

ISOLATION OF KERATINOLYTIC ORGANISMS FROM POULTRY WASTE AND THEIR APPLICATION FOR SILVER EXTRACTION FROM USED X-RAY FILMS

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Abstract:

Poultry waste represents one of the largest reservoirs of keratin-rich materials such as feathers, which are highly resistant to degradation and contribute significantly to environmental pollution. At the same time, discarded X-ray films pose additional environmental concerns due to their gelatin layer containing silver halides, and conventional chemical methods for silver extraction are hazardous and inefficient. This study was designed to address both waste streams through a microbial and enzymatic approach. Soil and feather samples collected from various poultry sites yielded approximately 30 bacterial isolates, of which a few demonstrated significant proteolytic and keratinolytic activity on skim milk agar and feather meal agar. These selected isolates were further cultivated for keratinase production. The crude enzyme was partially purified using ammonium sulphate precipitation followed by dialysis. Enzyme characterization revealed optimum keratinase activity at pH 8 and 45°C, supporting its alkaline protease nature. The produced keratinase efficiently degraded feathers and was further applied for the biodegradation of the gelatin layer of used X-ray films, enabling the release of silver halide in an eco-friendly manner. Overall, the study demonstrates an effective strategy for the valorization of poultry waste and the sustainable recovery of silver, converting two problematic wastes into valuable resources.

Keywords: Poultry waste, keratinase, keratinolytic organisms, feather degradation, X-rays, silver halide, enzyme optimization, gelatin layer, silver recovery.

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Introduction

The rapid increase in the global population has significantly increased demand for protein-rich foods, leading to the expansion of the poultry industry (Bischoff, 2017). This has resulted in the large-scale generation of feather waste, which poses serious environmental concerns due to its high keratin content (Cavello et al., 2013). Keratin is a tough, fibrous, and insoluble protein with a complex structure stabilized by disulfide bonds, hydrogen bonds, and hydrophobic interactions, making it highly resistant to degradation by common proteolytic enzymes. (Gupta & Ramnani, 2006). Traditional disposal methods such as incineration and landfilling are environmentally harmful and lead to resource loss. Therefore, eco-friendly alternatives are needed. (Sharma and Gupta, 2016; Aktayeva et al., 2022). Keratinolytic microorganisms, including bacteria (especially *Bacillus* species), fungi, and actinomycetes, play a crucial role in degrading keratin.

They produce keratinase enzymes, which are specialized proteases capable of breaking down keratin through a multistep process involving disulfide bond reduction and peptide bond hydrolysis, ultimately converting it into amino acids and peptides. (Anbesaw, 2022; Gupta et al., 2026). Keratinases have wide industrial applications. They are used for eco-friendly silver recovery from X-ray films by degrading the gelatin layer, in leather processing for enzymatic dehairing, in textiles for

wool treatment, in detergents for stain removal, and in pharmaceuticals, agriculture, and cosmetics. (Tiwary and Gupta, 2010; Brandelli et al., 2010; Aktayeva et al., 2022). These applications highlight their importance in sustainable waste management and industrial biotechnology. Overall, keratinases provide an efficient, eco-friendly, and cost-effective solution for converting keratin waste into valuable products, supporting environmental protection.

2. Materials and Methods

2.1. Study Site and Sample Collection

Soil and feather waste samples were collected from poultry waste disposal sites located in Panvel Taluka and Rasayani, Maharashtra, India, where feather waste has accumulated continuously over time. Samples were collected in sterile screw-cap containers (250 mL) using sterile spatulas and forceps, labelled, and transported to the laboratory at ambient temperature for immediate processing. (Hizam et al., 2025).

2.2 Enrichment and Isolation

Each sample was serially diluted and inoculated into Mineral Salt Medium (MSM) supplemented with 1% (w/v) Chicken feathers as the sole carbon and nitrogen source. The flasks were incubated at 37°C for 5–7 days with continuous shaking (150 rpm). Enriched cultures were plated on Feather Meal agar and Nutrient agar. Distinct colonies exhibiting zones of feather degradation (clear halos) were selected and sub-cultured to obtain pure isolates. (Debnath et al

2020, Zhang *et al.*, 2023).

2.3. Screening for Keratinase Activity

Qualitative screening was performed on Feather Meal agar plates (incubated at 37°C, 5 days); clear zones around colonies confirmed keratinase activity. Quantitative keratinase activity was determined by the tyrosine release method. Culture supernatants were incubated with 1% soluble keratin at 37°C for 20 min; the reaction was stopped with TCA, centrifuged, and absorbance of released tyrosine was read at 660 nm using Folin–Ciocalteu reagent. Activity was expressed as μmol of tyrosine released per mL per minute Subugade *et al.* (2017).

2.4. Morphological and Biochemical Characterization.

Potent isolates were characterized by Morphological & Biochemical analysis. Keratinase-positive isolates were selected for further optimization and detailed investigation. (Cappuccino & Sherman, 2014, Alamnie *et al.* 2023, Nnolim and Nwodo (2020).

2.5 Optimization of Culture Conditions.

The effects of temperature (15–60°C), initial medium pH (5.0–9.0), carbon source (glucose, sucrose, lactose at 1% w/v), nitrogen source (peptone, ammonium sulphate, sodium nitrate at 1% w/v), substrate concentration (0–1.4% w/v feather meal), and incubation time (24–120 h) on bacterial growth (OD600 nm) were evaluated using one-variable-at-a-time (OVAT) methodology. All experiments were performed in triplicate; results are expressed as mean $\pm$ standard deviation. (Stanier R. Y. *et al.* (1976).

2.6. Keratinase Production and Characterization

Enzyme production was conducted at 40°C using dextrose as a carbon source, peptone as a nitrogen source, and 1% feather meal as an inducer. Culture supernatants (crude enzyme) were used for characterization. The effects of temperature (15–80°C), pH (5–9), substrate concentration (0–1.4%), and metal ions (Ca^{2+} , Mg^{2+} , Cu^{2+} at 5 mM) on keratinase activity were evaluated using the tyrosine release method described above. (Rakesh Gupta and Pratibha Ramnani, 2006).

2.7 Application: X-Ray Film Silver Recovery

Used X-ray film strips were incubated with crude keratinase enzyme preparation (1 U/mL) in 50 mM phosphate buffer (pH 7.5) at 40°C for 24 hours. After incubation, the mixture was allowed to settle; the black precipitate was collected, washed, and air-dried. Qualitative confirmation of silver was achieved by the burn test (characteristic dark ash). Quantitative analysis of silver concentration in the treated solution was performed by Atomic Absorption Spectroscopy (AAS) against a silver calibration standard.

3. Results

3.1 Sample Collection, Isolation, Enrichment and Screening

Soil samples collected from different poultry areas from Panvel Taluka and Rasayani enriched in Mineral Salt Medium, yielded 15 microbial isolates.

3.2 Screening for Keratinase Activity

Primary screening on skim milk agar plates identified eleven isolates capable of producing clear zones around the colonies, indicating extracellular protease activity. These positive isolates were further subjected to secondary screening on feather meal agar (FMA) to evaluate their ability to degrade keratin. Among the screened isolates, S3IS3 and A2IS5 consistently produced the largest zones of clearance, reflecting their superior keratinolytic activity. Their ability to efficiently degrade feather meal suggested a higher potential for keratinase production. The feather substrate functioned both as an inducer for keratinase synthesis and as a nutrient source.

Based on these observations, S3IS3 and A2IS5 were selected for further biochemical characterization and detailed investigation.

3.3 Morphological and Biochemical Identification

Both isolates produced white to cream, opaque, irregular colonies with undulate margins. Gram staining revealed Gram-positive rod-shaped cells forming endospores, morphological features consistent with the genus *Bacillus* spp.

Table 1: Biochemical Test

Biochemical Test	S3IS3	A2IS5
Citrate Utilisation	Positive (+)	Positive (+)
Urease Test	Negative (–)	Negative (–)
Catalase Test	Positive (+)	Positive (+)
Oxidase Test	Positive (+)	Negative (–)
Starch Hydrolysis	Positive (+)	Positive (+)
Casein Hydrolysis	Positive (+)	Positive (+)
Lactose Fermentation	Acid	Acid
Sucrose Fermentation	Acid	Acid
Dextrose Fermentation	Acid	Acid
Voges–Proskauer	Positive (+)	Positive (+)
Methyl Red	Positive (+)	Negative (–)
Indole Test	Negative (–)	Negative (–)

3.4 Molecular Identification

The 16S rRNA gene sequence (~1500 bp) of isolate S3IS3 showed 100% similarity with *Bacillus subtilis* strain DSM 10 based on BLAST analysis, with 100% query coverage. Closely related species such as *Bacillus inaquosorum* and *Bacillus cabrialesii* showed >99.9% similarity.

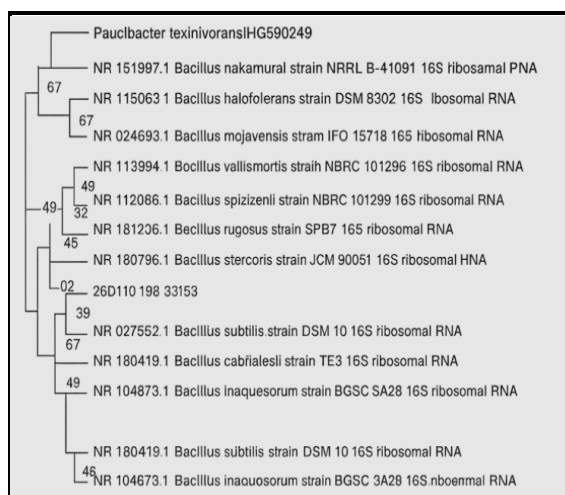
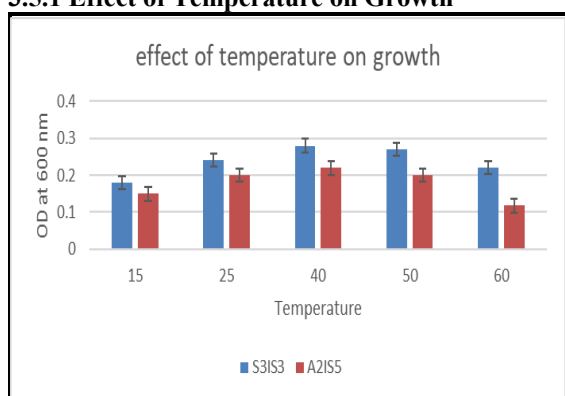


Figure: 1 (16s rRNA Sequencing)

3.5 Optimization of Culture Conditions for Growth

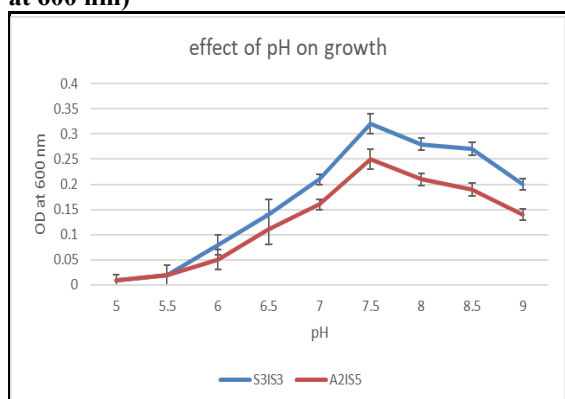
3.5.1 Effect of Temperature on Growth



Graph 1: Effect of Temperatures on Growth

The growth of isolates S3IS3 and A2IS5 was evaluated at temperatures ranging from 15–60°C. Both isolates exhibited maximum growth at 40°C, with OD₆₀₀ values of 0.28 and 0.22, respectively. Growth declined above 40°C, although both isolates retained moderate growth at 60°C. S3IS3 consistently showed higher growth than A2IS5.

3.5.2 Effect of Initial Medium pH on Growth (OD at 600 nm)

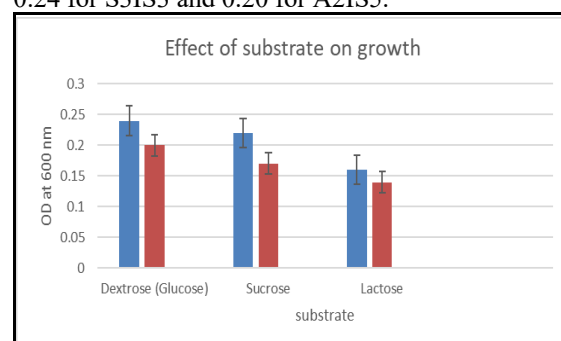


Graph 2: Effect of pH on Growth

Both isolates showed maximum growth at pH 7.5, recording OD₆₀₀ values of 0.32 (S3IS3) and 0.25 (A2IS5). Growth was lower under acidic conditions and declined above pH 8.0, indicating a preference for a slightly alkaline environment.

3.5.3 Effect of Carbon Source on Growth

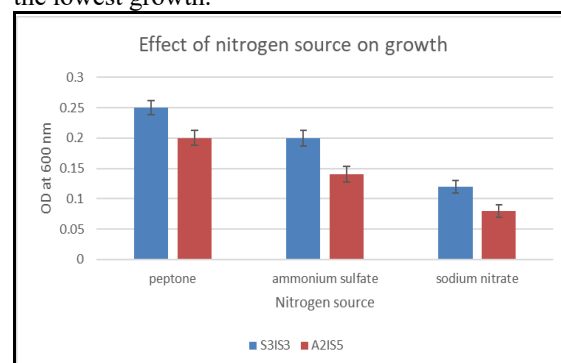
Among the carbon sources tested, dextrose supported the highest growth, followed by sucrose, while lactose resulted in the lowest growth. The maximum OD₆₀₀ values obtained with dextrose were 0.24 for S3IS3 and 0.20 for A2IS5.



Graph 3: Effect of Substrate on growth

3.5.4 Effect of Nitrogen Source on Growth

Peptone was the most suitable nitrogen source producing maximum OD₆₀₀ values of 0.25 for S3IS3 and 0.20 for A2IS5. Ammonium sulfate supported moderate growth, whereas sodium nitrate resulted in the lowest growth.

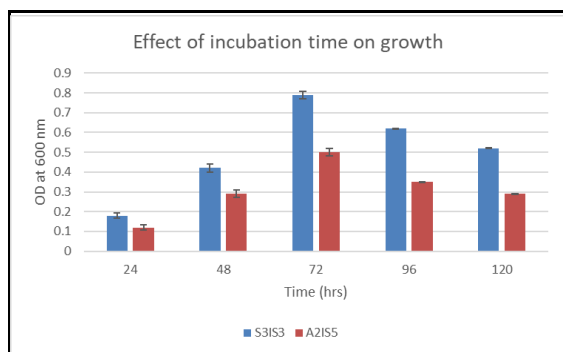


Graph 4: Effect of Nitrogen Source on growth

3.5.5 Effect of Incubation Time on Growth

Growth increased steadily with incubation time and reached its maximum at 72 hours, with OD₆₀₀ values of 0.79 for S3IS3 and 0.50 for A2IS5. A decline in growth was observed after 72 hours, indicating the onset of the stationary phase.

Based on the optimization studies, S3IS3 exhibited superior growth under all conditions tested. The optimum growth conditions were 40°C, pH 7.5, dextrose as the carbon source, peptone as the nitrogen source, and an incubation period of 72 hours. These conditions were selected for subsequent keratinase production and feather degradation studies.



Graph: 5 Effect of Incubation time on growth

4 Keratinase Enzyme Activity of Selected Isolates

A highly linear tyrosine standard curve was obtained over the concentration range of 0–200 µg/ml, with a slope of 0.0005 and an R^2 value close to 1.0, confirming the accuracy and reliability of the assay. This standard curve was used to estimate the amount of tyrosine released during the keratinase assay. Based on the standard curve, isolate S3IS3 exhibited higher keratinase activity (1.0066 µmol/ml/min), corresponding to 546.6 µg/ml of tyrosine released, whereas isolate A2IS5 showed lower activity (0.6872 µmol/ml/min) with 373.3 µg/ml tyrosine released. These findings indicate that S3IS3 is the more efficient keratinase-producing isolate.

5. Characterization of Keratinase Enzyme

The keratinase produced by isolates S3IS3 and A2IS5 was characterized by studying the influence of temperature, pH, substrate concentration, and metal ions on enzyme activity. Both isolates showed maximum keratinase activity at 40°C, with S3IS3 (2.4430 µmol/ml/min) exhibiting slightly higher activity than A2IS5 (2.2589 µmol/ml/min). A gradual decline in enzyme activity was observed at temperatures above the optimum, indicating reduced enzyme stability at higher temperatures.

The enzyme displayed optimum activity at pH 7.5, producing 2.3425 µmol/ml/min and 1.9521 µmol/ml/min for S3IS3 and A2IS5, respectively, suggesting that keratinase is most active under slightly alkaline conditions. Increasing the feather meal concentration enhanced enzyme activity up to 1.0% (w/v), where maximum activities of 2.9465 µmol/ml/min for S3IS3 and 2.6396 µmol/ml/min for A2IS5 were recorded. Beyond this concentration, no significant increase in activity was observed, indicating substrate saturation.

The effect of metal ions showed that Ca^{2+} markedly enhanced keratinase activity, followed by Mg^{2+} , whereas Cu^{2+} strongly inhibited the enzyme. Under all the conditions evaluated, S3IS3 consistently produced higher keratinase activity than A2IS5, demonstrating its superior keratinolytic potential and making it the more promising isolate for further studies and potential biotechnological applications.

6. Silver Recovery from Used X-Ray Films

Incubation of used X-ray film strips with crude keratinase resulted in visible degradation of the

gelatin emulsion layer, with gradual blackening of the solution indicative of silver release. A distinct black precipitate settled at the base of the reaction vessel post-incubation, confirming gelatin hydrolysis and silver liberation.

Qualitative confirmation via burn test produced characteristic dark ash, consistent with the presence of metallic silver. Quantitative AAS analysis detected silver at a concentration of 9.38 ppm in the treated solution, while the control (buffer-treated film without enzyme) yielded negligible silver release. These results confirm that crude keratinase from S3IS3 effectively hydrolyzed the protein-based binding matrix of the X-ray film, enabling silver recovery.

This outcome aligns with prior demonstrations by Cavello *et al.* (2013) and Shankar *et al.* (2010), and extends their findings to indigenous isolates from Indian poultry waste sites. The use of crude enzyme (rather than purified enzyme) demonstrates cost-effective potential for scale-up in industrial waste management contexts.



Figure: 2 X ray Film Degradation





Figure: 3 Burn Test for Silver detection

TEST REPORT					
Report No.	JIC/26/00791	Report Date	09/04/2026		
Name of Customer	Ummehani Sayyad Shah Nawaz.				
Customer Address	CKT College,Khanda Colony.				
Contact Person	Ms.Ummehani Sayyad				
Contact details	9637177169 uhanisayyad@gmail.com				
Name of Sample	Silver Halide				
Sample Conditions	Satisfactory				
Environmental Conditions	25.3°C				
Client Ref. No.	NM	Mfgd Lic No	NM		
Batch No	NM	Batch Size	NM		
Mfg. By	NM	Supplied By	NM		
Mfg. date	NS	Exp. Date	NS		
Sample Qty.	0.01gm	Sampling Date	31/03/2026		
Sample Location	NM	Sample Collected By	Customer		
Sampling Method/Plan	NM	Sample Packing	Sealed Pack Tube		
Sample Received Date	31/03/2026	Sample Ref. No	JIC/26/00791		
Date of Analysis Started	09/04/2026	Analysis Completed Date	09/04/2026		
Results of Analysis					
S.No.	Parameters	Results	Specifications	Units	Method Used
Chemical Parameters					
1	Silver (By AAS)	9.38	-	ppm	As Per USP

Figure: 4 AAS test for silver confirmation

7. Discussion

Keratinolytic *Bacillus subtilis* strains S3IS3 and A2IS5 isolated from poultry waste disposal sites showed significant keratin-degrading potential, with S3IS3 exhibiting comparatively higher keratinase activity than A2IS5.

Optimization studies demonstrated that both isolates achieved maximum growth and enzyme production at 40°C and pH 7.5, with dextrose and peptone serving as the most suitable carbon and nitrogen sources, respectively. Peak growth and keratinase activity were observed after 72 hours of incubation, corresponding to the late exponential growth phase. These findings are consistent with previous reports on *Bacillus* species producing alkaline keratinases under mesophilic to moderately thermotolerant conditions. Enzyme characterization revealed that Ca^{2+} enhanced keratinase activity, whereas Cu^{2+} significantly inhibited it, suggesting characteristics typical of alkaline serine proteases. Substrate concentration studies indicated maximum enzyme activity at 1.0% feather substrate concentration. The crude keratinase effectively degraded the gelatin layer of used X-ray films, enabling recovery of silver particles. Atomic Absorption Spectroscopy confirmed recoverable silver at 9.38 ppm, highlighting the potential application of the enzyme

in eco-friendly biomedical waste management and silver recovery. Overall, the findings indicate that *Bacillus subtilis* S3IS3 is a promising candidate for sustainable keratin waste degradation and related biotechnological applications.

8. Conclusion

The present study successfully isolated and identified *Bacillus subtilis* S3IS3 as an efficient keratinase-producing bacterium from poultry waste disposal sites. Optimization of culture conditions enhanced keratinase production, and the crude enzyme effectively degraded the gelatin layer of used X-ray films, enabling the recovery of 9.38 ppm silver. These findings demonstrate the potential of *Bacillus subtilis* S3IS3 as an eco-friendly and sustainable biocatalyst for feather waste bioconversion and silver recovery, highlighting its promising applications in environmental and industrial biotechnology.

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