After final incubation, liquid (0.1 ml) from the treatment sample was processed to cell enumeration process.

Enumeration of *Bacillus* sp. SLII-1 was conducted using Total Plate Count Method with serial dilution [14]. Liquid (0.1 ml) at the final step of *in vitro* model and Nutrient Agar (40°C liquid) was transferred to Petri dish. This solution was homogenized by shaking Petri Dish. This culture was incubated at room temperature for 48 hours. After incubation, the number of cells was calculated based on a formula.

$$\text{The number of bacteria (CFU/g)} = \frac{\text{the number of colonies x dilution factor x sample volume}}{\text{bead weight (g)}}$$

RESULTS AND DISCUSSION

Keratinolytic Potential of *Bacillus* sp. SLII-1

Comparison of the total soluble protein content of medium FMB (Feather Meal Broth) between *Bacillus* sp. SLII-1 treatment and without *Bacillus* sp. SLII-1 treatment is shown in Table 2.

TABLE 2. Total Soluble Protein Content

Treatment	Total soluble protein levels after 48 hours (mg / ml)
I (Medium FMB without *Bacillus* sp. SLII-1)	7.69 ± 0.35
II (Medium FMB + *Bacillus* sp. SLII-1)	16.74 ± 0.31

The total soluble protein content of treatment II (in the presence of *Bacillus* sp. SLII-1) is higher than the total soluble protein content of treatment I (without *Bacillus* sp. SLII-1). The result shows that *Bacillus* sp. SLII-1 can increase total soluble protein content (by 117.69%) in the FMB medium that contains insoluble feather meal keratin. *Bacillus* sp. SLII-1 synthesizes keratinase to hydrolyze keratin as the main source of carbon and nitrogen during its growth in the FMB medium [1]. Keratinase can break disulfide bonds and peptide bonds in keratin in order to release smaller peptides and amino acids which are soluble in water [5]. This result confirms that *Bacillus* sp. SLII-1 is keratinolytic bacteria and has the potential to increase the digestibility of feather meal keratin as feed.

Growth Profile of *Bacillus* sp. SLII-1

Growth profile of *Bacillus* sp. SLII-1 is shown in Fig. 1.

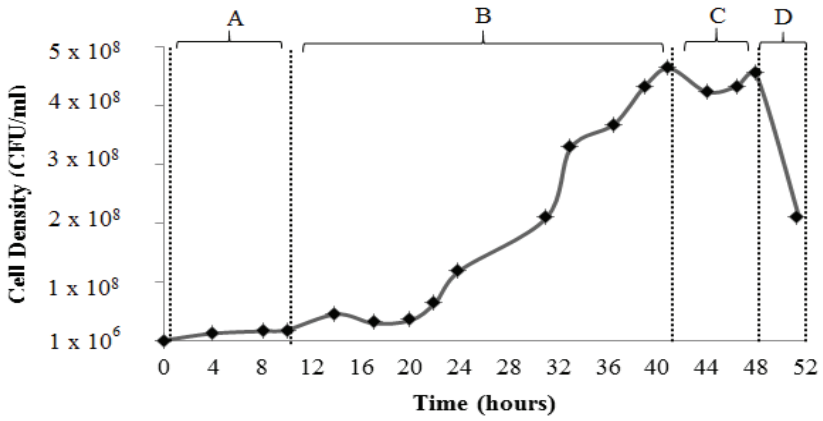

FIGURE 1. Growth Profile of *Bacillus* sp. SLII-1 on FMB medium. (A) Lag Phase, (B) Log Phase, (C) Stationary Phase, (D) Death Phase

Log phase occurs at the 10th-hour to 42nd-hour which is marked by a logarithmic increase in the number of cells. Bacteria synthesize keratinase to hydrolyze keratin in the medium as the main carbon and nitrogen source during the log phase [1]. The largest number of *Bacillus* sp. SLII-1 cells are reached at the end of the log phase or 42nd-hour , therefore, this time is used as an effective period for immobilization of *Bacillus* sp. SLII-1 in sodium alginate. [8] also used the end of the log phase as an effective time to immobilize bacteria.

Immobilization of *Bacillus* sp. SLII-1

The beads were evaluated in poultry digestive system simulations to determine the most effective concentration of sodium alginate in delivering *Bacillus* sp. SLII-1 to poultry intestines. The result of bead evaluation on poultry digestive system simulations is shown in Table 3.

TABLE 3. Test results of beads in poultry digestive system simulations

The concentration of sodium alginate	Number of cells released in the intestine (pH ±6,5) (x10^{7}CFU/g)
1% (w/v)	1.26±0.03
2% (w/v)	2.54±0.04
3% (w/v)	2.34±0.02

The most effective immobilization is to be able to maintain the highest number of cells after passing through the gizzard (pH 3.0) and releasing the highest number of cells in the intestine (pH 6.5). Based on Table 3, it is known that the concentration of sodium alginate 2% (w/v) results in the highest number of releasing cells in the intestine as much as 2.54x10^7 CFU/g bead. The concentration of sodium alginate affects the pore size of the bead. The higher the concentration of sodium alginate, the smaller the bead pore size produced [15]. The smaller the pore size of the bead, the slower rate of bacteria released from the bead. The concentration of sodium alginate 1% (w/v) is less effective because of the large pore size of the bead so that many cells are released from the bead before reaching the intestine. The concentration of sodium alginate 3% (w/v) is also less effective because the rate of cell release from the bead is smaller than the concentration of sodium alginate 2% (w/v) in the intestinal simulation of poultry digestion. This causes the number of cells released in the intestine with the concentration of sodium alginate 3% (w/v) is smaller than with the concentration of sodium alginate 2% (w/v).

This immobilization formulation also uses chitosan as a calcium alginate bead coating. The addition of 0.8% (w/v) chitosan increase bead resistance under acidic conditions. Cationic polymers such as chitosan are able to form gels

with sodium alginate through ionic crosslinking. This complex decreases the porosity of the bead and shows stability in low pH [16].

Research from [7] also showed that the concentration of sodium alginate 2% (w/v) is the most effective composition in immobilizing *Lactobacillus plantarum* TN8. The results of immobilization in the research of [7] was able to maintain the viability of *L. plantarum* TN8 in artificial intestinal fluid, artificial gastric fluid, the high temperature of 65^{o}C and the low temperature of 4^{o}C.[12] also used similar immobilization formulation as poultry feed and indicated that formulation can give effect to broiler performance. The results of this research indicated that immobilization of *Bacillus* sp. SLII-1 with 2% alginate and 0,8% chitosan can be promising feedstock supplement.

CONCLUSIONS

Bacillus sp. SLII-I as keratinolytic bacteria was able to increase soluble protein in feather meal liquid medium by 117.69%. The composition of 2% sodium alginate and 0.8% chitosan was most effective in delivering bacteria to poultry intestine as much as 2.54 ± 0.04 CFU per gram bead in poultry digestive system simulation. Immobilization of *Bacillus* sp. SLII-1 with 2% alginate and 0.8% chitosan can be promising feedstock supplement. This research provides a new approach in increasing the digestibility value of feather waste as animal feed.

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