# ISOLATION, IDENTIFICATION, AND CHARACTERIZATION OF KERATINASE ENZYME PRODUCING BACTERIA, COLLECTED FROM TANNERY SOIL

Submitted by

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Bachelor of Science in Biotechnology

Department of Mathematics and Natural Sciences
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August, 2022

## Declaration

It is hereby declared that, the thesis submitted is our own original work while completing degree at Brac University. This does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution. We have acknowledged all main sources of help.

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## Approval

The thesis/project titled "Isolation, Identification, and Characterization of Keratinase Enzyme Producing Bacteria, collected from Tannery Soil" is submitted by Iritra Siddiqua (18136005) and Md. Izhar Tahmid (18136080) of Fall 2022 has been accepted as satisfactory in
partial fulfillment of the requirement for the degree of Bachelor of Science in Microbiology.

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## Ethics Statement

No animals, birds or human being were harmed while conducting this experiment. This research is purely based on the microbial production of keratinase enzyme.

## Dedication

We dedicate our work solely for the sake of humanity. We pledge our hard work to our parents, faculty members, our brothers and sisters, who believe and have the potential to make the world better for the future.

## Acknowledgement

To begin with, we offer our heartiest gratitude to the Almighty for giving us the strength for successfully completing our thesis.

We would like to express our most humble recognition to Professor A F M Yusuf Haider, PhD, Chairperson, Department of Mathematics and Natural Sciences and all faculty members of Brac University's Mathematics and Natural Science Department for equipping us with the resources required for conducting this project.

We are immeasurably thankful to our supervisor, Akash Ahmed (Senior Lecturer, Department of Mathematics and Natural Science) for his support, guidanceand cooperation. Additionally, our sincere appreciation goes to our supervisor for sharing his experience and knowledge with the course ofthe thesis.

We would like to show our sincere gratefulness to Md.Mahmudul Hasan (Lab Officer), Shamim Akhter Chowdhury (Lab Officer) and Asma Binte Afzal (Lab Officer) for providing usthe necessary materials and equipments during the thesis work.

Moreover, we would like to extendour special thanks to Nabila Khan (Teaching Assistant), Nishat Tasnim Anonna (Student) to direct us, support us, encourage us and assist us to conduct our thesis whenever we needed. We are obliged to use all the necessary machinery and vital materials to complete the thesis in thelaboratory of Biotechnology in Brac University. Also, we would like to deliver our respect and gratitude towards ourparents for their support, love and proper guidance.

This report's perfect completion of the thesis work was impacted by the guidance and support of these valued persons. We see this chance as a major step forward for our professional development.
We will undoubtedly strive to put the knowledge and expertise we have gained to the best use in the future.

**Iritra Siddiqua**

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August, 2022

## Abstract

Keratin rich waste by-products from animal skin and other parts are being produced every year in a huge amount. These wastes are required to be degraded. Otherwise, it can become a hazardous to the environment. This study focuses to find potential bacteria from the soil sample of leather manufacturing industry located at Hazaribagh of Dhaka City, which can produce keratinase enzyme to degrade the keratin wastes. 16 individual colonies of bacteria were selected to observe the keratinolytic activity of those bacteria. Those bacteria were inoculated in minimal salt media and tested with chicken feathers onto it and results were observed with the changes in the smoothness and degradation of the feather. Only 8 bacterial colonies showed changes after keeping the solution in rotatory shaker incubator. Changes in the feather were assumed to be keratin degradation. To verify the degradation of keratin, the absorbance of the optical density of keratinolytic assay was observed of those bacteria. Different biochemical tests were done for the identification of the bacteria and the process was done with different temperature and pH separately for optimization of the experiment. Finally, extraction of DNA of those bacteria was done, along with the PCR (Polymerase Chain Reaction) to get the potential bacteria for further research.

**Keywords:** Keratin; Minimal salt media; Keratinolytic assay; Soil sample; Biochemical tests; *Bacillus licheniformis, Bacillus cereus, Bacillus carboniphilus, Serratia marcescens.*

## Table of Contents

## List of Tables

## List of Figures

## List of Acronyms

| | |
|---|---|
| **pI/pKi** | Isoelectric Point |
| **kDa** | Kilo Dalton |
| **MW** | Molecular Weight |
| **KFAP** | Keratin Filament Associated Protein |
| **OD** | Optical Density |
| **MSM** | Minimal Salt Media |
| **TSI** | Triple Sugar Iron |
| **MR** | Methyl Red |
| **VP** | Voges Proskauer |
| **MIU** | Motility Indole Urease |
| **LB** | Luria Bertani |
| **PCR** | Polymerase Chain Reaction |

# Chapter 1

# INTRODUCTION

# Chapter 1

## 1.0Introduction

Feathers are formed of a protein known as beta-keratin on a microscopic level. The same protein is responsible for avian beaks and nails, as well as reptile scales and shells. Its near (but less stiff) relative, alpha-keratin, is found in animals' nails, claws, and hair. Humans rely heavily on chicken for protein. Currently, the global output of chicken is at 96 million tons per year. Poultry meat consumption has reached a global average of about 14 kilograms per person annually. Feathers account for between 5% and 7% of a chicken's total weight; hence, roughly 6 million tons of feathers are generated as a byproduct of the poultry business. It is difficult to dispose of or process this by-product. Keratin (80-90% of feather) is the primary component. Keratins are proteins that have a high cysteine content that is cross-linked by disulfide bonds. These linkages render keratins mechanically stable and immune to enzymatic lysis by prominent peptidases such as trypsin, pepsin, and papain. Aside from the cysteine content, keratin resistance is connected with its compact structure, such as α-helices (α-keratins) or β-sheets (β-keratins). This lysis resistance has been a significant barrier in the processing of keratin. Furthermore, the management of feather waste has serious environmental consequences too. (Mazotto et al., 2017)

**Figure 1**: Origin of keratin

Keratin is also known to be the family of scleroproteins. The alpha-keratins are mainly found in vertebrates as it is the infrastructure of nails, hairs, scales, horns, feathers, hooves, claws and also the outer layer of the skin of the vertebrates. Keratins do not dissolve in water and it is only organic solvent. It also helps in protecting the epithelial cells from getting damaged. The monomers of keratin assemble in such a way that it forms a strong intermediate filament

which is found among mammals, reptiles, amphibians and birds. There are many kinds of keratin found in human body, 54 kinds to be specific.

Among these 54 kinds, it is divided into 2 types.

1) **Type 1:** Among the 54 kinds, 28 kinds reside in type 1. In these 28 kinds, 17 are skin cell keratins and the rest of 11 kinds are hair keratins. These keratins are mostly low weight proteins and acidic.
2) **Type 2:** The remaining 26 kinds of keratins belong to the second type. 6 among these kinds are hair keratin and the rest of 20 are cell keratins.

Moreover, keratins are found in two different types of forms, one is alpha-keratin and the other is beta-keratin. Mostly type 1 and type 2 are alpha-keratins and beta-keratins are found in birds and reptiles.Keratins mostly play role in protecting and helping the human body as the amount of keratin signifies the overall health of our hair, nails and skins. ("Keratin: Protein, Structure, Benefits, Uses & Risks", 2022)

Keratins are used as a treatment of hair also, which is known as Keratin treatment. Keratin softens the cells that overlap to produce hair strands, resulting in reduced frizz and more manageable hair. This results in hair that dries frizz-free and has a shiny, healthy appearance. Moreover, Keratin treatments can last up to 6 months if proper care is followed, which includes your hair not being washed too often (2 to 3 times per week). Besides, Keratin may thicken and reinforce hair, preventing it from breaking off readily. Because the ends aren't breaking off, the hair seems to grow faster.

**Figure 2**: Before and After Keratin Treatment of Hair

However, many (but certainly not all) keratin treatments include formaldehyde, which is hazardous if breathed. Formaldehyde is what makes the hair appear straighter. According to an examination conducted by the Environmental Working Group, some firms would deliberately try to cover up the fact that their keratin solution includes the toxin. Furthermore, each treatment might cost between $300 and $800, with tip. There are cheaper expensive at-home choices, but the outcomes will not be as long-lasting. Some people may find it more difficult to maintain if they wash their hair less frequently and avoid swimming.("Keratin Hair Treatment Pros and Cons", 2022)

If we consider the molecular formation of keratin, it is sometimes misinterpreted as a single material, despite being made up of a complex combination of proteins such as keratins, KFAPs, and enzymes derived from epithelia. Keratins are exclusively present in epithelial cells and have distinct physicochemical features. They are insoluble in dilute acids, alkali, water, and organic solvents and are unable to be digested by the protease's pepsin and trypsin. Keratins are insoluble in aqueous salt solutions, but are permeable in solutions containing denaturing agents like urea. Keratins may rebuild intermediate filaments in aqueous solution. Keratins are categorized based on their molecular composition, physicochemical properties, the epithelial cells that produce them, and the epithelial type that contains the keratin-producing cells. It is worth mentioning that the electric charge, size, and immunoreactivities of keratins generated by human and cattle tissues are highly comparable. Furthermore, the molecular quantities of histidine, lysine, and arginine in keratins of hard-cornified tissue and tooth enamel are surprisingly stable. (Bragulla&Homberger, 2009) Keratins may be removed from different tissues using reducing chemicals that break disulfide bonds, such as thioglycolate, dithiothreitol, or mercaptoethanol. Keratin nomenclature is based on standards established by the Human and Mouse Genome Nomenclature Committees and is an adaption of many prior keratin nomenclatures.(Bragulla&Homberger, 2009)

The physical and chemical properties of keratins inside epithelial cells and tissues are determined by their physicochemical qualities that is the Molecular Weight (MW) in kDa and isoelectric point (pI) in diverse tissues and species. To determine their physicochemical properties, keratins must first be extracted from epithelial cells utilizing urea solvents (at a specific pH and concentration) and reducing agents (like, mercaptoethanol or dithiothreitol) to split the disulfide bonds that connect these keratins to one another and Keratin Filament-Associated Proteins (KFPA). These soluble forms of keratins are subsequently sorted by MW and pI utilizing 1- and 2-dimensional gel electrophoresis. Differences in the MW and pI of

orthologous keratin proteins in different species are due to minor differences in keratin genes, post-transcriptional processing of mRNA (Messenger RNA), post-translational processing of the protein, or differences in the amount of phosphorylated or glycosylated amino acid residues. (Eichner, Bonitz& Sun, 1984).In mammals, the MW of keratins ranges from 40 to 70 kDa. Separating and comparing human and bovine keratins using two-dimensional gel electrophoresis and immunoblotting revealed that while certain keratins in homologous tissues had the same MW, others did not. Keratins are classified based on their pI. The range of pH at which the proteins are neutral distinguishes type I keratins from type II keratins. This pH is known as the pI and can be shortened as pI or pKi. Type I keratins have a pI of 4.9-5.4 in humans, whereas type II keratins have a pI of 6.5-8.5. Type I keratins in bovines have a pI of 5.6, whereas type II keratins have a pI greater than 6.0. Keratins with a pI of 4.7-5.4 are unique to hair, nails, or wool. Keratins' pI can be altered by post-translational alterations to their amino acids.(Bragulla&Homberger, 2009)

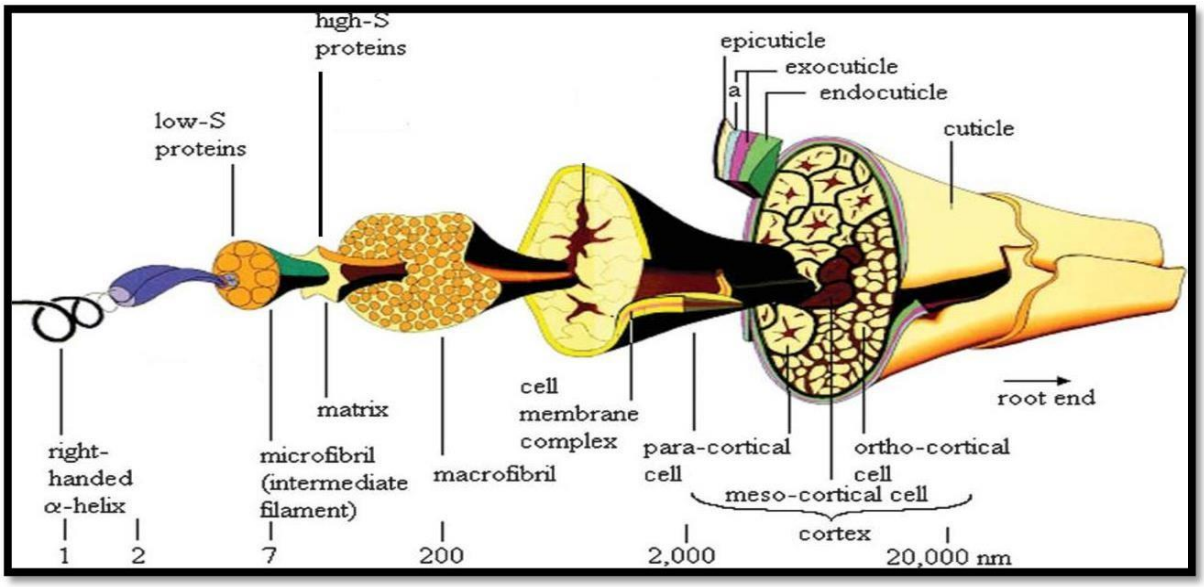



**Figure 3**: Structure of keratin

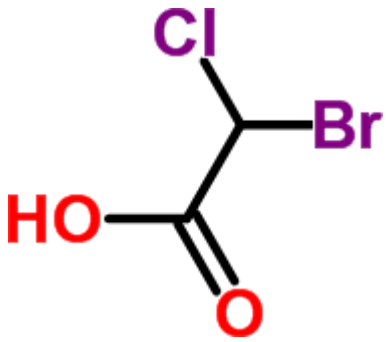



**Figure 4**: Molecular structure of keratin

In this study, with the help of different biochemical tests and processes, keratinase enzyme degrading bacteria are being found with which keratin treatment and other use of keratin can be done without artificial production of keratin rather with keratins produced from living beings. By studying these bacteria which can produce keratinase enzyme, it will be possible to extract the keratinase and use it for various purpose. Bacillus stains are the most common bacterium capable of producing keratinase. *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, and *Bacillus cereus* are among the species. Other bacteria are capable of degrading feathers with high effectiveness. Keratin-degrading microorganisms can be isolated from human and animal tissues, as the keratinase generated may be vital for fungal infection.Streptomyces is the most common actinobacterium capable of producing keratinase. Several studies have demonstrated that keratinolytic actinobacteria may be isolated from a variety of environments. Some actinobacteria can manufacture thermally stable keratinase, which have a high potential for usage in industry. Despite the fact that numerous keratinase producers have indeed been separated and characterized, keratinase-producing microbe isolation and characterization remains a crucial challenge.(Li, 2021)

Table 1. Bacterial and fungal keratinolytic enzymes.

| Microorganism | Enzyme | Substrates | Temp. (°C) | pH | Unit | Ref. |
|---|---|---|---|---|---|---|
| *Bacillus licheniformis* PWD1 | PE | Azokeratin | 50 | 7.5 | $\Delta A_{450} = 0.01$ | [25] |
| *Fervidobacterium pennavorans* | PE | Native feather meal | 80 | 9.0 | The residual dry weight of the remaining feather meal substrate | [21] |
| *Streptomyces* sp. $S.K_{1-02}$ | EE | Keratin azure | 50 | 8.5 | $\Delta A_{595} = 0.1$ | [26] |
| *B. subtilis* KS-1 | EE | Azokeratin | 30 | 7.5 | $\Delta A_{450} = 0.001$ | [40] |
| *Thermoanaerobacter keratinophilus* | CE | feather meal | 70 | 7.0 | 1 μmol of aromatic amino acids | [41] |
| *Stenotrophomonas* sp. D1. | EE | keratin powder | 30 | 8.0 | $\Delta A_{660} = 0.01$ | [42] |
| *Chryseobacterium* sp. kr6 | CE | Azokeratin | 50 | 8.0 | $\Delta A_{440} = 0.01$ | [43] |
| *Microbacterium arborescens* kr 10 | PE | Azokeratin | 45 | 7.5 | $\Delta A_{420} = 0.01$ | [44] |
| *B. subtilis* S 14 | CE | Azokeratin | 24 | 9.0 | $\Delta A_{450} = 0.1$ | [45] |
| *B. subtilis* NRC 3 | PE | aazokeratin | 50 | 7.5 | $\Delta A_{450} = 0.01$ | [46] |
| *Actinomadura keratinilytica* Cpt29 | PE | keratin azure | 70 | 10 | $\Delta A_{440} = 0.1$ | [47] |
| *B. safensis* LAU 13 | EE | feather powder | 40 | 7.5 | $\Delta A_{280} = 0.01$ | [48] |
| *B. pumilus* AT16 | PE* | azokeratin | 55 | 7.5 | $\Delta A_{450} = 0.01$ | [49] |
| *Actinomadura viridilutea* DZ50 | PE | keratin azure | 80 | 11 | $\Delta A_{440} = 0.01$ | [50] |
| *Thermoactinomyces* sp. RM4 | EE | keratin azure | 60 | 10.0 | $\Delta A_{595} = 0.01$ | [51] |
| *B. subtilis* DP1 | PE | chicken feather | 37 | 10.0 | increases absorbance by 0.1 | [52] |
| *Caldicoprobacter algeriensis* | PE | keratin azure | 50 | 7 | $\Delta A_{595} = 0.01$ | [53] |

PE, purified enzyme
CE, crude extract
EE, extracellular enzyme
*. recombinant

**Figure 5**: Microorganisms that shows keratinolytic enzyme activities .(Jin et al., 2017).

These bacteria, as shown above (**Figure 5**) show significant amount of keratinolytic enzyme activities at a certain temperature and pH.(Jin et al., 2017).These bacteria which can produce keratin enzymes can be used for various purpose such as, keratin treatment, keratin waste degradation, industrial production health care products and cosmetics, etc.

This study helps to identify bacteria from soil sample that can produce keratin enzyme and isolate it through different process and optimize the keratin enzyme to break the disulphide bond of the keratin found in hair. Through this process, curly hairs can be converted into silky hair and industrial production of it will be feasible as it is done with the help of bacteria. Since the stronger disulphide bonds of keratin in hair is the reason behind the formation of curly hair, by breaking the bonds through keratinase enzyme produced by bacteria itself will make the whole process cheaper, easier, hygienic (as it is not chemically produced rather from a living organism) and environment friendly. Industrial production of it can also be done after going through human trials and seeing the results. The study shows the amount of keratinolytic activity found from those keratin enzymes producing bacteria and the bacteria which gives the highest value of enzymatic activity.

Besides, the amount of keratin waste has increased a lot globally (like feather, skin, hooves and claws of different animals). In this case, these bacteria that can produce keratinase enzyme can help to degrade those keratin containing feathers and other parts of the animals that contain keratin and get rid of the garbage waste problem for many countries of this world. The main focus of this study was to isolate, characterize and identify keratinolytic bacteria from soil samples of leather producing industry that can degrade keratin of hair and other parts of animals.

# Chapter 2
# Materials and Methods

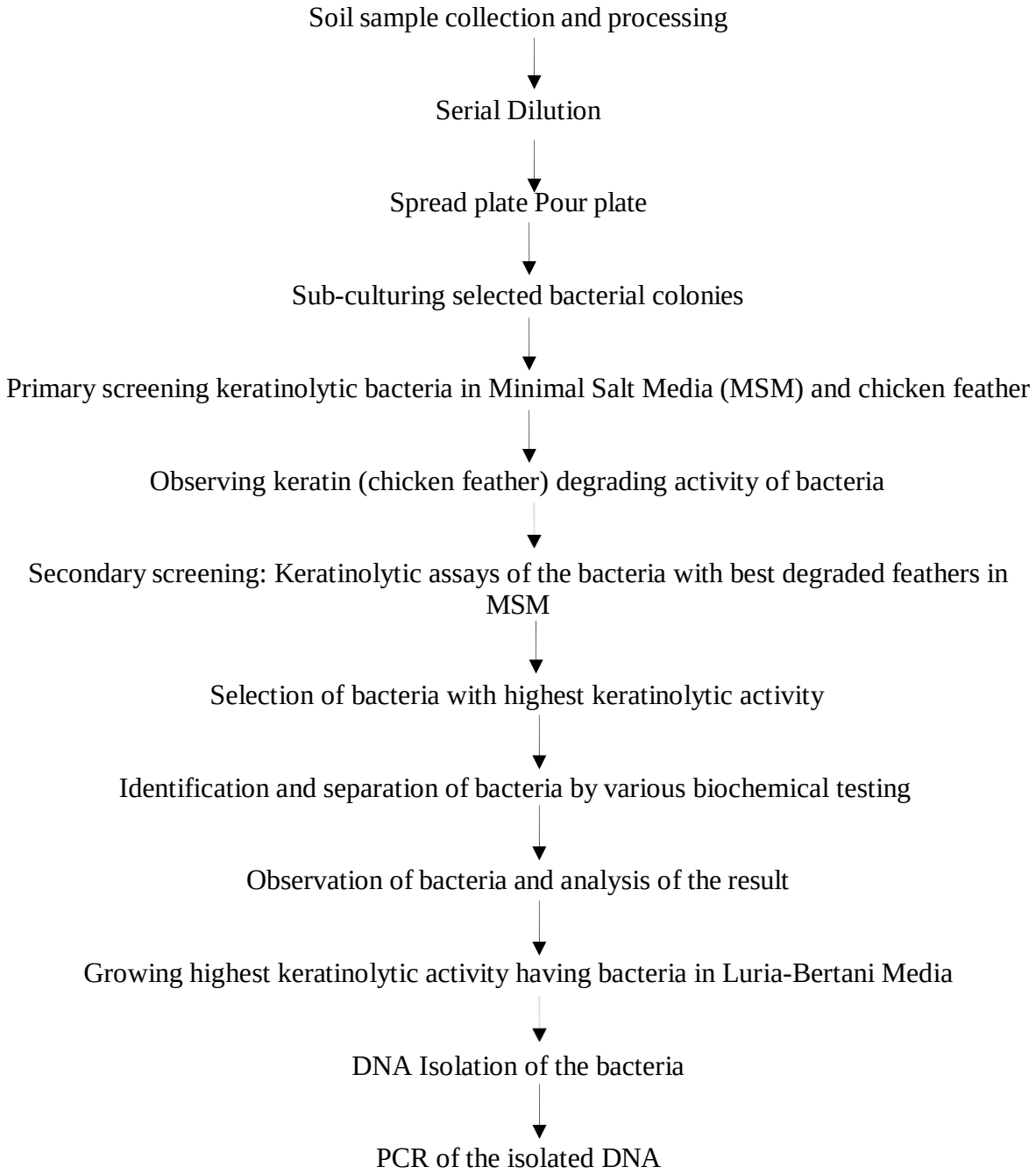
# Chapter 2

## Materials and Methods

### 2.1 Place of the research:

This research has been conducted in the Biotechnology and Microbiology laboratory in BRAC University, under the Department of Mathematics and Natural Sciences.

### 2.2 A brief illustration of the research conducted shown in a flowchart:



## 2.3 Sterilization and cleaning all necessary equipment's for the research:

Necessary equipment's like, sample collection plastic bags, Falcon tubes, Test tubes, Conical flasks, Beakers, Petri dishes, Spatulas, Glass pipettes, Micropipette tips, Eppendorf, and PCR tubes were cleaned with liquid dish cleaner and were sterilized by autoclaving for 2 hours at 121℃, except for the plastic zip lock bags, as those might melt and get clumped inside the autoclave machine.

## 2.4 Sample collection:

From Hazaribagh, Dhaka, various locations of skin processing and dumping zones were mainly selected for collection of soil samples. Since, the skin and fur or animals have keratin and the soil in that locations are composed of numerous bacteria that have or gained the ability to degrade keratin due to frequent dumping of skin and fur residues there. For this, soil samples were collected and placed in clean plastic zip lock sample collection bags.

**Figure 6**: Sample collection zone Hazaribagh Tannery, Dhaka

A total of four soil samples were collected using clean spatula and gloves at very early morning of February 10, 2022, since the processed skins were just removed from the selected areas during that particular day. Two of the samples were collected from slightly wet zone near sewage and the other two were collected from skin processing zones and skin dumping zones of the Tannery. Moreover that, each of the

specimen were placed in separate plastic bags, otherwise, each can get contaminated by the other.

### 2.5 Collected sample processing:

On the very same day, 10th of February, 2022, the collected four soil samples were brought to the laboratory for further processing and analysis. From each soil samples, 1 gram was measured in a sample weighting machine, placing an aluminum foil on the scale. For each case an individual aluminum foil piece was used. Each 1 gram of soil from the four samples were suspended in 9 ml of sterile saline (autoclaved and cooled down few hours earlier) in a 10 ml falcon tube. The total solution for each sample was prepared as a 10 ml solution and labeled as Sample 1, Sample 2, Sample 3, and Sample 4 respectively.


**Figure 7**: Serial Dilution of Samples

### 2.6 Screening and selecting bacterial colony:

The four falcon tubes with four different soil samples were serially diluted from 10¯1 to 10¯6 and each of the 24 tubes were labeled properly and carefully. In each test tube 9 ml of saline was taken with sterile glass pipette and 1 ml from four stock solution of soil was taken using 1ml micropipette in the first four test tubes labeled as 10¯1. Each of the four samples was diluted up to 10 ¯6 fold specifically in 20 other test tubes. The last four test tube (10¯6) of each sample contained 10 ml of solution and the remaining contained 9 ml as per the dilution.

Nutrient agar was prepared, autoclaved and plated in Petri dishes and the suspensions were cultured by spread plate method in nutrient agar (NA). In each plate, 100µl from each test tube were inoculated and the plates were labeled accurately for each specimen. The prepared media was incubated in an incubator for 48 hours at 37°C. A total of 24 Petri plates were cultured for the four samples, each sample having 6 plates labeled from $10^{-}1$ to $10^{-}6$.

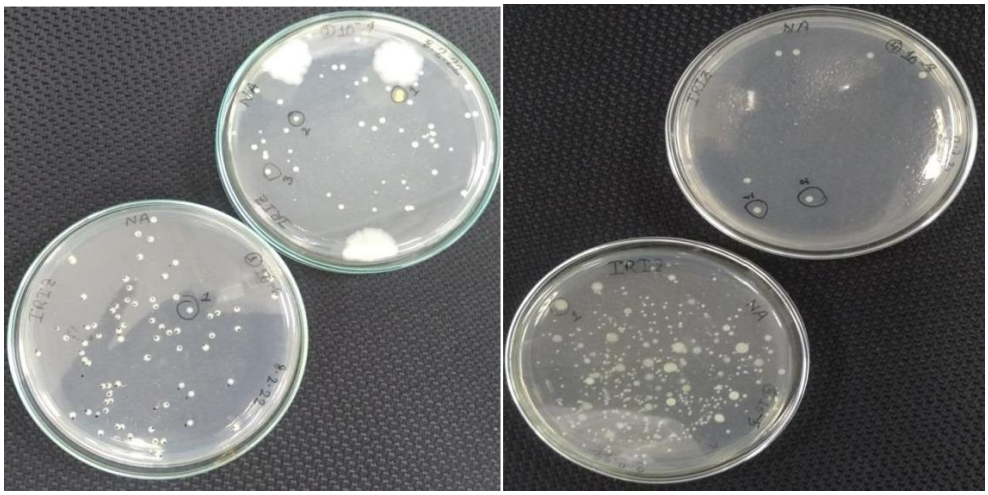


**Figure 8**: Selected bacterial colonies cultured in Nutrient Agar plates

After 48 hours, the cultured plates were found to have bacterial growth. The plates with $10^{-}1$, $10^{-}2$ and $10^{-}3$ suspensions were found to have excessive bacterial colonies; however, $10^{-}4$, $10^{-}5$ and $10^{-}6$ had countable clear different colonies of bacteria. From Sample 1, 2, 3, and 4, among countable colonies, 4, 4, 5, and 3 colonies were selected respectively from the mentioned samples. A total 16 colonies of bacteria were selected for further screening of keratinolytic activity.

### 2.7 Subculture of the selected colonies:

The 16 selected colonies were sub-cultured from time to time in nutrient agar media to ensure the availability of the bacteria throughout the research. Moreover, the bacteria were stocked in T1N1 media, incubated for 24 hours and autoclaved paraffin oil was poured in the cultured media. Lastly, the vials were tightly secured with paraffin paper and the stock was kept for further analysis.

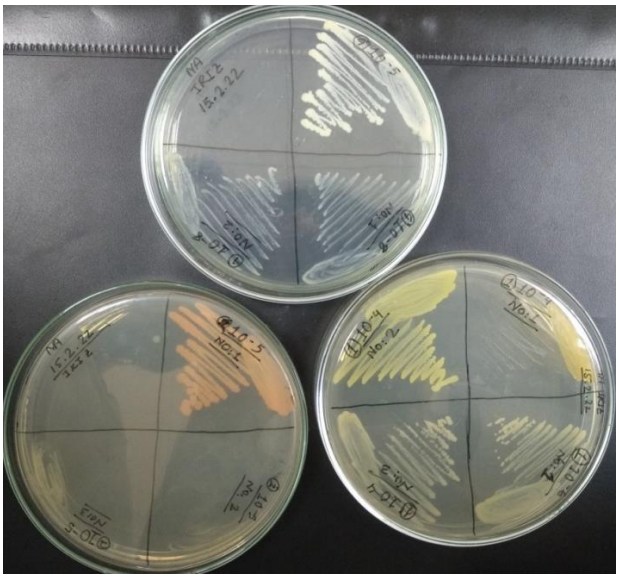


**Figure 9**: Subculture of the selected colonies

## 2.8 Preparation of chicken feather for testing keratinolytic activity:

From nearby butcher stores, numerous chicken feathers were collected and sundried. Afterwards, the chicken feathers were cleaned very well using liquid dish washer, ethanol and then air dried under laminar hood. Then, the chicken feather was wrapped very well in foil paper and kept in an incubator for some days at 50°C.

## 2.9 Preparation and inoculation of selected colonies of bacteria in Minimal Salt Media:

Primary screening was initiated by the preparation and culture of selected bacterial colonies in Minimal Salt Media.

A Minimal Salt Media of 1 Liter was prepared by using;

i) Potassium Dihydrogen Phosphate (3 gm)
ii) Dipotassium Hydrogen Phosphate (0.1 gm)
iii) Sodium Chloride (5 gm)
iv) Ammonium Chloride (2 gm)
v) Magnesium Sulphate Heptahydrate (0.16 gm)
vi) Calcium Chloride Dihydrate (0.1 gm)

These were dissolved in 1-liter distilled water and the media was autoclaved before use.

This media was specifically selected as it lacked in carbon source for the bacteria to grow. However, as the carbon source, some chicken feathers were given in the Minimal Salt Media for the bacteria to consume and find the potential ones with possible keratinolytic activity. In this media, the pH range was maintained from 5.6-6.0. Therefore, while preparing the media, the pH was adjusted to 5.7, using base (NaOH). This actual pH wasneeded for the media as to ensure the growth and activity of the keratin producing bacteria.

After preparation of 1 liter of minimal salt media, 20 clean, autoclaved conical flasks of 100 ml and the minimal salt media, along with small 1 inch cut pieces of chicken feathers were poured inside the 20 conical flasks separately and each of the flask contained 50ml of the media. After that, the flasks along with the media were sealed with aluminum foil and autoclaved to ensure no presence of unwanted microorganisms. After the media being cooled down, the selected 16 bacterial colonies were inoculated into the 16 flasks and labeled properly from 1 to 16. All of these procedures were carried out under laminar hood to ensure least possibility of contamination.


**Figure 10**: Bacteria inoculated in chicken feather and Minimal Salt Media

As soon as the 16 flasks were inoculated with 16 selected colonies, those were kept in rotary shaker incubator for about 30 days at 37°C and 130 rpm. Moreover, four distinct controls were set using the remaining 4 conical flasks with only the autoclaved minimal salt media and chicken feather piece in it, for four samples, with no bacteria inoculated in those and those were kept in rotary shaker as well. These four flasks were labeled as Control. All of the conical flasks were sealed properly with aluminum foil to assure there was no contamination within this period.

## 2.10 Selection of bacteria with keratin degrading ability in Minimal Salt Media:

Among 16 inoculated colonies, eight main bacteria were found to have the ability to degrade the chicken feather to some extent within the 30 days' time limit. Others were found to have slight or no degradation at all. Those eight bacteria were selected for secondary screening, by analyzing their keratinolytic activity.

## 2.11Preparation of keratin solution:

Keratin solution was prepared mainly by grinding 5 grams of clean, dried chicken feathers and dissolving the powdered feathers in distilled water. After powder formation, the 5 gram of powdered feather was weighed in the balance and diluted in 50 ml distilled water in an Erlenmeyer flask. The flask mouth was closed properly with aluminum foil. The solution was heated in 100ºC for about 120 minutes, vortexed for 2 minutes afterwards and then, the solution was kept in room temperature to cool down. After some time, the precipitated feather powder was filtered using filter paper, washed properly and dried to be dissolved in Tris Buffer. (Wawrzkiewicz et al., 1991)

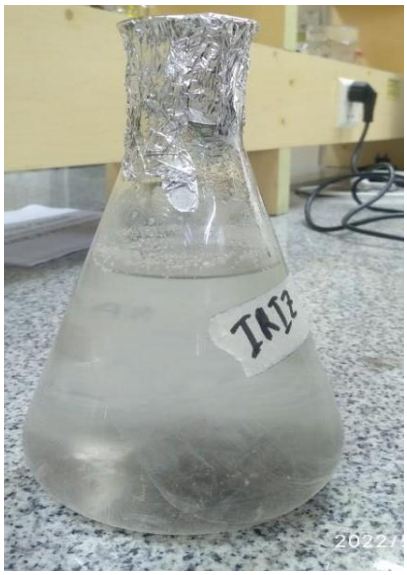


**Figure 11**: Keratin Solution

To dissolve the dried keratin, 0.1 M Tris-HCl buffer was prepared using 12.1 gm of Tris, dissolved in 1-liter distilled water. Afterwards, HCl was added for adjusting the pH to 7.5. Then, 0.05 M Tris-HCl was prepared using Tris-HCl 6.06 gm and Tris 1.39 gm, dissolved in 1-liter distilled water. In this buffer, HCl was added as well to adjust

the pH to 7.5. After preparing 0.1 M Tris-HCl, 9 ml of the buffer was taken to dissolve 1 gram of dried keratin precipitation. Total of 10 ml was prepared, which was further diluted into 90 ml of 0.05 M Tris-HCl buffer. The final keratin solution prepared was 100 ml.

### 2.12 Submerged fermentation:

The submerged chicken feather media with inoculated bacteria and kept for 1 month for fermentation in shaker incubator at 37ºC and 130 rpm. After 30 days, the chicken feathers were found to be degraded to some extent and the fermented broth was filtered using Whatman 40 and Millipore 0.4 µm. (Friedrich et al., 2002)

### 2.13Preparation of crude enzyme:

From the submerged fermentation, the filtered broth was collected in falcon tubes and, 5ml of it was centrifuged at 15000 rpm for 10 minutes to cause the unnecessary debris to settle at the bottom of the falcon tubes. Lastly, clear supernatant was isolated and taken in separate autoclaved falcon tubes which served as the crude enzyme for this experiment.


**Figure 12**: Crude enzyme

## 2.14Preparation of stop solution:

The stop solution using TCA was prepared using 0.4 M TCA diluted in 1-liter distilled water. 2 ml of 0.4 mol/L TCA was added to the falcon tubes to stop the reaction while conducting the keratin enzyme assay. (Wakil et al., 2011)

## 2.15 Analysis of keratinolytic activity:

The keratinolytic assay was conducted using1 ml of prepared crude enzyme from selected eight bacteria, diluting it in Tris-HCl buffer (0.05 M) and incubating those in 1 ml of keratin solution for each case. Eight falcon tubes were prepared for eight selected bacteria. Each tube had 2 ml of the total solution. After that, the tubes were placed in test tube holder and incubated at 50ºC in water bath for 10 minutes. A control was set as well for incubation. In the control, no crude enzyme was given, only 1 ml of keratin solution was placed for incubation. After incubation, the reaction was stopped using 2 ml of 0.4 M TCA in each falcon tubes. (Wakil et al., 2011) The tubes were centrifuged at 15000 rpm for 30 minutes. Finally, the supernatant was isolated and the absorbance was taken using Spectrophotometer, at 240 nm.

One unit of keratinolytic activity was defined as an increase of corrected absorbance at 240 nm (A240), calculated as U/ml. Absorbance of all the tubes along with the control was determined, keeping the cuvette at proper position in the spectrophotometer for about 0.01 minutes. (Gradisar et al., 2005)

The equation using which the calculation was done:

**$U = 2 \times n \times A240 / (0.01 \times 10)$**

Here, Dilution rate, n= 10

Final reaction volume= 2

Incubation time= 10 (min)

### 2.16 Preparation of Minimal Salt Media at different pH:

For optimization, the selected bacteria were cultured at different pHs, to estimate, at which pH, the bacterial Keratinase activity was highest. To identify that, the leading media for this experiment, which was the Minimal Salt Media, were prepared at different pH. Initially, the leading media was prepared at its required pH. Afterward, four main pHs were considered, pH-5.5, pH-6.5, pH-7.5, and pH-8.5. The adjustments of pH were made using Tris Base and HCl acid.


**Figure 13**: Minimal Salt Media at different pH

Using these Minimal Salt Media at different pH, the bacterial keratinolytic assays were conducted following the methods from 2.11 up to 2.15. Finally, the absorbance of all the samples was taken at 240 nm as well and the U/ml was calculated using the previously mentioned equation.

## Bacterial identification:

### 2.17 Morphological and microscopic testing of bacteria:

Morphological and microscopic identification of bacteria after carrying out various biochemical tests were done. Culturing in NA plates for 24 hours at 37ºC, the cellular structure, pigment, formation of spore etc. were analyzed from plates as well as various tests. The gram staining method was used to conduct the microscopic analysis of the selected bacteria. (Al-Dhabaan, 2019)

## 2.18 Biochemical testing for bacterial characterization:

Numerous biochemical tests were conducted to have a presumptive analysis of the selected bacteria. All these tests were carried out by following the provided laboratory procedure and information from authentic websites. For instance, Gram staining, Catalase, Oxidase, MIU (Motility Indole Urease), Nitrate Reduction, Indole, Methyl Red, Voges Proskauer, Citrate utilization, and TSI (Triple Sugar Iron) tests were done following proper methods, medias and reagents. (General Biochemical Tests - Microbiology Resource Center - Truckee Meadows Community College, n.d.)

## 2.19 Gram staining:

Necessary reagents i.e., Crystal Violet, Gram's Iodine, 95% ethanol (as decolorizer) and Safranin (Counter stain) were prepared. 24-hour pure cultures of bacteria were used to carry out the test. Lastly, under microscope, the stains were observed for each bacterium and results were interpreted following the protocol. (Gram Staining: Principle, Procedure, Interpretation, Examples and Animation, n.d.)

## 2.20 Catalase Test:

3% $H_2O_2$ was prepared and a clean sterile glass slide was taken for each bacterium. Using a sterile dropper, one drop of hydrogen peroxide was placed on the glass slide and using a sterile loop, a small number of bacteria of 24-hour pure culture was mixed slowly in the hydrogen peroxide. Immediate gas formation or no gas formation was observed. (Aryal, 2015)

## 2.21 Oxidase test:

Oxidase reagent was prepared and a small filter paper was soaked in the Oxidase reagent. Using a sterile toothpick, small amount of each bacterium was taken and small lines were drawn on the soaked paper to observe any color change to pink or pinkish red. Each bacterium was 24-hour pure culture. (Aryal, 2015)

### 2.22 MIU test:

This test ensures three results at a time that is Motility, Indole and Urease. MIU media was prepared and poured in 8 test tubes for 5 ml each and after autoclaving at 121°C, the media was cooled to certain temperature for about 50-55ºC. Then, 0.5 ml of Urease reagent was added aseptically to 5 ml base medium in each test tube. After that, the test tubes were kept unmoved to make sure that it forms a semi-solid media. Every time, ensuring sterile method, a small colony of each bacterium from 24-hour pure culture was stabbed into the tubes with an inoculation needle and the test tubes were incubated for 24 hours at 37ºC. ("MIU Test," 2019)

### 2.23 Nitrate Reduction test:

Nitrate broth was prepared and 6 ml in each test tube were poured and autoclaved at 121°C. In the cooled down broth, 24-hour pure culture of each bacterium were inoculated in the test tubes and incubated for 24 to 48 hours at 37ºC. Reagent A and Reagent B and Zinc powder were prepared to carry out the experiment. After the incubation period, at first reagent A (sulphanilic acid) was added to the eight test tubes for five drops and then reagent B (α-naphthylamine) was added to the test tubes for five drops. Lastly, on the basis of appearance of red color in the broth, zinc powder addition was carried out. That means, if there was no red color development in the broth, zinc powder in slight amount was added to the non-color developed test tubes. (Nitrate Reduction Test: Principle, Procedure, Results • Microbe Online, n.d.)

### 2.24 Triple Sugar Iron Agar Test:

TSI agar was prepared using TSI media and 5 ml of it was poured in nine test tubes. Eight were counted for eight selected bacteria and one was kept for control. All these test tubes with TSI agar were autoclaved at 121°C for 20 minutes and was cooled down to room temperature keeping in a slightly slanted position. After that, using a stabber, 24-hour culture of eight bacteria was inoculated in eight agar slants, following a stabbing and streaking on the slant. Lastly, the test tubes were kept for incubation of 24 hours at 37°C. (Tankeshwar, 2013)

**2.25 Indole Test:**

To carry out this test, tryptophan broths were prepared and poured in test tubes about 5 ml and were autoclaved afterwards at 121°C. Kovac's reagent was also prepared for this test that was to be added after 48-hour culture. The Kovac's reagent was stored in refrigerator at 4°C to ensure the reagent's stability. Using 24 hours culture, the bacteria were inoculated in the broth, after cooling down the broth in room temperature. The cultures were incubated at 37°C for 48 hours. After 48 hours, 5 drops of Kovac's reagent were added to the broth directly to check for any visible color change. (Aryal, 2015)

**2.26 Methyl Red Test:**

The Methyl red test was carried out by preparing MR broth and was poured in test tubes, about 6 ml. The broth was autoclaved at 121°C and was cooled down to room temperature before use. After that, 24 hours pure cultures of eight selected bacteria were inoculated in the broth and were incubated at 37°C for 24 hours. After 24 hours, 3 ml of the cultured broth were transferred to clean autoclaved test tubes for VP (Voges Proskauer Test). The remaining broth was kept for another 24 hours and by this time, the Methyl Red indicator was prepared. After 48 hours of culture, the Methyl Red indicator was added to the eight cultured broths and color change and red-ring formation was noted for analysis. (3.8, 2018)

**2.27 Voges Proskauer Test:**

The 3 ml broth kept aside from MR- test with 24 hours culture was used for this VP test. Two reagents were prepared named, Barritt's reagent A and Barritt's reagent B, which are 5% alpha-naphthylamine and 40% KOH respectively. 12 drops of reagent A and 4 drops of reagent B was added respectively to the eight cultured test tubes, sequentially. The tubes were shaken slightly to ensure some oxygen flow and then, the media were kept unmoved for some time. After 30-45 minutes, the color change was observed for analysis of result. (3.8, 2018)

### 2.28 Citrate Utilization Test:

Simmons citrate agars were prepared for 8 samples and 2 ml in each vial were poured. After autoclaving at 15 psi, 121°C and keeping the agar vials in slanted position, the media was cooled down to room temperature making sure that the agar has solidified. After that, 24-hour pure cultures of each bacterium were streaked on the vial agar slants and incubated for 48 hours at 37ºC. (Aryal, 2015)

### 2.29 Preparation of Luria-Bertani broth for growth of the selected bacteria:

Eight autoclaved test tubes with 10 ml of LB broth were prepared and autoclaved for 2 hours at 121°C. The selected 8 bacterial colonies were individually grown for 48 hours in autoclaved fresh LB broth at 37ºC. A control was set as well to compare there was bacterial growth. After 24 hours, the media became turbid, except for the control which had no bacteria inoculated in it.

### 2.30 Bacterial DNA isolation:

The method followed for this portion in the boil extraction method. Here, the Phosphate buffered Saline (PBS) and TE buffer was prepared, autoclaved and cooled down at room temperature before use.

After growing in LB broth for 48 hours, 700 µl of broth was collected in Eppendorfand was centrifuged at 3000 rpm for 10 minutes. A pellet was formed at the bottom of the eppendorf tube and the remaining supernatant was discarded. The pellet was washed with 300µl of PBS and re-pipetting was done to clean the pellet properly. Afterwards, the mixture was vortexed slightly and then, centrifuged at 14000 rpm for 5 minutes. Again, the supernatant was discarded for each bacterium containing eppendorf and TE buffer was added to each tube for 200µl and re-pipetting was done to mix. The tubes were placed in a floater and heated in water bath for 15 minutes at 100ºC. Immediately after removing from the water bath after 15 minutes, cold shock was given to the bacterial cells by placing those tubes in crushed ice for 10 minutes. Lastly, the tubes were centrifuged at 14000 rpm for 5 minutes and the supernatants were collected in a fresh sterile eppendorf. The bacterial genomic DNA was dissolved in the supernatant and it was stored at -20ºC.

## 2.31 PCR and Gel electrophoresis:

After extraction of genomic DNA from the bacteria, the PCR was carried out to amplify the amount of isolated DNA for further study and 16srRNA sequencing. Universal primers were used; forward primer 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and reverse primer 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). This primer amplifies the 1500bp 16srDNA fragment of the bacteria. (Piater, 2010). For PCR preparation, 10µl master mix, 2.5 µl forward primers and reverse primer each, nuclease free water of 5µl and lastly, 5µl of extracted DNA template were taken in each sterilized PCR tubes. PCR was carried out in the automated thermo-cycler adjusting the temperature in the following manner; initial denaturation at 95ºC for 5 minutes, denaturation at 95ºC for 30 seconds, annealing at 52ºC for 45 seconds, extension at 72ºC for 1 minute 30 seconds and final annealing at 72ºC for 10 minutes. (Piater, 2010). The final reaction volume for each PCR tube was 25µl and total of 30 cycles were carried out.

**Figure 14**: PCR tubes with samples inside the thermo-cycler

After completion of PCR, 1.5% concentration of agarose gel was prepared using TBE buffer and the PCR products were run at 100 volt for 30 minutes. The ladder used here was of 1500 BP. As soon as the gel run was accomplished, the gel was taken out from the apparatus (Cleaver) and placed on UV transilluminator very carefully. Finally, after observation of the band the gel was discarded by folding it in a foil paper and the PCR products were stored in -20ºC.

# Chapter 3
# Results

# Chapter 3

## Results

### 3.1 Keratin degradation test:

The feathers were incorporated in Minimal Salt media and inoculated with bacteria. Results were showing evident degradation of the chicken feathers after 30 days of placement in shaker incubator.

**Figure 15:** Minimal salt media along with chicken feather as **Control**, showing **no degradation** after 30 days.

**Figure 16:** Minimal salt media with feather and **inoculated bacteria**, showing **degradation** after 30 days

### 3.2 Culture characteristics:

The selected bacteria were cultured on nutrient agar plates for 24 hours at 37ºC for characterization.

| Bacterial No. | Size | Pigment | Colony Formation | Elevation | Texture | Spore |
|---|---|---|---|---|---|---|
| 1 | Small | Off-white | Circular | Slightly convex | White granular | Spore formation |
| 2 | Very small | White | Small circular | Convex | White granular | Slight spore formation |
| 3 | Very small | White | Small circular | Convex | White granular | Slight spore formation |
| 4 | Small | Orangish yellow | Small circular | Highly convex | Mucoid, sticky | Non-spore forming |
| 5 | Very small | White | Small circular | Convex | White granular | Slight spore formation |
| 6 | Slightly enlarged | Cream | Circular | Convex | Non-granular | Non-spore forming |
| 7 | Slightly enlarged | Cream | Circular | Flat | Mucoid, granular | Huge spore formation |
| 8 | Slightly enlarged | Cream | Circular | Flat | Mucoid, granular | Huge spore formation |

**Table 1**: Characteristics of selected bacteria

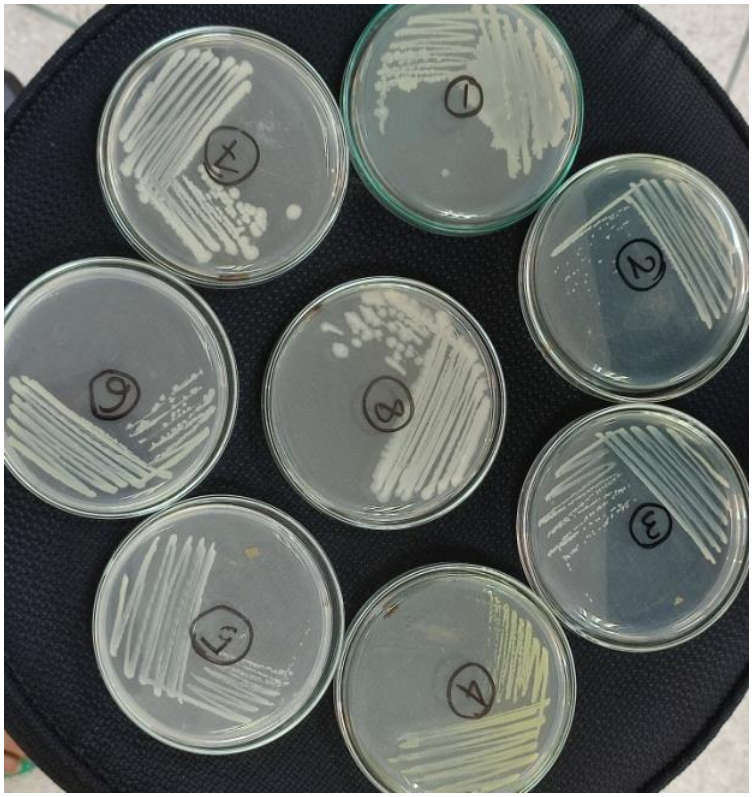



**Figure 17**: Pure culture of selected bacteria in nutrient agar plates after 24 hours at 37ºC.

### 3.3 Microscopic observation:

Gram staining was carried out to identify the characteristics of the bacteria under microscope.

#### 3.3.1 Gram staining:

| Bacteria No. | Color | Shape | Result |
|---|---|---|---|
| 1 | Purple | Rod | Gram Positive |
| 2 | Purple | Rod | Gram Positive |
| 3 | Purple | Rod | Gram Positive |
| 4 | Pink | Rod | Gram Negative |
| 5 | Purple | Rod | Gram Positive |
| 6 | Pink | Rod | Gram Negative |
| 7 | Purple | Rod | Gram Positive |
| 8 | Purple | Rod | Gram Positive |

**Table 2**: Gram staining of the samples

### 3.4 Biochemical tests:

The biochemical tests that have been conducted have been enlisted below:

i. Catalase test
ii. Oxidase test
iii. Citrate Utilization test
iv. Nitrate Reduction test
v. MIU test
vi. Indole test
vii. Methyl red test
viii. Voges Proskauer test
ix. TSI test

The results for each sample with each enlisted biochemical test have been illustrated below:

### 3.4.1 Catalase test:

| Bacteria Sample No. | Gas Formation | Result |
|---|---|---|
| 1 | Yes | Positive |
| 2 | Yes | Positive |
| 3 | Yes | Positive |
| 4 | Yes | Positive |
| 5 | Yes | Positive |
| 6 | Yes | Positive |
| 7 | Yes | Positive |
| 8 | Yes | Positive |

**Table 3:** Catalase test

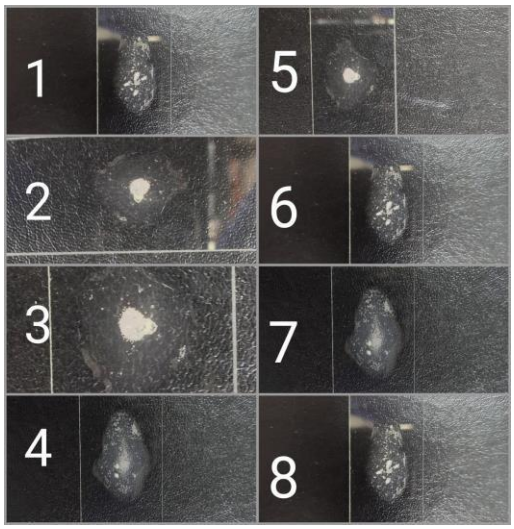



**Figure 18**: Catalase test; all samples have produced **oxygen gas**in the form of bubbles.

### 3.4.2 Oxidase test:

| Bacteria Sample No. | Color Change | Result |
|---|---|---|
| 1 | No | Negative |
| 2 | Yes; Purple | Positive |
| 3 | Yes; Purple | Positive |
| 4 | No | Negative |
| 5 | Yes; Purple | Positive |
| 6 | Yes; Purple | Positive |
| 7 | Yes; Purple | Positive |
| 8 | Yes; Purple | Positive |

**Table 4**: Oxidase test

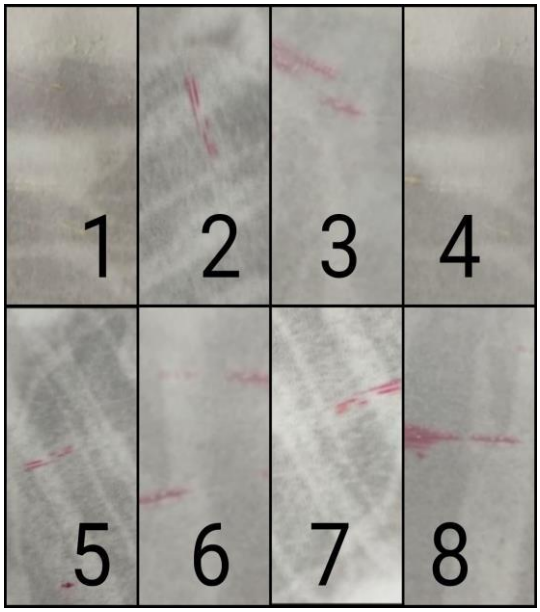



**Figure 19**: Oxidase test; among the 8 samples, **1 and 4** are showing **negative** result by not changing any color. Rest of the samples changed their color to purple which indicates the **presence of cytochrome C** oxidase enzyme and shows **positive** result.

### 3.4.3 Citrate Utilization test:

| Sample | Color | Result |
|---|---|---|
| Control | Green | Negative |
| 1 | Blue | Positive |
| 2 | Green | Negative |
| 3 | green | negative |
| 4 | blue | positive |
| 5 | Green | Negative |
| 6 | green | negative |
| 7 | blue | positive |
| 8 | blue | Positive |

**Table 5**: Citrate utilization test

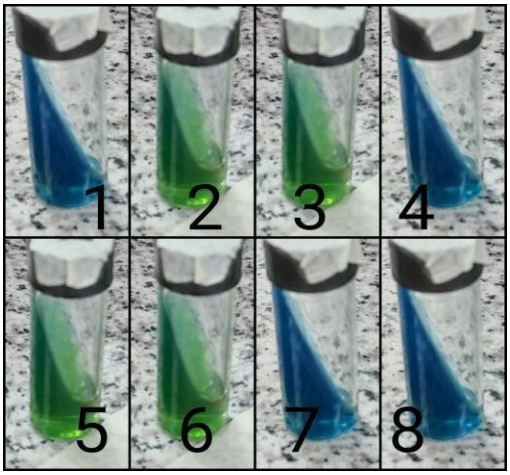



**Figure 20**: Citrate Utilization test; **1**, **4**, **7** and **8** indicates **positive** result by the**utilization of citrate** in the media and the increase of pH due to the presence of indicator Bromothymol blue. **2**, **3**, **5** and **6** shows **negative** result due to no citrate utilization.

### 3.4.4 Nitrate Reduction test:

| Sample | Color After Reagent 1 | Color After Reagent 2 | Color After Zinc Powder | Result |
|---|---|---|---|---|
| Control | Colorless | Colorless | Red | Negative |
| 1 | Colorless | Colorless | Red | Negative |
| 2 | Colorless | Colorless | Red | Negative |
| 3 | Colorless | Colorless | Red | Negative |
| 4 | Colorless | Red | Not Added | Positive |
| 5 | Colorless | Colorless | Red | Negative |
| 6 | Colorless | Colorless | Red | Negativ |

| | | | | e |
|---|---|---|---|---|
| 7 | Colorless | Red | Not Added | Positive |
| 8 | Colorless | Red | Not Added | Positive |

**Table 6**: Nitrate reduction test

### 3.4.5 MIU test:

| Sample | Motility | Color | Cloud | Result |
|---|---|---|---|---|
| Control | Non-motile | No color | No cloud | Negative |
| 1 | Motile | Pink red | Cloudy | Positive |
| 2 | Motile | No color | Cloudy | Negative |
| 3 | Motile | No color | Cloudy | Negative |
| 4 | Motile | No color | Cloudy | Negative |
| 5 | Motile | No color | Cloudy | Negative |
| 6 | Motile | No color | Cloudy | Negative |
| 7 | Motile | No color | Cloudy | Negative |
| 8 | Motile | No color | Cloudy | Negative |

**Table 7**: MIU test

### 3.4.6 Indole test:

| Sample | Color | Result |
|---|---|---|
| Control | No color | Negative |
| 1 | No color | Negative |
| 2 | No color | Negative |
| 3 | No color | Negative |
| 4 | No color | Negative |
| 5 | No color | Negative |
| 6 | No color | Negative |

| | | |
|---|---|---|
| 7 | No color | Negative |
| 8 | No color | Negative |

**Table 8**: Indole test

### 3.4.7 Methyl Red test:

| **Sample** | **Color** | **Result** |
|---|---|---|
| Control | No color | Negative |
| 1 | no color | Negative |
| 2 | No color | Negative |
| 3 | No color | Negative |
| 4 | No color | Negative |
| 5 | No color | Negative |
| 6 | No color | Negative |
| 7 | Red ring | Positive |
| 8 | Red ring | Positive |

**Table 9**: Methyl Red test

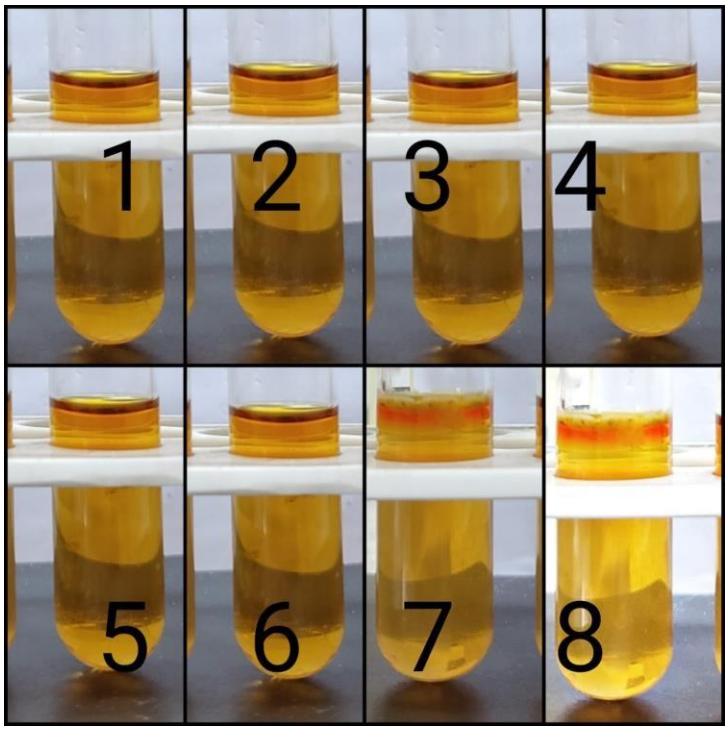



**Figure 21**: Methyl Red test; **7** and **8** shows **positive** result which indicates mixed acid fermentation and rest of the samples show **negative** result implying no mixed acid fermentation.

### 3.4.8 Voges Proskauer test:

| Sample | Color | Result |
|---|---|---|
| Control | No color | Negative |
| 1 | Red hue | Positive |
| 2 | No color | Negative |
| 3 | No color | Negative |
| 4 | Red hue | Positive |
| 5 | No color | Negative |
| 6 | No color | Negative |
| 7 | Red hue | Positive |
| 8 | Red hue | Positive |

**Table 10**: Voges Proskauer test

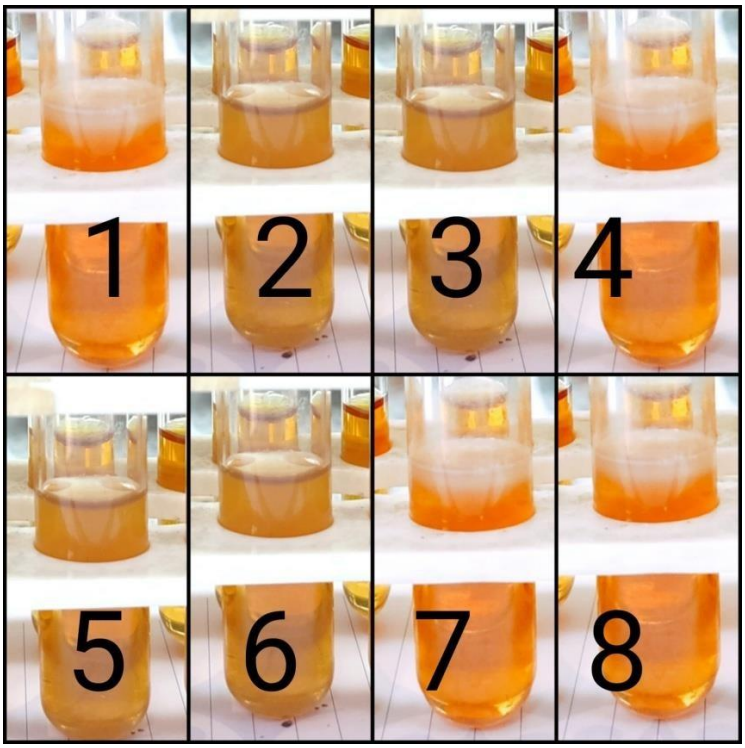



**Figure 22**: Voges Proskauer test; **1**, **4**, **7** and **8**showing **positive** result indicates the presence of acetoin, whereas **2**, **3**, **5** and **6** shows **negative** result due to absence of acetoin.

### 3.4.9 TSI test:

| Sample | Butt color | Slant color | Result |
|---|---|---|---|
| Control | Red | Red | No fermentation |
| 1 | Red | Yellow | Ferments glucose, sucrose but not lactose |
| 2 | Red | Red | No fermentation of glucose, sucrose and lactose |
| 3 | Red | Red | No fermentation of glucose, sucrose and lactose |
| 4 | Yellow | Red | Only glucose and sucrose fermentation, no lactose fermentation |
| 5 | Red | Red | No fermentation of glucose, sucrose and lactose |
| 6 | Red | Red | No fermentation of glucose, sucrose and lactose |
| 7 | Yellow | Yellow | Fermentation of glucose, sucrose, lactose |
| 8 | Yellow | Yellow | Fermentation of glucose, sucrose, lactose |

**Table 11**: TSI test


**Figure 23**: TSI test; Only 7 and 8 shows the complete fermentation of all the three sugars.

### 3.5 Keratinolytic activity of the selected bacteria at pH 5.6 and temperature 37ºC:

Minimal salt media was prepared with pH 5.6, using necessary amount of 0.05M Tris-HCl buffer. 1 ml of crude enzyme and 1 ml of keratin solution was mixed and incubated for 10 minutes at 50ºC. The reaction was stopped using 2ml of 0.4 M TCA and absorbance was taken at 240 nm in spectrophotometer, which has been represented here graphically. Here, X-axis shows Bacteria No. & Y-axis represents Enzymatic activity.

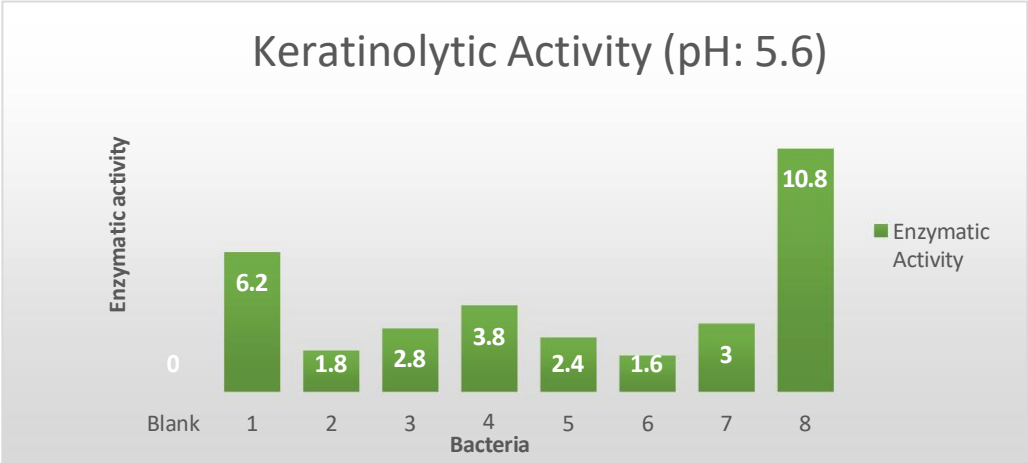



**Figure 24**: Keratinolytic activity of selected bacteria at pH: 5.6

| Temperature: 37°C; pH: 5.6 | | | |
|---|---|---|---|
| | **Sample no.** | **OD** | **Absorbance(U/ml)** |
| | Blank | 0 | 0 |
| | 1 | 0.031 | 6.2 |
| | 2 | 0.009 | 1.8 |
| | 3 | 0.014 | 2.8 |
| | 4 | 0.019 | 3.8 |
| | 5 | 0.012 | 2.4 |
| | 6 | 0.008 | 1.6 |
| | 7 | 0.015 | 3 |
| | 8 | 0.054 | 10.8 |

**Table 12**: Absorbance at specific temperature and pH

According to the graph and table, the highest enzymatic activity was found in bacteria labeled as No. 8 and the lowest activity was found from the bacteria labeled No. 6.

### 3.6 Keratinolytic activity of the selected bacteria at pH 6.5 and temperature 37°C:

Minimal salt media was prepared with pH 6.5, using necessary amount of 0.05M Tris-HCl buffer. Similarly, in this case as well, 1 ml of crude enzyme and 1 ml of keratin solution was mixed and incubated for 10 minutes at 50°C. Reaction was stopped using

2ml of 0.4 M TCA and absorbance was taken at 240 nm in spectrophotometer, which has been represented here graphically. Here, X-axis shows Bacteria No. & Y-axis represents Enzymatic activity.

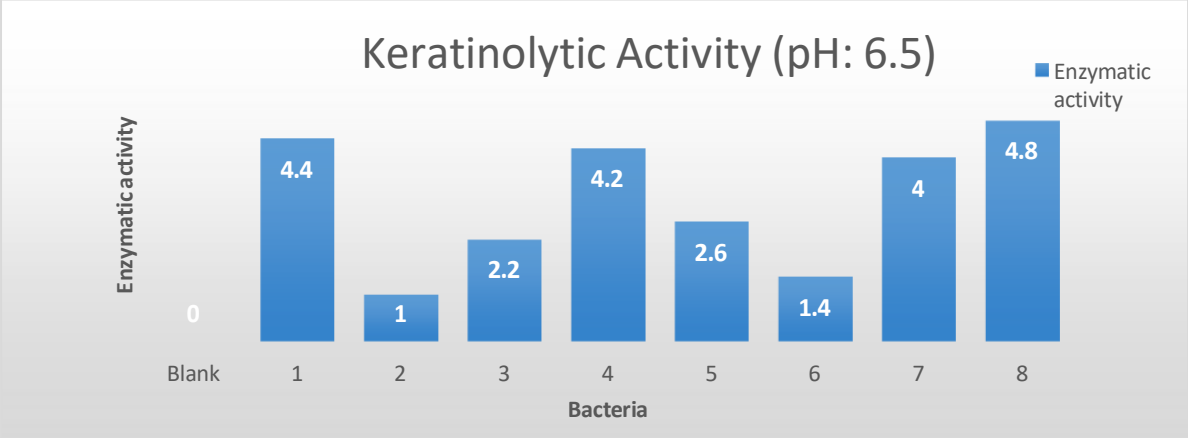



**Figure 25**: Keratinolytic activity of selected bacteria at pH: 6.5

| Temperature: 37°C; pH: 6.5 | | |
|---|---|---|
| **Sample no.** | **OD** | **Absorbance(U/ml)** |
| Blank | 0 | 0 |
| 1 | 0.022 | 4.4 |
| 2 | 0.005 | 1 |
| 3 | 0.011 | 2.2 |
| 4 | 0.021 | 4.2 |
| 5 | 0.013 | 2.6 |
| 6 | 0.007 | 1.4 |
| 7 | 0.02 | 4 |
| 8 | 0.024 | 4.8 |

**Table 13**: Absorbance at specific temperature and pH

According to the graph and table, the highest enzymatic activity was found in bacteria labeled as No. 8 and the lowest activity was found from the bacteria labeled No. 2.

### 3.7 Keratinolytic activity of the selected bacteria at pH 7.5 and temperature 37°C:

Following the above process, minimal salt media was prepared with pH 7.5, using necessary amount of 0.05M Tris-HCl buffer. Followed by the similar process, after incubation, absorbance was taken at 240 nm in spectrophotometer, which has been represented here graphically. Here, X-axis shows Bacteria No. & Y-axis represents Enzymatic activity.

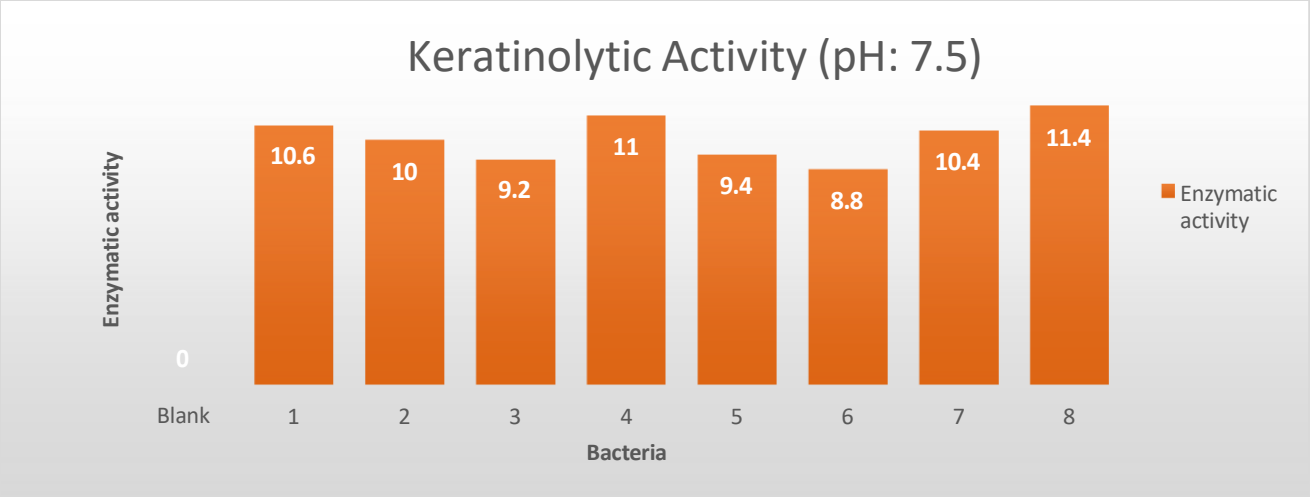



**Figure 26**: Keratinolytic activity of selected bacteria at pH: 7.5

| Temperature: 37°C; pH: 7.5 | | |
|---|---|---|
| **Sample no.** | **OD** | **Absorbance(U/ml)** |
| Blank | 0 | 0 |
| 1 | 0.053 | 10.6 |
| 2 | 0.05 | 10 |
| 3 | 0.046 | 9.2 |
| 4 | 0.055 | 11 |
| 5 | 0.047 | 9.4 |
| 6 | 0.044 | 8.8 |
| 7 | 0.052 | 10.4 |
| 8 | 0.057 | 11.4 |

**Table 14**: Absorbance at specific temperature and pH

According to the graph and table, the highest enzymatic activity was found in bacteria labeled as No. 8 and the lowest activity was found from the bacteria labeled No. 6.

### 3.8 Keratinolytic activity of the selected bacteria at pH 8.5 and temperature 37ºC:

Following the above process, minimal salt media was prepared with pH 8.5, using necessary amount of 0.05M Tris-HCl buffer. Followed by the similar process, after incubation, absorbance was taken at 240 nm in spectrophotometer, which has been represented here graphically. Here, X-axis shows Bacteria No. & Y-axis represents Enzymatic activity.

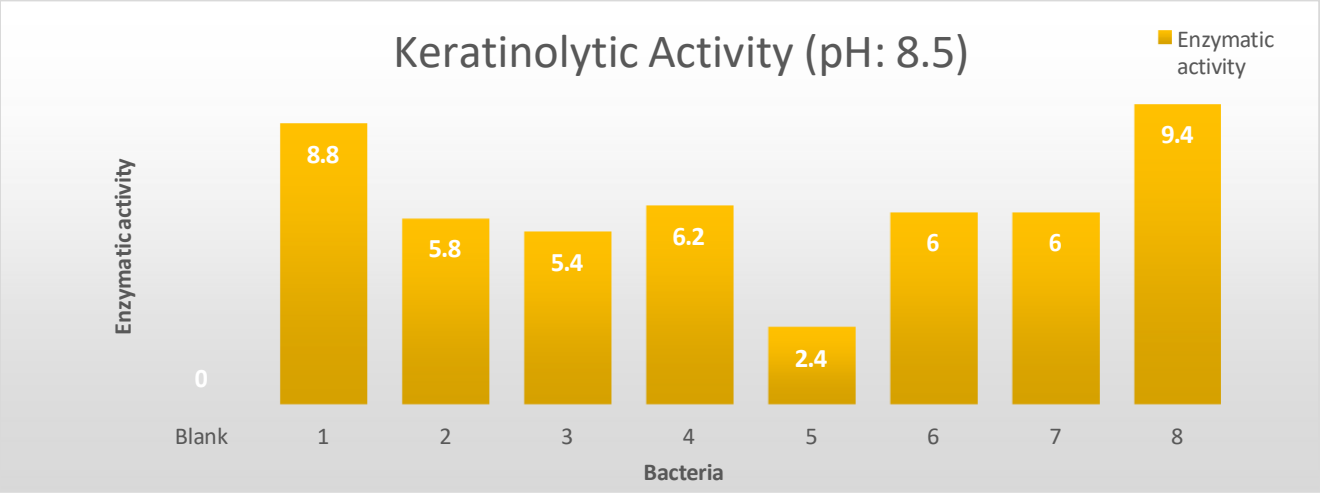



**Figure 27**: Keratinolytic activity of selected bacteria at pH: 8.5

| Temperature: 37ºC; pH: 8.5 | | | |
|---|---|---|---|
| | **Sample no.** | **OD** | **U/ml** |
| | Blank | 0 | 0 |
| | 1 | 0.044 | 8.8 |
| | 2 | 0.029 | 5.8 |
| | 3 | 0.027 | 5.4 |
| | 4 | 0.031 | 6.2 |
| | 5 | 0.012 | 2.4 |
| | 6 | 0.03 | 6 |
| | 7 | 0.03 | 6 |
| | 8 | 0.047 | 9.4 |

**Table 15**: Absorbance at specific temperature and pH

According to the graph and table, the highest enzymatic activity was found in bacteria labeled as No. 8 and the lowest activity was found from the bacteria labeled No. 5.

### 3.9 Keratinolytic activity testing at different pH with graphical representation:

Starting from an acidic pH 5.6 to a basic pH 8.5, the range was set to test the maximum enzymatic activity of the keratinase enzyme, produced by the selected eight bacteria. The graph below demonstrates a comparative analysis of the keratinolytic activity of bacteria at different pH.

Here, in the graph, the X-axis represents the Bacteria No. and the Y-axis represents the Enzymatic activity. The graph itself depicts the comparison of the keratinase enzyme activity at different pH, in different colors.

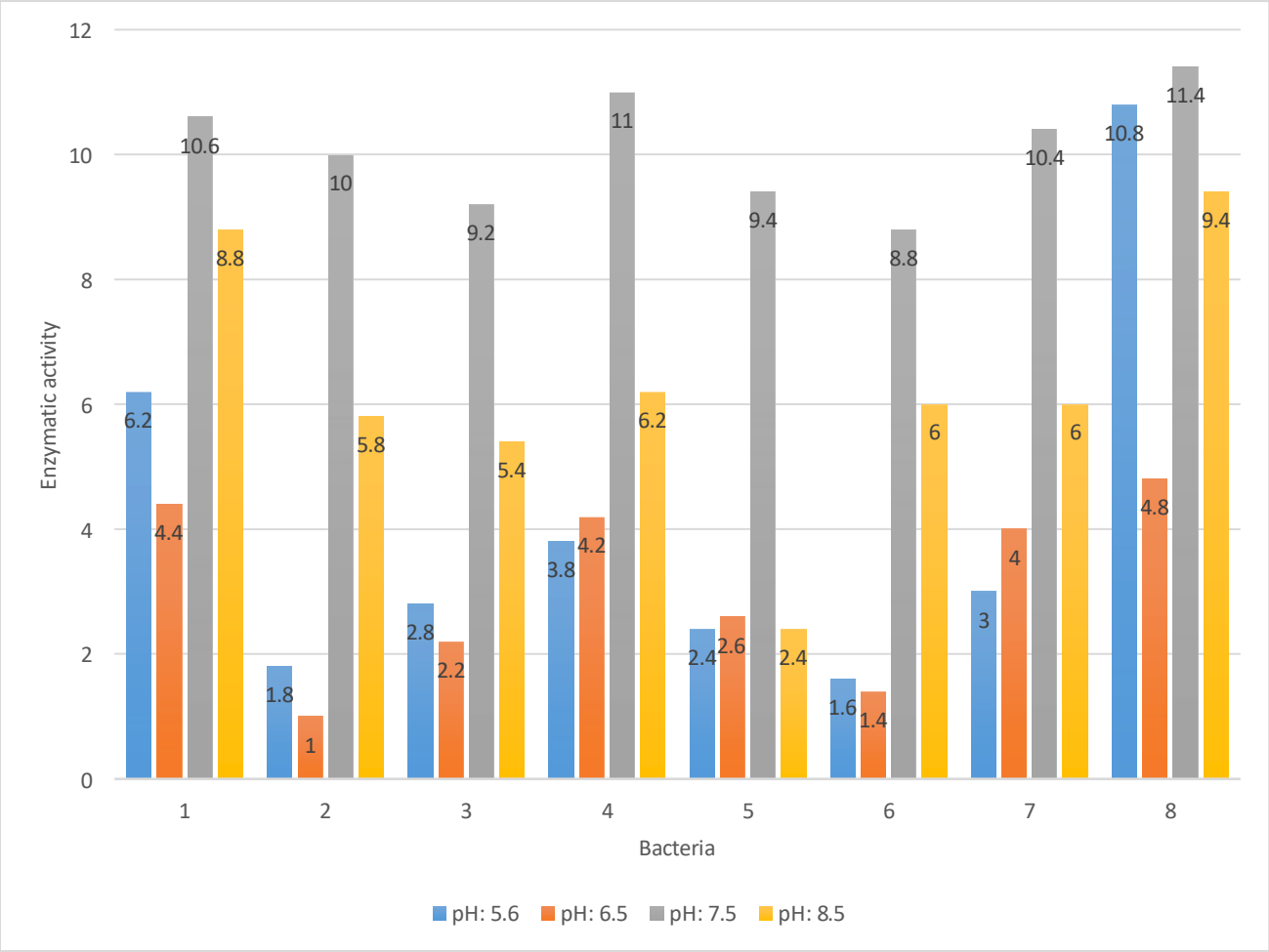



**Figure 28**: Comparison of the effects of different pH in keratinolytic activity of bacteria

### 3.10 Keratinolytic activity testing at room temperature (25°C) with graphical representation:

This test was conducted to check the keratinolytic activity at room temperature, since, if there was a possibility of keratin degradation and whether such temperature was suitable for keratinase production by bacteria. Here, in the graph, X-axis depicts the Bacteria No. and Y-axis shows the Enzymatic activity. Also, pH: 5.6 was kept as constant as it was mentioned for this media.

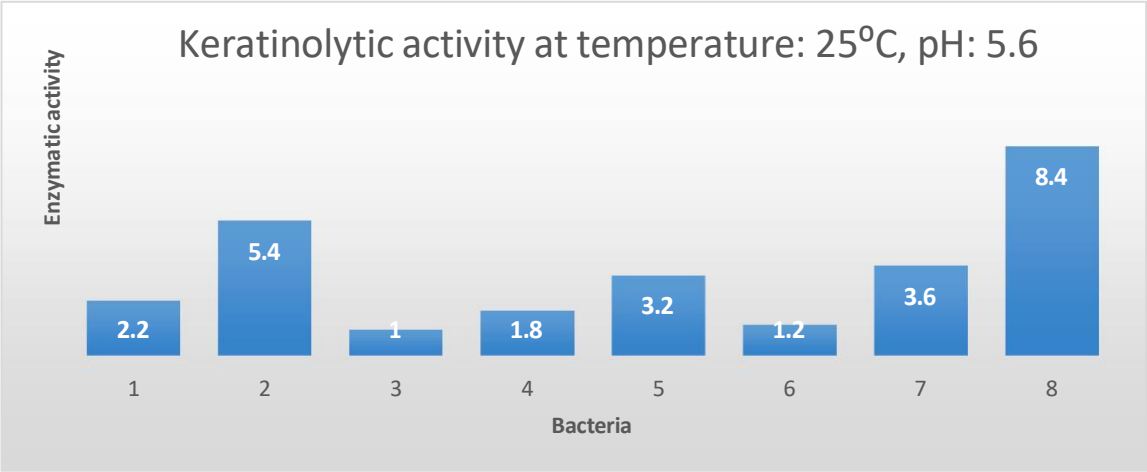



**Figure 29**: Keratinolytic activity at temperature 25°C and pH; 5.6 as constant.

| Temperature: 25°C; pH: 5.6 | | |
|---|---|---|
| **Sample no.** | **OD** | **U/ml** |
| Blank | 0 | 0 |
| 1 | 0.011 | 2.2 |
| 2 | 0.027 | 5.4 |
| 3 | 0.005 | 1 |
| 4 | 0.009 | 1.8 |
| 5 | 0.016 | 3.2 |
| 6 | 0.006 | 1.2 |
| 7 | 0.018 | 3.6 |
| 8 | 0.042 | 8.4 |

**Table 16**: Absorbance at specific temperature and pH

According to the graph and table, the highest enzymatic activity was found in bacteria labeled No.8 and lowest activity was found in bacteria labeled No. 3.

### 3.11 Identification of the bacteria:

After observation of morphological and biochemical tests conducted for the eight bacteria, the result concluded with the following findings:

| Bacteria Sample No. | Bacteria Names (presumptive) |
|---|---|
| 1 | *Bacillus cereus* |
| 2 | *Bacillus carboniphilus* |
| 3 | *Bacillus carboniphilus* |
| 4 | *Serratia marcescens* |
| 5 | *Bacillus carboniphilus* |
| 6 | *Pseudomonas putida* |
| 7 | *Bacillus licheniformis* |
| 8 | *Bacillus licheniformis* |

**Table 17**: Identification of bacteria

# Chapter 4

# Discussion

# Chapter 4

## 4.1 Discussion

Out of sixteen inoculated bacteria, eight potential bacteria were selected on the basis of visual observation of degradation of chicken feathers that were provided as the only carbon source for bacteria in the minimal salt media. Initially, the upper portion of the feather stick was selected and eventually, the middle and lastly, the end portion, which is the hardest part of a single feather, were successfully found to get degenerated after some period of time by the selected bacteria. For further confirmation, the remaining feather parts were visualized by Scanning Electron Micrograph (SEM) that showed visible keratin degradation on feathers and change in feather structure. (Cai et al. (2008). However, the time taken for complete degradation of the feathers was found to be different for different bacteria.

The findings evaluate that majority of the bacteria were found to be Bacillus and one was *Serratia marcescens* and another was *Pseudomonas putida*, which was expected as per the research conducted in the past studies. (*Frontiers | Microbial Keratinase: Next Generation Green Catalyst and Prospective Applications*, n.d.). Among the mentioned bacteria, the highest keratinolytic activity was found in case of Sample No. 8, i.e., *Bacillus licheniformis*. The second and third highest was staying in between *Bacillus cereus* and *Serratia marcescens*. All these bacteria were reported as effective to degrade keratin from environment, previously. (*Production and Purification of Keratinase Enzyme from Serratia Sp. Isolated from Poultry Wastes*, n.d.); (Singh & Masih, 2015); (Akhter et al., 2020).

In some cases, microbial keratinase has been found as an inducible enzyme, which means, bacteria can become adapted to a certain environment to produce keratinase enzyme for their survival. In this study, similar cases were evident as well. As long as there was availability of more keratin component, the keratinase enzyme production amount was also high. Alongside, the more the concentration of enzyme increased, the more the degradation process accelerated. Therefore, the relationship between, keratin availability with keratin degradation in presence of keratinase enzyme is found to be proportional. Nonetheless, for excessive incubation period, there was a possibility of increasing of bacteria concentration and eventually, resulting to the production of toxin. For this, chicken feathers as keratin were kept to be checking from time to time in the media, in order to make sure that the bacteria survived and produced enough keratinase enzymes for the assay.

Providing different pH and temperature condition to the inoculated bacteria, for keratinolytic assay, the results demonstrated incredible finding. Keeping temperature constant as 37ºC and pH- 5.6, 6.5, 7.5 and 8.5, the comparative analysis showed that at neutral pH, i.e., 7-7.5, the keratinolytic activity was found to be the highest, for most of the bacteria. Moreover that, the graph also showed that at room temperature, 25ºC, the keratinolytic activity was not found to be highest. These findings were also reported in papers previously. (Lateef et al., 2015). Bacillus cereus was found to have best keratinolytic activity at pH- 7.5, which was also reported in this paper. (Lateef et al., 2010). *Bacillus carboniphilus* and *Bacillus licheniformis* also showed active keratinolytic activity at the neutral pH. Furthermore, some studies also support that the media with basic pH up to 8.0-8.5 shows optimum production of keratinase enzyme by bacteria. This study also finds some similar analysis from the graph where from pH 7.0-8.5, some bacteria showed impressive keratinolytic activity by breaking down keratin using the produced keratinase enzyme. However, in case of acidic pH of the media, the enzymatic activity was not quite obvious. That is why, majority of the studies, along with the finding in this research has come up with the conclusion that the favorable condition for bacteria to proclaim better keratinolytic activity is at neutral to slightly alkaline pH and the favorable temperature is higher than 30ºC.

All these eight bacteria were found to have potential keratinase enzyme production to degrade the keratin from nature at a very low cost. The DNA these potential bacteria were extracted and to ensure further analysis for trial purpose after completion of 16S rRNA sequencing, precise identification was confirmed. Alongside, a small trial was carried out using crude enzyme on naturally curly human hair, where the crude enzyme with the hair was incubated for 2 hours at 50ºC. The finding brought a ray of hope by softening the hair follicle to some extent that was clearly visible. This certainly proved enzymatic hydrolysis of hair follicles. (Mazotto et al., 2010). However, the purification and characterization of the enzyme is necessary and the study is expected to be improved in the future to assure a phenomenal asset from nature, in the cosmetic, drug, and other essential industries of modern world.

# Chapter 5

# Conclusion

# Chapter 5

## 5.1 Conclusion

Among numerous fibrous proteins, keratin is the most available one. As much as it is essential for an organism, yet, the keratin-rich wastes are also necessary to be degraded. For this, the importance of keratinase enzyme has no similar alternative. Keratinase is quite an expensive enzyme and the industrial production of keratinase is often very exorbitant. However, isolation of keratinase enzyme from bacteria is not only environment friendly but also, the method is tremendously cost-effective. Expansion of this research to use the crude enzyme from bacteria in trials and purifying the enzyme from those successful trials in various industries can bring a revolutionary change. Therefore, this study is clearly a step forward in the production of this industrially vital enzyme, which is highly essential at pharmaceutical industries, cosmetic industries, and production of animal feed as well as degradation of keratin-rich wastes from earth.

# Chapter 6

# References

# Chapter 6

## 6.1 References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