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Culture optimization for keratinase production by thermophilic bacillus licheniformis AAL3 in keratin waste biodegradation

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ABSTRACT

Keratin waste is highly recalcitrant due to its complex protein structure, requiring efficient biodegradation strategies. This study aimed to evaluate and optimize keratinase production of thermophilic *Bacillus licheniformis* AAL3 isolated from the Air Putih hot spring, Lebong, Bengkulu. Qualitative keratinolytic activity was determined using feather meal agar, while quantitative enzyme activity was measured spectrophotometrically based on tyrosine release. The isolate exhibited a higher keratinolytic index (5.0) compared to *B. licheniformis* SCL2 (1.5). Quantitative analysis showed that AAL3 produced higher cell density (8.9×10^8 CFU/mL) and keratinase activity (1.0074 U/mL). Optimization studies revealed that maximum keratinase production occurred at 60 °C and pH 9. Among the tested carbon sources, 1% glucose resulted in the highest cell growth and enzyme activity, whereas 0.4% peptone was the most effective nitrogen source. The results demonstrate a strong positive correlation between bacterial growth and keratinase production. These findings indicate that *B. licheniformis* AAL3 has significant potential as a thermophilic keratinase producer for biotechnological applications, particularly in keratin waste biodegradation.

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INTRODUCTION

Keratin is a protein found in the skin, hair, and nails of humans and animals. Keratin is one of the most abundant proteins in nature and plays an important role in maintaining the structure and function of these tissues. Keratin consists of long and folded polypeptide chains, which form alpha (α-helix) and beta (β-sheet) structures (Vidmar & Vodovnik, 2018). Keratinolytic

microorganisms can break down keratin into peptides and amino acids, some of which are metabolized as carbon and nitrogen sources. Keratinase can be used to process textiles made from keratin, such as wool and silk, can remove excess keratin from skin and hair, and can be used in the development of drugs to treat keratin-related diseases(Li, 2021). In addition, this enzyme also has potential in processing waste containing keratin, such as textile and livestock industry waste (Vidmar & Vodovnik, 2018).

Thermophilic bacteria are a type of microorganism that can survive and thrive in extreme temperature conditions, generally between 45 °C and 80 °C. The keratinase enzyme produced by thermophilic bacteria has a major advantage in its stability at high temperatures. Additionally, thermophilic bacteria also have a high adaptability to the presence of toxins (Peng et al., 2019). This makes thermophilic bacteria more resistant to various hazardous compounds commonly found in industrial waste. Additionally, the efficient metabolic capability can accelerate the process of keratin biodegradation in a shorter time. Keratin waste, such as chicken feathers, is a significant environmental problem due to its difficult-to-decompose nature (Gerlicz et al., 2024). By using thermophilic bacteria, keratin waste can be transformed into more beneficial products, such as fertilizers, animal feed, and play a role in pharmaceutical and cosmetic industrial materials, as well as the leather and textile industries (de Menezes et al., 2021).

Thermophilic bacteria exhibit a variety of optimal conditions for keratinase enzyme activity (Sharma et al., 2022). Previous research by (Yohandini et al., 2015) showed that two thermophilic bacterial isolates from the hot springs of Tanjung Sakti (South Sumatra) exhibited qualitative keratinolytic activity, namely *Bacillus thermoamylovorans* and *Brevibacillus* sp., and had the same optimum temperature and pH, specifically at 70 °C and pH 7 with an incubation period of 28 hours, as well as the best carbon and nitrogen sources being 1% glucose and 0.4% casein. Another study by Koentjoro et al. (2018) reported that the bacterium *Bacillus* SL II-I grew optimally on chicken feather meal media with the addition of 1% peptone, and at pH 7, with the highest keratinase activity reaching 3.4 Units/ml. The capacity of bacteria to produce keratinase enzymes varies according to their type and environmental factors, such as temperature, pH, and nutrients like carbon and nitrogen present in the growth medium (Sharma et al., 2022).

The novelty of this study lies in the first quantitative analysis and culture optimization of keratinase production by *Bacillus licheniformis* AAL3, a thermophilic bacterium isolated from the hot springs of Air Putih Lebong, Bengkulu, which has previously been identified as a keratinolytic isolate based on the formation of clear zones around its colonies. However, quantitative data regarding the keratinase production capability of this isolate is still unavailable, thus its applicative potential in biotechnology and industry has yet to be maximized. Therefore, this study aims to optimize culture conditions, including temperature, pH, as well as carbon and nitrogen sources, to achieve optimal and quantitatively measurable keratinase production.

RESEARCH METHODS

Research Design

This study employed a laboratory-based experimental design to optimize keratinase production by *Bacillus licheniformis* AAL3 isolated from the Air Putih hot spring, Lebong, Bengkulu. The research was conducted through a stepwise optimization approach involving temperature (50–65 °C), pH (6–9), and different carbon (glucose, sucrose, galactose) and nitrogen sources (peptone, yeast extract, casein) under controlled conditions. Keratinolytic activity was evaluated qualitatively using the clear zone method and quantitatively by measuring cell density (CFU/mL) and keratinase activity (U/mL). Each experiment was performed in triplicate using a Completely Randomized Design to determine the optimal culture conditions that maximize bacterial growth and enzyme production.

Population and Samples

The sample in this study is the thermophilic bacterium *Bacillus licheniformis* AAL3, which was previously isolated and molecularly identified from the hot spring source of Air Putih Lebong, Bengkulu, with the highest keratinase activity qualitatively compared to other isolates.

Instruments

This research uses tools including an autoclave, Bunsen burner, Petri dishes, Erlenmeyer flasks, hot plate, incubator, inoculating needle, cuvette, magnetic stirrer, micropipette, microtube, analytical balance, pH meter, shaker incubator, centrifuge, UV-visible spectrophotometer, spin tube, spin column, centrifuge tube, test tube, thermometer, and vortex. The materials used include the bacterium *Bacillus licheniformis* AAL3, Luria-Bertani (LB) broth, Luria-Bertani (LB) agar, chicken feather meal, NH4Cl, NaCl, K2HPO4, KH2PO4, MgCl2.6H2O, bacto agar, phosphate buffer, 10% trichloroacetic acid (TCA), sodium carbonate, Folin reagent, glucose, sucrose, galactose, yeast extract, tryptone, peptone, casein, and filter paper.

Procedures

Characterization keratinase activity of *Bacillus licheniformis* Isolate

The *Bacillus licheniformis* AAL3 isolate was characterized by based on the potential keratinase activity qualitatively using the agar diffusion method. The tested colonies of Bacillus licheniformis AAL3 bacteria were first grown by inoculating them on the surface of agar media containing 2 grams of chicken feather substrate. The keratinolytic activity formed is marked by the presence of a clear zone around the bacterial culture (Yohandini et al., 2015).

$$CZ = \frac{VD + HD}{2}$$

Where, CZ: Clear zone (mm); VD: Vertical Diameter (mm); HD: Horizontal Diameter (mm)

Keratinolytic index was calculated based on the vertical and horizontal diameters of the degradation zone

$$KI = \frac{CZ}{A}$$

Where IK: Keratinolitic Index; D: Clear Zone Diameter (mm); A: Colony diameter (mm).

Preparation of a Standard Curve for Bacterial Cell Count

The culture of *Bacillus licheniformis* AAL3 was serially diluted 1:1, 1:2, 1:4, 1:8, and 1:16 using physiological NaCl (0.85%). The optical density value was measured at 600 nm, while the number of viable cells was counted using the plate count method on PCA medium after incubation for 24 hours at 50 °C. The relationship between OD value and cell count (CFU/mL) was analyzed thru a standard curve and linear regression (Yohandini et al., 2015).

Linear Regression Equation: $y = ax + b$

where: y: Absorbance value or OD; x: Number of bacterial cells; a and b: Constants obtained from the linear line.

Preparation of a Tyrosine Standard Curve

A total of 0.1 g of tyrosine was dissolved in distilled water and diluted to 100 mL to prepare a stock solution. Tyrosine standard solutions were prepared at concentrations of 60, 120, 180, 240, 300, and 360 ppm.

From each concentration, 1 mL was taken and mixed with 2.5 mL of 0.5 M Na_2CO_3 and 0.5 mL of Folin–Ciocalteu reagent, then homogenized thoroughly. The absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 578 nm. The obtained data were used to construct a standard curve based on a linear regression equation (Wang & Tong, 2022)

The linear regression equation is expressed as: $y = ax + b$.

Where: y : absorbance; x : concentration (mg/mL); a : slope b : intercept

Keratinase Enzyme Production

Bacillus licheniformis AAL3 was inoculated (one loopful each) into 100 mL of LB broth medium and incubated in a shaker incubator at 50 °C and 120 rpm for 24 hours. Cell density was measured as OD_{600} using a UV–Vis spectrophotometer at a wavelength of 600 nm (Safitri et al., 2020).

A total of 2 mL of *Bacillus licheniformis* AAL3 culture, with cell densities of 1.18×10^9 CFU/mL and 3.27×10^8 CFU/mL, respectively, were inoculated into 200 mL of production medium (the preparation procedure is described in Appendix 1.3). The cultures were then incubated at 50 °C for 48 hours in a shaker incubator at 120 rpm.

Enzyme extraction was carried out by centrifugation at 8000 rpm and 4 °C for 15 minutes. The supernatant obtained was considered the crude keratinase enzyme extract and was used for subsequent analysis (Abdul Gafar et al., 2020).

Quantitative Assay of Keratinolytic Activity

Keratinolytic activity was quantitatively determined by adding 200 µL of enzyme solution to 8 mg of chicken feather powder in 800 µL of phosphate buffer (pH 7.5). The mixture was incubated at 60 °C for 60 minutes. The enzymatic reaction was stopped by adding 1 mL of 10% trichloroacetic acid (TCA), followed by incubation at room temperature for 30 minutes and centrifugation at 10,000 rpm for 10 minutes. A 500 µL aliquot of the supernatant was mixed with 2.5 mL of sodium carbonate and incubated for 10 minutes, then 500 µL of Folin reagent was added and incubated for 30 minutes. Absorbance was measured at 660 nm using a UV-Vis spectrophotometer (Yohandini et al., 2015).

One unit of keratinase activity was defined as the amount of enzyme required to release 1 µmol of tyrosine per minute under the assay conditions. Keratinase activity was calculated using the following formula:

$$EA = \frac{[Tirosin]}{(BM\ Tirosin)} \times \frac{v}{p \times q} \times 1000 \frac{\mu mol}{mmol}$$

Where, AE : Enzyme activity (U/mL), BM Tirosin: molecule weight of Tirosin (181 mg/mmol); v: Total sample volume per reaction tube (mL); p: Volume of enzyme (mL); q : incubation time (minutes).

Optimization of Bacterial Growth for Keratinase Production

Temperature and pH: The culture of *Bacillus licheniformis* AAL3 was inoculated into keratin medium containing chicken feather substrate, then incubated at temperatures of 50, 55, 60, and 65

°C with an agitation speed of 150 rpm. The temperature that produced the highest keratinase production was selected for pH optimization. pH optimization was performed by incubating the culture in keratin medium adjusted to pH 6, 7, 8, and 9 at the previously determined optimum temperature, using a shaker incubator at 150 rpm (Yohandini et al., 2015).

Carbon and Nitrogen Sources: Carbon source optimization was carried out by growing *Bacillus licheniformis* AAL3 in keratin medium at the optimum pH, followed by the addition of 1% glucose, 1% sucrose, and 1% galactose as carbon sources. The cultures were incubated at the optimum temperature with an agitation speed of 150 rpm. Nitrogen source optimization was performed by regrowing the bacteria in keratin medium at the optimum pH, supplemented with 0.4% yeast extract, 0.4% peptone, and 0.4% casein as nitrogen sources, along with the best carbon source previously determined. The cultures were incubated at the optimum temperature with an agitation speed of 150 rpm (Koentjoro et al., 2018).

Data Analysis

The data obtained were analyzed using analysis of variance (ANOVA) to determine significant differences among the tested variables. When significant differences were observed, the analysis was followed by the Least Significant Difference (LSD) test at a 5% significance level. The observed variables included: (1) keratinolytic index, (2) bacterial cell density (CFU/mL), (3) keratinase activity (U/mL), and (4) the effects of temperature, pH, and nutrient sources (carbon and nitrogen) on bacterial growth and enzyme production.

RESULTS

Qualitative Keratinolytic Activity

The bacteria *Bacillus licheniformis AAL3* and *Bacillus licheniformis* SCL2 used in this study were isolated from the Air Putih hot spring in Lebong, Bengkulu, and are classified as thermophilic bacteria. The qualitative assay of keratinolytic activity was performed by growing *Bacillus licheniformis* AAL3 and *Bacillus licheniformis* SCL2 on keratin medium containing chicken feather substrate at 50 °C, pH 7, for 48 hours. Keratinolytic activity was indicated by the presence of a clear zone surrounding the bacterial colonies, as shown in Figure 1.

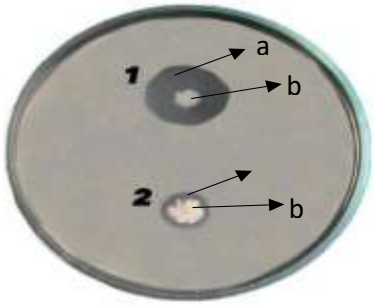

Figure 1. Qualitative keratinolytic activity of *Bacillus licheniformis* AAL3 and *Bacillus licheniformis* SCL2 on feather meal agar medium: (1) *Bacillus licheniformis* AAL3, (2) *Bacillus licheniformis* SCL2; (a) clear zone, (b) bacterial colony.

The clear zones formed around the colonies of *Bacillus licheniformis* AAL3 and *Bacillus licheniformis* SCL2 were measured by comparing the diameter of the clear zone to the diameter of the colony, resulting in keratinolytic index values as presented in Table 1. A higher keratinolytic index value indicates a greater potential for keratinase production.

Table 1. Keratinolytic Index of Thermophilic Keratinase-Producing Bacteria

Name of bacteria	Clear zone (mm)	D: Colony Diameter (mm)	Keratinolitic Index;
B. licheniformis AAL3	25	5.0	5.0
B. licheniformis SCL2	15	10	1.5

Based on Table 1, *Bacillus licheniformis* AAL3 exhibited a higher keratinolytic index (5.0) compared to *Bacillus licheniformis* SCL2, which showed a keratinolytic index of 1.5. The keratinolytic index reflects the relative capacity of bacterial isolates to hydrolyze keratin substrates. A higher index value indicates a greater keratin-degrading capability. These results suggest that *Bacillus licheniformis* AAL3 possesses a superior potential for keratinase production and/or exhibits higher enzymatic efficiency in hydrolyzing keratin into smaller peptides and amino acids than *Bacillus licheniformis* SCL2. Consequently, AAL3 may represent a more promising candidate for applications requiring effective keratin biodegradation.

Quantitative Keratinolytic Activity

The results of the quantitative assay of keratinolytic activity of *Bacillus licheniformis AAL3* and *Bacillus licheniformis* SCL2 are presented in Table 2. The table includes two primary parameters, namely bacterial cell density (expressed in CFU/mL) and keratinolytic enzyme activity (U/mL).

Table 2. Results of the Quantitative Assay of Keratinolytic Activity

Name of Bacteria	Total of cells (CFU/mL	Enzyme activity (U/mL)
B. licheniformis	8.9 x 10^8	1.0074 U/mL AAL3
B.licheniformis	1.8 x 10^8	0.7493 U/mL SCL2

Optimization of Thermophilic Bacterial Isolates for Keratinase Production

Temperature Optimization

The results of temperature optimization for Bacillus licheniformis AAL3 at temperature variations of 50 °C, 55 °C, 60 °C, and 65 °C are presented graphically in Figure 2. The data indicate that *Bacillus licheniformis* AAL3 exhibited a similar metabolic response to temperature adaptation, in which increasing temperature corresponded with an increase in cell density and keratinase activity up to a maximum level. Both bacterial cell density and keratinase enzyme activity increased progressively up to 60 °C, after which a decline was observed at 65 °C. These findings demonstrate that 60 °C represents the optimum temperature for keratinase production, as it yielded the highest enzymatic activity.

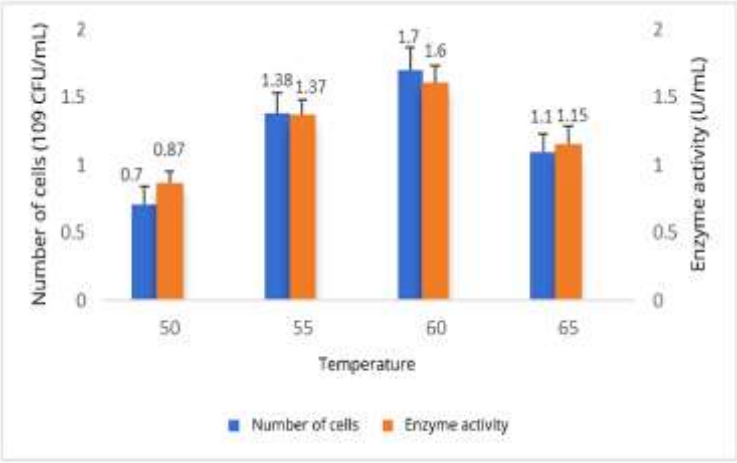

Figure 2. Temperature Optimization on Cell Growth and Keratinase Production of *Bacillus licheniformis* AAL3

pH Optimization

Based on the results of pH optimization presented in Figure 3, *Bacillus licheniformis* AAL3 was able to grow in medium at pH 6. This condition likely supports adequate nutrient solubility, thereby facilitating bacterial growth. A marked increase in both cell density and keratinase activity was observed at pH 7. At this pH level, nutrient availability appears to be optimal, enabling efficient cellular proliferation as well as enhanced enzyme synthesis and activity. Consequently, *Bacillus licheniformis* AAL3 exhibited improved keratinase production under neutral conditions.

Furthermore, both cell density and enzyme activity continued to increase significantly up to pH 9, indicating a strong adaptive capacity of the isolate to alkaline conditions and suggesting that alkaline pH favors keratinase production.

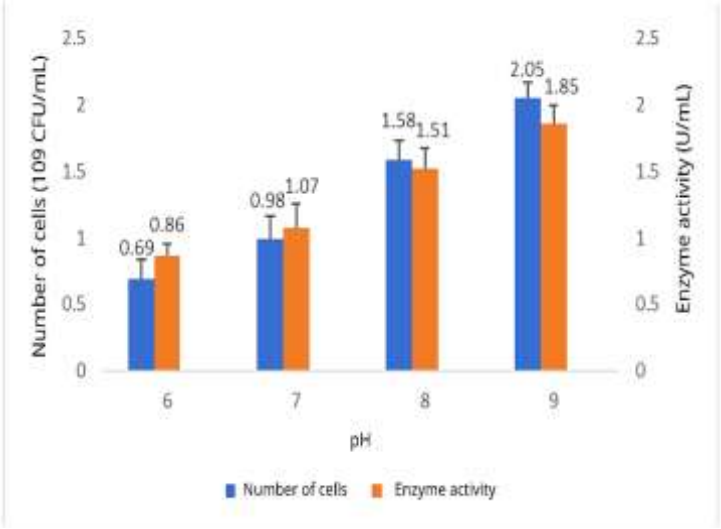

Figure 3. pH Optimization on Cell Growth and Keratinase Production of *Bacillus licheniformis* AAL3

Carbon and Nitrogen Optimization

Carbon and nitrogen sources play a crucial role in bacterial growth and metabolism. An appropriate balance between carbon and nitrogen sources is essential to maximize cell proliferation and enzymatic activity in bacteria (Deba et al., 2023). The results of carbon source optimization are presented graphically in Figure 4.

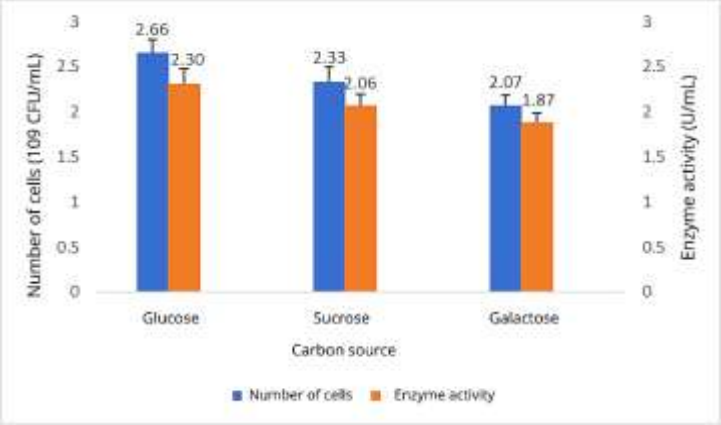

Figure 4 Optimization of Carbon Sources on Cell Growth and Keratinase Production of *Bacillus licheniformis* AAL3

The results of carbon source optimization in *Bacillus licheniformis* AAL3 demonstrated that the highest cell density and keratinase activity were observed in the medium supplemented with glucose. As a simple monosaccharide, glucose is readily metabolized by microorganisms, thereby providing sufficient energy to support rapid growth. Moreover, the energy generated from glucose metabolism contributes to enhanced enzyme synthesis, resulting in increased keratinase activity.

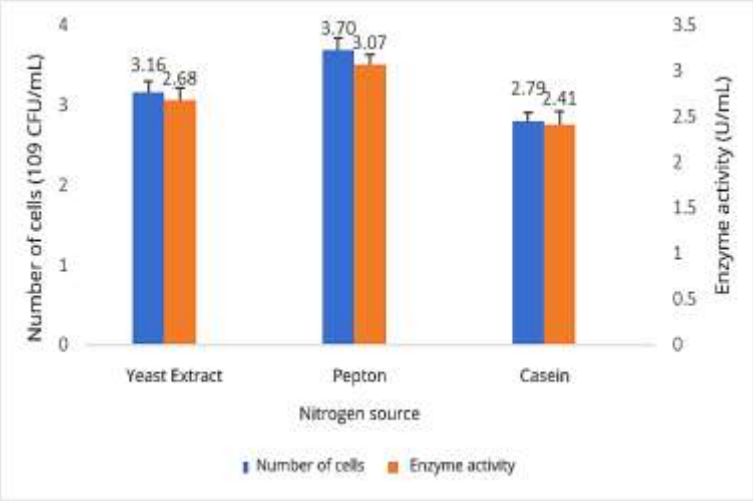

Figure 5. Optimization of Nitrogen Sources on Cell Growth and Keratinase Production of *Bacillus licheniformis* AAL3

Based on Figure 5, the results of nitrogen source optimization for *Bacillus licheniformis* AAL3 indicate that the highest cell density and keratinase activity were observed in the medium supplemented with peptone as the nitrogen source. Peptone, being a protein hydrolysate, provides readily assimilable amino acids that are more easily utilized and rapidly available to the cells, thereby supporting enhanced growth and enzyme production.

DISCUSSION

Keratinolytic activity reflects the ability of bacteria to degrade keratin, a complex and recalcitrant structural protein. Keratinase produced by bacteria hydrolyzes keratin into simpler compounds (Kumar and Singh, 2023). Gerlicz et al., (2024) reported that keratin degraded by keratinase is broken down into smaller peptides and amino acids, which can subsequently be utilized by microorganisms as nutrient sources to support growth.

According to (Yohandini et al., 2015), keratinolytic activity is indicated by the formation of a clear zone surrounding bacterial colonies on agar medium containing feather meal. The keratinolytic index provides further insight into the extent of keratin degradation by bacterial isolates. A higher keratinolytic index value indicates that *Bacillus licheniformis* AAL3 has a greater potential for keratinase production and/or exhibits higher enzymatic efficiency in hydrolyzing keratin into smaller peptides and amino acids compared to *Bacillus licheniformis* SCL2. These findings are consistent with previous research byde Paiva et al., (2019), who reported that certain bacterial strains, such as *Bacillus* sp., can achieve keratinolytic index values of up to 4.5, indicating a strong keratin-degrading capability. In contrast, other bacterial strains may exhibit different index values due to species- or strain-specific characteristics. Similarly, Aktayeva et al., (2022)reported that a higher proteolytic index value (≥ 3) reflects a substantial potential of an isolate to produce extracellular enzymes. The differences in keratinolytic index values observed between *Bacillus licheniformis* AAL3 and *Bacillus licheniformis* SCL2 may be attributed to genetic variation and the specific enzymatic capacity of each isolate to produce keratinase. Genetic variability among bacterial strains can influence growth and enzyme production through differences in metabolic

capability, environmental adaptability, and gene regulation mechanisms. Even within the same species, regulatory differences in genes encoding keratinase may occur, thereby affecting the level of enzyme production and its catalytic activity(Sharma et al., 2022).

The quantitative assay results (Table 2) demonstrated that *Bacillus licheniformis* AAL3 exhibited a higher cell density (8.9×10^8 CFU/mL) compared to *Bacillus licheniformis* SCL2 (1.8×10^8 CFU/mL). This finding indicates that *B. licheniformis* AAL3 possesses a greater capacity for growth and proliferation under the tested culture conditions, likely due to more efficient nutrient utilization and enhanced metabolic adaptability. According to Ningthoujam et al., (2019), a higher cell population can contribute to increased enzyme production. As the number of cells increases, the total amount of enzyme synthesized also rises, thereby enhancing the overall keratinolytic potential. Consistently, the keratinase activity of B. *licheniformis* AAL3 (1.0074 U/mL) was higher than that of *B. licheniformis* SCL2 (0.7493 U/mL), indicating that the keratinase produced by the AAL3 isolate exhibits superior keratin-degrading capability. These results further suggest a positive correlation between cell density and enzyme activity, where isolates with higher biomass yield greater enzymatic output. This relationship reflects that the bacteria were in favorable growth conditions and actively engaged in primary metabolic processes. In addition to primary metabolism, bacteria are also capable of producing secondary metabolites that contribute to survival under competitive environmental conditions (Mamangkey, et al., 2020). Aktayeva et al., (2022) stated that elevated enzyme activity indicates efficient nutrient utilization and active biochemical processes necessary for cellular growth and reproduction.

The capacity of bacteria to produce keratinase varies depending on the species and environmental factors, including temperature, pH, and the availability of nutrients such as carbon and nitrogen in the growth medium(Sharma et al., 2022). *Bacillus licheniformis AAL3* and *Bacillus licheniformis* SCL2 are thermophilic bacteria isolated from the Air Putih hot spring in Lebong, Bengkulu. Both isolates exhibited qualitative keratinolytic activity, as indicated by the formation of clear zones surrounding bacterial colonies. However, their quantitative keratinase production had not yet been determined. Therefore, optimization of temperature, pH, and carbon and nitrogen sources is necessary to evaluate and enhance keratinase production quantitatively, particularly in *Bacillus licheniformis* AAL3, which demonstrated the highest keratinolytic index among the tested isolates (de Paiva et al., 2019).

Temperature optimization in *Bacillus licheniformis* AAL3 demonstrated a metabolic response in which increasing temperature was accompanied by an increase in both cell density and keratinase activity until a maximum level was reached. Cell growth and keratinase production progressively increased up to 60 °C, followed by a decline at 65 °C. These results indicate that 60 °C represents the optimum temperature for keratinase production, as maximum enzymatic activity was achieved under this condition. This finding is consistent with previous studies. Gerlicz et al., (2024) reported that the optimal temperature for keratinase production by *Bacillus* sp. strain FK7 was 60 °C. Similarly, Hassan et al., (2020)demonstrated that thermophilic bacteria generally exhibit optimal keratinase production at temperatures ranging from 55–60 °C. The results also revealed that cell density increased proportionally with enzyme activity, suggesting a positive correlation between growth and keratinase production. Enhanced enzyme activity enables more efficient nutrient utilization, thereby supporting increased biomass formation. In addition, elevated enzymatic activity promotes the generation of primary metabolites necessary for cellular growth and reproduction. Conversely, when enzyme activity decreased at 65 °C, bacterial cell density was also adversely affected. Temperature plays a critical role in regulating biological processes, including the rate of enzymatic reactions. According to Vidmar & Vodovnik, (2018), increasing temperature generally accelerates enzymatic reactions and metabolic rates. However, excessively high temperatures may lead to enzyme denaturation, which likely explains the reduction in

keratinase activity observed at 65 °C. Beyond enzyme instability, elevated temperatures may also induce thermal stress in bacterial cells. Hassan et al., (2020) noted that although thermal stress can trigger cellular defense responses, prolonged exposure to supra-optimal temperatures may ultimately result in cellular damage or death.

Bacillus licheniformis AAL3 was able to grow in medium at pH 6, likely due to adequate nutrient solubility under mildly acidic conditions, which supports bacterial growth. A marked increase in both cell density and keratinase activity was observed at pH 7. At this neutral pH, nutrient availability is presumably optimal, enabling efficient cellular proliferation as well as enhanced enzyme synthesis and activity. Consequently, *B. licheniformis* AAL3 produced keratinase more efficiently under these conditions. Both cell density and enzyme activity continued to increase significantly up to pH 9, indicating strong adaptability to alkaline conditions. Although the solubility of certain nutrients may begin to decline at higher pH levels, *B. licheniformis* AAL3 was able to maintain growth and achieve maximal keratinase production. This suggests the presence of effective regulatory mechanisms that enable the bacterium to utilize available nutrients even under suboptimal environmental conditions. Overall, pH was shown to significantly influence cell growth and enzyme activity in both *Bacillus licheniformis* AAL3 and *Bacillus licheniformis* SCL2. According to (Deba et al., 2023), pH affects bacterial cell density, while Javier-López et al., (2025) reported that enzyme production may be inhibited when environmental pH falls outside the optimal range, resulting in reduced metabolic rates and cell growth. Extreme pH conditions can disrupt enzyme structure, alter protein conformation, and interfere with substrate–enzyme interactions, thereby diminishing catalytic efficiency.

Carbon and nitrogen sources play essential roles in bacterial growth and metabolism. An appropriate balance between these nutrients is crucial to maximize cell proliferation and enzymatic activity (Revankar et al., 2023). The results of carbon source optimization in *Bacillus licheniformis* AAL3 demonstrated that the highest cell density and keratinase activity were achieved in medium supplemented with glucose. As a simple monosaccharide, glucose is readily assimilated by microorganisms, providing sufficient energy to support rapid growth. The energy generated through glucose metabolism also promotes enzyme synthesis, thereby enhancing keratinase activity. According to Mousavi et al., (2013), certain bacteria possess regulatory systems that modulate enzyme production in response to substrate availability. When high glucose concentrations are detected, genes encoding enzymes required for glucose metabolism may be upregulated, leading to increased enzyme production concurrent with cell growth. Although media supplemented with sucrose also supported relatively high cell density and enzyme activity, the results were not as optimal as those observed with glucose. As a disaccharide, sucrose must first be hydrolyzed into glucose and fructose before utilization, which may slightly delay metabolic processes compared to direct glucose assimilation. In contrast, media containing galactose resulted in lower cell density and keratinase activity compared to glucose and sucrose, indicating less efficient utilization of this carbon source by *B. licheniformis* AAL3. According to Koentjoro et al., (2018), galactose requires additional metabolic steps to be converted into forms that can be readily utilized by the cell. This extended metabolic pathway may slow bacterial growth and consequently limit keratinase production.

Nitrogen source optimization in *Bacillus licheniformis* AAL3 demonstrated that the highest cell density and keratinase activity were achieved in medium supplemented with peptone. As a protein hydrolysate, peptone provides readily assimilable amino acids and short peptides that are rapidly available to the cells. This facilitates optimal protein synthesis and cellular metabolism, resulting in enhanced biomass production(Tesfaye et al., 2018). In addition to supporting robust growth, peptone supplies essential components required for enzyme synthesis, thereby promoting keratinase production. These findings indicate that peptone provides an optimal balance between

cell proliferation and enzyme production in *B. licheniformis* AAL3. Ningthoujam et al., (2019) reported that peptone is a nitrogen source rich in amino acids and peptides that can be efficiently absorbed by bacteria. Its availability enables rapid growth and increased protein synthesis, including enzymes such as keratinase. Although yeast extract also supported substantial cell growth, its performance was slightly lower than that of peptone. Despite being nutritionally rich, yeast extract may not be as readily metabolized by *B. licheniformis* AAL3 as peptone, resulting in comparatively lower growth and enzyme production. In contrast, casein supplementation resulted in the lowest cell density and keratinase activity. Casein requires additional hydrolysis before its amino acids become available for cellular utilization, thereby slowing growth rates and limiting enzyme production within a shorter incubation period, ultimately leading to reduced enzymatic activity (Peng et al., 2019).

CONCLUSION

This study demonstrates that the thermophilic bacterium *Bacillus licheniformis* AAL3 isolated from the Air Putih hot spring possesses strong keratinolytic potential, as evidenced by its high keratinolytic index and superior quantitative keratinase activity compared to *B. licheniformis* SCL2. Optimization of culture conditions significantly influenced enzyme production. The optimum temperature and pH for keratinase production were 60 °C and pH 9, respectively, indicating that alkaline and moderately high-temperature conditions favor enzymatic activity. Among the tested nutrients, 1% glucose and 0.4% peptone were identified as the most effective carbon and nitrogen sources for maximizing cell growth and keratinase production. A positive correlation between bacterial biomass and enzyme activity was observed, suggesting that enhanced growth conditions directly support increased keratinase synthesis. Overall, *B. licheniformis* AAL3 shows promising potential as a thermophilic keratinase producer for sustainable keratin waste biodegradation and related biotechnological applications.

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