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BIODEGRADATION OF CHICKEN FEATHER WASTE USING BACILLUS SUBTILIS FOR THE PRODUCTION OF KERATINASE, ITS PURIFICATION AND IMMOBILIZATION WITH POLY (VINYL ALCOHOL)-ASSISTED SILVER NANO-PARTICLES

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ABSTRACT

The escalating accumulation of chicken feather waste, a byproduct of the poultry industry, has posed significant environmental and public health concerns due to its resistance to conventional degradation methods. Microbial degradation using keratinolytic bacteria has emerged as an eco-friendly and sustainable solution, offering the added advantage of producing valuable enzymes. In this study, *Bacillus subtilis* was employed for the microbial degradation of chicken feathers via liquid state fermentation to produce extracellular keratinase. Response Surface Methodology (RSM) was utilized to optimize the fermentation parameters, revealing that a pH of 10, inoculum size of 2.5 mL, incubation period of 120 hours, and temperature of 30 °C yielded the maximum keratinase activity of 225.6 U/mL. The enzyme was subsequently purified through ammonium sulfate precipitation, ion exchange chromatography, and gel filtration, resulting in a 5.27-fold

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purification with 68.19% enzyme recovery and a final specific activity of 250 U/mg. SDS-PAGE analysis indicated a molecular weight of 32 kDa. Furthermore, keratinase was immobilized with silver nanoparticles, leading to a 4.7-fold enhancement in enzymatic activity compared to the free enzyme. FTIR analysis was conducted to compare structural changes in free and immobilized enzymes. The study demonstrates the promising potential of microbial keratinase production from poultry waste for industrial and pharmaceutical applications.

Introduction

The demand for poultry meat, especially chicken, has surged globally due to its affordability, easy accessibility, and perceived health benefits compared to red meat. As a result, the poultry processing industry has expanded significantly, giving rise to a large volume of slaughterhouse waste. Among this waste, chicken feathers constitute approximately 5–7% of the total body weight of slaughtered poultry(Verma, Yadav, Kumar, Singh, & Saxena, 2017). Given the scale of poultry production, this translates to millions of tons of chicken feather waste generated annually across the globe(Sangali & Brandelli, 2000). Unfortunately, the improper disposal of this keratin-rich waste not only poses a severe environmental threat but also serves as a breeding ground for pathogenic microorganisms, contributing to air, water, and soil pollution(Brandelli, Daroit, & Corrêa, 2015).

Chicken feathers are predominantly composed of β-keratin, a highly stable fibrous protein characterized by a high concentration of cysteine residues that form strong disulphide bonds(Pourjavaheri et al., 2019). These disulphide linkages, along with hydrogen bonds and hydrophobic interactions, confer remarkable resistance to degradation under natural environmental conditions. As a result, chicken feather keratin is insoluble in most organic and inorganic solvents and resistant to hydrolysis by conventional proteolytic enzymes like trypsin and pepsin(Tesfaye, Sithole, & Ramjugernath, 2017). This recalcitrance makes the waste difficult to manage and environmentally persistent.

Traditional methods employed to dispose of chicken feather waste—such as landfilling, incineration, and chemical treatment—have proven ineffective or even harmful in the long term. Landfilling contributes to soil and water pollution, potentially causing diseases like cholera and hepatitis in humans and animals consuming contaminated water(Xu et al., 2009). Incineration and chemical treatments not only degrade the nutritional value of feather proteins by destroying essential amino acids such as lysine, methionine, and tryptophan, but they also contribute to air pollution and greenhouse gas emissions(Okoroma, Garelick, Abiola, & Purchase, 2012). Furthermore, the labor and energy costs associated with these conventional techniques make them economically unsustainable(Wang et al., 2011).

In contrast, biological methods for the treatment and valorization of feather waste have emerged as eco-friendly, cost-effective, and sustainable alternatives(Tesfaye et al., 2017). One of the most promising approaches involves the microbial degradation of keratin using keratinase enzymes. Keratinase is a specific class of proteolytic enzymes capable of breaking down the rigid keratin structure by hydrolyzing its disulphide and peptide bonds(Mercer & Stewart, 2019). These enzymes are produced by a variety of microorganisms, including bacteria, fungi, and actinomycetes(Anbu, Jayanthi, & Velusamy, 2021). Among them,

bacterial species, particularly from the genus *Bacillus*, are favored for their high efficiency, non-pathogenic nature, and capacity to secrete keratinase directly into the culture medium (Agrahari & Wadhwa, 2010).

Several studies have highlighted the biotechnological potential of microbial keratinases in various industries. The amino acids and peptides derived from keratin hydrolysis have valuable applications as feed additives, organic fertilizers, leather dehairing agents, and in cosmetic and pharmaceutical formulations(Brandelli et al., 2015). Keratinases have also been explored for their roles in treating dermatological conditions like acne, psoriasis, and nail infections, owing to their ability to degrade hard keratin barriers, thus enhancing drug penetration (Estruch et al., 2013).

While microbial degradation offers multiple benefits, the practical application of keratinase in industrial processes requires enzymes that are stable under a wide range of environmental conditions(Verma et al., 2017). Enzyme immobilization techniques have thus been developed to enhance the operational stability, reusability, and catalytic efficiency of keratinase. Immobilization involves binding the enzyme to a solid support material, which helps preserve its activity and makes it suitable for continuous or large-scale use(Sarathi & Palaniappan, 2019). Among the various supports explored, chitosan—a biocompatible and non-toxic polymer derived from chitin—has gained prominence due to its high porosity and hydrophilicity(Tarique et al., 2023).

Further innovations have incorporated nanotechnology into enzyme immobilization. The use of silver nanoparticles (AgNPs) as supports for enzyme immobilization has shown significant promise, thanks to their high surface area, stability, and potent antimicrobial properties. AgNPs not only enhance the catalytic activity of immobilized enzymes but also protect against microbial contamination during storage and application(P. K. Rai & Singh, 2020). To improve the stability and dispersibility of AgNPs, polymers like poly(vinyl alcohol) (PVA) have been employed, creating hybrid systems that combine the advantages of nanotechnology and biopolymer supports(Khanam, Sultana, & Mushtaq, 2023).

In this context, the current study focuses on the biological degradation of chicken feather waste using *Bacillus subtilis*, a potent keratinase-producing bacterium known for its non-pathogenic nature and efficient degradation capabilities(NONKONZO, 2021). The study also involves the purification of the keratinase enzyme followed by its nano-immobilization using PVA-assisted silver nanoparticles. The dual objective is to explore an effective strategy for managing poultry waste while simultaneously producing a stable and industrially useful biocatalyst. This approach aims not only to minimize environmental pollution but also to generate value-added products with applications in agriculture, medicine, cosmetics, and beyond(Bala et al., 2023).

2. MATERIAL AND METHOD

The present study was conducted at the Enzyme Biotechnology Laboratory, Department of Biochemistry, University of Agriculture, Faisalabad. The research comprised two major parts: (i) the production and purification of keratinase enzyme from *Bacillus subtilis* using chicken feather waste via liquid-state fermentation, and (ii) nano-immobilization of the enzyme using poly(vinyl alcohol)-assisted silver nanoparticles to enhance thermostability and catalytic efficiency.

2.1 Sample Collection

Chicken feathers were obtained from a local poultry meat shop in Faisalabad, Pakistan. The raw feathers, contaminated with residual blood, meat particles, and dust, were initially washed with tap water followed by detergent treatment for thorough cleaning. After drying at room

temperature and under sunlight, the feathers were oven-dried at 50 °C until brittle, then ground into a fine powder known as feather meal for use as a substrate in fermentation (Khodayari, 2018).

2.2 Microbial Strain and Culture Maintenance

The bacterial strain *Bacillus subtilis*, known for its efficient keratinase production and ease of cultivation, was selected. The pure culture was obtained from the Enzyme Biotechnology Laboratory and grown on nutrient agar plates (pH 7.0) at 37 °C for 3–4 days under aseptic conditions (Khodayari, 2018).

Table 2.1. Composition of Nutrient Agar (per 100 mL)

Ingredient	Amount (g)
Yeast extract	0.2
Peptone	0.5
NaCl	0.5
Agar	1.5
Distilled water	To 100 mL

Plates were autoclaved (121 °C, 15 min), cooled, and streaked with the strain under laminar airflow before incubation.

2.3 Inoculum Preparation

Luria Bertani (LB) broth was prepared and autoclaved. After cooling, inoculation was performed in a sterile environment using an inoculation loop from previously incubated nutrient agar plates.

Table 2.2. Composition of LB Medium (per 100 mL)

Ingredient	Amount (g)
Tryptone	1.0
Yeast extract	0.5
NaCl	1.0
Distilled water	To 100 mL

The inoculated flasks were incubated in a shaking incubator at 37 °C and 120 rpm for 24 hours. A control (uninoculated) flask was also maintained.

2.4 Keratinase Production via Fermentation

Fermentation medium was prepared using chicken feather meal as substrate and the inoculum was added in various volumes. Optimization of conditions such as pH, temperature, inoculum size, and incubation time was performed using Response Surface Methodology (RSM) based on Central Composite Design (CCD) (Pandian et al., 2012).

Table 2.3. Fermentation Medium Composition (per 100 mL)

Ingredient	Amount (g)
NH_4Cl	0.05
NaCl	0.05
K_2HPO_4	0.03
KH_2PO_4	0.04
$MgCl_2$	0.024
Yeast extract	0.1
Chicken feather meal	1.0

Post-incubation, the fermentation broth was filtered and centrifuged at 1000 rpm for 10 minutes. The supernatant containing crude keratinase was collected for further analysis.

2.5 Keratinase Activity Assay

Keratinase activity was measured spectrophotometrically using 20 mg chicken feather in 3.8 mL Tris-HCl buffer (100 mM, pH 7.8) and 0.2 mL of enzyme source. The reaction mixture was incubated at 37 °C for 1 hour and absorbance was

recorded at 280 nm. One unit of enzyme activity was defined as the amount of enzyme causing an absorbance increase of 0.1 (Anbu et al., 2005).

Table 2.4. Tris-HCl Buffer Preparation (100 mM, pH 7.8)

Component	Amount
Tris base	0.972 g
HCl (adjust pH)	As required
Distilled water	To 100 mL

2.6 Purification of Keratinase

2.6.1 Ammonium Sulfate Precipitation

Crude enzyme was partially purified using salting-in (30% saturation) and salting-out (80% saturation) techniques with ammonium sulfate at 4 °C. The precipitate obtained was dissolved in 20 mM Tris-HCl (pH 8.0) for further purification.

2.6.2 Dialysis

The solution was dialyzed using a cellulose membrane against 20 mM Tris-HCl buffer for 6 hours at 4 °C to remove salts and impurities.

2.6.3 Ion Exchange Chromatography

A DEAE-cellulose column was prepared and equilibrated with Tris-HCl buffer (pH 8.0). The dialyzed enzyme sample was applied and 25 fractions were collected using the same buffer.

2.6.4 Gel Filtration Chromatography

Further purification was achieved using Sephadex G-200 column equilibrated with Tris-HCl buffer. The highest protein-containing fraction was selected for characterization.

2.6.5 SDS-PAGE Analysis

Purified enzyme was analyzed using SDS-PAGE (12% resolving, 5% stacking gel) to determine molecular mass. Coomassie Brilliant Blue was used for staining and destaining was done to visualize protein bands (Suntornsuk et al., 2005).

2.7 Immobilization of Keratinase

2.7.1 Synthesis of PVA-Assisted Silver Nanoparticles

Poly(vinyl alcohol) (5% w/v) solution was prepared by dissolving PVA in water and stirring at 60 °C for 6 hours. Silver nanoparticles were synthesized via in situ reduction of silver nitrate ($AgNO_3$) using hydrazine hydrate as a reducing agent in the PVA matrix.

2.7.2 Immobilization Procedure

Keratinase (2.0 mg/mL) was immobilized on AgNPs using cyanamide activation. The activated NPs were incubated with enzyme under sonication at 4 °C. The immobilized complex was washed repeatedly to remove unbound proteins, and activity was assessed using a keratin-based substrate at pH 9.0 (Rai et al., 2020).

3. Results

Chapter 3: Results

3.1 Microbial Growth Maintenance

Bacillus subtilis, a prominent bacterium for industrial biotechnology, was employed in this study due to its ability to produce extracellular enzymes such as keratinase. The strain was obtained from the Enzyme Biotechnology Laboratory, Department of Biochemistry, University of Agriculture, Faisalabad. It was cultured on nutrient agar under optimal conditions (pH 7, 37 °C, 24 h) (Khodayari, 2018), yielding characteristic colonies (Fig. 3.1).

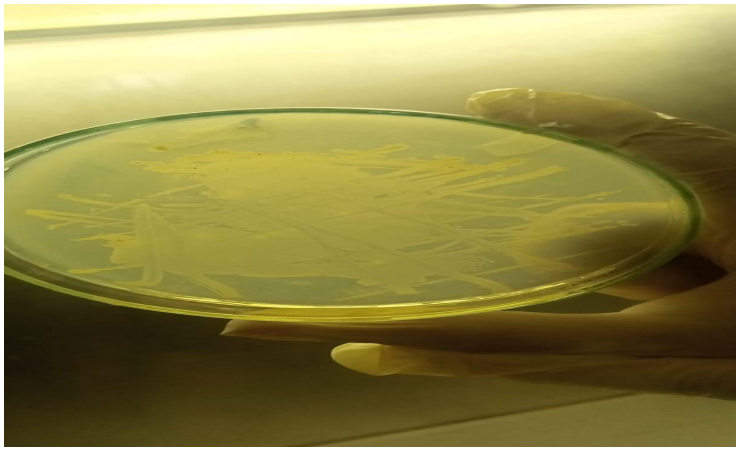


Figure 3.1 Growth of *Bacillus subtilis* on nutrient agar.

These results align with those of Pant et al. (2015), who isolated *B. subtilis* through serial dilution and sub-culturing techniques.

3.2 Inoculum Preparation

Inoculum was prepared using LB (Luria-Bertani) medium in a temperature-controlled orbital shaker at 37 °C and 120 rpm for 24 h. A control flask was also maintained under identical conditions without inoculation for comparative analysis (Khodayari, 2018) (Fig. 3.2).

Figure 3.2 Flask showing visible microbial growth.

Patrick and Kearns (2009) used similar LB medium preparations for evaluating the swarming motility of *B. subtilis*.

3.3 Optimization of Fermentation Medium

To enhance keratinase production, Response Surface Methodology (RSM) under Central Composite Design (CCD) was applied. A total of 27 experimental runs were conducted with varying levels of pH, temperature, inoculum size, and incubation time. The keratinase activity ranged from 93.8 to 225.6 U/mL, with the highest activity observed at pH 10, 30 °C, 2.5 mL inoculum, and 120 h (Table 3.1).

Table 3.1 Experimental design matrix and observed keratinase activity (U/mL) using RSM.

Run Order	pH (5.5-11.5)	Temperature (22.5-52.5°C	Inoculum size (0.25-3.25mL)	Incubation period (12-156 hrs.)	Enzyme activity (U/ml)

1	10	30	1	120	186.6
2	10	45	2.5	48	147
3	8.5	37.5	1.75	12	107.4
4	7	30	1	120	173
5	10	30	2.5	120	**225.6**
6	7	45	1	120	142
7	7	30	1	48	160
8	7	30	2.5	48	156
9	10	45	1	48	132.7
10	7	30	2.5	120	184.2
11	5.5	37.5	1.75	84	127.5
12	8.5	37.5	0.25	84	180.7
13	10	45	2.5	120	162
14	10	45	1	120	158.3
15	8.5	52.5	1.75	84	104.9
16	10	30	1	48	180
17	7	45	1	48	105.7
18	7	45	2.5	48	119.2
19	11.5	37.5	1.75	84	159.2
20	8.5	37.5	1.75	84	160.1
21	7	45	2.5	120	150.4
22	10	30	2.5	48	205.3
23	8.5	37.5	1.75	84	165.2
24	8.5	37.5	3.25	84	159.5
25	8.5	37.5	1.75	156	177
26	8.5	37.5	1.75	84	165.2
27	8.5	22.5	1.75	84	93.8

3.4 Analysis of Variance (ANOVA)

ANOVA (Table 3.2) revealed that pH ($p = 0.000$), temperature ($p = 0.000$), and incubation time ($p = 0.000$) significantly affected enzyme activity. Interaction terms such as pH × temperature were also significant ($p = 0.007$), indicating synergistic effects.

Table 3.2 ANOVA table for keratinase activity generated through RSM.

Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Model	14	999.96	71.426	23.53	0.000
Linear	4	859.09	214.773	70.76	0.000
pH	1	172.91	172.914	56.97	0.000
Temperature	1	499.23	499.229	164.49	0.000
Inoculum size	1	37.15	37.151	12.24	0.004
Incubation time	1	149.80	149.800	49.36	0.000
Square	4	79.06	19.765	6.51	0.005
pH*pH	1	3.19	3.193	1.05	0.325
Temperature*Temperature	1	1.56	1.562	0.51	0.487
Inoculum size*Inoculum size	1	42.60	42.601	14.04	0.003

Incubation time*Incubation time	1	5.56	5.562	1.83	0.201
2-Way Interaction	6	61.81	10.302	3.39	0.034
pH*Temperature	1	32.60	32.604	10.74	0.007
pH*Inoculum size	1	12.01	12.006	3.96	0.070
pH*Incubation time	1	0.28	0.276	0.09	0.768
Temperature*Inoculum size	1	10.63	10.628	3.50	0.086
Temperature*Incubation time	1	3.42	3.423	1.13	0.309
Inoculum size*Incubation time	1	2.87	2.873	0.95	0.350
Error	12	36.42	3.035		
Lack-of-Fit	10	34.58	3.458	3.76	0.228
Pure Error	2	1.84	0.920		
Total	26	1036.38			

3.4.1 Model Summary

Regression analysis demonstrated an R^2 of 96.49%, indicating a strong model fit. The adjusted R^2 and predicted R^2 were 92.39% and 80.38%, respectively, affirming the reliability and predictability of the model (Table 3.3).

Table 3.3 Regression analysis values for model.

S	R-sq	R-sq(adj)	R-sq(pred)
1.74215	96.49%	92.39%	80.38%

3.4.2 Coefficients and Regression Equation

Coded coefficients showed that pH, temperature, inoculum size, and incubation time had significant individual effects ($p < 0.05$). The regression model was derived as:

Specific Activity = $-40.1 + 8.33(\text{pH}) + 1.229(\text{Temperature}) - 9.56(\text{Inoculum size}) + 0.193(\text{Incubation time}) - 0.172(\text{pH}^2) - 0.00481(\text{Temperature}^2) + 2.512(\text{Inoculum size}^2) - 0.000394(\text{Incubation time}^2) - 0.1269(\text{pH} \times \text{Temperature}) + 0.770(\text{pH} \times \text{Inoculum size}) - 0.00243(\text{pH} \times \text{Incubation time}) - 0.1449(\text{Temperature} \times \text{Inoculum size}) - 0.00171(\text{Temperature} \times \text{Incubation time}) + 0.0157(\text{Inoculum size} \times \text{Incubation time})$

Table 3.4 Coefficients for coded factors with significance levels.

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	18.67	1.01	18.56	0.000	
pH	5.368	0.711	7.55	0.000	1.00
Temperature	-9.122	0.711	-12.83	0.000	1.00
Inoculum size	2.488	0.711	3.50	0.004	1.00
Incubation time	4.997	0.711	7.03	0.000	1.00
pH*pH	-1.55	1.51	-1.03	0.325	1.25
Temperature*Temperature	-1.08	1.51	-0.72	0.487	1.25
Inoculum size*Inoculum size	5.65	1.51	3.75	0.003	1.25
Incubation time*Incubation time	-2.04	1.51	-1.35	0.201	1.25
pH*Temperature	-5.71	1.74	-3.28	0.007	1.00
pH*Inoculum size	3.46	1.74	1.99	0.070	1.00
pH*Incubation time	-0.52	1.74	-0.30	0.768	1.00
Temperature*Inoculum size	-3.26	1.74	-1.87	0.086	1.00
Temperature*Incubation time	-1.85	1.74	-1.06	0.309	1.00

Inoculum size*Incubation time	1.70	1.74	0.97	0.350	1.00

3.4.3 Residual Analysis

Residual plots (Fig. 3.3) confirmed normal distribution of data with no significant deviations, validating model assumptions.

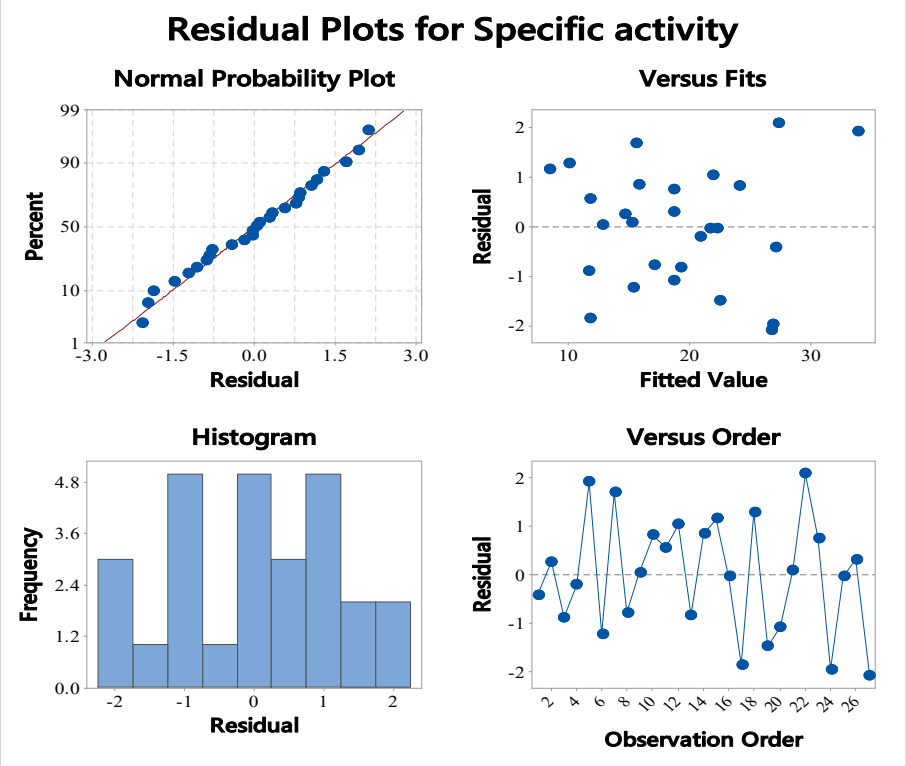


Figure 3.3 Residual plots for model validation.

3.4.4 Response Optimization and Predicted Activity

Response optimization aimed to achieve maximum specific activity. RSM predicted an optimum activity of 57.08 U/mL under conditions: pH 11.5, 22.5 °C, 3.25 mL inoculum, and 156 h (Table 3.4).

Table 3.4 Predicted optimized parameters and specific activity.

Response	Goal	Lower	Target	Upper	Weight	Importance
Specific activity	Maximum	9.62	35.69		1	1

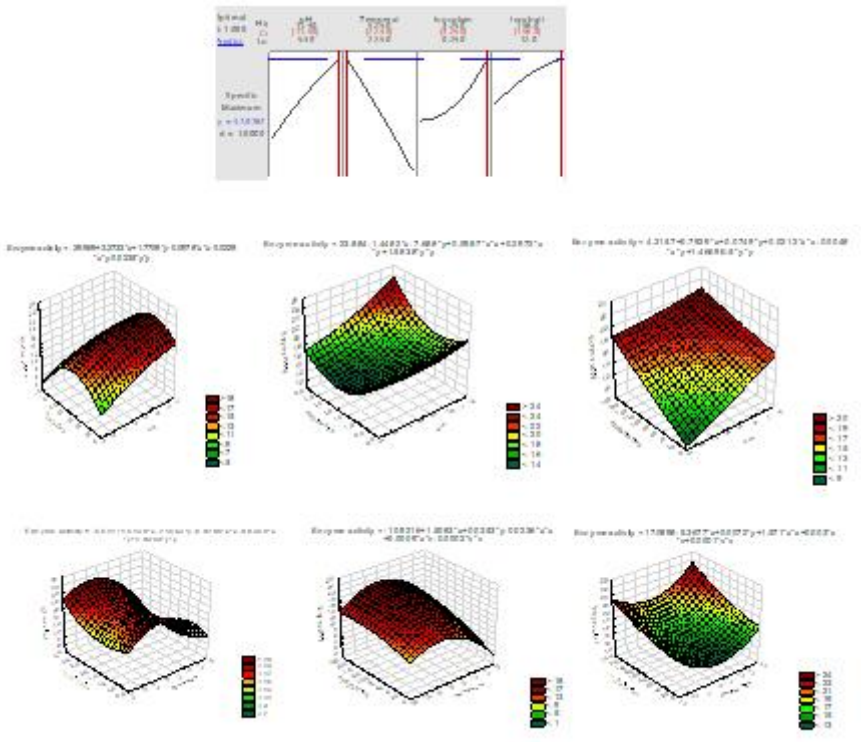
Observed maximum activity under experimental conditions was 225.6 U/mL (Table 3.5), indicating model robustness despite variability in prediction.

Table 3.5 Predicted vs. observed activity values.

Solution	pH	Temperature	Inoculum size	Incubation time	Specific activity Fit	Composite Desirability
1	11.5	22.5000	3.25	156	57.0767	1.00000
2	11.5	22.5000	3.25	12	41.0433	1.00000
3	11.5	39.3883	3.25	156	35.6900	1.00000

3.4.5 Optimization Plots

Optimization plots (Figs. 3.4 to 3.10) illustrated that keratinase activity increased with higher pH, lower temperature, prolonged incubation, and larger inoculum size.



Figures 3.4 – 3.10 2D and 3D response surface plots for various parameter combinations.

3.5 Effect of pH

Among all variables, pH was the most influential factor. Maximum activity (225.6 U/mL) was obtained at pH 10. Sanghvi et al. (2016) similarly reported optimal keratinase activity at pH 10 for *Bacillus subtilis* DP1 isolated from poultry soil.

3.6 Purification of Keratinase

Crude keratinase obtained after fermentation was subjected to purification steps including:

3.6.1 Ion Exchange Chromatography

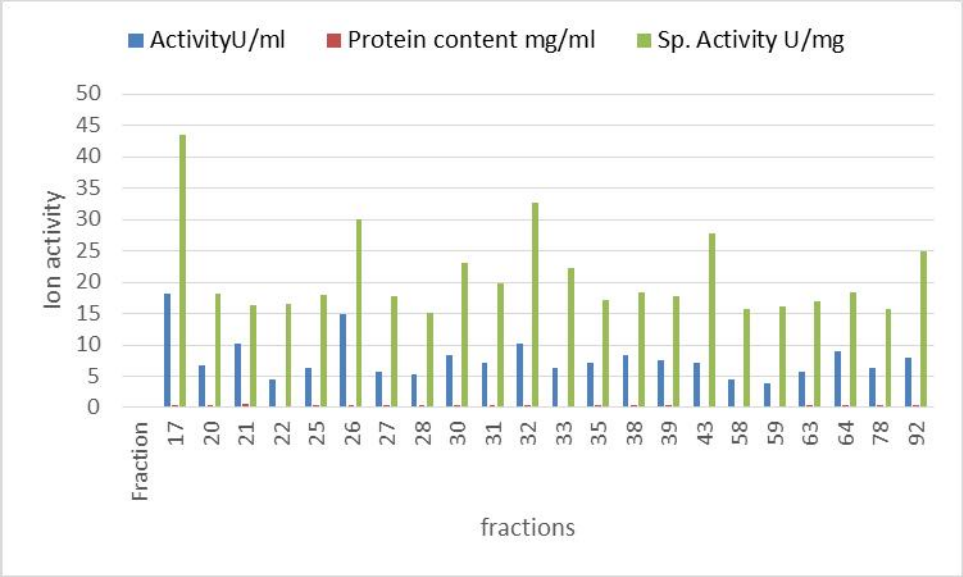
Using carboxymethyl cellulose (CMC), 100 fractions were collected. Selected fractions with high activity (Table 3.6) were moved to gel filtration. This step resulted in 39.3-fold purification with 44.08% yield.

Table 3.6 Selected fractions from ion exchange chromatography.

Fraction No.	Enzyme activity (U mL^{-1})	Protein content (mg mL^{-1})	Specific activity (U mg^{-1})
1	6.66	0.3679	18.10
2	10.2	0.624	16.34
3	4.48	0.2704	16.56
4	6.41	0.3568	17.96
5	15	0.4996	30.02

6	5.76	0.3230	17.83
7	5.38	0.3568	15.07
8	8.33	0.3614	23.04
9	7.05	0.3549	19.86
10	10.2	0.3113	32.76
11	7.05	0.4095	17.21
12	8.33	0.4537	18.36
13	7.56	0.4238	17.83
14	7.05	0.2537	27.78
15	4.48	0.286	15.66
16	8.97	0.4862	18.44
17	**18.15**	**0.4167**	**43.55**
18	7.94	0.3191	24.88
19	6.95	0.387	17.95
20	4.93	0.230	21.43
21	8.33	0.421	19.78
22	10.11	0.381	26.53
23	7.09	0.312	22.72
24	6.64	0.294	22.58
25	5.85	0.337	17.35

Figure 3.11 Chromatogram of ion exchange fractions.



3.6.2 Gel Filtration Chromatography

Using Sephadex G-200, further purification led to a specific activity of 796.31 U/mg with 30.6% yield and 43.9-fold purification (Table 3.7).

Table 3.7 Fractions from gel filtration chromatography.

Fraction No.	**Enzyme activity (U mL^{-1})**	**Protein content (mg mL^{-1})**	**Specific activity (U mg^{-1})**
1	3.20	0.0969	33.023
2	2.94	0.3529	8.3309
3	2.56	0.3549	7.2132
4	2.56	0.3100	8.2580
5	2.94	0.2879	10.211
6	3.46	0.2795	12.379
7	2.94	0.1865	15.764
8	2.69	0.2502	10.751
9	3.07	0.2314	13.267
10	1.79	0.2671	6.7016
11	1.92	0.2801	6.8546
12	1.92	0.2132	9.0056
13	2.94	0.3516	8.3617

14	3.84	0.2366	16.229
15	2.56	0.3516	7.2810
16	**7.94**	**0.0976**	**81.352**
17	1.92	0.4004	4.7952
18	2.69	0.3620	7.4309
19	2.56	0.2749	9.3124
20	2.82	0.3347	8.4254
21	2.94	0.2561	11.479
22	2.30	0.1495	15.384
23	2.05	0.3717	5.5152
24	2.05	0.3003	6.8265
25	2.05	0.4166	4.9207

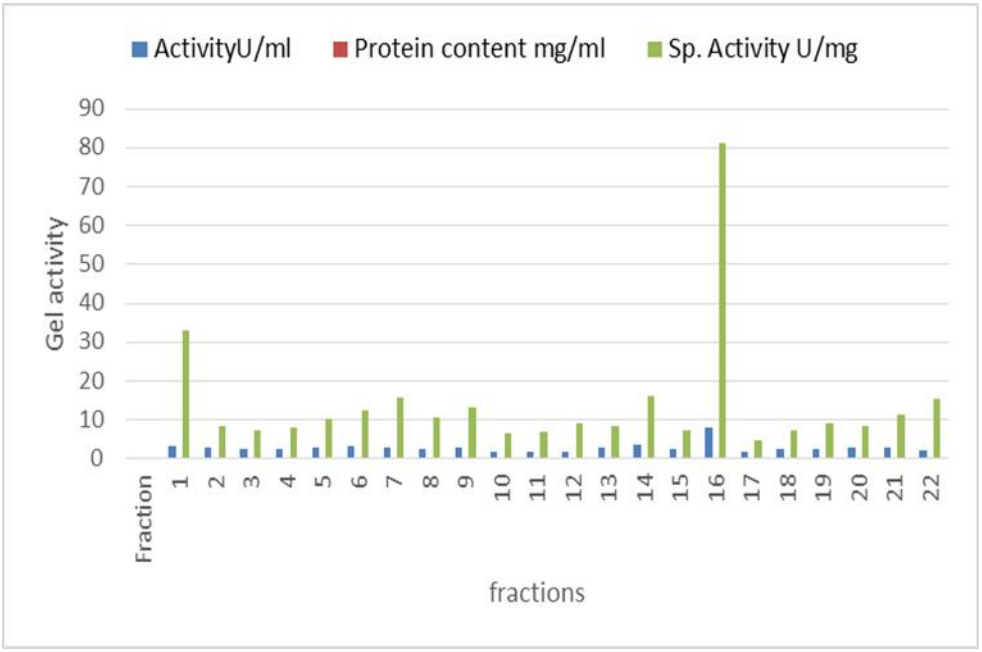


Figure 3.12 Chromatogram of gel filtration fractions.

3.6.3 Purification Summary

Table 3.8 Summary of keratinase purification.

Step	**Activity (U/mL)**	**Protein (mg/mL)**	**Specific Activity (U/mg)**	**Fold Purification**	**Yield (%)**
Crude	15,938	172	92.66	1	100

Desalted	13,955	71	196.54	3.6	87.55
Ion Exchange	6,152	11	559.27	39.3	44.08
Gel Filtration	4,539	5.7	796.31	43.9	30.60

3.7 SDS-PAGE Analysis

SDS-PAGE confirmed the molecular weight of purified keratinase to be ~35 kDa, consistent with findings by Kareem et al. (2017) and Zheng-Yu et al. (2007), confirming the identity and purity of the enzyme.

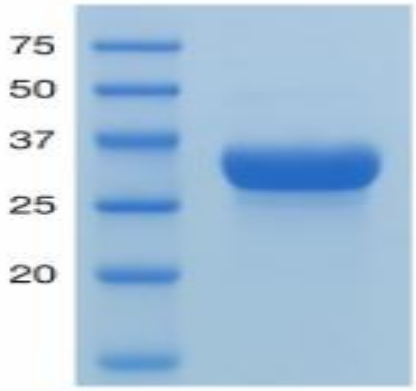


Figure 3.13 SDS-PAGE profile of purified keratinase.

4. Discussion

Keratinases are a unique class of proteolytic enzymes with the ability to hydrolyze the highly recalcitrant fibrous protein keratin into its constituent amino acids. Due to this property, keratinases have become highly valuable for various industrial sectors including leather processing, animal feed, detergent formulation, pharmaceuticals, and cosmetics(Ayele et al., 2021). The current study explored the production, purification, and nano-immobilization of keratinase using *Bacillus subtilis* with chicken feather waste as the primary substrate. The fermentation process was optimized using response surface methodology (RSM), and enzyme activity was significantly enhanced under specific conditions. Subsequent purification and nano-immobilization further improved the catalytic efficiency, demonstrating the biotechnological potential of this approach(Kim, Lee, & Lee, 2018).

In this study, *Bacillus subtilis* produced keratinase with a maximum specific activity of 27.11 U/mg under optimized conditions: 2 ml inoculum size, pH 8.5, temperature 37.5ºC, and 84 hours of incubation(Özalpar, Demirkan, & Sevgi, 2024). These findings are consistent with previous reports emphasizing the suitability of *Bacillus* species for keratinase production due to their efficient secretion of extracellular enzymes. For example, Brandelli and Riffel (2005) also reported that *Bacillus subtilis* strains could effectively degrade keratin-based substrates and highlighted optimal growth in alkaline pH and mesophilic temperatures. Similarly, Sangali and Brandelli (2000) achieved maximum keratinase activity at 37ºC and pH 8.0, which aligns closely with the current study's parameters(Jaouadi, Abdelmalek, Jaouadi, & Bejar, 2011).

Chicken feather waste served as an excellent and cost-effective substrate for enzyme production. The use of such agricultural and slaughterhouse waste materials not only lowers production costs but also offers a sustainable waste management strategy(Koul, Yakoob, & Shah, 2022). Tesfaye et al. (2017) emphasized that feather meal provides a rich keratin source, and when used as a substrate, it supports high yields of keratinolytic enzymes. The present study supports this

finding, showing that *Bacillus subtilis* can efficiently utilize untreated chicken feather meal in submerged fermentation(El Salamony, Hassouna, Zaghloul, & Abdallah, 2024).

Purification of the produced keratinase was achieved through a multi-step process, beginning with ammonium sulfate precipitation, which yielded a partially purified enzyme with a specific activity of 35.828 U/mg(Zhao et al., 2020). This technique is a widely adopted initial step in protein purification due to its simplicity and effectiveness in removing water-soluble impurities through differential solubility. Similar protocols have been employed by Riffel et al. (2003), where 60–80% saturation of ammonium sulfate was found optimal for keratinase concentration from bacterial cultures(Mehta, Jholapara, & Sawant, 2014).

Further purification via ion exchange chromatography enhanced the specific activity to 43.556 U/mg, which is comparable to the work by Anbu et al. (2005), where ion exchange chromatography improved the purification fold and recovery yield of keratinase from *Bacillus pumilus(Sun, Li, Liu, Chi, & Liu, 2021)*. Gel filtration chromatography followed, resulting in a highly purified keratinase with a specific activity of 81.352 U/mg and an overall 2.59-fold purification with 29.63% yield recovery. These values are in line with those reported by Gupta and Ramnani (2006), who used similar chromatographic methods and reported a purification fold of 2.5–3.0 with recovery between 25–30%(Thakur, Kumar, Sharma, Bhalla, & Kumar, 2018).

The molecular weight of the purified keratinase enzyme was estimated at approximately 20.1 kDa by SDS-PAGE, which is consistent with earlier findings(Mwanza, 2018). According to Martínez et al. (2013), keratinases from *Bacillus* species generally range between 20–30 kDa, and smaller enzymes tend to demonstrate greater diffusivity and stability during industrial applications(Vidmar & Vodovnik, 2018).

A novel aspect of this study was the nano-immobilization of purified keratinase using silver nanoparticles (AgNPs) prepared with a poly(vinyl alcohol) (PVA) matrix. The AgNPs had a narrow size distribution (8.0 ± 2.0 nm), and enzyme conjugation was optimized using Plackett–Burman factorial design. The analysis revealed that buffer pH, AgNP concentration, and enzyme concentration significantly influenced the binding efficiency. Immobilized keratinase showed a 5.6-fold increase in specific activity, suggesting that AgNPs provided a stable and catalytically favorable microenvironment for the enzyme(S. K. Rai, Roy, & Mukherjee, 2020).

This result correlates with previous research by Basso and Serban (2019), who noted that immobilization of enzymes onto nanoparticles could significantly enhance their stability, activity, and reusability. Similarly, Konwarh et al. (2009) demonstrated that magnetic and metallic nanoparticles could offer improved enzyme recovery and stability due to their high surface-area-to-volume ratio and strong binding affinity. Moreover, the PVA-assisted matrix contributed to improved enzyme dispersion and compatibility, as reported by(Khanna et al., 2005)

In addition to enhancing catalytic performance, AgNPs confer antimicrobial properties, reducing microbial contamination during enzyme storage and use(Priyadarshini, Sethi, Rout, Mishra, & Pradhan, 2023). This property is particularly advantageous for keratinase applications in cosmetics and dermatology, where enzyme integrity must be preserved over extended periods(S. K. Rai et al., 2020).

5. Conclusion

In conclusion, the current study reinforces the potential of *Bacillus subtilis* as a powerful biocatalyst for keratinase production using chicken feather waste(Das et al., 2024). Through a well-optimized fermentation strategy, effective purification,

and advanced nano-immobilization, the study achieved not only higher enzyme yields but also significantly improved activity and stability. The findings are consistent with and expand upon previous studies, suggesting that this integrated approach could pave the way for sustainable waste management and enzyme production for a broad range of industrial applications(Sharma et al., 2022).

6. References

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