# ***Isolation, characterisation and identification of keratinase producing microorganisms from feather waste dumping sites***

A Project Report Submitted to University of Calicut for the Partial Fulfillment of the requirements for the award of Degree, Bachelor of Science in Microbiology (March 2020)


Department of Microbiology

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# CERTIFICATE

**B. Sc., MICROBIOLOGY**
**Project Report: MB6B16 (Pr)**

**ISOLATION, CHARACTERISATION AND IDENTIFICATION OF KERATINASE PRODUCING MICROORGANISMS**

**FROM FEATHER WASTE DUMPING SITES**

By


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# CERTIFICATE

Certified that the project report entitled ***"Isolation, characterisation and identification of keratinase producing microorganisms from feather waste dumping sites"*** is an authentic record of the work carried out by **Reg. No GNAUSMB001, GNAUSMB002, GNAUSMB003, GNAUSMB004** and **GNAUSMB005, Anjitha Prabha K, Aparna M P, Arya S, Asna K B** and **Fathima Hiba** at **Department of Microbiology, Government Arts & Science College Kozhinjampara, Palakkad, Kerala, India**. This work has been carried out as a partial requirement for the completion of B. Sc. Degree in Microbiology from Government Arts and Science College Kozhinjampara, affiliated to University of Calicut.

**Head of the Department** **External Examiner**

# DECLARATION

We do hereby declare that the project entitled " ***Isolation, characterisation and identification of keratinase producing microorganisms from feather waste dumping sites***" is the original record of work carried out by us at Govt. Arts & Science College, Kozhinjampara, under the guidance of **Mrs. Ameena I,** Assistant Professor of Microbiology, during the academic year 2022-23 in partial fulfilment of requirements for the award of Degree of Bachelor of Science in Microbiology( B.Sc. Microbiology) under University of Calicut.

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# INDEX

# CHAPTER I

# INTRODUCTION

## 1.1 Keratin

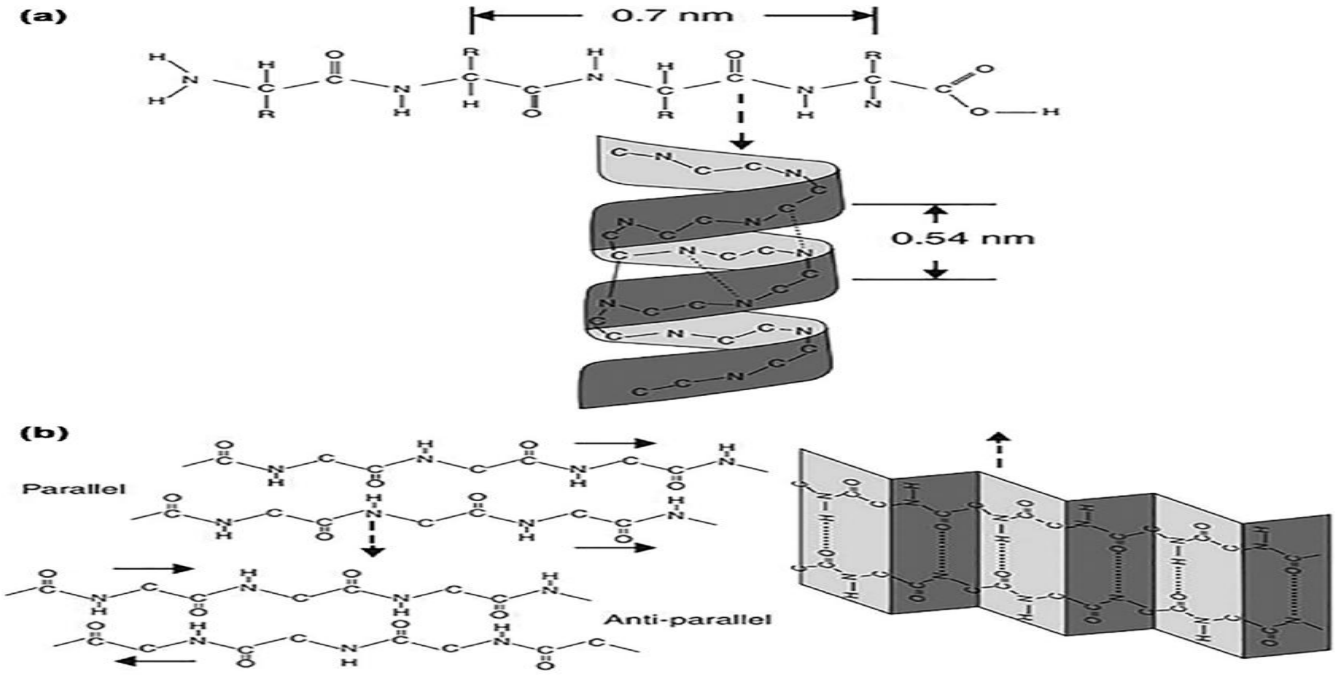
Keratin is a protein which belongs to the family of structural fibrous proteins, also known as scleroproteins. It is the key structural material making up scales, hair, nails, feathers, horns, claws, hooves, and the outer layer of skin among vertebrates. Keratin also protects epithelial cells from damage or stress. Keratin is extremely insoluble in water and organic solvent. Keratin can also be found in internal organs and glands. Keratin is a protective protein, less prone to scratching or tearing than other types of cells your body produces. Keratin is synthesized by specialized cells called keratinocytes, which are found in the skin, hair follicles, and nails of animals. These cells produce and secrete keratin proteins, which then become cross-linked and organized into the tough, fibrous structures that make up hair, nails, and other keratin-containing tissues. It is a complex protein with a high sulfur content and is particularly resistant to degradation. Keratin is responsible for the strength, rigidity, and elasticity of these structures." (**Hassan et al., 2020**)

Since keratin is the structural building block of hair, it is widely used to make products and treatments can help strengthen your hair and make it look healthier. Certain keratins may be found in higher-than-normal amounts in patients with different types of epithelial cell cancers, including lung, breast, colorectal, bladder, head and neck cancers. Due to the structure of keratin stabilized by disulfide bonds and hydrogen bonds, keratin is resistant to degradation by common proteases such as trypsin and pepsin.

**Composition:**

Keratin K75 (K6hf): K75 has a MW of 59.5 kDa and an isoelectric pH of 7.9; it is characteristic of the cells forming the companion layer of the inner root sheath of hair follicles (**Gu & Coulombe, 2007**). The keratin filaments containing K75 are oriented perpendicularly to the longitudinal axis of the hair like the ‘hoops of a barrel’ **(Winter et al. 1998)**. This basic (type II) keratin consists of 551 amino acids and 80% of the sequence of these amino acids is the same as

in the keratin K5. In contrast, the amino acid sequence of K75 is only 74% similar to that of K6a and K6b **(Winter et al. 1998)**




**Molecular structure of keratin protein; α-keratin (α-helix) and β-keratin (β-pleated sheet)**

## 1.2 SOURCES OF KERATIN:

Keratin is the type of protein that makes up our hair, skin, and nails. Keratin can also be found in internal organs and glands. Keratin can be derived from the feathers, horns, and wool of different animals and used as an ingredient in hair cosmetics. Our body produces keratin naturally. Animal fur, feathers, hooves and horns also consist of keratin. Keratin is found in many vegetables naturally. They include kale, onions, garlic, broccoli, and leeks. Other good sources of keratin include fish, low-fat milk, yogurt, and liver. Including these foods in your meal is a great way to increase keratin in your hair naturally.

## 1.3 SIGNIFICANCE OF KERATIN

**Structural role**: Keratin is the primary component of many important animal tissues, including hair, nails, feathers, hooves, and horns. It provides strength, rigidity, and elasticity to these structures, allowing them to withstand physical and chemical stresses.

**Protective role**: Keratin-containing tissues such as skin, hair, and nails act as a barrier against harmful substances such as bacteria, viruses, and UV radiation. Keratin helps to maintain the integrity of these barriers and protect the body from external threats.

**Functional role**: Keratin has a range of functional properties that make it important in various biological processes. For example, it helps to regulate the water content of skin and hair, and it can act as a scaffold for the attachment and growth of cells.

Applications in biotechnology: Keratin has numerous applications in biotechnology, including as a source of high-value proteins and as a material for biomedical applications such as wound dressings and tissue engineering scaffolds.

- Keratin is a protein that helps form hair, nails and skin's outer layer (epidermis).
- It helps support our skin, heal wounds and keep nails and hair healthy.
- Keratin provides support and protection in our body.
- Protect epithelial cells and strengthen the skin.
- Hydrolyze hair, feathers, and collagen in the sewage system during wastewater treatment.
- Useful in the food industry and the manufacturing of livestock feed, etc.

Overall, keratin is a versatile and important protein that plays a vital role in the structure, function, and protection of many different types of animal tissues. Its unique properties make it a valuable resource for a range of applications in biotechnology and other fields.

## 1.4 KERATINASE ENZYME

Keratinases belong to a class of proteases that are able to degrade keratins into amino acids. Keratinases play important roles in turning keratin-containing wastes into value-added products by participating in the degradation of keratin-rich wastes such as feathers from poultry industry into diverse products applicable to many fields. keratinase is responsible for the cleavage of peptides, making it attractive in pharmaceutical and feather industries. Despite of some progress made in isolating keratinase-producing microorganisms, structural studies of keratinases, and biochemical characterization of these enzymes, effort is still required to expand the biotechnological application of keratinase in diverse fields by identifying more keratinase understanding the mechanism of action and constructing more active enzymes through molecular

biology and protein engineering. Keratinases are produced only in the presence of keratin-containing substrate. They mainly attack the disulfide (-S-S-) bond of the keratin substrate. Keratinase is an enzyme that can break down keratin, the fibrous protein that makes up hair, nails, feathers, and other tough structures in animals.

Keratinases are of significant interest in biotechnology, as they have a range of potential applications in areas such as agriculture, waste management, and biomedicine.

Agriculture: Keratinases can be used to improve the nutritional value of animal feed by breaking down keratin in feathers, which is otherwise indigestible.

Waste management: Keratinases can be used to degrade keratin-containing waste products such as hair, feathers, and nails, reducing their volume and facilitating their disposal.

Biomedicine: Keratinases have potential applications in wound healing, as they can break down the tough outer layer of skin and promote the growth of new tissue. They may also be useful in the treatment of certain skin conditions, such as psoriasis, which are characterized by an overproduction of keratin.

Researchers have identified and characterized many different types of keratinases from a variety of microorganisms. Some of these enzymes are highly specific for keratin, while others can also digest other proteins. Ongoing research aims to improve our understanding of the structure and function of keratinases, and to develop new applications for these enzymes in biotechnology.

## 1.5 KERATIN-DEGRADING MICROORGANISMS

A vast variety of bacteria, fungi, and actinomycetes have been recognized as keratin degrading microorganisms (KDM). Keratinases are produced by microorganisms such as bacteria and fungi, which use them to digest keratin-rich materials in their environment. They degrade keratins mainly with their keratinases, which sometimes act synergistically with other enzymes like disulfide reductases and cysteine dioxygenase for effective degradation of keratin. To isolate a keratinase-producing microorganism, keratins or keratin- containing wastes such as feathers are usually used. Feathers are one of the most commonly used substrates in screening, and degradation of feathers can be readily monitored by observing the changes in shapes and releasing of proteins into the solution. It has been noted that several factors such as location,

water content, keratin composition, and weather of the local environment need to be considered to make sure that a collection of microorganisms can be obtained.

## 1.6 APPLICATIONS OF KERATINASE ENZYME

Keratinase enzymes have a wide range of industrial and biotechnological applications because of their propensity to break down keratins. They have potential uses as below,

- Used in the Livestock feed industry.
- Useful in the leather industry.
- In the detergent industry - traditional synthetic detergents.
- In the fertilizer industry – organic fertilizers are produced.
- In the pharmaceutical & cosmetic industry.

## 1.7 ROLE OF KERATINASE IN HAIR DEGRADATION

Human hair is a material considered useless in most societies and therefore is found in the municipal waste streams in almost all cities and towns of the world. Due to slow degradation, it stays in the dumps/waste streams for long occupying large volumes of space. It causes discomfort to people near them and, if inhaled in large amounts, can result in several respiratory problems.

Keratinases are significant in hair degradation because they are enzymes that can break down the tough, fibrous protein keratin that makes up hair. Hair is composed mainly of a type of keratin known as alpha-keratin, which is particularly resistant to degradation. However, certain microorganisms such as bacteria and fungi are capable of producing keratinase enzymes that can break down this protein. Keratinases break down hair by cleaving the disulfide bonds that hold together the individual keratin molecules, causing them to unravel and become more susceptible to further degradation. Once the keratin molecules have been broken down into smaller fragments, other enzymes can act on them to break them down into their constituent amino acids.

Hair degradation is important in various applications, such as waste management, animal feed production, and the production of high-value compounds such as amino acids and peptides. Keratinases have been shown to be effective in breaking down hair in all of these applications, making them a valuable tool in the processing of keratin-containing materials.

In summary, keratinases are significant in hair degradation because they are capable of breaking down the tough, fibrous protein keratin that makes up hair. This ability has important applications in various fields, making keratinases a valuable resource for the processing of keratin-containing materials.

## CHAPTER II

# REVIEW OF LITERATURE

Keratinases enzymes have been proved that they can degrade feathers and other keratin substances very efficiently, therefore it can be utilized for economical generation of animal feed and fertilizers. Their applications can be extended to leather, detergent, textile, pharmaceutical (**Gradisar et al., 2005**), cosmetic industries, prion decontamination, and biogas production, and also to improve the quality of silk and wool (**Arokiyaraj et al., 2019** and **Sousa et al., 2015**).

**Moniloa, W S et. al in 2011** studied that keratinase producing bacteria from poultry farm waste and screened for Proteolytic activity and feather degradation. From 10 isolates, B. *Licheniformis*(E1) had the highest keratinolytic activity of 10.604u/ml at incubation time of 72 hours, pH7.2 at 200 rpm.The isolated bacteria were capable of degrading other keratin-containing material including feather, hair, wool, and nail. A pH of 7.2 and incubation time ranged from 48 hours to 96 hours favors the production of keratinase in most of the isolates.

**Godbole, S et al. in 2017** observed that keratinolytic microorganisms having great importance in poultry waste degradation and its bioconversion to compost or animal feed. Five bacterial cultures were isolated using basal media with feathers meal and showed feather degradation capacity. Among identified isolates, *Aeromicrobium spp*. (KD1) shows the highest feather degradation of 72.5% in temperature 30°C and pH 7.0

**Jeevana, L. P et al., in 2013** studied about Efficient degradation of feather by keratinase producing *Bacillus sp* and characterized two strains. BF 11 (*Bacillus subtilis*) and BF21*(Bacillus cereus*) having more than 10 ku/ml production of enzymes. Strain improvement with BF11&BF 21 and resulted in isolation of MBF11 &MBF21.Its optimal conditions are about pH of 8.5, temperature of 45-55°C.
Screening and isolation of keratinase producing strains of bacteria from various region

of Bangalore using feather powder agar plate assay and biochemical characteristics of selected strains lead to their identification as *Bacillus licheniformis*, *Bacillus cereus* and *Staphylococcus aureus.* **(Naga Raju, E.V et al. in 2013).**

**Peng, Z et al. in 2019** investigated that the characteristics of feather degradation by B. *Licheniformis* is BBE II-1 & *S. maltophilia* BBEII-1 along with cultivation of keratinase producing strains. Concluded that cocultivation of these strains can efficiently degrades 50g/L chicken feather waste and produce large number of amino acids and antioxidant substances as a conversion rate of 70.0%.

Novel feather degrading isolated from different keratinolytic bacteria. KerS13uv+ems and kerS26uv presented high keratinolytic activity, thermostability & environmentally friendly alternative to the unconventional chemicals used in keratin hydrolysis **(Ali, A. A et al.)**

**Gandhe, B et al. in 2018** observed that microbial keratinases and other prospective agro industrial applications,which have never been considered as proteases due to their ability to act on the tough,rigid, insoluble structural protein, keratin.

In another study, keratinolytic probiotic *Bacillus licheniformis bacteria* has been identified. Only the *Bacillus licheniformis pvkr15* produced acceptable keratinase enzyme and demonstrated potential survival in the gut environment with ability to produce spores. And result revealed this strain can be employed as probiotic in animal and chicken feed industry (**Vanak, P et al.)**

Result of a study by **Ahalya, B. A et al. in 2002** strongly suggest that feather degrading bacteria and their proteolytic enzymes could be used for production of value-added materials and B. *licheniformis* appeared to be most efficient strain based on their investigations.

In a study by **Ahmed, M et.al in 2019,***bacillus spp* showed maximum enzyme production.Keratinolytic bacteria were isolated from soil of poultry farm and different cultural conditions were optimized to maximum enzyme yield. The isolates which made clear zones on skimmed milk agar plates were considered as protease producing isolates and then evaluated for keratinase production by using raw feather as substrate in submerged fermentation.

**Shen, N et. al in 2022,**studied about screening of keratinase strains from marine environment, fermentation condition, enzymatic properties and feather degradation mechanism that are crucial for efficient degradation of feathers. A novel efficient feather degrading bacteria, Gxun-17 isolated from soil sample was identified as *bacillus tropicus.*

**Casarin, F et.al in 2008,**studied that the production of keratinolytic enzyme by *Chryseobacterium spp* isolated from the poultry industry and tested on different growth substrates.

In another study by **Chandra, B. N et. Al in 2017** A good keratinase positive bacterium isolated from soil sample and identified as Arthrobacter spp, by sequencing 16SrRNA gene. Keratinase production was more than 5.0-folds increased when all optimized parameters were applied simultaneously.

A study by **lakshmipriya,T et.al in 2015** ,expansion of information about microbial keratinases and important considerations in keratinase production are discussed. Keratinase are essential in preparation of animal nutrients, protein supplement, feather manufacture, textile processing, detergent formulation, feather meal processing for feed and fertilizer, the pharmaceutical and biomedical industries and waste management.

Production of keratinases and degradation of keratin from microorganisms isolated from poultry soil in Trichy.Study concluded that *pseudomonas Aeruginosa* gmp could degrade waste dyed keratin in 12 days. **(Gowdhaman, D et.al in 2014).**

## CHAPTER III

# OBJECTIVES

1. Isolation of keratinase producing microorganisms from peafowl and poultry feather dumping soil.
2. Analysis of keratin degradation efficiency of isolates under various cultural conditions
3. Cultural and biochemical characterization of keratinase producing microorganisms
4. Molecular level identification of the most efficient strains
5. Study on cellulolytic, amylolytic and phosphate solubilizing properties of different keratinase producing bacteria.
6. Applications of keratin degrading bacteria on human hair degradation

# CHAPTER IV

# MATERIALS AND METHODS

## *4.1 Isolation of Keratinase Producing Bacteria*

### 4.1.1 Sample collection and preparation

Soil samples were aseptically collected from poultry farms and feather dumbing sites located near Kozhinjampara and Kannimari. Samples were stored at $4^0$C for microbiological analysis.

### 4.1.2 Enrichment of microorganisms

For the enrichment of Keratinase producing microorganisms, **Feather Meal Broth** with the following composition was used.

**Composition (g/l)**

$NH_4Cl$ - 0.5g

NaCl - 0.5g

$K_2HPO_4$ - 0.3g

$KH_2PO_4$ - 0.4g

$MgCl_2$ - 0.1g

Yeast extract - 0.1g

Feather powder - 10g; Distilled water - 1000ml

**Media preparation:** 1g feather powder is added to 100ml distilled water and dissolved all the components of feather meal broth media. This enriched media is then autoclaved at $121^0$C for 15lbs pressure.

After sterilization, 10g of sample was added and kept at rotary shaker for 24hrs.

**a) Serial dilution of the sample**

The enriched samples were serially diluted up to $10^{-7}$ using sterile saline solution.

**b) Inoculation and Incubation**

1 ml of enriched samples were inoculated in to **Nutrient agar media** and incubated at $37^0$C for 24hrs. The following composition was used for Nutrient agar preparation.

**Composition (g/l)**

Peptone - 5g

Beef extract - 3g

NaCl - 5g

Agar - 20g; Distilled water - 1000ml; pH – **7.0**

**c) Identifying similar colonies of microorganisms**

Colonies observed on Nutrient Agar media were tabulated according to cultural characteristics and similar colonies were grouped together.

**d) Purification of Bacterial Isolates**

Phosphate solubilizing microorganisms were selected based on the formation of clear halo/zone around the colonies on agar medium. The keratin digesting bacteria were streaked on Nutrient Agar plates to have single isolated colonies (Streak Plate Method-Quadrant streaking)

**4.1.3 Preservation of organisms**

Nutrient Agar slants were prepared and streaked with single isolated colonies, then the tubes were incubated at room temperature and stored in refrigerator after microbial growth.

**4.2 *Study on keratinase producing efficiency of different isolates***

Skimmed Milk Agar media was used for the efficiency study of keratinase producing bacteria

**4.2.1 Media preparation**

**Skimmed milk agar** was used to study keratinase producing efficiency of different isolates. The following composition was used.

**Composition (g/l)**

Skimmed milk - 3.0g

Casein enzyme hydrolyzate - 5g

Yeast extract - 2.5g

Dextrose - 1g

Agar - 15g; pH - 7+/- 0.2

**4.2.2 Inoculation and incubation**

Isolated colonies of all keratinase producing microorganisms were spot inoculated in to **Skimmed milk agar.** The plates were then incubated at $37^0$C for 24hrs and observed for zone formation around the colonies. **Keratin Degradation Index (KDI)** was then calculated for each organism.

**4.2.3 Keratin Degradation Index**

Keratin Degradation Index was calculated using the following equation.

$$\textbf{Keratin Degradation Index (KDI)} = \frac{\textbf{Zone diameter + Colony diameter}}{\textbf{Colony diameter}}$$

**4.3 *<u>Study on keratinase producing efficiency at various cultural conditions</u>***

**a) Temperature**

Two isolates showing the highest **Keratin Degradation Index** were selected and spot inoculated on to **Skimmed milk agar** and incubated at $25^0$C, $35^0$C and $37^0$C for 24hrs, 48hrs and 72hrs.

**b) pH**

Two isolates showing the highest **Keratin Degradation Index** were selected and spot inoculated on to **Skimmed milk agar** with pH 6.0, 7.0 and 8.0, and incubated at $37^0$C for 24hrs, 48hrs and 72hrs.

## 4.4 *Cultural and Biochemical Characterization of various isolates*

The following studies were conducted for the identification of all **Keratin Degrading Bacteria (KDB). KDB** grown on nutrient agar were observed for Colony morphology, Gram's reaction, Motility and Biochemical characteristics.

### A) Morphological Characteristics

Morphological characteristics of isolates viz. shape, size, elevation, surface form, margins, surface texture, and color were observed

### B) Gram Staining

The isolate was microscopically observed for its gram staining characteristics. The staining was performed as per standard protocol (James G. Cappuccino and Natalie Sherman, 10th edition) using the primary stain Crystal violet, Mordant Grams Iodine, Decolorizing agent Ethanol and Counterstain Safranin

### C) Motility Test

Motility of the bacterial cultures were studied by Hanging Drop Method with cavity slide and Microscope.

### D) Biochemical Identification

#### *a) Indole test*

Peptone broth was prepared and sterilized in test tubes. The tubes were inoculated with KDB isolates and incubated at $37^0$C for 24hrs. After incubation 5-6 drops of Kovac's reagent was added to each test tubes and observed for purple ring formation.

#### *b) Urease test*

Christensen's urea agar slants were prepared and inoculated with KDB and then incubated at $37^0$C for 24hrs. After incubation, the tubes were observed for color change to pink

### *c) Citrate test*

Simmons Citrate agar slants were prepared and inoculated with KDB and then incubated at $37^0$C for 24hrs. After incubation, the tubes were observed for color change to prussian blue

### *d) Nitrate Reduction test*

Nitrate broth was prepared and sterilized in tubes. The tubes were inoculated with KDB isolates and incubated at $37^0$C for 24hrs. After incubation, Nitrate reagent was added to the tubes. Zinc dust was added to tubes which did not show color change and results were observed.

### *e) Triple Sugar Iron agar test (TSI)*

Triple Sugar Iron Agar slant were prepared and streak inoculated with each KDB. This was kept under incubation at $37^0$C for 24hrs. Results were observed.

### *f) Methyl Red test*

MR-VP broth was prepared and sterilized in test tubes. The tubes were inoculated with KDB isolates and incubated at $37^0$C for 24hrs. After incubation 5-6 drops of Methyl Red was added to each test tubes and mixed well. Tubes were observed for color change.

### *g) Voges-Proskauer test:*

MR-VP media was prepared and sterilized in test tubes. The tubes were inoculated with KDB isolates and incubated at $37^0$C for 48 hours. After incubation 10 drops each of Baritt's reagent A and B were added and mixed well. The colour change was recorded.

### *h) Catalase test*

One drop of 3% H2O2 was taken on a slide. A loopful broth culture of KDB was taken and mixed with H2O2 on slide. Observed for bubble formation.

### *i) Oxidase Test*

Oxidase disc was taken using sterile loop, each KDB was streaked on to the disc. Color change on streak line was observed.

### *j) Carbohydrate Utilization test*

Fermentation media with specific carbohydrates including glucose, fructose, lactose and sucrose with phenol red indicator was prepared and sterilized separately. Each of the tube was inoculated with KDB cultures. All the tubes were incubated at $37^0$C for 24hrs and color change was observed

Bacterial isolates were identified using **Bergey's Manual of Systematic Bacteriology** and an online identification software **www.microrao.com**, based on the above test results

### 4.5 *Molecular identification of the most efficient strain using nucleotide sequencing method*

Pure culture of the selected bacterial strain having the highest value for KDI was send to the Regional Facility for DNA finger printing (RFDF), Rajiv Gandhi Centre For Biotechnology, Thiruvananthapuram for molecular level sequencing using 16sRNA

#### 4.5.1 16S rRNA- PCR

The identification of the selected isolate was done using 16S rRNA-PCR, which is a molecular method that identifies organisms based on their genetic sequences. The steps involved genomic DNA isolation, PCR reactions in isolated genomic DNA to get amplicons of 16S rRNA, Agarose gel electrophoresis, Purification of the PCR product and Sequencing using BigDye Terminator v3.1

#### 4.5.2 Sequencing and BLAST analysis

The 16S rRNA sequence is compared to a database of bacterial sequences to identify the genus and species of the bacteria. Sequencing involves determining the order of nucleotides in a DNA or RNA molecule, while BLAST analysis is a powerful bioinformatics tool used to compare a newly obtained sequence to a database of known sequences. Bidirectional sequencing has been carried out using Sanger's method. The sequences are then analyzed and identified using the BLAST software of GenBank databases which is the most commonly used database.

The percentage similarity has been determined using the sequence alignment of the test isolate with the GenBank sequence of the type strain by BLAST program, a bioinformatics tool to check the sequence similarity with the existing sequences in the NCBI website. Valuable insight into the genetics of the organism has been elucidated using gene sequencing and BLAST methods.

#### 4.5.3 NCBI ((National Center for Biotechnology Information) Submission

The details of the identified organism have been submitted to NCBI, which is a critical component of scientific research and collaboration, allowing researchers to share and access biological and genetic data in a way that benefits the entire scientific community

## 4.6 *Study on other metabolic activities of various isolates*

### 4.6.1 Cellulase production

The bacterial isolates were inoculated on **Carboxy Methyl Cellulose (CMC) agar** having the following composition.

**Composition (g/l)**

Peptone - 10.0

$K_2HPO_4$ - 2.0

$MgSO_4.7\ H_2O$ - 0.3

$(NH_4)_2SO_4$ - 2.5

Agar - 20.0; Distilled water - 1000ml

The samples were spot inoculated and the plates were incubated at $37^0$C for 48hrs. After incubation, the plates were flooded with **1% Iodine solution** with the following composition.

**Composition (g/l)**

Iodine - 3.0g, Potassium iodide - 6.0g, Distilled water - 1000ml

### 4.6.2 Amylase production

The bacterial isolates were inoculated on to **Starch agar medium** with the following composition.

**Composition (g/l)**

Beef extract - 3g

Peptone - 5g

Soluble starch - 10g

Agar - 20g: Distilled water - 1000ml; pH - 7.2 +/- 0.1

The samples were spot inoculated and the plates were incubated at room temperature for 48-72 hrs. After incubation, **2ml Gram's iodine solution** was added to the plates and spreaded over the entire surface of the plates.

### 4.6.3 Phosphate solubilization

**National Botanical Research Institute Phosphate agar (NBRIP)** with the following composition was used for the phosphate solubilizing efficiency study of various isolates

| | |
|---|---|
| Glucose | -10g |
| $Ca_3(PO_4)_2$ | -5g |
| $MgCl_2.6H_2O$ | -5g |
| $MgSO_4.7H_2O$ | -0.25g |
| KCl | -0.2g |
| $(NH_4)_2SO_4$ | -0.1g |
| Bromophenol Blue | -0.025g |
| Agar | -15g; Distilled water -1000ml, pH **-7.0 to 7.2** |

NBRIP agar was sterilized, poured into Petri dishes and then stored aseptically.

Isolated colonies of keratin digesting bacteria were spot inoculated into NBRIP agar. The plates were then incubated at Room Temperature for 72 hours and observed for clear zone formation around the colonies.

## 4.7 *Application of keratinase producing bacterial isolates on human hair degradation*

10g quantity of human hairs were weighed and taken in 3 conical flasks each. Overnight grown culture suspension of selected bacterial isolates having maximum potential for keratin digestion (**KDBI** and **KDBVIII)** have been inoculated into the flasks labelled I and II respectively. The third flask was inoculated with a mixture of inoculants with equal amounts of **KDBI** and **KDBVIII.** The inoculated samples were kept for incubation at room temperature and subjected

for biodegradation studies. The flasks were periodically sprayed with sterile water and observed physically for degradation pattern

# CHAPTER V

## RESULTS AND DISCUSSION

### *5.1 Isolation of keratinase producing microorganisms*

Keratinase producing microorganisms were isolated from soil samples of the selected poultry farms' feather dumping sites

The following pure isolates were obtained from spread plates and streak plates of enriched samples

**KDB I, KDB II, KDB III, KDB IV, KDB V, KDB VI, KDB VII, KDB VIII, KDB IX, KDB X, KDB XI** and **KDB XII.**

### *5.2 Selection of strains based on Keratin Degrading Index*

Among the colonies that were spotted on skimmed milk agar media some showed zone of clearance, colonies with maximum KDI were selected for further studies. **(Table 5.1.A)**

| Colony name | Zone diameter (cm) | Colony diameter (in cm) | Keratin Degrading Index (KDI) |
|---|---|---|---|
| KDB I | 3.8 | 0.7 | ***6.43*** |
| KDB II | 3.8 | 1.1 | 4.45 |
| KDB VI | 3.4 | 0.7 | 5.86 |
| KDB VIII | 3.4 | 0.65 | ***6.23*** |
| KDB IX | 4 | 1.5 | 3.67 |
| KDB X | 3.5 | 0.7 | 6 |

***(Table 5.1 : KDI of various isolates)***

KDI was observed to be different for different isolates. Keratin Degrading Efficiency is directly proportional to KDI. Based on the above data, KDB I and KDB VIII were found to be the most efficient Keratin degraders among the isolates.

***5.3 Keratin degradation efficiency under various cultural conditions***

The selected strains (KDB I and KDB VIII) were established with various conditions so as to optimize the optimum cultural conditions where they produce maximum KDI. Following are the parameters considered;

pH

Temperature

Incubation period

***5.3.1 Keratin degradation at various pH***

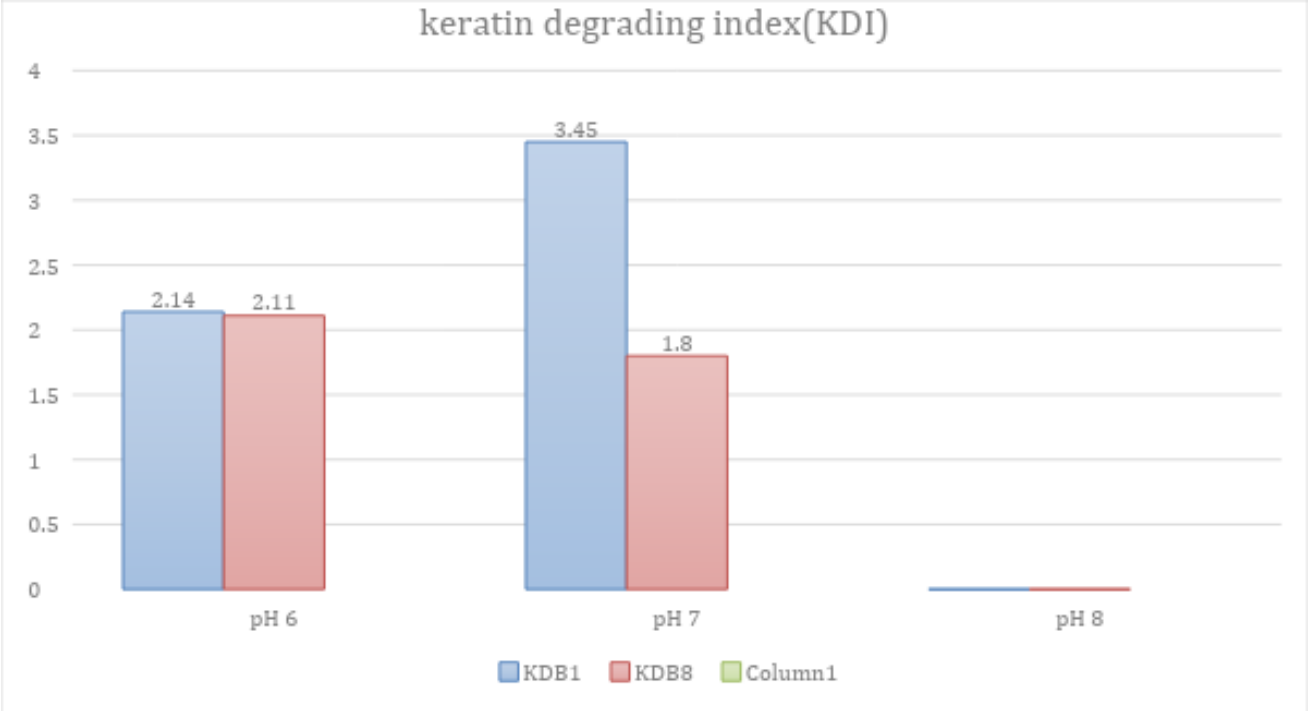
| pH | Incubation period (hr) | **KDB1** | | | **KDB8** | | |
|---|---|---|---|---|---|---|---|
| | | Zone Diameter (in cm) | Colony Diameter (in cm) | KDI | Zone Diameter (in cm) | Colony Diameter (in cm) | KDI |
| 6 | 24 hr | No zone | No zone | --- | No zone | No zone | --- |
| | 48 hr | 1.6 | 1.4 | 2.14 | 1.9 | 1.7 | 2.11 |
| | 72 hr | 2 | 1.6 | 2.25 | 2.6 | 2.4 | 2.08 |
| 7 | 24 hr | 1.5 | 0.9 | 2.67 | 2 | 1.6 | 2.25 |
| | 48 hr | 2.7 | 1.1 | **3.45** | 3.3 | 2.1 | 2.57 |
| | 72 hr | 3.5 | 1.5 | 3.33 | 4.1 | 2.4 | **2.71** |
| 8 | 24 hr | No zone | No zone | --- | No zone | No zone | --- |
| | 48 hr | No zone | No zone | --- | No zone | No zone | --- |
| | 72 hr | No zone | No zone | --- | No zone | No zone | --- |

***(Table 5.2) Keratin Degrading Index at various pH (skimmed milk agar)***

Both KDB I and KDB VIII had their highest KDI value at pH 7.0. Within this pH range itself, when incubation hours were changes, varying results were obtained; KDB I had its maximum KDI value (3.45) at 48 hours of incubation at pH 7.0, whereas KDB VIII had its maximum value of KDI (2.71) after 72 hours of incubation at pH 7.0

It can be concluded that KDB I is more efficient than KDB VIII in degrading keratin.

The graphical illustration of KDI at various pH levels are demonstrated in **Graph 5.3.**




***(Graph 5.3: KDI at various pH)***

***5.3.2 Keratin degradation at various temperatures***

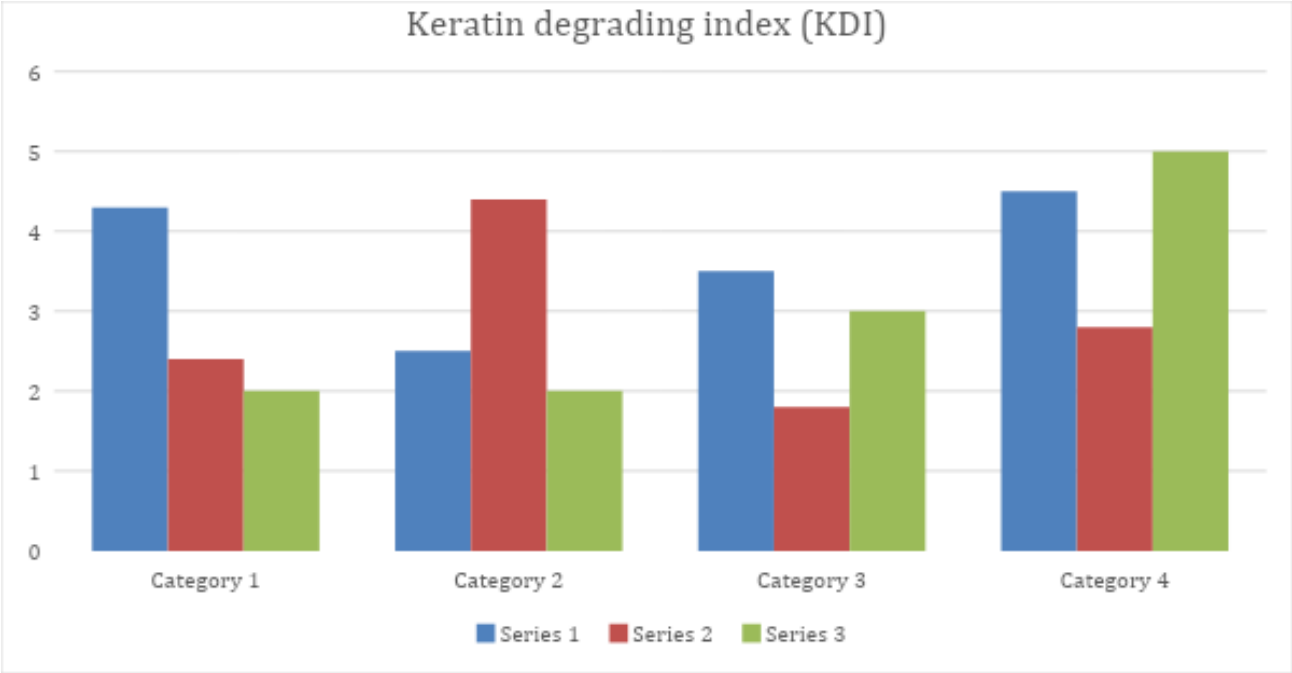
| Temperature (In $^0$C) | Incubation period (hr) | KDB1 | | | KDB8 | | |
|---|---|---|---|---|---|---|---|
| | | Zone diameter (cm) | Colony diameter (cm) | KDI | Zone diameter (cm) | Colony diameter (cm) | KDI |
| 25$^0$C | 24 hr | 1.2 | 0.7 | 2.71 | 1.6 | 1.3 | 2.23 |
| | 48 hr | 2.4 | 1 | 3.4 | 2.8 | 1.8 | 2.55 |
| | 72 hr | 2.9 | 1.3 | 3.2 | 3.5 | 2.1 | 2.66 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 32$^0$C | 24 hr | 2.1 | 1.5 | 2.4 | 1.9 | 1.2 | 2.58 |
| | 48 hr | 3.1 | 1.6 | 2.93 | 3 | 1.6 | 2.87 |
| | 72 hr | 3.9 | 1.7 | 3.29 | 3.8 | 1.8 | **3.11** |
| 37$^0$C | 24 hr | 1.5 | 0.9 | 2.66 | 2 | 1.6 | 2.25 |
| | 48 hr | 2.7 | 1.1 | **3.45** | 3.3 | 2.1 | 2.57 |
| | 72 hr | 3.5 | 1.5 | 3.33 | 4.1 | 2.4 | 2.70 |

***(Table 5.3.a) Keratin degrading index at different temperatures (pH 7.0)***

Maximum KDI value for was obtained at 37$^0$C and that for was found to be 32$^0$C The KDI values are 3.45 and 3.11 respectively for KDB I and KDB VIII respectively **(Table 5.3.a).**

Graphical representation of Keratin Degrading Index against variable temperatures are plotted **(Graph 5.3.b)**




**(Graph 5.3.b)** KDI at various temperatures

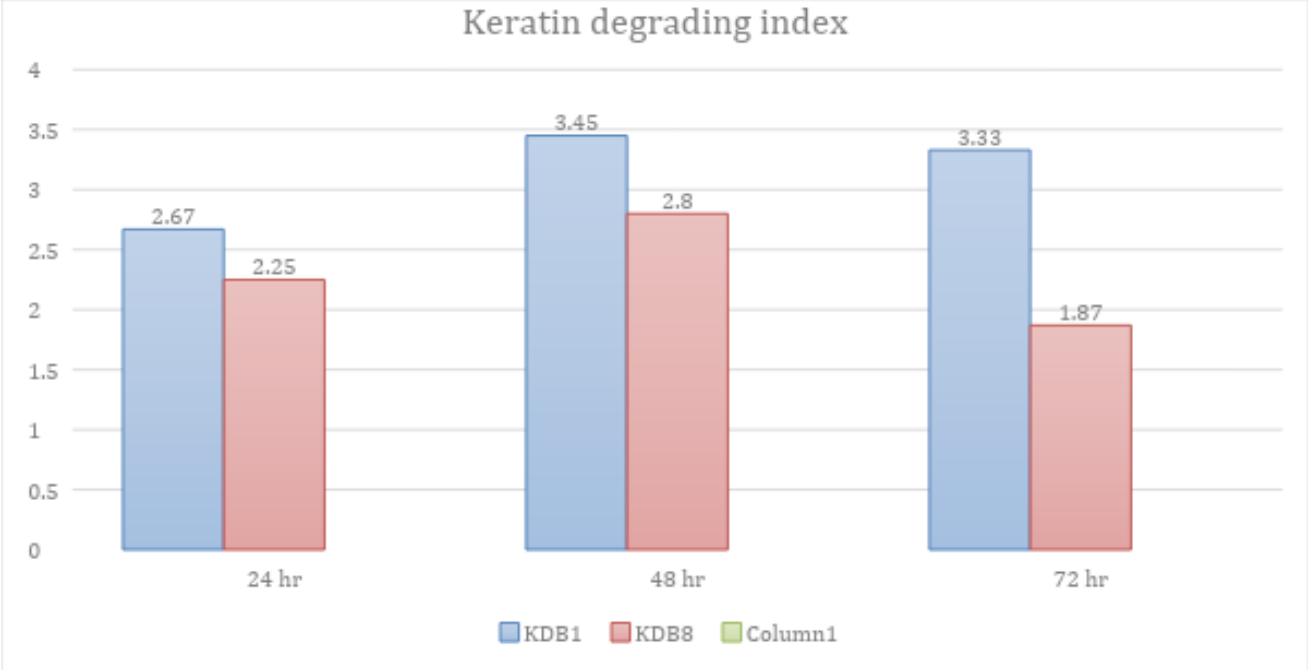
***5.3.3 Keratin degradation at various incubation hours***

Keratin degradation was observed on KDB I and KDB VIII at fixed temperature($37^0$C) and pH(7.0) ***(Table 5.3.c)***

| KDB I | | | | KDB VIII | | |
|---|---|---|---|---|---|---|
| Incubation period (hr) | Zone Diameter (cm) | Colony Diameter (cm) | KDI | Zone diameter (cm) | Colony diameter (cm) | KDI |
| 24 hr | 1.5 | 0.9 | 2.67 | 2 | 1.6 | 2.25 |
| 48hr | 2.7 | 1.1 | **3.45** | 3.3 | 4.1 | **2.80** |
| 72 hr | 3.5 | 1.5 | 3.33 | 2.1 | 2.4 | 1.87 |

***(Table 5.3.c) KDI at various incubation hours (temperature- $37^0$C, pH-7)***

Both strains showed maximum keratin degradation at 48 hours of incubation. The maximum KDI value for KDB I was 3.45 and that for KDB VIII was found to be 2.80). Hence 48-hour incubation is considered to be optimum for keratin degradation activity **(Graph 5.3.c)**




***(Graph 5.3.c: KDI at different incubating hours)***

## *5.4 Morphological and Biochemical Characterization of Efficient Keratin Degrading Bacteria*

### 5.4.1. Morphological Characteristics

Morphological characteristics including colony characters, gram stain and motility were observed and recorded. **(Table 5.4 a)**

| KDB | Colony Morphology | | | | | Gram staining | Motility |
|---|---|---|---|---|---|---|---|
| | size | Pigmentation | Form | margin | Elevation | | |
| KDBI | Large/ moderate | Creamy white | circular | entire | umbonate | Gram negative rod | Motile |
| KDBII | Large/ moderate | Creamy white | irregular | undulate | convex | Gram negative rod | Motile |
| KDBVI | Large/ moderate | Creamy white | circular | Entire | convex | Gram negative rod | Motile |
| KDBVIII | Large/ moderate | Creamy white | Circular | lobate | umbonate | Gram positive rod | Motile |
| KDB IX | Large/ moderate | Creamy white | filamentous | filamentous | Flat | Gram negative rod | Non motile |
| KDB X | Large/ moderate | Creamy white | circular | entire | flat | Gram negative rod | Non motile |

***(Table 5.4 a) Morphological characteristics of Keratin degrading bacteria***

### 5.4.2. Biochemical Properties

Biochemical properties of different Keratin degrading bacteria were observed and recorded as follows (**Table 5.4 b)**

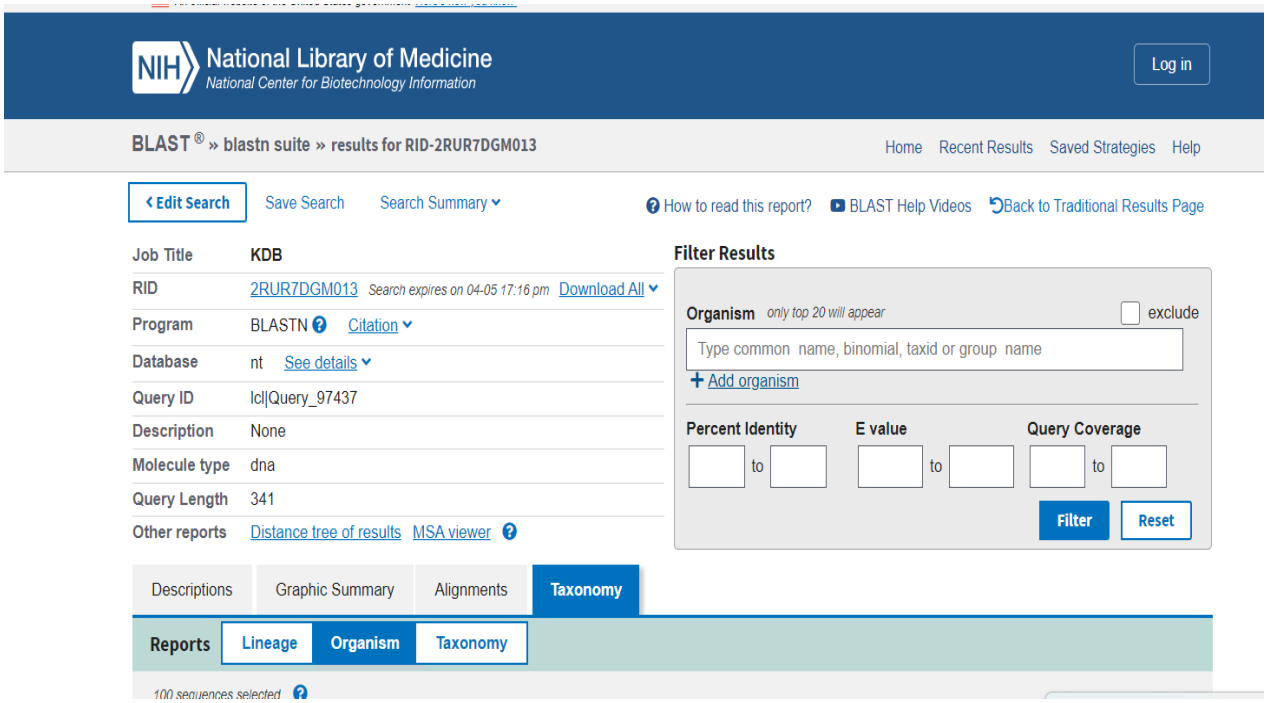
| Biochemical tests | KDB I | KDB II | KDB VI | KDB VIII | KDB IX | KDB X |
|---|---|---|---|---|---|---|
| Indole | - | - | - | - | - | - |
| Methyl Red | + | - | - | - | - | - |
| Vogues Proskauer | - | - | - | - | - | - |
| Citrate utilization | - | - | + | - | + | + |
| Nitrate reduction | + | - | - | + | + | + |
| Oxidase | + | + | - | + | + | - |
| Catalase | - | - | - | - | - | - |
| Urease | - | - | + | - | - | + |
| Glucose fermentation | A | A | A | A | AG | A |
| Lactose fermentation | A | A | A | A | AG | A |
| Sucrose fermentation | A | A | A | A | AG | A |
| Fructose fermentation | A | A | A | A | AG | A |
| Triple Sugar Iron | - | - | - | - | Gas production (no H2S) | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| ORGANISM IDENTIFIED (Using www.microrao.com) | *Bacillus paramycoides* | *E.coli(inactive ~54.69%)* | *Providencia rettgeri(~59.19%)* | *E.coli (97.88%)* | *Klebsiella pneumoniae (60.34%)* | *Providencia rettgeri (63.1%)* |

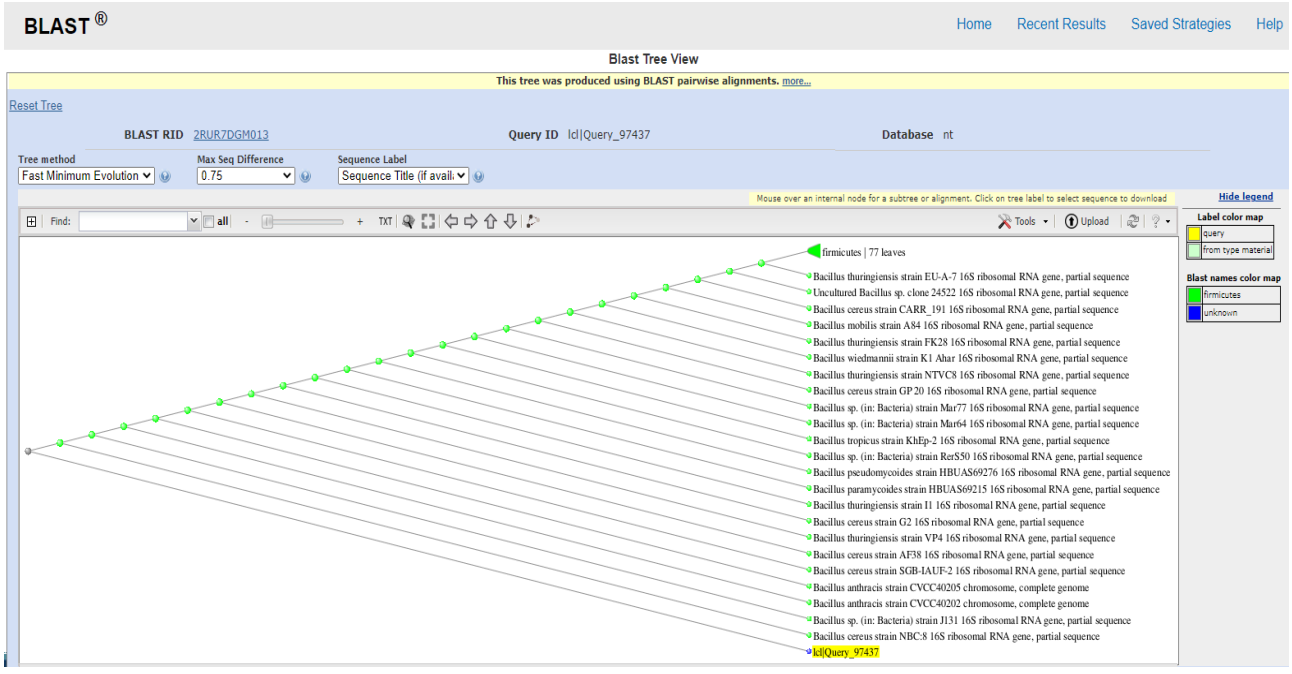
***(Table 5.4 b) Biochemical properties of different KDB isolates***

## ***5.5 Molecular Identification***

KDB I was selected as the most efficient keratin degrading bacteria among the isolates. The organism has been identified as ***Bacillus paramycoides*** strain ***RPA 11***



| Description | Score | E value | Accession |
|---|---|---|---|
| **Bacillus paramycoides [firmicutes ]** ▼Next ▲Previous ⏮First | | | |
| Bacillus paramycoides strain RPA11 16S ribosomal RNA gene, partial sequence | 630 | 4e-176 | OQ581573 |
| Bacillus paramycoides strain RDE9 16S ribosomal RNA gene, partial sequence | 630 | 4e-176 | OQ361861 |
| Bacillus paramycoides strain RDE17 16S ribosomal RNA gene, partial sequence | 630 | 4e-176 | OQ361859 |
| Bacillus paramycoides strain RDE7 16S ribosomal RNA gene, partial sequence | 630 | 4e-176 | OQ361858 |
| Bacillus paramycoides strain RDE6 16S ribosomal RNA gene, partial sequence | 630 | 4e-176 | OQ361857 |
| Bacillus paramycoides strain RDE2 16S ribosomal RNA gene, partial sequence | 630 | 4e-176 | OQ361855 |
| Bacillus paramycoides strain HBUAS69215 16S ribosomal RNA gene, partial sequence | 630 | 4e-176 | OP420614 |
| **Bacillus thuringiensis [firmicutes ]** ▼Next ▲Previous ⏮First | | | |
| Bacillus thuringiensis strain BtG4 16S ribosomal RNA gene, partial sequence | 630 | 4e-176 | OQ581574 |
| Bacillus thuringiensis strain Rhs0-L7 16S ribosomal RNA gene, partial sequence | 630 | 4e-176 | OQ581530 |
| Bacillus thuringiensis strain Rhs-L20 16S ribosomal RNA gene, partial sequence | 630 | 4e-176 | OQ581527 |
| Bacillus thuringiensis strain Rhs0-P38 16S ribosomal RNA gene, partial sequence | 630 | 4e-176 | OQ581521 |
| Bacillus thuringiensis strain Rhs-P20 16S ribosomal RNA gene, partial sequence | 630 | 4e-176 | OQ581511 |
| Bacillus thuringiensis strain B11 16S ribosomal RNA gene, partial sequence | 630 | 4e-176 | OP900044 |
| Bacillus thuringiensis strain M7 16S ribosomal RNA gene, partial sequence | 630 | 4e-176 | OP900004 |
| Bacillus thuringiensis strain OTG001 16S ribosomal RNA gene, partial sequence | 630 | 4e-176 | OP897664 |
| Bacillus thuringiensis strain CPO MNS22-Y16 16S ribosomal RNA gene, partial sequence | 630 | 4e-176 | OP897617 |
| Bacillus thuringiensis strain EU-A-7 16S ribosomal RNA gene, partial sequence | 630 | 4e-176 | OP450840 |
| Bacillus thuringiensis strain FK28 16S ribosomal RNA gene, partial sequence | 630 | 4e-176 | OP434617 |
| Bacillus thuringiensis strain NTVC8 16S ribosomal RNA gene, partial sequence | 630 | 4e-176 | OP430815 |
| Bacillus thuringiensis strain I1 16S ribosomal RNA gene, partial sequence | 630 | 4e-176 | OP420552 |




**<u>NCBI SUBMISSION</u>:** GenBank accession number for the nucleotide sequence:

SUB13027681 SR3476-KD-RSF1_C05.ab1 **<u>OQ733389</u>**

## 5.6 Other Enzymatic Activities Exhibited by Cellulose Degrading Bacterial Isolates

### *5.5.1 Amylase Production by various KDB*

Zone of clearance shown as an indication of amylolytic activity was measured for all the isolates on starch agar medium at 37°C. **(Table.5.5).** Keratin degrading bacteria degrade starch by production of **amylase** enzyme. Zone of clearance was better visualized by addition of Iodine solution on the agar plates with culture. Colony diameter as well as clear zone diameter were recorded for all the bacterial isolates and it is found out that all organisms subjected to the study are exhibiting great amylolytic activities, **KDB VI** with maximum value. Keratin degrading ability along with amylolytic activities of these microorganisms can be exploited in the bioprocess industries for the production of value-added materials from waste materials.

**Detection of amylase activity on Starch Agar medium after 24 hrs of incubation @ 37°C**

| Serial No. | Isolate Name | Colony Diameter (mm) | Zone Diameter (mm) | Ratio of Colony Diameter to Zone Diameter |
|---|---|---|---|---|
| 1 | KDB I | <2 | No zone | - |
| 2 | KDB II | <2 | No zone | - |
| 3 | KDB VI | 5 | 19 | 0.26 |
| 4 | KDBVIII | <2 | No zone | - |
| 5 | KDB IX | <2 | No zone | - |
| 6 | KDB X | 3 | 14 | 0.21 |

***(Table.5.5) Measurement of zone of clearance produced by Keratin Degrading Bacteria (KDB) on Starch agar medium at 37°C, for their amylolytic activity study***

### 5.5.2 Cellulase production by isolated strains

Zone of clearance was obtained for KDB on CMC Agar at 37ºC after 48 hrs. Based on the above experiment, it is observed that none of the bacterial isolates showed cellulase activity in the provided cultural conditions. Further studies may be conducted to optimize cultural conditions to detect the cellulase enzyme activity of keratin digesters isolated from feather dumping sites

| **Detection of cellulase activity on CMC Agar medium after 48 hrs of incubation @ 37ºC** | | | | |
|---|---|---|---|---|
| **Serial No.** | **Isolate Name** | **Colony Diameter (mm)** | **Zone Diameter (mm)** | **Ratio of Zone Diameter to Colony Diameter** |
| **1** | **KDB I** | **<1** | **No zone** | **-** |
| **2** | **KDB II** | **<1** | **No zone** | **-** |
| **3** | **KDB VI** | **<1** | **No zone** | **-** |
| **4** | **KDBVIII** | **<1** | **No zone** | **-** |
| **5** | **KDB IX** | **<1** | **No zone** | **-** |
| **6** | **KDB X** | **<1** | **No zone** | **-** |

**5.6.3 Phosphate Solubilization activity by selected bacterial strains**

In the above experiment, we have attempted cultivation of Keratin digesting bacterial isolates on NBRIP agar to check the presence of Phosphate solubilizing activity. Zone of clearance was obtained only for KDB IX on NBRIP Agar at 37ºC after 48 hrs., however variations in the cultural conditions may provide valuable information on this enzyme activity which shall be studied in the due course.

| **Detection of Phosphate Solubilization activity on NBRIP Agar after 48 hrs of incubation @ 37ºC** |
|---|

| Serial No. | Isolate Name | Colony Diameter (mm) | Zone Diameter (mm) | Ratio of Colony Diameter to Zone Diameter |
|---|---|---|---|---|
| 1 | KDB I | <1 | No zone | - |
| 2 | KDB II | <1 | No zone | - |
| 3 | KDB VI | <1 | No zone | - |
| 4 | KDBVIII | <1 | No zone | - |
| 5 | KDB IX | 1 | 2.5 | 0.4 |
| 6 | KDB X | <1 | No zone | - |

## FIGURES

**Figure 1.Sterilized feathers**

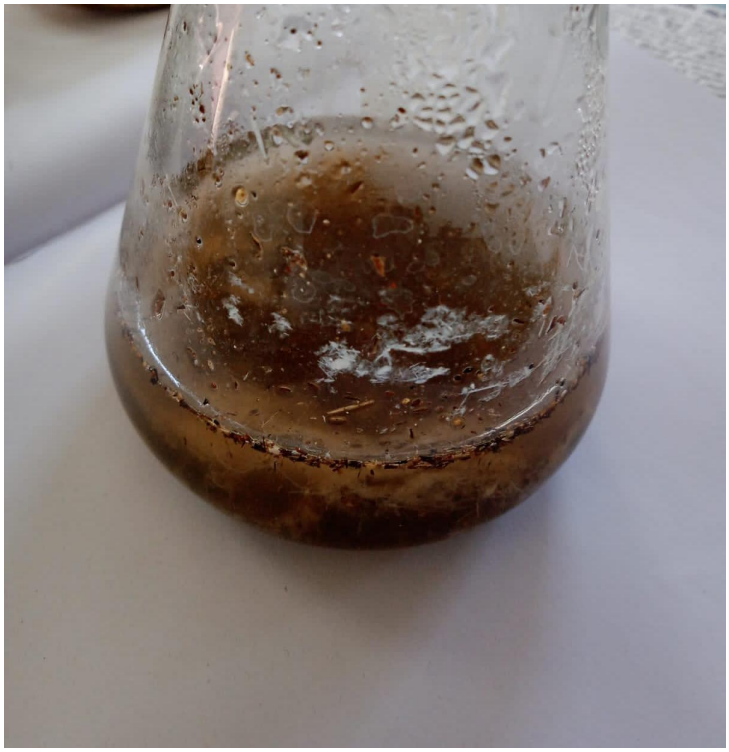


**Figure 2.Feather meal broth**

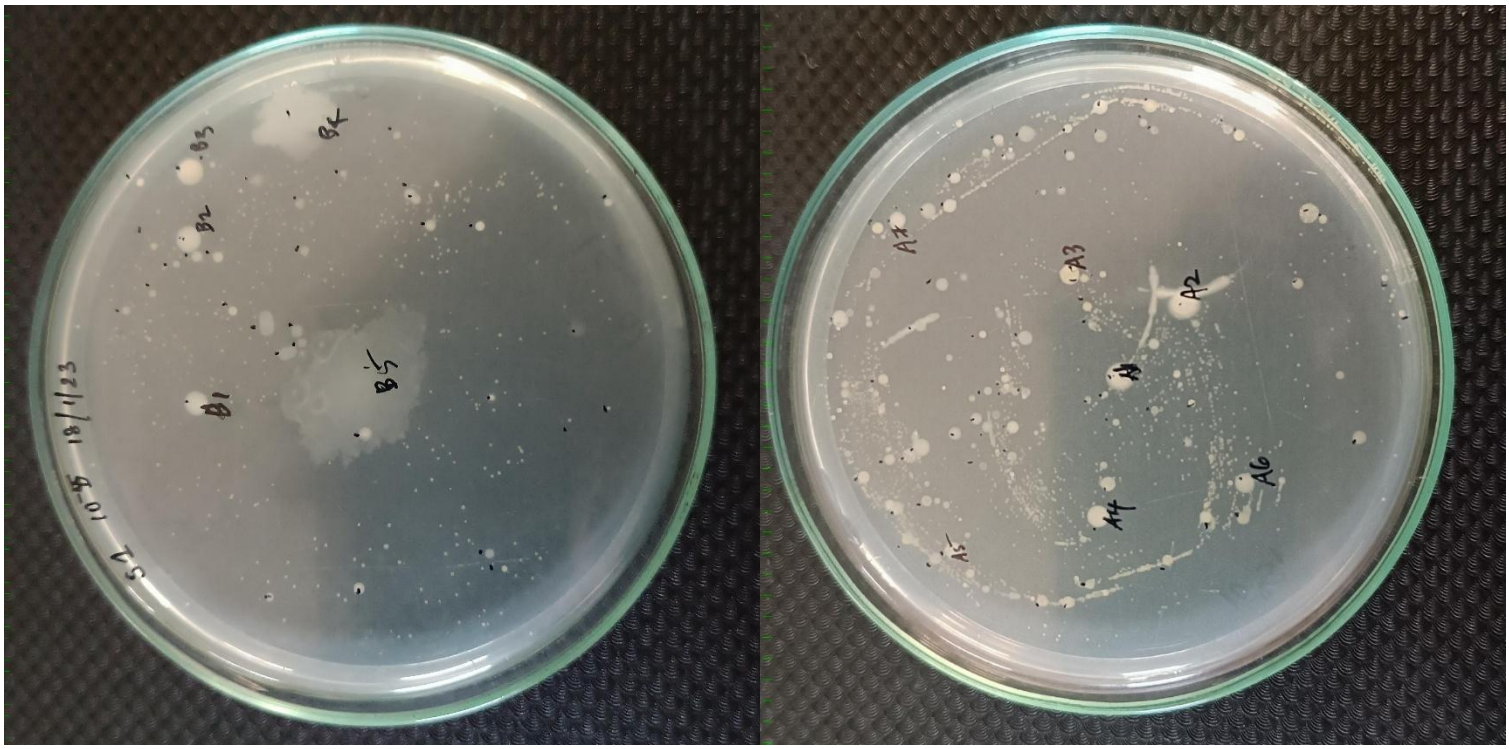


**Figure 3.Colonies formed in nutrient agar plates after enrichment with feather meal broth**

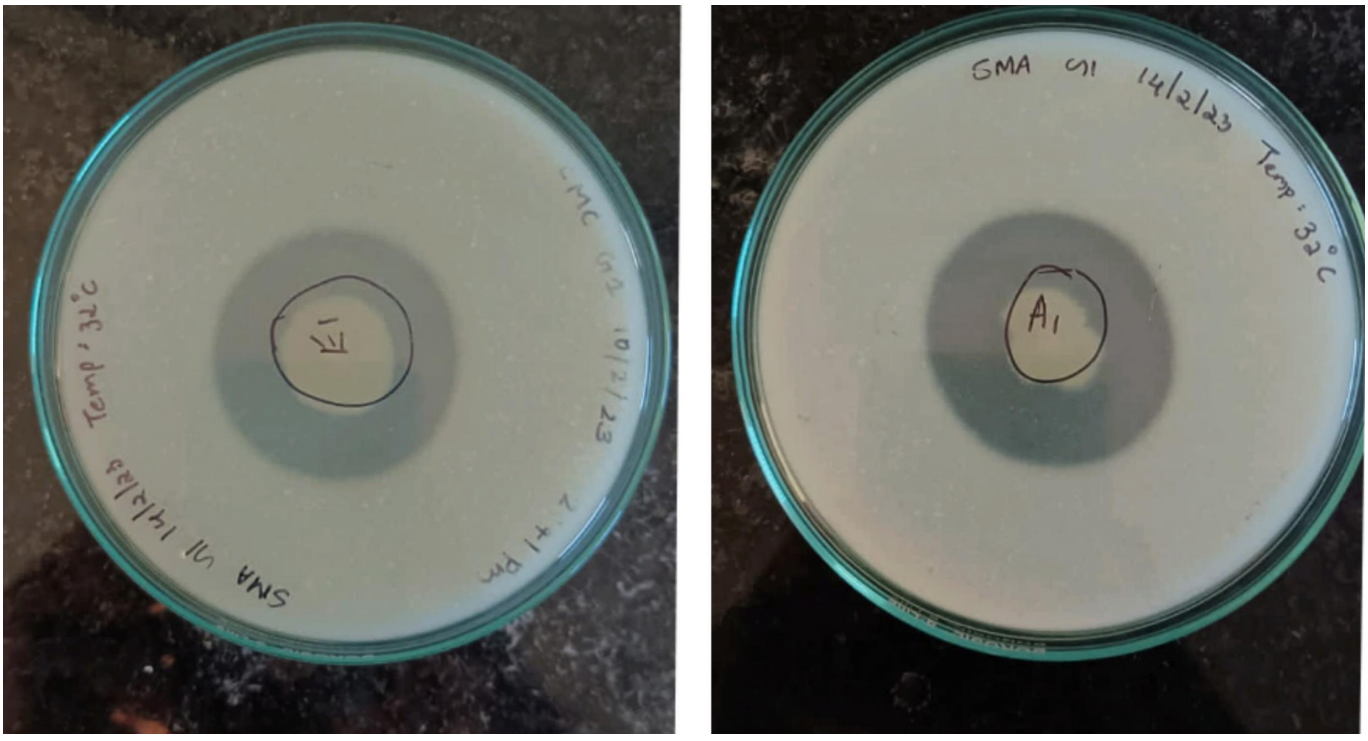


**Figure 4.Zone formation on skimmed milk agar media by KDB8 and KDB1**

**Figure 5.Indole test**

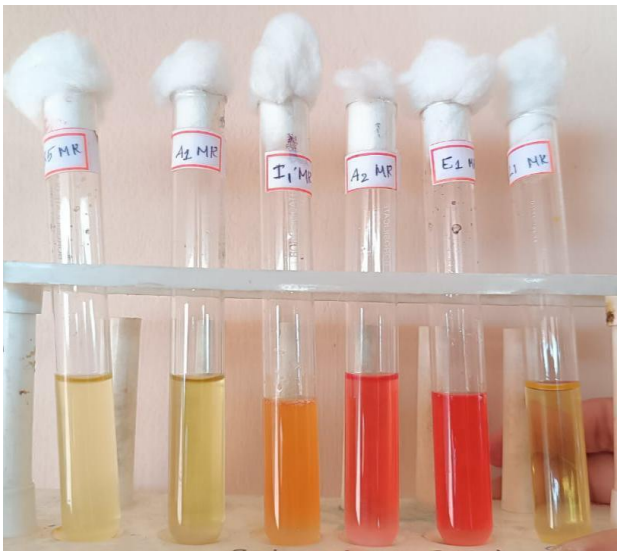


**Figure 6.Methyl red test**

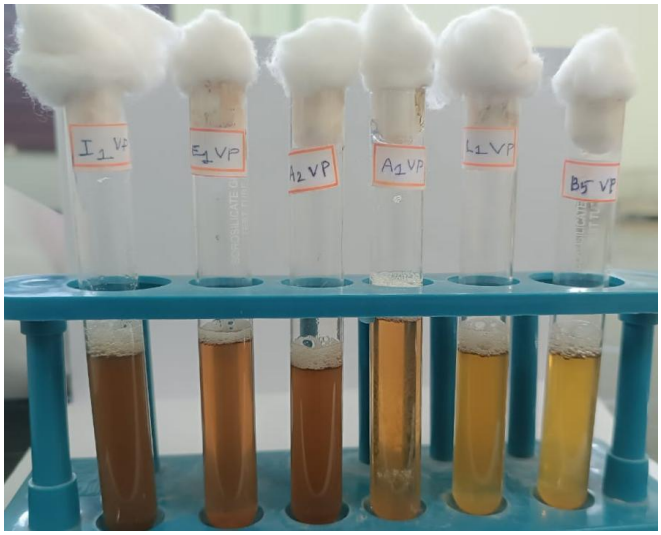


**Figure 7.Voges Proskauer test**

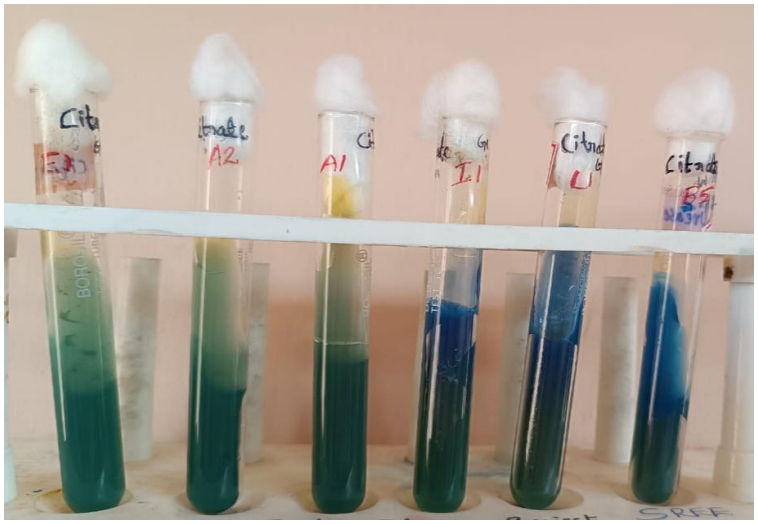


**Figure 8.Citrate utilisation test**

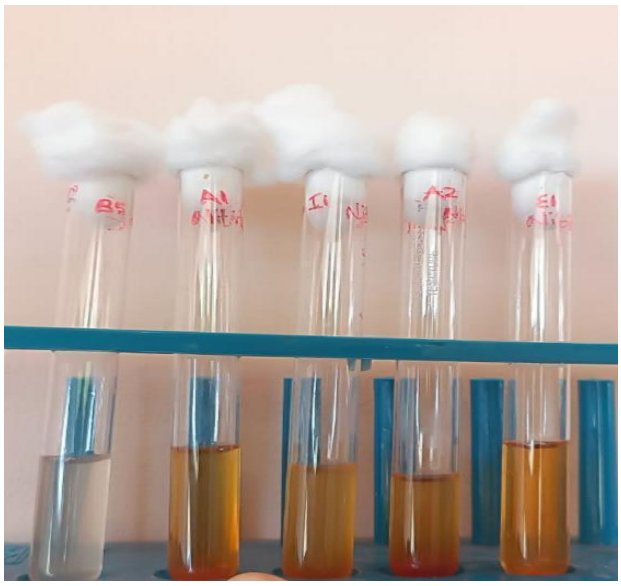


**Figure 9.Nitrate reduction test**

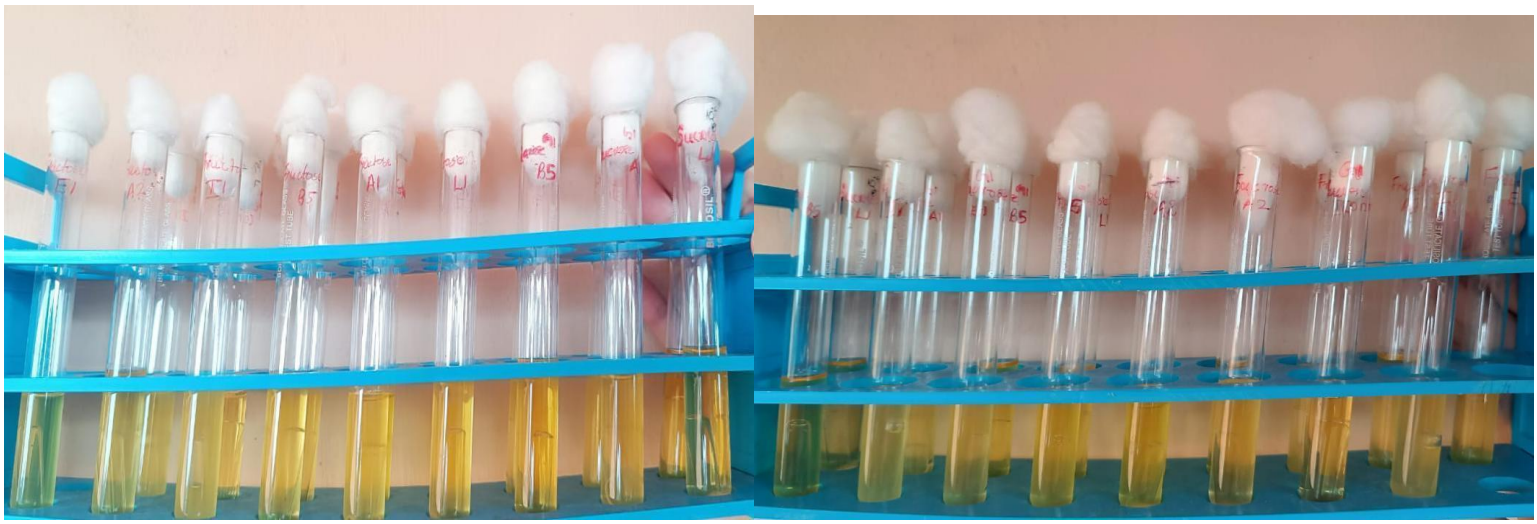


**Figure 10.Carbohydrate utilisation test**

**Figure 11.Triple sugar iron test**

## CHAPTER VI

# SUMMARY AND CONCLUSION

Keratinases are significant in hair degradation because they are enzymes that can break down the tough, fibrous protein keratin that makes up hair. Hair is composed mainly of a type of keratin known as alpha-keratin, which is particularly resistant to degradation. However, certain microorganisms such as bacteria and fungi are capable of producing keratinase enzymes that can break down this protein.

Keratinases break down hair by cleaving the disulfide bonds that hold together the individual keratin molecules, causing them to unravel and become more susceptible to further degradation. Once the keratin molecules have been broken down into smaller fragments, other enzymes can act on them to break them down into their constituent amino acids.

Hair degradation is important in various applications, such as waste management, animal feed production, and the production of high-value compounds such as amino acids and peptides. Keratinases have been shown to be effective in breaking down hair in all of these applications, making them a valuable tool in the processing of keratin-containing materials.

In summary, keratinases are significant in hair degradation because they are capable of breaking down the tough, fibrous protein keratin that makes up hair. This ability has important applications in various fields, making keratinases a valuable resource for the processing of keratin-containing materials. In the present study, we have aimed at isolating keratin digesting bacteria from various local sources of feather dumping area and we have identified the potential isolates belong to *Bacillus sp.* Studies shall be further elaborated on the mass production of keratinase enzymes from potent sources as well as their molecular characterization for industrial applications. In many of the recent studies, researchers consider Keratinase as an emerging green tool in bioremediation because of its proteolytic activities. Biowaste degradation, being a major challenge in the present scenario where the immense potential of keratinase enzymes could be exploited individually or along with other degradative enzymes for accelerated degradation processes, thereby addressing the prevailing issues of municipal solid waste management.

## CHAPTER VII

# REFERENCES


- Alahyaribeiki, S., Sharifu, S.D., Tahanden,F., Honarbakhsh, S. and Ghazanfari, S(**2020)** "Bioconversion of chicken feather waste by keratinolytic bacteria ", *Procon Safety and Environmental protection.*
- Almahasweer,A.A., Mahmoud,A. and Borgio,J.F "Novel feather degrading keratinase from Bacillus cereus Group, Biochemical Genetic and Bio informatics Analysis " *Multidisciplinary Digital Publishing Institute (MDPI)https://pubmed.ncbi.nlm.nih.gov/35056542/*
- Arokiyaraj, S., R, Varghese, A. Ahmed, V. Duraipandiyan, and N. Al-Dhabi, (**2019**) "Optimizing the fermentation conditions and enhanced production of keratinase from Bacillus cereus isolated from halophilic environment" Saudi J. Biol. Sci., 26(2): 3
- Bergey's Manual of Systematic Bacteriology, Vol 3
- Cladera-Olivera, F. & Brandelli, A (**2008) "**Use of poultry byproduct for production of keratinolytic enzymes " *Food Bioproc. Tech.*
- Godbole,S., Pattan,J., Gaikwad,S. and Jha,T **(2017)** "Isolation, identification and characterization of keratin degrading microorganisms from poultry soil and their feather degradation potential ", *International journal of Environment ,Agriculture and Biotechnology.*
- Gradisar, H., Friedrich, J., Krizaj I. & Jerala, R (**2005)** "Similarities and Specificities of Fungal Keratinolytic Proteases: Comparison of Keratinases of Paecilomyces marquandii and Doratomyces microsporus to Some Known Proteases" Appl. *Environ. Microbiol.,* 71: 3420-3426.
- Hassan, M., A., Abol-Fotouh, D., Omer, A., M., Tamer, T., M., and Abbas., E. **(2020)** "Comprehensive insights into microbial keratinases and their implication in various biotechnological and industrial sectors:" A review, *International Journal of Biological Macromolecules*, Volume 154, Pages 567-583, ISSN 0141-8130, https://doi.org/10.1016/j.ijbiomac.2020.03.116.
- http://www.tgw1916.net/Pseudomonas/alcaligenes.html for Bacterial Identification
- Jeevana,L.P., Kumari,C.M. and Lakshmi,V.V **(2013)** "Efficient degradation of feather by keratinase producing *Bacillus* sp", *International Journal of microbiology.* https://doi.org/10.1155/2013/608321

- Suntornsuk, W., Tongjun, J,. Onnim, P., Oyama, H.,Ratanatanokchai,K., Kusamran, T & Oda, K (**2005) "**Purification and Characterization of Keratinase from a Thermotolerant Feather degrading bacteria ",*World J. Microbial Biotechnol.*
- Sahoo, D., Das, A.,Thatoi,H.,Mondal, K. and Mohapatra, P (**2012)"**Keratin production and biodegenvironment whole chicken feather keratin by a newly isolated bacterium under submerged fermentation ", *App. Biochem Biotechnol*
- Monilia,W.S., Dada,M.T. and Onilude,A.A **(2011)** "Isolation and characterization of keratinase producing bacteria from poultry waste ", *Journal of pure and applied microbiology.*
- Naga Raju, E.V. and Divakar,G (**2013)** "Screening and isolation of keratinase producing Bacteria from poultry waste", *International journal of pharmaceutical Research and Allied Science (IJPRAS).*
- Online bacterial Identification software (www.microrao.com) http://www.microrao.com/identify.htm
- Peng, Z., Mao, X., Zherny, J & Janchen, G. D. (**2019)** " Efficient biodegradation of chicken feather waste by co-cultivation of keratinase producing strains. *Microb Cell Fact* 18, 84 (2019). https://doi.org/10.1186/s12934-019-1134-9
- Ramnani, P., Singh, R. & Gupta, R (**2005) "**Keratinolytic potential of Bacillus licheniformis RG1: structural and biochemical mechanism of feather degradation", *Can. J. Microbio.* https://doi.org/10.1139/w04-123
- Ratanakanokchai, K., Kusamran, T. & Oda, Riffel, A. & Brandelli, A(**2006) "** Keratinolytic bacteria isolated from feather waste" *Braz. J.*
- Sousa, M., Souza, O., Maciel, M., Cruz, R., Rêgo, M. G., Magalhães, O. & Motta, C. S., (**2015)** "Keratinolytic potential of fungi isolated from soil preserved at the Micoteca URM" *Eur. J. Biotechnol. Biosci.*, 3: 10-15.
- Suntornsuk, W., Tongjun, J., Onnim, P., Oyama, H., Vanaki,P., Zaboli,F., Kaboosi,H., Izadi,AB. and Savadkooni,F, "Isolation and identification of keratinolytic probiotic Bacillus licheniformis bacteria from the soil below Slaughter house waste", *Brazilian journal of Biology.*
- Winter,H., Langbein,L., Praetzel,S., Jacobs,M.,Rogers, M.A.,Schweizer, J., Leigh, I. M & Tidman, N (**1998) "**A novel human type II cytokeratin, K6hf, specifically expressed in the companion layer of the hair follicle. *J Invest Dermatol. 1998;111:955–962. https://www.sciencedirect.com/science/article/pii/S0022202X15403033#bb0055Gu*

- Gu LH, Coulombe PA (**2007)** "Keratin function in skin epithelia: a broadening palette with surprising shades" *Curr Opin Cell Biol. 2007;19:13–23.* *https://www.sciencedirect.com/science/article/abs/pii/S0955067406001888?via%3Dihub*