

# **Isolation and identification of extracellular keratinase producing bacteria from soil**



A dissertation submitted to the Department of Mathematics and  
Natural Sciences, BRAC University in partial fulfillment of the  
requirement for the degree of Bachelor of Science in Microbiology

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For my beloved parents, sister and brother

## Declaration

I hereby declare that this thesis entitled “**Isolation and identification of Extracellular Keratinase Producing Bacteria from soil**” is submitted by me, Md.Mahmud Al Hasan, to the Department of Mathematics and Natural Sciences under the supervision and guidance of Dr. M. Mahboob Hossain, Professor, Department of Mathematics and Natural Sciences, BRAC University. I also declare that the thesis work presented here is original, and has not been submitted elsewhere for any degree or diploma.

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## Abstract

Feathers, horns, cows skin are by product waste of cows skin, poultry and skin processing plants and produced in huge amount of waste every year. This horns, skin hair, chicken feather waste can be modified to make animal dietary protein. This conversion can be done by Keratinolytic bacteria, High protein content of keratin waste can be used as a good source of protein and amino acids by systemic recycling. Feather recycling can provides a cheap and alternative protein feed stuff. Further this can be used for many other purposes more likely animals feed fish feed. Some Bacteria, Fungi and Actinomycetes have been reported to produce keratinase in the presence of keratin substrate. In the present study Keratinase producing bacteria was isolated from soil and their Keratinolytic enzyme production was investigated. Soil sample was collected from Hazaribagh skin processing site Dhaka, Bangladesh, an organic waste dumping site. Soil samples were inoculated in eight nutrient broth media enriched with hair horns, feather powder and inoculated bacteria was isolated from soil sample. Only two organisms were able to degrade hair horns and feather powder indicating that these isolates produced Keratinase enzyme. In this study it was observed that keratinase and protease activity were detected in the culture supernatant and optimal medium for extracellular production of keratinase and protease is Keratin powder milk media at pH (7.5) and temperature (37°C). There was complete degradation of keratin after 168 hour of incubation in rotary shaking incubator 150 RPM at 37°C. Finally, biochemical test was done to identify the organism and it was found to be *Bacillus licheniformis*.

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# Chapter1

## Introduction

## **1.1 Introduction:**

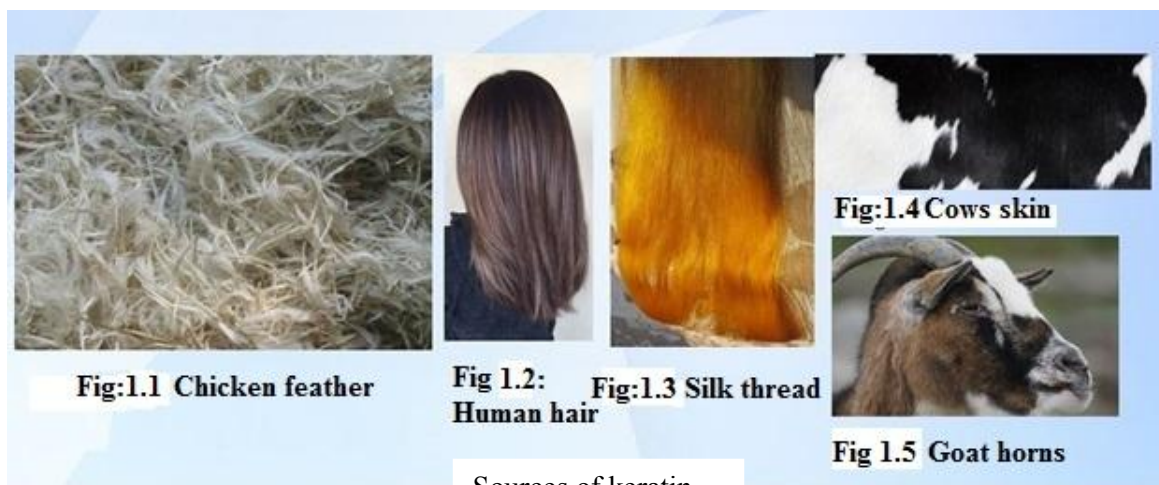
Feathers, horns, hair skin are produced worldwide in huge volumes as a waste composed of over 90% protein. Large quantities of feathers waste will lead to environmental pollution and protein depletion (Onifade et al., 1998; Gousterova et al., 2005). Keratin wastes are arising the worldwide issue of gathering by-products mainly at the slaughterhouse. Every year over 5 million tons of feathers are produced globally from poultry-processing plants as a waste product (Poole et al., 2009). Keratinolytic microorganisms trigger scientific interest by means of bioutilization of keratin. This bioconversion methods approach offers a constructive alternative to the currently employed energy-consuming techniques or toxic reagents. Traditional ways to degrade feathers such as hydrolysis in alkali and pressure by steam not only destroy the amino acids but this process also consume huge amounts of energy. Biodegradation of feathers by microorganisms may provide a viable alternative. Bacteria e.g.: *Bacillus* (Williams et al., 1990; Riffel et al., 2003; Manczinger et al., 2003; El-Refai et al., 2005), fungi (Gradišar et al., 2000; Friedrich et al., 2005) and Actinomycetes (Ignatova et al., 1999; Gousterova et al., 2005) have previously been shown to be able to produce feather-degrading keratinases. Keratinase and related products have many applications (Gupta and Ramnani, 2006). For example, *Bacillus licheniformis* PWD-1 and *Vibrio sp.* strain kr2 (Williams et al., 1991; Grazziotin et al., 2006) can be used as feed additives by hydrolysates of a feather, while the keratinase from *Bacillus subtilis* S14 exhibits significant dehairing capabilities (Macedo et al., 2005).

Moreover, feather degrade in the presence of detergents and heat treatment (Langeveld et al., 2003), *B. licheniformis* PWD-1 can degrade the infectious form of prion, PrPsc, which is very important for an animal meal as feed by means of utilization of Keratin. Usually, it is important to improve the enzyme yield for application purposes and so various methods including the optimization of cultural conditions and medium composition, or gene expression in heterologous condition have been applied (Ramnani and Gupta, 2004; Anbu et al., 2005). Given the effectiveness of traditional mutagenesis approach for isolating mutants that produce improved yields of various microbial enzymes such as lipase and  $\alpha$ -galactosidase (Tan et al., 2003; Wang et al., 2004) and in addition to that, it is possible that take some strategy to increase the production of this important enzyme by improving the keratinase producing strains. Keratinases are almost all inducible and different keratin-rich materials include feathers, hair, and horns. For keratinase production, feathers, hair, and horns can be used as substrates (Gupta and Ramnani, 2006).

Especially, *Bacillus* sp can utilize feather substrate mostly, while rarely utilized was human hair. Another keratin-containing materials, nail, and horn as well as feathers and hair, are mostly produced in China and these may also be potential substrates for keratinase production. The aim of this study was to identify a newly isolated feather-degrading bacterium strain, to characterize keratinase production and keratin degradation in feathers, hair, and horn and to optimize the conditions for keratinase production in feather substrates.

## 1.2 Keratin:

Keratin is highly stable and an insoluble fibrous protein due to the presence of high degree of cross-linkages (S-S) disulfide and hydrogen bonds. This protein consist of a variety of amino acids, mostly serine cystine, lysine, and proline. Considering the conformation secondary structural, keratins have been classified into  $\alpha$ - ( $\alpha$ -helix of hair and wool) and  $\beta$ -keratins ( $\beta$ -sheets of a feather). High disulfide bond keratins are found in hair, feather, nails, and wool, etc. Soft keratin having low disulfide bond content present in the skin.



Keratin rich wastes are creating environmental pollution the form of feathers. It is release as byproducts from agro-industrial processes and increasing day by day. Hair, nails, and horns is an insoluble and fibrous structural protein. Feathers and wools are constituent of this protein.

The byproduct of keratinous wastes is abundantly available, representing a valuable source of essential amino acids and protein, which could be useful for the source of nitrogen for plants or as animal feeds. Nevertheless, the keratin-containing substrates and materials have rigid, high mechanical stability recalcitrant and hence are difficult to be degraded by common proteases. specific proteolytic enzymes like Keratinases are capable of degrading insoluble keratins.

‘Keratin’ is not a single substance, it is misunderstood as single substrate as it is composed of a complex mixture of proteins, such as keratins, KFAPs and enzymes extracted from epithelia (Tomlinson et al., 2004). Keratins are found only in hair, nail, horns, mostly epithelial cells and categorically unique physicochemical properties (Steinert et al., 1982; Sun et al., 1983). They

are resistant to digestion or degradation by the proteases pepsin or trypsin and are insoluble in alkalines, dilute acids, organic solvents and water (Block, 1951; Steinert et al., 1982). Keratins are soluble in urea but insoluble in denaturing agents containing aqueous salt solutions, (Steinert et al., 1982). Keratins reassemble intermediate filaments in aqueous solution (Steinert et al., 1982; Sun et al., 1983). Keratins are classified specifically according to their physicochemical characteristics and molecular structure, they are produced by epithelial cells (Steinert et al., 1982).

Keratins can be extracted by using reducing agents from various tissues, such as mercaptoethanol, thioglycollate or dithiothreitol, which cleave disulfide bonds (Brown, 1950; Sun & Green, 1978; Steinert et al., 1982). The keratin protein nomenclature was first published by (Moll et al., 1982) and it has been updated in recent years (Hesse et al., 2001, 2004; Schweizer et al., 2006) to accommodate the results of ongoing research in other vertebrates and humans. The guidelines issued by the Human and Mouse Genome Nomenclature Committee follows the comprehensive nomenclature of keratins (Schweizer et al., 2006) and various older keratin nomenclatures also consider for adaptation. Szeverenyi et al. (2008) this committee published a comprehensive catalog of the human keratins, their nucleotide sequence, the amino acid sequence of the keratin genes in various vertebrate species as well as the human same data of the orthologue keratins and keratin genes in.

### **1.3 Molecular structure of keratin:**

Keratin is a ubiquitous biological material, high-sulfur content and filament-forming proteins, constituting the bulk of epidermal attachments such as hair, nails, claws, turtle scutes, horns, whale baleen, beaks, and feathers. They are among the toughest biological materials, serving as a wide variety of interesting functions, e.g. scales to armor body, horns to combat aggressors, hagfish slime as a defense against predators.

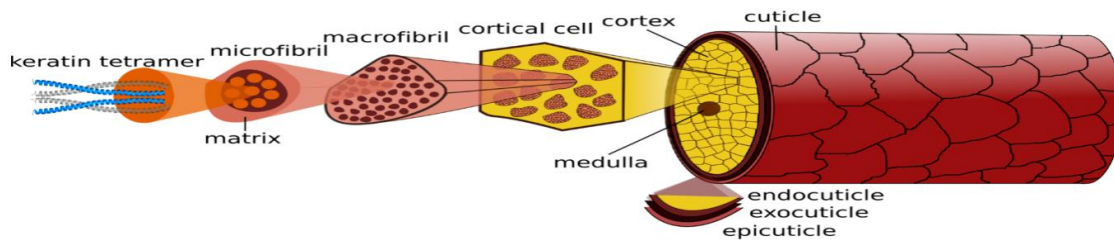


Figure 1.6: cross-section of a keratin hair fiber as example.

Keratins can be classified as  $\alpha$ - and  $\beta$ -types. Both show a characteristic filament-matrix structure: 7 nm diameter intermediate filaments for  $\alpha$ -keratin, and 3 nm diameter filaments for  $\beta$ -keratin. Both are embedded in an amorphous keratin matrix. The molecular unit of intermediate filaments is a coiled-coil heterodimer and that of  $\beta$ -keratin filament is a pleated sheet. The mechanical response of  $\alpha$ -keratin has been extensively studied, in some cases, a high reversible elastic deformation.  $\beta$ -keratin has not been investigated as comprehensively.

#### 1.4 Keratin under scanning electron micrograph:

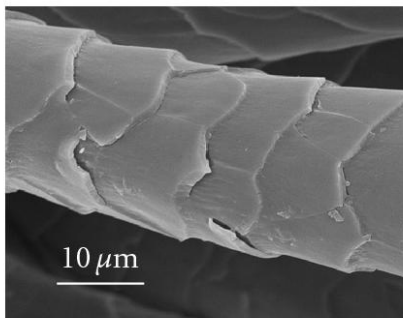


Fig 1.7: Human hair under SEM

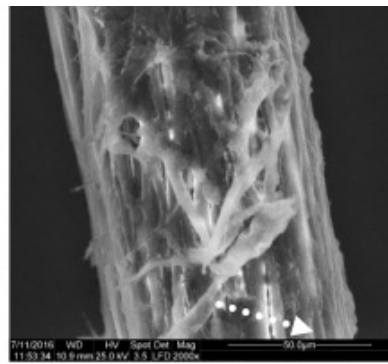


Fig 1.8: Hair is degraded by fungi under SEM



**1.5 Physicochemical characteristics of keratins** To determine their physicochemical properties, at a particular pH and a specific concentration. Extracting Keratin from cells using solvents such as urea and reducing agents, to break the (S-S) disulfide bonds that link these keratins to each other. mercaptoethanol or dithiothreitol has to be used. (Moll et al., 1982; Sun et al., 1983).

### **1.6 Molecular weight of Keratin**

The molecular weight of keratins in animals ranges from 40 to 70 kDa (Sun et al., 1983). Measured by immunoblotting method and two-dimensional gel electrophoresis, (Cooper & Sun 1986) separated and compared human and bovine keratins and found that although some keratins in corresponding tissues had the same Molecular weight.

### **1.7 Isoelectric point of Keratin**

Isoelectric points of keratinase are varied. (Moll et al., 1982). The variance is either acidic or basic properties.

Type I (acidic or subfamily A)

Type II (basic or subfamily B)

Keratins that are found in hair, nail or wool they have an isoelectric point pI of 4.7–5.4 (Marshall, 1983).

### **1.8 Keratinase:**

Keratinases are the unique group of proteolytic enzymes adept to hydrolyze different keratin substrates viz. a-keratins (hair, hooves, nail etc.) and b-keratins (Feather and silk fibrions, b-amyloid) into simple polypeptides and amino acids. The capricious activity of keratinase towards different keratin substrates is due to their extracellular secretion from microorganisms like bacteria, fungi, and actinomycetes. Since the skin and nails composed of keratins,

**1.9 Molecular modeling of Keratinase:** Molecular modeling of keratinase enzyme retrieved from expassy server, fig 1.9 and fig 1.10

**F8UFT3 (F8UFT3\_9BACI) *Bacillus sp MKR1***  
 Keratinase UniProtKB [InterPro](#) [Interactive Modelling](#)

379 aa; Sequence (Fasta) [Identical sequences: \*Bacillus licheniformis\*: Q6PNN5](#)

**F8UFT3**  
 (X-RAY) heteromer, 106-379  
 monomer, 36-379  
 monomer, 37-104

PDB 3qtl ["Structural Basis for Dual-inhibition Mechanism of a Non-classical Kazal-type Serine Protease Inhibitor from Horseshoe Crab in Complex with Subtilisin"](#)

Method: X-RAY DIFFRACTION 2.60Å  
 Released: 2011-06-01  
 Bioassembly File: [↓](#)

RCSB [PDBsum](#) [PDBe](#)

Sequence Features

[InterPro](#)

44-102	Peptidase S8 propeptide/proteinase inhibitor I9 <a href="#">IPR010259</a> <a href="#">PF05922</a>
128-370	Peptidase S8/S53 domain <a href="#">IPR000209</a> <a href="#">PF00082</a>

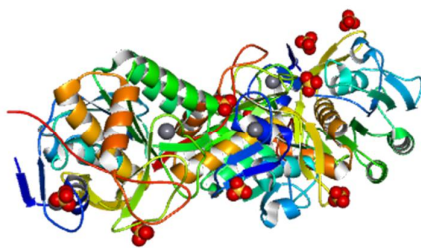


Figure 1.9:Keratinase molecular modeling

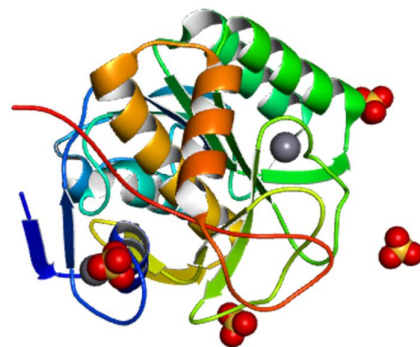


Figure 1.10:Keratinase molecular modeling 2

## 1.10 Keratinase producing microorganism

Table 1.1: Keratinase producing organism

Fungus		Keratinase Ku/ml Substrate		
		Pig hair*	Peacock feather**	Chicken feather+ Human hair***
<i>Acrodontium album</i>	IMI179837	182.2	15	190.2
<i>Aspergillus quercinus</i>	IMI179848	32.2	39	60.5
<i>Aspergillus ustus</i>	IMI179847	47.2	55	60.4
<i>Botryotricum keratinophilum</i>	IMI185322	93.2	21	110.4
<i>Chaetomium globosum</i>	IMI179869	12.2	21	30.4
<i>Chrysosporium crassitunicatum</i>	IMI185320	130.2	31	160.5
<i>Chrysosporium tropicum</i>	IMI179840	77.2	88	92.4
<i>Chrysosporium indicum</i>		75.0	-	85.9
<i>Curvularia indica</i>	ITCC1879	132.2	33	140.4
<i>Gliocladium agrawalii</i>	IMI179846	118.9	88	128.8
<i>Gliocladium rorseum</i>	IMI179845	68.2	78	75.5
<i>Keratinophyton terreum</i>	IMI185313	107.5	165	118.7
<i>Malbranchea pulchella</i>	IMI179843	146.2	73	170.2
<i>Microsporium fulvum</i>	IMI194744	86.7	78	96.9
<i>Microsporium gypseum</i>	IMI194745	69.6	2	85.8
<i>Penicillium lilacinum</i>		141.2	40	158.4
<i>Trichoderma harzianum</i>	IMI179833	34.1	15	60.3
<i>Trichoderma hematum</i>	IMI185323	28.8	64	58.6
<i>Trichophyton mentagrophytes</i>	IMI194747	112.4	4	115.6
<i>Verticillium lecanii</i>	IMI185318	187.8	95	195.4

\*Kushwaha and Nigam (1996)  
\*\*Kushwaha (1983)  
\*\*\*Kushwaha (2007 Unpublished).

# Chapter 2:

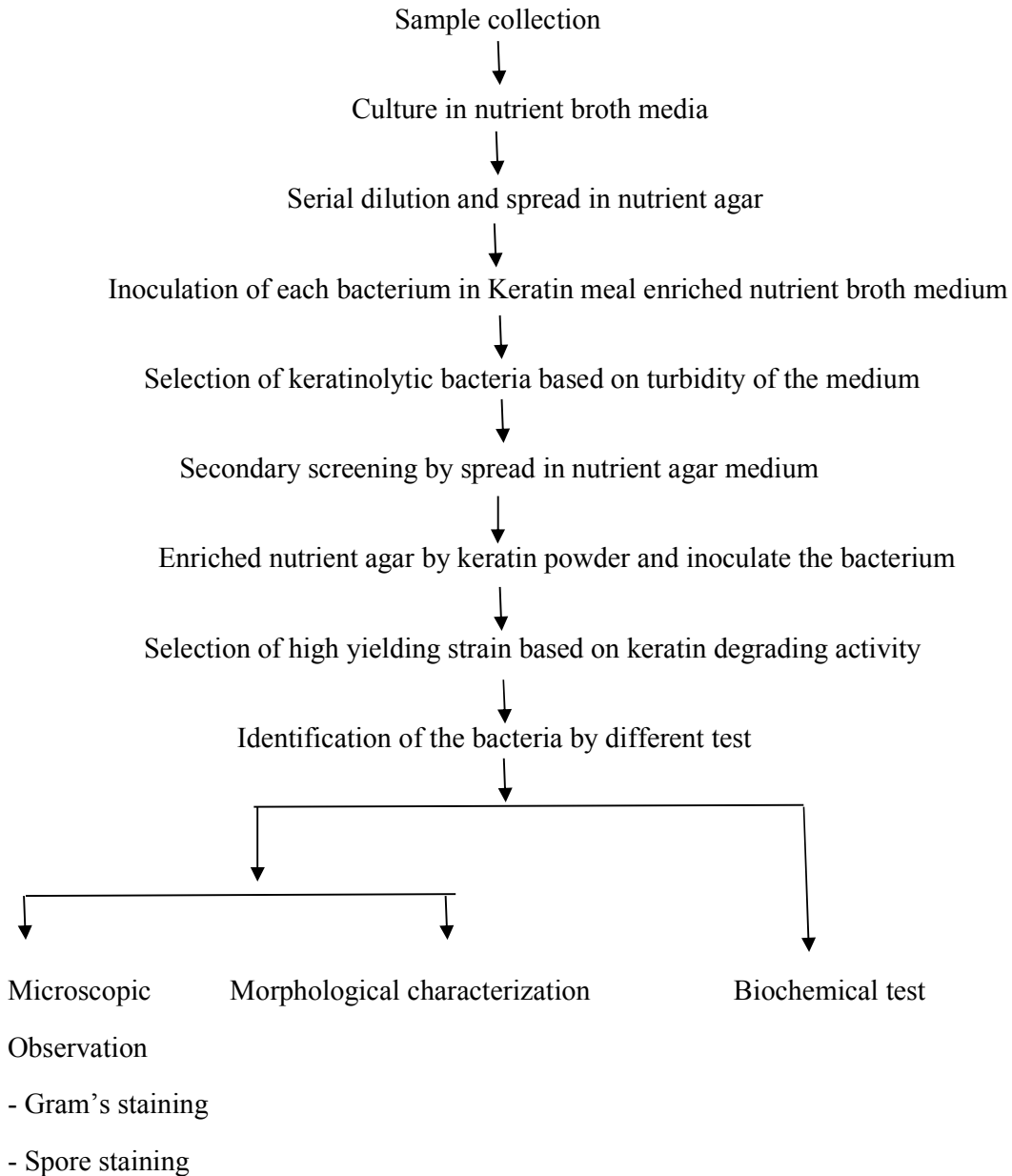
## Materials and method

## Materials and method

### 2.1 Place of Research:

The present study was carried out in the microbiology and biotechnology laboratory of the department of Mathematics and Natural sciences at BRAC University.

### 2.2 Flow Diagram of the Study Design



### 2.3 Handling of laboratory glassware and apparatus

Liquid detergents are used to wash all glassware's, rinsed with distilled water at least three times and allowed to air dry in dust free area. Before the use of the Petri dishes, they were dry heat sterilized at 160°C for two hours in the sterilizer (Oven, Model: MH6548SR, LG, China). Microcentrifuge tubes, Micropipette tips, and glass pipettes were sterilized by autoclaving at 121°C for 20 min at 15 psi (Wise Clave WAC-60, USA).

### 2.4 Specimen collection and Processing

The soil samples were collected from skin processing area Hazaribagh, Dhaka. Where the skin waste are dumped very close to sewage. Two soil samples were collected from the bank of the Rayer Bazar Lagoon and another two samples were collected from very close to sewage where dumped waste was composting (see fig 2,3) . These soil samples were kept into plastic zip lock bag by wearing hand gloves. These bags were entirely new. Sampling was done on 26 April 2017 when canal and lagoon had low water level and soil was almost dry. The sample was taken by stainless steel spatula after taking first samples and the second samples were collected 30 meters from the Tannery zone near Buriganga river.

### 2.5 Isolation and screening of keratinase producing bacteria:

The soil sample was collected from expected cows skin waste dumping site of Hazaribagh leather processing zone Dhaka. In sterilized sampling bags. The samples were brought to the laboratory and processed for analysis on a subsequent day. Two grams of soil taken from each sample finally total eight grams of soil suspended into the 10 ml sterile saline contained in a test tube. The saline sample was serially diluted to  $10^{-1}$  to  $10^{-8}$  fold after the test were labeled based on dilution number. These suspensions were reinoculated in nutrient agar media by spread plate technique. Only 100  $\mu$ l sample each suspension was taken. Inoculated nutrient agar media was incubated for growth at 37°C for 48 hours. Total eight nutrient agar plates were labeled  $10^1$  to  $10^8$  respectively and inoculated with the bacterial suspension. Two days later too numerous colonies were found in  $10^1$  to  $10^4$  labeled plate. Plate  $10^6$  had 21 separated colonies and  $10^7$  and  $10^8$  had only three colonies (Figure: 3) finally,  $10^6$  labelled plate was selected for isolation of keratinolytic

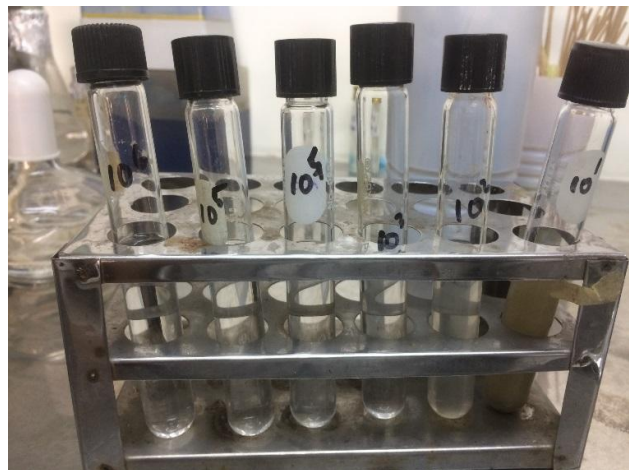


Figure 2.1 serial dilution  $10^1$  - $10^8$

bacteria. In addition to that nutrient broth enriched with keratin media was prepared, total eight 100 ml Erlenmeyer flask was labelled C1 to C8 and media were poured into it after that sample suspensions were added to them. Then eight bacterial colonies were separately inoculated individually into the media, incubated at 150 RPM rotary shaker incubator at 37 °C for 2 days. After 2 days in compared to control only c1 and c3 samples completely utilized keratin. Others are partially utilized, some are not degrade keratin.

## **2.6 Feather meal powder preparation:**

Skin hair, nail, horns poultry feathers were washed extensively by tap water, after that dried in open air, this mixer was pulverized and made powder further used as keratin meal.

## **2.7 Screening the keratinase positive bacteria**

Skim milk agar and feather meal agar plate were prepared for culturing Keratinase positive bacteria. The composition of skim milk agar medium was agar, 15 g/L for solidifying and casein enzymic hydrolysates, 5 g/L glucose monohydrate, 1 g/L skim milk powder, 1 g/L yeast extract, 2.5 g/L. The pH was adjusted to 7.4 by adding NaOH. Total 8 bacterial isolates were inoculated onto medium size petri dish and incubated at 37 °C for 48 hours. The clear zone formation indicates that isolates can degrade keratin, those are selected as keratinase producer. 10% trichloroacetic acid (TCA) was added to milk agar plate to observe clear zone. That clear zone is more distinguishable after adding this acid (Saran et al., 2007). Finally, it was further confirmed that the organism was keratinase producer by using feather meal powder in the medium instead of keratin. The isolates those produced clear zones on both media were considered as keratinase producers.

Initially, the primary selection was done based on their turbidity. Breakdown of keratin meal was increased with the increase of Keratinase and more turbid of the media. A control was set to compare their appearances. If the organism cannot degrade the keratin, our milk media would be intact as previously said keratinase enzyme is fallen under the group of serine or metalloproteases and these are predominantly extracellular enzymes produced by microorganisms only when basal medium contain keratinous substrates.( Gupta, P. Ramnani) hence, primary screening was done afterwards.

## **2.9 Identification of keratinase positive bacteria:**

Morphological and a range of biochemical tests were performed in order to identify the isolate. The isolate was identified based on appearance, biochemical characteristics morphological, as described in the Bergey's manual of systematic bacteriology.

## **2.10 Secondary screening:**

Ten grams of keratin feather were surface sterilized with ethanol (5%), washed thoroughly under running water, then air dried at 25°C room temperature under laminar airflow. The feather were autoclaved at 120°C for 15 min and then the selected microorganisms were inoculated in 200-ml Erlenmeyer flasks containing 100 ml LB medium and two gram keratin substrates. The flasks were inoculated with sample 1 organism.(see fig 8 and 9) with inoculation loop. The control contained only the basal mineral solution and keratin substrate, without any microorganism.

## **2.11 Morphological studies of isolated bacterial strains:**

Bacterial identification was conducted on morphological, physiological and biochemical tests. Results were compared with Bergey's Manual of Determinative Bacteriology, 8th edition (Buchanan and Gibbons, 1974). Genus *Bacillus*: Agriculture Handbook No. 427 (Gordon et al., 1973). The strain were also identified by chromogenic method on the *Bacillus* differential agar from Himedia, India, M1651 recommended for rapid identification of *Bacillus* species from a mixed culture. The medium contains peptic digest of animal tissues and meat extract, which provide nitrogenous compounds. Mannitol serves as the fermentable carbohydrate, fermentation of which can be detected by the pH indicator phenol red. Mannitol fermenting organisms like *B. megaterium* yield yellow coloured colonies, *B. thuringiensis* will grow as blue colonies and *B. pumilis* will also grow as green colonies on this medium. Results are summarized in Table 1. Growth determination of bacteria was done taking absorbance at 600 nm of bacterial growth media (Fig. 4) at regular intervals.

## **2.12 Keratinolytic activity of the purified keratinase on native keratinaceous materials:**

The keratinolytic activity of the crude enzyme on the previously prepared native powders of chicken feathers, human nails and human hair was determined by the method of Friedrich et al., (1999). The crude enzyme was incubated with the test substrate (4 mg/ml reaction mixture) at 57°C for 1 h. The reaction was stopped by adding 2 ml of 10% trichloroacetic acid (TCA) and



the samples were put in the refrigerator at 4 °C for 30 min. The reaction mixture was then centrifuged at 9000 rpm for 20 min in a cooling centrifuge (Hettich Zentrifugen, Universal 16/16 R). The absorption of the supernatant fluid was measured spectrophotometrically at 280 nm (Perkin–Elmer, Hitachi 200). Blanks were prepared in the same way except for the addition of TCA before the enzyme reaction. An increase of 0.1 in absorbency was taken to indicate one unit of enzyme activity. The obtained data represent the mean values of three determinations.

### **2.13 Maintenance of pure culture:**

The selected colonies subculture in nutrient agar medium, sealed with wax paper and incubated at 37°C for 24 hours and then stored at 4°C for further studies. (Immanuel et al., 2006).

### **2.14 Inoculum preparation**

The strains were cultivated on basic salt nutrient agar slants containing 10g at 500 ml pulverized chicken feather, horns, nail, hair incubated at 37 °C for five days. After incubation at 37 °C for 5 days, the culture medium were used for submerged and solid-state fermentation.

### **2.15 Submerged fermentation:**

The medium used for keratinase production by submerged fermentation contained the following composition 10 grams of feather, horns, nail, hair powder at pH 7.0. Cultivation was performed using 200 ml Erlenmeyer flasks containing 100 ml medium, five days incubation at 37 °C at different growth phases to check the production of enzymes. Successively, the cultures were filtered and used for enzymatic assays (Whatman 40 and Millipore 0.2 µm). The crude enzyme extract was developing from filtered culture supernatant. That was being used for advance enzyme activity study.

### **2.16 Solid-state fermentation**

Whole chicken feather, horns, nail, hair powder used for solid-state fermentation. 0.2 g of feather was mixed with 2 gram of a cellulose powder mixture taken in a 100 ml Erlenmeyer flask. After sterilization, whole media was inoculated and incubated at 37 °C for 5 days. Finally the whole media was filtered through a Whatman 40 filter paper and a Millipore 0.2 µm (Couri et al., 2000). The solid residue was separated from the crude enzymatic solution.

### **2.17 Preparation of keratin solution for enzymatic assay:**

Keratinolytic activity was measured with soluble keratin as substrate. Soluble keratin was prepared from white chicken feathers by the modified method of Wawrzekiewicz et al.(1987). Native chicken feathers was cut into small pieces and then to powder form and 8 gm of it in 50 ml of distilled H<sub>2</sub>O were heated in Erlenmeyer flask at 100 °C for 2 h. followed by vortexed for 2 min. Soluble keratin was then precipitated , The resulting precipitate was washed twice with distilled water and dried by Bunsen burner . One gram of keratin quantified was dissolved in 10 ml. The pH was adjusted to 7.5 with 0.1 mol/L Tris and 0.1 mol/L HCl and the solution was diluted to 100 ml with 0.05 mol/L Tris-HCl buffer (pH 7.5).

### **2.18 Preparation of crude enzyme**

Following five days of incubation in nutrient broth media, 200 ml of the fermented broth was centrifuged at 16000 rpm (Scan speed 1730R, Denmark) for 10 minutes in order to remove unwanted materials. After centrifugation the clear supernatant was collected. Finally, 1 ml of supernatant serve as crude enzyme source and utilized for determination of enzymatic activity.

### **2.19 Keratinolytic activity determination**

The keratinolytic activity was assayed as follows: One ml of crude enzyme properly diluted in Tris-HCl buffer (0.05 mol/L, pH 7.5) was incubated with 1 ml keratin solution at 50 °C in a water bath for 10 min, and the reaction was stopped by adding 2.0 ml 0.4 mol/L trichloroacetic acid (TCA). After centrifugation at 16000 RPM for 30 min, the absorbance of the supernatant was determined at 240 nm (Spectrophotometer, UV mini - 1240) against a control. The control was prepared by incubating the enzyme solution with 2.0 ml TCA with the addition of keratin solution. One unit (U/ml) of keratinolytic activity was defined as an increase of corrected absorbance of 240 nm (A<sub>240</sub>) (Gradišar et al., 2005) with the control for 0.01 per minute under the conditions described above and calculated by the following equation:

$$U=2 \times n \times A_{240} / (0.01 \times 10),$$

Where n is the dilution rate=1; 2 is the final reaction volume (ml); 10 is the incubation time (min).

## **Identification of the bacteria**

### **2.20 Morphological characterization of the bacteria**

Using sterile technique, an NA plate was streaked to obtain isolated discrete colonies. The plates were then incubated at 37°C for 24 hours. After incubation, the bacterial colonies were evaluated for size, pigmentation, form, margin, elevation, and texture (Cappuccino & Sherman, 2005).

### **2.21 Microscopic Observation of the bacteria**

The potential bacteria were observed under the microscope in order to study their properties stain their cell and spore were done.

### **2.22 Gram stain and Spore Stain**

Gram stain and spore staining were done following the manual of (Cappuccino & Sherman, 2005).

### **2.23 Biochemical characterization of the bacteria**

Several biochemical tests were carried out in order to have a presumptive identification of the potential bacteria chosen before. Most of the methods were done according to the microbiology laboratory manual (Cappuccino & Sherman, 2005). The biochemical tests performed were; Carbohydrate fermentation (Sucrose, fructose, glycerol, maltose and D-xylose), Triple sugar iron agar test, IMViC test (Indole production test, Methyl red test, Voges- Proskauer test, Citrate utilization test), Urease test, Nitrate reduction test, Catalase test, Oxidase test, Casein hydrolysis test, Motility test, Gelatin hydrolysis test, Mannitol Salt Agar, milk agar test, kertain hydrolysis, Starch hydrolysis, growth at 25°C, 65°C and 7% NaCl media and anaerobic growth.

### **2.24 Hichrome agar appearance:**

hi, chrome agar is a chromogenic differential medium for identification, differentiation, and conformation of enteric bacteria from specimens such as urine, water, or food which may contain a large number of *Proteus* species as well as potentially pathogenic Gram-positive organisms. Based on these characteristics, hI ChromoAgar is suggested for use in place of MacConkey Agar.

### **2.25 Carbohydrate Utilization test**

Phenol red sucrose, fructose, glycerol, maltose and D-xylose broths were prepared by autoclaving at 15 psi 121°C for 15 minutes (Autoclave, SAARC) in separate test tubes. Using sterile technique, a small amount of the experimental bacteria from 24-hour pure culture was inoculated into the broths by means of loop inoculation. All the tubes were incubated for 24 hours at 37°C (Cappuccino & Sherman, 2005).

### **2.26 Triple Sugar Iron Agar test**

Triple sugar iron slants were prepared in the test tubes and autoclaved at 15 psi 121°C. Using sterile technique, a small amount of the experimental bacteria from 24-hour old pure culture was inoculated into the tubes by means of a stab and streak inoculation method. The tubes were incubated for 24 hours at 37°C (Cappuccino & Sherman, 2005).

### **2.27 Indole Production test**

Tryptophan broth of 5 ml in each test tube was prepared by autoclaving at 15 psi, 121°C. Using sterile technique, small amount of the experimental bacteria from 24-hour pure culture was inoculated into the tubes and the tubes were incubated for 48 hours at 37°C. In order to test for indole production, 5 drops of Kovac's reagent were added directly into the tubes (MacWilliams, 2009).

### **2.28 Methyl red test**

The MR-VP broth of 7 ml in each test tubes was prepared by autoclaving at 15 psi 121°C. Using sterile technique, a small amount of the experimental bacteria from 24-hour pure culture was inoculated into the tubes and the tubes were incubated for 24 hours at 37°C. After 24 hours 3.5 ml of the culture tubes were transferred to clean test tubes for Voges- Proskauer test and the remaining broth were re-incubated for additional 24 hours. After 48-hour incubation 5 drops of methyl red indicator was added directly into the remaining aliquot of the culture tubes to observe the immediate development of a red Colour. (Cappuccino & Sherman, 2005)

### **2.29 Voges Proskauer test**

To the aliquot of MR-VP broth after 24-hour incubation, 0.6 ml (12 drops) of 5% alpha-naphthol (Barrit's reagent A) was added followed by 0.2 ml ( 4 drops) of 40% KOH (Barrit's reagent B). The tube was gently shaken to expose the medium to atmospheric oxygen (30seconds to 1 minute) and the medium was allowed to remain undisturbed for 10-15 minutes. The test was read but not beyond, one hour following the addition of the reagents (McDevitt, 2009).

### **2.30 Citrate utilization test**

Simmons citrate agar slants of 2 ml in each vial were prepared by autoclaving at 15 psi, 121°C. Using sterile technique, small amount of the experimental bacteria from 24-hour pure culture

was inoculated into the vials by means of a streak inoculation method with an inoculating needle and the vials were incubated for 48 hours at 37°C (Cappuccino & Sherman, 2005).

### **2.31 MIU (Motility- Indole- Urease) test**

MIU media was prepared by autoclaving at 15 psi, 121°C and the media was cooled to about 50- 55°C and 100 ml of urease reagent was added aseptically to 900 ml base medium. After that, the 6 ml solution was transferred to each sterile test tube and allowed to form a semi-solid medium. Using sterile technique, a small amount of the experimental bacteria from 24-hour pure culture was inoculated into the tubes by means of a stab inoculation method with an inoculating needle and the tubes were then incubated for 24 hours at 37°C (Acharya, 2015).

### **2.32 Nitrate reduction test**

Nitrate broth of 6 ml in each test tubes was prepared by autoclaving at 15 psi, 121°C. Using sterile technique, a small amount of the experimental bacteria from 24-hour pure culture was inoculated into the tubes by means of a loop inoculation method with an inoculating loop and the tubes were incubated for 24 to 48 hours at 37°C. After incubation, 5 drops of reagent A and Five drops of reagent B was added to each broth. If there was no red colour development, a small amount of zinc was added to each broth (Cappuccino & Sherman, 2005).

Note: Caution was maintained during the use of powdered zinc since it is hazardous.

### **2.33 Catalase test**

A microscopic slide was placed in a petri dish. Using a sterile inoculating loop, a few bacteria from 24-hour pure culture was placed on the microscopic slide. 1 drop of 3% H<sub>2</sub>O<sub>2</sub> was placed onto the organism on the microscopic slide using a dropper and observed for immediate bubble formation (Reiner, 2010).

### **2.34 Oxidase test**

A small piece of filter paper was soaked in Gaby and Hadley oxidase test reagent and let dry. Using an inoculating loop, a well-isolated colony from pure 24-hour culture was picked and rubbed onto filter paper and observed for colour change (Shields & Cathcart, 2010).

### **2.35 Gelatin hydrolysis test**

All the ingredients of the nutrient gelatin medium were mixed and gently heated to dissolve. 3 ml from the media was dispensed in glass vials. The glass vials with the medium were then autoclaved at 121°C, 15 psi. The tubed medium was allowed to cool in an upright position before use. Using sterile technique, a heavy inoculum of 24-hour old culture bacteria was stab inoculated into the tubes with an inoculating needle. The glass vials were then incubated at 37°C and observed up to 1 week (Cruz & Torres, 2012).

### **2.36. Mannitol Salt Agar test**

Using sterile technique, a plate of MSA agar was streaked by picking a loopful colony of 24-hour old pure culture to obtain isolated colonies. The plates were then incubated at 37°C for 24 hours (Shields & Tsang, 2013).

### **2.37 Starch hydrolysis test**

Using sterile technique, a starch agar plate was streaked by picking a loopful colony of 24-hour old pure culture with an inoculating loop. The plates were then incubated at 37°C for 48 hours and the hydrolysis was observed using gram's iodine (Cappuccino & Sherman, 2005).

### **2.38 Casein hydrolysis test**

Distilled water and agar solution was taken in separate conical flasks and both were autoclaved at 121°C, 15 psi. Skim milk powder (28 g/L) was then added to the autoclaved distilled water aseptically and boiled for 1 minute to dissolve completely. After that, the milk solution was mixed with agar solution. The media was dispensed into sterile plates while liquid and left for a while to solidify. Using sterile technique, a milk agar plate was inoculated by 24-hour old culture by means of streak plate method. The plates were then incubated at 37°C for 24 hours (Sturm, 2013).

# Chapter 3

## Results

### 3.1 Results:

In this study one bacteria was selected to study its keratinase activity. The result for the identification and characterization of isolated organisms are shown in Table 3.1.

**Table 3.1: Biochemical test result.**

Tests		
Morphology	Rods	Yellow
Gram staining		Positive
Endospores		Blue spore
Motility		Motile
Growth in pH	5	Slow Grow
	6	
	7	Grow
Growth at temperature	25°C	Grow
	30°C	Grow
	37 °C	Fastest Grow
Growth in NaCl	2%	Good growth
	5%	Quite grow
	7%	Less growth
Growth in carbohydrates	Glucose	yes
	Sucrose	yes
	Mannitol	no
	Citrate	yes
Hydrolysis of	Starch	yes
	Caesin	yes
	Gelatin	yes
Biochemical tests	Lipid test	+ve
	Catalase	-ve
	VP test	-ve
	Nitrate reduction	-ve
	Indole	-ve
	Methyl red	+ve
	citrate utilization	+ve
	H <sub>2</sub> S	-ve
	Oxidase test	-ve
	Urease test	-ve
	Gelatin Liquefaction	-ve
	Lactose	-ve
	Glucose	+ve
	Sucrose	-ve
	dextrose	-ve
	TSI	-ve

Morphological and biochemical test results shown in table 3.1 indicates that the organism is *Bacillus licheniformis* (Ref: ABIS online software).



### 3.2 Cultural characteristics

The well isolated colonies on the nutrient agar plates were evaluated in the following manner:

Parameters	Results shown by the isolate C1
Size	Moderate
Pigmentation	No pigmentation (Opaque and Cream Colour)
Form	Circular (unbroken, peripheral edge)
Margin	Undulate (wavy indentations)
Elevation	Convex (valley- shaped elevation)
Texture	Muroid (with an underlying muroid matrix)

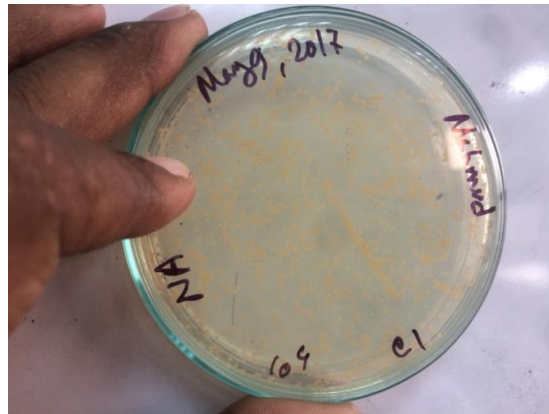


Fig 3.1: Organism in nutrient agar medium

### 3.3 Microscopic observation

#### Gram stain

The cells were observed under light microscope (Kruss, Germany). The cells were found to be gram positive. fig 3.2.



Figure 3.2: Cells under light microscope

### 3.4 Result Gram stain see fig 3.2.

#### Gram staining

Cell shape: Cylindrical (rod)

Cell arrangement	Single <i>Bacillus</i>
------------------	------------------------

Cell Colour:	Purple
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Gram reaction:	Positive
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### 3.5 Spore stain

The cells were observed under light microscope (Kruss, Germany). The addition of  $MnSO_4 \cdot H_2O$  in the nutrient agar media stimulated the sporulation creating nutrient deficient condition for the bacteria. Spores were visible under immersion oil field.

Result of spore stain (fig in 3.3).

Colour of spores:	Blue
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Colour of vegetative cells	: Purple
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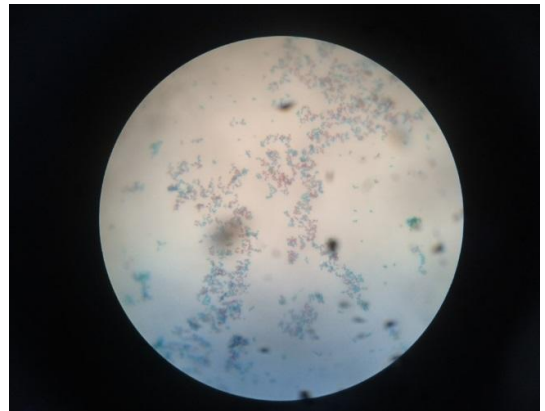


Figure: 3.3 Spore stained bacteria under light microscope

### 3.6 Results shown in different media:

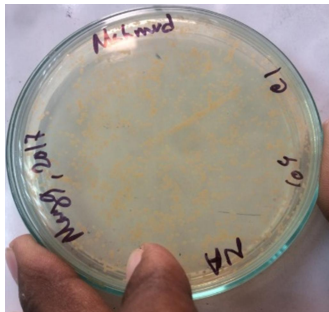


Figure 3.4: Organism in NA medium



Figure 3.5: Organism in Nutrient broth medium

**Culture in Agar medium:** The unknown organism in nutrient agar medium (fig 3.4) that appeared as cream colour.

**Nutrient broth enriched with Keratin:** organism degraded keratin thus media became turbid.

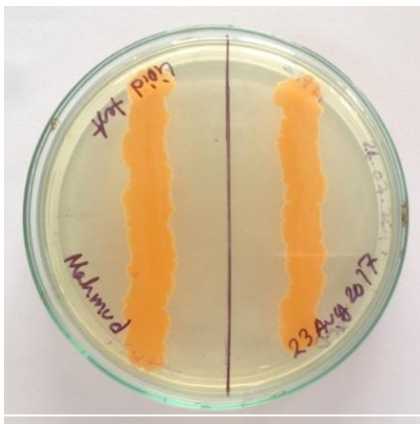


Figure 3.6: organism in Tributyrin agar

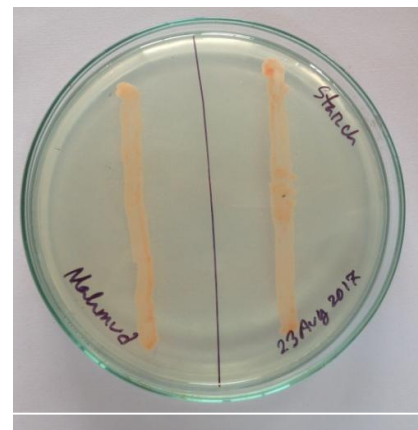


Figure 3.7: organism in Starch

**Lipid test result:** Organism cannot lyse lipid. So result was negative.

**Starch test result:** this organism that were not able to hydrolyze starch. So result was negative.

#### Nitrate test:

Negative result found.



Figure 3.8: Addition of nitrate reagent. Nitrate negative



Figure 3.9: After the addition of Zinc. So result was nitrate negative



Figure 3.10: Organism in Simmons citrate

#### Citrate test result :

This is a positive result for the citrate test.as the colour turned blue.



Figure 3.11: Lactose test :( control and sample)

**Lactose test:**

The inoculated tube was incubated our test organism unable to utilize lactose, so result was negative.



Figure 3.12: Dextrose test control and sample

**Dextrose test:** The organism was unable to ferment dextrose as sole source of carbon.



Figure 3.13: Sucrose test: Control and sample

**Sucrose test:**

The organism was unable to ferment sucrose so result was negative



Figure 3.14: Gelatin test result

**Gelatin liquefaction Test:**

After 48 hour of incubation at 37° C examined the tubes, No gelatin was liquefied so the result was negative.



Figure 3.15:TSI test both:(control and sample)

**TSI test:** TSI negative.



Figure 3.16: Indole test both control and sample

**Indole test:**

This organism was indole negative.



Figure 3.17: VP test both control and sample

**VP test:**

Organism did not produce cherry red Colour.  
So VP negative.



Figure 3.18: Appearance in Hichrome agar

**Appearance in Hichrome agar:** Selected organism cultured in Hichrome agar. The organism appeared as yellow.



Figure 3.19: MSA result

**Bacterial growth in MSA plate:**

The media Colour remain same. So  
MSA negative



Figure 3.20: MR test both control and

**Methyl red test:**

The formation of red colour after the addition of Methyl red reagent indicates the accumulation of acidic end products in the medium and is an indicative of MR positive test.



### 3.7.1 Keratin degraded test:

Nutrient broth medium along with feather was autoclaved and C1 organism was inoculated to it. Another flask was not inoculated with organism both were incubated at 37°C for 5 days at 150 RPM Control remained the feather but sample 1 can degrade all feather. Media became turbid. Fig 3.21 A control figure 3.21B was test sample. Feather was found to have degraded in the flask inoculated by the organism.



Figure 3.21 A: Control

Figure 3.21 B: sample

### 3.7.2 Feather meal powder (Keratin) degrading assay:

One drop of crude enzyme was put into the feather meal media that we prepared in the lab, in four different section, Clear zone formation indicates that enzyme can degrade keratin. So this test conclude that this enzyme was keratinase(Cai et al., 2008) Figure 3.22.

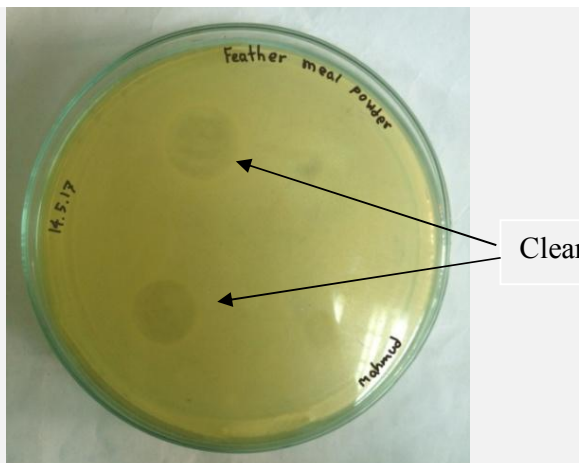


Figure 3.22: Clear zone in the media.

### 3.7.3 Keratinase producing organism screening:

The isolates C1 formed more clear zone after incubation for 2 days at 37°C The clear zone formation indicates that the isolate can produce keratinase, among them C1 was selected as best keratinase producer (figure 3.23).

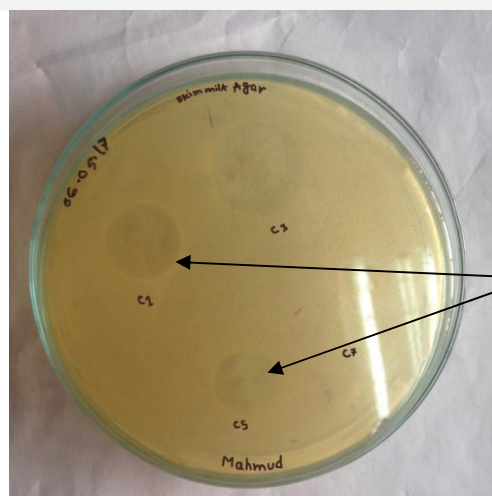


Figure 3.23: C1,C3,C5 and C7 organism in skim milk agar plate

### 3.8 Effect of pH in enzymatic activity:

To determine the enzyme activity at different pH, 0.25 ml of crude enzyme and 1.75 ml of keratin substrate was mixed together and incubated at 40°C for 10 minute, later the reaction was stopped by using 0.4 M 2 ml trichloroacetic acid (TCA). The optimum pH for enzyme activity was determined by incubating the reaction mixture at various pH, ranged from 5 to 10 in manual shaking water bath for 10 minute at 40 °C in 0.05 M Tris–HCl buffer solution. Then the solution was allowed to cool to the room temperature. The absorbance of each of the sample was measured at 280 nm in a spectrophotometer. A curve was prepared with pH versus absorbance. Keratin breakdown was increased with the increase of enzyme concentration. The pH was adjusted by using 0.1 N HCl or 0.1 N NaOH. More the enzyme activity more breakdown of keratin and tyrosine liberated into the media thus increased the turbidity of the media (Cupp-Enyard, C. 2008). The amino acid tyrosine was liberated into the media and absorbance also increased. The optimum pH was 7.7. Figure 3.24 and table 3.2.

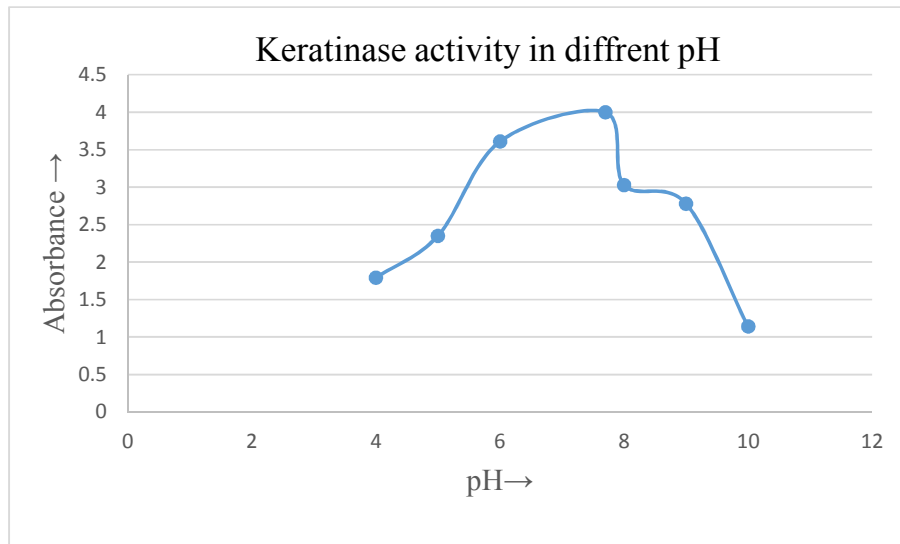


Fig 3.24: Tyrosine absorbance in different pH

Table 3.2: pH-absorbance data.

pH	Absorbance
4	1.79
5	2.35
6	3.61
7.7	4.00
8	3.03
9	2.78

### 3.9 Effect of temperature on enzymatic activity:

To determine the enzyme activity at different temperature 0.25 ml of crude enzyme and 1.75 ml of keratin substrate was mixed together and incubated at different temperature for 10 min, later reaction was stopped by using 0.4 M 2ml trichloroacetic acid (TCA).

By using 0.1 N HCl ( or 0.1 N NaOH) pH was adjusted to 7.7. Then the absorbance of each of the sample was measured at 280 nm in a spectrophotometer. A curve was prepared to plot the result into the graph. The more was the enzyme activity the more was the breakdown of keratin and the increased tyrosine discharged into the media. As a result, the turbidity of the media had increased (Fig 3.25 and table 3.3).

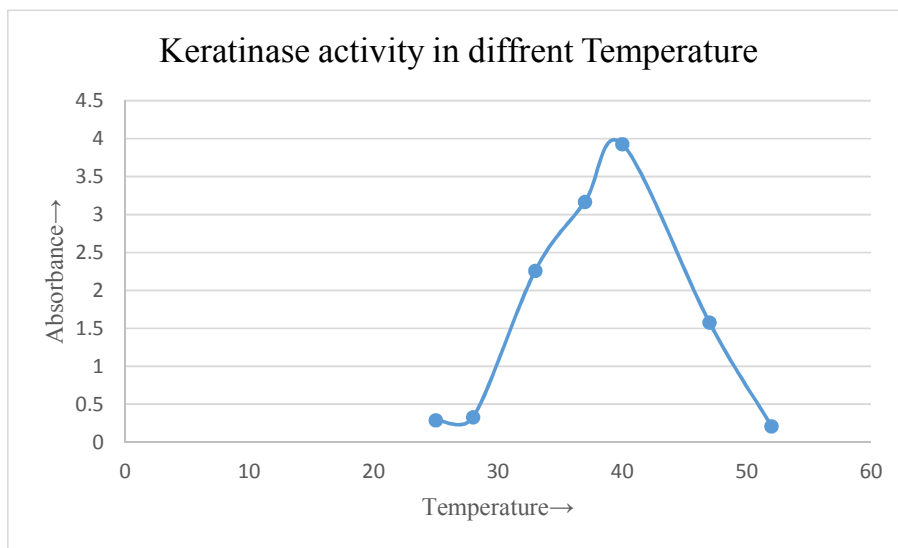


Fig3.25: Enzymatic activity at different temperature.

Table 3.3: Absorbance at different Temperature.

Temperature	Absorbance
25	0.29
28	0.33
33	2.26
37	3.17
40	3.93
47	1.58
52	0.21



### 3.10 Effect of Enzyme concentration in enzyme activity:

To determine the enzyme activity at different crude keratinase concentration, distilled water was used to dilute the crude keratinase and the final volume was accurately measured to 0.25ml ( table 3 is given below). 1.75 ml of keratin substrates was mixed together and incubated at 37°C for 10 minutes after that reaction was stopped by using 0.4 M 2ml trichloroacetic acid (TCA). At the same time, pH of the mixture was adjusted to 7.7 by using 0.1 N HCl or 0.1 N NaOH.

The absorbance of each of the sample was then measured at 280 nm in a spectrophotometer.

A curve was prepared plotting the result into graph. More the enzyme concentration more was the breakdown of keratin and tyrosine liberated into the media thus increased turbidity of the media. More amino acid tyrosine was liberated into the media more the absorbance was increased (Figure 3 and table 3). Breakdown of keratin increased with the increase of enzyme concentration

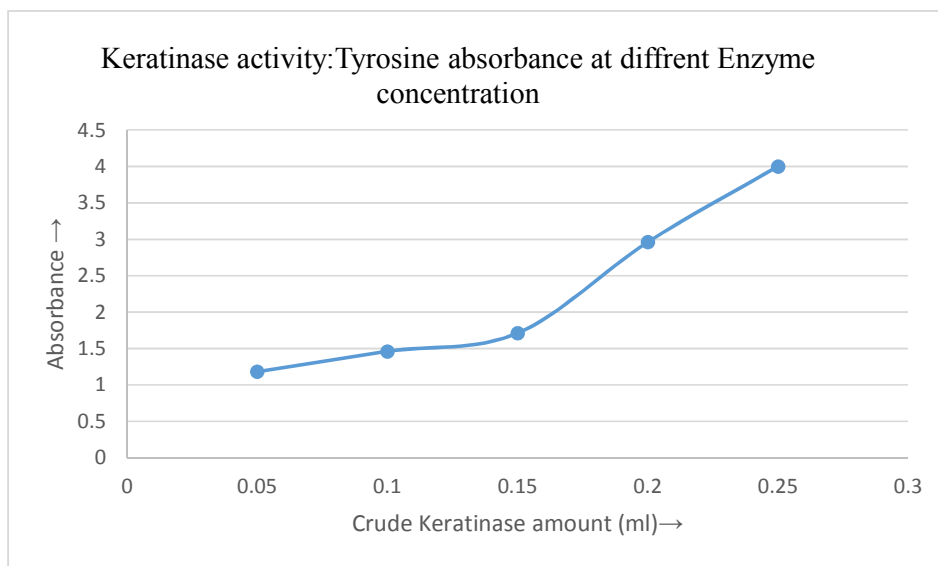


Fig 3.26: Graph Enzymatic activity in different concentration

Table 3.4: Absorbance and solution volume

Enzyme amount in (ml)	Distilled water added in (ml)	Absorbance
0.25	0	4.00
0.2	0.05	2.96
0.15	0.10	1.71
0.1	0.15	1.46
0.05	0.20	1.18

# **Chapter 4**

## **Discussion**

## 4.1 Discussion

In the present study, clear zone forming bacteria were isolated from the collected soil samples and screened for keratinase producing capability. Larger the zone formation more is the keratinase production. The C1 isolate was added to the raw chicken feather containing the nutrient broth samples which were kept at and its keratin degrading activity was measured by measuring the turbidity at room temperature so the keratin degrading ability was observed. This keratin activity can be observed through Scanning electron micrograph (SEM) image it is also a useful tool to detect keratinase activity by observing keratin texture and physical structure (Cai et al., 2008).

However, the bacterial isolate was gram-positive and was *Bacillus licheniformis*. Keratin degradation was executed by gram-positive bacteria in most cases, (Gupta and Ramnani 2006). It was also found in previous study that keratinases are produced by *Bacillus sp.* (Kumar et al., 2008). *Bacillus licheniformis* has been reported as better keratinase producer (Shih et al., 1992). Thus *Bacillus sp.* appears to be a potential candidate for keratinase production.

Microbial keratinase is an inducible enzyme. The isolated *Bacillus licheniformis* produced maximum keratinase when keratinous protein elements were present in the media. Similar result was found for *Bacillus subtilis* that gave the highest keratinase production in presence 20% feather powder and also achieved the maximum keratinase production in presence of 10% feather meal with *Bacillus sp.* The production of keratinase depends on the presence of keratin and its concentration. Enzyme production might be declined in the presence of higher concentration of feather meal indicating catabolic suppression (Saibabu et al., 2013).

Yeast extract as an organic nitrogen source and potassium nitrate as inorganic nitrogen sources gave the maximum amount of enzyme production. It has been reported previously, maximum keratinase production was achieved in yeast extract supplemented media as organic nitrogen source (Sivakumar et al., 2013). In each case, a lower amount of keratinase production was achieved from peptone containing media. Temperature, pH and other culture parameters play a vital role in enzyme production. Hence, optimum enzyme production by *Bacillus sp.* was found at 37 °C. Kate and Pethe (2014) reported that maximum keratinase production was achieved by *Bacillus licheniformis* at 37 °C. The higher enzyme production was also found at 35 °C for 24 h by *Bacillus sp.* The maximum temperature for keratinase production was recorded at 40 °C for some other keratinase positive bacteria such as *Bacillus subtilis* and *Bacillus pumilis* in the previous result (Suh et al., 2001). The pH of the media affects the reaction environment, enzymatic process and transport of nutrients across the cell membrane of bacteria. Maximum production takes place when a suitable pH in culture media was maintained. The optimum pH for keratinase production by *Bacillus sp.* Some *Bacillus* strain was observed at pH 7.0. A

similar finding was achieved from *Bacillus* by (Kate and Pethe). The bacterium had a neutral pH range for keratinase production. The maximum enzyme production was also found at moderate pH by others that support the present study (Matikeviciene et al., 2011; Kim et al., 2001).

In the present investigation, different inoculum volumes were tested for the production of keratinase. The production was found to increase with increasing size of inoculum and found to be optimal at 5%. Further increase in the inoculum size, greatly decreased the production might be due to rapid growth of bacteria and depletion of essential nutrients by bacteria in the early stages. Normally higher shaking rates (150–180 rpm) provided good growth of bacteria with possibly low keratinase production due to high dissolved oxygen. In contrast, substrates and bacterial cells were not well mixed at low shaking rate (100 rpm) and produced heterogeneous formation and lower dissolved oxygen meaning in low keratinase production. The optimum activity of the keratinase enzyme was found at pH 7.7 alongside temperature of 37 °C. Similarly, preferred pH 8.0 for the activity of keratinase has been reported by others (Deivasigamani and Alagappan 2008; Inamdar et al., 2012; Saranya et al., 2015). The extracellular crude keratinases exhibited more than 50% activity at pH 7.0. Keratinases from most bacteria have optimum pH ranging from neutral to alkaline (7.5–9.0) (Meng et al., 2013) reported keratinase exhibits its optimum activity at 40 °C. Usually, keratinase positive bacteria such as *B.subtilis* exhibited optimal production at temperatures ranging from 30 to 50 °C (Mazotto et al., 2011). So the properties of this enzyme increase the probability in the industrial application at high temperature. *Bacillus licheniformis* sp. might be a good candidate for keratinase production and may be employed for feather-degrading and dehairing purposes. Further studies should be carried out to purify and fully characterize the enzyme and to determine the sequence of this keratinase gene for future improvement for industrial application through genetic engineering approaches.

# **Chapter 5**

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## Appendix-I

### Media

#### composition

Composition soft himedia used in this study are provided below. The media were auto claved at 121°C for 15 min at 121psi.

#### 1. Nutrient Agar(HiMedia, India)

Ingredients	Amounts (g/L)
Peptic digest of animal tissue	5.0
Beef extract	1.5
Sodium chloride	5.0
Yeast extract	1.5
Agar	15.0

#### 2. Mueller-Hinton Agar(HiMedia, India)

Ingredients	Amounts (g/L)
Beef infusion	300
Casamino acids	17.5
Starch	1.5
Agar	17.0

## Appendix– II

### Reagents

The reagents used in the above procedures were made using the following compositions:

<b>LB medium</b>	1%tryptone 0.5%yeast extract 200 mMNaCl
<b>TE buffer</b>	10 mMTris-Cl (pH8.0) 1 mM EDTA(pH8.0)
<b>Lysis buffer</b>	(10 ml) 9.34 ml TE buffer 600 µl of 10%SDS 60 µl of proteinaseK (20mgml <sup>-1</sup> )
<b>TBE buffer (1x)</b>	5.4 gTris-HCl 2.75 g Boric acid 2ml0.5M EDTA Adjust volumewith distilled water pH: 8.0

Appendix – III

**Instruments**

Autoclave	Wisd Laboratory Instruments Made in Korea
WaterBath WiseBath <sup>®</sup>	Wisd Laboratory Instruments DAIHAN Scientific Co., Ltd Made in Korea
Shaking Incubator	Model:JSSI-1000C JSRESEARCH INC. Made in Rep. of Korea
Incubator	Model:DSI3000 Digisystem Laboratory Instruments Inc. Made in Taiwan
VortexMixer	Model:VM-2000 Digisystem Laboratory Instruments Inc. Made in Taiwan
Table Top Centrifuge	Model:DSC-200A-2 Digisystem Laboratory Instruments Inc. Made in Taiwan
ElectronicBalance	RADWAGWagi ELEktroniczne Model: WTB200
Refrigerator (4 <sup>0</sup> C)	Model:0636 Samsung