

# Determination of Keratinase Activity using Chicken Feather DMSO extract and its optimization with Response Surface Methodology

Fazria Rizqi Maharani<sup>1</sup> and Suharti Suharti<sup>1</sup>

<sup>1</sup>Department of Chemistry, Faculty of Mathematics and Natural Science, State University of Malang

**Abstract.** Keratinase is an enzyme capable of degrading keratin, exhibiting two activities: protease activity, which cleaves peptide bonds, and disulfide reductase activity, which reduces disulfide bonds (-S-S-). However, keratinase activity is often measured using casein as a substrate, even though casein lacks disulfide bonds and is therefore less suitable. The limited availability of effective keratin substrates highlights the need to develop a more appropriate alternative. This study aimed to extract keratin from chicken feathers, characterize the extract—focusing on peptide and disulfide bonds—and evaluate its effectiveness as a keratinase substrate using keratinase produced by *Bacillus* sp. MD24. The research was conducted in three stages: (1) extraction of keratin from chicken feathers, (2) production of keratinase, and (3) evaluation and optimization of chicken feather keratin as a substrate using Plackett-Burman Design (PBD) and Central Composite Design-Response Surface Methodology (CCD-RSM). Keratin was extracted with DMSO, and ATR-FTIR and EDX analyses confirmed the presence of characteristic functional groups (-C-O, -N-H, -C-N, -C-H, -C-S, -S-H, and -S-S), indicating successful extraction. Keratinase production was optimized using the One-Factor-at-a-Time (OFAT) method, with optimal conditions identified at a temperature of 37°C and pH 8. Keratinase activity was then tested with casein, chicken feather keratin, and azure keratin. CCD-RSM analysis predicted the following optimal conditions: 56.64 °C and pH 8.02 for casein, 63.07 °C and pH 8.14 for chicken feather keratin, and 53.07 °C and pH 7.97 for azure keratin. Although additional data are needed in PBD to address the lack of fit and in CCD-RSM to evaluate the significance of temperature and pH interactions, the findings demonstrate that keratin extracted from chicken feathers can serve as a viable substrate for keratinase activity assays.

## 1 Introduction

Global chicken production continues to increase annually. The United States Department of Agriculture Foreign Agricultural Service (USDA-FAS) and the Global Alliance for

\*Corresponding author: [suharti.fmipa@um.ac.id](mailto:suharti.fmipa@um.ac.id)

Improved Nutrition (GAIN) have reported that chicken meat production reached 102.7 million tons in 2023 and is projected to continue rising [1]. This increase has led to a corresponding rise in chicken feather waste, which accounts for approximately 8-10% of the body weight of chickens, resulting in an estimated 8.5 million tons of waste discarded each year. The Food and Agriculture Organization (FAO) projects that global poultry meat consumption will reach 181 million tons by 2050, potentially generating around 14.48 million tons of feather waste. Such a substantial amount of waste presents significant environmental and health issues, necessitating the development of effective and environmentally friendly processing methods [2].

Chicken feathers contain approximately 90% keratin, a structural protein characterized by disulfide bonds between cysteine residues, which endows it with strength and insolubility in water. Proximate analysis indicates that chicken feathers have a crude protein content of 82.36%, crude lipids of 0.83%, crude fiber of 2.15%, ash content of 1.49%, and a moisture content of 12.33%. Ultimate analysis shows the primary elements to be carbon (64.47%), nitrogen (10.41%), oxygen (22.34%), and sulfur (2.64%). Given its composition, chicken feathers have the potential to serve as a source of high-value raw materials if the keratin can be degraded or modified into a more soluble and reactive form [3].

Various approaches have been employed to convert chicken feather waste, including physical, chemical, and enzymatic methods. Physical methods, such as hydrothermal processing, are associated with high costs and the generation of toxic by-products, while chemical methods using strong acids are environmentally unfriendly. Enzymatic methods are considered the most eco-friendly, as they utilize keratinase enzymes, which are specific proteases capable of degrading keratin by breaking peptide and disulfide bonds. Keratinase is generally produced by microorganisms, particularly bacteria from the *Bacillus* genus. Several *Bacillus* species, including *B. cereus*, *B. subtilis*, *B. licheniformis*, and *B. pumilus*. They have been reported to effectively degrade chicken feathers. Indonesia, with its high biodiversity, has the potential to be a source of new keratinase-producing isolates [4,5].

Previous research successfully isolated *Bacillus* sp. MD24 from soil and demonstrated its ability to utilize chicken feathers as the sole source of carbon and nitrogen for growth while producing keratinase [6]. The highest activity was recorded at pH 8 and 37°C over three days of fermentation. However, activity assays in that study were conducted using casein as a substrate, which does not contain disulfide bonds and is water-soluble, thereby failing to accurately reflect keratinase activity specifically. Consequently, a more appropriate substrate, namely keratin, is required for testing.

Various substrates have been utilized for measuring keratinase activity, including casein, keratin azure, and mechanically ground chicken feathers. However, each has its drawbacks: casein solely detects protease activity, keratin azure exhibits low dye homogeneity, and mechanically ground feathers possess a complex structure that is difficult for enzymes to break down [7]. Therefore, developing a substrate based on pure keratin is essential for obtaining accurate measurement results.

Keratin in chicken feathers consists of an alpha-helical structure reinforced by disulfide bonds, rendering it strong and water-insoluble. Attempts to dissolve it using strong acids, bases, or organic solvents such as ethanol and benzene have proven ineffective as these tend to hydrolyze keratin into amino acids. Dimethyl sulfoxide (DMSO) has been shown to effectively dissolve proteins while maintaining their stability; it can even penetrate keratin-rich tissues. Thus, DMSO holds potential for extracting keratin from chicken feathers to serve as a stable and specific substrate for keratinase activity assays [8]. This study describes the preparation of a keratinase substrate derived from chicken feathers by dissolving them in DMSO and subsequent precipitation using acetone. The obtained keratin powder was then assessed for its suitability as a keratinase substrate, providing a reproducible and sustainable material for future studies on keratinase production, characterization, and application.

Enzyme activity, including that of keratinase, is influenced by pH and temperature conditions, which determine the enzyme's three-dimensional structure. Previous optimization of enzyme working conditions was conducted using the One Factor at a Time (OFAT) approach, which does not account for interactions between factors. This limitation can be addressed with statistical approaches such as Plackett-Burman Design (PBD) for significant factor selection and Response Surface Methodology (RSM) for simultaneous parameter optimization.

## 2 Materials and Methods

### 2.1 Materials

The reagents included NaCl, MgSO<sub>4</sub>, peptone, yeast extract, Bacto agar, casein, K<sub>2</sub>HPO<sub>4</sub>, citric acid, Keratin Azure, acetone, DMSO, HCl, NaOH, sodium citrate, Tris base, TCA, BSA, Na<sub>2</sub>CO<sub>3</sub>, and Folin–Ciocalteu reagent. Distilled water, domestic chicken feathers (*Gallus domesticus*), and skim milk medium were also used as substrates and growth media.

### 2.2 Preparation of Keratin Powder Using DMSO

Cleaned and dried chicken feathers were refluxed with DMSO at 100°C for 3 h. The yellowish-brown extract was filtered, and the filtrate was precipitated by adding acetone (2:1, v/v) and stored at 4°C for 48 h. The mixture was centrifuged at 10,000 rpm for 10 min, and the pellet was washed with distilled water and re-centrifuged. The precipitate was dried using a freeze dryer.

### 2.3 Keratinase Production in SmF Medium

Keratinase production was conducted in 250 mL Erlenmeyer flasks containing 1 g chicken feathers, 0.5 g NaCl, 0.1 g MgSO<sub>4</sub>, and 0.05 g K<sub>2</sub>HPO<sub>4</sub> dissolved in 100 mL buffer (pH 8). The medium was sterilized at 121°C and inoculated with 1% *Bacillus* sp. MD24 starter culture (OD<sub>600</sub> = 0.6–0.8). The culture was incubated at 37°C and 100 rpm for 65–72 h. After incubation, the hydrolysate was separated from the residue and analyzed for keratinase activity.

### 2.4 Keratinase Activity Assay Using Casein

Keratinase activity was determined by the Anson method, which measures the tyrosine released from casein hydrolysis. Two milliliters of keratinase hydrolysate were centrifuged at 4°C and 10,000 rpm. The reaction mixture contained 0.2 mL of 50 mM Tris-HCl buffer (pH 8), 0.5 mL of 1% (w/v) casein, and 0.01 mL of enzyme supernatant, and was incubated at 57°C for 5 min. The reaction was stopped with 1 mL of 10% (w/v) TCA, vortexed, and centrifuged at 4°C and 10,000 rpm for 10 min. One milliliter of the supernatant was mixed with 2.5 mL of 0.5 M Na<sub>2</sub>CO<sub>3</sub> and 0.5 mL of Folin–Ciocalteu reagent, vortexed, and incubated in the dark for 30 min. Absorbance was measured at 660 nm. A negative control was prepared by adding TCA prior to enzyme addition to prevent keratin hydrolysis. Tyrosine concentration was determined using a standard curve, and keratinase activity was expressed in units per milliliter (U/mL). All assays were performed in triplicate, and the results are presented as mean ± standard deviation.

## 2.5 Keratinase Activity Assay Using Keratin Powder/Keratin Azure

Keratinase activity was determined by the Anson method, based on the tyrosine released from keratin powder hydrolysis. Two milliliters of keratinase hydrolysate were centrifuged at 4°C and 10,000 rpm. The reaction mixture contained 0.2 mL of 50 mM Tris-HCl buffer (pH 8), 20 mg keratin powder, and 0.02 mL enzyme supernatant, and was incubated at 57°C for 5 min. The reaction was stopped with 1 mL of 10% (w/v) TCA, vortexed, and centrifuged at 4°C and 10,000 rpm for 10 min. One milliliter of the supernatant was mixed with 2.5 mL of 0.5 M Na<sub>2</sub>CO<sub>3</sub> and 0.5 mL of Folin–Ciocalteu reagent, vortexed, and incubated in the dark for 30 min. Absorbance was measured at 660 nm. A negative control was prepared by adding TCA prior to enzyme addition to prevent keratin hydrolysis. Tyrosine concentration was determined using a standard curve, and keratinase activity was expressed in units per milliliter (U/mL). All assays were performed in triplicate, and the results are presented as mean ± standard deviation.

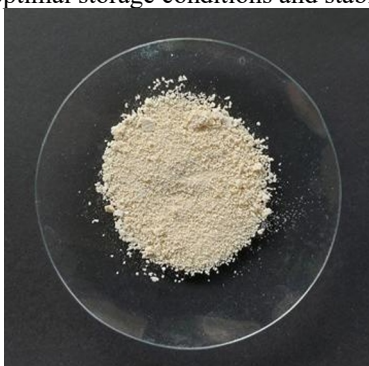
## 2.6 Characterization

Partially purified keratin powder was analyzed using Attenuated Total Reflectance– Fourier Transform Infrared (ATR-FTIR) spectroscopy to identify functional groups. The spectra were recorded in the wavenumber range of 4000–400 cm<sup>-1</sup> using the KBr pellet method. The extracted white keratin powder was analyzed using Energy Dispersive X-ray (EDX) spectroscopy to determine its elemental composition, based on the principle that each element exhibits a unique atomic structure producing characteristic electromagnetic emissions.

# 3 Results and Discussion

## 3.1 Extraction of Keratin Powder from Chicken Feathers

Keratin, an insoluble structural protein, requires high temperature and reducing agents for efficient extraction. The DMSO-extracted keratin from chicken feathers, subsequently freeze-dried, yielded a stable, white, odorless powder suitable as a substrate under elevated temperatures as shown in Figure 1. The extracted keratin powder was evaluated as a substrate for keratinase activity using the Anson method. The extracted keratin powder exhibited slight yellowing upon prolonged storage, suggesting chemical changes due to air exposure. Proper storage in airtight containers, preferably in a desiccator or at 4°C, is recommended. Further studies are needed to establish optimal storage conditions and stability parameters.



**Fig 1.** Keratin powder extracted from chicken feathers using DMSO

### 3.1.1 Energy Dispersive X-Ray (EDX) Analysis

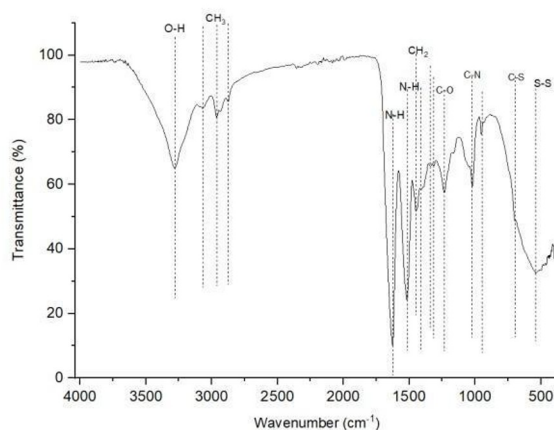
The Energy Dispersive X-Ray (EDX) analysis was performed to determine the elemental composition of the extracted keratin powder, with a particular focus on the sulfur content. The result of EDX analysis in this research confirmed that the keratin powder extracted using DMSO primarily contained carbon, oxygen, and sulfur, as presented in **Table 1**.

**Table 1.** Elemental composition of keratin powder extracted using DMSO

Element	Weight (%)	Atomic (%)
C (K)	76.9	82.2
O (K)	21.4	17.2
S (K)	1.7	0.6

### 3.1.2 Attenuated Total Reflectance Fourier-Transform Infrared Spectroscopy (ATR-FTIR) Analysis

The Attenuated Total Reflectance (ATR) technique allows direct analysis of solid or liquid samples without prior preparation. The combination of ATR with FTIR enables infrared spectra acquisition efficiently without mixing the sample with KBr, as required in conventional FTIR methods. The chemical structure of keratin extracted from chicken feathers was analyzed using ATR-FTIR at Brawijaya University, Malang.



**Fig 2.** ATR-FTIR spectrum of keratin powder extracted from chicken feathers

ATR-FTIR spectra of the extracted keratin (4000–400  $\text{cm}^{-1}$ ) revealed characteristic absorption peaks corresponding to amide and sulfur-containing functional groups typical of keratin. Major peaks included amide A (3500–3200  $\text{cm}^{-1}$ ,  $\alpha$ -helix), amide I (1700–1600  $\text{cm}^{-1}$ ,  $\text{C}=\text{O}$  stretching), and amide II (1480–1580  $\text{cm}^{-1}$ ,  $\text{N-H}$  bending), along with signals for  $\text{C-S}$  and  $\text{S-S}$  bonds (700–400  $\text{cm}^{-1}$ ). These results confirm the presence of amino acids such as cysteine and glutamine, supporting the integrity of the keratin's peptide and disulfide structures [9]. The combined SEM-EDX and ATR-FTIR analyses demonstrate that the extracted keratin powder maintains the chemical characteristics of native keratin, making it suitable as a keratinase substrate.

## 3.2 Production of Keratinase

Keratinase production was conducted using Submerged Fermentation (SmF), which allows better control of culture conditions. Compared with Solid State Fermentation (SSF), SmF

yielded higher keratinase activity for *Bacillus* sp. MD24; hence, it was employed as the production method in this study. Figure 3a shows keratinase production media using the SmF technique, using chicken feathers as the sole source of carbon and nitrogen. Figure 3b shows keratinase production media using the SmF technique, using chicken feathers as the sole source of carbon and nitrogen after being fermented for 3 days with *Bacillus* sp. MD24.



**Fig 3.** The keratinase production medium prepared using submerged fermentation (SmF) with chicken feathers as the sole carbon and nitrogen source (a), the medium after three days of fermentation by *Bacillus* sp. MD24 (b).



**Fig 4.** Reaction mixtures from keratinase activity assays using casein control (ai), sample (a), chicken feather keratin (b), and keratin azure (c) as substrates.

Keratinase activity was evaluated using casein, chicken feather keratin, and keratin azure as substrates. Casein and feather keratin assays employed the Anson method, which quantifies tyrosine release at 660 nm after Folin–Ciocalteu reaction. In contrast, keratin azure activity was determined by measuring the release of Remazol Brilliant Blue R dye at 595 nm [10]. The results of the keratinase activity test using the three substrates are shown in Figure 4.

### 3.3 Optimization of Keratinase Production Using the One Factor at a Time (OFAT) Approach

#### 3.3.1 Optimization of Temperature for Keratinase Production

The effect of temperature on keratinase production by *Bacillus* sp. MD24 was evaluated using the OFAT approach. Maximum enzyme activity (573.1 U/mL) was achieved at 37°C, while activity decreased by 8.52% and 24.08% at 32°C and 42°C, respectively, and was completely

inhibited at 47°C. The loss of activity at high temperatures is attributed to protein denaturation and cell damage, whereas reduced activity at lower temperatures results from slower metabolic rates [11]. These results indicate that *Bacillus* sp. MD24 is a mesophilic bacterium, favoring moderate temperatures (15–55°C), and its optimal growth at 37°C aligns with other mesophilic *Bacillus* species such as *B. cereus* IIPK35, *B. cereus* YQ15, and *B. pumilus* GRK.

### 3.3.2 Optimization of pH in Keratinase Production

The effect of pH on keratinase production by *Bacillus* sp. MD24 was evaluated within a pH range of 6–10. Maximum activity (689.7 U/mL) was observed at pH 8, indicating that the strain is neutrophilic. Enzyme activity decreased by 30.4% and 53.3% at pH 7 and 9, respectively, and was negligible at pH 6 and 10. The reduced keratinase activity at acidic pH (6–7) is attributed to protein protonation, which alters amino acid side-chain interactions and disrupts protein structure and function, leading to impaired metabolism and reduced enzyme production. At alkaline pH (9–10), molecular deprotonation similarly affects structural integrity and enzyme activity. Consistent with previous findings, *Bacillus* sp. MD24 exhibits optimal activity at pH 8, comparable to other keratinolytic *Bacillus* strains such as *B. cereus* (pH 8), *Bacillus* sp. NKSP-7 (pH 7.5), and *B. cereus* HD1 (pH 8) [12].

### 3.3.3 Optimization of Inoculum Size for Keratinase Production

The influence of inoculum size on keratinase production by *Bacillus* sp. MD24 was evaluated using inoculum concentrations of 1% and 5%. Maximum enzyme activity (454.6 U/mL) was achieved with a 1% inoculum, whereas a 24.4% reduction occurred at 5% (343.6 U/mL). The decrease at higher inoculum levels may result from oxygen depletion and premature enzyme production. Similar variability in optimal inoculum sizes has been reported among keratinolytic *Bacillus* species, including *Bacillus* sp. UPM-AAG1 (5%), *B. pumilus* GRK (4%), and *Bacillus* sp. Nnolim-K1 (3%) [13,14]. Based on OFAT optimization, the optimal conditions for keratinase production by *Bacillus* sp. MD24 were 37°C, pH 8, and 1% inoculum.

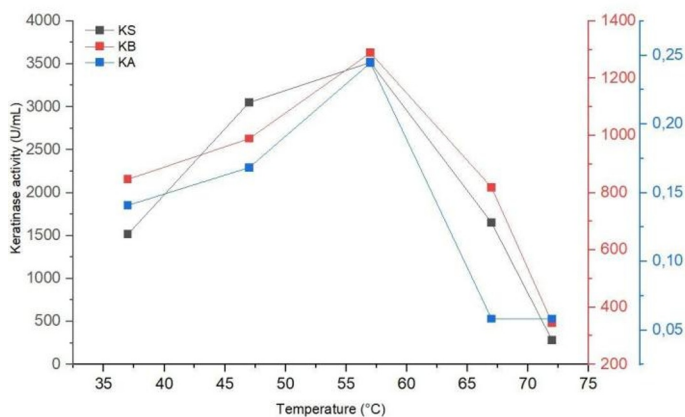
## 3.4 Trial of Substrate Utilization of Keratinase from Chicken Feather Extraction

Keratin extract analysis revealed peptide and disulfide bonds, confirming its suitability as a substrate for keratinase activity assays. Keratinase activity was validated using keratin azure, a dyed sheep wool keratin, where enzymatic degradation releases Remazol Brilliant Blue R dye, measurable at 595 nm. Casein, lacking disulfide bonds, was also used; its degradation releases tyrosine, which forms a blue complex with Folin reagent detectable at 660 nm. Activity with both substrates confirmed active keratinase presence. Keratinase activity is influenced by temperature and pH. To determine optimal conditions, three sequential optimization approaches were applied: (1) OFAT to estimate preliminary optimal temperature and pH, (2) Plackett-Burman Design to assess factor significance, and (3) CCD-RSM to precisely identify the optimal temperature and pH. All substrates were expected to exhibit similar optimal conditions since they are acted upon by the same enzyme.

### 3.4.1 Keratinase Activity Assay with pH and Temperature Variations Using One Factor at a Time (OFAT)

#### 3.4.1.1 Effect of Temperature on Keratinase Activity

Temperature significantly influences enzyme activity, as enzymes are biological catalysts that accelerate chemical reactions in cells. Each enzyme has an optimal temperature at which its activity is maximized. Temperatures that are too low reduce molecular kinetic energy, decreasing enzyme-substrate interactions and slowing the reaction rate. Conversely, high temperatures can denature the enzyme, disrupting its active site and impairing substrate binding. Enzymes vary in thermal stability; some, like those from thermophilic microorganisms, tolerate extreme temperatures, while others, such as amylase, function optimally near room temperature. In this study, the effect of temperature on the activity of keratinase from *Bacillus* sp. MD24 was evaluated to determine its optimal working temperature. Assays were conducted at 37, 47, 57, 67, and 77 °C.



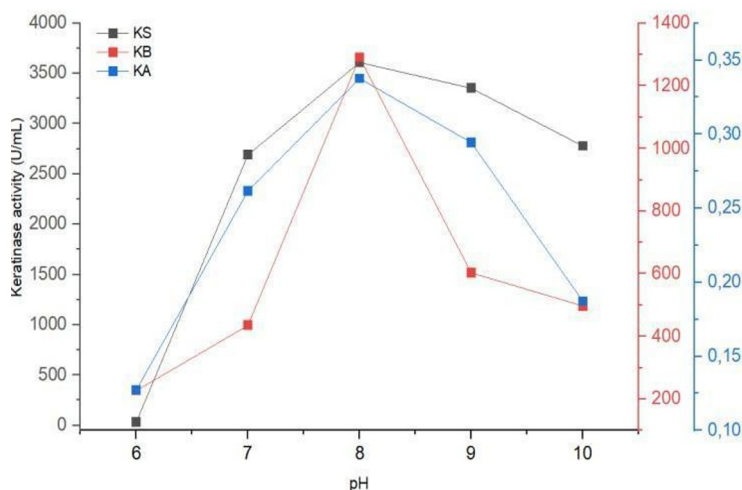
**Fig 5.** Curve showing the effect of temperature on the activity of *Bacillus* sp. MD24 keratinase using casein, chicken feather keratin, and keratin azure as substrates.

Figure 5 illustrates the effect of temperature on *Bacillus* sp. MD24 keratinase activity using casein (KS), chicken feather keratin (KB), and keratin azure (KA) as substrates. All substrates exhibited maximal activity at 57 °C. Casein showed the highest activity (3516.69 U/mL), followed by chicken feather keratin (1288.45 U/mL) and keratin azure (0.24 U/mL). The superior activity with casein is attributed to its flexible globular structure composed solely of peptide bonds. In contrast, KB and KA are fibrous and contain both peptide and strong disulfide bonds, which stabilize their protein structure and make them more resistant to enzymatic degradation. KA showed the lowest activity due to its more complex structure and higher cysteine content, rendering it particularly resistant to keratinase.

#### 3.4.1.2 Effect of pH on Keratinase Activity

pH significantly affects enzyme activity, as enzymes are composed of proteins whose structure can be altered by ionization. Changes in pH can influence protein side chains and overall enzyme conformation, potentially affecting substrate binding at the active site. Each enzyme has a specific optimal pH, and activity decreases when conditions deviate from this optimum. Extreme pH can denature the enzyme and disrupt the ionic balance around it, affecting functional groups such as carboxyl (-COOH) and amino (-NH<sub>2</sub>) groups. In this

study, the effect of pH on the activity of *Bacillus* sp. MD24 keratinase was evaluated using casein, chicken feather keratin, and keratin azure as substrates. Assays were conducted at pH 6, 7, 8, 9, and 10 to determine the enzyme's optimal pH.



**Fig 6.** Effect of pH on the activity of *Bacillus* sp. MD24 keratinase using casein, chicken feather keratin, and keratin azure as substrates

**Figure 6** shows the activity of *Bacillus* sp. MD24 keratinase using casein (KS), chicken feather keratin (KB), and keratin azure (KA) as substrates. The results indicate that the enzyme exhibits optimal activity at pH 8.

### 3.4.2 Significance Test of pH and Temperature Effects on Keratinase Activity Using Plackett-Burman Design (PBD)

In this study, Plackett-Burman Design (PBD) was used to identify factors that significantly affect the activity of *Bacillus* sp. MD24 keratinase. Temperature and pH, identified as influential factors from the OFAT screening, were selected for the analysis. Each factor was tested at two levels—low (−1) and high (+1)—corresponding to the minimum and maximum keratinase activity observed in the OFAT experiments, as summarized in **Table 2**. The ANOVA results in **Table 2** show the F- and P-values for the model, temperature, pH, and lack-of-fit on keratinase activity using casein (KS), chicken feather keratin (KB), and keratin azure (KA). Significance was evaluated at a 5% confidence level. Both temperature and pH were significant factors for all three substrates ( $P < 0.05$ ), indicating they have a substantial effect on keratinase activity. F-values indicate the relative influence of each factor. For KS, temperature ( $F = 136.82$ ) had a stronger effect than pH ( $F = 30.10$ ). For KB, pH ( $F = 287.81$ ) was more influential than temperature ( $F = 37.86$ ). For KA, temperature ( $F = 158.48$ ) again had a greater effect than pH ( $F = 122.98$ ) was more influential than temperature ( $F = 37.86$ ). For KA, temperature ( $F = 158.48$ ) again had a greater effect than pH ( $F = 122.98$ ).

Optimization of keratinase activity with respect to temperature and pH was conducted using CCD-RSM. Five levels of temperature (−21.64, 32, 57, 82, and 92.35°C) and pH (5.17, 6, 8, 10, and 10.82) were tested in 13 experimental runs generated by Minitab 20. Data were analyzed using ANOVA and fitted to a quadratic model to obtain  $R^2$ , P-value, and F-value. Pareto and residual analyses were performed to evaluate factor significance and model adequacy. The derived quadratic regression equation was used to construct surface plots and predict the optimal temperature and pH for maximum enzyme activity.

**Table 2.** Analysis of Variance (ANOVA) of Plackett-Burman Design (PBD) for Three Substrat

Source	KS Substrate		KB Substrate		KA Substrate	
	F-Value	P-Value	F-Value	P-Value	F-Value	P-Value
Model	83,46	0,000	162,83	0,000	140,73	0,000
Linear	83,46	0,000	162,83	0,000	140,73	0,000
Temperature	136,82	0,000	37,86	0,000	158,48	0,000
pH	30,10	0,000	287,81	0,000	122,98	0,000
Lack of Fit	190,32	0,000	22,29	0,002	31,27	0,000

### 3.4.3 Optimization Test of pH and Temperature on Keratinase Activity Using CCD-RSM

**Table 3.** ANOVA Data from CCD-RSM for Keratinase Activity Using Casein, Chicken Feather Keratin, and Keratin Azure Substrates

Source	KS Substrate		KB Substrate		KA Substrate	
	F-Value	P-Value	F-Value	P-Value	F-Value	P-Value
Model	2946.72	0.000	1505.21	0.000	1095.66	0.000
Linear	11.26	0.007	728.33	0.000	99.83	0.000
Temperature	7.99	0.026	1406.15	0.000	194.00	0.000
pH	14.52	0.007	50.52	0.000	5.67	0.049
Quadratic	7355.51	0.000	3033.74	0.000	2638.75	0.000
Temp*Temp	8484.14	0.000	5023.61	0.000	1864.92	0.000
pH*pH	8145.19	0.000	1703.87	0.000	4038.74	0.000
Two-Way Interaction	0.08	0.783	1.88	0.213	1.12	0.325
Temperature*pH	0.08	0.783	1.88	0.213	1.12	0.325
Lack of Fit	0.99	0.481	0.26	0.848	2.60	0.190

A significance level of 95% ( $P < 0.05$ ) was applied in the ANOVA analysis (Table 3). All parameters were significant except for the two-way interaction between temperature and pH. The F-values indicated that quadratic terms exerted the greatest influence on *Bacillus* sp. MD24 keratinase activity. For the KS and KB substrates, temperature<sup>2</sup> and pH<sup>2</sup> were the most influential factors, followed by the linear effects of temperature and pH. In contrast, for the KA substrate, pH<sup>2</sup> had the highest impact, followed by temperature<sup>2</sup> and the corresponding linear terms. The lack-of-fit test was used to assess the adequacy of the regression model relative to experimental data. All three substrates showed non-significant lack-of-fit values (KS = 0.481, KB = 0.848, KA = 0.190; all > 0.05), indicating good model agreement with observations. Among all parameters, only the temperature–pH interaction was non-significant, which is theoretically inconsistent since both factors are expected to influence keratinase activity through effects on amino acid protonation and protein folding.

**Table 4.** R-square ( $R^2$ ) values of the three substrates from CCD-RSM analysis of keratinase activity.

R-square Value
99.95%
99.88%
99.87%

The next parameter obtained from the CCD-RSM analysis is the coefficient of determination ( $R^2$ ), which indicates how well the regression model fits the observed data.  $R^2$  values range from 0–100%, where values closer to 100% signify a stronger fit between the

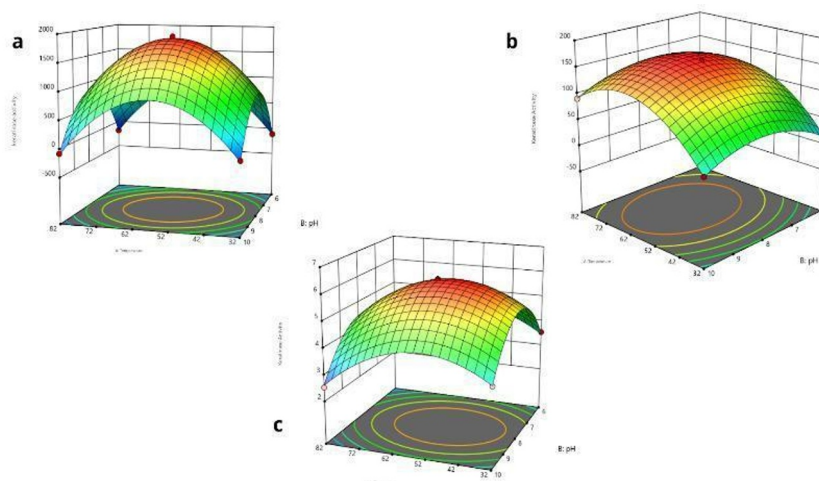
model and experimental observations. As shown in **Table 4**, the  $R^2$  values for the casein (KS), chicken feather keratin (KB), and keratin azure (KA) substrates were 99.95%, 99.88%, and 99.87%, respectively, with corresponding model errors of 0.05%, 0.12%, and 0.13%. These results demonstrate an excellent agreement between the regression model and experimental data. However, the nonsignificant lack of fit suggests potential improvement through additional experimental observations, which could further reduce residual deviations. The multivariate linear regression fitting describing the relationship between keratinase activity, temperature, and pH yielded the following regression equations for substrates KS (Equation 1), KB (Equation 2), and KA (Equation 3)

$$\text{Activity} = -18983 + 179.82 (\text{Temperature}) + 3917 (\text{pH}) - 1.5931 (\text{Temperature}^2) - 243.90 (\text{pH}^2) + 0.082 (\text{Temperature} \times \text{pH}) \quad (1)$$

$$\text{Activity} = -905.6 + 13.512 (\text{Temperature}) + 157.61 (\text{pH}) - 0.10484 (\text{Temperature}^2) - 9.540 (\text{pH}^2) - 0.0334 (\text{Temperature} \times \text{pH}) \quad (2)$$

$$\text{Activity} = -28.117 + 0.21328 (\text{Temperature}) + 7.165 (\text{pH}) - 0.001944 (\text{Temperature}^2) - 0.44700 (\text{pH}^2) - 0.000785 (\text{Temperature} \times \text{pH}) \quad (3)$$

The regression equations were employed to construct 3D surface plots (**Figure 7**) illustrating the combined effects of temperature and pH on keratinase activity. The red peaks on the surfaces represent the predicted optimal conditions yielding maximum enzyme activity.



**Fig 7.** Surface plots of CCD-RSM keratinase activity using casein (a), chicken feather keratin (b), and keratin azure substrates (c).

## 4 Conclusion

Keratin extracted from chicken feathers using DMSO retained its peptide and disulfide bonds, confirming its integrity as a substrate for keratinase assays. Enzyme activity patterns across temperature and pH variations followed a typical bell-shaped curve, as observed in both OFAT and CCD optimization results. The optimal pH values for keratinase activity using casein, feather keratin, and keratin azure were 8.02, 8.14, and 7.97, respectively, while the optimal temperatures were 56.64°C, 63.07°C, and 53.07°C. These slight variations are consistent with previous reports describing substrate-dependent differences in the preferred pH and temperature conditions for enzyme activity, such as those observed for bromelain

and laccase [36,37]. This confirms that pH and temperature optimization must be tailored to the specific substrate used. Residual analysis from both PBD and CCD-RSM models indicated deviations from normality and residual values distant from zero, suggesting data inconsistency. Additional replicates are therefore necessary to enhance the robustness of the statistical analysis and ensure model reliability.

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