https://doi.org/ 10.33472/AFJBS.6.5.2024. 2265-2290

African Journal of Biological Sciences

Isolation, characterization and comparative analysis of three Keratinolytic Bacteria from poultry waste for sustainable waste management and their Biotechnological applications

Taniya mary martin[1], Jabir padathpeedika khalid[2], Manikandan selvaraj[1], Lavanya prathap[1], Meenakshi sundaram kishore kumar*[1]

[1]Department of Anatomy, Saveetha Dental College and Hospital, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, India

[2]Department of Physiology, Saveetha Medical College and Hospital, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, India

Corresponding author: meenakshisundaram.sdc@saveetha.com

Article History
Volume 6, Issue 5, Apr 2024
Received: 01 May 2024
Accepted: 09 May 2024
doi :*10.33472/AFJBS.6.5.2024. 2265-2290*

Abstract

Feather waste is a significant byproduct of raising hens and poses a threat to the environment due to its abundance and resistance to degradation. Keratinase enzyme is the natural solution for degrading such kind of the wastes. The objective of this study was to isolate and characterize keratinolytic bacteria from poultry waste in Chennai. Soil samples from five locations were collected, and bacterial strains were isolated and characterized through various biochemical tests. Keratinolytic bacteria were screened by growth on skim milk agar and secondary screening on feather meal agar. Keratinase enzyme activity and feather degradation at different pH levels were assessed. Molecular identification of keratinase species was performed by extracting total DNA, partially amplifying 16S rRNA genes, and conducting sequence analysis

Three bacterial colonies were isolated and they had a rod-like morphology, stained positively with gram stain, and bore similarities to *Bacillus* species. Out of three, two colonies demonstrated efficient caseinolytic and keratinolytic activities in feather meal agar and skim milk agar. Their keratinolytic nature was verified by biochemical studies. After a 96-hour incubation period, keratinase synthesis was found. The bacterial genus C1, C2 and C3 were identified as *Bacillus subtilis* *Staphylococcus lentis* and *Mammaliicoccus lentus* respectively using 16S rDNA sequencing. Among three isolates, *Mammaliicoccus lentus* showed excellent keratinase production in the study. These findings present opportunities for contamination-free disposal.

Keywords: Poultry waste, keratin, Keratinolytic bacteria, *Mammaliicoccus lentus*

Introduction

India generates a huge amount of human hair, poultry feather waste, and wool waste each year. These wastes are dumped, buried, used for landfilling, or incinerated, increasing environmental hazards, pollution, and threat to public health and increasing greenhouse gas emissions (1). As a byproduct of processing, chicken, chicken feathers pose serious environmental problems; each year, some 8.5 billion tons are produced globally, with 350 million tons coming from India alone. Because so much feather waste pollutes the air, water, and soil, it endangers the integrity of the ecosystem (2). However, chicken feathers may be fermented with bacterial cells to provide an organic nitrogen supplement, providing a long-term way to improve field farming methods. Feathers from chickens are taken from plants that treat poultry; initially, they are considered waste. After the feathers are collected, they are cleaned to remove any dirt, debris, and any remaining tissue or blood. This ensures that the feathers are hygienic and ready for processing. Feathers are then sorted according to size, quality, and kind in order to make it easier to separate them for different uses and guarantee uniformity in the finished product. An essential stage is hydrolysis, which involves treating feathers with heat, pressure, chemicals, or both to break down the keratin protein into smaller, more manageable pieces. This process is frequently made easier by enzymes like keratinase (3). Filtration may be performed after hydrolysis to get rid of any leftover solid particles or contaminants, producing a solution that is rich in amino acids and peptides generated from keratin. This solution can be concentrated to increase the amounts of peptides and amino acids, which will improve its applicability for a variety of uses. After concentration, surplus moisture can be removed by air drying, freeze drying, or spray drying processes, which will result in a dry, powdered product. In the end, the product made from dried keratin is packaged for distribution to other sectors, and it could even go through additional processing to satisfy certain application needs. There are a number of difficulties in the processing of chicken feathers, even with the possibility of turning them into products with additional value (4). The primary protein in feathers, keratin, has a great resistance to breakdown, which presents a considerable problem. Effective keratin breakdown necessitates specific procedures like hydrolysis, which can be expensive and energy-intensive. Furthermore, extensive cleaning and sterilizing procedures are required due to the presence of pollutants in feathers, including dirt, oil, and microbes (5). However, implementing these procedures on a wide scale might present difficulties. To maintain uniformity in the finished product, sorting feathers according to size, quality, and kind needs careful handling and adds complexity to the process. Moreover, there are logistical and environmental difficulties in expanding feather processing plants to satisfy demand while preserving sustainability and efficiency. In order to overcome these obstacles, more research and creativity are needed to discover new uses for keratin-derived products, enhance resource efficiency, and optimize processing methods.

Value-added items such as chicken feathers may now be produced with keratin-rich materials thanks to recent breakthroughs in keratin processing technology. One important advancement is the creation of new microbial and enzymatic activities that increase the efficiency of keratin breakdown.

Improved keratinolytic activity in engineered enzymes makes it easier for keratin to be hydrolyzed into peptides and amino acids. (6). These enzymes are produced by a few actinomycetes, thermophilic Fervidobacterium pennavorans, some strains of Bacillus licheniformis, and several saprophytic and parasitic fungi (7). Comparable rates of growth were seen in chickens fed isolated soybean and feather meal supplemented with methionine and fermented using Streptomyces fradiae (8). Furthermore, the identification of microbial consortiums that have the ability to degrade keratin in various environmental settings has encouraging prospects for extensive processing. Further facilitation of keratin hydrolysis has been made possible by improvements and optimizations in bioreactor design, which have increased productivity and decreased expenses. Cutting-edge techniques like continuous-flow reactors and solid-state fermentation have improved process productivity and resource efficiency. New uses for keratin-derived products have been discovered in biomedicine, biodegradable plastics, agriculture, and cosmetics. Furthermore, research with a sustainability focus prioritizes eco-friendly solvents and renewable energy sources to reduce environmental impact and increase resource efficiency. These developments point to a change in the economy to one that is more circular and sustainable, and they emphasize the need for continuous multidisciplinary cooperation in order to optimize and innovate keratin processing technologies.

Because of their varied metabolic capacities and cooperative relationships, microbial consortia play a crucial role in the processing of keratin, particularly in the synthesis of keratinase enzyme. These consortiums provide a number of benefits: First off, the variety of microorganisms present in them allows for the effective breakdown of intricate keratin structures that are difficult for a single strain or enzyme to break down. Second, by using metabolic byproducts as substrates, cooperative relationships between consortium members improve keratin breakdown and keratinase enzyme synthesis. Thirdly, microbial consortia exhibit environmental flexibility, rendering them appropriate for keratin processing in a range of industrial contexts. Furthermore, compared to monocultures, the inclusion of many microbial species in consortia frequently results in larger quantities of keratinase enzymes, which helps with keratin breakdown and downstream processing. Keratin proteins are classified into two categories based on their sulphur content: (a) soft keratin, found in the skin and callus; and (b) hard keratin, found in the feathers, hair, and hooves. These proteins are members of the scleroprotein group, also known as fibrous proteins (9). Beta Keratin (β- Keratin) is a member of structural protein family which found in the epidermis of birds as well as reptiles. The term β Keratin is replaced by “corneous Beta-protein” or “Keratin associated Beta protein” (10). Tailored consortia can also be created to maximize product yields and enzyme activity for certain keratin-rich substrates or processing circumstances. In the end, using microbial consortia provides economical and environmentally friendly keratin processing options, lowering energy usage, waste production, and environmental effect in a range of industrial applications. Nature’s gift of Keratinolytic fungi and bacteria is the largest group of organisms which are capable of degrading Keratin waste (11).

Traditional methods for the disposal of keratin waste are buried, burning, incineration, composting. According to studies, certain bacteria are capable of breaking down this kind of waste by secreting enzymes called keratinases, which are both proteolytic and keratinolytic (12).

It is difficult to identify microbial consortia for keratinase synthesis because of their varied metabolic profiles. These microorganisms can be labor-intensive to cultivate and isolate from their native habitats, requiring specific procedures. Furthermore, the identification of important keratinase producers is made more difficult by the geographical and temporal variability displayed by microbial consortia. Extensive experimentation is required to determine the ideal growing conditions for the synthesis of enzymes, taking into account variables like pH and temperature. It takes a lot of work to screen several isolates for keratinase activity, and sophisticated analytical techniques are needed to comprehend the intricate microbial interactions within consortia. Maintaining consortium stability and productivity presents additional hurdles when scaling up from lab to industrial production. Interdisciplinary strategies combining molecular biology, bioprocess engineering, and microbiology are needed to overcome these challenges. Metagenomics is an advanced technology that helps discover important microbial actors. Fermentation process optimization increases the yields of enzymes that may be used in industry.

MATERIALS AND METHODS

Identification of keratinolytic Bacterial colonies from Poultry soil

At five different locations in Chennai—Porur (13.038200°N 80.156500°E), T-Nagar (13.041800°N 80.234100°E), Madambakkam (12.903848°N 80.15861°E), Medichur (12.910203°N 80.071712°E), and Chetpet (13.07412°N 80.24238°E) —soil samples contaminated with chicken feather from poultry farms were collected in sterile polythene bags. The raw, fresh chicken feathers were removed from the samples using a sieve and repeatedly cleaned with distilled water. Subsequently, the cleaned feathers are left to air dry at room temperature for a period of 3-4 days, ensuring complete moisture removal. Isolation of the bacterial strains was made by serial dilution. All the dilutions were plated on Nutrient Agar medium and incubated at 37^0C for 24 hours. Among the different bacterial colonies obtained on the spread plate agar plate, ten morphologically different bacterial colonies were identified and was quadrant streaked on the Nutrient Agar Plate, and incubated at 37^0C for 25 hours. The colonies appeared was checked for the presence of spore and streaked onto Nutrient Agar for further characterization. Spore production and localization were examined by microscopic observation. Three colonies are streaked onto Skim Milk Agar plates, a nutrient-rich medium that contains skim milk powder as a substrate for caseinolytic activity, in order to isolate keratinolytic bacteria. The process of incubation at about 37°C for a full day facilitates the growth of bacteria and the measurement of enzyme activity. After the bacterial

colonies have been incubated, the plates are examined for clear zones surrounding them, which signify caseinolytic activity. Prominent clearance zone-exhibiting colonies are selected as possible candidates for keratinolysis with few modifications adopted from previous studies (13). To generate pure cultures for further examination, these chosen colonies are subculturing onto new agar plates. The strain with clear and distinct zone was selected marked as Colony C 1- 3, respectively. The bacteria were identified and confirmed by carrying out tests like Gram staining, Spore staining, Oxidase Test, Lactose fermentation Test, Sucrose fermentation Test.

Extraction of Enzyme

Whatman No. 1 filter paper was used to filter the culture media in order to remove any residues that had not decomposed. The filtrate was then centrifuged for 10 minutes at 10,000 rpm in order to remove any remaining germs. The supernatant's keratinase activity was measured after centrifugation.

Determination of feather degradation

Investigating feather deterioration was done in accordance with the previous procedure Kumar et al. (14) Described. Five different bacterial isolates (C1, C2 and C3) were cultivated in a broth made of feather meal that included 1% feathers as the main source of carbon. After that, the cultures were cultured for a week on a rotating shaker. The residual feathers were then measured gravimetrically by filtering the culture broth and weighing the filter paper both before and after the filtering process. By contrasting the starting weight with the weight attained during one week of incubation, the percentage drop in feather mass was ascertained.

Assay for keratinase activity

Keratinase assay was performed according to previous method. Briefly, Centrifugation was performed on every culture filtrate for 30 minutes at 5000 rpm. A mixture was made composed of 0.2 ml of the culture filtrate supernatant, 3.8 Tris HCl buffer, and 20 mg of feather meal. Additionally, 0.2 milliliters of distilled water were used in place of the culture supernatant to provide a control sample. After an hour of incubation at 30°C, the tubes were chilled in ice water for ten minutes. The optical density (OD) was measured at 280 nm following filtering. After that, the OD readings were translated into enzyme units per milliliter using the following formula: Enzyme Units per Milliliter = (Optical Density × 4 × dilution rate) / (0.01 × T), where T stands for the assay's total volume and 4 for the incubation period.

Characterization and optimization of keratinase enzymes

Keratinase assay was done using Krishna Rayudu, 2014. The enzyme optimization was done with different media composition and pH.

Phylogenic determination of Bacterial Isolates

The bacterial isolates were identified using 16srRNA method using Forward 27F 5′-AGAGTTTGATCMTGGCTCAG - 3′ and Reverse 1492R 5′- GGTTACCTTGTTACGACTT - 3′ as primers. Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) based on the manufacture's protocol. The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using MEGA 7. The amplified sequences belong to 16Sregion were confirmed by similarity index built in the NCBI's BLAST program.

RESULTS

Bacterial Selection and Identification

In this study, keratin-degrading bacteria were identified from part of the poultry contaminated with the chicken manure. Using nutrient Agar plates contaminated with five distinct soil samples, colonies exhibiting discernible morphological alterations were found. Based on microscopic, biochemical, and cultural features, the bacteria were identified as rod-shaped, Gram-positive, sporulating Bacillus species-like. These bacteria were able to break down one percent of the feather in just seven days. To aid in future study, colonies that displayed unique qualities were designated as C1, C2 and C3 respectively (Figure, 1).

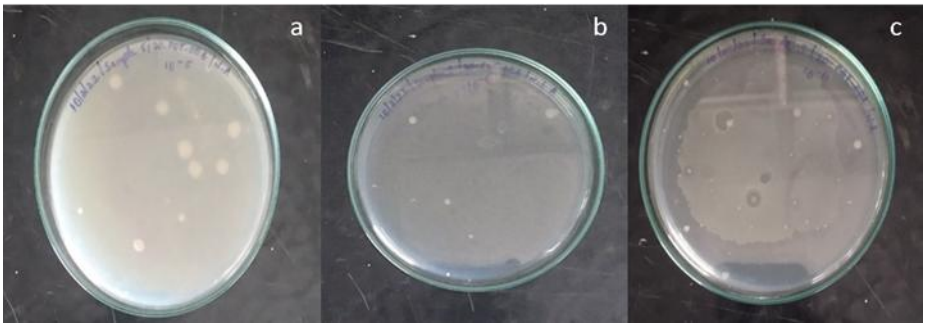


Figure. 1. Three bacterial isolates were selected for the study based on their endospore formation. (a, b and c – C1, C2 and C3 respectively)

Preliminary Screening for Keratinase Production

Skimmed milk agar medium was inoculated with the microbial isolates. The plates were inoculated, and then they were incubated for 24 hours at 37°C. Proteolytic activity was revealed by the clear zones that surrounded the colonies. The protease test was used for preliminary screening in order to lower the number of isolates and to verify the existence of proteolytic activity. Three of the five isolates formed clearing zones that were suggestive of proteolytic activity when the isolates were cultivated on skim milk agar. Out of all of them, isolates C3, C2 and C1 had the biggest clearing zones (Figure. 2). These chosen isolates were then recovered and put through further testing.

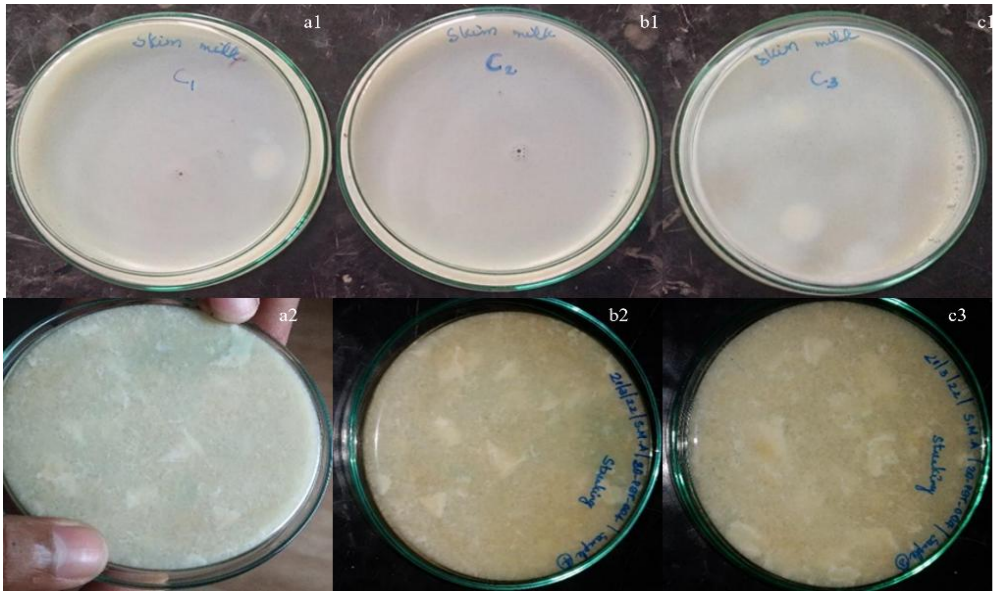


Figure. 2. Preliminary screening of selected bacterial isolates for Keratinase production. The bacterial isolates (C1, C2 and C3) were inoculated on skimmed milk agar (pH – 7 ± 0.2) and incubated for 24h at 37ºC.

Confirmation for Keratinase Production

Feather meal powder was used in place of keratin in the growing medium to further confirm the presence of keratinase-positive bacteria. A distinct zone on both media identified the isolates as keratinase makers. The bacterial isolates that showed a clean zone surrounding the inoculum were then put through a further screening process. Following a pH adjustment to 8.0, the isolates were placed into a base medium supplemented with chicken feathers and incubated at 37°C (Figure.3). With 72% feather degradation, C3 demonstrated the maximum efficacy in the feather degradation experiment, according to the results shown in Table 1. C3 was degraded at a rate of 72%, C2 at 67% and C3 at 58%, KD4 at 58.82%, and KD3 at 55%.

Table. 1. Feather degradation by the isolated bacterial cultures

Isolates	**Filter paper weight (g)**		**Reduction of feathers (%)**
	Initial	**Final**	
C1	1.25 ± 0.02	0.58 ± 0.02	58.00
C2	1.26 ± 0.03	0.67 ± 0.04	67.00
C3	1.29 ± 0.02	0.72 ± 0.05	72.00

On both skimmed milk agar and feather milk agar, bacterial colony-forming unit (CFU) counts were performed to evaluate the development and activity of the bacterial isolates under investigation. Feather milk agar only promotes the growth of bacteria that can use feathers as a source of carbon, whereas skimmed milk agar offers a nutrient-rich medium ideal for the development of a wide variety of microorganisms. To calculate the population density of the bacterial isolates, the number of bacterial colonies that developed on each agar medium was counted after incubation. While CFU counts on feather milk agar offers information on the isolates' particular capacity to degrade feathers, CFU counts on skimmed milk agar serves as a broad indication of bacterial growth (Table. 2). The C3 isolated showed higher CFU followed by C2 and C1.

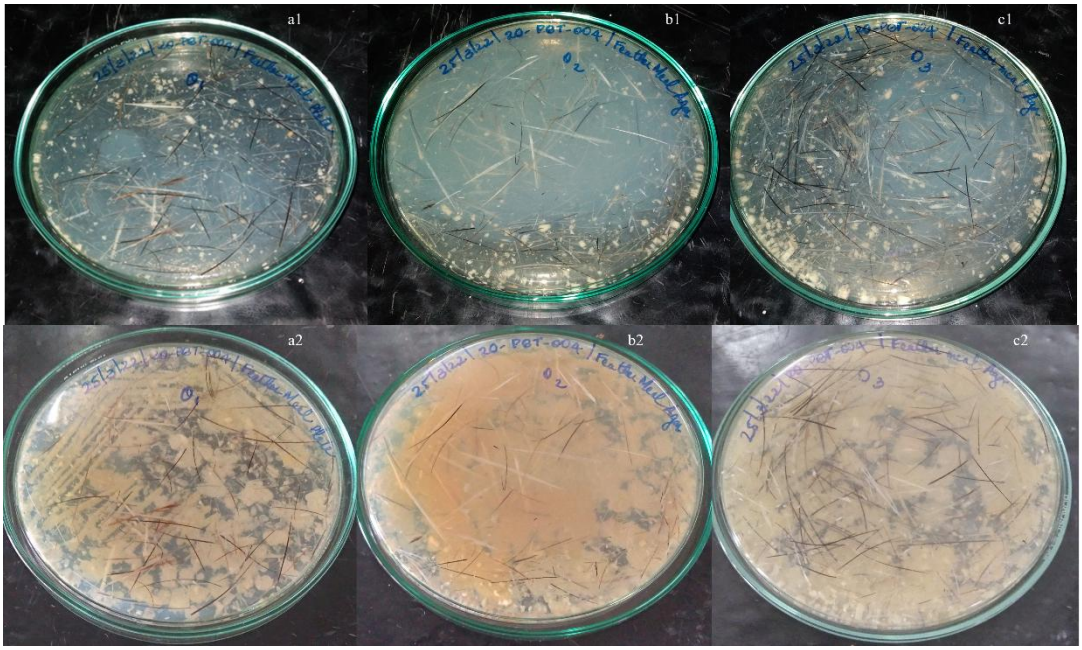


Figure. 3. Confirmation of selected bacterial isolates for Keratinase production.

The bacterial isolates (C1, C2 and C3) were inoculated on Feather meal agar (pH – 9 ± 0.2) and incubated for 24h at 37ºC.

Table. 2. Bacterial Colony count of Different isolates

	Skim Milk Agar (CF/ ml)			**Feather Meal agar (CF/ ml)**		
Dilution	10^4	10^5	10^6	10^4	10^5	10^6
C1	$2.1*10^4$	$1.8*10^3$	$1.1*10^3$	$1.8*10^4$	$1.2*10^3$	$0.87*10^3$
C2	$1.8*10^4$	$1.31*10^3$	$0.98*10^3$	$1.51*10^4$	$1.24*10^3$	$0.81*10^3$
C3	$2.64*10^4$	$6.0*10^3$	$2.4*10^3$	$1.81*10^4$	$1.72*10^3$	$1.4*10^3$

The distinct enzymatic properties of keratinases and their prospective uses in a variety of sectors support the selection of keratinase-producing bacteria for biochemical characterization throughout a population. Specialized enzymes called keratinases may break down keratin-rich substrates like hair, horns, and feathers into smaller, easier-to-manage molecules. Researchers can target organisms with the capacity to effectively break down these normally resistant materials by choosing bacteria that generate keratinases. Moreover, the biochemical characterization of microorganisms that produce keratinase offers important insights into the substrate selectivity, regulatory processes, and enzymatic characteristics of keratinases.

The characterization of all three keratinase-producing microbes involved a comprehensive assessment based on both morphological and biochemical tests. All of them were shown Gram-positive, rod-shaped bacteria (Table 3). The morphological and biochemical traits of these isolates were evaluated in accordance with the guidelines outlined in Bergey's Manual of Determinative Bacteriology for accurate identification and classification.

Table. 1. Morphological and Biochemical characteristics of the Isolated Bacterial Colonies (C1, C2 and C3) from Poultry soil contaminated with chicken feathers

S. No	**Tests**	**Colony 1**	**Colony 2**	**Colony 3**
1.	Gram staining	Purple	Purple	Purple
2.	Shape	Rod and round	Rod and round	Rod and round
3.	Arrangements	Single &chain	Single and chain	Single and chain
4.	Endospore staining	Green	Green	Green
5.	Catalyze Test	Formation of bubbles	Formation of bubbles	Formation of bubbles
6.	H_2S production	Black	Black	Black
7.	Oxidase Test	Negative (No red color)	Negative	Negative
8.	Glucose fermentation	Yellow	Yellow	Yellow
9.	Lactose fermentation	Negative (No Red-Red color)	Negative	Negative
10.	Sucrose fermentation	Negative (No Yellow color)	Negative	Negative
	Identified Organism	*Staphylococcus* sp.,	*Staphylococcus* sp.,	*Staphylococcus* sp.,

Based on morphological and biochemical analyses, all three keratinase-producing bacteria were characterized. Gram-positive organisms were found in all three isolates. They were discovered to be

spherical and rod-shaped morphologically. They were set up in chains and as singles. Additionally, they were displayed as endospores. The results of the H_2S production test, glucose fermentation test, and catalyst test were positive. However, the results of the lactose fermentation, sucrose fermentation, and oxidase test were negative. The results were summarized in Table. And Figure. 4A - E.

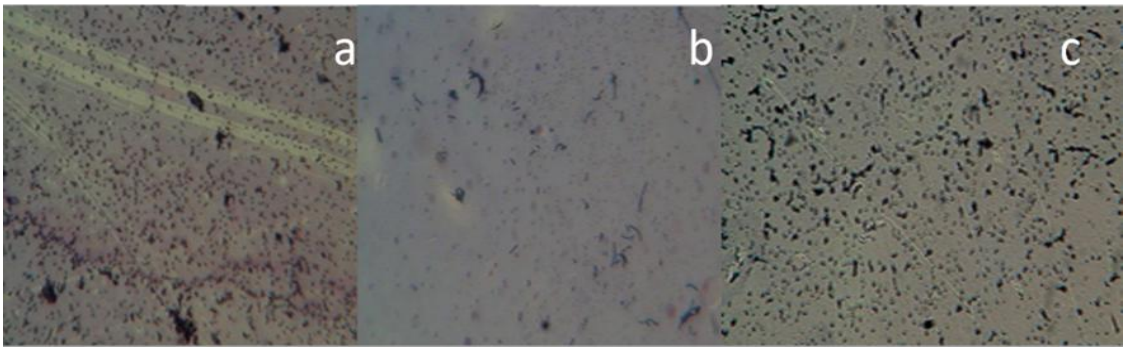


Figure. 4A. Gram positive rods of C1, C2 and C3

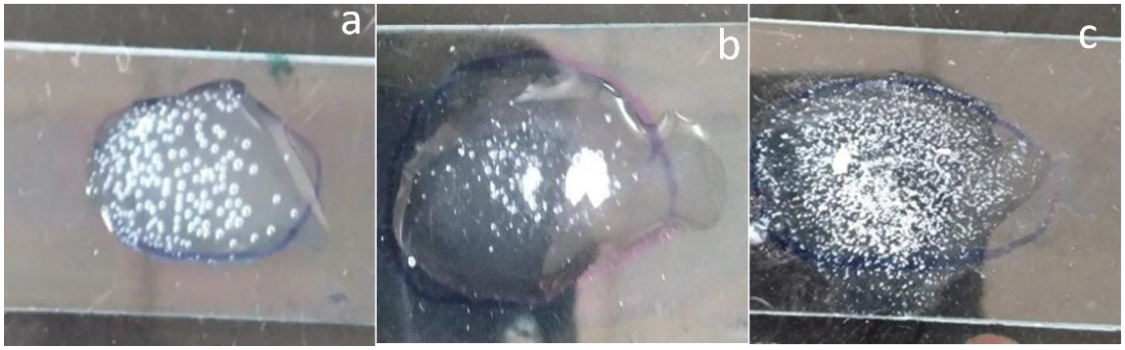


Figure. 4B. Catalyze Test of C1, C2 and C3

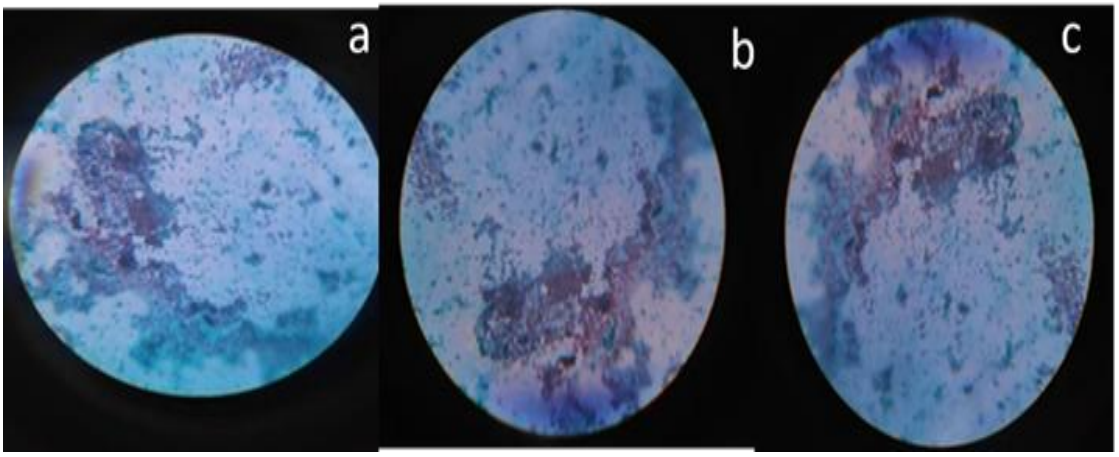


Figure. 4C. Endospore staining of C1, C2 and C

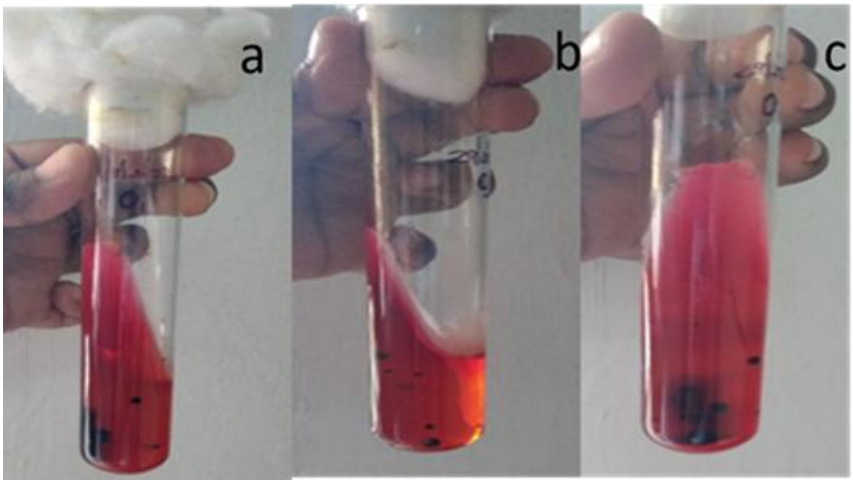


Figure. 4D. Hydrogen sulphide (H_2S) test for C1, C2 and C3

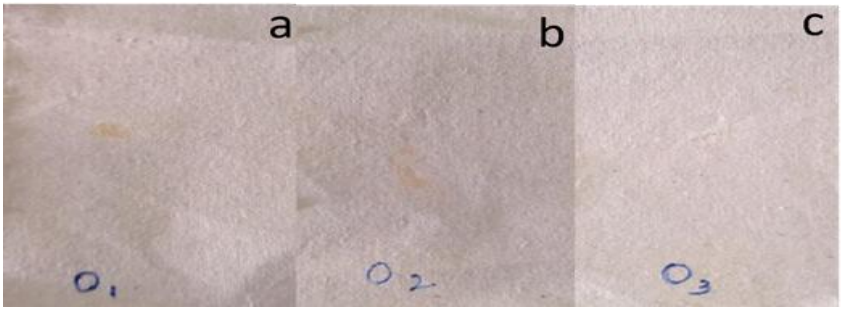


Figure. 4E. Oxidase test for C1, C2 and C3

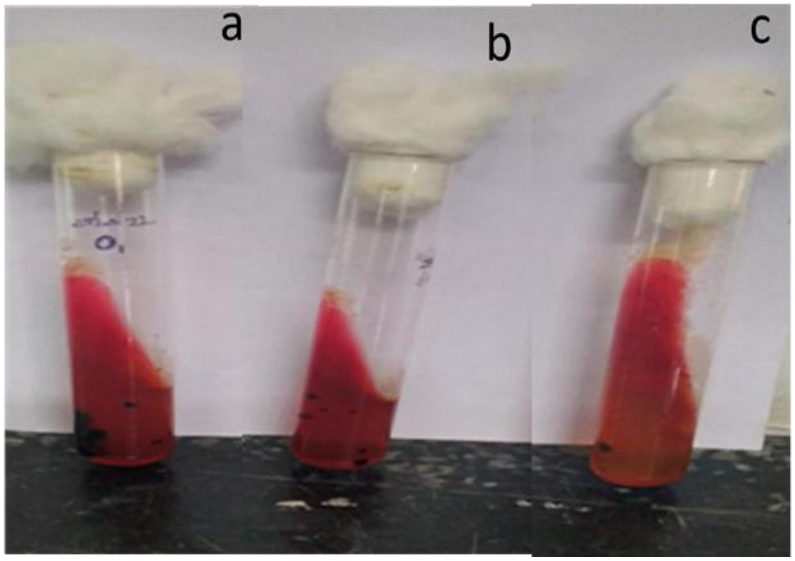


Figure. 4E. Triple sugar ion for C1, C2 and C3

The findings showed that, in a time-dependent manner, the C3 isolate produced keratinase enzymes with greater efficiency than C1 and C2. This implied that, in contrast to the other isolates, C3 showed a gradual rise in keratinase synthesis over time. C3's capacity for sustained enzymatic performance is highlighted by its time-dependent increase in keratinase activity. This might be useful for applications that call for extended enzymatic action (Figure. 6).

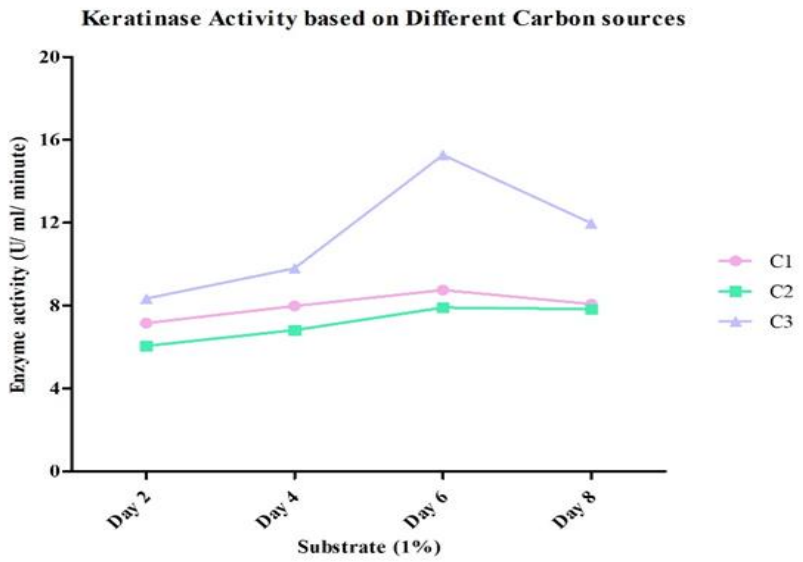
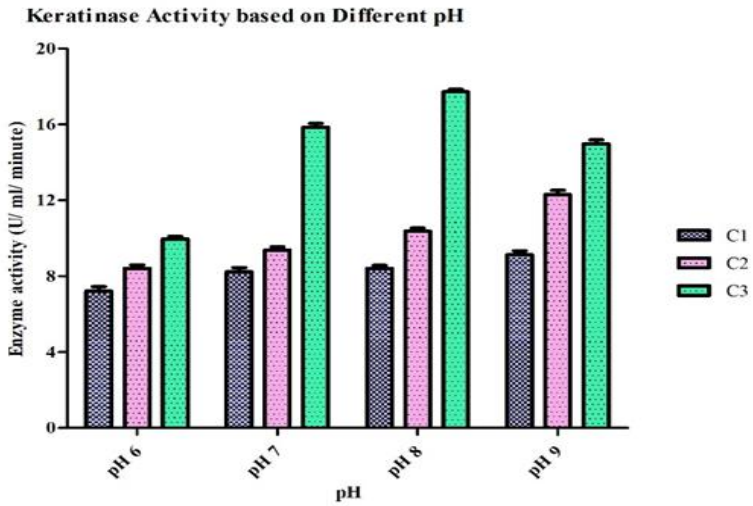


Figure.6: Effect of Incubation Time on keratinase production

The Figure 7 represented the influence of pH on the keratinase production by the isolates. The ideal pH was found to be 8 for C3, and 9 for bacterial isolates C3 and C2 (Figure. 7). Comparatively, C1 showed little variation on selected pH for keratinase production. According to Inamdar et al.'s findings, the majority of isolates showed increased enzyme activity at alkaline pH values. Enzymes that function best at an alkaline pH have clear benefits for a variety of uses, such as the leather industry and the decomposition of feathers.



. **Figure.7: Effect of pH on keratinase production**

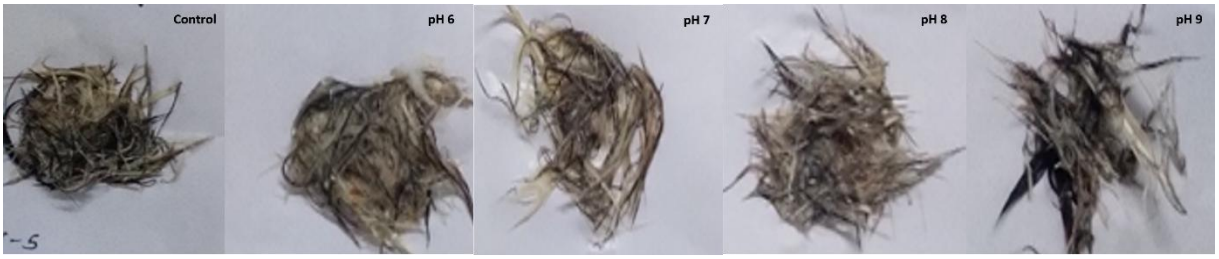


Figure. 7a. Effect of pH on keratinase production

Different carbon sources such as feather meal, skim milk, peptone and Casein were used for the production of keratinase enzyme. Among the isolates, C3 showed better enzyme production on casein followed by feather meal, peptone and skim milk. C2 showed better activity over C1 (Figure. 8). The isolates scarcely metabolize the skim milk but, efficiently degraded the feather meal. This showed that they already adopted for feather meal.

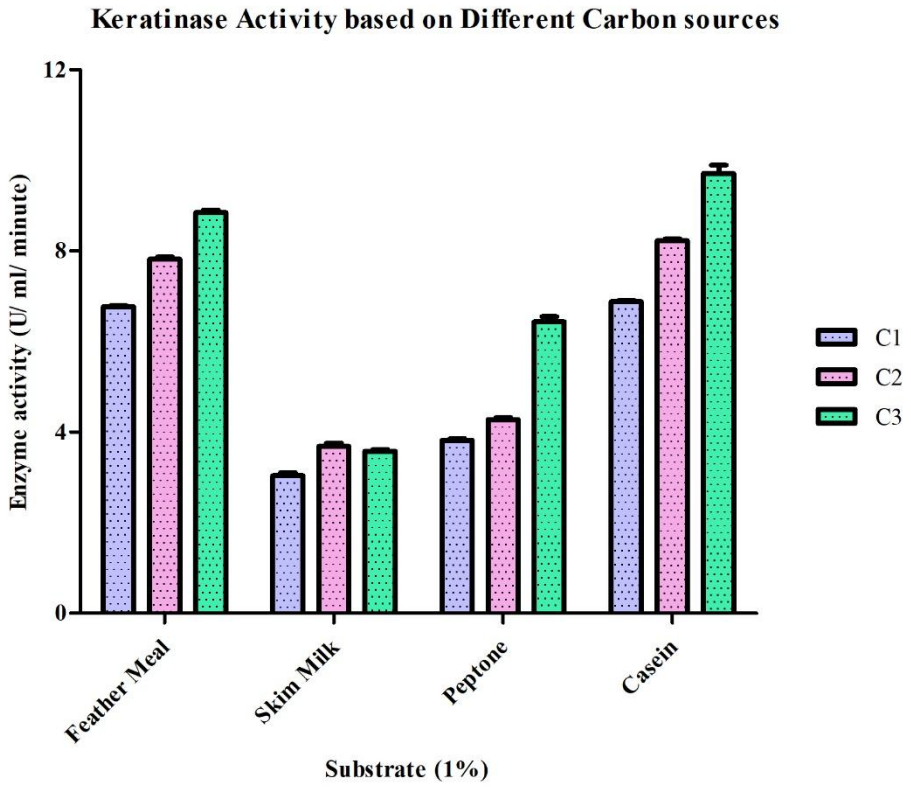


Figure.8: Effect of different carbon source on keratinase production

Molecular identification of the isolates using Sequence Analysis

The two isolates underwent DNA isolation, which was followed by PCR amplification using primers specific to 16S rRNA. The ensuing 1.5 kb sized amplicon on the agarose gel electrophoresis showed that the amplification process was effective. This finding attests to the DNA samples' integrity and appropriateness as PCR amplification templates. An essential stage in the identification and phylogenetic study of bacteria is the amplification of the 16S rRNA gene region, which yields important data for taxonomic categorization. The target region's effective amplification suggests that the isolate genomes include conserved 16S rRNA gene sequences. The bacterial isolates will be accurately identified and characterized by further sequencing and analysis of these amplicons, which will advance our knowledge of their genetic diversity and phylogenetic connections. Sequencing of the PCR products was conducted using the same set of primers. The highest hits from the BLAST analysis indicated that the isolates are members of the, *Bacillus subtilis* strain (Figure. 9), *Staphylococcus cohni* strain. (Figure. 10), and *Mammaliicoccus lentus* strain. (Figure. 11), The sequence details given in Table 4. This identification provides valuable insights into the taxonomic classification of the isolates, shedding light on their genetic relatedness and potential physiological characteristics associated with the *Bacillus cereus* species.

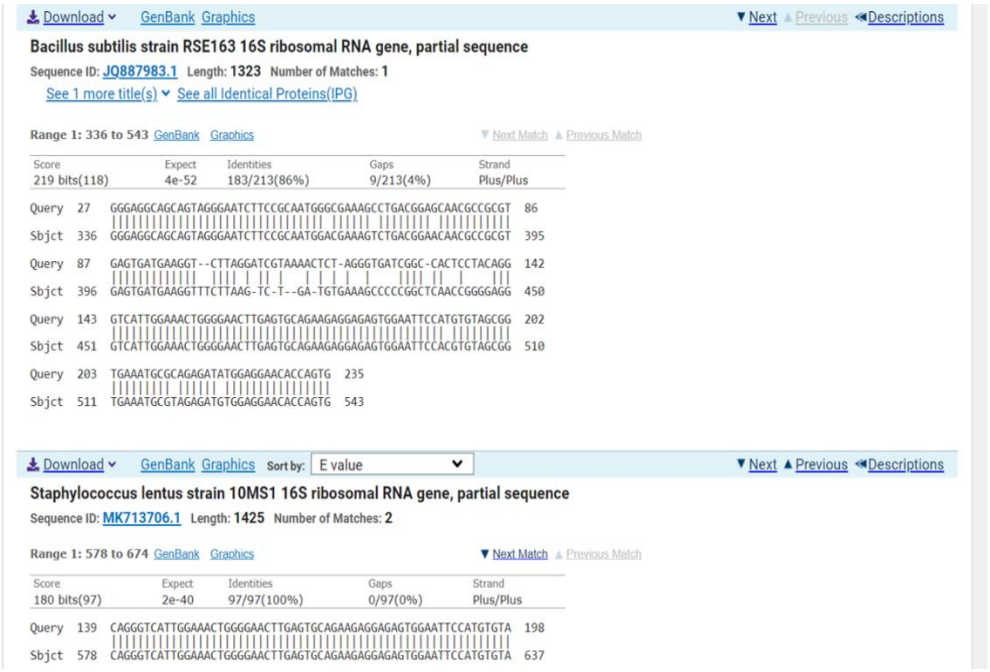


Figure. 9. C1, isolate was identified as *Bacillus subtilis* strain using BLAST

Figure. 10. C2, isolate was identified as *Staphylococcus lentils* strain using BLAST

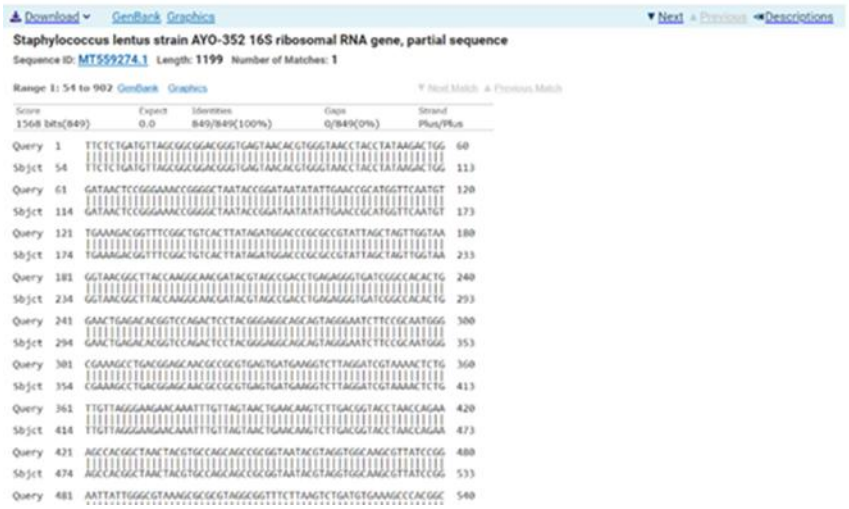


Figure. 11. C3, isolate was identified as *Mammaliicoccus lentus* strain using BLAST

Isolate C1 *Bacillus subtilis* strain	GCTTGCTTCTCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGGTCTGTAAGT TGTTGG TTCAATGTTGAAAGACGGTTTCGGCTGTCACTTATAGATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGG CTTACC AAGGCAACGATACGTAGCCGACCTGAGCTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACC CTGGTAG TCCACGCCGAGGGAAGAACAAATTTGTTAGTAACTGAACAAGTCACTGGAACTGAGACACGGTCCAGACTTGACG GTGATA ACTCCGGGAAACCGGGGCTAATACCGGATAATATATTGAACGCGAAGGCGGCTCTCTGACCTAACCAGAAAGCC ACGGCTA ACTACGTGCCAGCAGCCGCGGTAATACGTAGGCGCATTAAACGATGAGTGCTAAGTGTGGGAGGCAGCAGTAGG GAATCTT CCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTAGGATCGTAAAACTCTAGGGT GATCGG CCACTCCTACAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTG AAATG CGCAGAGATATGGAGGAACACCAGTGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTT TCTTA AGTCTGATGTGAAAGCCCACGGCTCAACCGTGGTAGGGGGTTTCCGCCCCTTAGTG
Isolate C2 *Staphylococcus cohni* strain	GCTTGCTTCTCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGGATAACTCC GGGAAACC GGGGCTAATACCGGATAATATATTGAACCGCATGGTTCAATGTTGAAAGACGGTTTCGGCTGTCACTTATAGATG

	GACCCGCG CCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCA CTCCTACA GGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAG AGATATGG AGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAG GATTAGAT ACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCG AAAGCCT GACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTAGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAATTTGTT AGTAACT GAACAAGTCACTGGAACTGAGACACGGTCCAGACTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGC CAGCAGC CGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTCT GATGTGA AAGCCCACGGCTCAACCGTGGTAGGGGGTTTCCGCCCCTTAGTG
Isolate C3 *Mammaliicocc us lentus* *Mammaliicocc us lentus SIMATS ZB TMS 1* (C3)	TTCTCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGGATAACTCCGGGAAA CCGGGG CTAATACCGGATAATATATTGAACCGCATGGTTCAATGTTGAAAGACGGTTTCGGCTGTCACTTATAGATGGACCC GCGCCG TATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACAC TGGAACT GAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAA

	CGCCGCGT GAGTGATGAAGGTCTTAGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAATTTGTTAGTAACTGAACAAGTCTT GACGGTAC CTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTA TTGGGCG TAAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTG GGGAACT TGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGC GAAGGCG GCTCTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCAC GCCGTAA ACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGA GTACGACC GCAAGGTTGAAACTCAAAGGAATTGACGG

Table. 4. 16srRNA sequence and identification of isolated bacterial species

Discussion

After screening soil samples from five poultry farms in Chennai, more than twenty isolates that degrade feathers were found; three of these isolates with unique characteristics were chosen for more investigation. The colonies, which were gram-positive bacteria with the ability to keratinolyze, were observed in both round and rod shapes. These bacterial colonies were streaked in both Feather meal Agar and Skim milk Agar. These bacteria displayed a distinct zone on a skim milk Agar plate, indicating that the organism is active in caseinolysis (15). Additionally, it demonstrated the use of keratin feathers by the Keratinolytic bacteria to clean the feather meal pate.

Studying the significance of different substrate concentrations for the synthesis of enzymes, it was shown that an increase in feather content was accompanied by an increase in enzyme activity and the release of amino acids. The optimal feather concentration was found to be 0.75%, which is consistent with earlier research by (16). That found that 0.7% chicken feather meal produced the largest amount of keratinase. It has been shown by earlier research that a feather concentration of 1% to 2% is ideal (17). Therefore, feathers that contain keratin act as an inducer and substrate for the synthesis of keratinase. Thus, keratin and its concentration in the medium are necessary for keratinase synthesis, supporting the enzyme's inducible characteristic. However, any further increases in substrate concentration will cause the enzyme to become saturated or inhibited once it has achieved its optimal level of substrate concentration.

The active range of pH 5.0 and 7.0 whereas the optimum pH for the Keratinase was found to be at pH 7.0. The enzyme was stable at the range of 6-8. This is similar to the study conducted by Nonso E. Nnolim and Uchechukwu U. Nwodo on *Bacillus* sp. CSK2 who conducted keratinolytic characterization (18).

The activity decreased at pH 3.0 and 8.0. Keratinolytic bacteria often exhibit optimal growth and activity at higher temperature. This was consistent with the study conducted by Geun-Tae Park and Hong-Joo Son. They found that the strain they isolated was able to grow from 15 to 40 °C and the optimum growth temperature was determined to be 25–40 °C (19). Some mesophilic bacteria exhibit the optimal enzyme production and activity ranging from 20 to 30°C. Here the keratinase secreted by the isolate was active in a broad range of temperature and maximum activity continued beyond ambient temperature. By sequencing Isolates 1 and Isolate 3 are identified. In our study we have done genomic sequencing and found the isolates are *Mammaliicoccus lentus* which belongs to *Staphylococcus* family. Previous study conducted by Richa Vema and Vijay Kumar isolated

Bacillus genera. Isolates TP1, JP1, and PP1 were identified as Bacillus cereus, whereas isolate VP4 was confirmed as Bacillus thuringiensis (20). These bacteria are more seen in the poultry soil which has pathogenic as well as non-pathogenic in nature. The microbiota present in the intestines of poultry is diverse and comprises an extensive range of microbial species, predominantly anaerobic bacteria. The specific microorganisms present in the intestines of poultry are significantly influenced by their diet and habitat. Similarly phylogenetic analysis in our study revealed the isolated microorganism is related to staphylococcus. Muhammad Ali Syed et al also concluded the same in their study that staphylococcal population isolated among farmed and household chickens.

These bacteria from the gut flora may aid digestion and protect animals against harmful bacteria. However, the presence of bacteria in the intestines of chicken poses a risk of contamination of the final product if the contents of the intestines become distributed. Infection of chickens is with *Staphylococcus* aureus which belongs to the same family. Condition of the skin, bones, joints, and navel is prevalent with this infection. There's a chance of getting food poisoning as a result of this. Chemical and physical treatments have little effect on *Staphylococcus* species. They are very resistant to large quantities of common table salt (NaCl) (7.5%), which can be utilised to separate the agent for severely polluted material. Chlorine-based disinfectants are efficient against the organism (in the absence of abundant organic matter). These bacteria are capable of degrading Keratin containing feathers means have high Keratinolytic activity.

The isolated bacterium can potentially be used for degradation of feather waste, thus bringing about effective waste management of feather waste using an eco-friendly approach. The optimized parameters can be implemented, and the process can be scaled up to use this microbial technology. Thus, the study provides an alternative approach for poultry waste management.

Conclusion

As a conclusion that the feather-degrading bacteria in chicken waste may create a new avenue for the poultry business to dispose of waste without contaminating it. Keratin degradation can be carried out by a variety of microbes. Further studies are needed to evaluate the different aspects of keratinase enzyme including its stability, separation and purification, kinetics and activators of the enzyme.

References

1. Li Q. Progress in Microbial Degradation of Feather Waste. Front Microbiol. 2019 Dec 5;10:2717. doi: 10.3389/fmicb.2019.02717.

2. Rahman MdM, Hassan A, Hossain I, Jahangir MMR, Chowdhury EH, Parvin R. Current state of poultry waste management practices in Bangladesh, environmental concerns, and future recommendations. J Adv Vet Anim Res. 2022 Sep 30;9(3):490–500.

3. Nandini Rajput, Heenu Sharma, Jasveen Bajwa. Potential role of keratinase in the environmental remediation, Materials Today: Proceedings,2023. https://doi.org/10.1016/j.matpr.2023.02.221.

4. Joardar JC, Rahman MM. Poultry feather waste management and effects on plant growth. Int J Recycl Org Waste Agricult. 2018 Sep 1;7(3): 183–8.

5. Chilakamarry CR, Mahmood S, Saffe SNBM, Arifin MAB, Gupta A, Sikkandar MY, et al. Extraction and application of keratin from natural resources: a review. 3 Biotech. 2021 May; 11(5): 220.

6. Nandini Allurea, Madhusudhan D.N. b and Dayanand Agsarc. Enhanced Production, Purification and Characterization of Alkaline Keratinase from Streptomyces minutiscleroticus DNA38. International Letters of Natural Sciences. Online: 2015-07-22 Vol. 43:27-37 doi: 10.18052/www.scipress.com/ILNS.43.27 2015

7. Muhammad Ashraf M, Asad W, Kiran T. Isolation of keratinolytic bacterial strains from poultry farm soil samples; Production, optimization and thermal characterization of the keratinase enzyme(s). Journal of the Chemical Society of Pakistan. 2018 Jan 1;40:953–60.

8. Bohacz J. Biodegradation of feather waste keratin by a keratinolytic soil fungus of the genus Chrysosporium and statistical optimization of feather mass loss. World J Microbiol Biotechnol. 2017 Jan; 33(1): 13. Do: 10.1007/s11274-016-2177-2.

9. Jaouadi B, Abdelmalek B, Zarai N, Bejar S. The Bioengineering and Industrial Applications of Bacterial Alkaline Proteases: the Case of SAPB and KERAB [Internet]. Progress in Molecular and Environmental Bioengineering - From Analysis and Modeling to Technology Applications. InTech; 2011. Available from: http://dx.doi.org/10.5772/23850

10. Sharma V, Kr. Kumawat T, Sharma G, Garg R, Biyani M. Contribution of Environmental Constituents in the Genomic Disruption of Cytokeratins [Internet]. Cytogenetics - Classical and Molecular Strategies for Analysing Heredity Material. IntechOpen; 2021. Available from: http://dx.doi.org/10.5772/intechopen.96877

11. Hong SJ, Park GS, Jung BK, Khan A, Park YJ, Lee CH, et al. Isolation, Identification, and Characterization of a Keratin-degrading Bacterium Chryseobacterium sp. P1-3. Journal of Applied Biological Chemistry. 2015 Sep 30;58:247–51.

12. Meyers MA, Chen PY, Lin AYM, Seki Y. Biological materials: Structure and mechanical properties. Progress in Materials Science. 2008 Jan 1;53(1): 1–206.

13. Kumar A. J. M., Lakshmi A. , Sangita R.V. Isolation and characterization of further degrading bacteria from poultry waste. Journal of Research in Biology.2012, vol 2(7), pp 676- 682

14. Sousa M, Souza O, Maciel M, Cruz R, Rêgo MG, Magalhães O, et al. Keratinolytic potential of fungi isolated from soil preserved at the Micoteca URM. European Journal of Biotechnology and Bioscience. 2015;3(4):10–5.

15. Matikevičienė V, Grigiskis S, Levisauskas D, Sirvydytė K, Dižavičienė O, Masiliūnienė D, et al. Optimization of Keratinase Production By Actinomyces Fradiae 119 and Its Application in Degradation of Keratin Containing Wastes. Environment Technology Resources Proceedings of the International Scientific and Practical Conference. 2015 Aug 5;1:294.

16. Kanagaraj J, Velappan KC, Babu NKC, Sadulla S. Solid waste generation in the leather industry and its utilization for cleaner environment. A review. 2006;65.

17. Nnolim NE, Nwodo UU. Bacillus sp. CSK2 produced thermostable alkaline keratinase using agro-wastes: keratinolytic enzyme characterization. BMC Biotechnol. 2020 Dec 14;20:65.

18. Park GT, Son HJ. Keratinolytic activity of *Bacillus megaterium* F7-1, a feather-degrading mesophilic bacterium. Microbiological Research. 2009 Jan 1;164(4): 478–85.

19. Vema R. Isolation and characterization of keratinolytic bacteria from poultry waste soils of Himachal Pradesh. J App Biol Biotech [Internet]. 2022 [cited 2024 Feb 21]; Available from: https://www.jabonline.in/abstract.php?article_id=949&sts=2

20. Syed MA, Ullah H, Tabassum S, Fatima B, Woodley TA, Ramadan H, Jackson CR. Staphylococci in poultry intestines: a comparison between farmed and household chickens. Poult Sci. 2020 Sep; 99(9): 4549-4557. Do: 10.1016/j. psj. 2020.05.051.