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MICROBIAL PROTEOLYTIC ENZYME-MEDIATED DECOMPOSITION AND VALORIZATION OF KERATIN- RICH WASTE

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Abstract

The biodegradation and valorization of keratin-rich waste represent a sustainable strategy for agro-industrial waste management and provide an environmentally friendly alternative to synthetic fertilizers through the production of keratin hydrolysates. The research objective was to isolate bacterial strains exhibiting both keratinolytic and proteolytic activities. Eight bacterial isolates were screened from soil samples collected from the Hide Market, Lahore, Pakistan. Bacterial isolates demonstrated resistance to a broad range of antibiotics and heavy metals and exhibited high tolerance to NaCl. The enzymatic assays revealed that bacterial isolates S3 and S5 exhibited the highest keratinase (S3 = 44.3 U mL-1 and S5 = 59.7 U mL-1) and protease (S3 = 190.6 U mL-1 and S5 = 196.8 U mL-1) activity. Maximum enzymatic activity by bacterial strains S3, S5, S6 and S8 was observed at 45˚C, pH (7.0-9.0), using fructose and lactose as carbon sources and yeast extract as the nitrogen source. Proteolytic strains S3 and S5 successfully biodegraded feather waste and dehaired hides within 48-72 h of incubation. Microbial processing of keratin waste into keratin hydrolysates enhanced the germination rate (100% by hide hydrolysate and 80% by feather hydrolysate) and other growth parameters of Zea mays compared to the control. 16S rRNA gene sequencing identified isolate S5 as Priestia aryabhattai, and the sequence is submitted in GenBank under accession number PQO69771. The enzymatic bioconversion of keratinaceous waste offers an eco-friendly and industrially relevant approach.

Keywords

keratin-rich waste, enzymatic assays, biodegradation, dehairing, keratin hydrolysate, eco-friendly

1. Introduction

The structural biopolymer, keratin, is ubiquitously distributed in nature. Keratin is a recalcitrant biomolecule and cannot be easily degraded, resulting in the environmental accumulation of keratinous waste (Lai, Wu, Zheng, Li, & Wang, 2023). As the global human population continues to expand, keratin waste has emerged as a significant environmental concern due to the large quantities generated annually by the poultry, leather, and meat-processing industries (Anbesaw, 2022). Effective management and valorization of these waste streams are essential for environmental sustainability. The high abundance and slow degradation rate of keratinous waste generated by leather processing industries have substantially contributed to environmental pollution (Nnolim, Udenigwe, Okoh, & Nwodo, 2020). The leather industry, which dates back to early human civilization, provides valuable products like hides and skins for the manufacture of upholstery, clothing, and accessories and is closely associated with the textile industry (Yorgancioglu, Başaran, & Sancakli, 2020). The leather industry valued at approximately USD 100 billion annually, contributes significantly to the global economy stability and serves as an important component of the circular economy. More than 99% of the leather is processed from raw animal hides owing to increased demand for leather in developing countries(Vidaurre-Arbizu, Pérez-Bou, Zuazua-Ros, & Martín-Gómez, 2021). Leather processing units generate enormous quantities of solid waste including raw and finished trimmings, fleshings, buffing dust, hair waste, splitting scraps and chrome shavings, resulting in considerable environmental pollution due to inadequate waste-management practices. Improper waste disposal also contributes to unpleasant odors and greenhouse-gas emissions (Mella, Benvenuti, Oliveira, & Gutterres, 2019). The disposal of chromium-containing solid waste into water bodies poses significant risks to environmental and public health. Adoption of sustainable management of wastes strategies, prudent use of chemicals, and adoption of waste minimization techniques are some measures to mitigate the environmental impacts of hide and skin processing (Yorgancioglu *et al*., 2020). Moreover, traditional methods of dehairing in leather industries use more than 75 chemicals, including lime, sulfide and other alkalis (Akhter, Wal Marzan, Akter, & Shimizu, 2020). Leather industry is one of Pakistan's largest revenue-generating sectors and provides employment and export earnings. About half a million people rely on this sector for their livelihood (Ali *et al*., 2022). Although the sector is economically significant, it generates huge amounts of waste approximately 40 million skins and 7.6 million hides are produced annually. Lahore is one of Pakistan's major industrial hubs and a hotspot for hide-processing units, producing substantial quantities of keratinous waste each year (Butt *et al*., 2021). The poultry industry is the second-largest agricultural industry in Pakistan, and the country is one of the leading poultry producers worldwide. According to the Pakistan Poultry Association (PPA), approximately 2.27 billion broilers, 89.7 million layers and 18.3 billion breeding-stock birds are produced annually (Government of Pakistan, 2026). This level of production generates large quantities of broiler litter, with feathers constituting

a substantial proportion of the waste stream. Consequently, the continued expansion of the leather and poultry industries presents a growing challenge to environmental sustainability (Shaukat, Sattar, & Munir, 2020). The characterization of indigenous keratinolytic microorganisms are therefore important, particularly in areas where conventional keratin-waste management practices are limited, as locally adapted strains may provide stable and efficient bioconversion solutions (Park *et al*., 2023). Microbial protease-producing organisms, including actinomycetes, fungi, and bacteria, are widely distributed in soil, industrial residues, and waste-rich environment. Bacterial species, in particular have shown remarkable keratinolytic ability and have been extensively investigated (Lai *et al*., 2023). Microbial proteases offer an eco-friendly solution for the management of keratinous waste. Compared to traditional chemical treatments used for leather processing, enzymatic degradation minimizes amino-acid loss, valorize the waste, and has applications in industries from many sectors (Gahatraj *et al*., 2023). Enzymatic remediation is an eco-friendly alternative for waste valorization, which generates fewer toxic by-products and enhances recovery (Oyewole, Idris, Bello, Yakubu, & Saidu, 2022). The keratinase discovery represented a major milestone in microbial enzymology and highlighted the ability of certain microorganisms to utilize keratin as a nutrient source (Mohaghegh, Hosseini, Moosavi-Nejad, & Qamsari, 2026). This discovery opened new avenues for biotechnological applications, including feather bioconversion, hair waste valorization into digestible protein products, sustainable leather processing, and the production of bioactive peptides (Mokrani & Nabti, 2026). Subsequent investigations into keratinase diversity, regulation, and catalytic efficiency have further expanded its industrial significance, particularly in animal-feed production, leather processing, and sustainable agricultural practices (Thadiyan, Sharma, & Gupta, 2025). Bacterial proteases are highly effective biocatalysts and provide a green biocatalytic process for the bioconversion of keratinous waste materials. Their application has gained considerable attention as an effective strategy contributes to the reduction of keratin waste and the recovery of valuable resources (Park *et al*., 2023). Keratin hydrolysates produced through microbial biodegradation exhibit reduced environmental persistence and enhanced nutrient bioavailability, making them suitable a eco-friendly biofertilizers. These hydrolysates promote plant growth and soil biological activity owing to their high nitrogen content and diverse amino-acid composition (Możejko & Bohacz, 2023). The use of proteolytic enzymes has transformed conventional industrial practices by enabling bio-based production systems characterized by high substrate specificity and minimal generation of toxic by-products (Nnolim *et al*., 2020). Enzymatic bioremediation has been increasingly adopted across various industrial sectors, including the leather, plastic and textile industries, where it contributes to pollution mitigation and process efficiency (Niyonzima *et al*., 2023). The application of proteolytic enzymes for the management of keratin waste reduces dependence on hazardous chemicals provides a sustainable alternative to conventional treatments methods (Ekeoma *et al*., 2023).

Environmentally friendly enzymatic processes minimizes ecological impacts while maintaining operational efficiency and reducing potential health risks associated with toxic chemicals (Gupta, Chakraborty, & Sarkar, 2023). Detoxification, bioremediation and bioconversion are among the major industrial applications of microbial enzymes. The high enzymatic specificity and sustainability contributed to reduced environmental impact and enhanced production efficiency (Adetunji, Olaitan, Erasmus, & Olaniran, 2023).The continuous exploration of keratinolytic microorganism is expected to facilitate the discovery of more stable proteases capable of improving degradation rates, process efficiency and sustainability in waste-management systems. Several bacterial species are known for their remarkable keratinolytic activity and making them promising candidates for industrial exploitation.In the present study, we isolated and characterized keratinase-producing bacteria obtained from a keratin-rich environment. Impact of different environmental conditions and substrate availability on enzyme production was evaluated. Bacterial isolates were also examined for tolerance to varying salt concentrations and heavy metals, as a measure of their robustness and potential suitability for agro-industrial applications. To evaluate the agricultural relevance, keratin hydrolysates produced through microbial degradation were tested as biofertilizers, with effects assessed on the growth parameters of *Zea mays.* This work links environmental bioremediation with waste valorization, offering a sustainable route for keratinous-waste management while generating useable agricultural input. The findings provide valuable insight into sustainable agricultural practices based on keratinase- mediated biodegradation and support the development of circular bioeconomy strategies.

2. Methodology

2.1 Screening and Biochemical Profiling of Keratin-Degrading Bacteria

Soil samples were collected from the Hide Market, Lahore (31° 17' 27" N, 74° 20' 11" E) and transported in sterile plastic bags (Figure 1**).**



Figure 1: Soil sample collection site from Hide Market, Lahore.

For isolation of keratinolytic and protease-producing bacteria, soil samples were serially diluted and screened on skim milk agar to evaluate protease activity and feather meal agar to evaluate keratinase activity. The plates were incubated at 37°Cfor 24-48 h (Latafat, Siddiqui, Ashish, Vimal, & Bhargava, 2024). Skim milk agar was prepared by dissolving 28g of skim milk powder and 15g of agar in 1000mL

of distilled water. Protein hydrolysis was indicated by the formation of clear zones around the bacterial colonies. Feather meal agar medium (1.0% feather meal, 0.05% NH4Cl, 0.05% NaCl, 0.04% K2HPO4, 0.03% KH2PO4, 0.01% MgCl2, and 0.01% yeast extract, pH 7.5) was used for the growth of keratinolytic bacteria (Moonnee, Foysal, Hashem, & Miah, 2021).Biochemical characterization was performed based on Gram staining, catalase and oxidase production, starch hydrolysis, and growth on MacConkey agar and Mannitol salt agar (Yadav & Khosla, 2021). In addition, resistance to a broad range of antibiotics and heavy metals as well as halotolerance to NaCl, was evaluated. The disc diffusion method was used to determine resistance against five broad-spectrum antibiotics including ampicillin, chloramphenicol, rifampin, streptomycin and gentamicin (Shah *et al*., 2017). The plate dilution method was used to assess resistance against different concentrations of heavy metals ($PbCl_2$, $K_2Cr_2O_7$, $HgCl_2$ and $CuSO_4$) (El-Sayed, Abdellatif, Mostafa, Elsehemy, & Kobisi, 2024). Luria agar (L-agar) supplemented with different concentrations of NaCl was used to assess the halotolerance of bacterial strains (Masi, Gemechu, & Tafesse, 2021).

2.2 Enzyme Activity Assays and Optimization

Crude enzyme extract was used for the determination of keratinase and protease activities. For this purpose, 72-h-old bacterial cultures were prepared by inoculating basal medium with 2 % bacterial inoculum under continuous shaking conditions, followed by centrifugation at 8,000 rpm for 15 min at 4°C. The resulting cell-free supernatant was used as the crude enzyme extract for enzymatic assays (Nachimuthu & Kathirvel, 2023). For keratinase activity determination, the reaction mixture consisted of crude enzyme extract, Tris-HCl buffer, and keratin azure as the substrate. The reaction mixture was incubated at 45˚C and subsequently centrifuged at 10,000 rpm. The absorbance of the supernatant was measured at 595 nm. One unit (U mL^{-1}) of keratinolytic activity was defined as an increase in absorbance of 0.01 under the assay conditions (Batool, Akhthar, & Saleem, 2021).For protease activity determination, a gelatin solution (0.65% w/v) was incubated with crude enzyme extract in 0.1 M Tris-HCl buffer. The reaction was terminated using 10% trichloroacetic acid, followed by incubation for 10 min and centrifugation at 10,000 rpm for 15 min at 4°C. The resulting supernatant was mixed with sodium carbonate and Folin–Ciocâlteu phenol reagent and incubated at 37°Cfor 30 min. The absorbance of the reaction mixture was measured at 660 nm. One unit of protease activity was defined as the amount of enzyme required to release 1 µmol of tyrosine mL^{-1} min^{-1} from gelatin under the assay conditions (Mushtaq *et al*., 2021). Physicochemical parameters were optimized to obtain maximum bacterial growth and enzyme production using One Factor at a Time (OFAT) approach (Sudarsanam & Frey, 2011). Proteolytic activity was evaluated over a pH range of 6-9 and at different temperatures (25°C, 37°C, 45°C). The effects of different nitrogen sources (yeast extract, beef extract, peptone and gelatin) and carbon sources (lactose, glucose, fructose and sucrose) on enzyme production were also investigated (Beryl *et al*., 2021).

2.3 Application of Keratinase in Agro-Industrial Processes

Keratinase enzymes provide a biological alternative to conventional chemical methods and contribute to the biodegradation of keratin-rich waste materials, including feathers and hair and bio-convert them into animal-feed supplements and biofertilizers (Ben Elhoul *et al*., 2021). Fresh sheep hides were collected from a hide market in Lahore and chicken feathers from a local poultry shop, respectively, for the evaluation of keratinase activity. The hides were cut into pieces, washed thoroughly, and preserved to prevent spoilage, whereas the feathers were cleaned, autoclaved, and stored in sterile jars at room temperature. Basal medium containing autoclaved feathers was inoculated with keratinolytic strains (S3 and S5) and incubated at 45°Cunder continuous shaking (80–100 rpm) at pH 8.0. Following 72 h of incubation, feather degradation was assessed and compared with the uninoculated control to evaluate keratinolytic efficiency (Almahasheer *et al*., 2022). Pieces of sheep hide were incubated with crude enzyme extract at 37˚C for 72 h. Additional hide samples incubated with Phosphate-buffered saline (PBS) as the control treatment. The extent of hair removal was assessed following incubation (Moonnee *et al*., 2021).

2.4 Eco-Friendly Hydrolysate Production and Application in Zea mays Cultivation

Conventional methods for processing keratinous waste, including feathers and hides, are often unsustainable and may result in the loss of valuable nutrients. Microbial hydrolysis using keratinase and protease-producing bacteria represents an environmentally sustainable alternative for the valorization of keratin-rich waste and its application in agriculture. Submerged cultivation was employed for the biodegradation of keratin present in feathers and hides using keratinolytic bacterial strains. Minimal salt medium supplemented with feathers or hide material served as the substrate for proteolytic enzyme activity and was inoculated with bacterial isolates S3 and S5. The reaction mixtures were incubated at 30°Cfor 3-4 days. Hydrolysates were prepared through filtration, autoclaving and centrifugation following incubation (Moktip *et al*., 2025).*Zea mays* seeds were cultivated in clay pots under controlled greenhouse conditions. Experimental plants were irrigated with feather hydrolysates, hide hydrolysates, and bacterial suspensions of each isolate. Control plants received tap water only. Growth parameters including germination rate, vigor index, root length, shoot length, root number and leaf number, were recorded one month after germination and compared with the control treatment (Saleem *et al*., 2025).

2.5 Statistical Analysis

All experiments were performed in triplicate to ensure reproducibility (Tang *et al*., 2020). Data were analyzed using analysis of variance (ANOVA) to determine statistically significant differences among treatments. Differences were considered statistically significant at $p < 0.05$.

2.6 Identification of Proteolytic Strains

The most potent bacterial isolate S5 was selected for 16S rRNA gene sequencing and was submitted to Macrogen Inc. (Seoul, South Korea). Basic Local Alignment Search Tool (BLAST), was used to analyze the obtained sequence, available at the National Center for Biotechnology Information

(NCBI). The nucleotide sequence of isolate S5 was deposited in the GenBank database for the accession number.

3. Results

3.1 Screening and Biochemical Profiling of Keratin-Degrading Bacteria

Microbial proteases are predominantly isolated from keratinous waste or soil contaminated with keratin-rich materials. Sevaral bacterial species have previously been isolated from keratinous-waste particularly feather waste (Akhter *et al*., 2020). In present study, eight bacterial isolates (S1, S2, S3, S4, S5, S6, S7 and S8) were recovered from keratin-rich environments and subsequently screened for keratinolytic and proteolytic activities on skim milk agar and feather meal agar. Among the eight isolates, four strains (S3, S5, S6 and S8) hydrolysed both skim milk agar and feather milk agar (Figure 2).

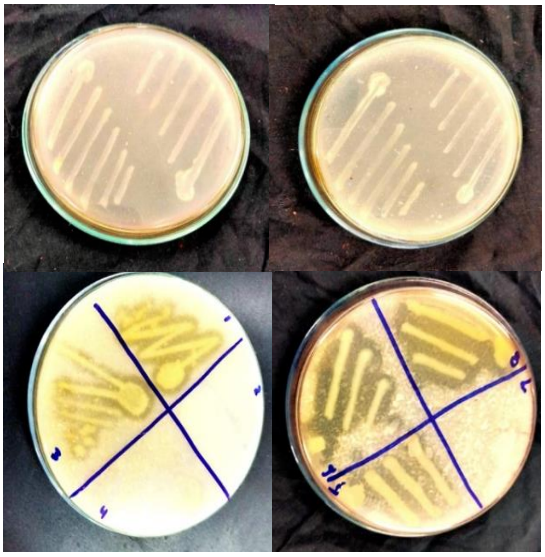


Figure 2: Hydrolyzing zones on (a) Skim Milk Agar indication of protease activity (b) Feather Meal Agar indicating keratinase activity**.**

All bacterial isolates exhibited diverse cellular and colonial morphologies. Biochemical characterization was performed using gram staining, catalase and oxidase production, starch hydrolysis, and growth on MacConkey agar and Mannitol salt agar. Isolates S3 and S4 were identified as Gram-negative rods whereas Isolates S1, S2, S5, S6, S7 and S8 were identified as gram positive rods or cocci. All bacterial isolates were positive for catalase and oxidase activities, whereas isolate S2 was negative for oxidase activity. Triple Sugar Iron (TSI) test results indicated that isolates S3 and S4 fermented glucose, whereas no sugar fermentation and gas production was observed in the remaining isolates. Methyl Red-Voges Proskauer (MRVP) test results varied among the isolates. Approximately 50% of the isolates (S1, S2, S4, S6) were positive for the Methyl Red test, indicating stable acid production following glucose fermentation whereas isolates S3, S5, S7 and S8 were negative. Similarly, isolates S3, S4, S6 and S8 exhibited positive Voges-Proskauer reaction, indicating acetoin production, whereas isolates S1, S2, S5 and S7 were negative. Starch hydrolysis was observed only in S5 isolate. Several biological factors including genetic variability, biofilm formation and horizontal gene transfer may

contribute to the development of antibiotic resistance in proteolytic bacterial species. Antibiotic susceptibility profiling revealed 100% resistance to gentamicin and chloramphenicol among the tested isolates. Resistance to ampicillin and streptomycin was observed in approximately 75% of the isolates, whereas sensitivity to rifampin was recorded. Proteolytic bacterial species frequently exhibit tolerance to heavy metals because they are often associated with contaminated environments. The highest tolerable concentrations of heavy metals exhibited by the selected bacterial isolates were 2500 μg mL^{-1} for $PbCl_2$, 1000 μg mL^{-1} for $K_2Cr_2O_7$, 500 μg mL^{-1} for $HgCl_2$, and 1500 μg mL^{-1} for $CuSO_4$. Proteolytic bacterial species are capable of tolerating elevated salt concentrations and have frequently been reported from saline and marine environments. In the present study, the highest NaCl tolerance (2.4M) was observed in isolates S1, S2, S4, S5, and S8 (Table 1).

Table 1: Antibiotic and heavy metal resistance profiling of bacterial isolates

Antibiotics/Heavy metals	Bacterial strains							
	S1	S2	S3	S4	S5	S6	S7	S8
Antibiotics (μg mL^{-1})								
AM	10	10	10	10	10	0	0	10
CHL	30	30	30	30	30	30	30	30
S	10	10	10	10	10	10	10	10
CN	10	10	10	10	10	10	10	10
RA	0	0	0	0	0	0	0	0
Heavy metals (μg/ml)								
$PbCl_2$	2500	1500	2500	2500	1500	2500	250	2500
$K_2Cr_2O_7$	1000	1000	1000	750	250	1000	100	1000
$CuSO_4$	250	1000	1000	250	250	1500	1500	1500
$HgCl_2$	250	500	500	100	100	500	100	500

AM= Ampicillin, CHL= Chloramphenicol, S= Streptomycin, RA= Rifampin

3.2 Enzyme Activity Assays and Optimization

Approximately 80 % of industrial enzymes are used as biological catalysts, with hydrolases, particularly proteases, representing a major enzyme group. Enzymatic assays revealed that isolates S3 and S5 exhibited the highest keratinase and protease activities among all tested bacterial strains. Isolate S5 demonstrated the maximum keratinase activity (S5 = 59.7 U mL^{-1}) and protease activity (S5 = 196.8 U mL^{-1}) whereas isolate S3 exhibited comparatively lower keratinase (S3 = 44.3 U mL^{-1}) and protease (S3 = 190.6 U mL^{-1}) activities Therefore, isolate S5 was selected as the most promising keratinolytic and proteolytic bacterium for further characterization

and molecular identification. The findings were further supported by optimization studies in which isolate S5 consistently exhibited superior enzymatic performance under the tested environmental conditions (Figure 3).

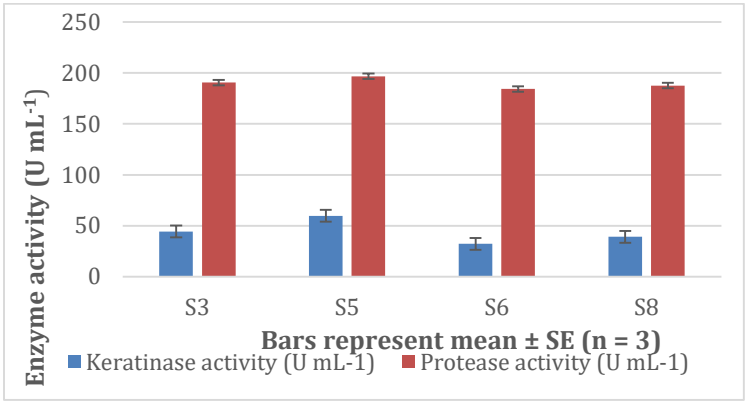


Figure 3: Keratinase and protease activities in bacterial isolates.

The proteolytic enzyme production significantly influenced by environmental conditions. Maximum keratinolytic activity of isolates S3, S5, S6 and S8 were observed at 45 °Cand pH 9.0 (Figures 4 and 5).

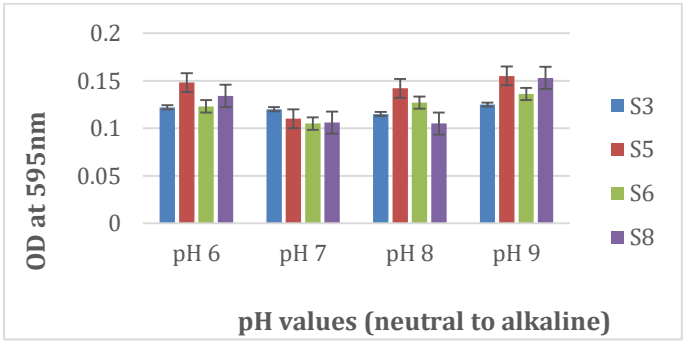


Figure 4: Optimization of proteolytic activity at variable pH values

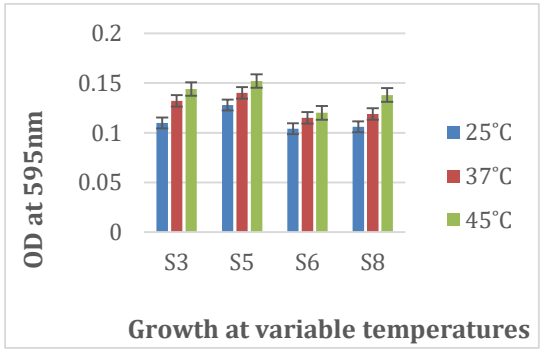


Figure 5: Optimization of proteolytic activity at different temperatures.

Different carbon sources (glucose, lactose, sucrose, fructose) and nitrogen sources (gelatin, peptone, yeast extract, beef extract) were evaluated to determine the optimal nutritional

requirements of the selected isolates. The results demonstrated that bacterial growth was significantly influenced by the carbon source provided. Fructose and lactose supported the highest bacterial growth among the tested substrates. A clear difference in carbon source preference was observed between Gram-positive and Gram-negative isolates. Fructose supported maximum growth in Gram-positive isolates (S5, S6, S8), whereas lactose supported maximum growth in Gram-negative isolate S3. Bacterial growth also varied in response to different nitrogen sources. Yeast extract supported the highest growth of all tested isolates because it provides a rich source of organic nitrogen, vitamins and growth-promoting nutrients as shown in Figures 6 and 7.

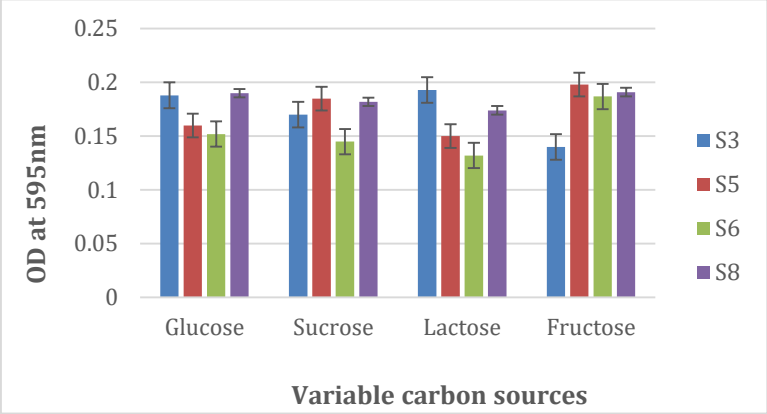


Figure 6: Optimization of proteolytic activity using variable carbon sources

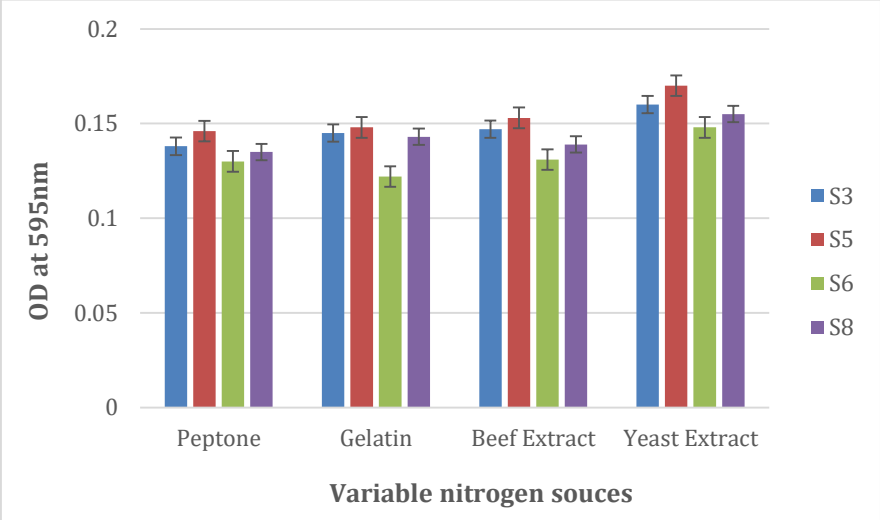


Figure 7: Optimization of proteolytic activity using variable nitrogen sources.

3.3 Application of Keratinase in Agro-Industrial Processes:

Keratinase has emerged as environmentally sustainable tool for the management of keratinous waste in agro-industrial systems. Bacterial

keratinases possess the ability to biodegrade feathers and facilitate the biological dehairing of animal hides within a relatively short incubation period. In the present study, isolates S3 and S5 successfully biodegraded feather waste and dehaired hides within 48-72 h of incubation (Figures 8 and 9).



Figure 8: Biodegradation of keratin in feathers: (a) Complete degradation of feather keratin by S3 isolate (b) S5 isolate completely degraded the feather keratin as shown in picture.

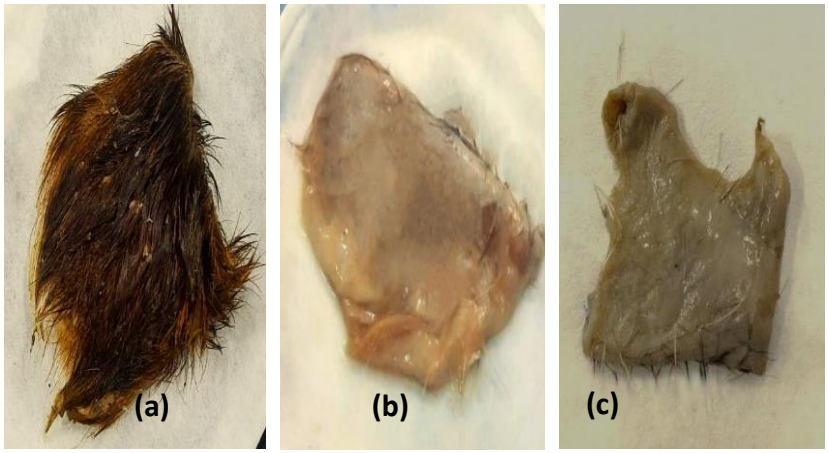


Figure 9: (a) Control - incubated with PBS and without bacterial inoculum (b) Dehairing of raw hide on incubation with S5 strain (c) Dehairing of raw hide on incubation with S3 strain

3.4 Eco-Friendly Hydrolysate Production and Application in Zea mays Cultivation

Microbial conversion of keratin waste into keratin hydrolysates enhanced germination and growth parameters of Z*ea mays* compared with the control treatment. The hydrolysates demonstrated potential as biofertilizers, particularly hide hydrolysates, provided improved nutrient availability following biodegradation. Hide hydrolysates appeared to provide a more favorable substrate than feather hydrolysates, resulting in enhanced plant-growth responses. Plants treated with Hide Hydrolysate (HH) exhibited increases in vigor index of 50.6% in S3 treated plants and 71.7% in S5 treated plants

compared with plants treated with Feather Hydrolysate (FH), indicating the superior performance of HH (Table 2).

3.5 Statistical Analysis:

One-way ANOVA revealed significant differences among treatments, with the HH treatment exhibiting the best overall growth performance, while the control showed the lowest values.

Table 2: Evaluation of growth parameter of corn plants treated with keratin hydrolysates

Sr. no	Bacterial Strain	Vigor Index	Germination %	Seedling Length (cm)	Shoot Length (cm)	Root Length (cm)	No of roots	No of shoots
1	Control	832	40	20.8± 0.4	13.6± 0.9	7.1±0. 6	6.6± 0.4	3.3± 0.3
2	S3+ FH	240 0	80	30.0±1 .5	19.5±0 .7	10.5±0 .8	7.6± 1.6	3.6± 0.3
3	S3+ HH	412 0	100	41.2± 1.2	25.9±1 .0	15.3± 0.3	8.6± 2.6	4±0. 0
4	S3 Bacteria	166 2	60	27.7± 1.5	17.3± 0.9	10.4± 0.5	7.6± 1.5	3.6± 0.3
6	S5+ FH	308 8	80	38.6± 1.6	24.4± 1.6	14.2± 0.3	7±1. 5	3.6± 0.3
7	S5+ HH	465 0	100	46.5± 2.6	30.5 ±0.7	16±0. 8	8.6 ±1.2	4±0. 0
8	S5 Bacteria	181 6	80	22.7± 1.8	14.8± 1.9	7.8±0. 3	6.6 ±1.8	3.3± 0.3

Each value is a mean (±SE) of three replicates, statistically analyzed by one-way ANOVA

Each value is a mean (±SE) of three replicates, statistically analyzed by one-way ANOVA

3.6 Identification of Proteolytic Strains

Isolate S5, which showed the strongest keratinolytic activity, was identified by 16S rRNA gene sequencing as *Prestia aryabhattai*. The nucleotide sequence was deposited in the NCBI GenBank database under accession number PQO69771.

4. Discussion

The present study demonstrated that soils collected from leather-processing environments harbor diverse keratinolytic bacterial populations, highlighting the ecological adaptation of these microorganisms to keratin-rich habitats (Park *et al*., 2023). Hydrolases, particularly proteases such as keratinases, represent one of the major categories of enzymes used in agro-industrial applications because of their combined industrial and ecological significance, accounting for approximately 65% of enzyme applications (Sobucki *et al*., 2019). Several proteolytic bacterial strains were isolated from keratinous waste-contaminated soils, demonstrating the favorable adaptation of these bacteria to keratin-rich environments (Naseer, Fayyaz, Sharif, & Sarfraz, 2022). Similarly, *Bacillus cereus* KP015746 (Lr3/2), isolated from spoiled leather, exhibited strong keratin-degradation potential (Kate, 2023). In addition, *Bacillus cereus* recovered from feather substrate demonstrated considerable bioremediation potential through the keratinase activity (Butt *et al*., 2021). Industrial keratinous wastes generated by poultry and leather processing industries represents

abundant, inexpensive and readily available substrates for microbial enzyme production (Chu *et al*., 2026). The successful isolation of efficient keratin-degrading bacteria in the present study indicates the adaptive enrichment of those microorganisms in the keratin-rich environment. The simultaneous production of keratinases and associated proteolytic enzymes indicates synergistic enzymatic system is required for efficient keratin degradation. Keratinases target the condensed structure of keratin by breaking disulfide bonds and a high-order protein fold and exposing more cleavage sites that can be cleaved by broad spectrum proteases (Luo *et al*., 2026). Studies on feather degrading microorganisms have reported that combinations of keratinase, protease and accessory proteins, including disulfide reductases, results in higher degradation efficiency than individual enzymes alone, highlighting the importance of complementary enzyme systems for efficient keratin biodegradation (Chu *et al*., 2026). These observations support the effectiveness of the isolates exhibiting both keratinolytic and proteolytic activities, evaluated in the present research and further demonstrate their potential for agro-industrial applications. Available literature suggests that keratin degradation is more frequently observed among Gram-positive bacterial genera as compared with Gram-negative bacteria due to their efficient extracellular enzyme secretion systems and efficient utilization of complex keratin-containing substrates, consistent with the present study, the isolate with highest keratinolytic activity was identified as Gram-positive bacteria (Mpaka, Nnolim, & Nwodo, 2025).Antibiotic resistance is a frequently reported characteristic of keratinolytic and proteolytic bacteria. Species belonging to the genera *Pseudomonas* and *Bacillus* are known have been known to have mechanisms of antibiotic resistance and this can be attributed to their environmental adaptation, genetic diversity, biofilm formation and horizontal gene transfer (Nnolim *et al*., 2020). Consistent with these observations, keratinase producing isolates from the present study showed resistance to a range of broad-spectrum antibiotics. Furthermore, the observed tolerance to heavy metals and salt concentrations, indicating their viability and enzymatic activity in metal contaminated environments and ensuring effective keratin degradation under saline conditions, highlighting their potential for biotechnological processes (Venkatachalam, Rathinam, Rao, & Krishnan, 2022).Variations in proteolytic enzyme production among microorganisms are influenced by genetic variability, nutritional requirements and environmental adaptation, and sensitivity to physicochemical parameters such as iconic strength, pH and temperature (Koutb, Hassan, Morsy, & Bagy, 2023). Microbial keratinases exhibit optimal activity within a temperature range of 45–65°C, and under alkaline pH range of 8.0-10.0 (Chu *et al*., 2026). *Bacillus genus* emerged as a most extensively characterized and industrially relevant sources of proteases, primarily due to its ability to produce high enzyme yields. Consequently, *Bacillus* species have frequently been served as reference models for enzyme optimization studies (Maduahasova, Baltin, Bizhanova, Khassenov, & Aktayeva, 2025). Prior studies have reported, optimum enzyme production among bacillus species within the temperature range

of 40-45 °C including *Bacillus licheniformis Strain NBRC 14206* at 40 °C and pH 9.0 (Ire & Onyenama, 2017), *Bacillus velezensis* is t 42 °C within a pH range of 7-8 (Khan *et al*., 2024), *Bacillus cytotoxicus* LT-1 at 40 °C and pH 7.0 and *Bacillus aerius NSMk2* at 45 °C with pH 9.0. In the present study, the isolates exhibited maximum enzyme production at 45 °C across neutral to alkaline pH conditions, consistent with previously studied keratinase-producing bacteria.Influence of various carbon (lactose, glucose, sucrose, fructose) and nitrogen sources (gelatin, peptone, yeast extract, beef extract) are observed on enzymes yield and activity. Optimal proteolytic activity was observed with fructose and lactose as carbon source in conjunction with yeast extract as nitrogen source, highlighting the synergistic effect of specific carbon-nitrogen combination on enzyme production. The present study results consistent with those reported in prior studies, simple sugars and complex nitrogen combination often upregulate keratinase genes in bacteria (Derhab, Mabrouk, El-Metwally, & Mohammed, 2024). Comparable findings have been documented elsewhere, optimal production obtained particularly when supplemented with lactose as carbon source and yeast extract as the nitrogen source, suggesting that the rapid metabolization of sugars support bacterial growth and essential amino acids and vitamins in nitrogen complex, provide favorable conditions for enzyme synthesis (Ire & Onyenama, 2017). The present study results highlight the synergistic effects of carbon-nitrogen combinations for maximum proteolytic enzyme production under controlled conditions.The keratinase producing Isolates have shown significant potential in feather biodegradation and facilitate dehairing corroborating previous research, highlighting their industrial capability for keratinous waste management. *Bacillus species* such as *Bacillus pumilus, Bacillus cereus, Bacillus lichenifomis* and *Bacillus subtilis* have demonstrated effective feather degradation, making it suitable for managing poultry solid waste through feather degradation (Li, 2021). In addition to feather degradation, keratinases have also been employed in the leather industry for dehairing hides, presents an eco-friendly alternative. Keratinase-producing bacteria, such as *Bacillus tropicus LS27 (Liya et al., 2023), B. cereus KJ72442 (Kate, 2023), Bacillus sp. KLP1* (Venkatachalam *et al*., 2022), and *Actinomadura viridilutea DZ50* (Ben Elhoul *et al*., 2021), have shown significant efficacy in dehairing operations. Microbial hydrolysis of keratin-rich substrates such as feathers and hides produces keratin hydrolysates, have demonstrated potential as biofertilizers for maize cultivation. Compared to feather keratin, the superior solubility of hide-based hydrolysates, facilitates their application and integration into soil and plant systems, providing essential nutrients to plants more efficiently, resulted in increased germination of seeds (Mazotto, Cedrola, de Souza, Couri, & Vermelho, 2022). Plants treated with Hide Hydrolysate (HH) showed increase in vigor index by 50.6% in S3 inoculated plants and 71.7% in S5 inoculated plants, compared to plants treated with Feather Hydrolysate (FH), reflecting the better performance of HH over FH.16S rRNA gene sequencing identified isolate S5, as closely related to *Priestia aryabhattai,* providing a molecular basis for interpreting its potential functional potential. The

sequence was deposited in NCBI GenBank under accession number PQO69771. This species is recognized for its keratinolytic and proteolytic activity, halotolerance and plant growth-promoting traits, a functional profile consistent with previous studies (Dhar *et al*., 2024). The observed characteristics of isolate S5 suggest that it is physiologically robust strain with real potential for sustainable agricultural practices.The study is not without limitations. Enzymatic activity depends largely on environmental conditions and enzyme specificity towards particular keratin substrates, which may alter its catalytic capability with diverse waste materials. Scaling enzyme production to an industrial process may present practical challenges that fall outside the scope of this work. Even so, the rapid biodegradation of feathers and hides by bacterial isolates suggests promising opportunities in the industrial and agricultural sectors including its use as microbial biofertilizers. The development of more robust engineered microbial strains would allow biotechnological innovations, green technological waste management and promote circular bioeconomy.

5. Conclusion

The present work concludes that microbial proteolytic enzymes have a great potential in the biodegradation and sustainable valorization of keratin-rich wastes, but their activity can be influenced by substrate specificity and environmental conditions. Therefore, optimal enzymatic conditions should be identified for bioconversion of agro-industrial waste and their scalable applications. Furthermore, the effective degradation of hides and feathers by bacterial isolates, coupled with their potential to enhance the growth of *Zea mays* as biofertilizers, highlights their relevance for sustainable biobased solutions.

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