Exploring the potential of proteolytic bacteria for keratinase enzyme synthesis: Study on isolation and screening

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Abstract. Keratinase enzymes have garnered interest for their ability to degrade keratin-rich substrates. In this study, we focus on isolation and screening proteolytic bacteria capable of producing keratinase enzymes. By identifying novel bacterial sources of keratinases, This study aims to explore the potential proteolytic bacteria to produce keratinase enzymes, through isolation and screening processes, in order to harness their capability for keratinase synthesis. Proteolytic bacteria were isolated from the storage warehouse of hide and skin in Politeknik ATK Yogyakarta. Screening for keratinase producing bacteria were conducted by selection of proteolytic activity test and keratin degradation test. Six of the twelve isolates exhibited halo zones when they grew on proteolytic media. Among the bacteria isolates, bacterium ATK3 demonstrated the highest percentage of keratin degradation and keratinase activity. Based on the study, bacteria isolate of ATK3 had the most potential to be explored as a keratinase enzyme producing bacteria.

1 Introduction

The burgeoning demand for sustainable and eco-friendly solutions in biotechnology has directed scientific endeavors toward the exploitation of microbial enzymes. Among these, keratinases, a subset of proteolytic enzymes, have drawn significant attention due to their remarkable ability to degrade recalcitrant keratinous substrates such as feathers, wool, and human hair [1]. Keratin, an insoluble and fibrous protein, poses a substantial challenge in waste management and environmental conservation due to its highly stable and resistant structure. Consequently, the bioconversion of keratin-rich waste into value-added products through microbial keratinase activity represents a promising area of research with far-reaching implications [2].

Proteolytic bacteria, especially those producing keratinases, have been isolated from diverse environments, including soil, animal hides, poultry waste, and feather waste sites. These microorganisms have demonstrated proficiency in keratin degradation, thereby presenting

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potential for industrial applications in sectors such as leather processing, detergent formulation, animal feed production, and waste management. Recent advances in microbial biotechnology have underscored the significance of isolating high-yield, keratinase-producing bacteria as a strategic step towards large-scale enzyme production [3,4].

The isolation and screening of potent keratinase-producing bacteria involve several methodological approaches. Enrichment culture techniques, coupled with selective media and biochemical assays, facilitate the identification of microbial strains capable of robust keratin degradation [5]. This study aims to harness proteolytic bacteria to produce keratinase enzymes through a systematic isolation and screening process. By exploring various environmental samples and employing rigorous screening methodologies, we seek to identify and characterize bacterial strains with high keratinolytic potential. The findings from this research are anticipated to contribute to the development of efficient and sustainable biotechnological processes for waste valorization and industrial enzyme applications

2 Materials and Methods

2.1 Bacterial Isolation

Bacterial isolation samples were collected from the leather warehouse at Politeknik ATK Yogyakarta. The sampling method was based on Ogino et al. (2008) with slight modifications, specifically by using nutrient broth as the bacterial growth medium. Samples were collected from the skin of hide salt preservation. Skin and fur were used as sample materials. Ten milliliters of sterilized nutrient broth was used to suspend a sample taken with a sterile loop. The samples were incubated for 72 hours at 30°C [6].

2.1.1. Bacterial Growth in Nutrient Agar

Nutrient Agar was initially used to culture and isolate bacteria. After obtaining distinct colonies, the isolates were further evaluated for proteolytic activity using a selective medium specifically designed for detecting protease activity. Bacteria were isolated by the serial dilution method using nutrient agar. One milliliter of the culture was diluted in nine milliliters of distilled water, the first tube which has dilution of 10^{-1} and serially diluted to 10^{-5}. The spread plate method was then used to place the serial culture dilution on nutrient agar media. Isolates were incubated at 30°C for 48 hours and observed for microbial growth. The differences in colony morphology are then isolated and used streak plate method to obtain a pure colony. The nutrient agar medium was prepared and sterilized, and the different colonies were streaked and incubated at 30°C for 48 hours. The proteolytic activity of the pure colony was further evaluated by growing it in selective medium.

2.2 Bacterial Screening

2.2.1. Bacterial Screening in Skim Milk Agar

Proteolytic activity is obtained by screening bacteria on Skim milk agar medium (2,5 % w/v) in 100 milliliters of distilled water. A loopful bacteria from each isolate were transferred to skim milk agar medium (SMA). The bacteria were placed in the center of a petri dish containing sterile SMA (the point technique) then incubated at 28°C for 48 hours. After the incubation period, the formation of the halo zone was measured. The halo zone indicates

proteolytic activity and is an indicator of keratinase enzyme production. The following formula was used to determine the halo zone's diameter:

$$\text{Proteolytic index (\%)} = \frac{\text{Halo zone diameter}}{\text{Colony diameter}}$$

The bacterial isolates that possess the highestProteolytic Index (IP) can proceed to the stage of keratinase fermentation. Proteolytic Index (IP) is a measure that shows the ratio between the diameter of the halo zone to the diameter of the colony [7].

2.2.2. Keratinolytic Bacteria Screening

The screening of keratinolytic bacteria was carried out according to Nnolim, Okoh, et al. (2020). Based on skimmed milk agar plates, bacteial strains exhibiting proteolytic activity were cultured in a basal medium supplemented with 10-gram whole chicken feathers as the only source of carbon and nitrogen, and contained (g/L) KH2PO4, 0.4; K2HPO4, 0.3; CaCl2, 0.22; MgCl2, 0.2. Prior to sterilization using autoclave at 121 °C for 15 minutes, the initial pH of the medium was corrected to 8.0. The submerged fermentation was conducted in triplicate using 125 mL Erlenmeyer flasks filled with 50 mL of working media. The fermentation was carried out for 96 hours at a temperature of 30 °C, with agitation provided by an orbital shaker set at 130 revolutions per minute. A fresh inoculum for the submerged cultivation was prepared using a bacterial suspension with a concentration of 2% (v/v). Following incubation, flasks containing chicken feathers that had undergone either complete or significant breakdown were chosen for subsequent investigations, and the corresponding culture broths were further analyzed. The cultures underwent filtration, and the remaining chicken feathers were utilized to calculate the percentage of degradation. Afterwards, the liquid above the cultured cells was utilized to measure the amount of keratinase activity. The bacterial strains with significant keratin degradation ability were preserved on chicken feather powder (CFP) agar slants at 4 °C to create fresh inoculum, and in 20% glycerol at -86 °C for long-term storage [8].

2.2.3. Percentage of Keratin Degradation

The degradation of chicken feathers by isolated bacterial strains was assessed using the feather weight loss method Ramakrishna Reddy et al. [9]. Unused feathers were collected by passing the culture broth through a Whatman no. 1 filter paper. Subsequently, the leftovers were rinsed with distilled water to eliminate bacterial cells, and subsequently dehydrated in an oven at 60 °C for 24 hours until a consistent weight was attained [9]. The dry weight of the remaining feathers was measured, and the deterioration % was computed using the following formula.

$$\text{Percentage feather degradation (\%)} = 1 - (RF/WF)) \times 100$$

Where RF = dry weight of residual feathers after fermentation; WF = dry weight of intact feathers before fermentation.

2.2.4. Keratinase Enzyme Activity Test

The method described by Mechri et al. (2017) was slightly modified, specifically related to the pH of buffer, to measure the keratinase activity. In a nutshell, the reaction mixture was composed of 0.5 mL of 10 g/L keratin azure (Sigma Aldrich, USA) in a 100 mM Phosphate buffer (pH 8.0) and 0.5 mL of diluted crude enzyme preparation. The mixture was shaken

(220 rpm) and incubated for one hour at 37 °C. To stop the reaction, the reaction mixture was then put on ice for ten minutes. By centrifuging at 15,000 g for 10 min and filtering through Millipore cellulose filters (0.45 mm), the leftover substrate was eliminated. Using a Perkin Elmer Lambda25 Spectrophotometer, the absorbance of the filtrate at 595 nm was used to calculate the free azo dye. The mixture in the control had the enzyme and buffer but not the substrate; otherwise, the procedure was the identical. According to the assay procedure provided, one unit of keratinase was defined as the amount of enzyme that caused an increase in absorbance of 0.01 at 595 nm per hour [10].

3 Results & Discussion

3.1. Bacterial Isolation

A total of 12 bacterial isolates were collected salt preservation hide skin. Figure 1 presents the isolation results. The isolate shows that there are variations in the colonies observed. Isolation basically is to separate a single microbe from many microorganisms that are formed from a complex mixture [11]. The isolation results for codes 2, 8, 9, 16, and 18 show a similar tendency for irregular colonies. The morphology of these bacterial colonies with codes 6, 7, and 15 was identical, consisting of a combination of spherical and irregular. The isolation coded 1 and 14 show irregular and filamentous bacterial colonies. Isolation results with code 3 show round morphology, whereas code 4 shows a combination of round and rhizoid colonies. Each of the types of bacterial colonies observed were re-selected for the purpose of determining the protease activity. This was done as a preliminary step in identifying the keratinase enzyme in the isolate. The keratinase enzyme is a protease enzyme that has keratinolytic activity [12] and thus can be defined as a specific group of proteolytic enzymes that demonstrate the capacity to degrade insoluble keratin substrates [5,13].

Fig. 1. Bacterial isolation from hide salt preservation in warehouse Politeknik ATK Yogyakarta

3.2. Bacterial Screening in Skim Milk Agar

The results of bacterial inoculation on SMA media are shown in Figure 2, and the percentage of halo zone can be seen in Table 1. The halo zone on SMA was the result of the protease enzyme's activity. The formation of a halo zone on skim milk media characterizes bacterial growth. Extracellular enzymes hydrolyze proteins in skim milk media into amino acids, which cells directly use as a source of nutrition, leading to the formation of halo zones [14]. Casein in skim milk contains phosphoproteins, which then bind to calcium to form calcium caseinate, giving it a white color. Bacteria produce the protease enzyme, which hydrolyzes casein into soluble amino acids, causing the white color to disappear and forming a halo zone around the bacterial colony [15]. Isolates with bacterial growth that form halo zones are categorized as protease enzymes that have proteolytic activity. The halo zone was formed, and the percentage was then calculated. Table 1 presents the halo zone percentage results. The highest halo zone percentage result is in isolate no. 3 with a percentage of 37.8%, while the lowest percentage is in isolate no. 18 with a percentage of 16.8%. Based on the halo zone percentage test, there are 8 isolates that have proteolytic properties, which are characterized by the presence of halo zones, while 4 other isolates have halo zones. Of the 8 isolates, there are 5 that have the highest percentage, namely isolates 3, 4, 6, 8, and 14. The five bacteria will continue keratinolytic selection.

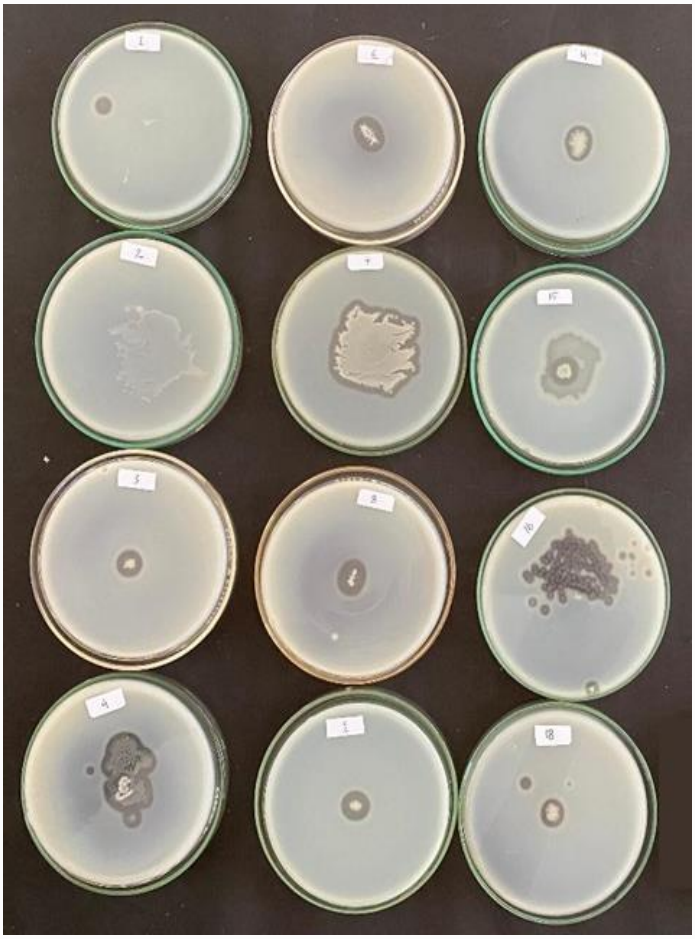

Fig.2. Isolation of bacteria on SMA media

Table 1. Isolate description and halo zone percentage

Isolate code	Halo zone percentage (%)	Protease enzyme	Description
ATK1	No Halo zone	-	No halo zone was formed, there is contamination in SMA media. Isolate 1 is not categorized as a protease enzyme.
ATK2	No Halo zone	-	No halo zone was formed. Isolate 2 has bacterial growth, but it does not form a halo zone. Isolate 2 cannot be categorized as a protease enzyme.
ATK3	37,8	+	A halo zone was formed. Isolate 3 is categorized on protease enzyme
ATK4	24,5	+	The formation of a halo zone was characterized by its irregular shape and the presence of several bacterial colonies with varying shapes. Isolate 4 is categorized by protease enzymes.
ATK6	34,5	+	Formed a halo zone, bacterial colonies, bacterial colonies are different from isolate 3. Isolate 6 is categorized on protease enzymes
ATK7	18,7	+	A halo zone was formed., the halo zone area is smaller than the bacterial growth. Bacterial colonies that grow are irregular in shape. Isolate 7 is categorized on protease enzyme
ATK8	35,7	+	A halo zone was formed. Isolate 8 is categorized on protease enzyme
ATK9	32,2	+	A halo zone was formed. Isolate 9 is categorized on protease enzyme
ATK14	34,6	+	A halo zone was formed. Isolate 14 is categorized on protease enzyme
ATK15	22,3	+	A halo zone was formed. There are two layers of bacterial growth, isolate 15 is still categorized in protease enzymes
ATK16	No Halo zone	-	There was bacterial growth, and halo zone formed. However, the halo zone formed is contaminated. So that isolate 16 is not categorized as a protease enzyme
ATK18	16,8	+	A halo zone was formed, although the media area around the halo zone was contaminated. Isolate 18 is categorized in protease enzymes.

3.3. Bacterial Screening in Keratin Degradation

Keratinolytic selection aims to determine the ability of specific bacteria capable of degrading keratin [16]. Based on the research results, which are shown in Figure 3, it shows that there is a change in the shape of the simple keratin substrate in chicken feathers into soft granules and most of it has dissolved in water after fermentation by an inoculant of bacterial isolates

for 96 hours. According to Mousavi et al. (2013), this is due to the activity of the keratinase enzyme produced by keratinolytic bacteria, so it is able to degrade keratin [17].

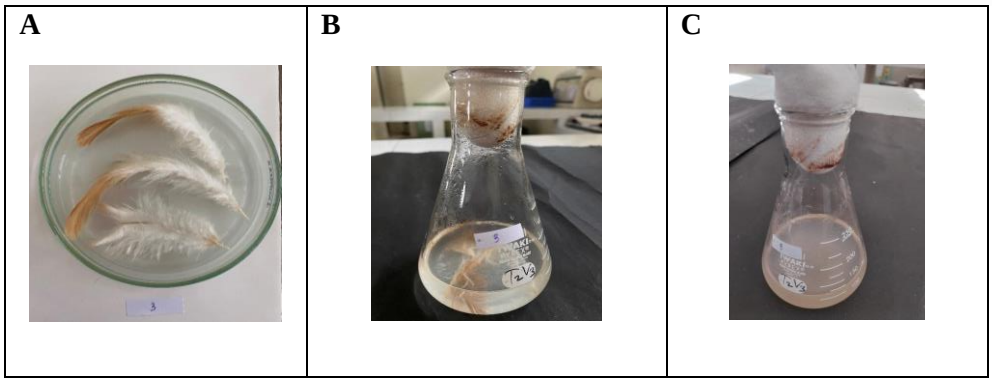

Fig.3. Screening process of keratinolytic bacteria. A) Keratin source (chicken feather); B) Medium of keratinase enzyme; C) Result of fermentation of bacteria isolate using chicken feather as substrate

The keratinase enzyme is specifically able to destroy the peptide bonds that normally make keratin very hard and resistant to degradation. This process helps break down the stiff keratin structure to make it softer and easier to break down. This is in accordance with research conducted by Nnolim et al. (2020) and Sivakumar et al. (2019). They stated that changes in the shape and properties of keratin due to keratinolytic properties were caused by the keratinase enzyme from bacteria which succeeded in breaking down the α-keratin helices in the structure of chicken feathers into a form that was more amorphous and easily broken down [8,18].

The degradation process by keratinase can increase the solubility of keratin which was previously difficult to dissolve to become more soluble in the enzymatic solution. This indicates that the keratinase enzyme effectively changes the physicochemical properties of chicken feather keratin [19]. The enzyme degradation percentage test is a test to determine the strength of the keratinase enzyme in degrading keratin quantitatively.

Five bacterial isolates from proteolytic selection were tested for the percentage of keratin degradation, which is shown in Table 2 Based on the test results, it shows that the percentage of bacterial degradation ranges from 26.83 ± 7.88 to 74.48 ± 3.93%. Isolate code ATK3 has the best keratin degradation value. This value is almost the same as research by Nnolim et al. (2020). They degraded keratin by 74.5 ± 2.12 % after fermentation with Arthrobacter sp bacteria. KA4-2 [8].

Tabel 2 Percentage of keratin degradation from isolated bacteria

Isolate Code	Percentage of Keratin Degradation (%)
ATK3	74,48 ± 3,93
ATK4	26,83 ± 7,88
ATK6	54,35 ± 2,00
ATK8	45,87 ± 5,91
ATK14	41,81 ± 4,84

3.4. Keratinase Enzyme Activity

Based on the data in Table 3, it shows that code 3 bacteria have the highest keratinase enzyme activity of 3.56 ± 0.61 U/ml, while code 6 bacteria have the lowest activity of 1.28 ± 0.64 U/ml. This difference shows that the bacteria tested have different abilities in producing keratinase enzymes with varying levels of activity.

Table 3. Activity of keratinase enzyme

Isolate code	Activity of keratinase enzyme (U/ml)
ATK3	3,56 ± 0,61
ATK4	2,17 ± 0,66
ATK6	1,29 ± 0,64
ATK8	2,68 ± 0,62
ATK14	1,58 ± 0,30

Differences in keratinase enzyme activity between bacteria can be influenced by various factors such as the type of bacterial species, genetic expression of the keratinase enzyme, environmental conditions, and enzyme regulatory mechanisms. Further analysis can be carried out to understand the factors that influence keratinase enzyme activity in each type of bacteria [20]. The discovery that code ATK3 bacteria have the highest keratinase enzyme activity has important implications for biotechnological applications in the leather tanning process.

4 Conclusions

A total of eleven isolates were identified from warehouse of skin in Politeknik ATK Yogyakarta, with six of them exhibiting halo zones during proteolytic activity test. Bacteria ATK3 exhibited the highest percentage of keratin breakdown and keratinase activity in the experiments. According to the study, the bacteria isolate ATK3 showed the highest promise for further investigation as a producer of keratinase enzymes.

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