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OPTIMIZATION OF KERATINASE PRODUCTION BY BACILLUS SLII-I BACTERIA IN CHICKEN FEATHER WASTE MEDIUM

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ABSTRACT

Keratin is an undissolved protein in water and difficult to transform by proteolytic enzymes because there is a cystine disulphide bond. One of the bacteria that produce keratinase enzyme is Bacillus sp. which is able to break the disulfide bond on the keratin protein into a soluble protein. The purpose of this research is to produce keratinase optimally through analysis of bacterial growth profile, protein content, and keratinase activity by varying pH conditions and composition of chicken feather waste medium. The optimization of keratinase was based on the growth curve of bacteria cultured on 3 medium types namely Nutrien broth, minimal medium feather meal, minimal medium feather meal pepton 1% and 3 types of pH 7, 8, and 9, for keratinase activity from crude enzyme extract, While for protein profile analysis using ammonium sulphate method, isoelectric point, and SDS-PAGE was done to find out protein profile. From the result of optimum keratinase production from minimal medium feather meal pH 7, the highest keratinase enzyme activity was 0.34 unit / ml. The isoelectric point of the keratinase enzyme is known at pH 5.3 while the molecular weight based on the SDS-PAGE yield is 38kDa.

Keywords: *Bacillus sp.*, keratin, keratinase optimization, protein profile.

INTRODUCTION

Livestock feed as a source of animal protein is still relatively expensive because Indonesia still relies on imported products. The number of livestock feed imports is still very high because domestic products have not been able to fulfill market needs. This is a major problem for farmers in their efforts to increase livestock production. Therefore, it is necessary to use organic waste as an alternative source of protein to reduce feed costs [1].

Chicken feather is one of the organic waste products from chicken butchery business which is still not utilized optimally. Chicken butchery produces feather of 4-9% of total chicken weight in average. [2]. In 1998, the solid waste load produced by the poultry industry in East Java was 121,793 tons [3]. In 1999 until 2011 chicken meat consumption in East Java increased from 25.924.000 kg [4] to 65.429.000 kg [5]. The increasing of people's need for chicken meat consumption will increase the waste of chicken feathers produced. Chicken feathers waste are usually thrown away, stacked as land filling or burned resulting in environmental pollution [6].Chicken feathers have a high enough protein content. The coarse chicken flour protein protein reached 86.5% and the metabolic energy was 3,047 kcal / kg, in addition the chicken feather contained much higher protein content than fish meal and had a high crude protein [7]. So, chicken feathers is a livestock waste that can be used as an alternative feed ingredients to substitute animal protein sources in chicken feed formulation (poultry).

Keratin is one type of protein found in chicken feather waste; keratin is the structure of insoluble proteins found in the skin, wool, hair, horns, stratum corneum layer. Keratin has cystine disulfide bonds, hydrogen bonds and hydrophobic interactions causing keratin to be rigid and stable, making it extremely difficult to overhaul and resistant to physical, chemical, and biological treatments [8].Keratin can be transformed with protease enzyme groups. Keratinase belongs to a group of protease enzymes that can hydrolyze keratin by breaking the disulfide cystine bonds in keratin, thus have an important role in the transformation of keratin into simple proteins [9].

Keratinase is produced by bacteria, such as the genus *Bacillus*. *Bacillus* genus is able to live in a wide range of temperatures and pH. *Bacillus genus* is one of the bacteria that have various capabilities that can be developed in the industrial world. *Bacillus* is very potential to be developed in the biotechnology industry because it has excellent properties such as wide growth range, spore formation, extensive habitat, and resistance to antiseptic compounds, aerobic or facultative aerobics, multiple enzymatic capabilities, and some biodegradation against many xenobiotic compounds [10]. Research [11], reported that the ability of bacteria in producing keratinase enzymes can be increased by adding 0.1% peptone to its growth medium. *Bacillus* is able to grow in a synthetic medium [12], i.e. a minimal medium feather meal. *Bacillus* SLII-I collection of Laboratory of Microbiology and Biotechnology Department of Biology FMIPA ITS allegedly able to get source C (carbon) from keratin chicken feathers contained in medium minimal feather meal. Then in this research is done optimization, production, and characterization of keratinase from Bacillus SLII-I.

MATERIALS AND METHODS

Time and place of study

This research was conducted at the Laboratory of the Institute of Tropical Disease Airlangga University and the laboratory of Microbiology and Biotechnology Department of Biology FMIPA ITS in April 2014 until January 2015.

Methods

Making chicken feathers flour

Chicken feather flour which has a source of keratin, is made by a modified method [13]. First, chicken feathers are washed and boiled for 2-3 hours, then burned for 8 hours at 50 ^{o}C. Chicken feathers that have been dried, grinded, crushed with mortar and filtered with a flour filter to become chicken flour.

Starter production

The *Bacillus* SLII-I isolate starter was prepared by gradual method [14]. Using 1 ose isolate of *Bacillus* SLII-I in the solid medium of Nutrient Agar (Appendix 1) was then included in 10 mL minimal feather meal medium (Attachment 1), medium minimal with 1% peptone addition (Appendix 1), while for nutrient broth medium Appendix 1) without starter. Then incubated with a rotary shaker at 120 rpm for 24 hours at room temperature. After that 5 ml of culture was transferred into both of the new medium as much as 45 ml and incubated again in the same condition as the previous. Then 10 ml of culture was transferred again into 90 ml of new medium and incubated with the same condition. The culture was taken 20 ml transferred again into a new medium of 180 ml and incubated under the same conditions. The end result of culture is used as starter culture.

Making growth curve

In the nutrient broth medium, *Bacillus* SLII-I isolates were inoculated on a medium of 250 ml. While for starter culture taken as much as 25 ml and put into 225 ml medium minimum feather meal (FM), medium minimum feather meal (FM) with addition of 1% pepton and incubated for 24 hours. Each hour is measured using spectrophotometer growth, by measuring the absorbance value of optical density at 600 nm wavelength. Measurements were made in the first, cultured way taken as much as 0.2 ml and diluted by adding 1.8 ml of sterile *aquades* and then homogenized. Then the culture is fed into the cuvette to measure the value of optical density by spectrophotometer. For the blanks was used medium without isolate with dilution 10 times using sterile *aquades*, i.e. 0.2 ml each modified medium without isolate plus 1.8 ml of sterile *aquades*. Sampling is done in every hour from hour 0 to 24 hours (Anitha *et al*., 2012).

Optimization of *Enzyme* production

In medium nutrient broth, the isolates were inoculated on 250 ml medium, while for 25 ml of Bacillus SLII-I isolate starter was inoculated into 225 ml of medium minimum medium feather meal (FM) and minimal feather meal medium (FM) with the addition of pepton 1 %. At Erlenmeyer 500 ml and incubated with an incubator shaker at 120 rpm for 24 hours and repeated three times [14]. According to [11], keratinase enzyme production may be affected by temperature, pH, and medium composition. In this study the temperature used is 40 ^{o}C with pH 7, 8, and 9. After got optimum enzyme production, then inoculation into 500 ml for the greater enzyme production.

Preparation of crude *Enzyme* extracts

The crude enzyme extract was extracted from the homogenized cultures and fed into a centrifugation tube of 30 ml culture from the three repeats of culture. The culture was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant obtained was a crude enzyme extract for the measurement of keratinase activity and stored in the freezer [15].

Isolation and purification of *enzymes*

The *ammonium sulphate* method

The partial purification of crude extract of protease enzyme is purified by gradual precipitation $(NH_4)_2SO_4$ with a fraction of 0-30%, 30% -45%, 45% -60%, 60-75%, the treatment is carried out in an ice box containing iced fragments stone. To create a 0-30% $(NH_4)_2SO_4$ fraction weighed 3.52 g, then added to 20 mL of a crude enzyme extract solution in a 50 mL beaker glass. The addition is done bit by bit while stirring using a stirrer glass. After all $(NH_4)_2SO_4$ added soluble then left it for 1 hour. The enzyme deposition is separated by centrifugation using a speed of 3000 rpm, at 40 ° C for 30 minutes. It produced supernatant and sediment. Then the 30% -45% fraction is followed, the supernatant is fed into a 50 mL beaker glass and then added with 1.88 g $(NH_4)_2SO_4$. After all the soluble then left for 1 hour. Deposition of enzyme in centrifugation. It produced supernatant and sediment. Then for a fraction of 45% -60%, a 30% -45% fractional supernatant is fed into a 50 mL beaker glass and then added with 1.97 g $(NH_4)_2SO_4$. After being dissolved, left it for 1 hour. The enzyme deposit is centrifuged. It produced supernatant and sediment. For the fractionation of 60-75%, the Supernatant of the fraction 45% -60%, put into beaker glass 50 mL then added with 2.06 g $(NH_4)_2SO_4$. After being dissolved, then left it for 1 hour. The enzyme deposit was centrifuged using a speed of 10000 rpm, at 40 ° C for 10 minutes and produced supernatant and sediment. The amount of ammonium sulfate salt added to each fraction is based on the ammonium sulfate precipitation table..

Isoelectric point method

Isoelectric point is a particular p region where the protein does not have difference charge or the number of positive and negative charge are same, so it does not move when placed in an electric field. At the isoelectric pH (pI), the solubility of protein is minimal, thus causing the protein settles. First prepared 5 clean and dry tubes, then put 1 ml of enzyme in each tube. In each tube 1 ml of acetate buffer solution was added each of pH 3.8; 4.7; 5.0; 5.3; and 5.9, the isoelectric point of the amino acid cysteine not far from pH 4.3. Then shaked, after that recorded the degree of turbidity after 0, 10, and 30 minutes. Observed how many tubes are formed the maximum sediment. Then all tubes are heated on the water

bath. Observed the results. The quickest or the most formation of turbidity deposits is an isoelectric point [16].

Enzyme characterization

Protein content

Determination of protein content was determined by method [17]. A total of 1 mL of enzyme solution was added to a tube containing 5 mL of a diluted Bradford reagent five times. The mixture is divorted and lefted at room temperature for 5 minutes. Treatment for the blanko solution, enzyme solutions replaced with *aquades*. Furthermore, the solution is homogenized and left for 20 minutes at room temperature. The absorbent of the solution is measured at a wavelength of 595 nm. The protein concentration of the sample was calculated based on the standard BSA curve (Bovine Serum Albumin). On standard protein curves, enzyme solutions are replaced by BSA with a concentration range of 0 to 1 mg / mL.

Electrophoresis *SDS-PAGE*

The initial preparation that needs to be done in electrophoresis is gel preparation. The method used in gel preparation is the method [18]. The material for separating gel is mixed one by one by inserting *tetramethylenediamine* (TEMED) at the end of the mixture. The solution is stirred and piped slowly into the glass plate up to 1.5 cm from the glass surface and then left it for about 15-20 minutes. In this process cultivated not to form air bubbles. After the gel becomes solid, the stacking gel mixture is gently dipipet into the glass plate and then immediately insert the comb (10 wells) as the place to enter the sample.

Samples that have been heated at 100 ^{0}C for 3 minutes are mixed with buffer sample and then loading the sample into the well as much as 12 µl. Unlike the case with the sample, the Marker is loaded into the well as much as 10 µl. Before running is done, the electrophoresis buffer is inserted into the chamber. Running electrophoresis is performed at 100 volts, 50 mA in cold conditions. The time required for running electrophoresis is about 1.5 hours

After separation process, the gel is removed from the glass plate then soaked in a fixation solution (25% methanol + 12% acetic acid) for 1 hour. Furthermore, the gel was immersed in 50% ethanol solution for 20 minutes and 30% ethanol solution for 2 x 20 minutes [18].

***Enzyme* activity**

Enzyme that obtained from centrifugation was added 20 mg of chicken feather flour which dissolved in buffer solution with enzyme ratio with buffer solution of 1: 4 (200 µl enzyme extract: 800 µl buffer solution). To make a pH of 7, 0.2 M *phosphate buffer* [15] was used. The solution was incubated for 1 hour at 37 ° C. After incubation, the solution was cooled in ice water for 10 min. The solution absorbance was measured using a spectrophotometer at a 280 nm wavelength [19].

Research design

Based on a combination of physical and chemical factors from previous research, the pH and the composition of the chicken waste medium determined the production of keratinase, so that the above variables were chosen in this study using a complete randomized factorial design with two factors: pH (7, 8, and 9) And medium type (nutrient broth, minimal medium feather meal and minimal medium plus 1% pepton) to obtain the most optimum keratinase production. The data is processed by using Analysis of Variance (ANOVA), if there is significant difference then continued with Duncan test with 95% level of confidence. The result is a bacterial growth profile in the form of growth curve and protein concentration profile and enzyme activity that explained by descriptive analysis.

RESULTS AND VERIFICATION

A. Preparation of Bacillus SLII-I isolate growth curve analysis

The growth curve was performed to determine the exact time of *Bacillus* SLII-I for culture crop (Figure-1). From the medium selection conducted on the basis of pH (7, 8 and 9), the most optimum condition was determined at pH 7. [20] Reported that the initial pH of the medium greatly influenced bacterial growth, percentage of feather degradation and keratinase production. It has been observed that *Bacillus* species are most active in neutral conditions (pH 7), the optimum pH for *B. cereus* is 7.0 [21], while for *B. pumilus* is 8.0 [22]. For *B. subtilis*, the highest enzyme production was obtained from pH 5 to pH 9.

Figure-1 shows that the profile of the bacterial growth curve generally consists of adaptation (lag), exponential (log), stationary and death phases. Measurements of optical density values of each medium were carried out at a maximum absorbance of 600 nm. The bacterial growth curve is made with the x axis as the incubation time and the y-axis as the optical density (OD) or absorbance value [15]. Medium nutrient broth is used as a standard medium, because it is included in the enriching medium, so that all types of bacteria can grow in nutrient broth medium. The most optimum medium for growth is at least the feather meal medium as seen from the bacterial growth curve; the medium has a short phase of adaptation (lag) and a high exponential phase (log). The growth curve of at least 1% peptone feather meal medium has a long phase of adaptation (lag), because *Bacillus* uses peptone first rather than the feather meal. If the peptone source is depleted, the bacteria will produce keratinase to break down keratin in chicken feathers for source C for its growth. In addition to peptone, modification of the medium can be accomplished by the addition of *glucose*, *phosphate*, and *glutathoinin* for its enrichment [23]. Figure-1 describes the best growth curve of each bacterial medium based on the analysis of the growth curve profile shown that the medium modified with nutrient broth resembles the profile of the standard medium growth

curve, so that modification of the minimum medium feather meal can be sure to replace the standard medium.

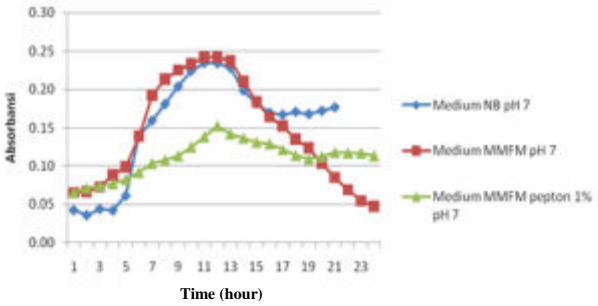

Figure-1. Growth curve isolate *Bacillus* SLII-I.

Measurement of keratinase activity (Figure-2), the highest activity occurred in a minimum feather meal medium with pH 7 at a susceptible 1 hour to the 14th hour. At that time the sampling for the crude enzyme extract was performed, as it was assumed to be the optimal time to produce keratinase at most and was directly proportional to the number of isolates in the medium. The activity of keratinase was measured using a quantitative spectrophotometer at a wavelength of 280 nm that could detect amino acid cystine in keratin [24]. The highest keratinase activity observed was 3.4 Units / ml at the 11th hour and lowest 1.5 Unit / ml at and 5th hour. Previous studies have shown the value of keratinase activity of 3.35 Units / ml.

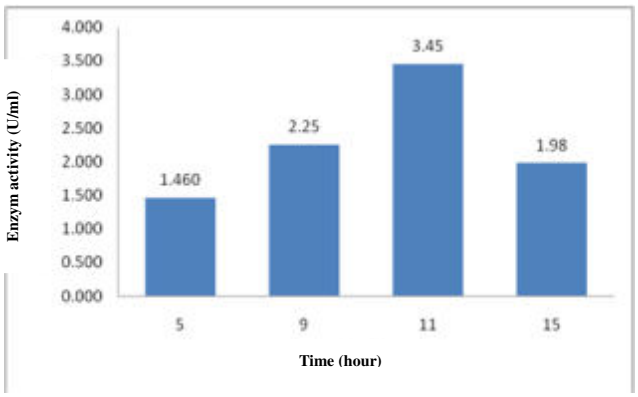

Figure-2. Keratinase activity at minimum medium feather meal pH 7 optimum conditions.

From the statistical test with Analysis of Variance (ANOVA) two-way (Appendix 2), on the growth curve of bacteria can be concluded that pH and medium factor have significant effect (p value <0.05). Then followed by Duncan test showed that there was no significant difference of treatment at pH 8 and 9 in medium nutrient broth and at least medium feather meal, while pH 7 in medium minimum feather meal pepton 1% there was significant treatment difference. At pH factor, there are 3 kinds of pH used in this research, that is 7,8, and 9. From the Duncan test results, at pH 7 was a significant treatment difference because pH 7 was the optimum condition for the growth of *Bacillus* SLII-I. The pH factor is fundamental for influencing the physiology of microorganisms by affecting nutrient and absorption solubility, enzyme activity, morphological cell membrane, by-product formation and oxidative-reductive reactions. During keratinase production, keratin utilization occurs more rapidly at a large pH of 7.5 [20]. [25] explains that the maximum keratinase production at base pH, the optimum pH reported for keratinase production of *B. cereus* is 7.0 [21], *Chryseobacterium sp*. Is 9.0 [26], while *B. pumilus* FH9 is 8.0 [22]. For *B. subtilis*, the highest enzyme production has been reported in the pH range of 7 to 9. It has been reported that maximum keratinase production occurs at an *alkaline* pH (base PH).

B. Ammonium sulfate method

The *ammonium sulphate* method aims to separate keratinase with other compounds. Precipitation using salt is based on the solubility of proteins that interact polar with water molecules, ionic interactions of proteins with salt and repulsive rejection of similarly charged proteins. Based on the results obtained in this study, keratinase can settle at a 75% fraction with keratinase activity of 0.375 Units / ml, which means to hydrolyze 1µmol of keratin required 1 ml of keratinase [27].

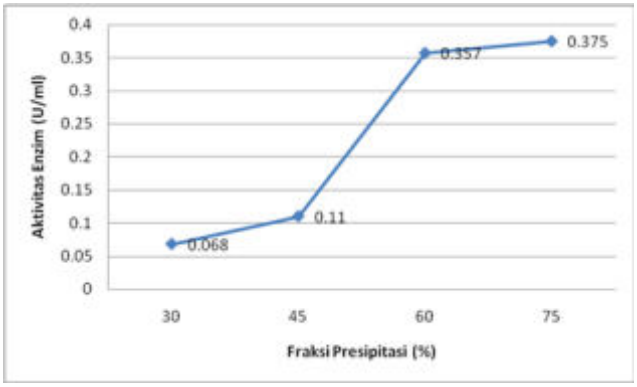

Figure-3. Keratinase activity for each precipitation fraction of ammonium sulphate.

The solubility of proteins (at a certain pH and temperature) increases as salt concentration increases (salting in). The addition of certain salts will cause the solubility of the protein decreases (salting out). Increased solubility of protein will increase the strength of the solution ions. Water molecules that bind to salt ions more and more cause the withdrawal of water sheath that surrounds the protein surface, therefore causing proteins to interact, aggregate, and then settle. *Ammonium sulfate* is the most commonly used salt for precipitating proteins because it has high solubility in water, otherwise it is relatively inexpensive [28]. Measurement of keratinase activity from *ammonium sulphate* precipitation results. From the results obtained, the activity of keratinase at the fraction of 75% is 0.375 units / ml, so that every 1 unit of keratinase enzyme is needed to liberate 1µmol of keratin from at least feather meal medium in standard condition [27]

C. MetodeIsoelektrik point

The isoelectric point is a condition when a protein has no charge difference or amount between the same positive and negative charge, so the protein solubility rate decreases and reaches the lowest number resulting when placed in an electric field will have the same number of cations and anions. PH is called the isoelectric point (pI) where the total charge on the molecule is zero, is a characteristic of each enzyme, where the solubility in *aqueous* solution is generally minimum. In solution, the charged group will interact with the polar water molecules and will stabilize the protein because it is hydrophobic. Most aliphatic or aromatic side chains are characteristic of less water-soluble proteins. One example of pI is that the number of ionized groups increases as the solubility tends to increase. Therefore an isoelectric point is important because it affects solubility and protein interactions [29]. The isoelectric point of the crude enzyme extract on the feather meal medium is pH 5.3. According to research [30] the isoelectric point on keratinase ranges from pH 3.5 to 9.5.

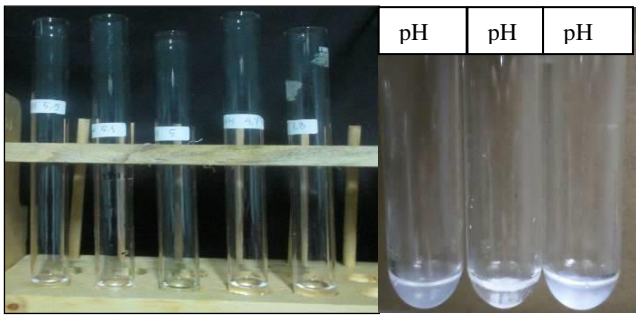

Figure-4. Isoelectric point Keratinase.

D. Elektrophoresis SDS-PAGE

Sodium dodecyl sulphate (SDS) - polyacrylamide gel electrophoresis (PAGE) is a method used to characterize enzymes and aims to determine the molecular weight of a protein. The use of SDS serves to denature the protein because SDS is a detergent that results in bonds in the interrupted protein forming a protein that can elute in gel [31]. The enzyme sample injected into the gel well is colored with ionized bromphenol blue. The function of the dye is to help monitor the process of electrophoresis [32]. Polyacrylamide gel with 4% stacking gel and 12% separating, at a voltage of 120 volts 28 A. The molecular weight of keratinases enzyme ranges from 15 to 240 [23]. However, according to [19] most of the keratinase enzyme's enzyme molecular weight is in the range of 20 to 50 kDa. From result of SDS-PAGE known that keratinae protein molecular weight in this research is 38 kDa.

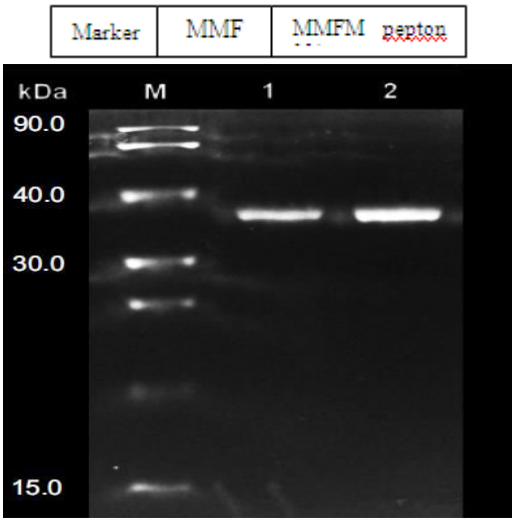

Figure-5. SDS-PAGE.

CONCLUSIONS

Based on the result of this research, it can be concluded that Bacillus SL II-I bacteria grow optimum at least feather meal medium pH 7 at hour to 11 with the highest keratinase activity reach 3.4 Unit / ml. Based on partial purification on crude keratinase extract using ammonium sulfate protein was achieved maximally at 75% concentration with keratinase activity of 0.375 Unit / ml. The characterization of the keratinase isoelectric point was achieved at pH 5.3 with a molecular weight of 38 kDa. Greater production needs to meet the market demand for animal feed production. In addition it is necessary to research other factors such as:

a) Calculate the total protein content, dissolved protein and the amount of degraded keratine.

b) Knowing the effect of protein source of chicken feathers waste in animal feed.

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