

Isolation And Characterization Of Keratinase-Producing Microbes From Chicken Slaughterhouse Waste Soil With Double Enrichment And Initial Characterization Of Keratinase

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Abstract: Every year, there is an increase in chicken meat consumption. The high consumption of chicken meat results in significant waste from chicken slaughter, including chicken feathers. The soil around the chicken slaughterhouse waste disposal has the potential to produce bacteria capable of producing high proteases and keratinases. This research aims to obtain microbial isolates capable of degrading chicken feather waste and to characterize the microbial isolates and the resulting enzymes. The microbes obtained were enriched twice, namely by adding soil with chicken feathers and using a liquid medium supplemented with chicken feathers. This is done by getting bacteria that are really able to degrade keratin from chicken feathers. From the results of double enrichment, 10 isolates were obtained, with MSF6 being the isolate that produces the highest levels of protease and keratinase enzymes, at 761.532 U/mL and 37.733 U/mL, respectively, at 57 °C and pH 8. Molecular identification using the 16S rRNA sequence, MSF6 can be identified as a *Bacillus paramycooides* with a resemblance as great 100%. Based on the results of SDS-PAGE and zymography tests, the protease produced from MSF6 has a molecular weight of 68.181 ± 0.068 kDa and 68.147 ± 0.068 kDa, respectively. The enzyme produced from the MSF6 isolate can be used for animal feed, glue, and other industries that require proteases and keratinases.

1 Introduction

Chicken meat consumption in Indonesia, according to the Central Statistics Agency/Badan Pusat Statistik (BPS) [1], shows a significant increase. This has led to an increase in slaughter waste, including chicken feathers, which contain about 91% protein in the form of keratin [2]. The importance of recycling chicken feathers is constrained by the properties of keratin that are difficult to degrade by other common protease enzymes, due to the presence of strong disulfide bonds [2]. This study highlights the potential of soil microbes, especially *Bacillus* species, in producing keratinase enzymes that can degrade keratin as a solution to treat chicken feather waste [2, 3]. Isolation is carried out using the enrichment method, in which microbes capable of degrading chicken feathers are multiplied. Some isolates have shown high ability to produce

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keratinase enzymes with different specific activities, which were measured using the ANSON method [4], and molecular weight analysis was performed with SDS-PAGE combined with the Zymography method [5].

In this study, microbial isolation measures were carried out by characterizing genotypes and phenotypes, followed by protease and keratinase enzyme activity tests, and identification of the molecular weight of the keratinase produced. The determination of nutritional and environmental factors, such as pH and temperature, contributes significantly to the production of these proteases and keratinases. The results of the study show that optimization under these conditions can increase the production efficiency of protease and keratinase, which have the potential to support the industry in waste management and the utilization of keratin as a source of nutrients on an industrial scale.

2 Materials and Methods

2.1 Materials

The materials used in this research are p.a (pro analysis) grade materials, specifically: pepton, yeast extract, bactoagar, sodium chloride (NaCl), sodium acetate (CH₃COONa), acetic acid (CH₃COOH), potassium hydrogen phosphate (K₂HPO₄), potassium dihydrogen phosphate (KH₂PO₄), hydrochloric acid (HCl), sodium hydroxide (NaOH), trisbase (C₁₄H₁₁NO₃), trichloroacetic acid (TCA), sodium carbonate (Na₂CO₃), potassium chloride (KCl), magnesium sulfate (MgSO₄), Folin-Ciocalteu reagent, crystal violet (C₂₅H₃₀ClN₃), safranin (C₂₀H₁₉ClN₄), 96% alcohol (C₂H₅OH), iodine (I₂), potassium iodide (KI), acrylamide/bis 30%, sodium dodesyl sulfate (SDS), ammonium persulfate (APS), TEMED, laemmli, β-mercaptoethanol, 10x electrophoresis buffer, Triton X-100, and casein (Amresco: E666-500G) as substrates, Coomassie gel staining solution and Coomassie gel destaining solution, except chicken feathers (broiler chickens (*Gallus domesticus*)), skimmed milk, and buffer samples for SDS Page and Zymography.

2.2 Preparation of Sample

Samples were taken from the wasteland of a chicken slaughterhouse in East Java, Indonesia. Soil samples were collected at three points at a depth of 5 cm from the surface, and pH measurements were carried out. Furthermore, stage I enrichment was carried out: soil samples were mixed with chicken feathers at a 1:1 ratio and incubated for ±7 months. During incubation, chicken feathers were washed with water; this is done when the chicken feathers in the soil have been completely degraded (±6 months). A small sample of soil (which is around the chicken feathers) and the incubated chicken feathers are put into a liquid enrichment medium (stage II enrichment) containing (g/100 mL): 0.05% NaCl, 0.1% MgSO₄, 0.05% K₂HPO₄, and 1% chicken feathers. Enrichment stage II: incubate for 10 days at 37°C with agitation at 100 rpm.

2.3 Isolation and Screening of Keratinase-Producing Bacteria

A total of 1 mL of liquid culture mixed microbes from incubation in stage II enrichment was dissolved with 9 mL of NaCl salt. The mixture is diluted in stages using the same saline solution until dilution levels of 10³, 10⁶, 10⁹, and 10¹² are obtained. This series of dilutions is carried out to avoid the accumulation of bacterial colonies during the isolation stage. Each dilution series was taken as much as 100 µL to be aseptically dispersed using a spreader in the LAF on sterile Skim Milk Agar (SMA) medium containing (g/100mL): 5% skim milk, 0.01% MgSO₄, 0.05%

NaCl, and 2% Bactoagar. Bacterial colonies that produce clear zones are bacteria that are capable of producing extracellular proteases. Colonies with a high Proteolytic Index (ratio of colony diameter and clear zone) were selected for further purification by the quadrant streak method. The colony was transferred to fresh SMA medium and scratched into quadrants. Bacterial cultures of 1 ose are then inoculated (scratched) into sterile SMA medium. The medium that contains the sample is incubated at a temperature of 37 °C for 24 hours. The colony is then purified by means of quadrant streaks on the SMA medium until a pure single colony is produced.

2.4 Optimization Enzymes Production and Enzyme Activity

The activity of protease and keratinase enzymes was measured using the modified ANSON method [4]. The substrates used are 1% casein (b/v) (protease enzyme) and keratin powder (keratinase enzyme). The resulting crude extract is taken as much as 2 mL and centrifuged. 0.02 mL supernatant was added with 0.2 mL buffer (pH buffer optimization variation of 5-10 was performed), and 0.5 mL of 1% casein. The mixture is vortexed until homogeneous and incubated at a temperature variation of 37-77 °C in a heat box for 5 minutes. 1 mL of 1% TCA is added to the mixture to stop the enzymatic reaction, then the mixture is vortexed and incubated for 10 minutes. The mixture is centrifuged at 10,000 rpm at 4 °C for 10 minutes. The supernatant was further added with 2.5 mL of 0.5 M Na₂CO₃ solution and 0.5 mL of Folin-Ciocalteu reagent, which was further incubated in a dark room for 30 minutes. The result is measured in absorbance at a wavelength of 660 nm.

2.5 Genomic identification of bacterial cells

This identification was carried out after obtaining a single colony that had been pure and then genotypically identified at Macrogen Inc., South Korea. This identification is carried out in 4 stages, namely total DNA isolation, sequencing using primers 785F 5'GGA TTA GAT ACC CTG GTA3', and 907R 5'CCG TCA ATT CMT TTR AGT TT3', and data analysis using bioinformatics applications. The test was carried out to determine whether the species of the isolated bacteria had kinship with other bacteria. The results of the sequences obtained are then analyzed with Bioedit by inserting sequences that use forward and reverse primers, then a consensus sequence is sought so that a total sequence of bacteria is produced. The resulting sequences were analyzed using the Nucleotide Basic Local Alignment Search Tool (BLASTN) program available on the National Library of Medicine (NCBI) website. Comparatively, the sequence data were processed to obtain phylogenetic trees.

2.6 Determination of Enzyme Molecular Weight with SDS-PAGE Combined with Zymography

The determination of protein molecular weight is carried out through SDS-PAGE electrophoresis, referring to the standard procedure of Bio-Rad Laboratories (2020). Before analysis, a crude extract of extracellular protein was concentrated using a Microcon device to achieve optimal protein concentrations. Furthermore, the protein samples in Microcon were washed with a Tris-HCl pH 8.0 buffer to remove salt contaminants that could interfere with electrophoretic separation. Further analysis by the zymographic technique was performed to characterize enzyme activity, whereby the SDS-PAGE gel was incubated under renaturation conditions with a specific substrate, allowing visualization of enzyme activity based on the hydrolysis zone formed. This integrated approach not only allows for accurate determination of molecular weights but also provides functional confirmation regarding the biological activity of the protein being analyzed.

3 Results and Discussion

3.1 Enrichment, Isolation, and Selection of Chicken Feather Degrading Bacteria from Chicken Slaughterhouse Waste Soil

The isolation of keratinase-producing bacteria from the waste soil of the chicken slaughterhouse aims to explore bacteria that are able to degrade chicken feathers optimally. Sampling was carried out by taking soil at 3 points of waste disposal locations from the chicken slaughterhouse at a depth of 5 cm from the ground level. The wastewater soil sample was then checked for soil pH, and the wastewater soil pH was 6.17. Chicken blood has a pH range of 5.81-6.30 [6]. Enrichment is one way to increase the bacteria that are specifically needed. Soil samples were enriched in two stages. Enrichment phase I, by adding chicken feathers and a little water (so that the soil does not dry out). The sample is added with chicken feathers periodically and incubated for 7 months. Every month, soil samples are checked, and added with chicken feathers and a little water are added if the chicken feathers have degraded. Figure 1a shows the condition of the soil after the addition of chicken feathers and water, and Figure 1b shows the degradation of chicken feathers.

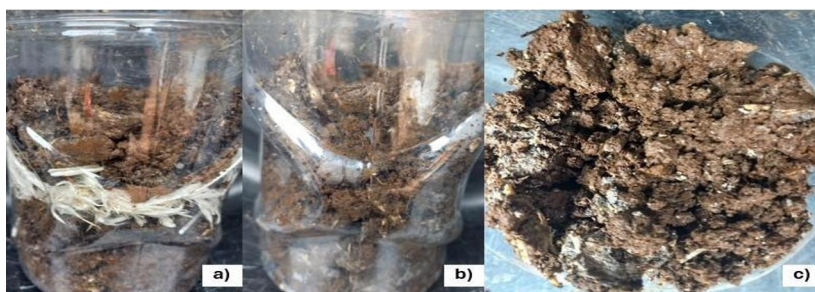


Fig 1. a) Mixture of chicken feathers and water to soil samples, b) Soil samples with degraded chicken feathers (side view), and c) Soil samples with degraded chicken feathers (top view)

Stage II enrichment is carried out in liquid chicken feather medium. Soil from the enrichment phase I, taken from close to degraded chicken feathers were put in the stage II enrichment medium aseptically. The mixture of this solution is then incubated in an incubator shaker for 10 days, with the temperature set at 37 °C at a speed of 100 rpm. Figure 2 shows the addition of soil samples from stage I enrichment that were put into liquid enrichment medium. There is a process of degradation of chicken feathers, which can be seen in Figure 2c.

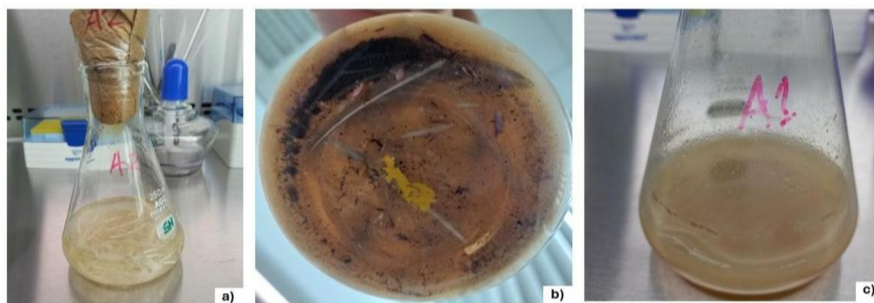


Fig 2. Second enrichment method: a) sterile liquid enrichment medium II, b) medium containing soil and chicken feather from the first enrichment medium, and c) chicken feather degradation after 10 days of incubation

3.2 Isolation of Enriched Keratinase-Producing Bacteria

The process of isolating protease bacteria from slaughterhouse waste begins with selection using SMA medium, followed by isolation and purification through the spread plate technique in multi-stage dilution. Dilution of 10^{12} was identified as the optimal condition for obtaining a single colony, where eleven colonies showing a high Proteolytic Index (PI) as an indicator of casein hydrolysis ability were further purified by the quadrant streak method. Based on this procedure, ten potential isolates (MSF1-MSF7 and MSF9-MSF11) were successfully obtained for advanced characterization, while one isolates (MSF8) were excluded as it showed sub-agar growth. Table 1 shows the IPs of the 10 colonies, and the isolates are named MSF1, MSF2, MSF3, MSF4, MSF5, MSF6, MSF7, MSF9, MSF10, and MSF11. Where the MSF code itself comes from the word Malang Soil Feather.

Table 1. Proteolytic index data isolated from wastewater soil

Code of Isolate	PI	Code of Isolate	PI
MSF1	3.50	MSF6	2.88
MSF2	10.00	MSF7	5.29
MSF3	3.52	MSF9	1.60
MSF4	2.82	MSF10	3.75
MSF5	3.13	MSF11	1.77

3.2.1 Purification of Bacteria by the Quadrant Streak Method

Purification of bacterial isolates is confirmed by the quadrant streak method, the homogeneity of which is verified based on the uniformity of the colony morphology and the Gram staining characteristics. The results of Gram staining were obtained with nine isolate codes (MSF1, MSF2, MSF4, MSF5, MSF6, MSF7, MSF9, MSF10, and MSF11) as Gram-negative (red), while one isolate (MSF3) was Gram-positive (purple color). This difference is due to variations in the structure of the cell wall, where Gram-positive bacteria have a thick peptidoglycan layer that retains the violet crystal complex, while the thin peptidoglycan layer in Gram-negative bacteria causes the dye to wash away [7]. Morphologically, all isolates exhibit a bacilli shape, with MSF6 isolates exhibiting unique characteristics that indicate the presence of spore-like structures within their cells. Figure 3 shows the MSF6 isolates resulted from Gram staining.

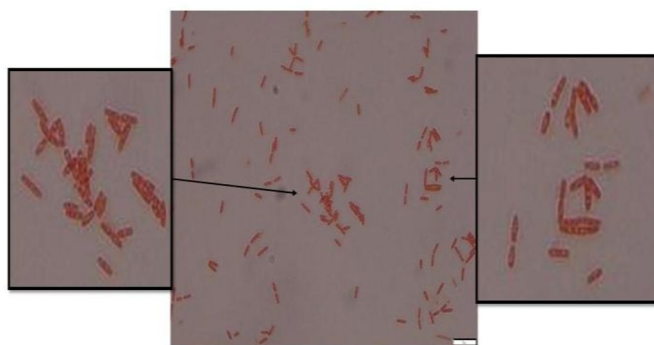


Fig 3. Gram staining result of MSF6 isolate

3.2.2 Isolate's Ability to Degrade Casein and Chicken Feathers

The highest keratinase-producing isolates were further screened to degrade the chicken feather and casein substrate. Keratinase activity was measured using keratin as a substrate. At the time of measurement, keratin has not been extracted from chicken feathers, which is used as a substrate

in the keratinase activity test. Keratinase has protease and disulfide reductase [8]. If the cell is grown using chicken feathers as the only source of carbon and nitrogen, then the cell produces keratinase, which will degrade the keratin in the chicken feathers and be used for energy needs and the material that makes up the cell. This can be evidenced by the degradation of chicken feathers during the fermentation process, as shown in Figure 4. Chicken feathers, especially the rachis (stem) part, are difficult to degrade because the rachis part contains more keratin in the form of a β -sheet [8, 9].



Fig 4. Degradation of chicken feathers by MSF6 isolate: a) Production medium, b) Production medium after 3 days of fermentation, and c) Dried leftovers of degraded chicken feathers.

Figure 5 shows the development of the isolate that grows in the skim milk medium. In the skim milk medium, which is initially yellowish-white in color, becomes orange after incubation for 1 day. Incubation using skimmed milk medium cannot be done for 3 days, like in chicken feather production medium, because on the 3rd day, the bacterial isolate has run out of nutrient sources, so that the activity produced is low or non-existent.



Fig 5: a) Sterile skimmed milk medium, and b) Skimmed milk medium that has been degraded by bacterial isolate

Table 2 shows that not all bacterial isolates that have been selected at the beginning can degrade chicken feathers, and the protease activity produced is different when grown on carbon

and nitrogen sources in the form of casein and chicken feathers. Interestingly, IP (in Table 1) does not guarantee high protease activity when grown in Liquid Skimmed Milk (LSM) medium. Table 2 shows no linear relationship between the activity of proteases grown in LSM medium and chicken feathers. The protease activity when the isolate is grown in the chicken feather medium is most likely the protease activity of keratinase.

Table 2. Degradation ability of chicken feathers, protease activity fermented using casein and feather substrates.

Code	%Degradation	Protease Activity with Skimmed Milk Medium (U/mL)	Protease Activity with Chicken Feather Medium (U/mL)
MSF1	22,03	4.680	363,257
MSF2	22,12	4.357	219,310
MSF3	21,17	0	222,860
MSF4	11,79	58.902	0
MSF5	13,83	0	0
MSF6	26,38	159.601	761,532
MSF7	13,39	4.357	0
MSF9	18,09	186.066	170,768
MSF10	20,74	22.754	391,013
MSF11	19,62	689.558	213,500

Protease activity was produced in skimmed milk medium using MSF11> MSF9> MSF6> MSF4> MSF10> MSF1> (MSF2 = MSF7) > (MSF3 = MSF5) isolates. MSF11 isolate has a high activity of 689.558 U/mL. Thus, the MSF11 isolate can be used to obtain high levels of protease activity. Degradation of chicken feathers by isolates was comparable to the results of protease activity tests using MSF6> (MSF2 = MSF1 = MSF3 = MSF10)> MSF11> MSF9> (MSF5 = MSF7 = MSF4). To confirm this, it is necessary to test keratinase activity using a keratin substrate. However, based on Table 2 data, the MSF6 isolate was selected for further analysis due to its highest degradation ability and the highest protease activity of the enzyme produced with chicken feather substrates.

3.2.3 Identification of Genetically Isolated Species

The results of the genotype test obtained from Macrogen Korea are in the form of nucleic acid sequences that encode 16s rRNA (sequence). The MSF6 isolate sequence was identified as "*Bacillus paramycoides*" with a 100% degree of similarity through 16S rRNA gene sequencing and BLAST analysis, following an established molecular workflow that is important for taxonomic precision. This process begins with the initial isolation and culture of bacterial colonies from environmental samples, a matrix that is often rich in diverse microbial populations relevant to biodegradation [10]. Genomic DNA is then carefully extracted from the purified bacterial isolate, ensuring the quality and high yields that are essential for subsequent amplification [10]. The 16S rRNA gene, a conserved but varied genetic marker found everywhere in bacteria, is amplified using Polymerase Chain Reaction (PCR) with a universal primer that targets a species-specific variable region [13]. After successful amplification, the PCR product undergoes Sanger sequencing to determine the sequence of its nucleotides [13].

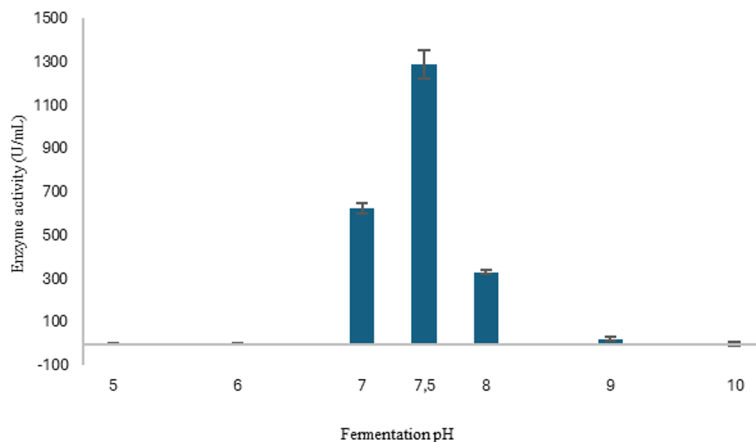


Fig 7. Effect of production pH on protease activity

Changes in the pH of the environment can affect the structure of enzymes, which greatly affects the enzyme activity produced later. Changes in enzyme structure will affect the performance of the enzyme, resulting in a decrease in metabolic rate, which leads to a decrease in keratinase production [11]. Within a narrow range of pH changes, structural and morphological changes of enzymes may be reversible, but if the pH changes are very extreme, as in Figure 7, at pH 5, pH 6, and pH 9, the production of keratinase is very low, indicating a slowdown in cell growth. The optimum activity was obtained at pH 7.5 with a protease activity of 1288.907 U/mL. Isolate growth is very sensitive to changes in environmental pH, where at pH 7 production decreases by 52%, while at pH 8 it decreases by 75%. Thus, the MSF6 isolate has different properties from those of the likely new strain of *Bacillus paramycooides*.

3.3.2 Effect of Fermentation Temperature on Enzyme Production

Each bacterium has an optimal temperature for growth based on its growing environment and the type of bacteria [2]. Figure 8 shows that the growth of the MSF6 isolate can occur at temperatures of 27-37 °C. At a temperature of 37 °C, the enzyme activity was 769.278 U/mL.

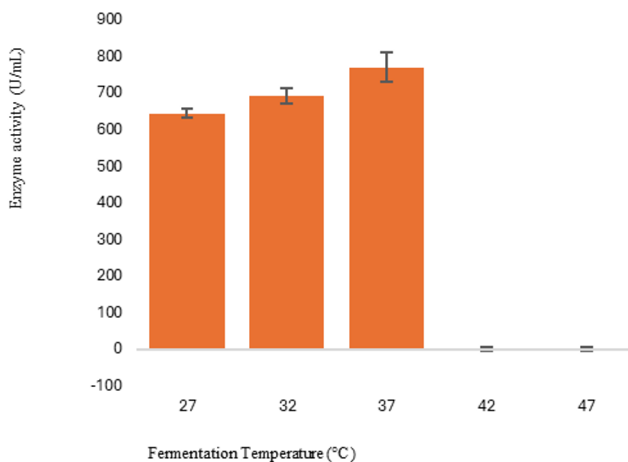


Fig 8. Effect of production temperature on protease enzyme activity

At this optimum temperature, the work of enzymes and metabolic processes runs most efficiently. At temperatures below 37 °C, enzyme activity decreases, and metabolic processes slow down [12], resulting in decreased keratinase production. Meanwhile, at temperatures above 37 °C, proteins undergo denaturation, which results in the disruption of cell metabolism. At 42 °C, the production of keratinase dropped by almost 100%, which indicates that the isolates are sensitive to temperatures above their optimal temperature. At 32 °C, there was a decrease in activity by 10%, while at 27 °C, it decreased by 16.5%.

3.4 Optimization of Enzymes Activity

3.4.1 Effect of Temperature on Keratinase and Protease Activity

Figure 9 shows the activity curve of keratinase and protease at various keratinase temperatures using the tris-HCl pH 8 buffer. Activity using powdered keratin and casein both showed optimum activity at a temperature of 57 °C. Keratin powder is a substrate to measure the activity of keratinase enzymes, and casein is a substrate to measure the activity of protease enzymes.

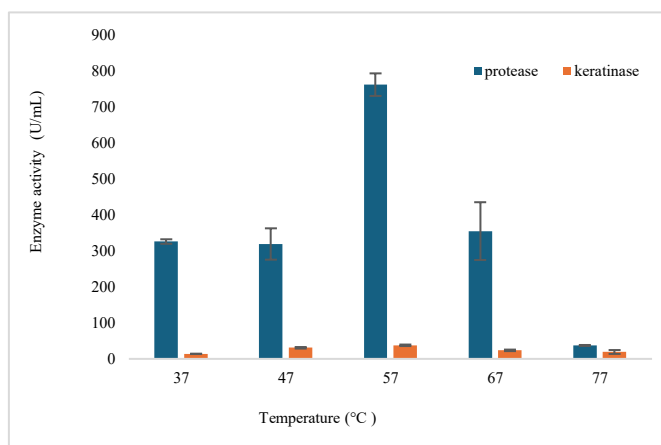


Fig 9. Effect of temperature on protease and keratinase enzyme activity of MSF6 isolate

However, protease activity above 57 °C is greater than that of keratinase activity, as well as below 57 °C. The keratin substrate, dominated by the helical structure, may stabilize the 3D structure of the keratinase, whereas the globular casein structure is not able to stabilize the 3D structure of the keratinase [2, 12].

3.4.2 Effect of pH on Keratinase and Protease Activity

The structure of the enzyme can be affected by the change in pH because the side protonation of the protein is affected by the pH, the change in pH is able to change the conformation of the protein in the enzyme [13]. Each enzyme has a different optimal pH; if the pH used is not at its optimal condition, it can cause enzymes to be denatured [13]. Figure 10 shows that both keratinase and protease activity have an optimal pH at pH 8, with protease activity of 505.267 U/mL and keratinase activity of 37.373 U/mL. The enzyme is relatively stable at alkaline conditions, although its activity is lower than at pH 8; thus, it can be concluded that the keratinase produced by MSF6 isolate is an alkaline protease group. Keratinase activity drops drastically at pH below 7. Both the pH and the optimum temperature of keratinase differ from the pH and optimum temperature of keratin fermentation.

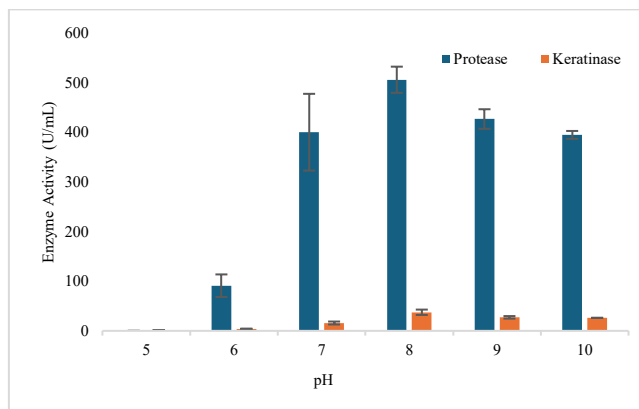


Fig 10. Effect of pH on protease and keratinase activity

3.5 Determination of Protein Molecular Weight and Protease Molecular Weight with SDS PAGE and Zymography

Determination of protein molecular weight can be done using SDS-PAGE, and bands that have protease enzyme activity are confirmed by zymography. Extracellular proteins obtained from the MSF6 isolate were compared with Promega Markers as standard protein markers. The sample and marker are loaded into the same volume of 10 μ L. The determination of the MW protein can be obtained from the standard protein bands, which are then included in the Log MW (Molecular Weight) vs. relative migration distance (R) graph plotted, based on the values obtained for the bands in the MW standard [14]. The molecular weight of proteins can be calculated by separating the proteins in the acrylamide gel through the process of electrophoresis. The final result of protein separation is blue protein bands with colorless gels. The protein band is blue, as shown in Figure 11, it shows the distance of the migration of the separated proteins. Before calculating the molecular weight, the standard protein migration is calculated first, resulting in the obtained regression equation, which is $y = 0.4209x + 5.5844$. The protein migration distance was calculated by interpolating into the equation obtained, so that the molecular weight of extracellular proteins was 68.181 ± 0.07 kDa. (Figure 11) Well numbers 2-8 show a blue band (faintly visible), which is due to the lack of concentration of the protein sample obtained [14, 15]. The protein band obtained needs to be confirmed to have protease enzyme activity. Therefore, it is necessary to confirm the presence or absence of protease activity produced by MSF6 isolates.

Zymography can be used to analyze the activity of an enzyme by adding a substrate to the gel. In addition, it can also be used to confirm protein bands from SDS PAGE to degrade substrates on gel separation in zymography [15]. Positive results from enzyme degradation on zymography indicate the presence of clear bands. Figure 12 shows the formation of a faint, clear band between No. 2 and No. 8. The observable clear band has a MW of 68.147 ± 0.07 kDa, which is included in the equation $y = -0.3762x + 5.4944$. There is a buildup of enzymes on top of the gel [14]. This is due to the concentration of enzymes being too high, leading to aggregation and large protein sizes [14]. The dilution factor reduces the aggregate, so it is necessary to optimize zymography to obtain a single band.

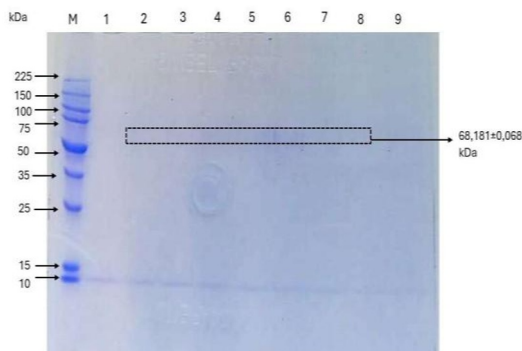


Fig 11. SDS-PAGE results from crude extract of MSF6 isolate, remarks; markers (M) and No. 2-10 are extracellular protein samples: Tris-HCl buffer pH 8 (No. 2 (1:0); No. 3 (9:1); No. 4 (8:2); No. 5 (7:3); No. 6 (5:5); No. 7 (4:6); No. 8 (3:7); No. 9 (1:9))

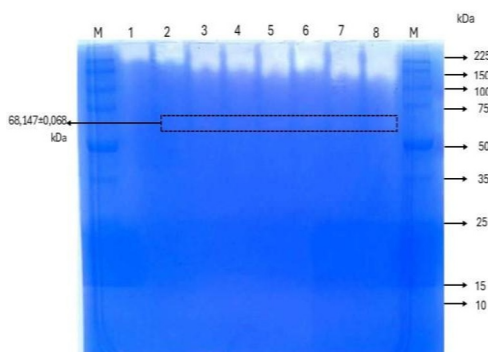


Fig 12. Zymographic results from MSF6 isolate crude extract, description: M = marker, as well as sample comparison: Tris-HCl buffer pH 8, which is 1) 20:0; 2) 18:2; 3) 16:4; 4) 14:6; 5) 12:8; 6) 1:1; 7) 8:12; and 8) 6:14

4 Conclusion

From the study, ten keratinase-producing microbial isolates were obtained from soil from the land where the Chicken Slaughterhouse was disposed of, with two stages of enrichment, and isolates that have keratinase enzyme activity and high ability to degrade chicken feathers, with the code MSF6 isolate. The isolate obtained is Gram-negative except for the MSF3 isolate, which is Gram-positive. All ten isolates are in the form of bacillus (rod-shape), and the bacterial species obtained in the MSF6 isolate have a similar kinship to *Bacillus paramycooides*. The optimum conditions during the enzyme production process were obtained at pH 7.5 with enzyme activity of 1288.907 U/mL, and the optimum temperature at 37°C with enzyme activity of 769.278 U/mL. The optimal condition of keratinase enzyme using a substrate in the form of chicken feather powder was obtained at pH 8 with an enzyme activity of 37.373 U/mL and an optimal temperature of 57°C with an enzyme activity of 37.733 U/mL. The optimum condition of protease enzymes using a substrate in the form of casein was obtained at pH 8 with enzyme activity of 505.267 U/mL and optimal temperature at 57°C with enzyme activity of 761.532 U/mL. The molecular weight of extracellular proteases using the SDS-PAGE method combined with the zymography method had almost the same MW of 68.181 ± 0.07 kDa and 68.147 ± 0.07 kDa.

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