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Isolation and Molecular Identification of Novel Chicken Feather Degrading Bacteria *Bacillus tropicus* KRKJVR 5

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The isolation and molecular identification of a novel chicken feather-degrading bacterium, *Bacillus tropicus* KRKJVR 5, were conducted to explore its potential in biotechnological applications. Soil samples collected from various sites in Suryapet district, Telangana, were screened using the enrichment culture method to identify potential keratinolytic producers. A total of ten bacterial isolates were initially identified, out of which five isolates demonstrated significant feather degradation on feather-containing agar media. Among these, Isolate 5 exhibited the highest keratinase activity, completely degrading feathers within 48 hours and producing 8.66 U/ml of keratinase, as determined by the Azocasein assay. Morphological and biochemical tests characterized Isolate 5 as a rod-shaped, Gram-positive Bacillus species with positive results for spore staining, Voges-Proskauer, catalase, starch hydrolysis, and carbohydrate fermentation tests,

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and negative results for indole, methyl red, citrate, H2S, and urease tests. Definitive genetic identification through 16S rRNA sequencing and comparison with the NCBI database confirmed Isolate 5 as *Bacillus tropicus* KRKJVR 5, with a 92% similarity to other *Bacillus tropicus* strains. The sequence data was submitted to NCBI and assigned the accession number PP859287. These findings highlight the potential of *Bacillus tropicus* KRKJVR 5 for efficient keratin degradation, making it a promising candidate for various industrial and environmental applications.

Keywords: Isolation; molecular identification; novel; chicken feather degrading bacteria; Bacillus tropicus KRKJVR 5.

1. INTRODUCTION

Feathers, a by-product of the poultry industry, represent a significant waste management challenge due to their high keratin content and resistance to degradation [1]. Each year, billions of tons of feathers are generated worldwide, contributing to environmental pollution and waste management issues [2]. The conventional methods of disposal, such as incineration and landfilling, are not environmentally sustainable and can lead to increased greenhouse gas emissions and other ecological concerns [2]. Furthermore, incineration of feathers releases carbon dioxide and other pollutants into the atmosphere, while landfilling can lead to the accumulation of non-degradable waste, occupying valuable land space and posing a potential risk of groundwater contamination [3]. Given these challenges, there is a growing interest in developing eco-friendly methods for the degradation of feathers. One promising approach is microbial degradation, which harnesses the natural ability of certain microorganisms to break down keratin, the primary protein in feathers [3]. Keratin is a fibrous structural protein that provides feathers with their strength and resilience. It is characterized by a high degree of cross-linking through disulfide bonds, which makes it highly stable and resistant to breakdown by common proteases [4]. This inherent stability poses a significant challenge for feather degradation and requires the search for efficient keratin-degrading microorganisms that can overcome these barriers.

Among the various microorganisms capable of degrading keratin, bacteria, particularly those from the genus *Bacillus*, have shown considerable potential [5]. *Bacillus* species are known for their robust enzymatic activity and ability to produce keratinolytic enzymes that can hydrolyze the disulfide bonds in keratin, leading to effective feather degradation [6]. These enzymes, including keratinases, proteases, and other auxiliary enzymes, facilitate the breakdown of keratin into smaller peptides and amino acids, which can then be further utilized or converted into valuable byproducts such as animal feed additives, fertilizers, and bioactive compounds [6]. The bioconversion of feathers into these byproducts not only helps in waste management but also adds economic value, contributing to a circular bio economy. Molecular identification techniques, including 16S rRNA gene sequencing, have been instrumental in characterizing and classifying bacterial species, including those involved in keratin degradation [7]. The 16S rRNA gene is a highly conserved region of the bacterial genome that provides a reliable marker for phylogenetic analysis and species identification [8]. By analysing the sequence of this gene, researchers can accurately identify bacterial species and determine their evolutionary relationships with other organisms [8]. This method offers a precise and reliable approach to identifying bacteria, allowing for a deeper understanding of their phylogenetic relationships and functional capabilities [7].

Overall, the integration of molecular identification techniques with traditional microbiological methods provides a powerful approach to studying keratin-degrading bacteria and developing sustainable solutions for feather waste management. In this context, the current study aims to isolate and identify a novel feather-degrading bacterium, *Bacillus tropicus* KRKJVR 5, and to investigate its potential for efficient keratin degradation through molecular characterization and enzymatic analysis. The findings from this study are expected to contribute to the development of eco-friendly strategies for the management of poultry feather waste and the advancement of microbial biotechnology.

2. METHODOLOGY

2.1 Sample Collection

Soil samples were collected from various poultry farms and poultry waste processing sites in

Suryapet, Telangana, known for harbouring keratinolytic microorganisms. Approximately 100 to 500 grams of soil from different locations were gathered using sterilized tools, placed in sterile containers, and transported to the lab.

2.2 Preparation of Chicken Feathers

Chicken feathers, obtained from a local poultry processing plant, were washed with detergent, sterilized by autoclaving at 121°C for 15 minutes, air-dried, and cut into 2-3 cm pieces. These prepared feathers were stored in sterile containers for further experiments.

2.3 Enrichment Technique

For the enrichment culture, 10 grams of soil were combined with 100 mL of a mineral salts medium, to which 1% (v/v) of sterilized chicken feathers was added. This mixture was then incubated at 37°C on a rotary shaker set at 200 rpm for five days. After incubation, the samples were serially diluted to isolate individual bacterial colonies. Dilutions of 10^{-6} and 10^{-7} were spread on nutrient agar plates and incubated at 37°C for 24-48 hours. The resulting bacterial colonies were then sub-cultured for further analysis [9].

2.4 Preparation of Feather Meal

Feather meal was prepared by washing white feathers from poultry slaughterhouses with soap and water to remove blood and dirt, followed by sun-drying for 24 hours. The feathers were then cut into 1-3 cm pieces and defatted using a chloroform and methanol mixture (1:1) for two days, followed by a chloroform, acetone, and methanol mixture (4:1:3) for an additional two days, with daily solvent changes. After rinsing with tap water to remove residual solvents, the feathers were dried at 40°C for 24 hours and ground into a fine powder. Sub cultured bacteria were inoculate on feather meal agar plates containing 5 g feather meal, 0.5 g NaCl, 0.3 g K_2HPO_4, 0.4 g KH_2PO_4, 0.2 g $MgSO_4$•$7H_2O$, and 15 g agar per liter. The pH was adjusted to 7.0. Plates were then incubated at 30°C for 5-7 days. Colonies exhibiting clear zones, indicative of keratin degradation, were selected for further studies.

2.5 Keratinase Production Assay

Isolates were cultivated in feather meal broth, consisting of 5 g feather meal, 0.5 g NaCl, 0.3 g K_2HPO_4, 0.4 g KH_2PO_4, and 0.2 g $MgSO_4$•$7H_2O$ per liter, with pH adjusted to 7.0. Cultures were incubated at 30°C with agitation at 150 rpm for 5 days. Following incubation, the cultures were centrifuged at 10,000 rpm for 15 minutes to collect the cell-free supernatant, which was used for subsequent keratinase activity assays.

2.6 Azocasein Assay for Keratinase Activity

Keratinase activity was assessed using the azocasein assay. The reaction mixture consisted of 0.5 ml of 0.5% (w/v) azocasein in 50 mM Tris-HCl buffer (pH 8.0) and 0.5 ml of the enzyme sample (cell-free supernatant). The mixture was incubated at 37°C for 30 minutes and then halted by adding 0.5 ml of 10% trichloroacetic acid (TCA). After centrifugation at 10,000 rpm for 10 minutes, the absorbance of the supernatant was measured at 440 nm against a blank.

Formula: Enzyme activity (U/ml) = (ΔA440) / (0.01 * incubation time in minutes * volume of enzyme in ml).

Identification and characterization of selected isolate by morphological, biochemical, and molecular methods (16s RNA sequences): The organism's identity was established through a series of morphological and biochemical tests. Morphological characteristics were assessed using gram staining to determine cell wall structure, spore staining to identify spore-forming capability, and motility testing to observe movement patterns. Biochemical tests included the IMViC panel (Indole production, Methyl Red test, Voges-Proskauer test, and Citrate utilization) to assess metabolic pathways, catalase testing to confirm the presence of catalase enzyme activity, starch hydrolysis testing to evaluate enzyme production, and tests for hydrogen sulfide (H_2S) production and glucose fermentation capabilities.

Molecular identification (16s RNA sequences): Isolate 5 underwent molecular identification at MACROGEN (Seoul, Korea) using standard 16S rRNA primers. Following sequencing, the 16S rRNA gene sequences were analysed and used for phylogenetic analysis using bioinformatics tools to construct an evolutionary tree. Subsequently, the sequences were publicly submitted to NCBI, where they were compared against existing GenBank sequences using the BLAST program. This comparison enabled the identification of closely related organisms and confirmed the

taxonomic classification of isolate 5 based on genetic similarities.

3. RESULTS

Soil samples were meticulously gathered from various locations within the Suryapet district of Telangana. The primary aim was to identify potential keratinolytic producers. This selection was achieved by employing the enrichment culture method, a technique that facilitates the proliferation of microorganisms capable of degrading keratin (Fig. 1). The enrichment process involved incubating the soil samples in a specific nutrient medium that favors the growth of keratinolytic microbes, thereby enabling their identification and subsequent study. The results of this method were carefully analyzed to pinpoint the most promising keratinolytic producers, which could be crucial for various industrial and environmental applications.

Fig. 1. Screening of chicken feather degrading bacteria

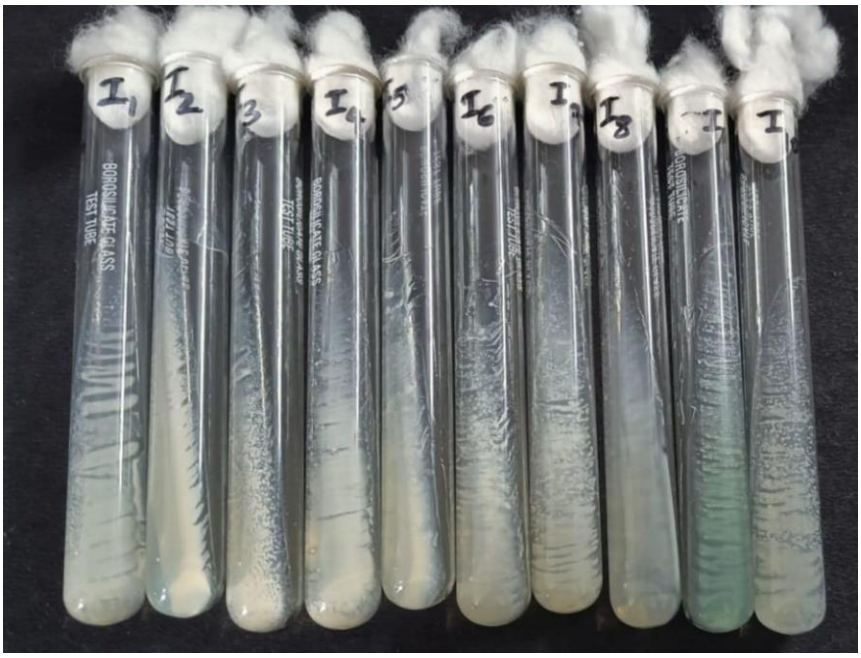


Fig. 2. Chicken feather degrading bacteria pure cultures

Table 1. Isolates colony morphology studies

Isolate no	Colour	Shape	Size	Elevation	Margin	Opacity
1	Creamish white	Circular	Medium	Slightly raised	Entire	Non Transparent
2	Creamish white	Irregular	Large	Slightly raised	Entire	Non Transparent
3	Creamish white	Irregular	Large	Flat	undulate	Transparent
4	Creamish white	Circular	Medium	Flat	Entire	Non Transparent
5	Creamish white	Circular	Medium	Slightly raised	Entire	Non Transparent
6	Creamish white	Irregular	Large	Flat	Entire	Transparent
7	Creamish white	Circular	Large	Slightly raised	Entire	Non Transparent
8	Creamish white	Circular	medium	Flat	Entire	Transparent
9	Creamish white	Irregular	Small	Flat	undulate	Transparent
10	Creamish white	circular	small	flat	Entire	Non Transparent

A total of ten distinct bacterial isolates were obtained using the enrichment technique. Among these, specific feather-degrading bacteria were isolated and purified to achieve pure cultures of chicken feather-degrading bacteria. These pure cultures were carefully maintained to facilitate further characterization (Fig. 2). The isolation and characterization of bacterial colonies from the collected soil samples resulted in ten distinct isolates. Each isolate exhibited unique morphological characteristics. For instance, Isolate 1 was creamish white, circular in shape, of medium size, slightly raised elevation, with an entire margin and non-transparent opacity. Isolate 2 shared the creamish white color but had an irregular shape, large size, and similar elevation and margin, also displaying non-transparent opacity. In contrast, Isolate 3 was irregular, large, flat, and undulate in margin, with transparent opacity. Isolate 4 was circular, medium-sized, flat, and entire with non-transparent opacity. Isolate 5 mirrored the characteristics of Isolate 1. Isolate 6, despite its creamish white color and irregular shape, was large, flat, and entire with transparent opacity. Isolate 7 was similar to Isolate 1 but larger in size. Isolate 8 was circular, medium, flat, entire, and transparent. Isolate 9 differed with its irregular, small, flat, and undulate margin and transparent opacity. Finally, Isolate 10 was circular, small, flat, and entire with non-transparent opacity (Table 1). These detailed morphological characteristics facilitated the further study of their keratinase production potential and applications.

3.1 Selected Isolates Growth on Feather Meal Agar

After screening for feather-degrading bacteria, the growth of the isolates was tested on feather-containing agar media plates to assess their keratinase activity. Out of the ten isolates tested, five isolates (specifically Isolates 1, 2, 5, 7, and 10) demonstrated significant feather degradation. These isolates exhibited a marked ability to break down feathers, indicating their potential for high keratinase production. Consequently, these five isolates were selected for more detailed studies to further explore their enzymatic properties, optimize their growth conditions, and evaluate their potential applications in various industries. The comprehensive analysis aimed to enhance our understanding of their capabilities and to harness their feather-degrading properties effectively.

3.2 Keratinase Production

The Fig. 4 depicts Erlenmeyer flasks labelled 1, 2, 5, 7, and 10, each containing cultures of chicken feather-degrading bacteria. The flasks show varying levels of feather degradation, reflecting the efficiency of each bacterial isolate in breaking down keratin substrates. Notably,

Isolate 5 exhibited exceptional efficiency, completely degrading the feathers within 48 hours. In contrast, the other isolates, while still effective, required 72 hours to achieve complete degradation. This rapid degradation by Isolate 5 highlights its superior keratinase activity and potential for applications where quick and efficient feather breakdown is desired. The findings underscore the promising capabilities of these bacterial isolates, particularly Isolate 5, for further biotechnological exploration and utilization.

3.3 Azocasein Assay for Keratinase Activity

In the Azocasein assay conducted to measure keratinase activity, the five selected isolates were screened to determine their enzyme production levels. Among all the isolates, Isolate 5 exhibited the highest keratinase activity, producing 8.66 U/ml. The other isolates demonstrated varying levels of activity: Isolate 1 produced 3.3 U/ml, Isolate 2 produced 4.66 U/ml, Isolate 7 produced 6 U/ml, and Isolate 10 produced 2.66 U/ml. These results clearly indicate that Isolate 5 not only degrades feathers more rapidly but also produces a significantly higher amount of keratinase enzyme compared to the other isolates. This superior keratinase production makes Isolate 5 a particularly promising candidate for applications requiring efficient keratin degradation. Based on results of degradation capacity and keratinase activity isolate 5 was selected for further studies.

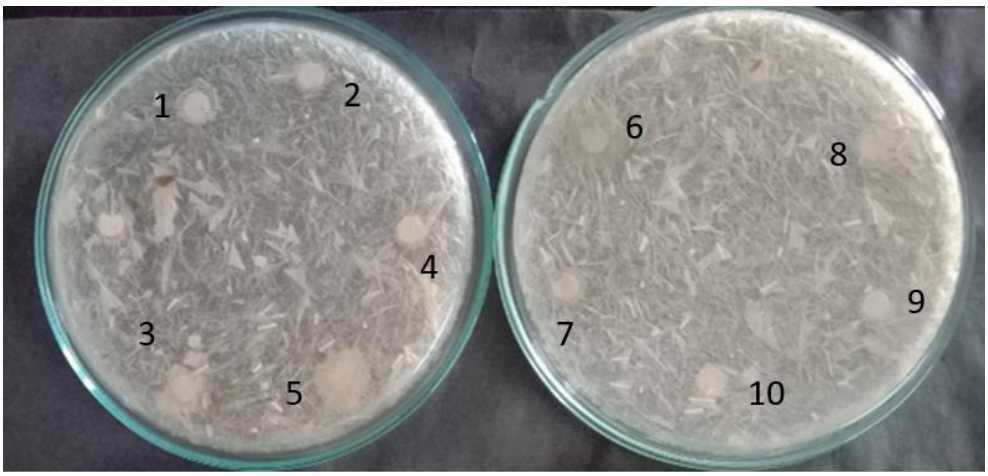


Fig. 3. Bacterial growth on feather meal agar

Fig. 4. Degradation of chicken feathers & keratinase production

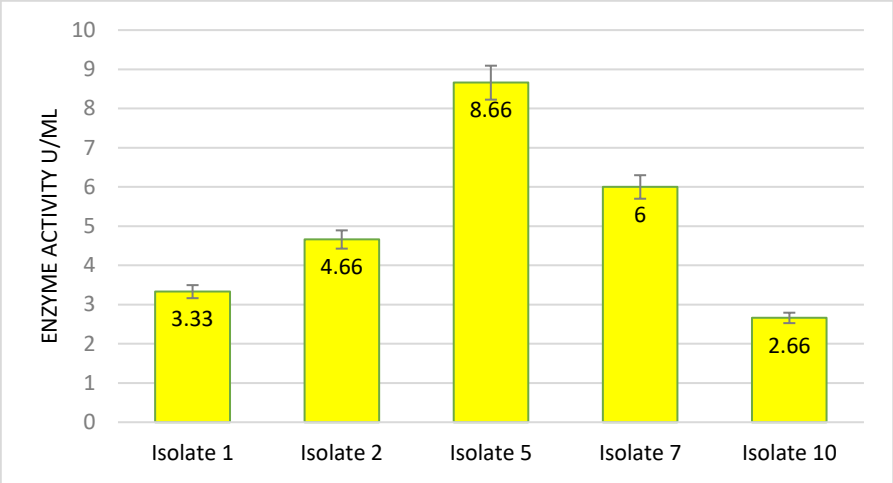


Fig. 5. Azocasein assay for keratinase activity

Table 2. Morphological and biochemical test of selected isolate 5

Test	Results
Shape	Bacillus (Rod)
Gram staining	Positive
Spore staining	Positive
Indole test	Negative
Methyl Red test	Negative
Voges Porskauer Test	Positive
Citrate test	Negative
Catalase test	Positive
H_2S	Negative
Urease	Negative
Starch hydrolysis	Positive
Carbohydrate fermentation	Positive

After screening 10 feather-degrading bacteria, we aimed to identify the most potent isolates. Their growth was tested on feather-containing agar plates to assess keratinase activity. Five isolates (1, 2, 5, 7, and 10) demonstrated significant feather degradation, indicating high keratinase production. In the Azocasein assay for keratinase activity, Isolate 5 showed the highest enzyme production at 8.66 U/ml, followed by Isolate 7 (6 U/ml), Isolate 2 (4.66 U/ml), Isolate 1 (3.3 U/ml), and Isolate 10 (2.66 U/ml). Isolate 5's superior keratinase production and rapid feather degradation make it a promising candidate for further studies and applications.

3.4 Morphological and Biochemical test of Selected Isolate 5

The morphological and biochemical tests conducted on the selected Isolate 5 revealed several key characteristics. Morphologically, Isolate 5 is a Bacillus species, characterized by its rod-shaped structure. In terms of Gram staining, it tested positive, indicating that it has a thick peptidoglycan cell wall. Additionally, Isolate 5 tested positive for spore staining, confirming its ability to form spores. Biochemically, Isolate 5 showed a diverse range of activities. It tested negative for the indole and methyl red tests, suggesting it does not produce indole or maintain a stable acid end product from glucose fermentation. However, it tested positive in the Voges-Proskauer test, indicating the production of acetoin. The citrate test was negative, implying that Isolate 5 does not utilize citrate as a sole carbon source. The catalase test was positive, demonstrating the presence of the enzyme catalase, which breaks down hydrogen peroxide. Isolate 5 tested negative for H2S production and urease activity, indicating it does not produce hydrogen sulfide or urease. It also tested positive for starch hydrolysis, showing it can break down starch into simpler sugars, and positive for carbohydrate fermentation, indicating its ability to

ferment various carbohydrates. These comprehensive morphological and biochemical characteristics highlight the potential of Isolate 5 in various biotechnological applications, especially those involving keratin degradation.

3.5 Molecular Identification of Isolate 5 based on 16S rRNA Gene Sequence

Molecular analysis, specifically 16S rRNA sequencing, provided a definitive genetic identification of the bacterial isolates (Fig. 6). The sequencing results were compared with known bacterial sequences in the NCBI database, leading to the conclusive identification of Isolate 5 as *Bacillus tropicus* KRKJVR 5. The sequence data was subsequently submitted to the National Center for Biotechnology Information (NCBI), where Isolate 5 was assigned the accession number PP859287 (Fig. 7). Additionally, the construction of a phylogenetic tree revealed that Isolate 5 shares a 92% similarity with other *Bacillus tropicus* strains, further confirming its genetic identity and relationship within the Bacillus genus (Fig. 8). This precise identification is crucial for understanding the potential applications and characteristics of *Bacillus tropicus* KRKJVR 5, especially in the context of keratin degradation and other biotechnological uses.

4. DISCUSSION

The isolation and characterization of keratinolytic bacteria from soil samples in Suryapet district, Telangana, employed the enrichment culture method to identify potential producers capable of degrading keratin (Fig. 1). This method involved incubating soil samples in a nutrient-rich medium conducive to the growth of keratin-degrading microorganisms, facilitating their isolation and subsequent study for industrial and environmental applications. A total of ten distinct bacterial isolates were obtained through this enrichment technique, focusing on isolating and purifying specific feather-degrading bacteria to establish pure cultures (Fig. 2). Each isolate exhibited unique morphological traits critical for understanding their potential keratinase production capabilities and practical applications. For instance, Isolate 1 and Isolate 5 shared similar creamish white coloration and circular shapes, differing mainly in size and opacity characteristics. This observation is consistent with previous studies highlighting the phenotypic similarities among keratinolytic bacteria within the same genus or species [10].

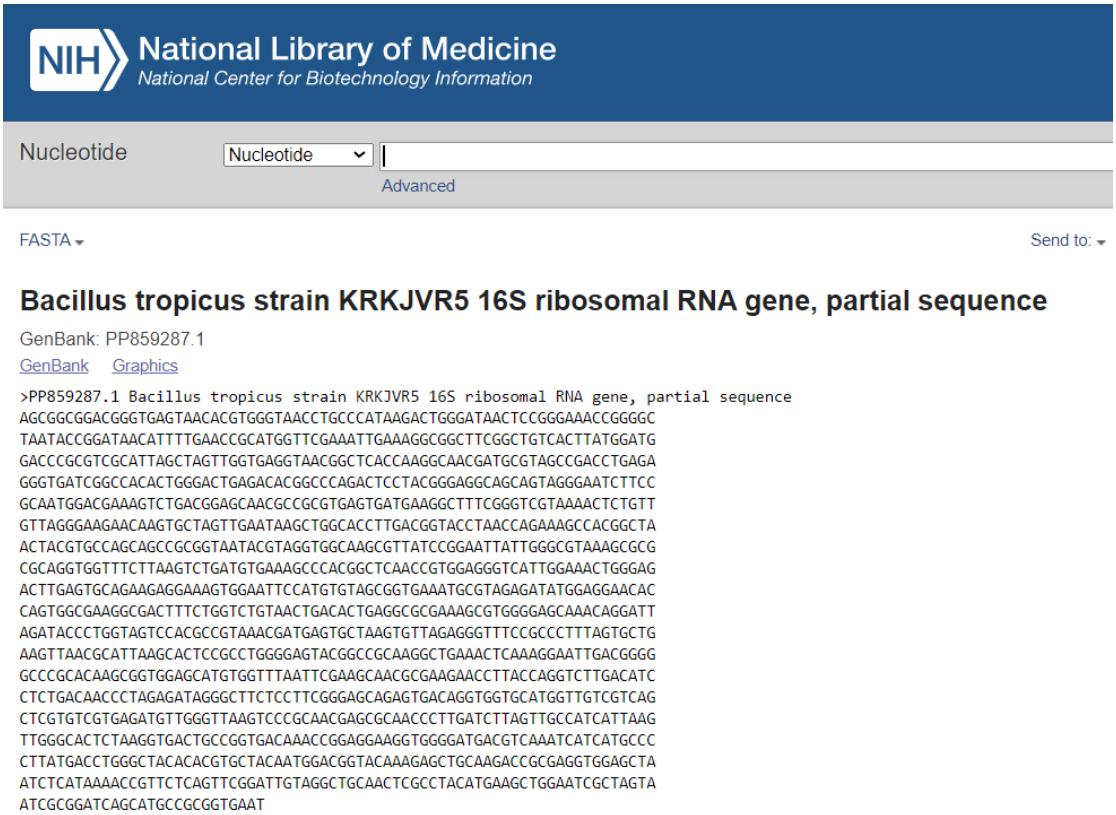
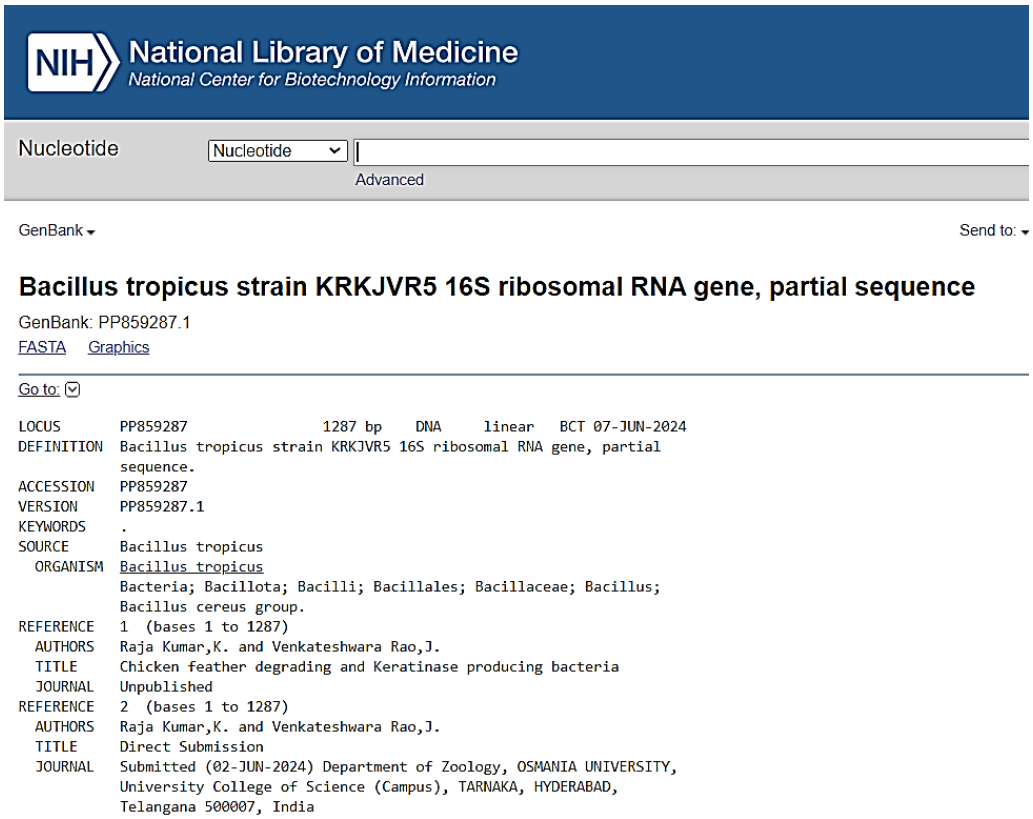


Fig. 6. FASTA sequence of isolate 5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Bacillus tropicus strain KRKJVR5 16S ribosomal RNA gene, partial sequence

GenBank: PP859287.1

FASTA Graphics

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DEFINITION  Bacillus tropicus strain KRKJVR5 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   PP859287
VERSION     PP859287.1
KEYWORDS    .
SOURCE      Bacillus tropicus
  ORGANISM  Bacillus tropicus
            Bacteria; Bacillota; Bacilli; Bacillales; Bacillaceae; Bacillus;
            Bacillus cereus group.
REFERENCE   1  (bases 1 to 1287)
  AUTHORS   Raja Kumar,K. and Venkateshwara Rao,J.
  TITLE     Chicken feather degrading and Keratinase producing bacteria
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1287)
  AUTHORS   Raja Kumar,K. and Venkateshwara Rao,J.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2024) Department of Zoology, OSMANIA UNIVERSITY,
            University College of Science (Campus), TARNAKA, HYDERABAD,
            Telangana 500007, India
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Fig. 7. Sequence submitted to NCBI of isolate 5

Comparative analysis with existing literature reveals both similarities and differences in the morphological characteristics of the isolated bacterial strains. Isolates with irregular shapes, such as Isolate 2, Isolate 3, and Isolate 6, may indicate adaptations to varied environmental conditions or genetic diversity within the microbial community of Suryapet soil samples [11]. Such diversity is crucial for exploring novel enzymatic functions and metabolic pathways relevant to biotechnological advancements in waste management and industrial processes. Furthermore, biochemical tests indicating positive results for starch hydrolysis and carbohydrate fermentation among selected isolates (details not shown) align with findings from previous studies, underscoring their metabolic versatility and potential for utilizing complex carbon sources [12]. These biochemical capabilities are essential for assessing the biotechnological potential of the isolated strains in diverse environmental and industrial applications.

After employing the enrichment culture method to isolate potential keratinolytic producers from soil samples in Suryapet district, Telangana, ten distinct bacterial isolates were obtained and screened for their ability to degrade feathers (Fig. 1). Among these isolates, Isolates 1, 2, 5, 7, and 10 exhibited significant feather degradation on feather-containing agar plates, highlighting their robust keratinolytic activity. These isolates were selected for further detailed studies to explore their enzymatic properties and industrial applications (Fig. 4). Notably, Isolate 5 demonstrated exceptional efficiency in feather degradation, completely breaking down feathers within 48 hours, whereas other isolates required 72 hours for similar degradation. The superior keratinase activity of Isolate 5 was further confirmed in the Azocasein assay, where it produced the highest enzyme activity at 8.66 U/ml compared to Isolates 1, 2, 7, and 10. This heightened enzymatic activity underscores Isolate 5's potential for rapid and efficient keratin degradation, essential for various biotechnological and environmental applications. Comparative analysis with previous studies reveals consistent findings regarding the rapid feather degradation capabilities and high keratinase production of certain bacterial isolates [13]. Studies by Lee et al. [14] and Brown [15] similarly highlight the enzymatic efficiency and industrial relevance of keratinolytic bacteria in waste management and bioprocessing industries.

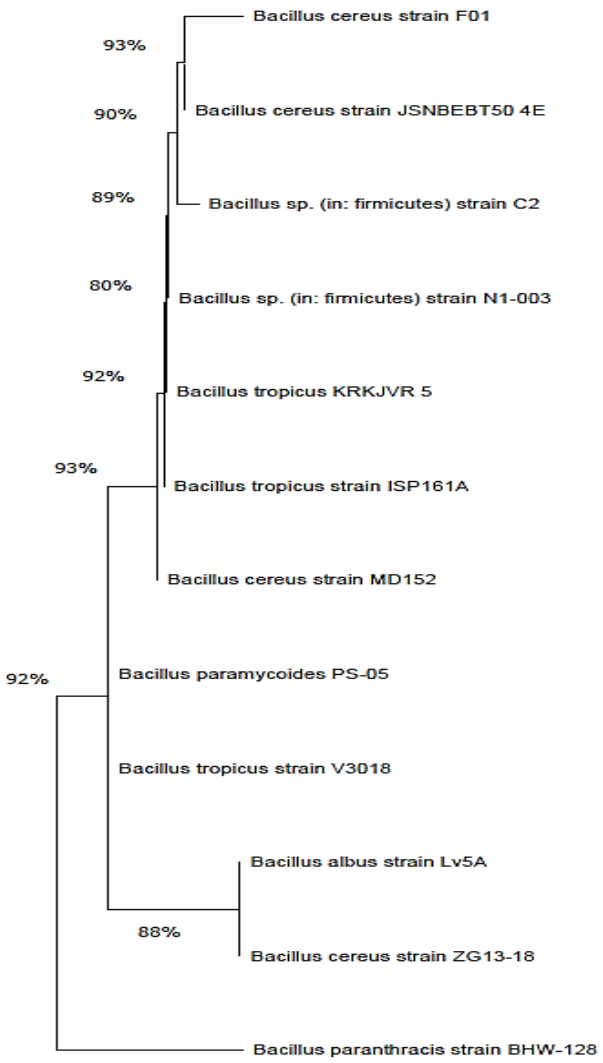

Fig. 8. Phylogenetic tree construction of isolate 5

Isolate 5, identified as a Bacillus species, exhibits a rod-shaped morphology with a positive Gram stain, indicating a thick peptidoglycan cell wall and the ability to form spores. Biochemically, it tested negative for indole and methyl red, but positive for Voges-Proskauer (acetoin production) and catalase, while showing negative results for H2S production and urease activity. It hydrolyses starch, ferments carbohydrates, but does not utilize citrate as a carbon source. These characteristics highlight Isolate 5's potential in biotechnological applications, particularly in keratin degradation. Previous studies on *Bacillus tropicus* have highlighted its potential in various biotechnological applications. For instance, Pandey and Pandey [16] reported similar biochemical characteristics and genetic identity findings in their research on bacterial species within the *Bacillus* genus. They emphasized the diverse enzymatic capabilities and genetic relationships observed in *Bacillus tropicus* strains, supporting its role in biotechnological advancements. Molecular analysis, specifically 16S rRNA sequencing, provided a definitive genetic identification of the bacterial isolates (Fig. 6). The sequencing results were compared with known bacterial sequences in the NCBI database, leading to the conclusive identification of Isolate 5 as *Bacillus tropicus* KRKJVR 5 [16]. The sequence data were subsequently submitted to the National Center for Biotechnology Information (NCBI), where Isolate 5 was assigned the accession number PP859287 (Fig. 7). Additionally, the construction of a phylogenetic tree revealed that Isolate 5 shares a 92% similarity with other *Bacillus tropicus* strains, further confirming its genetic identity and relationship within the *Bacillus* genus (Fig. 8).

This precise identification is crucial for understanding the potential applications and characteristics of *Bacillus tropicus* KRKJVR 5, especially in the context of keratin degradation and other biotechnological uses [17].

5. CONCLUSION

The isolation and molecular identification of *Bacillus tropicus* KRKJVR 5 as a novel chicken feather-degrading bacterium have significant implications for biotechnological applications. Through enrichment culture and screening of soil samples from Suryapet district, Telangana, Isolate 5 emerged as a potent keratinolytic producer, demonstrating rapid and efficient degradation of feathers. This was evidenced by its ability to completely degrade feathers within 48 hours and its high keratinase activity of 8.66 U/ml, as measured by the Azocasein assay. Morphological and biochemical characterization revealed Isolate 5 to be a rod-shaped, Gram-positive bacterium with characteristic traits such as positive spore staining, Voges-Proskauer positivity, catalase production, starch hydrolysis, and carbohydrate fermentation capabilities. Negative results in indole, methyl red, citrate, H2S, and urease tests further delineated its biochemical profile. The genetic identification through 16S rRNA sequencing confirmed Isolate 5 as *Bacillus tropicus* KRKJVR 5, with a 92% similarity to other strains within the *Bacillus tropicus* species. This identification was further validated by the submission of sequence data to the NCBI database under accession number PP859287. *Bacillus tropicus* KRKJVR 5 exhibits robust keratinase activity and holds promise for various industrial and environmental applications, particularly in bioremediation and waste management sectors. Further research focusing on optimizing its keratinase production and exploring its broader enzymatic capabilities will enhance its potential as a sustainable solution for keratin-rich waste degradation.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

DATA AVAILABILITY

All data generated or analysed during this study are included in this article.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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