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SCREENING, CHARACTERIZATION AND EVALUATION OF A POTENT KERATINOLYTIC BACTERIA FOR BIODEGRADATION OF POULTRY FEATHER WASTE IN AND AROUND VIJAYAWADA

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Abstract

Keratinases are proteolytic enzymes capable of degrading keratinous proteins, which are highly resistant and insoluble structural proteins abundantly present in feathers, hair, wool, nails, and other biological materials. Unlike conventional proteases, keratinases possess the ability to attack the complex structure of keratin; however, most keratinases reported to date are unable to completely solubilize native keratin. The unique mechanism of keratin degradation therefore remains an intriguing and incompletely understood phenomenon in the field of proteolytic enzymes. Keratinases have gained considerable attention due to their wide range of potential applications in feed, detergent formulations, leather processing, pharmaceutical industries, waste management, and other biotechnological processes. Poultry feather waste is produced in large quantities by the poultry industry and represents a significant environmental concern because of its high keratin content, resistance to degradation, and accumulation in the environment. Microbial degradation of feather waste using keratinolytic microorganisms offers an eco-friendly and sustainable alternative to conventional disposal methods and may facilitate the conversion of feather waste into valuable products. The present investigation focuses on the isolation and characterization of keratinolytic bacteria capable of producing extracellular keratinase and their potential for keratin degradation. Soil samples collected from areas surrounding poultry farms in and around Vijayawada were screened for keratinase-producing bacteria. The selected isolates were evaluated for keratinase production and feather degradation efficiency, followed by biochemical and molecular characterization for identification. The study aims to identify efficient extracellular keratinase-producing bacterial strains with potential applications in sustainable poultry feather waste management and biotechnological processes.

***Keywords*:** *Keratinase, Keratinolytic bacteria, Poultry feather waste, Feather degradation, Microbial biodegradation, Sustainable waste management.*

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1. INTRODUCTION

Keratin the insoluble protein of feather is known for its high stability. However, feathers do not accumulate in nature suggesting the existence of natural microbial decomposers of feathers. The development of bio-technological approach to chicken feather hydrolysis is the main goal of this project. The hypothesis is that if keratin degrading microorganism do exist in nature, then it will be possible to isolate them and use them for keratin hydrolysis. Feathers are composed of keratin protein in large amounts as a waste by poultry processing worldwide. Accumulation of feathers will lead to environmental pollution [1–3], to prevent this pollution we require keratolytic organisms which produces keratinase. Keratolytic activity has been reported for various bacterial genera such as Bacillus [4–6], Thermoanaerobacter [7], Chrysobacterium [8], Flavobacterium [9, 10] and vibrios [11]. Keratinases from microorganisms have attracted a great deal of attention in the recent decade, due to their industrial applications in feed, detergent, leather and pharmaceutical industries. So the main objective of project is to isolate keratinase producing microorganisms from soil.

OVER VIEW OF THE AREA OF STUDY

Poultry is one of the leading industries in and around Vijayawada. The soil around the poultry farms is

contaminated with poultry waste like feathers and chicks' droppings, which has to be degraded by keratinolytic microorganisms. So, it is very much essential to isolate the keratinolytic organisms and study their rate of degradation to avoid pollution and further infections in chicks which might result in new diseases in chicks as well as in human beings who consume it. And also, keratinases have various industrial applications in feed, detergent and pharmaceuticals.

OBJECTIVES

So, the main objective is to isolate keratin degrading organism from different soil samples in nearby poultry farms in and around Vijayawada

1. Isolation of keratinolytic organisms capable of producing keratinase enzyme.
2. Assessment of their ability to degrade keratin.
3. Identification of isolated organism through biochemical and molecular characterization

MATERIALS AND METHODS

BACTERIAL ISOLATION AND SCREENING:

COLLECTION OF SOIL SAMPLE

Collection of Samples Chicken feathers and feathers dumped soil/ compost samples were collected from the selected chicken poultry farms in Vijayawada for the isolation of keratin degrading microorganisms. Samples were collected in sterilized polythene bags and were stored in refrigerator at 4°C for further processing.

PREPARATION OF FEATHER MEAL

The collected chicken feathers from poultry farm washed thoroughly under tap water, and sterilized with mild detergent and followed by autoclaving. The sterilized feathers are dried in hot air oven for 2 hours at 50℃. Fine powder prepared in blender and used as sole source of carbon and nitrogen in screening media 12].

Composition of Feather meal broth

- NaCl - 0.5 g
- K2HPO4 - 0.3 g
- KH2PO4 - 0.4 g
- Magnesium Chloride Hexahydrate - 0.1 g
- Distill water – 1000ml
- Feather Meal powder-10gm

Feather meal broth was prepared as per the composition and sterilized in autoclave at 121°C for 15 minutes

ISOLATION OF KERATINOLYTIC MICROORGANISMS BY ENRICHMENT METHOD

Pre-enrichment was done by transferring 10 g of soil sample into 90 mL Feather Meal Broth and afterwards incubated at 37 ºC for 48 hours. After 48 hours, sample was then serially diluted at 10^{-3}, 10^{-5} and 10^{-7} dilutions and plated into Feather Meal Agar (FMA). Plates were incubated at 37 ºC for five days. Distinct colonies were streaked into FMA and incubated for two days.

STOCK CULTURE PREPARATION

Preparation of Luria Bertani (LB) Agar

Composition of Luria Bertani (LB) Agar

- Tryptone -10g
- yeast extract -5g
- NaCl – 10g
- Agar-agar -15g
- Distilled water – 1000ml

Luria Bertani (LB) Agar was prepared as per the composition and sterilized in autoclave at 121°C for 15 minutes. Well isolated colonies from feather meal agar plates were streaked into Luria Bertani (LB) Agar and incubated at 37 ºC for 24 hours. These served as culture stocks for the succeeding steps and stored at -4ºC.

PRELIMINARY SCREENING OF PROTEASE POSITIVE CULTURE

Preparation of skimmed milk agar

Composition of skimmed milk agar

- Skim milk powder: 28.0 g
- Casein enzymic hydro lysate (Tryptone): 5.0 g
- Yeast extract: 2.5 g
- Dextrose (Glucose): 1.0 g
- Agar: 15.0 g
- Distilled water – 1000ml

Initial screening of isolates was done by proteolytic activity assay using Milk Agar plates. Bacteria were inoculated onto plates and incubated for 24 hours. Isolates with zones of clearing are protease positive and were used for the succeeding steps.

SECONDARY SCREENING OF KERATINOLYTIC BACTERIA

Feather broth containing was prepared and transferred into big tubes. Instead of adding cut feathers, each broth was added with single whole feather and then sterilized. Added feathers were of approximately the same sizes. Loopful of proteolytic positive isolate from primary screened culture was inoculated into separate feather broth. Cultures were then incubated at 37 ºC for 14 days and were monitored for the presence of feather degradation. Isolates that exhibited signs of feather utilization were selected and subjected to keratinase assay, biochemical and molecular characteristics.





Figure 01: Feather meal broth inoculated with a whole feather

PRODUCTION AND ASSAY OF KERATIN

For enzyme production, feather meal broth was prepared by dissolving feather meal (10 g), KH_2PO_4 (0.3 g), K_2HPO_4 (0.4 g), NaCl (0.5 g), and $MgSO_4 \cdot 7H_2O$ (0.1 g) in 1 L of distilled water. The pH was adjusted to 7.5, and the medium was sterilized by autoclaving at 121°C for 15 min. A 100 mL aliquot of sterile feather broth was inoculated with 1 mL of an overnight bacterial culture obtained through secondary screening and incubated at 37°C on an orbital shaker at 150 rpm for 72 h. After incubation, the culture was centrifuged at 10,000 rpm for 15 min at 4°C, and the clear supernatant was collected as the crude extracellular keratinase enzyme.

LOWRY METHOD FOR KERATINASE ASSAY

Extracellular keratinase activity of potent bacterial isolate obtained from poultry farm soil was estimated by the Lowry method using L-tyrosine as the standard**.** The assay is based on the ability of keratinase to hydrolyse insoluble feather keratin into soluble peptides and amino acids, which react with the Lowry reagent followed by Folin–Ciocalteu reagent to produce a blue-coloured complex. The intensity of the colour is directly proportional to the concentration of soluble protein released and was measured spectrophotometrically at 750 nm**.**

The keratinase assay was carried out by mixing 1.0 mL of feather suspension (10 mg/mL), 1.0 mL of crude enzyme, and 1.0 mL of 50 mM phosphate buffer (pH 7.5) in a test tube. The reaction mixture was incubated at 50°C for 30 min to allow enzymatic hydrolysis of feather keratin. The reaction was terminated by boiling the mixture for 5 min (or alternatively by centrifugation), and the resulting supernatant containing soluble peptides was collected for protein estimation.

A 100 µg/mL L-tyrosine stock solution was prepared and used to construct a standard curve. Standard solutions containing 0, 20, 40, 60, 80, and 100 µg of L-tyrosine were prepared by appropriate dilution with distilled water. To each standard and sample, 5 mL of freshly prepared Lowry reagent was added, mixed thoroughly, and allowed to stand for 10 min at room temperature. Subsequently, 0.5 mL of Folin–Ciocalteu reagent was added rapidly, the contents were mixed immediately, and the tubes were incubated for 30 min for colour development. The absorbance of the standards and samples was measured at 750 nm against a reagent blank using a UV–Visible spectrophotometer. A calibration curve was prepared by plotting L-tyrosine concentration (µg) on the X-axis against absorbance at 750 nm on the Y-axis. The concentration of soluble peptides released in the enzyme-treated samples was determined by interpolating their absorbance values from the standard curve and expressed as tyrosine equivalents. Keratinase activity was calculated using the equation:

Keratinase Activity (U/mL) = Tyrosine released (µg) / [Incubation time (min) × Enzyme volume (mL)]

If a different unit definition was adopted from the reference method, such as defining one unit of keratinase activity as the amount of enzyme causing an increase of 0.01 absorbance units at 280 nm under specified assay conditions [13, 14], the corresponding calculation was performed accordingly.

GRAM'S STAINING

On a clean dry slide the isolated colony was placed by marking smear area with help of inoculating loop. Now the smear is heat fixed by keeping near the flame. Now the fixed smear was flooded with crystal violet for 1 minute. Then the excess stain was washed off with distilled water. Again, the smear was covered with gram's iodine solution for 1 minute. Using the distilled water, the iodine was washed off. Now the smear was decolorized rapidly with 95% ethyl alcohol and washed immediately with distilled water. Then the smear was again covered with safranin for 1 minute. This stain was washed off with distilled water and the slide was allowed to air dry and was viewed under microscope for identification.

SPORE STAINING

A thin smear of the bacterial culture was prepared on a clean glass slide, air-dried, and heat-fixed. The smear was flooded with 5% malachite green and steamed for 5–7 minutes, keeping the stain moist throughout the process. The slide was then washed gently with distilled water and counterstained with 0.5% safranin for 1 minute. After rinsing with water and air-drying, the slide was observed under the oil immersion objective (100×) of a microscope.

MOTILITY TESTING

A sterile inoculating needle was used to pick a small amount of the bacterial culture and inoculate the center of a tube containing semi-solid motility agar (0.4% agar) by making a single straight stab to about

two-thirds the depth of the medium. The inoculated tube was incubated at 37°C for 24–48 hours. After incubation, the tube was examined for bacterial growth.

BIOCHEMICAL TESTS

The colony was selected from the plate and then screening was done to find the isolated colony.

Indole production test

Initially tryptone broth was prepared and it was transferred in to two test tubes one serving as control. Using sterile technique, a loop full of organism was inoculated into the test tube. The test tube was incubated for 48hrs at 37°C. Then Kovac's reagent was added and gently shaken and result was observed.

Methyl red

Buffered Glucose broth was prepared and it was transferred in to two test tubes one serving as control. Using sterile technique, a loop full of organism was inoculated into the test tube. The test tube was incubated for 48hrs at 37°C. Then 0.5ml of Methyl Red solution was added and gently shaken and results were observed immediately.

Voges-proskauer

Glucose phosphate broth was prepared and it was transferred in to two test tubes one serving as control. Using sterile technique, a loop full of organism was inoculated into the test tube. The test tube was incubated for 48hrs at 37°C. Then 0.5ml of Methyl Red solution was added and gently shaken and results were observed immediately

Citrate utilization test

Simmon citrate agar was prepared and transferred in to two test tubes one serving as control. Using sterile technique, a loop full of organism was inoculated into the test tube. The test tube was incubated for 48hrs at 37°C. Then the growth and color change was observed.

Catalase test

The organism was transferred into a clean glass slide using sterile technique, then three to four drops of 3% hydrogen peroxide was allowed to overflow the entire surface of the culture and formation of brisk effervescence was observed.

Gelatin hydrolysis test

Gelatin broth was prepared and transferred in to two test tubes one serving as control. Using sterile technique, a loop full of organism was inoculated into the test tube. The test tube was incubated for 24hrs in refrigerator. Then the solidified medium was observed.

Starch utilization test

Starch agar plates was prepared and allowed to solidify. Using sterile technique, a loop full of organism was streaked into the petri plate. The petri plate was incubated for 48hrs at 37°C. Then 20ml of 5% iodine solution was added and starch utilization was observed.

Carbohydrate fermentation test

Carbohydrate broth was prepared with different carbohydrate source like (Lactose, maltose, mannitol, glucose) and transferred in to two test tubes one serving as control. And Durham's tube was transferred to the test tube to analyze the gas formation. Using sterile technique, a loop full of organism was inoculated into the test tube. The test tube was incubated for 48hrs at 37°C. Then the color change and was observed and checked if gas formation is present.

MOLECULAR ANALYSIS

Definitive identification of the isolated strain is confirmed by molecular method such as 16S rRNA gene sequencing

RESULTS AND DISCUSSIONS

Isolation and Screening of Bacteria

Serial Dilution of Soil Sample

In this study, a targeted sampling approach was utilized in order to isolate microbial populations with keratinolytic potential. Soil with degrading feathers was used as microbial source in order to increase the chances of isolating bacteria with keratinase activity. Furthermore, samples were pre-enriched in FMB for two days in order to magnify the population of the target microorganisms from the soil samples. Initial isolation yielded 4 bacterial isolates that was capable of thriving in FMA with clear zones around colonies. The morphology characteristics of the 4 isolates were provided.



Figure 02: colony morphology of screened isolates

Table 01: The Morphology characteristics of the 4 bacterial isolates

S.NO.	Morphology character's	Isolate no-1	Isolate no-2	Isolate no-3	Isolate no-4
1	Colour	yellow	white	Creamy shine	creamy
2	Colony shape	Round	Irregular		pinpoint
3	Cell shape	Short rods	Rod	Rod	Rod
4	Motility	positive	positive	positive	positive

Preliminary screening of keratinolytic bacteria

Skimmed milk plate assay was carried to check the zone and to check if protease is present in the sample and is capable of caseinhydrolysis. This was carried out

since keratinase is type of protease enzyme. There was zone formation of 1cm in diameter around the well.



Figure 03: preliminary screening of keratinolytic isolates

Secondary screening of keratinolytic bacteria

In secondary screening, the 4 isolates were grown in FMB with single chicken feather. Out of the four isolates, isolate-2 shows more turbidity and also degrades the feather rapidly when compared to other isolates. The isolate-2 selected as potent keratinolytic bacteria for keratin enzyme production. The isolate-2 was subjected to biochemical tests and enzyme production.

Keratinase Assay

For standard Tyrosine assay the following concentration of tyrosine in distilled water is taken:

Table 02: Tyrosine standard assay

Tube	Tyrosine (µg)	Distilled water (mL)
Blank	0	1.0
S1	20	0.8
S2	40	0.6
S3	60	0.4
S4	80	0.2
S5	100	0

To every tube 5 mL of Lowry reagent was added and allowed to stand for 10 mins. Then 0.5 Folinreagent is added and kept for 30mins. Absorbance is read at 750nm. The following values are obtained:

Table 03: Absorbance at 750 nm

Tyrosine (µg)	Absorbance (750 nm)
0	0.000
20	0.107
40	0.213
60	0.324
80	0.437
100	0.546

The selected isolate-2 with potent keratinolytic activity showed the following absorbance at 750nm.

Table 04: Absorbance at 750 nm for isolate-2

Test sample	Absorbance (750 nm)
Isolate-2	0.452

A plot of **Tyrosine concentration (µg)** on the X-axis versus **Absorbance at 750 nm** on the Y-axis produced an approximately straight line. Using the standard curve, absorbance of Isolate-2 is interpolated to estimate the keratinase activity equivalent of tyrosine.

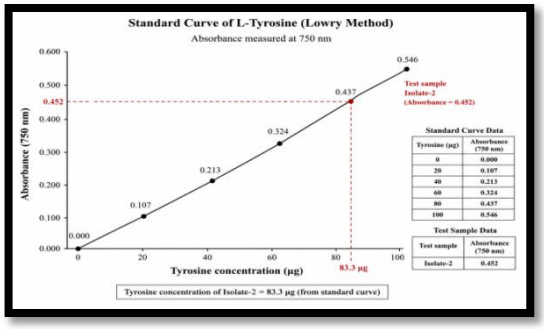


Figure 04: Standard curve of thyrosine

Calculation of Keratinase activity:

Keratinase Activity (U/mL) = Tyrosine released (µg) / [Incubation time (min) × Enzyme volume (mL)]

Keratinase Activity (U/mL) = 83.3 (µg) / [30 (min) × 1 (mL)]

Keratinase Activity (U/mL) = 2.776

Biochemical Test for Characterization

For the isolated bacteria different biochemical test was carried out to find out the bacteria.

Gram's Staining

Gram staining was done and it was observed as gram positive and rod shaped, so from this this is found that it is bacillus species due to aerobic and spore forming of bacteria. Later different test was done to find the species.



Figure 05: Gram's Staining of isolates

Spore Staining

Spores appeared green, while vegetative cells appeared pink or red. The isolated bacterium is positive for Spore formation.

Motility Testing

The isolated bacterium shows diffused growth spreading away from the stab line indicated a positive motility test.

Indole Production Test

Indole production test is observed to be negative here since there so no formation of red ring after addition of kovac's reagent to the inoculated organism.



Figure 06: 2nd image-indole production test

METHYL RED TEST

Methyl red test is observed to be negative here since there is no formation of red color after addition of methyl red reagent to the inoculated organism.

Voges-Proskauer Test

VP test is observed to be positive here since there is formation of yellow color after addition of methyl red reagent to the inoculated organism

Citrate Utilization Test

Citrate utilization test is observed to be positive here since there is change of colour from green colour to blue colour after the growth of bacteria in the medium.

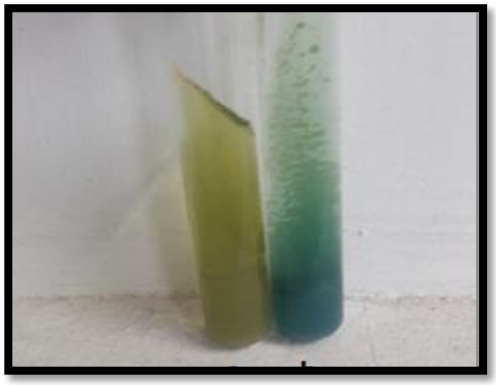


Figure 07: citrate utilization test

Catalase Test

Here when the 3% hydrogen peroxide is added to the inoculum there is formation of bubbles which indicates it is positive reaction.

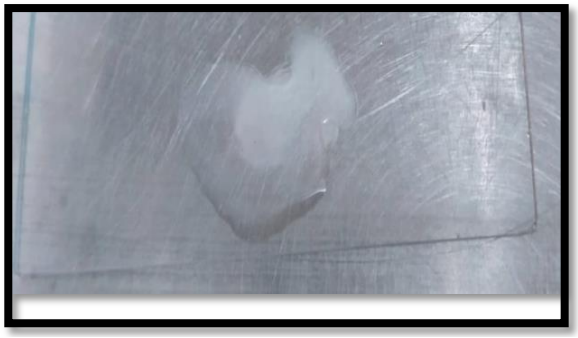


Figure 08: catalase test

Gelatin Hydrolysis Test

Here when the inoculated tube was taken from the refrigerator and the result was observed. The tube inoculated with gelatin got solidified resembling the control tube. This showed that bacteria did not hydrolyze the gelatin and it is negative for Gelatin hydrolysis.

Starch Utilization Test

Here after the growth of bacteria iodine was poured and left for 10mins, the clear zone was seen around the bacteria, which shows the bacteria has hydrolysed the starch and it is a positive result.



Figure 09: Starch hydrolysis

Carbohydrate Fermentation Test

Here the bacteria were tested for four different carbohydrate sources such as glucose, lactose, Sucrose and maltose. The isolate was positive for glucose, sucrose and maltose and negative for lactose. Also there is no formation of gas in Durham's tubes.



Figure 10: Carbohydrate fermentation test

Table 05: Result of biochemical characterization of bacterial isolate -2

S.NO.	Biochemical test	Isolate no-2
1	Gram's staining	Positive rod
2	Indole	-
3	Methyl red	-
4	Voges-proskauer	+
5	Citrate utilization test	+
6	Catalase test	+
7	Gelatin hydrolysis test	-
8	Starch utilization	+
9	Glucose	+
10	Maltose	+
11	Sucrose	+
12	lactose	-

Molecular Analysis

Based on morphological and biochemical characteristics, the keratinase-producing isolate was presumptively identified as *Bacillus cereus*. Definitive identification confirmed by molecular methods such as 16S rRNA gene sequencing

Report of Microbial Identification Experimental Method

1. DNA was isolated from the culture. Its quality was evaluated on 1.0 % agarose gel, a single band of high-molecular weight DNA has been observed.
2. Fragment of 16S rRNA gene was amplified by 16SrRNA-F and 16SrRNA-R primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on agarose gel.
3. The PCR amplicon was purified to remove contaminants.
4. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 16SrRNA-F and 16SrRNA-R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.
5. Consensus sequence of 16S rRNA gene was generated from forward and reverse sequence data using aligner software.
6. The 16S rRNA gene sequence was used to carry out BLAST with the 'nr' database of NCBI GenBank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix and phylogenetic tree was constructed was constructed using MEGA 11.

Result: Sample was found to be Bacillus cereus, as it showed high similarity based on nucleotide homology and phylogenetic analysis.

DISCUSSION

The present study was undertaken to isolate and characterize keratinase-producing bacteria from poultry farm soil collected from different locations in and around Vijayawada for the sustainable management of poultry feather waste. Poultry feathers constitute a major by-product of the poultry industry and are composed primarily of keratin, a highly stable and insoluble structural protein that is resistant to degradation by common proteolytic enzymes. The accumulation of feather waste poses significant environmental and public health concerns. Therefore, isolation of efficient keratinolytic microorganisms capable of converting feather waste into useful products provides an eco-friendly and economically sustainable alternative to conventional disposal methods.

In the present investigation, poultry farm soil containing feather residues was selected as the source material because such environments naturally harbour microorganisms adapted to utilize keratin as a nutrient source. The enrichment culture technique using feather meal broth effectively increased the population of keratin-degrading bacteria. Four morphologically distinct bacterial isolates capable of growing on Feather Meal Agar (FMA) were obtained.

Isolate-2 showed rapid feather degradation and maximum turbidity compared to the remaining isolates, indicating efficient utilization of keratin as the sole source of carbon and nitrogen. This observation agrees with previous reports that highly keratinolytic bacteria produce extracellular enzymes capable of complete feather degradation under submerged fermentation conditions.

Keratinase production by the selected isolate was quantified using the Lowry method with L-tyrosine as the standard. Morphological examination and biochemical characterization revealed that the selected isolate closely resembles that reported for *Bacillus cereus***,** one of the most extensively studied keratinase-producing bacterial species. Members of the genus *Bacillus* are widely recognized for producing extracellular hydrolytic enzymes with considerable industrial importance due to their rapid growth, high enzyme productivity, and ability to secrete thermostable alkaline proteases.

The isolation of a potent keratinase-producing ***Bacillus cereus*** from poultry farm soil highlights the ecological significance of indigenous microorganisms in the biodegradation of keratin-rich agricultural waste. Biological degradation of feathers offers several advantages over conventional chemical and physical processing methods, including reduced energy consumption, lower environmental pollution, preservation of amino acid quality, and production of value-added protein hydrolysates suitable for animal feed, fertilizers, and other industrial applications.

Although the biochemical characteristics strongly suggest that the isolate belongs to *Bacillus cereus***,** species-level identification based solely on conventional tests cannot be considered definitive. Molecular identification through 16S rRNA gene sequencing**,** followed by phylogenetic analysis, was done to confirm the taxonomic identity of the isolate and it is confirmed as *Bacillus cereus***.** Furthermore, purification and characterization of the keratinase enzyme, optimization of production parameters, determination of enzyme stability under different physicochemical conditions, and evaluation of industrial applicability will provide additional insight into its commercial potential.

CONCLUSION

The present study successfully isolated keratinase-producing bacteria from poultry farm soil collected in and around Vijayawada using feather meal enrichment techniques. Among the four isolates obtained, Isolate-2 demonstrated superior keratinolytic activity by rapidly degrading chicken feathers and exhibiting maximum extracellular keratinase production. Keratinase activity determined by the Lowry method using L-tyrosine as the standard was 2.776 U/mL, confirming the isolate as an efficient producer of keratin-degrading enzyme. Morphological and biochemical characterization indicated that the isolate was presumptively identified as *Bacillus cereus*, and confirmed by 16S rRNA gene sequencing as *Bacillus cereus***.**

The findings demonstrate that indigenous keratinolytic bacteria from poultry farm soils can effectively biodegrade feather waste and produce valuable keratinase enzyme. Therefore, the isolated strain has considerable potential for environmentally sustainable management of poultry feather waste and may be exploited for applications in the animal feed, fertilizer, leather, detergent, pharmaceutical, and

biotechnological industries after further optimization and molecular characterization.

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AUTHOR CONTRIBUTION STATEMENT

Contribution of N. Saritha: Conceptualization, Methodology, Investigation, Supervision, Data Interpretation, and Manuscript Review.

Contribution of Ch. Meghala: Methodology, Experimentation, Data Collection, Analysis, Interpretation of Results, and Manuscript Preparation. Both authors contributed to the revision and final approval of the manuscript.

CONFLICT OF INTEREST

We, N.Saritha and Ch. Meghala hereby declare that we have no conflict of interest related to the publication of this research work.

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