Journal of Molecular Science

Journal of Molecular Science
Volume 36 Issue 2, Year of Publication 2026, Page 549-557
DoI-10.004687/1000-9035.2026.065

Journal of Molecular Science

www.jmolecularsci.com ISSN:1000-9035

Sampling, Isolation and Profiling of Keratinase Producing *B licheniformis* from Poultry Environment Setting and Enzyme Characterization Study

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Article Information
Received: 18-05-2026
Revised: 04-06-2026
Accepted: 21-06-2026
Published: 07-07-2026

Keywords
Keratin, keratinase, microbes, B licheniformis, enzyme activity, specific activity, kinetic constant.

ABSTRACT
Keratin-rich wastes are challenging to break down because the protein chains are tightly packed and strongly supported by numerous hydrogen bonds and hydrophobic interactions. Considering the increasing global demand of proteolytic enzymes and precisely keratinase, isolation, purification and characterization studies were carried out. Hydrolysing the peptide bonds in keratin's polypeptide chains is how keratinases work. The enzyme's ability to interact with the hydrophobic and stiff structure of keratin depends on certain structural characteristics. In the present study (2022-2025) of five poultry farms from districts Jagtial, Sircilla, Karimnagar, Nizamabad and Hyderabad districts of poultry litter, poultry water, and commercial feed. In total, 125 samples were acquired from each farm, including soil, litter, feed and water samples. B *lichenformis* is key bacterium in all the poultry samples profoundly present in all the regions of sampling. Bacillus licheniformis strain wx1, Bacillus licheniformis strain DAS-2, Bacillus licheniformis strain GS01, and Bacillus licheniformis strain GS3 had nucleotide sequences of 16s rRNA genes that were 98.6%, 98.5%, 98.4%, and 98.4% like those of the closest bacterial relatives. The protein content of crude and culture supernatant keratinase enzyme was reported 1.68 and 1.24 mg/ml. The purified enzyme had shown 70.89 and 60.31% activity at temperature between 40 and 50^{0}C which further declined after 70^{0}C. The enzymatic activity and stability were declined in lower temperature below 20^{0}C.

1. INTRODUCTION:

Keratin-rich wastes are challenging to break down because the protein chains are tightly packed and strongly supported by numerous hydrogen bonds and hydrophobic interactions **(Sypka et al. 2021).** Additionally, polypeptide chains are mechanically resistant and cannot be broken down by conventional proteolytic enzymes because to the many disulfide linkages that cross-links them (**Fang et al. 2016**). Waste is produced in addition to the enormous expansion of the chicken industry for food and food products (**Nnolim et al. 2020**). Primary waste materials generated in chicken processing operations include feathers, soft flesh, blood, deboning residue, and arrival dead. These resources are currently rendered into fats and oils, meat and bone meal, feather meal, and blood meal (**Pei et al. 2025**). Feathers are a common waste product in the poultry industry since they provide for 90% of an adult chicken's environment. Keratinases are essential for managing keratin waste because they are the only proteolytic enzymes that can break down strong, insoluble proteins. In recent decades, researchers have concentrated on creating and characterizing keratinases as well as identifying keratinase makers (**Chu et al. 2026**). The feed, fertilizer, leather, detergent, cosmetic, and pharmaceutical sectors have all investigated the possible uses of keratinases. Wool, feathers, and hair are examples of highly stiff keratin wastes that can be hydrolysed by the special enzyme keratinase (**Qiu et al. 2020**).

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Due to their tight structure and high disulfide-bond content, these significant agroindustry by-products are produced in massive amounts worldwide each year, but they are challenging to eliminate (**Zhang et al. 2022**). The poultry industry might lessen its influence on the environment and generate new economic prospects in the expanding fields of waste-to-value and biotechnology applications by concentrating on the effective manufacture and use of keratinase. Keratinases may break down keratin to produce useful bioactive peptides, amino acids, and proteins that are used in a variety of sectors, such as animal feed, pharmaceuticals, and cosmetics. For example, animal feed contains feather meal as a source of protein (**Li, 2021**). By converting keratin into forms that are easier for animals or fowl to digest, keratinase may enhance the nutritional value of this product. Chicken business provides a home for mesophilic microorganisms, such as fungi and bacteria, which aid in the recycling and breakdown of poultry waste. Amount of trash generated expanded along with the chicken industry's fast growth (**Su et al. 2020**). Current study's objective is to separate microorganisms and assess their capacity to generate keratinase enzymes.

Microbes are tiny factories producing a wide range of molecules as primary and secondary metabolites for their physiological need and against the defense system (**Zhang et al. 2025**). These molecules include proteins, enzymes, complex sugar and lipid derivatives with immense potential in commercial applications. It has been reported that microbes from different genus and species are characterized by specific molecules with unique properties. The market for industrial enzymes was valued at $3.3 billion in 2010 and is expected to reach 4.4 billion by 2015 (**Traverso et al. 2010).** Among them, technical enzymes are frequently used as bulk enzymes in the textile, biofuels, pulp and paper, and detergent industries, among others. Subsequently, it is evident that the burden of keratin waste is raised tremendously and posing risk to water and soil pollution. Keratin as fibrous protein is resistance to break and degrade **(Moktip et al. 2025)**. Hence there is an immense need of robust and catalytic effective enzyme/s. Microbes capable in producing such enzymes habitat in different climatic conditions. Hence, present study aimed to profile microbes from the poultry environmental settings. Study also emphasizes the characterization of keratinase from isolated and profiled microbes.

2.0 MATERIALS AND METHODS:

2.1 Sampling:

The present study was carried out during the year 2022-2025. Here is the present study of five poultry farms from districts Jagtial, Sircilla, Karimnagar, Nizamabad and Hyderabad districts of poultry litter, poultry water, and commercial feed. Litter samples from chicken farms were chosen at random and feed and water samples were gathered directly from the chicken farms feeding operations. In total, 125 samples were acquired from each farm, including soil, litter, feed and water samples.

2.2 Microbial screening:

All the samples collected and brought to laboratory were subjected to serial dilution. Solid and semisolid forms of samples (soil and feed) 2 gm where litter and water samples 2 ml were aliquoted in separate sterile tubes. Using sterile water, the samples were diluted 1:10, 1:100, 1:1000 and 1:10000 times. Samples were mixed with sterile water and filtered via Whatman filter paper. The filtrate was collected in separate sterile tube and stored at 4^0C. To screen the microbes from the samples collected from the poultry environment LB media used both broth and plates. The bacterial isolates were subjected to pure culture and characterization using biochemical and genomic studies (16S RNA analysis).

2.2.1 Biochemical Characterization:

The biochemical examination allowed us to ensure precise metabolic activity of screened bacterium. Here we have tested various capabilities of screened bacterium Indole production, Methyl red test, Voges-proskauer test, Citrate utilization, Catalase test, Hydrogen sulphide, Oxidase, Starch hydrolysis, Casein hydrolysis, Urease test and Gelatine liquefaction (**Cai et al., 2008**).

2.2.2 DNA isolation and PCR based gene identification:

Total genomic DNA of isolated microbes was isolated using CTAB method. All the biochemically and microscopically confirmed *B licheniformis* samples were subjected to the total genomic DNA isolation. The isolated genomic was used for the template for PCR based 16S rRNA amplification. Here in the present study, primer used for amplification of 16S RNA gene includes 27F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R: 5′-GGTTACCTTGTTACGACTT- 3′. The primers used in the study were designed (gene specific) and amplified via polymerase chain reaction with the specific thermal cycler program denaturation (94^0C for 4 mins), 30 cycles denaturation (95^0C for 30Sec,) annealing (54-60^0C based on the annealing temperature) and extension (72^0C for 1 mins) and final extension (5mins at 72^0C).

2.2.2.1 Agarose gel electrophoresis, Gel amplicon extraction and sequencing

The amplicon (16s rRNA) was visualized on 1.2% w/v agarose against the standard DNA ladder. The gel containing amplicon of interesting size (bp against the DNA ladder) was extracted and purified.

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The amplified products were sequenced in present study using 3730 Genetic Analyzer. A 10µl Hi-Di formamide was added to each tube on DNA pellet and seal the plate with Septa. All the samples were denatured by heating to 96^0C for 3 minutes in the thermo cycler and immediately placed on ice. The sequencing was carried using 27F: 5′-AGAGTTTGATCCTGGCTCAG-3′ primer.

2.3 Purification of Keratinase

A chromatography column with a diameter of 2.5 cm and height of 50 cm, containing Sephadex G-75, was used as the gel filtration matrix. Eppendorf tubes were used as containers to collect the fractions during chromatography. Cell-free culture supernatant was collected, and the protein was precipitated at 80 % ammonium sulphate saturation on ice. The sample was then transferred to a refrigerator for 24 h. The sample was centrifuged at 8000 rpm for 15 min, and the supernatant was discarded. The pellet was suspended in 10 mL buffer (20 mM Tris-HCl and NaCl at pH 8.0) and loaded onto the column. The elution was allowed to proceed under gravity, and fractions (1.5 mL) were collected. A total of 80 fractions were collected, and for each fraction, enzyme activity and total protein estimation were performed using the Lowry protein estimation assay. The active fractions were pooled and analysed for purity and molecular weight using Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2.3.1 Quantification of Keratinase and Enzyme activity

The total protein content was estimated during purification. The concentration of protein was estimated by Lowry method using Bovine Serum Albumin (BSA) as standard. The concentration of the keratinase enzyme was calculated by standard BSA graph and by following calculation-

$$Conc.\ of\ Protein\ \left(\frac{mg}{ml}\right) = \frac{OD\ of\ Test}{OD\ of\ Std.} \times \frac{Conc\ of\ Std.}{Vol.\ of\ sample\ used\ ml}$$

Keratinases break peptide bonds and keratinase assay is based on liberation of amino acids from digested substrates. The released amino acids from proteolytic degradation of substrate measured and define capacity of enzymatic catalysis. Here, casein acts as a substrate. When the keratinase digests casein, the amino acid tyrosine is liberated along with other amino acids and peptide fragments. Folin & Ciocalteus Phenol or Folin's reagent primarily reacts with free tyrosine to produce a blue colored chromophore, which is quantifiable and measured as an absorbance value on the spectrophotometer. The more tyrosine that is released from casein, the more the chromophores are generated and the stronger the activity of the keratinase. To get the activity of enzyme in units/ml, perform the following calculation-

$$Enzyme\ activity\ \left(\frac{U}{ml}\right) = \mu M\ of\ tryosine\ released\ x\ \frac{V}{pqr}$$

Where V is the total volume of assay in ml, p - volume of enzyme used, q denotes the time of assay and r is volume used for colorimetric determination. Specific activity was calculated

$$Specific\ activity\ of\ enzyme\ \left(\frac{U}{mg}\right) = \frac{Enzyme\ activity}{Protein\ content\ (mg)}$$

To further identify keratinolytic bacteria, isolates were cultured in minimal broth media containing (g/L): $MgSO_4 \cdot 7H_2O$ 1, K_2HPO_4 1, KH_2PO_4 1, NaCl 1, $CaCl_2 \cdot 2H_2O$ 0.1, yeast extract 0.1, and pH 10. After a week of incubation at 30 °C, the tubes were visually inspected for feather deterioration. Additionally, feather degradation rates were assessed through weight loss measurements. Feather weight loss was measured by incubating them in a 250-mL Erlenmeyer flask containing 100 mL of sterilized minimal medium supplemented with white feather meal (10 g/L) at 30 °C. Keratinolytic bacterial isolates showing maximum feather degradation were selected for further experimental studies. The degree of feather degradation was calculated and expressed as a percentage, as shown in Equation

$$\text{Percentage of feather degradation } (\%) = \frac{\text{Initial Weight - Final Weight}}{\text{Initial Weight}} \times 100$$

2.4 Kinetic studies

The kinetic parameters Km, Vmax and Kcat for enzyme were calculated using casein as substrate for the enzyme. The amount (µM) of tyrosine released by the reaction between casein and keratinase per unit time was considered as the velocity of the reaction. The kinetic study was performed at optimal conditions such as temperature 40^0C and pH 8.0. The different kinetic plots were made to calculate kinetic constant including Michalis–Menten plot, *Lineweaver–Burk plot, Hanes–Woolf plot and Eadie–Hofstee diagram. Turnover number Kcat (Vmax/E_T) and* catalytic efficiency of purified keratinase *(Kcat/Km)* were calculated to determine keratinase efficiency.

2.4.1 Effect of temperature the enzymatic activity and stability

The purified enzyme was analysed for activity and stability in the temperature range of 30°C-130°C. The enzyme in 50mM phosphate buffer, pH 6.0 was incubated for 1 hour in the different temperatures range from 30°C-130°C. After the incubation

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enzymatic activity was analysed by incubating with casein as substrate and relative keratinase activity was measured at 440 nm. The activity profile of purified keratinase under different temperature condition has shown in the graph where activity was measured in the term of residual activity.

2.4.2 Effect of pH on the enzymatic activity and stability

The activity and stability of keratinase assessed in different pH, range 4-12 by incubating keratinase in different buffer. The buffer used in this study, Citrate buffer 0.05M (pH 4.0-6.5), Phosphate buffer 0.05M (pH 7.0-7.5), Tris-HCl Buffer 0.05M (pH 8.0-8.5), Glycine NaOH 0.05M (pH 9.0-12.0). Keratinase was incubated for 1 hour with each of the buffer and then incubates with casein for relative keratinase activity at 440 nm. The activity profile of purified keratinase under different pH condition has shown in the graph where activity was measured in the term of residual activity.

2.4.3 Effect of keratinase inhibitors on the enzymatic activity

Activity and stability of purified keratinase were also evaluated in the presence of different keratinase inhibitors. PMSF, Sodium azide, EDTA, Indoacetamide *and aprotinin were used for analysis. Purified keratinase was incubated for 1 hour at 80°C with these keratinase inhibitors (1%). After 1 hour keratinase was incubated with casein to calculate relative keratinase activity measured at 440 nm.* The activity profile of purified keratinase under different inhibitors has shown in the graph where activity was measured in the term of residual activity.

3.0 RESULTS AND DISCUSSION:

Present study was carried out at Department of Microbiology Chaitanya Deemed to be University, Hyderabad, Telangana state, India during 2021-2025. The study was designed and carried out after approval from the University and consent from the sample sources (poultry farm owners). The study was performed as per guidelines of standard microbiology practices for sample collection, microbial isolation, characterization and storage of microbial culture. The chemical and consumables used in the study were procured from the Sigma Alrich, HI Media and Merck India Ltd. The samples collected were grown and maintained in the prescribed medium as per recommend growth condition. Enzyme activity and estimation of keratinase enzyme was carried out in triplicate and mean of result were included in the study.

3.1 Microbial screening

Microbial screening of all the samples was carried out under standard microbiological procedure. The samples were subjected to the serial dilution 1:10, 1:100, 1:1000 and 1:10000. The samples were allowed for growth at LB media, NB media and others. The culture was growth at different incubation conditions; temperature range 20-45^{0}C, pH 6-8 under aerobic condition. All the samples showed significant number of microbes primarily bacteria belonging to genus Bacillus species **(Table 1).** As the data showed in the table 1, B *lichenformis* is key bacterium in all the poultry samples profoundly present in all the regions of sampling **(Figure 1).** In fact, Bacillus genus reported a common bacterium from soil, water, feed and litters collected from the poultry farms in Jagtial, Sircilla, Karimnagar, Nizamabad and Hyderabad.

Table 1: Summary of bacterial isolates from the poultry setting collected from the regions in Telangana state i.e. Jagtial, Sircilla, Karimnagar, Nizamabad and Hyderabad.

Bacterial species	Bacterial Isolates of Poultry Setting				
	Jagtial	Sircilla	Karimnagar	Nizamabad	Hyderabad
B. subtilis	++	+	+++	++	+++
B. licheniformis	+++	+++	++++	+++	++++
B. velezensis	+	++	++	++	++
B. cereus	++	++	++	++	++

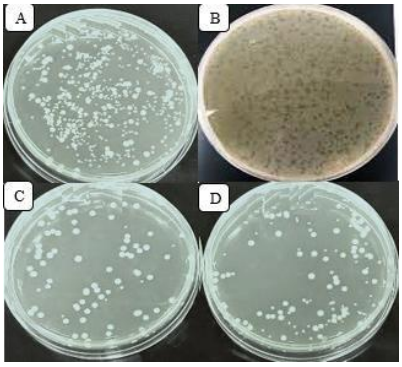

Figure 1: The bacterial isolates from the samples collected from poultry setting in Jagtial, Sircilla, Karimnagar, Nizamabad and Hyderabad. The samples showed prevalence of B. subtilis (A), B. licheniformis (B), B. velezensis (C), and B. cereus (D).

3.2 Biochemical Characterization:

Out of 125 samples majority of isolates were B. licheniformis from soil, water and feature samples. Based on the results, B licheniformis samples were further characterized using biochemical and genomic tools. The samples analyzed for complete characterization showed 63 *B licheniformis* confirmed samples mostly from soil and feathers. The confirmation (biochemical) of samples showed

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positive result for the Gelatin Hydrolysis, Methyl Red test, Citrate test, Voges Proskauer, Casein Hydrolysis and Chitin utilization **(Figure 2)**. The nutrient utilization of B licheniformis aligned with previous studies. The excellent antibacterial activity of the crude extract of bacterial bacteriocins of Gram-positive bacteria is likely caused by an ion channel-produced film that can alter cell permeability, allow amino acid infiltration, and ultimately cause cell death (**Traverso et al. 2010**). Because the susceptible membrane receptor is activated when bacteriocins attach to it, their powerful antibacterial activity is closely linked to general microorganisms.

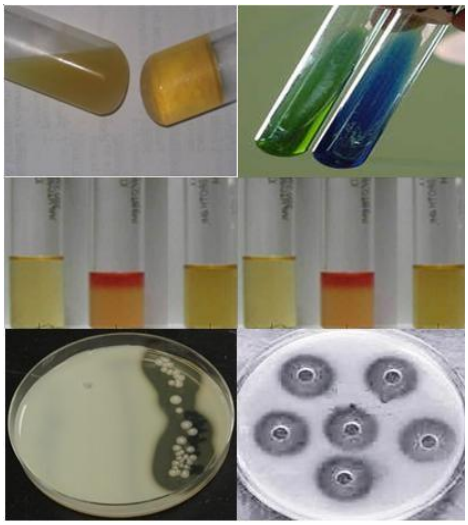

Figure 2: Biochemical characterization of microbial isolates as major bacterium through A-Gelatin Hydrolysis, B-Methyl Red test, C-Citrate test, D- Voges Proskauer, E-Casein Hydrolysis and F- Chitin utilization. Additionally, isolates showed negative test for oxidase test, catalase test, starch hydrolysis and Urease.

3.3 Genomic DNA Isolation

Genomic DNA was isolated form the *B lichenformis* bacterium isolated from poultry farms in Telangana region, India Jagtial, Sircilla, Karimnagar, Nizamabad and Hyderabad. **Figure 3** illustrate agarose gel visualization of genomic DNA of *B licheniformis* The 16S rRNA gene of *Bacillus licheniformis* is a crucial ~1500 base pair (bp) DNA sequence used for its precise identification, classification, and phylogenetic analysis, featuring conserved and variable regions (V1-V9) that help differentiate it from other *Bacillus* species, often showing high sequence identity (e.g., 99.9%) with other *B. licheniformis* strains in databases like GenBank (**Abdel-Naby et al. 2017**). Here in the study 16S rRNA gene using universal primers (like 27F/1492R) via PCR, then sequence it to confirm isolates' identity and study their functions, like enzyme production or phosphate solubilisation, relating these traits to specific gene clusters **(Figure 4).**

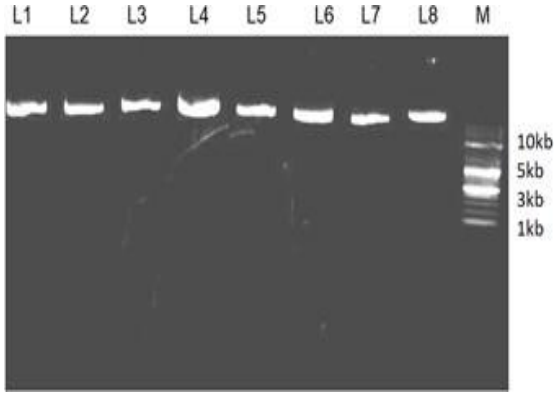

Figure 3: illustration of genomic DNA of B lichenformis collected from poultry samples in Jagtial, Sircilla, Karimnagar, Nizamabad and Hyderabad.

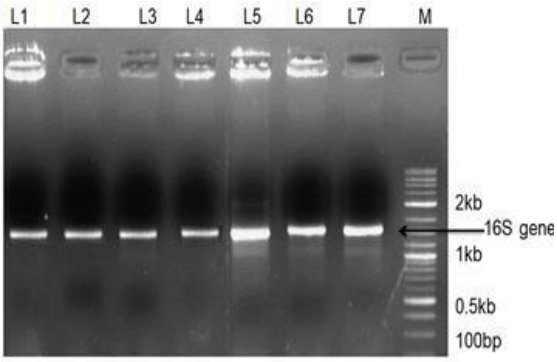

Figure 4: Illustration of 16S RNA gene of B lichenformis (1500 bp)

GTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGG
GTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGA
AACCGGGGCTAATACCGGATGCTTGATTGAACCGCATGGTTCAATCATA
AAAGGTGGCTTTTAGCTACCACTTACAGATGGACCCGCGGCGCATTAGC
TAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTG
AATTCTCTAACCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG
GGAGGCAGGGAATGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGC
AACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTA
GGGAAGAACAAGTACCGTTCGAATAGGGCGGCACCTTGACGGTACCTAA
CCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
TGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTT
TCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGG
AAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAG
CGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTC
TCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTA
GAGGGAAAGCCCCTTTAGTGCTGGTAAGCATTGCATTAAGCACTCCGCC
TGGGGAGTACCTTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCT
TACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCCCCTTC
GGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGA
GATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCC
AGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGA
AGGTGGGGATGACGTCAAATCATCATGCCCCGTGCACCGAGCCTTACAC
ACGTGCTACAATGGGCAGAACAAAGGGCAGCGAAGCCGCGAGGCTAAG
CCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACT
GCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAA
TACGTTCCCGGGCCTTGTACACACCGCCCGTCACAGAGGTAGAGTTTGT
AACACCCGAAGTCGGTTAATGAACCTTTTGGAGCCAGC

Figure 5: *Bacillus licheniformis* strain 16S ribosomal RNA gene, partial sequence

3.4 Kinetic Constant:

The kinetic constants of keratinase enzyme were determined using different kinetic plots including Michaelis–Menten plot. Km and Vmax of keratinase enzyme by Michaelis–Menten plot 2.3 g/ml and 55.17 g/ml/min. The kinetic constant was found similar while using Lineweaver–Burk plot 0.07 g/ml and 27.0 g/ml/min respectively. Further, turn over number and catalytic efficiency of purified enzyme shown similar variation among different kinetic

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plots. Previously **Abdel-Naby et al. (2017)** determined kinetic efficacy of keratinase enzyme from four different iso-forms isolated. The Km and Vmax reported 0.09 g/ml and 24.63 g/ml/min. the study emphasizes commercial implication of proteolytic enzymes including keratinase. The study also demonstrates higher catalytic efficiency of keratinase. In recent study, **Al-Bedak et al. 2026** isolated keratinase enzyme possesses not only higher catalytic efficacy but also specificity for the substrates. As the result shown in table and figure keratinase isolated from soil samples in the present study possess significant catalytic efficacy and capable to catalytic proteolytic breakdown **(Figure 6)**.

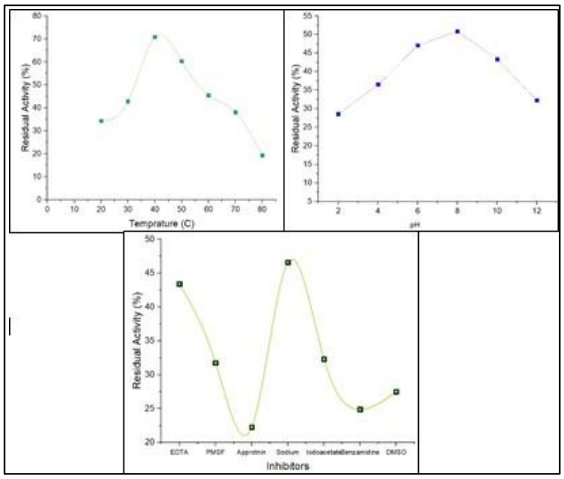

Figure 6 Effect of external factors (pH Temperature and inhibitors) on the activity of keratinase enzyme

Keratinase enzyme kinetics studies demonstrate how the enzyme cleaves keratin, revealing optimal conditions (pH 8-9, 40-60°C) and characteristics like Km and $Vmax$, crucial for its industrial use in waste management, showing dependence on pH, temperature, and metal ions with kinetics often analysed using Michaelis-Menten principles for free and immobilized forms (Figure 5). Leather tanning, detergent additives, silver recovery, and protein hydrolysis are just a few of the many industrial uses for keratinolytic proteases, which convert keratin-rich substrates into proteins and amino acids (**Nnolim et al. 2020**). The isolation and characterization of novel bacterial strains that generate potentially beneficial enzymes for industrial applications has been the subject of numerous investigations (**Jin et al. 2017**). According to **Saeed et al. (2024)** microbial enzymes are the most popular industrial enzymes and have drawn the attention of the global enzyme market because of their broad range of pH and temperature stability, great variety, and numerous application possibilities in different bioindustries (**Mazotto et al. 2022**).

The most promising characteristics of microbial enzymes are their wide range activity and stability. Therefore, assessing the wide range of biotechnological uses of novel powerful bacteria that produce keratinolytic proteases requires a detailed study of their features. According to **Kokwe et al. (2023)** a keratinase intended for industrial application needs to be highly selective, have a long shelf life, and be active and stable over a broad pH and temperature range. As a result, many microbially derived enzymes remain undiscovered, and there are several chances to discover more extensive industrial uses for these enzymes. However, a significant barrier to large-scale applications is the sensitivity of enzymes to harsh environmental conditions that shorten their half-lives.

3.5 Keratinase purification using Sephadex G-75 Gel Filtration Chromatography

Sephadex G-75 gel filtration chromatography was used to purify the extracellular keratinase after it had first been concentrated using 80% ammonium sulphate precipitation. Eighty fractions in all were collected, and each fraction was examined for keratinase activity and protein content using the Lowry technique. High enzyme activity fractions were chosen for further examination.

The purity and molecular weight of the isolated keratinase were assessed using SDS-PAGE analysis **(Figure 7)**. The protein molecular weight marker was found in Lane A, Fraction 12 was represented by Lane B, and Fraction 32 from the Sephadex G-75 column was represented by Lane C.

A strong protein band of around 27 kDa was seen in fraction 12, along with a few weak higher molecular weight bands that suggested the presence of small contaminating proteins. Fraction 32, on the other hand, demonstrated better purification by gel filtration chromatography with a more pronounced and strong protein band at about 27 kDa and a discernible decrease in contaminating bands. The purified enzyme seems to be keratinase based on the presence of a prominent protein band at about 27 kDa.

The extracellular keratinase was successfully purified since the estimated molecular weight of about 27 kDa is within the range reported for keratinases from Bacillus subtilis and similar Bacillus species, which normally fluctuate between 27 and 33 kDa.

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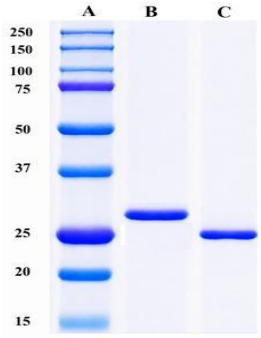

Figure 7 Keratinase extracted by Sephadex G-75 gel filtration chromatography was analysed using SDS-PAGE. Protein molecular weight marker (15–250 kDa) in Lane A; Fraction 12 from Sephadex G-75 gel filtration chromatography in Lane B; and Fraction 32 from Sephadex G-75 gel filtration chromatography in Lane C. The purified keratinase is shown by the noticeable protein band at around 27 kDa.

3.5 Analysis of enzyme activity and stability

The keratinase enzyme produced from the microbes' samples collected poultry setting was evaluated for its activity and stability in different conditions. The keratinase enzyme was analysed in different temperature, pH and presence of inhibitors.

3.5.1 Effect of temperature on enzyme activity and stability:

The keratinase enzyme was reported active in the temperature range of 20 and 80°C with an optimum at 40°C (Figure 4). The purified enzyme had shown 70.89 and 60.31% activity at temperature between 40 and 50^{0}C which further declined after 70^{0}C. The enzymatic activity and stability were declined in lower temperature below 20^{0}C. These findings reported keratinase enzyme isolated from soil and other poultry settings in the present study is stable and retain enzymatic activity for broad temperature range.

3.5.2 Effect of pH on enzyme activity and stability:

The keratinase enzyme isolated from soil in the present study possesses strong enzymatic activity and stability. Enzyme was reported active between pH 4-10 with maximum activity and stability at pH 8.0. The activity of enzyme was fallen significantly at lower pH 2-6. Further, at higher temperature pH 10-14 activity was rapidly reduced (Figure 4). Several findings have reported structural reason for strong proteolytic activity of enzyme in different pH and other environment is due to protein secondary structure composed of several forms in defined orientation. The environment is key driver for the microbial adoption and production of enzymes with varying enzyme activity at different temperatures.

3.5.3 Effect of protease inhibitors on enzyme activity and stability:

The keratinase enzyme produced from the microbes' samples collected poultry settings was evaluated for activity in the presence of various protease inhibitors including, EDTA, PMSF, Approtnin, Sodium Azide, Iodoacetate, Benzamidine and DMSO. All these protease inhibitors were used at optimal incubation conditions pH 7.5 and temperature 37^{0}C in 1% w/v solution. The maximum inhibition of enzymatic activity in keratinase enzyme was reported with DMSO. Other protease inhibitors such as PMSF, Approtinin, Iodoacetate and Benzamidine were reported for significant inhibition. However, EDTA and Sodium azide had shown least inhibition (Figure 4). Enzyme application is becoming increasingly important in various industrial processes due to the increased demand for environmentally sustainable industrialization. The current isolate has successfully manufactured keratinase enzyme utilizing readily available, low-cost substrate (soil, feed, litter and water), and it is capable of large-scale production (**Cavello and Cavalitto, 2014**).Kinetic and thermodynamic characterisation of keratinolytic protease generated by B. lichenformis in a conventional tannery setting has never been reported before, as far as the authors are aware (**Bressollier et al. 1999**). Because it hydrolyses both soluble and insoluble substrates, this enzyme is extremely significant **(Sharna et al. 2022).**

When keratinous protein components were present in the media, the isolated Arthrobacter sp. NFH5 produced the most keratinase. The enzyme's kinetic data demonstrated its strong catalytic capabilities. The enzyme's relative thermostability and suitability for usage in various industries were proven by its thermostability properties (**Nnolim et al. (2020)**. According to **Sharma et al. (2022)**, microbial keratinase is an inducible enzyme. A similar outcome was observed for Arthrobacter creatinolyticus KP015744, which produced the most keratinase when 1% feather powder was present. Additionally, **Li et al. (2019)** found that Bacillus sp. JB99 produced the most keratinase when 1% feather meal was present. The amount and concentration of keratin are necessary for the synthesis of keratinase. When feather meal concentrations are higher, enzyme synthesis may decrease, suggesting catabolic inhibition. The keratinase enzyme was shown to be most active at pH 8.0 and 40 °C.

The poultry industry might lessen its influence on the environment and generate new economic prospects in the expanding fields of waste-to-value and biotechnology applications by concentrating on the effective manufacture and use of keratinase **(Moktip et al. (2025)**. Keratinases may break down keratin to produce useful bioactive peptides, amino acids, and proteins that are used in a variety of sectors, such as animal feed, pharmaceuticals, and cosmetics. For example, animal feed contains feather meal as a source of protein. By converting

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DoI-10.004687/1000-9035.2026.065

keratin into forms that are easier for animals or fowl to digest, keratinase may enhance the nutritional value of this product **(Traverso et al. 2010)**. The current study's objective is to separate microorganisms and assess their capacity to generate keratinase enzymes. The chicken business provides a home for mesophilic microorganisms, such as fungi and bacteria, which aid in the recycling and breakdown of poultry waste. The amount of trash generated expanded along with the chicken industry's fast growth (**Chu et al. 2026**).

Several enzymes, including keratinase, are produced by microbes that colonize the poultry environment. Keratinases are essential for managing keratin waste because they are the only proteolytic enzymes that can break down strong, insoluble proteins. In recent decades, researchers have concentrated on creating and characterizing keratinases as well as identifying keratinase makers. The feed, fertilizer, leather, detergent, cosmetic, and pharmaceutical sectors have all investigated the possible uses of keratinases. Wool, feathers, and hair are examples of highly stiff keratin wastes that can be hydrolysed by the special enzyme keratinase. Due to their tight structure and high disulfide-bond content, these significant agroindustry by-products are produced in massive amounts worldwide each year, but they are challenging to eliminate.

4.0 CONCLUSION:

Due to the growing need for environmentally sustainable industrialization, the use of enzymes in various industrial processes is becoming increasingly significant. The present isolate is capable of large-scale production and has effectively produced keratinase enzyme using easily accessible, inexpensive substrate (soil, feed, litter, and water). To the best of the authors' knowledge, the kinetic and thermodynamic characterization of keratinolytic protease produced by B. lichenformis in a traditional tannery setting has never been published before. This enzyme is very important since it hydrolyses both soluble and insoluble substrates. Here in the present study, B licheniformis is major microbe isolated from the samples collected from the poultry farms in the state of Telangana, India. The enzyme's potent catalytic capabilities were shown by its kinetic data. The thermostability features of the enzyme demonstrated its relative thermostability and potential for use in a variety of industries.

CONFLICT OF INTEREST:

Authors declare no conflict of interest

ACKNOWLEDGMENTS:

Authors thankful to the Management of Chaitanya Deemed to be University, Moinabad, Hyderabad, for providing the facilities for the accomplishment of this research.

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