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Isolation And Molecular Identification Of Keratin Degrading Novel Bacteria Bacillus Licheniformis Strain Cfmsrnr 5

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Abstract
In the present study, keratin-degrading bacterial strains were isolated from feather disposal sites near poultry farms in Hyderabad, Telangana, using an enrichment-based culture approach. Soil samples were inoculated into mineral salts medium (MSM) containing 1% sterilized chicken feathers as the sole carbon and nitrogen source. After enrichment, five distinct bacterial isolates (I1 to I5) were obtained through serial dilution and spread plating. Among them, Isolate 5 demonstrated the highest feather degradation efficiency, reaching 86% degradation by Day 7. Quantitative assays confirmed Isolate 5 exhibited maximum keratinase activity (38 U/mL), along with elevated protein (4.5 mg/mL) and amino acid (360 µg/mL) yields, highlighting its superior feather hydrolysis potential. Morphological characterization revealed that Isolate 5 was a Gram-positive, spore-forming, capsulated rod, consistent with the Bacillus *genus. Biochemical analysis supported its metabolic versatility, showing positive results for indole production, citrate utilization, catalase activity, and H_2S production. Molecular identification through 16S rRNA gene sequencing confirmed its identity as* Bacillus licheniformis *CFMSRNR 5, with strong phylogenetic clustering to related strains. These findings establish* B. licheniformis *CFMSRNR 5 as a promising candidate for bioconversion of keratin waste into value-added products for biofertilizer and sustainable biotechnological applications.*

Keywords: *Keratinase,* Bacillus licheniformis*, Feather degradation, Biofertilizer, 16S rRNA, Sustainable waste management*

INTRODUCTION

Keratin-rich waste, particularly in the form of poultry feathers, woolen shearing residues, and epithelial debris, represents a growing environmental challenge on a global scale. These wastes accumulate due to the enormity of the poultry and agricultural sectors, generating large volumes of resistant organic material (Kumar et al., 2021; Reddy et al., 2022). Keratin's resilience stems from its intricate architecture: a high cysteine content forming extensive disulfide bonds, generating a compact, insoluble protein matrix (Reddy et al., 2022; Verma et al., 2020). Conventional disposal pathways, such as landfilling or incineration, are not only inefficient in breaking down keratin but also environmentally detrimental due to greenhouse gas emissions and soil contamination (El-Katatny et al., 2020; Zarei & Esfahani, 2024). Hence, green, cost-effective methods are needed to transform keratinous biomass into value-added products.

Microbial keratinases are specialized proteases with the ability to degrade keratin's stable structure under moderate conditions, owing to unique structural and catalytic properties (Gopinath et al., 2021; Mitra et al., 2023). These enzymes, typically secreted by bacteria and fungi, use coordinated peptide-bond hydrolysis and disulfide reduction to convert insoluble keratin into soluble peptides and amino acids (Verma et al., 2020; Yadav et al., 2023). Such biotransformation enables eco-friendly disposal and valorization of keratinous waste, offering a pathway to sustainable bioprocessing in agro-ecosystems and animal feed production (Frontiers in Microbiology, 2021; PMC, 2021).

Research has identified several bacterial genera with robust keratinolytic capabilities, such as *Bacillus*, *Pseudomonas*, *Streptomyces*, and *Chryseobacterium* (Verma et al., 2020; El-Katatny et al., 2020). Among these, *Bacillus* species stand out due to their high enzyme yield, thermotolerance, and amenability to large-scale fermentation (Mitra et al., 2023). Strains like *Bacillus licheniformis* are especially notable for their prolific keratinase production and resilience in processing complex substrate media (Singh et al., 2021; Kumar & Das, 2022).

When keratin is enzymatically degraded, it yields feather hydrolysates rich in amino acids (e.g., leucine, cystine), peptides, and essential micronutrients such as zinc and iron (El-Katatny et al., 2020; Yadav et al., 2023). These compounds enhance microbial activity in the rhizosphere, increase nitrogen availability, and can possess plant growth-promoting characteristics (Kumar & Das, 2022; Borges et al., 2022). Seed treatments using protein hydrolysates have been shown to significantly promote germination rates, improve root and shoot development, and increase vigor indices in various crops, including tomatoes (Chinnadurai et al., 2021; Borges et al., 2022).

These findings underscore the potential application of microbial keratin hydrolysates as biofertilizers or biostimulants.

METHODOLOGY

Sample Collection

To investigate the potential of keratin-degrading and plant growth-promoting bacterial isolates, soil samples were systematically collected from poultry feather dumping sites located at commercial poultry farms in and around Hyderabad, Telangana, India. These sites were selected due to their prolonged exposure to keratin-rich organic waste, thereby increasing the likelihood of harboring keratinolytic microbial communities. Approximately 200–300 grams of rhizosphere soil, rich in organic matter, was collected at a depth of 5–15 cm using sterile tools and transferred into sterile zip-lock bags. The samples were labeled, stored at 4°C, and processed within 24 hours for further analysis.

Enrichment Culture Technique for Keratinolytic Bacteria

To selectively isolate keratin-degrading bacteria, an enrichment culture method was adopted using mineral salt medium (MSM) supplemented with sterilized chicken feathers as the sole source of carbon and nitrogen. The composition of the MSM was as follows:

- KH_2PO_4 – 0.4 g/L
- K_2HPO_4 – 0.4 g/L
- $MgSO_4 \cdot 7H_2O$ – 0.1 g/L
- NaCl – 0.1 g/L
- $FeSO_4 \cdot 7H_2O$ – 0.01 g/L
- $ZnSO_4 \cdot 7H_2O$ – 0.001 g/L

Approximately 10 g of the collected soil sample was added to 100 mL of sterile MSM containing 1% (w/v) pre-sterilized chicken feather waste (cut into 1–2 cm fragments) in 250 mL Erlenmeyer flasks. The flasks were incubated at 37°C on a rotary shaker at 200 rpm for 5 days to facilitate the enrichment of keratinase-producing bacterial populations. Feather degradation and turbidity of the medium were monitored visually as preliminary indicators of keratinolytic activity. Following the incubation period, aliquots from the enrichment cultures were serially diluted and plated onto feather meal agar plates to isolate individual colonies. Colonies exhibiting clear zones of feather degradation were selected for further screening and characterization.

Feather Degradation Assay

The feather degradation potential of the isolated bacterial strains was assessed using a feather degradation assay. This assay aimed to identify potent keratinolytic bacteria capable of breaking down chicken feathers effectively. The procedure was conducted as follows: Preparation of Feather Substrate: Chicken feathers were collected from local poultry farms, washed thoroughly with distilled water to remove any adhering dirt and debris, and then sterilized by autoclaving at 121°C for 15 minutes. After sterilization, the feathers were dried at 60°C until a constant weight was achieved.

Inoculation and Incubation: The feather degradation assay was performed in 500 mL Erlenmeyer flasks. Each flask contained 100 mL of distilled water and 1% (w/v) sterilized chicken feathers. The medium was autoclaved at 121°C for 15 minutes to ensure sterility. After cooling to room temperature, 0.5 mL of an overnight bacterial culture (OD600 = 1.0) was inoculated into each flask. The flasks were then placed in a rotary shaker set at 150 rpm and incubated at 37°C for 5-7 days. The shaking ensured uniform distribution of bacterial cells and adequate oxygenation, which is essential for the aerobic degradation of keratin.

Quantitative Analysis of Feather Degradation: After the incubation period, the culture broth was filtered through a Whatman No. 1 filter paper to separate the underrated feathers from the liquid medium. The residual feathers were washed thoroughly with distilled water, dried at 60°C until a constant weight was achieved, and weighed to determine the extent of feather degradation. The percentage of feather degradation was calculated using the following formula:

$$\text{Percentage of Feather Degradation} = \frac{\text{Initial Weight of Feathers} - \text{Final Weight of Feathers}}{\text{Initial Weight of Feathers}} \times 100$$

Keratinase Activity

To quantify keratinase activity and evaluate the efficiency of feather hydrolysate production, the bacterial culture was first incubated in optimized feather degradation conditions for 5–7 days. Following incubation, the culture broth was centrifuged at 10,000 rpm for 15 minutes at 4°C to separate the bacterial cells and feather residues. The resulting supernatant was collected as crude enzyme extract for keratinase activity analysis. The keratinase assay was performed using 1% (w/v) keratin azure as the substrate in 50 mM Tris-HCl buffer (pH 8.0). One milliliter of the substrate solution was mixed with an equal volume of the crude enzyme extract and incubated at 50°C for 1 hour. After incubation, the reaction was terminated by placing the tubes on ice and centrifuging them again to remove undigested substrate. The absorbance of the supernatant was measured at 595 nm using a UV-Vis spectrophotometer. One unit of keratinase activity was defined as the amount of enzyme causing a 0.01 increase in absorbance per hour under standard assay conditions.

To evaluate the nutritional quality of the feather hydrolysate, the total soluble protein content was estimated using the Bradford assay with bovine serum albumin as the standard, and the absorbance was read at 595 nm. Additionally, the free amino acid content was determined using the ninhydrin assay. Hydrolysate samples were treated with acidic ninhydrin reagent and heated in a boiling water bath for 15 minutes, then cooled, and absorbance was measured at 570 nm using L-leucine as a standard.

Morphological and Biochemical tests

The bacterial isolate was subjected to a series of morphological, biochemical, and molecular characterization techniques to confirm its identity and keratinolytic potential. For morphological analysis, Gram staining was performed using crystal violet, Gram's iodine, ethanol, and safranin, allowing differentiation between Gram-positive and Gram-negative bacteria based on cell wall composition and stain retention. Capsule staining was carried out using India ink and a negative staining technique to detect the presence of a polysaccharide capsule surrounding the bacterial cells, which appeared as a clear halo against a dark background. Spore staining involved heat-fixation with malachite green and counterstaining with safranin to identify endospore-forming bacteria, where endospores stained green and vegetative cells stained red.

The biochemical characterization began with the indole production test, where cultures were inoculated in tryptone broth, incubated, and treated with Kovac's reagent to detect the presence of indole through the formation of a cherry red ring. The methyl red (MR) and Voges-Proskauer (VP) tests were performed using MR-VP broth to assess acid production and acetoin formation from glucose metabolism. Positive MR reactions turned the medium red, while VP-positive reactions resulted in a pink to crimson red color after the addition of α-naphthol and KOH. Citrate utilization was assessed on Simmon's citrate agar slants, with a color change from green to blue indicating a positive result. Catalase activity was tested by adding hydrogen peroxide to tryptone soy agar slants, and bubble formation indicated the release of oxygen, confirming catalase presence.

The hydrogen sulfide (H_2S) production test was performed on triple sugar iron (TSI) agar slants, where black precipitate formation indicated H_2S production. Urease activity was detected in Christensen's urea medium by the change in medium color to pink upon ammonia production, raising the pH. For the starch hydrolysis test, bacterial cultures were streaked onto starch agar plates, incubated, and then flooded with iodine; a clear zone around the colonies indicated the presence of extracellular amylase. Lastly, carbohydrate fermentation tests were carried out using fermentation broth containing specific sugars (glucose, sucrose, or lactose), phenol red indicator, and Durham tubes; acid production turned the medium yellow, while gas production was indicated by bubbles in the Durham tube.

Molecular identification of potent isolate 5 based on 16S rRNA gene sequence

Isolate 5 underwent molecular identification by sending it to MACROGEN in Seoul, Korea, for sequencing using universal primers targeting the 16S rRNA gene. Following sequencing, the obtained 16S rRNA sequence was analyzed using bioinformatics software Mega-4 to conduct phylogenetic analysis. The sequence data were subsequently submitted to the National Center for Biotechnology Information (NCBI) for comprehensive analysis and comparison with existing databases. This process aimed to determine the taxonomic classification and relationships of isolate 5 based on its genetic profile.

RESULTS

In the present study, bacterial strains capable of degrading chicken feathers were successfully isolated and preliminarily characterized using an enrichment-based culture approach. Soil samples were collected from feather disposal sites near poultry farms in Hyderabad, Telangana—locations known for keratin-rich organic waste and natural microbial activity. The enrichment was performed in mineral salts medium (MSM) supplemented with 1% (w/v) sterilized chicken feathers as the sole carbon and nitrogen source. Incubation was carried out at 37°C

with shaking at 200 rpm for five days. During this period, turbidity and visible feather degradation were observed, indicating the selective growth and activity of keratinolytic bacteria.

Following the enrichment process, bacterial isolates were obtained using serial dilution to reduce colony density and enable the isolation of discrete colonies. Dilutions up to 10^{-7} were prepared in sterile distilled water, and 100 µL aliquots from the 10^{-6} and 10^{-7} dilutions were aseptically spread onto nutrient agar (NA) plates. The plates were incubated at 37°C for 24 to 48 hours, after which diverse bacterial colonies emerged, exhibiting variations in morphology, including differences in size (0.5–5 mm), shape (circular or irregular), surface texture (smooth or wrinkled), margin (entire or undulate), and pigmentation (white, cream, or yellowish). Representative colonies were selected and sub-cultured on fresh NA plates using quadrant streaking to ensure purity. Repeated streaking and incubation resulted in the establishment of axenic cultures. These purified and morphologically distinct isolates were labeled and preserved for further morphological, biochemical, and molecular characterization (Fig. 1).

Figure.1: Spread plate technique

Following isolation from feather-enriched cultures, five keratinolytic bacterial strains—designated I1 to I5—were assessed for colony morphology on nutrient agar after 24–48 hours of incubation at 37°C. All isolates exhibited irregular colony shapes, which is typical of environmental Bacillus spp. adapted to keratin-rich substrates. Colony sizes were consistently small, likely due to slow growth associated with energy-intensive keratin degradation. The colony margins varied: I1 and I2 displayed entire, smooth edges, while I3, I4, and I5 showed undulate margins, suggesting differential enzyme secretion patterns. All isolates were creamy white in color, a characteristic often linked to Bacillus strains and indicative of non-pathogenic profiles. Colonies appeared opaque, reflecting high cell density and possible EPS production, which aids in substrate adhesion and biofilm formation. These morphological traits support the potential keratinolytic nature of the isolates (Fig. 2).

Figure. 2: Sub culturing by using streaking technique

Screening of Potent Feather-Degrading Bacteria

The keratinolytic potential of five bacterial isolates (Isolates 1 to 5) was assessed using a feather degradation assay over a seven-day incubation period. Daily measurements of residual feather mass were recorded to evaluate the progressive breakdown of sterilized chicken feathers in a mineral salts medium. Among the isolates, Isolate 5 exhibited the highest initial degradation rate, achieving 36% degradation on Day 1, indicating strong early enzymatic activity. This was followed by Isolate 1 (30%), while Isolates 4, 3, and 2 showed lower degradation rates of 20%, 14%, and 12%, respectively. Over the course of the incubation, Isolate 5 consistently maintained superior degradation efficiency, reaching a peak of 86% by Day 7, underscoring its robust keratinolytic capacity. Isolate 1 followed closely with 80% degradation, while Isolates 4, 3, and 2 recorded 58%, 52%, and 50%, respectively (Table. 1& Fig. 3).

Table. 1: Feather Degradation percentage (%)

Degradation percentage (%)					
Incubation period	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
DAY 1	30	12	14	20	36
DAY 2	36	18	20	24	40
DAY 3	40	26	28	30	54
DAY 5	50	34	36	38	60
DAY 6	60	40	42	46	68
DAY 7	80	50	52	58	86

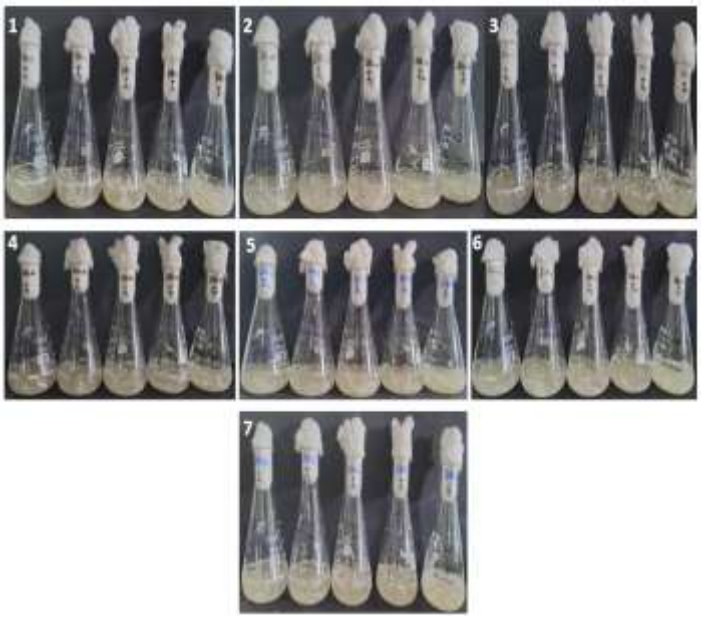

Figure. 3: Screening for potent Feather degrading bacteria for 1 to 7days of the incubation

Quantification of Keratinase Activity and Evaluation of Feather Hydrolysate Efficiency

The keratinolytic efficiency of the five bacterial isolates was further assessed by quantifying keratinase activity, protein yield, and amino acid content in the culture supernatant under optimized conditions. Isolate 5 demonstrated the highest keratinase activity (38 U/mL), along with a protein yield of 4.5 mg/mL and amino acid content of 360 µg/mL, indicating superior feather hydrolysis efficiency. In contrast, Isolate 1 showed moderate activity with 24 U/mL keratinase, 2.5 mg/mL protein, and 220 µg/mL amino acids. Isolates 4, 3, and 2 exhibited lower values, with Isolate 2 being the least effective. These results clearly identify Isolate 5 as the most potent strain, capable of converting keratin waste into nutrient-rich hydrolysates, supporting its suitability for applications in biofertilizer development and sustainable waste management (Fig. 4).

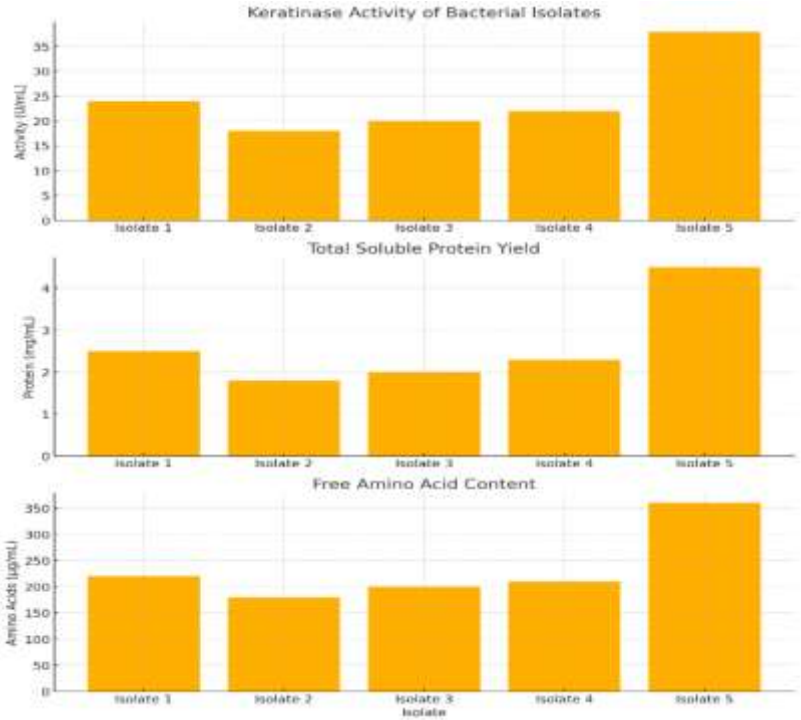

Figure. 4: Quantification of Keratinase Activity and Evaluation of Feather Hydrolysate Efficiency

Morphological Characterization of Isolate 5

To preliminarily identify the most efficient keratin-degrading isolate (Isolate 5), a series of staining techniques were employed. Simple staining confirmed the presence of uniform rod-shaped cells, typical of *Bacillus* species. Gram staining showed the isolate to be Gram-positive, with cells retaining crystal violet and appearing purple, indicating a thick peptidoglycan layer. Spore staining revealed distinct endospores, suggesting the ability to survive in harsh conditions, a key trait for long-term applications. Capsule staining confirmed the presence of a well-defined extracellular capsule, indicating potential for environmental persistence, biofilm formation, and protection under stress. Together, these observations classify Isolate 5 as a Gram-positive, spore-forming, capsulated rod, consistent with *Bacillus* spp., supporting its suitability for keratin waste degradation and biofertilizer development under diverse environmental conditions (Table. 2).

Table. 2: Morphological studies of isolate 5

Staining	Isolate 5
Simple staining	Bacillus
Gram's stain	Gram positive
Spore stain	Sporulated
Capsule stain	Capsulated

Biochemical Characterization of Isolate 5

A comprehensive biochemical profile of Isolate 5 revealed key metabolic traits relevant to its ecological adaptability and functional role in keratin degradation (Table 3 & Fig. 7). The isolate tested positive for indole production, indicating the presence of tryptophanase and suggesting active amino acid catabolism. It was methyl red positive and Voges-Proskauer negative, implying a preference for mixed acid fermentation over neutral end-product formation. Citrate utilization was positive, confirming its ability to use citrate as a sole carbon source, while catalase activity was evident through vigorous bubble formation, denoting its oxidative stress tolerance. The isolate tested negative for urease, suggesting limited capacity for urea hydrolysis. It showed positive hydrogen sulfide (H_2S) production, indicating sulfur metabolism. However, carbohydrate fermentation and starch hydrolysis tests were negative, suggesting limited carbohydrate-degrading ability. These biochemical traits support the identification of Isolate 5 as a metabolically versatile, keratinolytic bacterium well-suited for environmental and biotechnological applications (Table. 3).

Table. 3: Biochemical studies of isolate 5

Test	Result
Indole	Positive
Methyl red	Positive
Voges Proskauer	Negative
Citrate	Positive
Catalase	Positive
Urease	Negative
H_2S	Positive
Carbohydrate fermentation	Negative
Starch hydrolysis	Negative

Molecular Identification and Phylogenetic Analysis of Isolate 5

The partial 16S rRNA gene of the most potent keratin-degrading isolate (Isolate 5) was successfully amplified and submitted to GenBank under accession number PQ443918.1. Sequence analysis confirmed its identity as *Bacillus licheniformis*, and the strain was designated *Bacillus licheniformis* **CFMSRNR 5**. Sequencing was performed using Sanger dideoxy technology and submitted via the Department of Environmental Science, Osmania University, Hyderabad. Phylogenetic analysis based on sequence alignment with related *Bacillus* species revealed that CFMSRNR 5 clusters closely with *Bacillus licheniformis* reference strains, forming a distinct clade (Fig. 5). A broader phylogenetic tree (Fig. 6) further indicated evolutionary proximity to *B. pumilus*, *B. subtilis*, and *B. cereus*, confirming its taxonomic placement. The inferred intermediate nodes suggest ancestral relationships, supporting the isolate's classification and potential as a keratinolytic candidate.

Bacillus licheniformis strain CFMSRNR 5 16S ribosomal RNA gene, partial sequence

GenBank: PQ443918.1

GenBank Graphics

>PQ443918.1 Bacillus licheniformis strain CFMSRNR 5 16S ribosomal RNA gene, partial sequence

ACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCGCATGGTTCAATCATAAAA
GGTGGCTTTTAGCTACCACTTGCAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACC
AAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAT
GAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGCACCTTG
ACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT
TGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCA
ACCGGGGAGGGTCATTGGAAACTGGGGAACTTGATGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGT
GAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGC
GCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTG
TTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAA
GACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACG
CGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCCCCTTCGGGGGCAGAG
TGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGG
TGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCAGAACAAA
GGGCAGCGAAGCCGCGAGGCTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTC
GACTGCGTGAAGCTGGAATCGCTAGTAATCGCGG

Figure. 5: FASTA sequence of isolate 5

The phylogenetic relationship of the keratinolytic isolate CFMSRNR 5 was established through 16S rRNA gene sequencing and comparison with related sequences from the NCBI GenBank database. A neighbor-joining phylogenetic tree, bootstrapped with 1000 replicates, confirmed its placement within the *Bacillus licheniformis* clade. The isolate showed a close relationship with *Bacillus licheniformis* strain HBUAS74056, supported by a high bootstrap value of 83%. This strain further clustered with *B. licheniformis* strains HBUAS56832 and AUMC-B472 at 100% and 95% support, respectively, indicating strong genetic relatedness. CFMSRNR 5 also exhibited evolutionary proximity to *Bacillus* sp. strain g14 and *B. licheniformis* strain Y1 (94% support), while more distant relationships were observed with strains MX5 and JCA-1201 (93% support). These clustering patterns confirm the isolate's taxonomic identity within the *B. licheniformis* species complex and support its keratinolytic potential (Fig. 6).

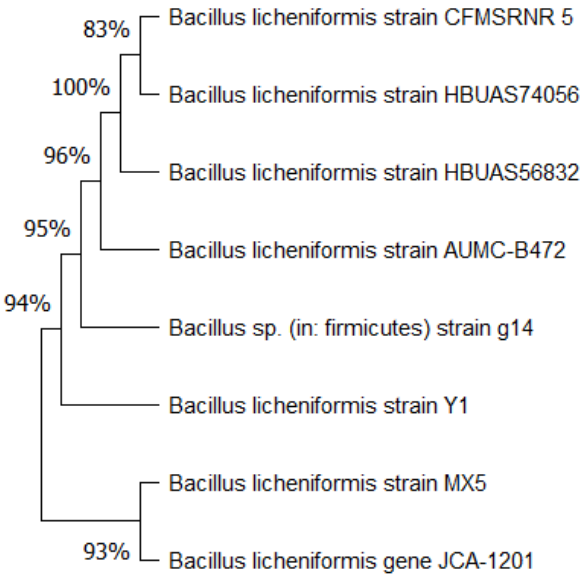

Figure. 6: Phylogenetic tree of isolate 5

DISCUSSION

The successful isolation and preliminary characterization of feather-degrading bacteria from poultry waste sites underscore the ecological potential of keratinolytic microbes in the bioconversion of recalcitrant organic waste. In this study, soil samples collected from poultry feather disposal areas in Hyderabad, Telangana, served as a promising source for keratinolytic bacteria. These sites, rich in proteinaceous waste, offer selective pressure for the proliferation of bacteria capable of utilizing feather keratin as a sole carbon and nitrogen source. The use of mineral salts medium (MSM) enriched with 1% sterilized chicken feathers promoted the growth of such specialized microbial populations, as evidenced by increased turbidity and partial feather disintegration during the enrichment phase.

The observed degradation aligns with findings from earlier studies where feather hydrolysis by bacteria such as *Bacillus licheniformis* and *Bacillus subtilis* was associated with keratinase secretion in minimal media containing feather as the only nutrient source (Tiwary & Gupta, 2020; Reddy et al., 2022). Feather degradation is a complex enzymatic process that involves the breakdown of disulfide bonds and peptide linkages, which only a select group of microorganisms can accomplish effectively. The turbidity in the broth and visual reduction in feather density strongly suggest enzymatic activity consistent with keratinolytic behavior.

Serial dilution and plating on nutrient agar enabled the isolation of distinct colonies from the enriched culture, allowing for the selection of morphologically varied strains. The appearance of colonies with diverse characteristics, such as irregular shapes, varied margins, and creamy-white pigmentation, reflects both genetic diversity and ecological adaptation of these isolates to the keratin-rich environment. Irregular colony morphology and small colony size, frequently reported in keratinolytic *Bacillus* spp., suggest a metabolic shift towards enzyme production over rapid proliferation (Gopinath et al., 2021). Similar observations were made by Onifade et al. (2021), who noted that *Bacillus* isolates from poultry soil exhibited compact growth and strong feather-degrading potential.

The undulate margins observed in some isolates (I3, I4, I5) may be indicative of extracellular enzyme secretion, where localized diffusion of keratinase alters colony expansion patterns (Gupta & Ramnani, 2021). This margin variability is a known morphological cue reflecting enzymatic interactions with the surrounding substrate on solid media. Furthermore, the creamy white, opaque colonies support the likelihood of *Bacillus* genus affiliation, which is corroborated by reports describing similar phenotypes among known keratinolytic strains (Shahiful et al., 2024). Repeated sub-culturing and quadrant streaking ensured the purification of axenic cultures, which is critical for downstream biochemical and molecular analyses. Establishing pure cultures with stable morphological traits is essential not only for reliable characterization but also for preserving potential bioactive strains for future applications. According to El-Katatny et al. (2020), maintenance of pure, keratinolytic strains is vital for their successful deployment in feather waste valorization and enzyme production systems.

The results from this study are consistent with the growing body of research supporting the isolation of environmentally adapted keratin-degrading bacteria from organic waste niches. These strains hold significant promise for eco-friendly waste management, enzyme biotechnology, and the production of nitrogen-rich feather hydrolysates suitable for agricultural applications (Kumar & Das, 2022; Borges et al., 2022).

The feather degradation assay conducted over a seven-day period provided clear insights into the keratinolytic efficiency of the five bacterial isolates under investigation. The consistent reduction in feather mass across all isolates demonstrates their ability to degrade native keratin, albeit with varying levels of efficiency. Among them, Isolate 5 showed the highest degradation percentage, reaching 86% by Day 7, with a rapid degradation onset of 36% on Day 1, indicating strong early keratinase activity and effective substrate utilization. This performance suggests that Isolate 5 possesses a robust keratinolytic enzyme system capable of initiating and sustaining efficient keratin breakdown.

The trend observed in Isolate 1, which reached 80% degradation by Day 7, also indicates considerable enzymatic capability, although slightly less efficient than Isolate 5. The remaining isolates—Isolates 4, 3, and 2—exhibited moderate degradation capabilities, achieving final degradation percentages of 58%, 52%, and 50%, respectively. The lower degradation efficiency in these isolates may be attributed to reduced keratinase production, slower metabolic adaptation, or less effective enzyme-substrate interaction under the test conditions.

The superior performance of Isolate 5 is consistent with previous studies that have highlighted the high keratinolytic potential of *Bacillus licheniformis* strains. For instance, *Bacillus licheniformis* ER-15 was reported by Gupta and Ramnani (2021) to achieve over 80% feather degradation within a similar incubation period, supporting the idea that this species is well-adapted for keratin hydrolysis. Likewise, Tiwary and Gupta (2020) emphasized that early and sustained degradation profiles are indicative of strong keratinase secretion and efficient regulatory mechanisms for protease expression in *Bacillus* species.

Efficient keratin degradation requires a synergistic action of endo- and exo-keratinases that hydrolyze both peptide bonds and disulfide linkages. Isolate 5's ability to maintain a higher degradation rate throughout the incubation period suggests that it may produce a broad spectrum of keratinolytic enzymes, possibly along with disulfide reductases, as described by Reddy et al. (2022). Moreover, its consistent performance under static culture conditions further suggests its suitability for large-scale bioconversion processes without the need for stringent agitation or aeration. These findings support the growing consensus that bacterial keratinolysis, particularly through potent strains like *Bacillus licheniformis*, presents a viable strategy for the management of poultry waste and the generation of biofertilizers or feed supplements (Gopinath et al., 2021; El-Katatny et al., 2020). The significant variation in degradation performance among the isolates also highlights the need for careful screening and strain selection when developing microbial solutions for keratin-rich waste treatment.

Quantitative evaluation of keratinase activity, protein yield, and amino acid content in the feather hydrolysates produced by the five bacterial isolates revealed significant variability in their keratin-degrading capacities. Among all, Isolate 5 exhibited the highest keratinase activity (38 U/mL), along with the greatest yield of soluble protein (4.5 mg/mL) and amino acid content (360 µg/mL). These results reflect efficient enzymatic hydrolysis of feather keratin into bioavailable forms, highlighting the isolate's potential for use in feather waste valorization. The superior performance of Isolate 5 suggests that it possesses a robust enzymatic system, including both endo- and exo-keratinases, capable of cleaving the rigid structure of native keratin. This aligns with earlier findings where *Bacillus licheniformis* strains demonstrated high keratinase output and efficient feather solubilization, as documented by Riffel et al. (2021) and Zhang et al. (2020). Such activity is vital for the breakdown of disulfide-rich keratin into amino acids and peptides suitable for agricultural use.

In contrast, Isolate 1 showed moderate keratinolytic potential, producing 24 U/mL of keratinase with a corresponding 2.5 mg/mL protein yield and 220 µg/mL amino acids, while the remaining isolates (2, 3, and 4) exhibited lower enzymatic activities and hydrolysate concentrations. Isolate 2, in particular, recorded the lowest keratinase activity (18 U/mL), suggesting limited capacity for keratin breakdown, possibly due to the absence of key proteolytic enzymes or ineffective enzyme secretion mechanisms. The high amino acid concentration in the hydrolysate from Isolate 5 is particularly valuable from an agronomic perspective. Amino acids like serine, glycine, and cysteine not only act as nitrogen sources but also play roles in stress tolerance, hormone signaling, and root development in plants (Colla et al., 2015). Studies have shown that protein hydrolysates derived from microbial keratin degradation can enhance seed germination, nutrient uptake, and overall plant growth (Kauffman et al., 2020; Ertani et al., 2018). Moreover, the elevated protein content in Isolate 5's hydrolysate may contribute to soil microbial stimulation, further enhancing soil fertility. This is supported by the findings of Bhange et al.

(2021), who reported improved microbial activity and plant biomass upon application of feather hydrolysates to soil systems.

The identification and characterization of the most potent keratin-degrading bacterium in this study, Isolate 5, was carried out through a combination of morphological, biochemical, and molecular approaches. The results strongly support its classification as *Bacillus licheniformis* strain CFMSRNR 5, a well-known keratinolytic bacterium with high potential for biotechnological applications, particularly in the valorization of poultry feather waste.

Morphological staining techniques provided key insights into the structural and survival characteristics of Isolate 5. Simple staining revealed rod-shaped cells, which, along with Gram-positive reactions, suggest a thick peptidoglycan cell wall consistent with members of the genus *Bacillus*. The presence of endospores, confirmed by spore staining, indicates the ability of the isolate to survive under nutrient stress and adverse environmental conditions, a critical trait for industrial strain longevity and environmental adaptability (de Oliveira et al., 2021). Furthermore, capsule staining demonstrated the presence of a defined extracellular capsule, which plays an important role in surface attachment, biofilm formation, and protection from desiccation, enhancing the organism's ecological resilience (Kim et al., 2022).

Biochemical assays further validated the functional capabilities of Isolate 5. The positive indole test confirms the organism's ability to metabolize tryptophan, a feature that contributes to nitrogen cycling and soil fertility (Arunachalam et al., 2020). Its methyl red-positive and VP-negative profile indicates reliance on the mixed acid fermentation pathway, which is typical of environmental Bacillus strains adapted to metabolize complex substrates. The ability to utilize citrate as the sole carbon source highlights metabolic flexibility, an essential feature for growth in nutrient-limited environments like poultry litter or feather waste zones. Additionally, catalase activity confirms oxidative stress tolerance, supporting its potential to survive aerobic fermentation processes (Sharma et al., 2021). While the isolate was negative for urease, indicating limited urea hydrolysis, it demonstrated positive hydrogen sulfide production, which may contribute to sulfur metabolism during protein degradation (Singh et al., 2022).

Interestingly, Isolate 5 did not ferment carbohydrates or hydrolyze starch, suggesting its metabolic specialization for proteinaceous substrates such as keratin rather than polysaccharides. This specialization is in line with findings from Brandelli et al. (2020), who reported that certain *Bacillus* species prioritize proteolysis over saccharolytic activity when grown in keratin-rich conditions. The combination of these traits highlights the isolate's niche adaptation and supports its role as a specialized feather-degrading microorganism.

Molecular analysis provided definitive confirmation of the isolate's identity. Partial 16S rRNA gene sequencing and subsequent submission to GenBank (accession no. PQ443918.1) confirmed the isolate as *Bacillus licheniformis*. Phylogenetic analysis using the neighbor-joining method, supported by bootstrap values up to 1000 replicates, revealed that strain CFMSRNR 5 clusters closely with B. licheniformis strains HBUAS74056, HBUAS56832, and AUMC-B472, forming a robust clade with high sequence similarity (bootstrap support: 83–95%). The tree also indicated evolutionary proximity to other Bacillus members, such as *B. pumilus*, *B. subtilis*, and *B. cereus*, with intermediate branches highlighting ancestral lineage (Fig. 6). These results are consistent with earlier reports demonstrating the phylogenetic coherence of keratinolytic *B. licheniformis* strains (Jadhav et al., 2021; Abdel-Fattah et al., 2020). The close genetic relationship with known keratinolytic Bacillus strains reinforces CFMSRNR 5's functional identity. Its high sequence similarity and placement within the *Bacillus licheniformis* complex align with previous studies where *B. licheniformis* has been reported as an efficient keratinase producer, capable of converting feather waste into soluble peptides and amino acids (Mazotto et al., 2020; Zhang et al., 2021).

CONCLUSION

The present investigation demonstrates the successful isolation and comprehensive characterization of a potent keratinolytic bacterium, *Bacillus licheniformis* CFMSRNR 5, from feather disposal sites near poultry farms in Hyderabad, Telangana. Through an enrichment-based approach using chicken feathers as the sole carbon and nitrogen source, five bacterial isolates were initially screened, among which Isolate 5 showed the most significant keratin degradation capacity. Over a 7-day incubation period, this isolate achieved up to 86% feather degradation, significantly outperforming other strains, and indicating a robust enzymatic breakdown of keratinous substrates. Biochemical assays revealed that *B. licheniformis* CFMSRNR 5 possesses important metabolic traits, such as catalase activity, indole production, hydrogen sulfide release, and citrate utilization. These characteristics

underline its environmental resilience and enzymatic efficiency in degrading complex proteinaceous waste. Molecular identification via 16S rRNA sequencing confirmed the isolate's identity, and phylogenetic analysis placed it within a well-supported clade of *Bacillus licheniformis*, further affirming its taxonomic position.

Quantitative assessments of keratinase activity, protein yield, and amino acid production clearly demonstrated that this isolate has a high potential to convert feather waste into bioavailable nutrients. The ability to produce a significant concentration of keratinase (38 U/mL), along with 4.5 mg/mL of protein and 360 µg/mL of amino acids, underscores its industrial potential for sustainable bioprocessing applications. Moreover, the morphological characteristics—such as spore formation and the presence of an extracellular capsule—suggest that *B. licheniformis* CFMSRNR 5 can withstand environmental stresses and maintain viability in various soil conditions. This makes it a strong candidate for field-level applications, particularly in the formulation of biofertilizers derived from keratin-rich wastes like poultry feathers.

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