SCREENING AND CHARACTERIZATION KERATINASE ENZYME FROM BACTERIA IN KERATINOUS WAST

MSc THESIS

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Production of Bacterial Keratinase from Keratinous Waste

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By

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As thesis research advisors, we hereby certify that we have read and evaluated the thesis entitled **"Production of Bacterial Karatinase from Keratinous Waste"** and prepared, under our guidance, by Yimeslal Atinafu and we recommend that it be submitted as fulfilling the thesis requirement.

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DEDICATION

I dedicate this thesis to my beloved family, who had given me the best advices in all respects to the success of my life.

STATEMENT OF AUTHOR

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LIST OF ABBREVIATIONS AND ACRONYMS

AA	Amino acid
BSA	Bovine serum albumin
DMSO	Dimethylsulfoxide
FC	Folin ciocalteau
Min	Minute
OD	Optical density
RPM	Revolution per minute
SMF	Submerged fermentation
SSF	Solid state fermentation
TCA	Trichloroacetic acid
TSI	Triple sugar iron
UV	Ultraviolet

TABLE OF CONTENTS

Continued

Continued

LIST OF TABLES

LIST OF FIGURES

Figure
page

LIST OF TABLES IN THE APPENDIX

Appendix Table
Page

LIST OF FIGURES IN THE APPENDIX

Appendix Figure

Page

Production of Bacterial Keratinase from Keratinous Waste

ABSTRACT

Microbial keratinases are keratinolytic enzymes widely used in many industrial processes and management of wastes. This study was conducted with the aim of isolation of efficient keratinase producing bacteria form the soils of chicken feather-dumping sites and human hair-dumping places, determining optimum keratinase production conditions and partially characterizing the stability and activity of keratinase with regards to some physicochemical parameters. Plating of soil sample suspensions on chicken feather and human hair modified meal agar media resulted in the growth of three keratinolytic bacterial isolates (Kf1and Kf2 from poultry farm and Kh from hair cut dumping site) that were later identified as Bacillus species on the basis of their biochemical characteristics. The optimum temperature of keratinase production for all isolates was recorded at 60 ^{0}C with activities of 16.4 U/ml/min, 15.1U/ml/min and 12.3 U/ml/min for isolates Kf1, Kf2 and Kh, respectively. In all cases, pH 7 was optimum for keratinase production resulting in activities corresponding to 16.4 U/ml/min, 15.1U/ml/min and 12.4/ml/min for Kf1, Kf2 and Kh, respectively. Among the various carbon sources tested, potato gave the activities 14.3U/ml/min, 12.3 U/ml/min and 10.1U/ml/min for isolates Kf1, Kf2, and Kh, respectively. Regarding nitrogen sources, Yeast Extract gave maximum activities corresponding to 25.2 U/ml/min, 20.5 U/ml/min and 16.37 U/ml/min for Kf1, Kf2, and Kh, respectively. Studies on the effect of pH on the activity and stability of keratinase enzyme revealed that the crude enzyme had a maximum stability at pH7 for all isolates with activities of 15.2 U/ml/min, 13.2 U/ml/min, and 11U/ml/min for Kf1, Kf2 and Kh isolates, respectively. The maximum keratinase stability time for the three isolates was found to be 48 hours with keratinase activity of 8.5U/ml/min, 6.5U/ml/min and 5.9 U/ml/min for Kf1, Kf2, and Kh . These results generally indicate that the keratinase obtained in this study belong to the class of hydrolases that are active and stable at neutral pH conditions. These enzymes were also less active and less stable at 80^{0}C for all isolates. Thus identification of the three Bacillus isolates at a molecular level and the purification as well as the detailed characterization of the types of keratinase is recommended for effective utilization in animal feed processing and other industrial applications.

Keywords: Bacillus, hydrolase, keratin, keratinase production, physico-chemical parameters.

1. INTRODUCTION

Microbial keratinase is an enzyme capable of degrading the insoluble structural protein called keratin. Keratin is a fibrous structural protein of hair, nails, horn, hoofs, wool, feathers, and of the epithelial cells in the outermost layers of the skin (Cai *et al*., 2008).

Keratin proteins are the major constituents of epidermal structures, whose function is connected with mechanical protection of skin surface. Keratin of epidermal stratum corneum is listed among "soft" cytoskeletal keratins. Its fibers build the largest group of intermediate filaments in cytoskeleton of epithelial cells, whose function exceed simple mechanical support and are associated with cell-to-cell communication, as well as cell cycle and apoptotic mechanisms (Coulombe and Omary, 2002).

Keratin present in feathers and human hair cannot be degraded by the usual proteolytic enzymes such as trypsin, pepsin and papain. This is because of the differences in composition and molecular conformation of the amino acids found in keratin (Janaranjani *et al*., 2015). Several strains of microorganisms produce intracellular and extracellular enzymes in their regular metabolism which have wide commercial applications. Animal wastes can be used as a powerful nutrient source for the growth of microorganism. But wastes like feathers and hair obtained as byproducts of meat and dairy processing cannot be degraded easily and hence they form a source of pollution. There are certain strains of microorganisms which produce extracellular enzymes which can serve as a substantial source of keratinase for the degradation of keratinous wastes (Adriano**,** 2007)**.**

Keratinases belong to a class of hydrolases. These are metallo-proteins and efficient proteolytic enzymes. The enzyme keratinase is a potential enzyme for removing hair and feathers in the tannery and poultry industry. A number of keratinolytic microorganisms have been reported including *Microsporum, Aspergillus, Bacillus*, *Streptomyces* and other Actinomycetes. Keratinase production from microorganisms is affected by media composition including carbon and nitrogen sources and their concentrations. Media containing substrates such as raw feather, feather meal,

powdered chicken nails, and bovine hair or wool have been reported to be suitable for isolation and growth optimization of keratinase producing microorganisms (Riffel *et al*., 2002).

In recent years, feather-treated with microbial keratinase is attracting wide attention with several applications. Keratinase–treated feather is considered as a viable source of dietary protein in food and feed supplements, as the enzyme-treated end product is thought to retain high nutritive value. Keratinases are projected to generate a potential worldwide market similar to other proteases. Keratinolytic enzymes have several current and potential applications in agro industrial, pharmaceutical, and biomedical fields. Their use in biomass conversion into biofuels may address the increasing concern on energy conservation and recycling (Andriano *et al.***,** 2009).

Keratinase enzymes are useful in the bio-conversion of keratin waste to feed and fertilizers. Other promising applications of keratinolytic enzymes include enzymatic deharing in leather and cosmetic industry, and detergent uses. The use of keratinase to enhance drug delivery in some tissues and hydrolysis of prion proteins arise as outstanding applications for enzymes (Adriano and Brandelli**,** 2007).

In Ethiopia isolation of protease was conducted by Gessesse *et al.*, (2011). This report described the isolation of *Bacillus* species AR009 and *B. pseudofirmus* AL-89 from an alkaline soda lake in Ethiopia, Rift Valley Area. Gizachew Haile and Amare Gessesse(2012) also done on a research on keratinase protease who were isolated a total 240 alkalophilic bacteria from Chitu, Ethiopian Soda Lake and they checked those bacilli bacteria on keratinolytic activity on cow hair .Though these two investigators checked keratinolytic activity of their isolates on cow hair , they did not evaluated the activity of the isolates against feather and human hair which are wastes. It is therefore crucial to continue the efforts of searching for potent isolates in the environment because there are plenty of potential applications of keratinase in the food industry, animal feed processing and management of wastes of various industrial activities and municipal household garbage wastes (Gessesse *et al*., 2011).

General objectives

To evaluate the *in-vitro* production of Keratinase from bacterial species grown on keratineous waste (chicken feather and human hair).

Specific objectives

- To isolate bacterial species from keratinous waste.
- To test keratinolytic activity of each isolates.
- To identify the preferred carbon and nitrogen sources required for production of keratinase.
- To identify the isolates using appropriate biochemical tests.
- To determine the physico-chemical parameters (temperature, pH, and concentrations of carbon and nitrogen) required for optimum production of keratinase under laboratory conditions.
- To evaluate the effect of different physicochemical factors on the activity and stability of keratinase (time, temperature and pH).

2. LITRATURE REVIEW

2.1. Enzymes

Enzymes are the biological substance or biological macromolecules that are produced by a living organism which acts as a catalyst to bring about a specific biochemical reaction. These are like the chemical catalysts in a chemical reaction which helps to accelerate the biological/biochemical reactions inside as well as outside the cell. These are generally known as "Biocatalyst." In 1877, Wilhelm Friedrich K¨uhne a professor of physiology at the University of Heidelberg first used the term enzyme. Even many centuries ago, enzyme and its use were well known to the mankind (Neelam *et al*., 2013).

Enzymes are very specific that this was because both the enzyme and the substrate to be catalyzed possess specific complementary geometric shapes that fit exactly into one another (Emil Fischer, 1894). This complementarities is often referred to as "the lock and key"model. However, while this model explains enzyme specificity, it fails to explain the enzymes are much larger than the substrates they act on, and only a small portion of the enzyme (around 2–4 amino acids) is directly involved in catalysis (Kirk *et al*., 2002). The region that contains these catalytic residues, binds the substrate, and then carries out the reaction is known as the active site. Enzymes can also contain sites that bind cofactors, which are needed for catalysis. Some enzymes also have binding sites for small molecules, which are often direct or indirect products or substrates of the reaction catalyzed. This binding can serve to increase or decrease the enzyme's activity, providing a means for feedback regulation. Like all proteins, enzymes are long, linear chains of amino acids that fold to produce a three dimensional product. Each unique amino acid sequence produces a specific structure, which has unique properties (Powers, 2006).

Individual protein chains may sometimes group together to form a protein complex. Most enzymes can be denatured, that is, unfolded and inactivated by heating or chemical denaturants, which disrupt the three dimensional structure of the protein. Due to their wide range of activities

based on their nature of reaction enzymes are being classified according to the chemical reactions they catalyze. The Enzyme Commission number (EC number) is a numerical classification scheme for enzymes, based on the chemical reactions they catalyze. As a system of enzyme nomenclature, every EC number is associated with a recommended name for the respective enzyme. Except for some of the originally studied enzymes such as pepsin, rennin, and trypsin, most enzyme names end in "ase." The enzyme nomenclature scheme was developed starting in 1955, when the International Congress of Biochemistry in Brussels set up an Enzyme Commission. The first version was published in 1961. The current sixth edition, published by the International Union of Biochemistry and Molecular Biology in 1992, contains 3196 different enzymes. The International Union of Biochemistry (I.U.B.) initiated standards of enzyme nomenclature which recommends that enzyme names indicate both the substrate acted upon and the type of reaction catalyzed. According to the enzyme commission, the enzymes are divided into 6 parts (Webb, 1992).

(i) Oxidoreductase (EC 1),

(ii) Transferase (EC 2),

(iii) Hydrolase (EC 3),

(iv) Lyase (EC 4),

(v) Isomerase (EC 5),

(vi) Ligase (EC 6).

2.1.1. Proteases

Proteases are the most important industrial enzymes of interest accounting for about 60% of the total enzymes market in the world and approximately 40% of the total worldwide enzyme sale (Chouyyok *et al.*, 2005). They are generally used in detergents (Barindra *et al.*, 2006), food industries, leather, meat processing, cheese making, silver recovery from photographic film, and certain medical treatments of inflammation and wounds. They also have other medical and pharmaceutical applications. Microbial proteases are derivative enzymes, which catalyze the total hydrolysis of proteins. The molecular weight of proteases ranges from 18 – 90 kDa. These enzymes are found in a wide diversity of sources such as plants, animals and microorganisms but they are mainly produced by bacteria and fungi (Paranthaman *et al.*, 2009).

2.1.2. Keratinases

Keratinases are key protease enzymes, they hydrolyse both 'soft' (cytoskeletal materials in epithelial tissues, containing up to 1% sulphur) and 'hard' (protective tissues in hairs and nails, containing up to 5% sulphur) keratins. Hence, in the past few decades, a number of research projects have focused on the activities of keratinases. The potential of keratinases in the biotechnological context has gained substantial and significant recognition since the beginning of the 21st Century: their substrate specificity and ability to attack highly cross-linked and recalcitrant structural proteins that resist common known proteolytic enzymes, such as trypsin and pepsin, make them valuable biocatalysts in industries that deal with keratineous materials (Karthikeyan *et al.*, 2007).

Keratinases are proteolytic enzymes that can hydrolyse keratins. Microbial keratinases are predominantly of the metallo, serine or serine-metallo type (Brandelli, 2008) with the exception of keratinase from yeast which belongs to aspartic protease (Negi *et al.,* 1984). Both metallo and serine peptidases are endoproteases that cleave peptide bonds internally within a polypeptide. Metalloproteases are highly diverse, having more than 90 families. A common feature of this type of enzyme is the involvement of a divalent ion (such as Zn^{2+}) for their catalytic activities which are inhibited by metal chelating agents, transition or heavy metals (Gupta and Ramnani 2006). Serine proteases fall into two broad categories based on their structure: chymotrypsin-like (trypsin-like) or subtilisin-like. The subtilisin subfamily is completely inhibited by PMSF (phenylmethane sulfonylfluoride), antipain and chymostatin (Tyndall *et al*., 2005).

2.1.3. Sources of Microbial Keratinases

Keratin degraders can be found among diverse groups of microorganisms: from fungi to bacteria. These microorganisms are frequently isolated from keratin-rich environments such as soil and waste water associated with the poultry industry and tannery (Monod *et al*., 2002). However, due to the potential risk of infection, biotechnological applications of these fungi have not been widely explored. A number of Gram-positive and Gram negative bacteria are also found to be important

keratinase producers. From the Gram positive category, members of the *Bacillus* genus are the most prominent and prolific keratin degraders (Brandelli *et al*., 2010).

2.1.4. Keratinous Substrates and their Specificities

Keratinases from fungi, actinomycetes and bacteria have a wide range of substrates: from soft keratin such as stratum corneum to hard keratin such as feather keratin (Mazotto *et al.*, 2013), sheep's wool (Farag and Hassan, 2004), human and animal hairs (Chen *et al*., 2011), nail, hoof and horn and azokeratin. Other substrates that are susceptible to keratinase degradation include: collagen, elastin, gelatine (Tork *et al.*, 2013), albumin and haemoglobin. The substrate specificity of keratinases is strongly influenced by the chemical properties of their substrates. As keratin is composed of 50-60% hydrophobic and aromatic amino acids (Gradišar *et al.*, 2005; Brandelli *et al.*, 2010), keratinases appear to cleave preferentially hydrophobic and aromatic amino acid residues at the P1 position (Gupta *et al.*, 2013a).

2.2. Mechanism of Keratinolysis

Over the years, a number of hypotheses have been proposed to explain the mechanism of keratin degradation by microbial keratinases (Korniłłowicz-Kowalskaand Bohacz, 2011). Broadly speaking, it is agreed that keratin degradation encompasses two main stages: deamination and keratinolysis. Deamination creates an alkaline environment for optimal enzymatic reaction by the alkaline proteases. The complex mechanism of keratinolysis that follows involves the cooperative action of sulphitolytic and proteolytic enzymes noted for their degradation activities on natural keratin substrates by purified keratinase from *Bacillus* sp. Sulphitolysis changes the conformation of keratin and exposes more active sites, making them accessible for further digestion by alkaline protease and resulting in the release of soluble peptides and amino acids (Monod, 2008).

It is noted that purified keratinases are generally less effective in hydrolyzing native keratin, probably due to the removal of disulphide bond reduction components during the purification process .A suitable redox environment may be necessary for effective degradation of keratin. The presence of reducing agents stimulates keratin hydrolysis by purified keratinase. In a cell-bound redox system, the bacterial cells probably provide a continuous supply of reductant (e.g. sulphite) to break disulfide bridges (Fang *et al*, 2013).

2.3. Optimization of Keratinase Production

Production of keratinase from a commercial perspective requires an integrated approach that combines optimal fermentation conditions, operational optimization and effective downstream processing. Medium composition and culture conditions are the two important factors that affect the yield of an enzyme in a fermentation process. The keratin source usually serves as the sole carbon and nitrogen sources in a growth medium. The addition of separate carbon and nitrogen sources has been shown to increase enzyme production in some microorganisms (Brandelli *et al*., 2010) but suppress production in others. It is suggested that as each microorganism has its own optimal set of growth parameters; these conditions should be treated on a case-by-case basis (Cai and Zheng, 2009). The most significant parameters that affect keratinase production can be investigated using a one factor-at-a-time method (Tiwary and Gupta, 2010).

2.4. Established Applications of Keratinases

The ability of microbial keratinases to degrade keratin and other recalcitrant materials holds much biotechnological potential and has generated a significant amount of research interest in the last couple of decades. One of the earliest reviews on the biotechnological applications of keratinases, written by Onifade *et al.,* (1998) documented the potential of these enzymes in producing livestock feeds. Subsequently, other potential biotechnological applications of keratinases have been identified. A number of excellent reviews have extensively examined the use of keratinases in the waste management industry, agro industry, pharmaceutical and biomedical industries, leather and bioenergy industries (Onifade *et al.,* 1998).

2.4.1. Keratinous Waste Management

Feathers are produced in large amount as waste by poultry product processing plants; it has reached millions of tons per year worldwide (Fernandes, 2010). They can be degraded by keratinolytic bacteria. A number of keratinolytic microorganisms have been reported, including some species of bacteria such as *Bacillus, Streptomyces* and other actinomycetes; fungi such as *Microsporum and Trichophyton* (Essien *et al.,* 2009).

A large number of keratinous wastes are generated every year mainly from poultry production and processing, as well as leather and textile industries (Suzuki *et al,* 2006).

Approximately 8.5 million metric tones of poultry waste was produced worldwide annually; India contributes about 3.5 million tones, the United States 1.8 million tones and the United Kingdom 1.5 million tones. Livestock and poultry farms and slaughter houses also produce a significant number of keratinous wastes in the form of feather, bristles, hair, down, horns and hooves. Keratinous wastes can be processed and used for livestock, pet and fish food, and for composting where organic keratinous wastes are ultimately degraded and converted to inorganic nitrogen (ammonium and nitrate) and sulphurs (sulphates) that can be easily absorbed by plants. Nevertheless, the rate of degradation in compost may be slow due to the recalcitrant nature of keratins and their resistance to normal proteolytic enzymes. Within the compost, the succession is dominated by bacteria and actinomycetes during the first two to four weeks of composting; this is then gradually replaced by fungi (Okoroma *et al.*, 2012).

The addition of keratinase producing microorganisms as inoculums could, in theory, accelerate and enhance the process. Adding *B. licheniformi s*and a *Streptomyces* sp. isolated from the plumage of wild birds to compost bio-reaction vessels had shown that the bacteria-soaked feathers were degraded more quickly and more completely than the controls (Nayaka and Vidyasagar, 2013). The same authors also demonstrated that the addition of *Streptomyces albus* helped to enhance the degradation of chicken feather compost and the release of valuable byproducts acceptable in land use applications. However, Tiquia *et al*., (2005) failed to observe significant changes in the rate of feather degradation when *B. licheniformis* (OWU 1411T) and *Streptomyces* sp. (OWU 1441) were used in co-composted poultry litter and straw. The microbial community structure over time was also found to be very similar in inoculated and uninoculated waste feather composts (Tiquia *et al.*, 2005).

2.4.2. Animal Feed and Feed Supplements

Feather waste contains large amounts of amino acids such as cystine, glycine, arginine and phenylalanine (Onifade *et al.*, 1998) but they have to be hydrolyzed to release these valuable amino acids. The processing methods commonly employed to hydrolyse feather waste include thermal, chemical and enzymatic treatments. The use of keratinases or keratinolytic microorganisms in the treatment of feather meal overcomes some of the limitations posed by thermal and chemical treatments. Keratinase PWD1 was found to improve the digestibility of keratin and significantly enhanced the growth of poultry. The application of *Kocuria rosea* in the

production of feather meal has shown to improve the digestibility of the fermented products; increase the lysine, histidine and methionine content and boost the availability of these amino acids. The commercial products Versazyme produced by BBI and Cibenza DP100™ by Novus International have been marketed as additives to feed to improve their nutritional values. The nutritional values of animal feeds can also be enriched by the introduction of a hydrolysate supplement, produced by keratinolytic microorganisms (Papadopoulos, 1985).

2.4.3. Leather and Textile Industry

Leather processing involves three major processes: pre-tanning (beam-house operation) where hides or skins are cleaned using sodium sulphate (Na_2SO_4) and lime; tanning where the leather materials are stabilised with chromium sulphate ($CrSO_4$), solvent and lime; and post-tanning and finishing where aesthetic value is added. During the conventional lime-sulphide dehairing process, large amount of Na_2SO_4 is involved and the waste generated by this operation causes serious environmental and waste disposal problems. The use of kertinlolytic microorganisms with good de-hairing action has been hailed as a promising and viable alternative to chemical de-hairing (Dettmer *et al.*, 2013).

Enzymatic depilation generally only requires small quantities of Na_2SO_4 and could be an eco-friendly alternative to the chemical process. Keratinases from *B. subtilis* and *Trichoderma harzianum* could even be applied in the absence of Na_2SO_4. Thus the use of a keratinase-assisted tanning process can significantly reduce the impact of dehairing waste in the environment. Keratinases produced by a number of *Bacillus* strains also have important applications in the textile industry. A number of microbial keratinases including those from: *B. licheniforms, B. cereus, Chryseobacterium* and *Pseudomonas spare* able to improve felt-shrink resistance and dyeing characteristics with no loss of fiber weight. It is reported that keratinase acting in combination with other enzymes such as cutinase, lipase and trans-gultaminase can be used to further improve the wool processing (Lv *et al*., 2010).

A number of consumer products have been known to involve keratinases, from formulation of detergents to personal care products such as shampoo, cosmetics and acne treatment (Brandelli *et al.*, 2010; Gupta *et al.*, 2013b).

2.4.4. Consumer Products

A number of consumer products have been known to involve keratinases, from formulation of detergents to personal care products such as shampoo, cosmetics and acne treatment (Brandelli *et al.*, 2010; Gupta *et al*, 2013b).

2.4.5. Detergent

Due to their substrate specificity, keratinases can clean within a short period of time without damaging the fibre strength and structure (Paul *et al* 2014) and a number of keratinases are shown to be capable at hydrolysing keratinous materials that fix on soiled collars and cuffs. The alkaline keratinase of *Paenibacillus woosongensis* TKB2 is effective at removing blood stains from surgical garments and composite stains of blood, egg yolk and chocolate from conventional clothes in a short period without changing the texture of the cloth and cloth fibres. Similarly, keratinase of *B. thuringiensis* TS2 are also effective in the removal of blood and egg stains as well as depilation of goat hide (Sivakumar *et al*, 2013). Another application of keratinases in the detergent industry involves their uses in cleaning up drains that are clogged with keratinous waste and keratineous dirt associated with laundry (Brandelli, 2008).

2.4.6. Biological Control

The potential for keratinases to act as a biological control agent has been explored by several research groups recently. Keratinase produced by *Stenotrophomonas maltophilia* R13 is effective against several fungal pathogens including *Fusarium solani*, *F. oxysprum*, *Mucor* sp. And *A.nigar* that cause diseases in valuable plants and crops (Jeong *et al*, 2010). Similarly, keratinase produced by *Thermoactinomyces* also showed antifungal properties against these plant pathogens. Yue *et al.,* (2011) reported that the keratinase produced by *Bacillus* sp. has the ability to work effectively against agricultural pests such as toot-knot nematodes (*Meloidogyne incognita)*. In insects, the tracheae are found on the exoskeleton and each tracheal tube is lined with a thin strip of cuticle called the *taenidia* which reinforces the tracheae to maintain the structure of the tracheal walls. As insect tracheal taenidia contains a protein similar to the vertebrate keratins (Yue *et al*, 2011) this protein may present a possible target for keratinase

hydrolysis to control harmful insects such as mosquitoes that are the major vectors of a number of serious tropical diseases (Gousterova *et al*, 2012).

The use of two recombinant baculoviruses containing the *ScathL*gene from *Sarcophagacylindric*(vSynScathL) and the keratinase gene from *Aspergillus fumigates* (vSynKerat) has been successful in destroying the larvae of an agricultural pest, *Spodoptera frugiperda*, by degrading extracellular matrix proteins and interfering with the phenoloxidase activity of the insect host. Tangentially, keratinase hydrolysate can be used as a substrate for pesticide production. Poultry waste is a low-cost and effective substrate to cultivate *Bacillus sphaericus* and *B. thuringiensis* sero var*israelensis* to produce mosquito-cidal toxin (Gramkow *et al*, 2010).

2.4.7. Green Energy

Conversion of keratinous waste into bio-fuel is a promising application to generate green energy that may address some of the global demand for energy. In a two-step formation process, keratineous waste was first hydrolysed by *B. lichenifomis* and the hydrolysate was subsequently utilised by *Thermococcus litoralis* to produce bio-hydrogen gas (Bálint *et al*, 2005. Production of methane can also be achieved by combining the biological degradation of keratin-rich waste with keratinase in an anaerobic digester. Chicken feather waste pre-treated with a recombinant *B. megaterium* strain showing keratinase activity prior to biogas production, was able to produce methane (Forgács *et al.*, 2013).

3. MATERIALS AND METHODS

3.1. Description of the Study Area

The experiment was conducted at the School of Biological Sciences and Biotechnology, Haramaya University, Oromia Region, Ethiopia. Haramaya University is located at $43^{0}3$'E longitude, $9^{0}26$'N latitude at an altitude of 900m above sea level and 510 km east of Addis Ababa, the capital city of Ethiopia (Agricultural and Rural Development Office of Haramaya District, 2006).

3.2. Research Design

The design of the research involved a laboratory based study of isolation, screening, biochemical characterization of bacterial isolates. And also the performance of bacterial isolates in terms of keratinase production and its stability of the activity were done. The xperiments were conducted first by collecting two soil samples from different two keratinous duping sites. The first was from poultry dumping area and the second soil sample was taken from human hair dumping sites .Those two sites were found in the campus of Harmaya University. All experiments were done in triplicates and their values were recorded and used for subsequent analyses (Daniel *et al.*, 2014).

3.3. Soil Sample Collection

Soil samples were collected from poultry feather and human hair cut dumping sites at Haramaya University. The samples were collected using sterile plastic bags and stored in the laboratory until used for isolation of bacteria.

3.4. Preparation of Feather Meal and Human Hair Cuts Meal

The feather and human hair cuts meal were prepared from native chicken feathers and human hair collected from poultry farm and barber's shop at HU, respectively, as described by Tork *et al.,* (2008) with slight modifications. The feathers and human hair cuts were further broken into smaller pieces of 2 cm long with scissors and 5gm of each was washed several times with tap water. Defatting of feather and hair pieces was done by soaking in 250ml mixture of pure chloroform: methanol (1:1) for 2 days followed by treatment with chloroform: acetone: methanol (4:1:3) solution for 2 days. The solvent was replaced every day. The feathers and hairs were finally washed several times with tap water to eliminate the solvent, dried for 24 h in sunlight, ground using an electrically operated blender and used as feather and human hair cuts powder.

3.5. Isolation, screening, and identification of keratinase Producing Bacteria

3.5.1. Isolation of bacteria from soil

Isolation of bacteria was performed by serial dilution and plating method on nutrient agar medium. One gram of soil sample was transferred to 10 ml of sterilized distilled water and mixed properly. Serial dilution was made up to 10^{-6}. The diluted sample (0.1 ml) was inoculated by spreading on to nutrient agar media plates from each dilution. The Petri plates were rotated clockwise and anticlockwise to spread the samples uniformly. Plates were incubated at 37ºC for 24 to 48 hours. The bacterial isolates were further sub cultured on nutrient agar media to obtain pure culture. Pure isolates were maintained in nutrient agar slants at 4ºC for further studies (Harison and Sandeep, 2014).

3.5.2. Screening of keratinase producing bacteria

About 0.1 ml of the pure culture of each isolate was uniformly spread plated on the surface of an agar medium supplemented with finely ground and powdered chicken feather and human hair cuts as a sole carbon and nitrogen source separately. That is, isolates obtained from poultry site was spread on modified chicken feather agar, whereas the isolate from soil samples collected from human hair cut dumping sites were spread on modified human hair cuts meal agar. Then colonies

that appeared on the media were picked and sub-cultured on nutrient agar (Harison and Sandeep, 2014).

The modified feather meal agar contained the following ingredients ($g.L^{-1}$): NaCl (0.5), KH_2PO_4 (0.7), K_2HPO_4 (1.4), $MgSO_4$ (0.1) and feather meal (10). The modified human hair cuts meal agar contained all the above-listed ingredients except that the feather meal was replaced with human hair cuts meal. The pH of the medium was adjusted to 7 using sodium carbonate in order to isolate alkalophilic microorganisms. The plates were incubated at 37°C till the colonies appeared. The presence of keratinase producing bacteria was indicated by growth and formation of colonies on modified chicken feather meal agar or on human hair cuts meal agar media. Colonies on such media were sub-cultured and pure cultures were maintained on nutrient agar plate slants at 4°C (Shabaan et al., 2014).

3.6. Preparation of Keratin Solution

Soluble keratins were prepared from human hair cuts and chicken feathers separately. For all 5gm of native chicken feathers and 5gm human hair, separately mixed with 250 ml of dimethylsulfoxide (DMSO) and heated in a reflux condenser at 100°C for 2 h. Soluble Keratin of each was precipitated by addition of chilled acetone at −20°C for 2 h, followed by cooling and centrifugation at 10000 rpm for 10 min. The resulting precipitate was washed twice with distilled water and dried at 40°C in a hot air oven. The keratin solution was prepared by dissolving 1gm of soluble keratin in 20 ml of 0.05 M NaOH, Adjusting the pH to 7 with 0.1 M HCl and diluting the resulting solution to 200 ml with 0.05 M phosphate buffer pH 7(Vigneshwaran *et al.*, 2010).

3.7. Production of Crude Keratinase using the Bacterial Isolates

Keratinase was produced by employing submerged fermentation using two types of sterile media. The first one was prepared from chicken feather meal (1%), yeast extract (0.01%), NaCl (0.05%), KH_2PO4 (0.03%), K_2HPO4 (0.04%) and $MgCl_2$ (0.01%), at a pH of 7. And the second media composed of human hair cuts meal (1%), yeast extract (0.01%), NaCl (0.05%), KH_2PO4

(0.03%), K_2HPO4 (0.04%) and $MgCl_2$ (0.01%), at a pH of 7. These media were inoculated by 2-3 inocula taken from pure cultures of isolates, shaked in rotary shaker for 3hours and incubated for 3 days at 37℃. After incubation, portions of the broth cultures were centrifuged at 10,000 rpm for 10 min and the supernatants were used as source of crude enzymes (Lin and Yin, 2010). The amount of keratinase was estimated using the keratinolytic (keratinase) assay method described in section 3.8.

3.8. Assay for Keratinolytic Activity

The keratinolytic activity was assayed by the modified method of Cheng *et al.,*(1995) by incubating 1.0 ml of crude enzyme which was diluted in 0.05 M Tris-HCl buffer (pH 8.0) with 1 ml of 1% soluble keratin solution at 50℃ in a water bath for 20 min. The reaction was stopped by adding 2.0 ml of 0.4 M trichloroacetic acid (TCA). The precipitate formed by the addition of TCA was removed by centrifugation at 10,000 rpm for 10 min. After centrifugation, 0.2 ml of the supernatant was taken and diluted with 1.0 ml of distilled water. To the resulting dilution, 5.0 ml of alkaline copper reagent was added, shaken well, and incubated for 10 min. After incubation, 0.5 ml of Folin Ciocalteau (FC) reagent was added and incubated again in the dark for 30 min to allow blue color development. The control was prepared by incubating enzyme solution with 2 ml of TCA without the addition of keratin solution. The absorbance was measured at 660 nm using a UV-spectrophotometer. One unit of alkaline keratinase was defined as the amount of enzyme required to liberate 1 µg of tyrosine per min per ml under the standard assay conditions (Vigneshwaran *et al.*, 2010).

3.9. Assay for Proteolytic Activity

Proteolysis refers to the excretion of proteases by the bacterial cells during growth. Proteolytic activity of the isolates was determined by hydrolysis of gelatin that was due to the growth the three bacterial isolates on gelatin media became liquid at room temperature for positive result. But for negative result the media remains solid (Thiery and Frachon, 1997).

3.10. Determination of the Amount of Protein

The amount of protein present in the fermentation sample was assayed by the Lowry method using bovine serum albumin (BSA) as standard. The blue color developed was read using a spectrophotometer at 660 nm (Lowry *et al*, 1951).

3.11. Determination of the Percentage of Keratin Degradation (PKD) by Isolated Bacteria

For studying the bio-degradation of different keratinous materials, the keratinous wastes (chicken feather and human hair) were fragmented into pieces of about 1cm long and added to the fermentation media as a sole source of carbon and nitrogen. These sources were added separately to the fermentation media at 1% w/v. The percentage of keratinous waste degradation was determined following the method used by Harison and Sandeep (2014). The residual keratinous waste obtained at the maximum growth of the bacterial isolate was washed, dried and measured to calculate PKD using the following equation: PKD (%) = TKW-RKW ×100/TKW; where, TKW is the total keratinous waste and RKW is the residual keratinous waste (Kim *et al.*, 2001).

3.12. Effect of Physico-chemical Parameters on Keratinase Production

3.12.1. Effect of pH, temperature, and concentration of substrate on keratinase production

The effect of three different physicochemical parameters, i.e. pH, temperature, and substrate concentration, on keratinase production was assessed for the isolate. The effect of pH and temperature on keratinase production were individually tested by adjusting the production media to different pH values (pH 5, 6, 7, 8, 9 and 10) and incubating to varying temperatures (50^0C, 60^0C, 70^0C,and 80 ^{0}C). Similarly, the effect of substrate concentration on the production of keratinase was assessed by growing the isolates in media containing different concentrations of feather meal and human hair as a substrate (2mg/ml, 4mg/ml, 6mg/ml, 8mg/ml and 10mg/ml) were

used. These fermentation media were assayed every day for keratinase production till a decline is observed in the enzyme activity (Saibabu *et al.*, 2013).

3.12.2. Effect and types of carbon and nitrogen source on the production of keratinase

Wheat flour, corn flour, sorghum flour, and potato were used as carbon source in place of glucose to find out their effect on keratinase production. The cultures were incubated at 37^{O}C and pH 7 for 48 h and the sources of carbon used in the production medium were varied by replacing glucose with the above-mentioned substrates (Akcan, 2012)

The effect of different nitrogen sources were tested by adding nitrogen-containing substrates such as tryptone, peptone, and yeast extract in the production medium, the amount to be added was found on each container. The fermentation media having the three isolates in different flask were incubated at 37^{O}C and pH 7 for 48 h and tested for keratinase production (Venkata *et al*., 2013).

3.12.3. Effect of pH, temperature and incubation time on keratinolytic activity and stability

The optimal pH for keratinolytic activity was determined over a pH range of 5, 6, 7, 8, 9, and 10. To determine the pH stability, partially purified enzyme was pre-incubated in buffers over a pH range of 5,6, 7,8,9 and 10 at 50°C for 60 min. Citric acid/sodium phosphate buffer was used for pH between 5 and 7; barbital/HCl for pH between 8 and 9 and sodium carbonate buffer for pH between 10. To determine the thermo-stability, the enzymes were pre-incubated in barbital buffer (pH 7) at temperatures over the range of 50, 60, 70 and 80°C (Minghai, 2012).

The time required to attain the maximum enzymes activity was optimized for each isolate. To do this, each isolate was inoculated into chicken feather and human hair cuts meal media and incubated at 37°C. The keratinase activity was measured at regular intervals for 5 days (24h, 48h, 72h, 96h, and 120h. From the graph, the time required to attain the highest enzyme production was determined (Anitha and Eswari, 2012).

3.13. Biochemical Characterization

The isolates were identified using: a) their cultural characteristics such as colony morphology with respect to shape, texture of colony and pigmentation b) microscopic observation of the isolates

under the high power magnifying lens of the compound light microscope after Gram staining. Motility tests were also performed to observe the morphology and motility of the cells and biochemical characteristics as described in Bergey's Manual of Systematic Bacteriology (Sneath *et al.*, 1986).

The isolates were biochemically characterized using catalase, carbohydrate fermentation, oxidase, starch hydrolysis, motility, indole production, methyl red, Vogas-Proskauer, citrate utilization, triple sugar iron, nitrate reduction and amino acid utilization tests for the purpose of preliminary identification of the isolates.

3.13.1. Catalase test

Two loop-full of the isolates' culture was mixed into a solution having 3% hydrogen peroxide in the test tube. The formation of bubbles displayed a positive test indicating the presence of the enzyme catalase. If no gas was produced, the isolate was considered as negative for the test (Mohsin *et al*, 2014).

3.13.2. Carbohydrate fermentation test

Carbohydrate fermentation test (test for glucose, sucrose, lactose fermentation) was carried out for each isolate under anaerobic condition in which a Durham tube was placed in an inverted position to trap the gas bubble formed due to production of gas. The fermentation broth contains ingredients of nutrient broth, a specific carbohydrate (lactose , glucose and sucrose separately) and a pH indicator (phenol red), which is red at neutral pH (pH 7) and turns yellow below a pH of 6.8 due to the production of an organic acid (Mohsin and Veena, 2014). The isolates showing color change from pink to yellow with accompanying production of gas bubble in the Durham's tube indicated that the sugars (glucose, sucrose, and lactose) were fermented **(**Tariq *et al.*, 2016).

3.13.3. Oxidase test

A piece of filter paper was placed in a sterile Petri dish, on to which a bacterial culture was placed using a swab. A few drops of tetra methyl-p-phenylenediamine were then added on the culture with the help of a sterile dropper. For positive result bluish color and for negative results colorless were observed within few seconds (Jackie, 2012).

3.13.4. Citrate test

In a sterile test tube, Simmons citrate agar was added and kept it in a slant position till the agar solidified. Then, a pure bacterial culture was streaked on the slant using a sterile cotton swab. The results were recorded after incubating the slant for 24 hours at 37ºC. The isolates were positive if they showed that blue color (Samuel *et al.*, 2012).

3.13.5. Starch hydrolysis test

The pure bacterial culture were inoculated on starch-containing solidified agar plate and incubated at 37ºC for 48 hours. After incubation, iodine solution was flooded on the plate. Results were observed in 10 minutes, for positive result clear zone were observed indicates that the isolates were hydrolyzed the starch (Bhaskara and Arthi, 2012).

3.13.6. Indole test

An inoculums taken from a pure culture of an isolate was added into a sterile peptone broth and incubated for 48 hours at 37ºC. Four-five drops of Kovacs Reagent (sulfanilic acid and α-naphthylamine) were added to the broth and gently shaked. Results were observed during the addition of α-naphthylamine**.** The results were positive if formation of a pink to red color in the reagent layer on top of the medium within seconds of adding the reagent and negative if no color change even after the addition of reagent.

3.13.7. Lactose utilization test

Phenol red lactose broth was transferred to a sterile test tube and inoculated with the bacterial culture. The culture were incubated at 37ºC for 24 hours and then observed for a change to yellow color which shows the fermentation of lactose (Sarita and Neeraj, 2010).

3.13.8. Methyl red

A sterile MR-VP broth was inoculated with pure culture of isolates using a sterile loop. The broth was then incubated for 48 hours at 37ºC. Following this, 5 drops of methyl red was added to the broth and the result was observed without mixing. The results were positive if formation of a pink

to red color in the reagent layer on top of the medium within 10-15 seconds of adding the reagent (methyl red) and negative if yellow color observed.

3.13.9. H_2S formation and motility test

Pure culture of each isolate was transferred aseptically to TSI agar slant. The resulting culture was incubated for 24 hours at 37ºC. After the incubation, the slant was observed for color change (Janaranjani *et al.*, 2015). Bacterial motility was observed directly from the examination of the culture tubes containing the semi-solid media of the nutrient agar and casein substrate. In the semi-solid medium, the growth of motile bacterial isolates was spread out from the line of inoculation while the growth of non-motile microorganisms occurred only along the stab line. And also for H_2S formation black color was detected (Murray *et al.*, 2007).

3.14. Data Analysis

All data gathered in this study were subjected to statistical analysis using SPSS 20 . In addition, the degradability and enzyme activities were analyzed by taking the mean values of triplicates. Microsoft office excels work sheet 2007 was used for the analysis of generated data and the preparation of graphs.

4. RESULTS AND DISCUSSION

4.1. Isolation and Screening of Keratinase Producing Bacteria

The purpose of this study was to isolate bacterial isolates which degrade human hair cut and chicken feather keratin. Soil samples were collected from poultry and human hair dump yards in Haramaya University. Three bacterial isolates (i.e. Kf1& Kf2 isolated from poultry farm site and Kh isolated from human hair-dumping site). The isolates were able to grow on the modified human hair cut and feather meal agar plates. Two isolates (Kf1 and) have circular, white and smooth texture. But for Kh isolate the morphology of the colony was white darkish, oval shaped and its texture was smooth like that of the other isolates. They were able to excrete extracellular keratinase enzymes that breakdown keratin and increase the amino acid (tyrosine) content of the medium from which they could satisfy their carbon and nitrogen requirements. The three isolates were then further studied. The isolates then screened for keratinase production by using feather and hair kertain meal.

4.2. Identification of keratinase producing bacteria

The results of the biochemical tests of the three isolates i.e., Kf1, Kf2, and Kh are summarized in Table 1. The data indicated that they all belonged to the Genus *Bacillus* according to the descriptions provided in Berge's Manual of Systematic Bacteriology (Brenner *et al*., 2004).

Table 1 Biochemical characterization of the bacterial isolates

Biochemical Characteristics	Bacterial Isolates		
	Kf1	Kf2	Kh
Catalase test	+	+	+
Indole test	-	-	-
Citrate utilization test	+	+	+
Starch hydrolysis test	+	+	+
Oxidase test	-	-	-
Methyl red test			
H_2S formation test(TSI) test	+	+	-
Slant color	Yellow(acid)	Yellow(acid)	-
Bottom color	Red(alkali)	Red(alkali)	Red(alkali)
Lactose utilization test	-	-	+
Carbohydrate fermentation test			
Glucose	+	+	+
Sucrose	+	+	+
Lactose	-	-	+

(+) Indicates positive result and (-) Indicates negative result

4.3. Production of Keratinase Using Bacterial Isolates

In the present study *in vitro* keratin degradation was found to be associated with the release of a Large amount of extracellular keratinase into the production medium by the grown isolates. The amount of Extracellular proteolytic keratinase in the culture fluid of the bacterial isolates grown on various keratin substrates were determined using spectrophotometer by measuring the amount of tyrosine released per ml per min under the standard assay conditions as described by

Vigneshwaran *et al.* (2010). The results of this study indicated that the amounts of kerstinase released were 13±0.45 U/ml/min, 10.7±0.35 U/ml/min and 9.9±0.34 U/ml/min for Kf1, Kf2 and Kh, respectively (Table 2). The highest and lowest keratinolytic activities were consistently recorded for isolates Kf1 and Kh, respectively.

Table 2 Assay for keratinolytic activity

No.	Isolates	Activity u/ml/min
1	Kf1	14.2±0.45
2	Kf2	11.9±0.35
3	Kh	10.9±0.34
4	Cf	1.2±0.45
5	Ch	1±0.26

(Cf) Indicates that control for feather and (Ch) control for hair

4.4. Assay for Proteolytic Activity

Qualitative study of the proteolytic activities revealed that the three isolates produce gelatinase. For this test, the three isolates were evaluated for growth on gelatin agar slant at 37^0C and pH 7 for 36 hours. Due to the production of a proteolytic enzyme (gelatinase), the media became liquid at 37^0C and room temperature whereas the control remained solid at the same temperature (Thiery and Frachon 1997).

4.5. Determination of the Amount of Protein

Estimation of the total protein content of the substrate was carried out by Lowry's method. The amount of protein content that was found in the fermentation medium after 48 hour , at pH 7 and temperature of 37^0C inoculated by Kf1 (2mg/ml) was higher than those inoculated with the other two isolates (Table 3).

Table 3 the amount of protein obtained using three different isolates.

Bacterial Isolates	Absorbance	Protein Concentration mg/ml
Kf1	0.219	2±0.17
Kf2	0.178	1.6±0.17
Kh	0.141	1.3±0.2

4.6. Determination of the Percentage of Keratin Degradation (PKD) by Isolated Bacteria

This result indicates that Kf1 showed highest percent of degradation than the other two isolates. But bacterial isolates that was isolated from human hair dumping sites showed least percentage of keratin degradation .

Table 4 Percentage of keratin degradation (PKD) by isolated bacteria

Bacterial Isolates	PKD
Kf1	12.8%
Kf2	11.2%
Kh	8.6%
Cf	0.8%
Ch	0.4%

(Cf) Indicates that control for feather and (Ch) control for hair

4.7. Effect of Physico- Chemical Parameters on Keratinase Production

4.7.1. Effect of pH on keratinase production

The highest keratinase production with 60^0C was recorded for Kf1 at pH 7 (showing a keratinase activity of 16.4 U/ml/min), but for the Kf1 and Kh, optimum keratinase production at pH 7 was 15.1 U/ml/min and 12.3 U/ml/min respectively. The lowest keratinase production was recorded at pH 10 in all isolates (Kf1, Kf2 and Kh had an activity of 4.5 U/ml/min, 4 U/ml/min, and 2.7 U/ml/min respecitively (Figure1). Srivastava *et al.* (2011) reported pH 8.5 as optimum for keratinase production in *Bacillus* spp. Cheng *et al*. (1995) also reported the highest keratinase production by *B. licheniform* was obtained at initial pH of 8.5. Similarly, *B. megatherium was identified as* a better alkalophilic keratinase producer by Srivastava *et al.,* (2011).

pH

Figure 1 Effect of pH on keratinase production

4.7.2. Effect of temperature on keratinase production

The highest keratinase production at pH7was recorded at 60°C for Kf1, Kf2 and Kh with an activity of 16.2 U/ml/min, 15.5 U/ml/min and12.3 U/ml/min respectively. The lowest keratinase production was recorded for Kf1, Kf2 and Kh at 80°C was 14.1, 13.2 and 8.7 respectively (Fig. 2). Brandelli , 2008 reported the production of Keratinase between 25 and 37℃, with maximum activity and yield at 30℃. The maximum temperature for keratinase production of 40℃ was recorded by with *Bacillus subtilis* and *Bacillus pumilis* (Brandelli and Riffel, 2005).

Temperature in ^{0}C

Figure 2 Effect of temperature on keratinase production

4.7.3. Effect of concentration of substrates on keratinase production

The highest keratinase production was recorded at 10 mg per ml of feather meal and human hair cut concentration (giving the specific activities of 10.5, 8.5 and 5.5 U/ml/min for Kf1, Kf2 and Kh, respectively). The lowest keratinase production was recorded at 2 mg/ml of feather meal and human hair concentration for all bacterial isolates (Fig. 3). Feather protein has been shown to be an excellent source of metabolizable protein (Klemersrud *et al*., 1998), and that microbial keratinases enhance the digestibility of feather keratin (Lee *et al*., 1991; Odetellah *et al*., 2003), these keratinolytic strains could be used to produce animal feed protein in addition to enhancement of the biodegradation of poultry wastes, including its application in composting (Korniłłowicz-Kowalska and Bohacz, 2011). In fact, large-scale composting of keratin wastes, especially poultry feathers that are produced in large amounts, may provide a solution to the problem of their utilization (Korniłłowicz-Kowalska, 1997)**.**

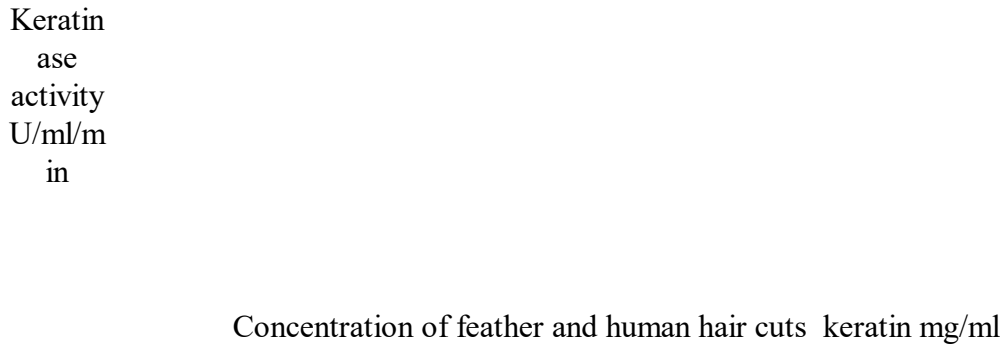

Figure 3 Effect of feather and human hair cut concentraton on keratinase production

4.7.4. Effect of the type of carbon and nitrogen source on the production of keratinase

The highest keratinase production was recorded with the addition of yeast extract for all isolates (with the activities of 25.2 U/ml/min, 20.5 U/ml/min and 16.4U/ml/min for Kf1, Kf2, and Kh, respectively). The lowest keratinase production was recorded on maize (with specific activities of 7.9 U/ml/min for Kf1 and Kh; and 8.5U/ml/min for Kf2) (Fig. 4).

Optimization of medium for keratinase production by *Bacillus* sp. glucose and peptone were found to have positive effects. The effects of nitrogen sources on keratinase production also vary. Supplementation of yeast extract resulted in maximal keratinase production by *Stenotrophomonas* sp (Ramnani and Gupta, 2004). The low-cost substrates were screened for the maximum production of keratinase. Some cost effective substrate such as soybean meal have been successfully used (Vidyasagar *et al*., 2007). However, keratinase production decreased

significantly in the absence of carbon source. Different bacteria have different preference for either organic or inorganic nitrogen for growth and enzyme production although complex nitrogen sources are usually used for alkaline protease production (Panday *et al*., 2000). The choice of carbon and nitrogen sources has a major influence on the maximum yield of enzymes. Johnvesly *et al*. (2002) reported maximum activity of keratinase in the presence of 2% yeast extract on the first day followed by a decrease thereafter. Highest keratin hydrolyzing activity was achieved at higher yeast extract concentrations in a shorter period than in the presence of lower yeast extract concentrations (Prakasham *et al*., 2006).

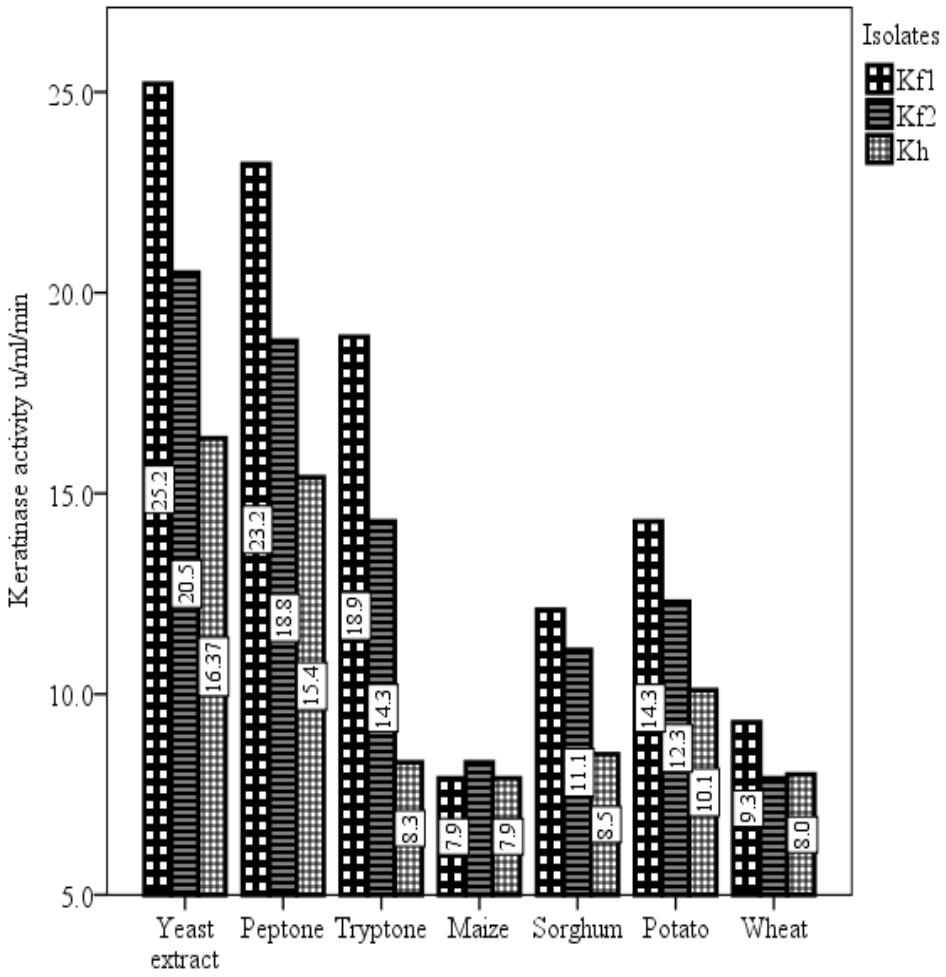

Figure 4 Effect and types of carbon and nitrogen source on keratinas production

4.8. Effect of Physico-Chemical Parameters on Keratinolytic Activity and Stability

4.8.1. Effect of pH on keratinolytic activity and stability

The effect of pH on the keratinolytic activity of crude extracellular keratinase of the three isolates induced by feather and human hair cuts was investigated and the results are depicted in Figure 5. As can be seen from the figure, keratinase activity increased with increase in pH from 5 to 7 attaining the maximum value at 7 and then followed by a decrease in activity with further increase in alkalinity. It has been previously reported that alkaline pH from 6 to 9 supports keratinase production in most keratinase producing microorganisms (Gupta and Ramnani, 2006). Furthermore, it has been reported that keratinases from most bacteria, actinomycetes, and fungi have their optimal pH values in the range of neutral to alkaline conditions (Riffel *et al*., 2003; Anbu *et al*., 2005), and are also known to be generally active and stable over a wide range of pH, i.e. from 5 to 13 (Farag and Hassan, 2004).

pH

Figure 5 Effect of pH on keratinolytic activity and stability

The stability of the keratinase in terms of percentage residual activity with increase in pH taking the activity at pH 7 as the maximum. So that %residual activity = activity at ph 8 / activity at ph 7 x 100. In this way the %residual activities for all other pH values greater equals to 7 were calculated using a table below.

Table 5 the percentage of residual keratinase activity obtained at pH 7-10

pH	Kf1		Kf2		Kh	
	A	%RA	A	%RA	A	%RA
7	15.2	100	13.2	100	11	100
8	12.4	81.5	11.2	84.8	9.3	84.5
9	6.5	42.5	5.9	44.6	3.4	30.9

10	4.1	26.9	3.1	23.4	2.2	20

A= Activity (U/ml/min), %RA = Percentage Residual Activity

4.8.2. Effect of temperature on keratinolytic activity and stability

The keratinolytic activity increased with increase in temperature from 50 to 60°C (Figure 6). However, keratinolytic activity decreased with further increase in temperature from 70 to 80 ^{0}C. The optimum temperatures for most keratinases have been reported by previous researchers to be in the range of 30 to 80°C (Gupta and Ramnani, 2006).

Figure 6 Effect of temperature on keratinolytic activity and stability

Table 6 The percentage of residual keratinase activity obtained at 60^{0}C -80^{0}c

Temperature (^{O}C)	Kf1		Kf2		Kh	
	A	%RA	A	%RA	A	%RA
60	12.4	100	11.2	100	9.3	100
70	10.3	83	9.9	88.3	7.4	79.5
80	8.5	68.5	6.5	58	5.9	63.4

A= Activity (U/ml/min), %RA = Percentage Residual Activity

4.8.3. Effect of incubation time on keratinolytic activity and stability

The highest keratinase production was recorded at 48 hrs of incubation (with specific activities of 9.5, 8.4 and 6.7u/ml/min for Kf1, K2 and Kh, respectively). The lowest keratinase production for all isolates was recorded at 96 hrs of incubation (with specific activities corresponding to 5.1, 3.5 and 3.3u/ml/min for Kf1 K2, and Kh, respectively). The keratinolytic activity of all isolates increased with the increase in incubation time up to 48hour (Figure 7). However, the rates of increase of the enzyme activity were not similar in pattern for all the isolates (Figure 7). The highest activity was demonstrated by strain Kf1 (9.5u/ml/min) after 48 h of cultivation on feather meal. *Bacillus* spp. is known to produce a number of hydrolytic enzymes including keratinase, which are able to degrade feather, wool and hair which was extracted from Amazon Basin fish (Daroit et al., 2009).

Time in hour

Figure 7 Effect of incubation time on keratinolytic activity and stability

Table 7. The percentage of residual keratinase activity obtained at incubation times 48 hours-96hours

Incubation Time (hrs)	Kf1		Kf2		Kh	
	A	%RA	A	%RA	A	%RA
48	9	100	8.1	100	7.1	100
72	8	88.8	4.7	58	5.2	73
96	4.3	47.7	3.3	40	2.9	40.8

A= Activity (U/ml/min), RA= Percentage Residual Activity

5. SUMMARY, CONCLUSION, AND RECOMMENDATIONS

5.1. Summary and Conclusion

Keratinase is one of the most important groups of industrial enzymes with considerable application in the animal feed processing leather industry, medical activity, beverage industry and others sectors. In this study keratinase produced by three bacterial isolates which were obtained from poultry and human hair damp site soil waste. On the basis of biochemical characteristics, these three isolates were identified and those belong to the genus of *Bacillus*.

Growth media adjusted to the pH7 were used to determine the optimum time for maximum keratinase production by those three isolates. The optimum incubation time was 48 hours at 60^0C. The optimum temperature was 60^0C for all isolates. In addition to this, the optimum pH 7 though the enzyme can be produced in the pH range of 5-10.

Among the various complex and available carbon source potato shows maximum enzyme production, better than other carbon sources to all isolates whereas maize shows the least enzyme production. Evaluation of the effect of nitrogen sources revealed that growth medium containing Yeast Extract produce maximum keratinase production while the media containing Tryptone shows least keratinase production.

The study on the effect of pH on keratinase activity and stability showed that it was active in the pH range of 5-10 and it exhibited maximum activity at 7 for all isolates. These results suggest that those keratinase produced by the three isolates are stable on its activity at pH of neutral. The effect of different temperature was studied to find out the temperature suitable for the activity and stability of keratinase. The result showed greater activity at 60^0C for all isolates. Evaluation of the carbon and nitrogen source on keratinase production indicated that Yeast Extract and potato were best for all isolates to produce more keratinase.

Generally the three bacterial isolates Kf1 and Kf2 isolated from soil of poultry site and Kh isolated from human hair cut damp sites are the efficient one in the production of the keratinase enzyme.

5.2. Recommendations

On the basis of the findings of this study the following recommendations are made:

- The three Bacillus isolates need to be fully characterized at the molecular level.
- The methods of large-scale production and purification of keratinase from each isolate need to be developed.
- The purified keratinase need to be carefully characterized.
- The suitability of the three isolates for use in animal feed processing and other industrial applications to be further studied.

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7. APPENDIX

Appendix A Standard curve

Tyrosine Standard Curve

To prepare the standard curve 0.5M of $NaCO_3$,50mMof sodium phosphate buffer ,pH 7diluted 1N Folin reagent and 10 mg/ml of Tyrosine stock solution were used .A required amount of buffer and Tyrosine were added in each test tube except blank. Then 2.5 ml of 0.5 M $NaCO_3$ was added in each test tube including blank. After 500µlof 1N Folin reagent was added in each test tube including blank, the solution was mixed immediately and kept for 30min at room temperature. Finally, the optical density (OD) was measured at 660nm using spectrophotometer and the standard curve was plotted (Fig 1.).

Based on these procedures and experimental results (data not shown), the following standard curve was obtained. So to determine keratinase activity of the three isolates the following calibration curve was used.

Absorb
ance

Appendix Figure 1 Tyrosine standard curve detremination of keratinase activity

BSA Standard Curve

Total protein content of the sample, degraded keratin obtained from production media was determined according to the method described by Lowery (Lowery *et al*., 1051). Bovine serum albumin was used as protein standard with 10mg/ml BSA stock solution. Working protein standard solution was prepared in the range 0.1- 1.1 mg/ml to obtain a standard curve (Fig 2.). The blank was prepared using the same procedure without sample (protein source).protein content was estimated by calibration with the standard graph. Absorbance of the sample was measured spectrophotometrically at 660nm.

Appendix Figure 2 BSA standard curve

Appendix B: The three bacterial isolates

Appendix Figure 3 cultures of three bacterial isolates

Appendix Figure 4 Biochemical characterization of the three isolates

Appendix c: Results of keratinase activity at various physic-chemical parameters

Appendix tables 1 Effect of physico-chemical parameters on keratinase production

Effect of carbon source on keratinase production						
	Kf1		Kf2		Kh	
Carbon sources	OD Value at 600 nm	Keratinase activity U/ml/min	OD Value at 600 nm	Keratinase activity U/ml/min	OD Value at 600 nm	Keratinase activity U/ml/min
Maize	0.094	7.9	0.112	9.3	0.094	7.9
Potato	0.411	14.3	0.312	12.3	0.201	10.1

Sorghum	0.301	12.2	0.251	11.1	0.121	8.5
Wheat	0.162	9.3	0.091	8.2	0.101	8
Effect of nitrogen source on keratinase production						
Peptone	0.852	23.2	0.631	18.8	0.466	14.3
Tryptone	0.633	18.9	0.411	14.3	0.321	8.3
Yeast extract	0.951	25.2	0.721	20.5	0.512	16.37
Effect of pH on keratinase production						
5	0.561	11.3	0.523	10.5	0.455	9.2
6	0.621	12.5	0.556	11.2	0.521	10.5
7	0.813	16.4	0.751	15.1	0.612	12.3
8	0.443	9	0.401	8.1	0.354	7.1
9	0.322	6.5	0.291	5.9	0.167	3.4
10	0.221	4.5	0.198	4	0.132	2.7
Effect of temperature on keratinase production						
50 ^{0}C	0.752	15.2	0.681	13.2	0.551	11
60^{0}C	0.813	16.4	0.751	15.1	0.612	12.3
70^{0}C	0.716	14.4	0.656	13.2	0.592	11.9
80^{0}C	0.697	14.1	0.654	13.2	0.431	8.7

Appendix tables 2 Effect of physico chemmical parameters on keratinase activity and stability

Effect of pH on keratinase activity and stability						
pH	Kf1		Kf2		Kh	
	OD Value at 600 nm	Keratinase activity U/ml/min	OD Value at 600 nm	Keratinase activity U/ml/min	OD Value at 600 nm	Keratinase activity U/ml/min
5	0.472	9.5	0.371	7.5	0.311	6.3

6	0.621	12.5	0.556	11.2	0.521	10.5
7	0.752	15.2	0.656	13.2	0.551	11
8	0.124	12.4	0.557	11.2	0.463	9.3
9	0.322	6.5	0.291	5.9	0.167	3.4
10	0.201	4.1	0.156	3.1	0.11	2.2
Effect of temperature on keratinase activity and stability						
50 ^{0}C	0.443	9	0.401	8.1	0.354	7.1
60 ^{0}C	0.613	12.4	0.557	11.2	0.463	9.3
70 ^{0}C	0.542	10.9	0.493	9.9	0.367	7.4
80 ^{0}C	0.421	8.5	0.321	6.5	0.294	5.9
Effect of incubation time on keratinase activity and stability						
24	0.5	10	0.291	5.9	0.234	4.7
48	0.443	9	0.4	8.1	0.361	7.1
72	0.395	8	0.234	4.7	0.254	5.2
96	0.212	4.3	0.164	3.3	0.145	2.9