Malaysian Journal of Chemistry, 2023, Vol. 25(5), 111-117
(Special Issue: *The 4th International Conference on Chemistry and Material Sciences 2022*)

Initial Characterization of Keratinase by *Bacillus proteolyticus* GAM 15 Isolated from Palangkaraya Peat Soil, Central Kalimantan and Its Application for Goat Skin Dehairing

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Dehairing is one important process in leather industry. The process was generally proceed chemically which is not environmental friendly. A greener process could be done using utilizing the keratinase secreted by bacteria. A preliminary study found that bacterial isolates, from the Peat Soil Palangkaraya, Central Kalimantan, were proven to decompose chicken feathers which proved its capability to produce This paper reports our work on the characterization of keratinase its potential to dehair goat skin. This research was conducted in several stages, they are (1) regeneration of *Bacillus proteolyticus* GAM 15, (2) keratinase production and determined protein content and feather weight loss, (3) determination of keratinase characteristics by pH, temperature, the effect of metal ions on the enzyme activity, the type of enzyme (4) identification of Gram type and the bacterial species based on 16S rRNA gen sequence, and (5) its application for dehairing agent. The results showed tha the isolate is a gram-positive bacteria with bacil cell shape. It has 100% homology with *Bacillus proteolyticus*. The keratinase enzyme secreted by *Bacillus proteolyticus* GAM 15 was proven to able decompose chicken feathers and produce the highest protein content on 3 days incubation time. The characterization results of the keratinase enzyme were metalloprotease and activity of keratinase increased when adding Ca^{2+}, Mn^{2+}, Co^{2+}, and Ni^{3+}.This enzyme can survive at pH 6,8, and 9. Furthermore, the optimum temperature of the keratinase enzyme activity at 52^{o}C. Crude extract of the keratinase enzyme was effective for dehairing goat skin with 48 hours of incubation time.

Keywords: Keratinase; bacillus; peat soil; dehairing

Received: September 2022; Accepted: January 2023

Peat soil of Palangkaraya, Central Kalimantan, Indonesia is one of the ecosystems composed of organic matter with a relatively long decomposition rate due to anaerobic conditions in the swamp. This triggers the number of microorganisms that can survive in the peat soil area, one of which is bacteria. Several bacterial isolates, especially protease bacteria, were isolated from peat soil at Balikpapan [1]. This shows that there is an abundance of biodiversity of microorganisms, especially bacteria in peat soil that can be utilized.

One of the bacteria that can be used is keratinolytic bacteria. Keratinolytic bacteria are bacteria that can secrete the keratinase enzyme which is useful for break disulphide bonds in keratin to convert into peptides and free amino acids. Several bacteria are capable of producing keratinase, including *Bacillus* sp. MD24 [2], *Bacillus weihenstephanensis* PKD-5 [3], *Bacillus substilis* [4], and *Bacillus pumilus* [5]. Keratinase enzymes can be secreted by microorganisms using a fermentation method with specific substrates such as chicken feathers.

Chicken feathers are waste from slaughtering industry that are difficult to decompose so that they can pollute the environment. Chicken feathers contain up to 90% keratin [6-7]. Keratin is a insoluble protein which can't dissolve in water, organic solvents, and acid solvents [8]. Keratin has a strong structure due to the presence of disulfide cross-links, hydrogen bonds, and hydrophobic interactions between molecules. The presence of disulfide bonds formed from the amino acid cysteine which has a thiol group (-SH) so it can form disulfide bridges structure [9]. Utilization of bacteria capable of secreting keratinase to decompose chicken feathers is one of the green degradation methods. The keratinase enzyme plays an important role in various industries such as the manufacture of detergents [10], adhesive [11], animal feed, organic fertilizers, and animal skin tanning [12].

The dehairing process is one of the most important processes in the animal skin tanning industry. Animal's skin covered by hair containing keratin. An effective method that is often used by the

†Paper presented at the 4th International Conference on Chemistry and Material Sciences 2022 (IC2MS2022)

leather tanning industry to break the disulfide bonds in keratin is by using sodium sulfide [2, 13]. However, despite its high effectiveness, the use of sodium sulfide has drawbacks including the presence of sulfide gas that formed which can damage respiratory organs. The long-term effects that can be experienced by inhaling this gas include central nervous system disorders, lymphoreticular, and even death [14].So the use of keratinase enzyme secreted by bacteria can be an alternative solution. The advantages of using the enzymatic method are that it can overcome pollution problems in the environment, inexpensive, and increases the yield of animal skin tanning.

In the present study, the isolation of keratinolytic bacteria obtained from the Palangkaraya Peatlsoil will be carried out. The ability of bacteria to degrade keratin was carried out by screening using chicken feathers as a substrate in the fermentation process. The crude extract of the enzymes produced during the fermentation process will l be applied for dehairing of goat skin.

EXPERIMENTAL DETAILS

Chicken Feather Preparation

The feathers used in this study were obtained from chicken slaughterhouses at Blimbing Market, Malang, East Java, Indonesia. Chicken feathers are washed with water for 4 to 5 washes. Furthermore, the chicken feathers are dried in the sun [9] and cut around 0.5 cm.

Isolation and Identification of Bacterial Strains

The sample was taken from peat soil in the Palangkaraya Central Kalimantan with coordinates 2º13'56.6''S113º52'001.1''E. Soil samples were taken from two different areas with a depth around 5 cm from the surface. Before sampling, the soil pH and temperature were measured.

One gram of peat soil was dissolved in 9 mL of 0.85% NaCl and serial dilutions were carried out (10^3, 10^6, 10^9, dan 10^{12}). The dilution series was taken 100 µL and suspended in Skim Milk Agar medium (1.5% bacto agar, 0.5% NaCl, 0.1% $MgSO_4$, 5.0% skim milk at pH 8) and incubated overnight at 37ºC [2].The isolates that formed the clear zone were inoculated in a medium containing keratin with a composition of 1 g/L chicken feather, 0.2 g/L KCl; 0.5 g/L K_2HPO_4; 20 g/L $CaCO_3$; 0.2 g/L $MgSO_4.7H_2O$; 0.5 g/L KH_2PO_4; and 1 g/L $(NH_4)_2PO_4$. The bacteria with the highest activity named GAM 15 was selected for further analysis.

Bacterial strain identification was carried out by phenotyping with gram staining and genotyping using the 16S rRNA gene. The sequencing amplification process was carried out at Macrogen (Seoul, Korea) using 785F and 907R primers. Sequencing results were processed with BioEdit and then entered into the BLASTN database program (http://www.ncb.nlm.nih.gov) to determine the relationship between GAM 15 species. A total of 10 bacteria with the highest percentage of similarity were selected for alignment using MEGA XI software and processed for the construction of phylogenetic trees by the neighbour-joining method.

Chicken Feather Degradation Ability and Dissolved Protein GAM 15 Isolate

The bacterial isolate GAM 15 was grown in Luria Bertani broth medium (1% peptone and 0.5% yeast extract) and incubated in a shaker incubator for 16 hours at 100 rpm. Then inoculated in 100 mL production medium (Tris-HCl buffer pH 8) containing 0.05% K_2HPO_4; 0.5% NaCl; $MgSO_4$ 0.1%; and 1 gram of chicken feathers as the only source of carbon and nitrogen. The fermentation medium was incubated in incubator shaker for 3 days. The resulting hydrolyzate was analyzed for enzyme activity, protein content, and feather weight loss during the fermentation process.

Determination of Protein Content

Protein content was determined using the Lowry method. A total of 0.5 mL of the centrifuged hydrolyzate was taken and 2.5 mL of Biuret reagent was added. The mixture was vortexed and allowed to stand for 10 minutes. Then 0.25 mL of Folin-Ciocalteu reagent (1:1) was added and incubated for 20 minutes. The absorbance of blue colour complex was determined with UV-Vis spectrophotometer at wavelength 750 nm.

Determination of Enzyme Activity

The activity of the keratinase enzyme secreted by GAM 15 was determined by the Anson method. A total of 0.2 mL of crude extract of the enzyme was centrifuged at 10,000 rpm at 4ºC for 15 minutes. The crude extract was added with 0.5 mL of 1% (w/v) casein substrate and incubated at 37ºC for 30 minutes. The enzymatic reaction was stopped by adding 1 mL of 10% TCA, then vortexed and allowed to stand at room temperature for 15 minutes. Then the mixture was centrifuged at 10,000 rpm at room temperature for 15 minutes. The supernatant was taken at 1 mL and added 2.5 mL of Na_2CO_3 and 0.5 mL of Folin-Ciocalteu reagent (1:1). Then it was incubated in a dark room for 30 minutes and the absorbance of the blue solution was measured using a UV-Vis spectrophotometer at a wavelength of 660 nm [9].

Optimization of enzyme catalytic activity was determined by adding divalent metal ions (Ca^{2+}, Co^{2+}, Mn^{2+}, Ni^{3+}, and Zn^{2+}) with concentrations of 1 mM and 5 mM. In addition, the determination of the type of keratinase enzyme was carried out by adding PMSF (phenazine methosulfate) and EDTA (ethylenediaminetetraacetic acid) at concentrations of 1 mM and 5 mM. The incubation temperature optimization was carried out at 32ºC, 37 ºC, 42ºC,

47ºC, and 52ºC while the optimum pH was determined in the optimum pH range of 6 to 10.

Goat Skin Dehairing

Goat skin dehairing is done by cutting goat hair that has been dried in the sun by 1x1 cm. Then 3 mL of crude extract with activity value 15,270 U/mL of the keratinase enzyme was added and incubated in a water bath shaker at 37 ºC. The incubation process was carried out for 24 hours and 48 hours. The goat skin is then scraped slowly to separate the hair with goat skin [2].

RESULTS AND DISCUSSION

Bacteria Strain Identification

Screening of proteolytic bacterial isolates was carried out using skim milk agar media. The skim milk agar medium was selected to confirm the growth and ability of bacteria isolated from the Palangkaraya Peat Soil to hydrolyze casein. Based on Figure 1, it can be seen that 15 bacterial isolates are capable of forming a clear zone in the growth medium. This clear zone is formed because bacteria can secrete protease enzymes that can hydrolyze proteins contained in casein into simple peptides and amino acids [15]. However, in this study, GAM 15 isolate was used in further research because it had a higher protease enzyme activity value in the preliminary study using the Solid State Fermentation method than others.

Phenotype and Genotype Characterization of GAM 15

The characterization of GAM 15 species was carried out using two methods, namely phenotyping and genotyping. Phenotypic characterization utilizes identification by gram staining. This staining aims to see the shape of the cell and the colour produced when given Gram staining reagent. Based on Figure 2, it can be seen that the GAM 15 isolate had the shape of a red *bacillus* or rod cell. This shows that GAM 15 belongs to the category of gram-negative bacteria.

Identification of GAM 15 species was then carried out by genotypic analysis using the sequencing method. The sequence of GAM 15 isolates with the gene marker 16S rRNA was then used for phylogenetic tree reconstruction. Phylogenetic trees are used to determine the kinship of the same species [2]. Based on Figure 3, it can be seen that there is a kinship relationship with *Bacillus proteolyticus* MCCC 1A00365 with a 100% similarity percentage. The majority of bacteria from the *Bacillus* family can secrete protease enzymes in their ecosystem, thereby increasing the productivity of the enzymes secreted by these bacteria in the industrial sector. Several studies suggest that bacilli are also capable of producing keratinase [16].

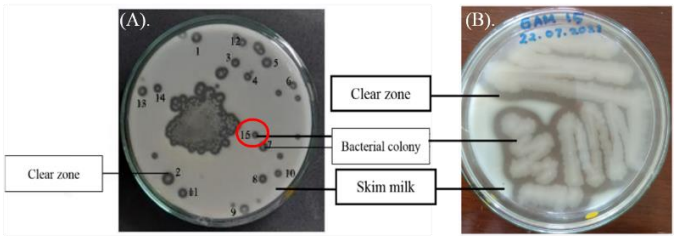

Figure 1. (A) Colonies of bacteria growing in Skim Milk Agar media with serial dilutions of 10^8 and red marks on GAM 15 isolates (B) Regeneration of GAM 15 isolates.

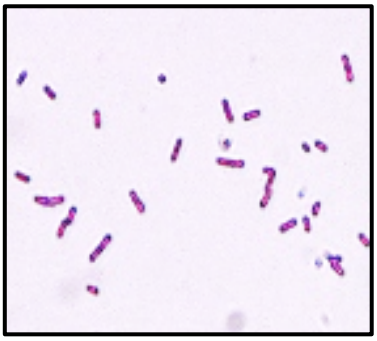

Figure 2. Gram staining of GAM 15 isolate.

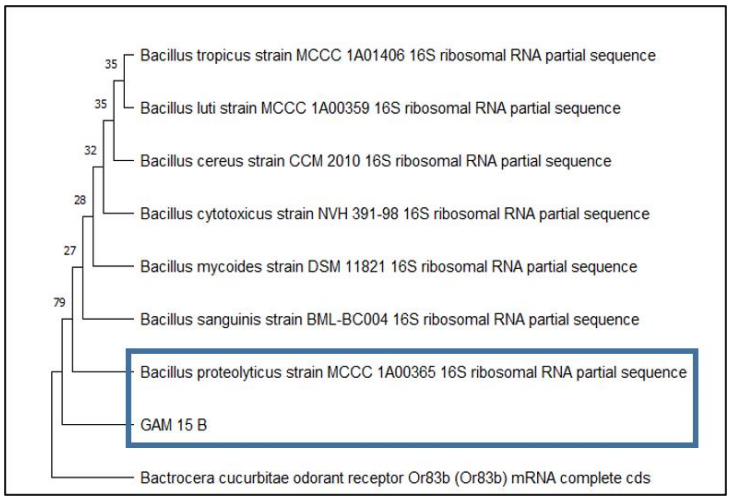

Figure 3. GAM 15 phylogenetic tree based on 16S rRNA sequences.

Table 1. Degradation of Chicken Feather by *Bacillus proteolyticus* GAM 15.

Incubation time (days)	% Weight loss	Protein Content (µg/mL)	Left over of chicken feather
1	8,2	29,44	
2	13,20	32,11	
3	31,80	142,78	

The Ability of *Bacillus proteolyticus* GAM 15 in Decomposing Chicken Feathers

Chicken feathers are one of the waste products from slaughterhouses. Chicken feathers can be used to trigger the performance of the keratinase enzyme secreted by *Bacillus proteolyticus* GAM 15 in the fermentation medium. The positive results of the decomposition of chicken feathers were indicated by the presence of turbidity in the fermentation medium and a decrease in the mass of chicken feathers with increasing incubation time. In addition, the decomposition process of chicken feathers produces dissolved protein in the fermentation medium. Table 1 presents the physical changes in chicken feathers and the increase in protein content with increasing

incubation time [2]. Based on the table, it can be understood that with increasing incubation time, the mass of chicken feathers decreases and there is an increase in protein levels in the fermentation medium.

Effect of Adding Inhibitors on Enzyme Activity

The types of protease enzymes secreted by *Bacillus proteolyticus* GAM 15 were identified using specific inhibitors, namely EDTA (ethylenediaminetetraacetic acid) and PMSF (methyl sulphonyl fluoride) with varying concentrations of 1 mM and 5 mM. Based on Figure 4, it can be seen that the addition of EDTA and PMSF at 1 mM was not able to inhibit the activity of the keratinase enzyme. Furthermore, with the addition of 5 mM EDTA it was found that the activity of the keratinase enzyme was strongly inhibited while at the same concentration PMSF was unable to inhibit the performance of keratinase. These results indicate that the type of protease secreted by *Bacillus proteolyticus* GAM 15 is a metalloprotease type. EDTA is a chelating agent capable of binding metal ions contained in protease enzymes. The binding of metal ions to the active site will cause a conformational change in the enzyme structure so that it is not optimal in binding the substrate. This is what causes a decrease in the activity of the protease enzyme [17]. Metalloprotease types have also been reported for some bacteria [16, 18-19].

Effect of pH and Temperature on Keratinase Enzyme Activity

Measurement of the effect of pH and temperature was carried out on fermentation days 1 until 4 with maximum enzyme activity on day two. In optimizing the effect of pH, it can be seen in Figure 5A that the keratinase enzyme secreted by *Bacillus proteolyticus* GAM 15 can survive in the pH range of 6-10. However, the optimum pH of protease activity increased at pH 9. Furthermore, Figure 5B presented the effect of temperature on keratinase activity. Based on the figure, it can be seen that the performance of the enzyme is relatively stable in the temperature range of 32ºC to 52ºC. The optimum activity of the keratinase enzyme was at an incubation temperature of 52ºC on the second day of fermentation.

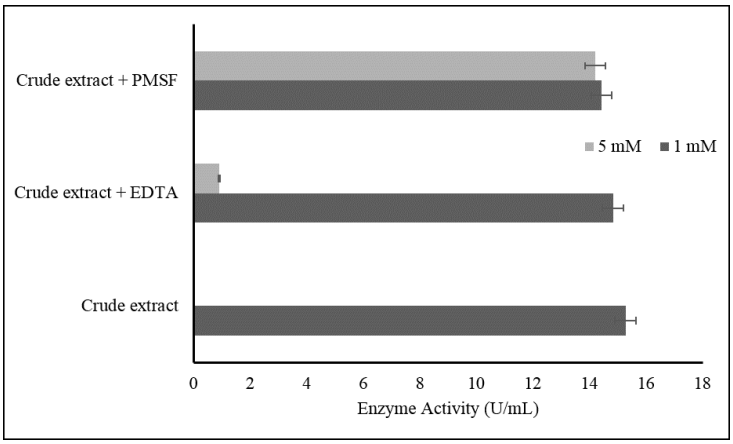

Figure 4. Effect of addition of inhibitor on keratinase activity.

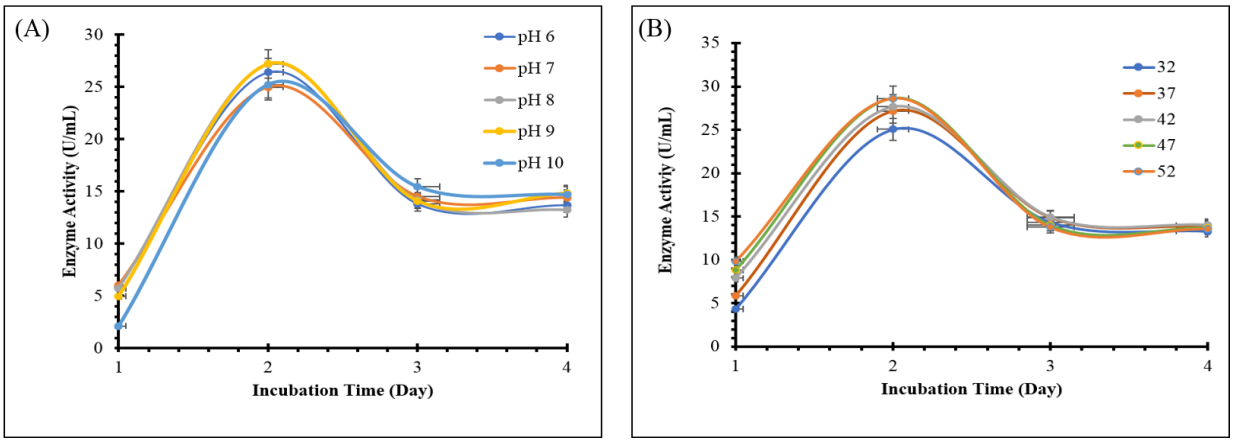

Figure 5. Effect of pH (A) and temperature (B) on keratinase activity.

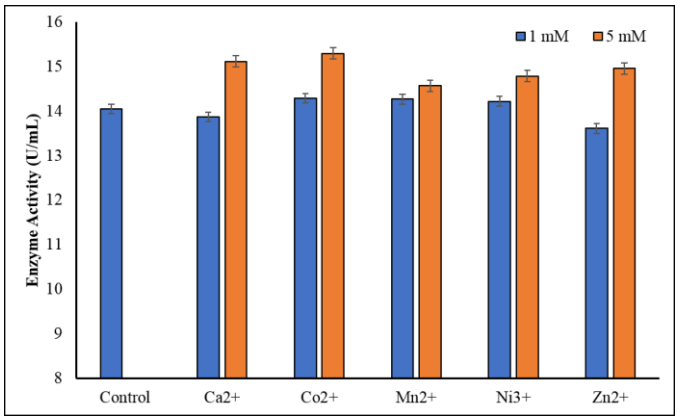

Figure 6. Effect of addition of metal ions on keratinase activity.

Effect of Addition of Metal Ions on Keratinase Activity

The addition of metal ions to the enzyme activity test is useful to determine the effect of metal ions on the stability of the enzyme structure. It is correlated with the activity of the keratinase enzyme in decomposing the substrate. Metal ions can act as cofactors or trigger the performance of enzymes and inhibitors that play a role in reducing enzyme performance. Figure 6 shows the effect of several additions of metal ions on the activity of the keratinase enzyme with different concentrations. The addition of metal ions with a concentration of 1 mM showed a not very significant change in keratinase activity. However, the addition of metal ions both Ca^{2+}, Co^{2+}, Mn^{2+}, Ni^{3+}, and Zn^{2+} with a concentration of 5 mM showed significant results where the divalent metal ions were able to increase the activity of the keratinase enzyme. Several studies stated that the addition of Ca^{2+}, Mn^{2+}, Co^{2+}, and Ni^{3+}, was able to increase keratinase activity [2, 20-22]. The increase in enzyme activity is due to metal ions being able to form salts to maintain the stability of the conformational structure of the enzyme-substrate [22].

Goat Skin Dehairing by Keratinase Enzyme Secreted by *Bacillus proteolyticus* GAM 15

The process of dehairing goat hair is carried out enzymatically by utilizing an enzyme secreted by *Bacillus proteolyticus* GAM 15. Figure 7 B and C show the comparison of goat hair before and after dehairing. Goat hairs begins to fall out of the skin during incubation with the enzyme for 24 hours in the keratinase enzyme with activity of 15.27 U/mL. The keratinase enzyme is effective for dehairing goat hair with an incubation time of 48 hours. Several previous studies have reported the effectiveness of the enzyme in dehairing goat hair and producing a smoother surface of goat skin when compared to conventional methods using sodium sulfide [23-25].

SUMMARY

The bacterial isolate of *Bacillus proteolyticus* GAM 15 was isolated from the Peat Soil of Palangkaraya, Central Kalimantan. This isolate can degrade chicken feathers containing keratin. The keratinase enzyme secreted by these bacteria can survive at pH 6, 8 and 9

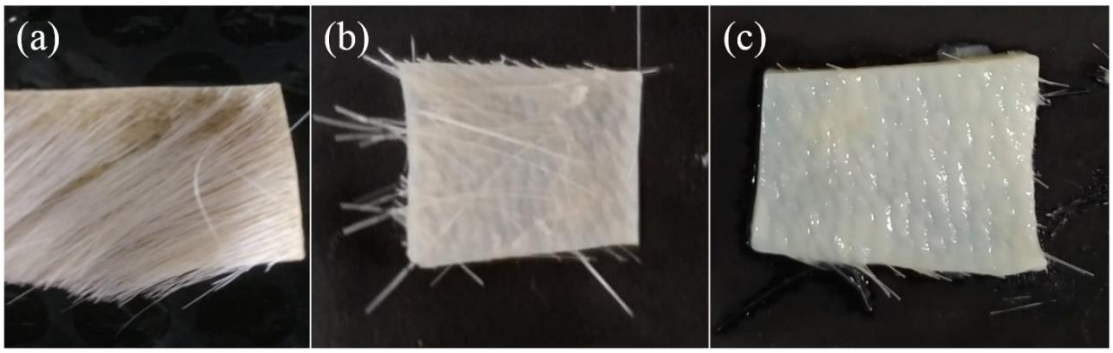

Figure 7. Dehairing goat skin by enzymatic method (A) before dehairing; (B) Changes in goat skin after 24 hours incubation; and (C) Changes in goat skin after 48 hours incubation.

at an optimum temperature of 52ºC. The addition of metal ions such as Ca^{2+}, Mn^{2+}, Co^{2+}, and Ni^{3+} has been shown to increase enzyme activity. This enzyme is categorized as a metalloprotease because its activity decreases when EDTA is added. The enzyme secreted by *Bacillus proteolyticus* GAM 15 can dehairing of goat skin with an incubation time of 48 hours.

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