Addis Ababa University
School of Graduate Studies
Biotechnology Program

Production, characterization, and potential application of a keratinolytic alkaline protease produced by alkaliphilic *Vibrio* sp.

A Thesis Submitted to the School of Graduate Studies Addis Ababa University
In Partial Fulfillment of the Requirement for the Degree of Master of Science in Biotechnology

By

Muhammed Seid

Advisor: Amare Gessesse (PhD)

May 2011

Addis Ababa

ACKNOWLEDGMENT

First of all, I would like to express my sincere gratitude to my advisor Dr. Amare Gessesse for his unlimited guidance, supervision, and constant encouragement at all steps of my study. I would also like to thank him for his financial support and material support required for this research work. Finally, I would also like to appreciate him for his brilliant ideas and opinions for my study all the time.

I would like to thank Addis Simachew (PhD candidate) for his assistance at all stages of this work.

In addition, I would like to express my gratitude for Yemisirach Mulugeta and Tirunesh Shiferaw for assisting me voluntarily in many ways and the whole staff at Laboratory of Biotechnology program.

I also acknowledge Addis Ababa University Biotechnology program for allowing me to join this program.

I am grateful to thank all my friends who helped me psychologically and morally during my study period.

I would like to thank my family for their moral and constant encouragement at all levels of my study. Especially, I am indebted to my mother for her deep interest and assistance for the successes of my study.

Finally, I would like to thank Aminat Seid for receiving and sending my salary on time every month during my stay at Addis Ababa University to complete my study.

Table of contents **Page No**

ACKNOWLEDGMENT....i

List of Tables....v

List of Figures....v

ABSTRACT....vi

1. Introduction....1

2. Objectives of the study....4

2.1. General objective....4

2.2. Specific objectives....4

3. Literature review....5

3.1. Alkaline soda lakes....5

3.2. Alkaliphiles....5

3.2.1. Special physiological features of alkaliphiles for existence in alkaline environments....6

3.2.2. Application of Alkaliphiles in Biotechnology....6

3.3. Keratin structure, classification and potential uses....7

3.3.1. Keratin structure and fiber histology....7

3.3.2. Keratin classification....8

3.4. Keratin degradation....9

3.5. Keratin degrading organisms....9

3.6. Proteolytic enzymes (proteases)....10

3.6.1. Source of proteolytic enzymes....10

3.6.2. Alkaline proteases....11

3.6.3. Keratinolytic proteases....11

3.6.3.1. General biochemical characteristics of microbial keratinolytic proteases....12

3.6.3.2. Source of keratinolytic proteases....13

3.6.3.3. Microbial keratinolytic protease production and culture conditions....13

3.6.3.4. Potential applications of keratinolytic proteases....14

3.6.3.4.1. Preparations of keratin hydrolysate as source of food....14

3.6.3.4.2. Dehairing application and environmental pollution control....15

3.6.3.4.3. Detergent application....16

3.6.3.4.4. Silver recovery....17

3.6.3.4.5. Other potential applications of keratinolytic proteases....17

4. Materials and Methods....20

4.1. Materials....20

4.2. Source of microorganisms....20

4.3. Preparation of starter culture....20
4.4. Culture conditions for selection of hair degrading isolates 20
4.5. Selection of hair degrading isolates 21
4.6. Morphological and biochemical characterization of strain R11 21
4.7. Effect of medium supplements on growth of the strain....22
4.8. Time course of cell growth and enzyme activity....22
4.9. Preparation of Tyrosine standard curve 22
4.10. Keratinolytic protease production and culture conditions 23
4.11. Determination of total soluble protein from degraded hair 24
4.12. Enzyme assay....25
4.13. Characterization of keratinolytic protease produced by *Vibrio* sp. R11 25
4.13.1. The effect of temperature on enzyme activity and stability....25
4.13.1.1. Effect of temperature on enzyme activity 25
4.13.1.2. Effect of temperature on thermal stability....25
4.13.2. The effect of pH on enzyme activity and stability....26
4.13.2.1. Effect of pH on enzyme activity....26
4.13.2.2. Effect of pH on enzyme stability....26
4.14. Effect of NaCl concentration on enzyme stability....26
4.15. Effect of protease inhibitors....26
4.16. Effect of detergents, oxidizing and bleaching agents on enzyme activity....27
4.17. Effect of methanol on enzyme stability 27
4.18. Stain removal efficiency of the crude enzyme and commercial powder detergent ...288
4.19. Testing crude enzyme for dehairing activity 28
4.20. Removal of gelatinous coating from used x-ray film 29
4.20.1. Effect of temperature and pH on enzyme activity to remove gelatin from used x-ray film....29
4.20.2. Reuseability of the enzyme for gelatin removal from used x-ray film 29
4.20.3. The effect of Glycine buffer, $CaCl_2$ and incubation period for removal of gelatin 30
4.20.4. Weight loss of used x-ray film during silver recovery process 30
4.21. Methods of data analysis....30
5. Results 31
5.1. Screening of keratinolytic microorganisms 31
5.2. Characterization and identification of *Vibrio* sp. strain R11 31
5.3. Effect of medium supplements and growth profile of *Vibrio* sp. strain R11 on different media....32
5.4. Time course of cell growth and enzyme activity profile 33
5.5. Amount of total soluble protein from degraded hair 34

5.6. Characterization of alkaline keratinolytic protease produced by *Vibrio* sp. R11 35
5.6.1. Effect of temperature on enzyme activity and stability 35
5.6.2. Effect of pH on enzyme activity and stability 36
5.7. Effect of sodium chloride concentration on stability of the enzyme 37
5.8. Effect of inhibitors and protease type 38
5.9. Effect of surfactants, detergents and oxidizing or bleaching agents on enzyme stability 38
5.10. Effect of sodium perborate on enzyme activity 39
5.11. Effect of methanol on enzyme stability 40
5.12. Stain removal efficiency of commercial detergent and keratinolytic protease produced by R11 40
5.13. Testing crude enzyme for dehairing activity 42
5.14. Removal of gelatinous coating from x-ray film 43
5.14.1. Effect of temperature on hydrolysis of gelatin from used x-ray film 43
5.14.2. Reuse of enzyme for gelatin hydrolysis from used x-ray film in the presence and absence of $CaCl_2$ 45
5.14.3. Effect of pH on hydrolysis of gelatin from used x-ray film 46
5.14.4. Enzyme concentration and time course of gelatin hydrolysis from x-ray film... 46
5.14.5. The effect of Glycine buffer, $CaCl_2$ and incubation period for removal of gelatin 47
5.14.6. Weight loss of the film during gelatin removal from used x-ray film 47
6. Discussion 48
7. Conclusion and Recommendations 57
7.1. Conclusion 57
7.2. Recommendations 57
References 58

List of Tables **Page No**

Table 1: Screening of media components for hair degradation with *Vibrio* sp. R11……......22

Table 2: Media compositions for determination of total soluble protein from degraded hair………24

Table 3: Morphological and Biochemical tests of R11 cells..32

Table 4: Total soluble protein from degraded hair by *Vibro* sp. strain R11............................35

Table 5: The effect of A) EDTA, B) 1, 10 phenanthroline and C) PMSF on alkaline keratinolytic protease activity…….……………..…………………………………38

Table 6: Time course and reuse of enzyme for complete removal of gelatin from used x-ray film at a temperature ranging from room temperature to 85^oC with 11.6U/ml enzyme in the presence and absence of 5mM of $CaCl_2$ ………………..................44

List of Figures **Page No**

Figure 1: Basic structure of hair...8

Figure 2: Higher order α-keratin structure..8

Figure 3: Applications of Bacterial keratinase in different sectors...19

Figure 4: Tyrosine standard curve for determination of keratinolytic activity………………23

Figure 5: BSA standard curve…………………………….....…...24

Figure 6: Hair degradation efficiency of bacteria strain *Vibrio* sp. R11……………………. 31

Figure 7: Growth of graphical representation of *Vibrio* sp. R11 in different media compositions...33

Figure 8: Time course of enzyme activity versus cell growth of *Vibrio* sp. strain R11 for 12 days of incubation in 12h interval at room temperature ……………………………..34

Figure 9: Effect of temperature on keratinase activity...…........35

Figure 10: Thermal stability of keratinase……………………………………………..........36

Figure 11: Effect of pH on enzyme activity…………………………………………………36

Figure 12: Effect of pH on the stability of keratinase…………………………....……………. 37

Figure 13: Effect of NaCl on stability of the crude enzyme………......................................37

Figure 14: Effect of hydrogen peroxide on enzyme stability………………………………...39

Figure 15: Effect of anionic and non-ionic detergents on enzyme stability..…………………39

Figure 16: Effect of sodium perborate on enzyme stability……………………………….....40

Figure 17: Effect of methanol on enzyme stability...40

Figure 18: Egg yolk stain removal efficiency of crude Alkaline keratinase ………………..41
Figure 19: Blood stain removal efficiency of crude Alkaline keratinase ……………………42
Figure 20: Dehairing efficiency of crude Alkaline keratinase …………………………....43
Figure 21: Sample pictures: before and after gelatin layer removal from the film..…………44
Figure 22: Effect of temperature on hydrolysis of gelatin from used x-ray film…………….44
Figure 23: Reuse of enzyme at 50°C 45
Figure 24: Effect of pH on stripping of the gelatin layer from used x-ray film.......................46
Figure 25: Time course (min) of complete removal of gelatin from used x-ray film in the absence of 5mM CaCl2 with pH value from 7-12 at 50°C.........................…………46
Figure 26: Effect of enzyme concentration and time course of gelatin hydrolysis from used x-ray film……………………………………………………………………...47

ABSTRACT

At present alkaline proteases are widely used in leather processing, detergent formulations, silver recovery process, and in the production of protein hydrolysates. All alkaline proteases are dervied from microbial sources that grow on expensive growth substrate. Many studies showed that nearly 40% of the production cost of alkaline proteases is accounted for by the growth substrate. To reduce the production cost it is important to search for microorganisms capable of growing and producing suffiecient amount of the enzyme using cheap substartes. In this regard keratinacious wastes released by poultry and leather tanning industries has an enourmous potential to serve as growth substartes for protease production. In this study, a protease producing *Vibrio* sp. capable of growing on bovine and sheep hair was isolated from Lake Arenguade. The organism produced appreciable level of keratinolytic protease using hair as the sole source of carbon and nitrogen. The enzyme was active in the pH range of 7.0-11.5 and 40-70°C with an optimum pH of 11.0 and 50°C. The enzyme showed good stability in the presence of oxidizing agents and detergents. Application of the enzyme at the inner side sheep skin at a dosage of 58U/ml, pH 10.0 brought about complete removal of hair within 24 h at room temperature and 12 h at 37°C. Protease R-11 was also tested for the recovery of silver from used x-ray films. At enzyme dose of 11.6 U/ml and at 55°C complete removal of the gelatin layer of used x-ray films was achieved within 3 min at pH 10.00. These results indicate the potential of protease R-11 for multipurpose industrial application. Because the organism produces the enzyme using cheap substrates, hair, its production cost is expected to be very low.

***Key words*:** Dehairing; Gelatin hydrolysis. Hair degrading; Keratinolytic protease; Keratinous wastes; Used x-ray film; *Vibrio* sp. strain R11

1. Introduction

Proteolytic enzymes constitute one of the most important groups of commercial enzymes with extensive application in different industrial sectors (Godfrey, 1996; Onifade *et al.,* 1998; Rao *et al*., 1998; Kumar and Takagi, 1999). Although animal and plant proteases are of important industrial applications, a large proportion of commercially useful proteolytic enzymes currently available in the market are from microorganisms. Microbial proteases represent one of the largest groups of industrial enzymes and account for approximately 60% of the total industrial enzyme sale in the world (Gupta *et al*., 2002). Considering this market demand, there is a need to effort to solve this problem by investigating new microorganisms because they are the major sources of all commercially important alkaline proteases, which have unlimited industrial applications in industries.

One area of application for proteases is as detergent additives. Protein stains such as blood and egg as well as keratinous soils that often encountered in the laundry are difficult to remove with surfactants commonly added to detergents. Therefore, addition of proteases in the detergent helps to degrade the protein and greatly improve the washing performance of standard detergents. Most commercially available detergents have an alkaline pH and contain chelating agents to overcome water hardness. This shows that enzymes used for detergent application need to be active and stable at alkaline pH and in the presence of different chemicals (Kumar and Takagi, 1999; Banik and Prakash, 2004). However, the majority of the alkaline proteases currently used for detergent applications face some limitations such as low activity and stability in the presence of surfactants, bleaches, and chelating agents (Kumar and Takagi, 1999; Joo *et al*., 2004; Gupta *et al.,* 2002). Bearing in mind this critical factor, effort should be directed to alkaline proteases with high stability in the environment of detergent formulations.

Another area of application for proteases is production of food and feed from proteinous wastes. The increasing of world population necessitates a bigger demand for protein sources from domesticated animals such as chicken, cows and pigs. The large scale production of meat and meat products require protein rich feed supplements in order to fulfill the protein demand of the world population. Keratin rich wastes such as animal hair and chicken feathers are among the well accessible and cheap protein sources with high protein content (approximately 92% - 96%) (Onifade *et al*., 1998). These keratin rich wastes are difficult to

degrade by animal and plant proteases because they are made from polypeptides, which are densely packed and strongly stabilized by several hydrogen bonds, hydrophobic interactions, and disulfide bonds. Despite their high resistance, keratins can be converted in to valuable products by the action of keratinolytic enzymes. Hence, it is crucial to find effective enzymes that are able to degrade these valuable protein resources in to valuable food products. Therefore, searching for new microorganism that produce keratinolytic proteases is considered to be highly promising.

In addition to washing purpose, food processing and protein sources, proteases are useful in dehairing process of leather manufacture. In the conventional dehairing process of leather manufacture, animal skins are subjected to a drastic chemical treatment using lime and sodium sulfide. But currently as an alternative to chemical dehairing, enzyme based dehairing processes using proteases avoiding the use of lime and sulfide are being developed because of their environmental benefits (Thanikaivelan *et al.* 2004). The use of sulfide during dehairing process produces an effluent with chemical oxygen demand (COD) of about 60,000 mg/L (Choudhary *et al.,* 2004). Thus, enzyme based dehairing processes using proteases help to reduce or even avoid the use of lime and sulfide and offer enormous environmental benefits (Anwar *et al*., 1998; Gupta *et al*., 2002; Thanikaivelan *et al.,* 2004; Sundararajan *et al.,* 2010). As Rao *et al.* (1998) and Tanksale (2001) reported, there is an increased usage of alkaline proteases for dehairing purpose. However, still there is a limited usage of these enzymes for such applications because they need high expense for their growth substrate, which makes expensive to use them.

Although microorganisms have a potential to produce enzymes, production cost of the enzyme is the critical issue for further application at industrial level. It is estimated that, growth media accounts for 30-40% of the production cost in enzyme industries (Kumar *et al.,* 2004). For production of keratinolytic protease, keratinous waste such as animal hairs removed from leather industries, poultry industries, and barber shops can serve as ideal substrate as it is cheap and available. Therefore, the feasibility of enzyme production on low-cost fermentable substrates needs to be studied (Amare Gessesse, 1997; Kumar *et al.,* 2004; Enshasy *et al.,* 2008). In addition, the removal of these non-degradable wastes could minimize environmental pollution.

Keratinolytic proteases are grouped under insoluble protein degraders. They have the capability to degrade insoluble keratin, which are insoluble protein found in chicken feathers, human and animal hair, animal horns and wool substrates more efficiently than other proteases (Onifade *et al.,* 1998). Keratinolytic proteases can be produced by different microorganisms (Riffel *et al*., 2007; Tatineni *et al*., 2008) including fungi and keratinolytic bacteria that are mostly represented by *Streptomyces* and *Bacillus* (Williams *et al*., 1990; Onifade *et al.,* 1998). *Vibrio* sp. are also used as sources of keratinolytic proteases (Sangali and Brandelli, 2000). Despite these, the industrial application and hence the market demand of keratinolytic proteases as compared to other industrial enzymes is still in the stage of infancy. Therefore, effort should be directed to investigate these enzymes from different microorganisms and employ them in the biotechnological processes involving keratin waste hydrolysis.

Industrial processes are carried out under specific physical and chemical conditions, which cannot always be adjusted to the optimal values required for the activity and stability of the available enzymes (Karbalaei-Heidari *et al.,* 2007). Therefore, it would be of great importance to have available enzymes showing better activities at different values of pH, salt concentrations, and temperatures. In view of these restrictions, attention to production and characterization of proteases from extremophiles is very important.

Microbial communities in natural alkaline environments such as soda lakes have attracted attention due to biotechnological uses of enzymes and metabolites from such organisms. A considerable number of studies have been conducted on microbial metabolites in Ethiopian soda lakes, particularly on alkaline enzymes, which are promising for human welfare in different aspects (Amare Gessesse, 1997; Amare Gessesse and Berhanu Gashe, 1997; Amare Gessesse, 1998; Amare Gessesse *et al*., 2003; Gashaw Mamo *et al*., 2006; Gashaw Mamo *et al*., 2009). Lake Arenguade is among the few soda lakes in Ethiopia. Some proteolytic alkaliphilic bacteria have been isolated and characterized from this lake (Gizachew Haile, 2009). These bacteria might have potential keratinolytic activity that needs to be assessed. Therefore, this study was designed to select an isolate, which has appreciable keratinolytic activity from among alkaliphilic bacteria identified for proteolytic activity. Keratinous waste (hair) was evaluated as a cheap substrate for enzyme production using the selected isolate designated as R11 isolated from this lake.

2. Objectives of the study

2.1. General objective

- To screen for effective keratinolytic protease production by different alkaliphilic bacteria isolated from different Ethiopian Soda Lakes, optimize the enzyme production by growing the selected bacterium on different carbon and nitrogen sources, characterize the enzyme and test for different applications.

2.2. Specific objectives

- To select and characterize alkaliphilic bacterial isolates with high efficiency of hair degradation;
- To characterize the keratinolytic protease enzyme; and
- To evaluate potential applications of the enzyme for detergent, leather dehairing and recovery of silver from used x-ray film.

3. Literature review

3.1. Alkaline soda lakes

Of the extreme environments, alkaline soda lakes are highly alkaline aquatic environments where evaporative concentration results in carbonate as a major dissolved anion. Therefore, alkaline soda lakes represent the major types of naturally occurring highly alkaline environments in which the indigenous microflora are subjected to a number of extreme ecological pressures. In alkaline soda lakes, sodium carbonate is generally the major source of alkalinity (Jones *et al.,* 1998; Kumar and Takagi, 1999; Jones and Grant, 1999). Prokaryotic photo-synthetic primary production is probably the driving force behind all nutrients recycling in alkaline soda lakes. Although alkaline soda lakes are widely distributed throughout the world, most of the detailed biological analysis has been limnological rather than microbiological. This shows few of such lakes have been explored from the microbiological point of view (Jones and Grant, 1999). East African soda lakes are among the most productive area of naturally occurring aquatic environments in the world, for the reason that there is unlimited supply of CO_2 combined with high ambient temperatures and high daily light intensity in the tropics (Jones and Grant, 1999).

3.2. Alkaliphiles

According to Grant and Tindall (1980), alkaline adapted microorganisms can be classified into two main groups: (1) Alkaliphiles (also called alkalophiles) and (2) Alkalitolerants. The term alkaliphiles is generally restricted to those microorganisms that actually require an alkaline media with pH of 9 or more for their growth and have an optimal growth pH of around 10. This indicates that, optimum growth rate of these microorganisms is observed in at least 2 pH units above neutrality (Horikoshi, 1999; Ulukanli, 2002). Organisms capable of growing at pH values more than 9 or 10 but with optimum growth rates at around neutrality or less are referred to as alkalitolerant. There are no precise definitions of what characterizes an alkaliphilic or alkalitolerant organism because several microorganisms exhibit more than one pH optimum for growth. This indicates that depending on the growth conditions, particularly nutrients, metal ions and temperature (Horikoshi, 1999). With the concept of other groups (Jones and Grant, 1998; Grant and Jones, 2000), alkaliphiles are organisms, which prefers high pH and require high sodium ion concentration for their survival and they are widely distributed throughout the world.

Alkaliphiles can be isolated from a place where microorganisms exist mainly from neutral environments, sometimes even from acidic soil samples and feces. Although they were once considered as junk, awareness of alkaliphiles has blossomed in recent years due to an interest in their physiological adaptation to high pH and their potential uses in biotechnological applications (Horikoshi, 1999).

3.2.1. Special physiological features of alkaliphiles for existence in alkaline environments

One of the most important and remarkable features of many alkaliphiles is their ability to optimize external pH for growth, exhibit a remarkable ability to maintain cytoplasmic pH much lower than external pH values of 10 to 11 (Horikoshi, 1999). Therefore, alkalophilicity can maintain by these organisms through bioenergetic membrane properties and transport mechanisms and does not necessarily depend on alkali-resistant intracellular enzymes (Krulwich and Guffanti, 1983).

The cell wall of alkaliphilic organisms contain peptidoglycan and certain acidic polymers (negatively charged), such as galacturonic acid, gluconic acid, glutamic acid, aspartic acid and phosphoric acid. The negative charges on the acidic nonpeptidoglycan components may give the cell surface its ability to adsorb Na^+ and H^+ ions and force away hydroxide ions and therefore may assist cells to grow in alkaline environments (Krulwich *et al.,* 1998). As Ito *et al*. (1997) speculated, the alkaliphilic property of the bacterium may be due to the behavior of the membrane towards charged substances admitted into the organisms and several open reading frames for Na^+/H^+ antiporter found in chromosomal DNAs may play roles in pH homeostasis of bacterial cells exist in alkaline environment.

3.2.2. Application of Alkaliphiles in Biotechnology

Alkaliphilic microorganisms provide an important resource for biotechnology and offer a multitude of actual or potential applications in various fields of biotechnology. For example, alkaline enzymes produced by those organisms have extensive domestic and industrial applications in detergent additives, wood-pulp treatment, dehairing of hides and silver recovery process (Grant *et al*., 1990; Horikoshi, 1999; Nakiboglu *et al.,* 2003). Particular properties of alkaliphiles also offer great potential for the treatment of waste or the bioremediation of hydrocarbons, both in naturally alkaline environments and in areas where the pH has been increased by industrial activities. Industrial applications of alkaliphiles have

been investigated and some enzymes have been commercialized. Of the enzymes now available to industry, enzymes such as proteases, cellulases and lipases are by far the most widely employed and they are the target biomolecules in industry (Horikoshi, 1999).

3.3. Keratin structure, classification and potential uses

3.3.1. Keratin structure and fiber histology

Keratins are the most abundant structural protein present in keratinous materials including animal skin, horn, hair, wool and feathers (Voet and Voet, 1995). They are extremely resistant to the action of physical, chemical, and biological agents. High mechanical stability and resistance to proteolytic degradation of keratins is because of their disulfide bonds, hydrogen bonds (Fig. 2), salt linkages, and other crosslinkings (Kunert, 1973; Kaluzewska *et al*., 1991). Higher order α-keratin structure can be build from keratin polypeptides (Fig. 2). Therefore, it is generally not recognized as substrate for commonly known proteases. Because of this, there is a limited use in practice for different applications (Letourneau *et al*., 1998; Rouse and Van Dyke, 2010).

Histologically, keratin fibers (hair) have three morphological regions (Fig. 1). These are: (1) the cuticle cells (about 10% of the fiber) or it is a translucent outer layer of the hair shaft (the portion of a hair between the root and the tip) consisting of scales that cover the shaft. (2) The cortical cells (about 88% of the fiber), which are long spindle shaped cells aligned parallel to the fiber direction and (3) the cell membrane complex (about 2% of the fiber), which separates each cuticle or cortical cell from its neighbor (Leon, 1972; Crewther *et al.,* 1965). Medulla is present in some types of fibers. It consists of a core of air-filled cells, which runs down the middle of the fiber and it is a central core of cells (Rouse and Van Dyke, 2010).

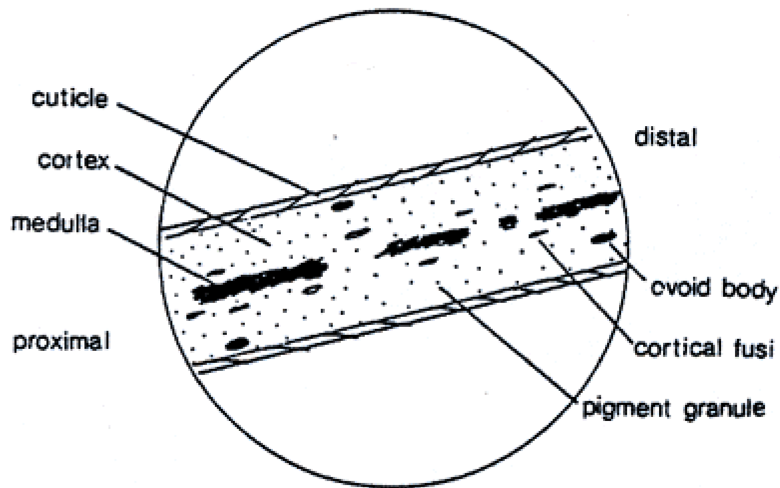

Figure 1: Basic structure of hair. Source: http://www.fbi.gov

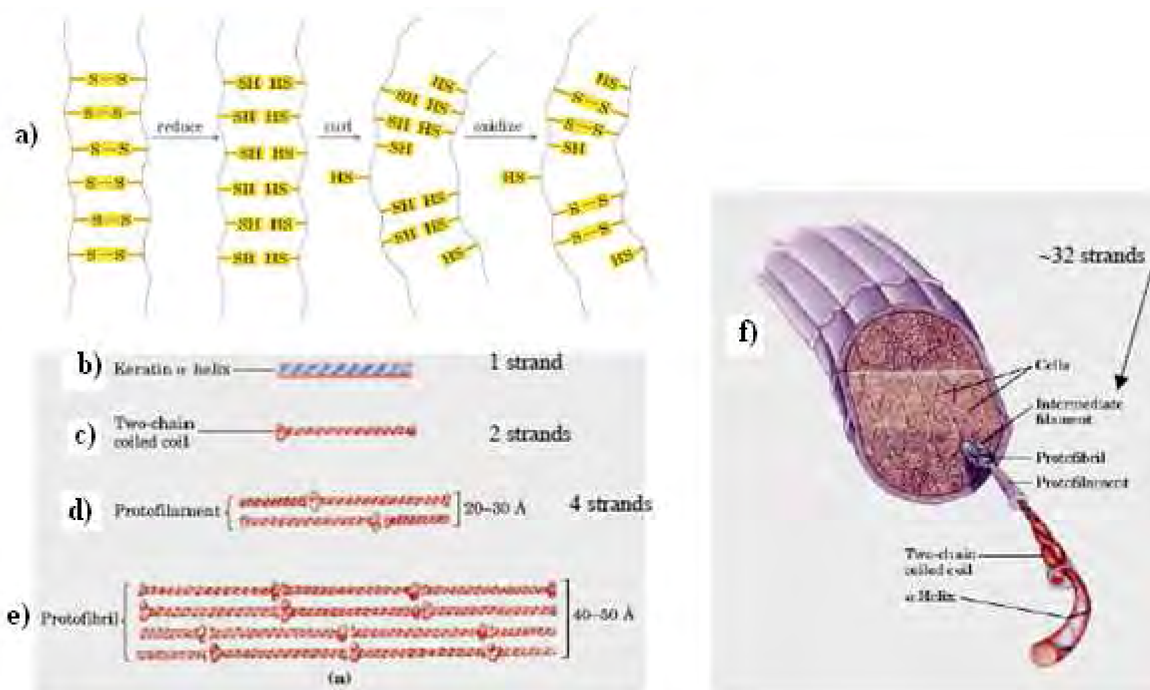

Figure 2: Higher order α-keratin structure. (a) Hair keratin with non helical sequences that are reach in cysteine residues. The shape of a hair shaf (The portion of a hair between the root and the tip) can altered by breaking and reforming the cysteine bonds between kertin and associated proteins (b) α-keratin helix c) Two keratin polypeptides form a dimeric coiled coil d) Prtofilaments are formed from two staggered rows of head to tail associated coiled coils e) Protofilaments diamerize to form a protofibril, four of which form a microfibril f) cross section of a hair.

Sources: From Voet and Voet (1995) Biochemistry, 2nd ed. John wiley and sons, inc. New York.

3.3.2. Keratin classification

Keratin is rich in cysteine residues, which form disulfide bonds that crosslink adjacent polypeptide chains. Depend on sulfur content keratins can be grouped into hard (5% sulfur) and soft (1% sulfur). Hard keratins found in appendages like feathers, hairs, hoofs and nails

that have high disulfide bond content. Whereas soft keratins found like in skin and callus that have low content of disulfide bonds (Voet and Voet, 1995). Keratins also exist as either α-keratins, which occur in mammals or β-keratins, which occur in birds and reptiles (Voet and Voet, 1995).

3.4. Keratin degradation

There are two approaches to convert keratin protein in to keratin hydrolysates, which are hydrothermal and biological treatments (Eggum, 1970; Papadopouos, 1986). Hydrothermal process usually employs high steam pressure (10-15 psi) and/or high temperature (80-140°C) in the presence of acid or base (Gousterova *et al*., 2003).

Globally, commercial poultry processing generates million tons of feathers per year that are currently converted to feather meal through steam pressure and chemical treatments (Shih, 1993). This process of chemical treatment makes keratin waste more digestible. Their expensiveness, partial or even complete destruction of certain amino acids, requirement of high-energy input (Papadopoulos *et al*., 1986), losses of essential amino acids (Lysine, Methionine and Tryptophan), and the formation of non-nutritive amino acids like Lycinoalanine and Lanthinonine (Wang and Parsons, 1997) are the main drawbacks of this process. Whereas, microbial degradation of keratin wastes provide a viable alternative for improving feathers utilization because it is ecologically safe, low-cost, offers mild reaction conditions and nutritional upgrade of feather meal with the treatment of microbial keratinase might significantly increase amino acid availability of feather keratin (Odetallah *et al.,* 2003; Grazziotin *et al.,* 2006).

3.5. Keratin degrading organisms

Keratin degrading microorganisms flourish under different ecological and environmental conditions and they reveal a wide range capacity to solubilize keratinous substrates as well as other compact proteinous substrates. As Kansoh *et al*. (2009) reported, several species of bacteria, actinomycetes and fungi produce keratinase enzymes to degrade keratinous material like hair. So, they are known as keratin degraders. Several insects like cloth moth larvae, carpet beetles and chewing lice are known to degrade keratin (Waterhouse, 1957).

Keratinolytic bacteria can be isolated using feather as a substrate (Amare Gessesse *et al.,* 2003) and most of them grouped under the genus *Bacillus* (Riffel and Brandelli, 2006). Gram-negative keratinolytic bacteria (Sangali and Brandelli, 2000; Riffel and Brandelli, 2006) including *Vibrio* sp. strain kr2 (Sangali and Brandelli, 2000) have also been isolated from chicken feathers in decomposition. The common occurrence in nature of microorganisms that readily and in some cases preferably grow on keratinous substrates has supported the general belief that keratinolytic enzyme producer microorganisms can degrade keratin. Classical examples of such microbes are the dermatophytes and the saprophytic *Onygena equina* (Ward, 1899).

3.6. Proteolytic enzymes (proteases)

Proteolytic enzymes are a group of enzymes, which hydrolyze peptide bonds in aqueous environment that hydrolyze proteins via the addition of water across peptide bonds (Beg *et al.,* 2003).They are categorized under hydrolase class of enzyme. Proteases are essential constituents of all forms of life on earth, including prokaryotes, fungi, plants and animals and they comprise one of the most important groups of commercial enzymes. These enzymes have ample utilization in industrial processes such as detergent industry (a major consumer of proteases) as well as food and leather industries (Kumar and Takagi 1999; Gupta *et al.* 2002).

3.6.1. Source of proteolytic enzymes

Because proteases are physiologically necessary for living organisms, they are ubiquitous found in a wide diversity of sources such as plants, animals and microorganisms. They are produced by wide range of microorganisms such as bacteria, fungi, viruses, archaea, and various plant and animal tissues (Rao *et al.,* 1998). Of these, microorganisms are the best preferable sources of proteases with compared to plant and animal sources. This is because of their short cultivation time, required small area for cultivation and easily genetically manipulated. Furthermore, most of the enzymes in microorganisms are extracellular, no need of cellular disruption (Rao *et al.,* 1998). Considering inability of the plant and animal proteases to meet current world demands, there is a need to paradigm shift to increase an interest towards microbial proteases. Therefore, proteases of bacteria and fungi are increasingly studied due to its importance and subsequent applications in industry and biotechnology.

3.6.2. Alkaline proteases

Alkaline proteases are defined as enzymes, which are active in a neutral to alkaline pH range. They are produced by a wide range of microorganisms including bacteria, molds and yeasts (Singh *et al.,* 2001). Alkaline proteolytic enzymes from bacterial origin are the most important industrial enzymes, which account for nearly 60% of the industrial enzyme market in the world (Enshaasy *et al.,* 2008). This indicates that they have many applications in a number of biotechnological processes and used in many industries due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures (Gupta *et al.,* 2002; Agrawal *et al.,* 2004,).

The optimum pH range of alkaline proteases is generally between pH 9 and 11 with a few exceptions of higher pH optima of 11.5 (Tobe *et al.,* 1975; Takami *et al.,* 1990), pH 11–12 (Kumar, 1997; Horikoshi, 1971), pH 12.3 (Kobayashi *et al.,* 1995) and pH 12-13 (Fujiwara *et al*., 1993). As Kumar and Takagi (1999) reported, the optimum temperature of alkaline proteases ranges from 50°C to 70°C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry (Rao *et al.,* 1998). But the enzyme from an alkalophilic *Bacillus* sp. B189 showed an exceptionally high optimum temperature of 85°C and *Fervidobacterium islandicum* AW-1 100°C.

3.6.3. Keratinolytic proteases

Keratinolytic proteases are proteolytic enzymes, produced by keratin degrading species of bacteria (Williams *et al*., 1990) and fungi (Friedrich *et al*., 1999). They have the capability of degrading insoluble keratin, which are an insoluble protein found in chicken feathers, human and animal hair, animal horns and wool substrates (Onifade *et al.,* 1998). Most keratinolytic proteases hydrolyse only keratin but some of them like keratinolytic protease from *Bacillus licheniformis* have also the ability to hydrolyse several protein substrates (Lin *et al.,* 1992).

Keratinolytic enzymes are active on keratin (Rao *et al.,* 1998). However, the exact mechanism of keratinolysis is not fully understood. The existence of complete keratin degradation efficiency of keratinolytic microorganisms are not only using keratinolytic protease enzyme rather than the cooperative action of keratinolytic protease and disulfide reductase enzyme produced by those organisms. Though some reports are available on the

presence of disulfide bond reducing factors in keratin utilizing microorganisms, no one has focused on the isolation and characterization of disulfide bond reducing enzymes with respect to keratin degradation (Yamamura *et al.,* 2002). Thiol formation by *Vibrio* strain kr2 has suggested the presence of disulfide reductase in the culture grown on feather keratin (Sangali and Brandelli, 2000).

3.6.3.1. General biochemical characteristics of microbial keratinolytic proteases

Microbial keratinolytic proteases are predominantly extracellular, alkaline or neutral proteases showing optima pH ranging 7.5- 9.0. However, some of them are optimally active outside this range even at extreme alkaline pH (Kobayashi *et al.,* 1995). Keratinolytic protease produced by *S. pactum* DSM 40530, *Streptomyces* sp. SK1-02, *Scopulariopris brevicaulis, B. licheniformis* and *K. rosea* have similar optimal temperature between 40 and 50°C (Bressollier *et al*., 1999; Bockle *et al*., 1995; Letourneau *et al.*, 1998; Bernal *et al*., 2006). Optimal temperature of these enzymes may also variable ranging from 30 to 80°C because of the source and origin of the organism. But the enzyme from an alkaliphilic *Bacillus* sp. B189 showed an exceptionally high optimum temperature of 85°C and *Fervidobacterium islandicum* AW-1 100°C (Nam *et al.,* 2002).

The molecular mass of several keratinolytic proteases have been determined but their weight is found within the range 18 to 240kDa. But most of these enzymes have often less than 50kDa. Higher molecular mass are often associated to keratinolytic proteases with metalloprotease character or those produced by thermophilic organism (Brandelli *et al.,* 2010).

The presence of divalent metal ions like Ca^{2+}, Mg^{2+}, and Mn^{2+} often stimulate keratinolytic protease activity. On the other hand, transition and heavy metals like Cu^{2+}, Ag^{2+}, Hg^{2+}, and Pb^{2+} generally cause inhibition of keratinolytic enzymes (Brandelli *et al.,* 2010).

Most of the microbial keratinolytic proteases reported to date belong to serine protease family as determined by specific substrates and inhibitors (Lin *et al*., 1992). But now days, many reports are available about keratinolytic metalloproteases and mostly associated with Gram negative bacteria. Keratinolytic protease produced by the Gram negative bacterium *Chryseobacterium* sp. kr6 belong to the metalloprotease type because it is inhibited by 1,10-

phenanthroline (Riffel *et al.,* 2003). As Tatineni *et al.* (2008) reported, keratinolytic protease produced by Streptomyces sp. S7 shows an exceptional character, which is serine-metallo in nature.

3.6.3.2. Source of keratinolytic proteases

Keratinolytic proteases can be harvested from plant tissues and microbial cells especially from several species of bacteria, fungi and actinomycetes, which are found frequently around soils or aquatic environments where keratinous materials are deposited (Rao *et al.,* 1998). *Aspergillus* sp. and *Rhizomucor sp.* (Friedrich *et al*., 1999), *Trichophyton mentagrophytes*, *Trichophyton rubrum, Trichophyton gallinae, Microsporum canis and Microsporum gypseum* (Wawrzkiewicz *et al*., 1991) are different species of fungi, which can produce keratinolytic proteases. Keratinolytic activity is well known among *Bacillus* sp. including several strains of *B.* licheniformis and *B.* subtilis (Lin *et al*., 1992; Lin *et al.,* 1999; Suh and Lee., 2001). *Streptomyces* such as *S. albidus E4* and *S. griseoaurantiacus E5* (Amany *et al.,* 2009), *S. albus* AZA (Esawy, 2007), *S. pactum, S. albus, S. thermoviolaceus,* and *S. albidoflavus* (Bressollier *et al*., 1999) are also bacterial species which can produce keratinolytic proteases. Few reports are available on Gram negative bacteria including feather degrading strains of *Vibrio* sp. as sources of keratinolytic proteases (Sangali and Brandelli 2000). Similarly, another study also reported by Riffel and Brandelli (2006) where keratinolytic bacteria from decomposing feathers were mostly Gram negative.

3.6.3.3. Microbial keratinolytic protease production and culture conditions

Keratinolytic proteases have been produced under submerged shaking conditions, except for a few thermopilic bacteria (Nam *et al.,* 2002) and fungi (Singh, 1999) where static submerged fermentation has been reported. Until 2006, there were no reports available on solid state fermentation for keratinolytic protease production (Brandelli *et al.,* 2010). However, recently the production of keratinolytic enzymes through solid state fermentation has been demonstrated (Gioppo *et al.,* 2009).

Keratinolytic proteases are largely produced in a basal medium (mineral salts) with a keratinous substrate. Most of the keratinolytic microorganisms are capable of using keratin as the sole source of carbon and nitrogen (Lin *et al.,* 1999). However, addition of a keratinous substrate is not always required for keratinolytic protease production (Cheng *et al.,* 1995;

Kim *et al.,* 2001). Other non keratinous substances have been also reported to act as inducers of keratinolytic protease production (Brandelli *et al.,* 2010).

Supplementation of keratin containing media with different carbon and/or nitrogen sources might result in higher levels of keratinolytic protease production. For instance, the addition of glucose (Ramnani and Gupta, 2004), molasses (Cheng *et al.,* 1995), and bagasses (Gioppo *et al.*, 2009) and additional nitrogen sources such as peptone, yeast extract, urea, tryptone, ammonium chloride and sodium nitrate are reported to enhance keratinolytic protease yields (Ramnani and Gupta, 2004). Conversely, the addition of supplementary substrates (carbohydrates; inorganic and/or organic nitrogen sources) often decreases keratinolytic protease production by some microorganisms, mainly due to catabolite repression mechanisms (Gioppo *et al.,* 2009).

3.6.3.4. Potential applications of keratinolytic proteases

3.6.3.4.1. Preparations of keratin hydrolysate as source of food

Currently food requirements have increased drastically. This phenomenon leads to a bigger demand for protein sources for domesticated animals. A good alternative includes animal hair and chicken feathers having high protein content (approximately 92% and 96%, respectively) in the form of keratin (Onifade *et al*., 1998). Since animal feed industry is the main consumer for keratin hydrolysates from agroindustrial byproducts, recycling of keratinous wastes is a subject of great interest for animal nutrition because of its potential as an inexpensive and alternative protein source (Takami *et al*., 1992). Despite the limited nutritional value of keratin, both the digestibility and amino acid balance of keratinous wastes protein might be improved by microbial fermentation (Lee *et al*., 1991; Williams *et al.,* 1990).

Feather waste represents a potential protein alternative to more expensive dietary ingredients for animal feed (Onifade *et al*., 1998). However, the commonly known proteases could not degrade the keratin to a large degree. But it can be broken down by some keratinolytic protease secreting microorganisms that turn native keratin into smaller molecular entities (protein hydrolysates) that in turn can subsequently be absorbed by cells (Bernal *et al*., 2006).

Versazyme is the only keratinolytic protease enzyme produced by *Bacillus licheniformis* PWD1, which is the first commercial keratinolytic protease developed by Shih and coworkers

at BRI (North Carolina) and gives a guarantees to convert keratinous wastes in to cash through feather meal production (Brandelli *et al.,* 2010).

Use of keratinolytic enzymes in the production of amino acids and peptides is attractive for biotechnological applications. With the help of these enzymes, hairs could be converted to defined products such as the rare amino acids serine, cysteine and proline. This enzymatic process is advantageous over commercial methods as large amounts of salts that need to be separated from the product would not be produced (Korkmaz and Dincer, 2004).

3.6.3.4.2. Dehairing application and environmental pollution control

The global environment is gradually deteriorating because of the socio-economic activities of humankind such as processing industries. Many industrial processes cause adverse changes in the immediate environmental change and therefore being challenged by society. Of these, leather industries and the increased amount of feathers generated by commercial poultry processing may represent a pollution problem and needs adequate management (Shih, 1993). Leather processing involves a series of unit operations. At each stage, various chemicals are used and varieties of materials are expelled (Thanikaivelan *et al.,* 2004).

Depilation or dehairing of hides and skins in leather industry is traditionally done with chemical methods using lime, sodium sulfide, etc, which contributes to 80-90% of the total pollution load in the leather industry and generates noxious gases as well as solid wastes, e.g. hydrogen sulfide and lime (Thanikaivelan *et al.,* 2004). Therefore, leather industry is one of the industries looking up to enzymes to reduce the impact of tanning processes on the environment (Sundararajan *et al.,* 2010).

The advantages of enzymatic dehairing include hair-saving dehairing process, a reduction of sulfide content in the effluent, recovery of hair which is of good quality and elimination of the bate in the de-liming (Choudhary *et al.,* 2004; Brandelli, 2008; Arunachalam and Saritha, 2009). Enzyme produced by *Bacillus subtilis* S14 has a potential to complete elimination of the need for toxic sodium sulfide during dehairing process in leather industry (Macedo *et al.,* 2005). Therefore, the ever increasing attention to the environmental impact of leather industry has necessitated for the development of enzyme based processes as potent alternatives to pollution causing chemicals.

Keratinolytic proteases lacking collagenolytic and having mild elastolytic activities are increasingly being explored for dehairing process because these enzymes would help in the selective breakdown of keratin tissue in the follicle, thereby pulling out intact hairs without affecting the tensile strength of leather (Macedo *et al.,* 2005). *B subtilis* S14 produces a keratinolytic protease (KerS14), which does not have any detectable effect on collagen that is a crucial property for an enzyme intended to be used in skin dehairing (Macedo *et al.,* 2008).

3.6.3.4.3. Detergent application

The idea of using proteases was first proposed by Rohm (German Patent GP283923, 1913), who incorporated pancreatic enzymes into a detergent (Saeki *et al*., 2007). The economic importance of alkaline proteases became known when bacterial alkaline proteases from *Bacillus* sp. were introduced in 1960s to the detergent industry (Kazan *et al*., 2005; Kumar *et al.,* 2008).

The use of enzymes in detergent formulations is now common in developed countries, with over half of all detergents presently available containing enzymes (Kumar *et al.,* 2008). Proteases are one of the standard ingredients of all kinds of detergents ranging from household laundering to reagents used for cleaning contact lenses or dentures. The use of proteases in the laundry detergents accounts for approximately 25% (around 13 billion tons per year) of the total worldwide sales of enzymes (Tanksale, 2001).

Even if there is limited information in the literature regarding detergent applications of keratinolytic protease, (Gupta and Ramnani, 2006) confirmed that keratinolytic proteases are used for detergent formulations. Unlike other proteases, keratinolytic proteases have the ability to bind and hydrolyze solid substrates like hair. This is an important property of detergent enzymes, as they are required to act on protein substrates attached to solid surfaces, making them attractive additives for hard-surface cleaners (Brandelli *et al.,* 2010). They could also help in the removal of keratinous soils that often encountered in the laundry, such as collars of shirts, on which most proteases fail to act (Amare Gessesse *et al.,* 2003).

The ideal detergent protease should possess broad substrate specificity to facilitate removal of a large variety of stains due to blood, food and other body secretions. Activity and stability at

high pH, temperature, compatibility with other chelating and oxidizing agents and high isoelectric points (pI) value, to withstand higher pH ranges added to the detergents are among the major prerequisites for the application of proteases in detergents (Tanksale, 2001). In general, the majority of the commercially available enzymes are not stable in the presence of bleaching or oxidizing agents. Hence, the latest trend in enzyme-based detergents is exploring microorganisms with high stability of those agents to produce bioengineered enzymes with better stability (Gupta *et al*., 2002).

3.6.3.4.4. Silver recovery

Silver is one of the expensive and gracious metals used in large quantities for many purposes, especially in the photographic industry. The waste (used) x-ray or photographic film containing silver spread in gelatin are very good source for silver recovery with compared to other types of film. With an increasing demand for silver in the world, recent attention is focused on used x-ray or photographic films as one of the sources (Shankar *et al.,* 2010).

Protein found on x-ray film (gelatin) is the derivative of collagen, which is insoluble protein and not degraded by common proteases. Keratinolytic proteases are one class of proteases and have the ability to degrade gelatin from x-ray film and therefore they are used for silver recovery process. Currently, many investigations have been reported to recover the silver from used x-ray film/photographic film wastes (Nakiboglu *et al.,* 2003). Silver is recovered by burning the films, which causes undesirable environmental pollution and base film made of polyester cannot be recovered using this method. Since the silver is bound to gelatin, it is possible to extract silver from the protein layer by proteolytic treatments. Therefore, enzymatic hydrolysis of gelatin not only helps in extracting silver, but also the polyester film base can be recycled (Nakiboglu *et al.,* 2003).

3.6.3.4.5. Other potential applications of keratinolytic proteases

Keratinolytic proteases are used for the production of medicine, biodegradable films, glues, development of biopolymers from keratin fibers (Riffel *et al.,* 2003) and cosmetics including hair care products, nail hardening products (Matsubara and Tabata, 1994 cited in Karthikeyan *et al.,* 2007). They are also used to produce slow release nitrogen fertilizers (Chikura *et al.,*1994). Hydrolysates of keratinous materials are used as additive in the preparations of concrete and ceramics (Kawashima, 1993 and 1994 cited in Karthikeyan *et al.,* 2007). They

are also used for removal of some polluting metals such as calcium, magnesium, iron and manganese from polluting industrial waste water by immobilization of active amino acids found in hydrolysate solution on silica surface as regenerable sorbents (Sayed *et al*., 2005). Fire fighting composition is prepared from a solution of organic colloid derived from the hydrolysis of horns and hoofs (Datta, 1993). Oxidation of keratinous materials cleaves and oxidizes some of the disulfide linkages to form water soluble peptides and this material is used as a wound healing agent (Van Dyke *et al.,* 2001).

Other promising applications have been associated with keratinolytic enzymes is that to enhance drug delivery in some tissues. For example, the effectiveness of topical therapy of nail diseases is usually limited by the very low permeability of drugs through the nail plate (Mohorcic *et al.* 2007). Hydrolysis of prion proteins (prp) (causative agents of fatal and transmissible neurodegenerative diseases and Transmissible Spongiform Encephalopathy (TSE) in humans) arise as novel outstanding applications for keratinolytic protease enzymes (Gupta and Ramnani, 2006; Brandelli, 2008; Sharma and Gupta, 2010; Vigneshwaran *et al.,* 2010). For example, a keratinolytic protease produced by *Bacillus licheniformis* PWD-1 was effective for full digestion of PrP^{Sc} (abnormal isoform of PrP) in infected cow or sheep brain stem (Priola, 2001; Langeveld *et al.,* 2003).

Keratinolytic proteases are used for developing feather-based culture media that is useful in the production of endotoxins to kill the mosquito larvae, (Poopathi and Abidha, 2007). As Kwon *et al*. (1994) reported the use of alkaline proteases from *Vibrio metschnikovii* RH530 used as an alternative for Proteinase K in DNA isolation.

Bioconversion of keratinous wastes used for subsequent production of bioenergy including methane and biohydrogen (Balint *et al.,* 2005; Brandelli *et al.,* 2010). Furthermore, keratinolytic fungi are a potential tool for assessment of petroleum hydrocarbon contamination in the soil and associated bioremediation progress (Ulfig *et al.,* 2003). Using keratinolytic proteases, keratin-based biomaterials or hydrolysates used to regulate hemostasis and nerve regeneration (Sierpinski *et al.,* 2008; Apel *et al.,* 2008; Aboushwareb *et al.,* 2009; Rouse and Van Dyke, 2010).

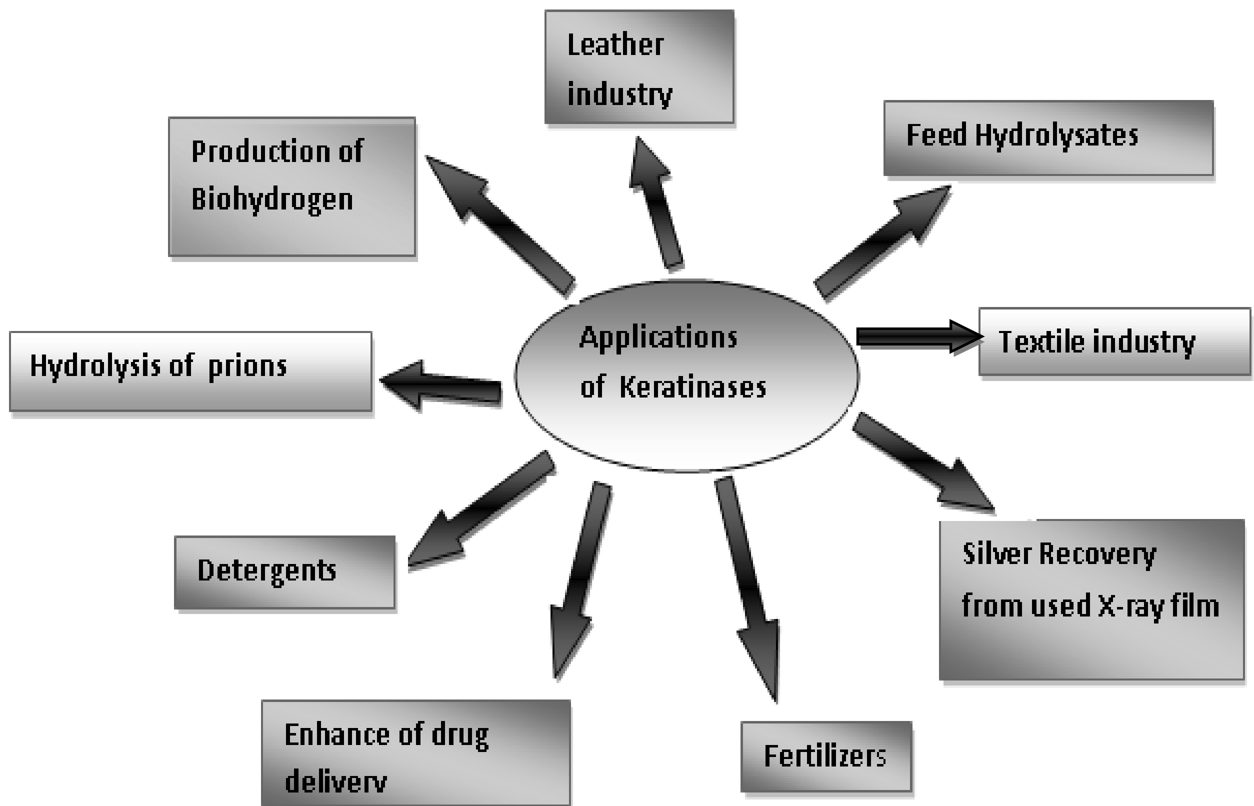

Figure 3: Applications of Bacterial keratinolytic protease in different sectors

Sources: Brandelli, A. (2008). Bacterial keratinase

4. Materials and Methods

4.1. Materials

Hair and sheep skins were provided by Dire Leather industry while blood was collected from Butchers around Arat kilo. Eggs and used x-ray films were purchased from local markets and medical centers, respectively.

4.2. Source of microorganisms

Ten microbial strains isolated from mud sample of from different Ethiopian Soda Lakes by Gizachew (2009) and preserved in the Biotechnology laboratory were used in this study. All isolates were previously screened for proteolytic activity on casein agar medium. In this study, they were also further checked for keratinolytic activity.

4.3. Preparation of starter culture

A loop full of 24 h grown cells from each 10 isolates was inoculated in 100 ml casein-yeast extract-peptone (CYP) medium in 500 ml Erlenmeyer flasks to refresh the isolates. The medium components were (g/l): peptone (5), casein (10), K_2HPO_4 (1), CaCl2 (0.13), yeast extract (1), $MgSO_4.7H_2O$ (0.2), agar (15) for plates and 1% Na_2CO_3. Inocula (1%) from the above medium were added in to working medium (Table 1. medium C). In this study, all media preparations were accomplished by following the procedures developed by Amare Gessesse *et al*. (2003). Therefore, after 1% Na_2CO_3 was prepared and autoclaved separately it was mixed with other media components after cooling.

4.4. Culture conditions for selection of hair degrading isolates

Hair medium with the same composition and preparation as starter culture (section 4.3) medium except casein replaced with hair was used for screening of keratinolytic isolates. Hair that was used for each experiment was washed gently with tap water until clear effluent was obtained and rinsed with distilled water. The washed hair was dried with a circulating air drying oven at 45°C. Then it was weighed with sensitive balance and mixed with broth medium before autoclaving. After autoclaving, 1% of refreshed cells taken from starter culture were inoculated into 500 ml Erlenmeyer flasks with a working volume of 100ml of medium. Cultivation was conducted at room temperature on orbital rotary shaker at a speed of 120 rpm. Complete degradation of the hair was examined visually.

4.5. Selection of hair degrading isolates

Active isolates having keratinolytic activity were screened qualitatively for their keratinolytic activities using starter culture except 1% of hair, used as a sole carbon and nitrogen source, replaced with casein. From 10 isolates, 5 showed complete hair degradation (keratinolytic activities) in shaken culture medium with a continuous incubation periods for 15 days. Among these, isolate designated as R11 showed high keratinolytic activities after 84h of incubation. Therefore, this isolate was selected for further investigation in this study.

4.6. Morphological and biochemical characterization of strain R11

Colony characterization such as configuration, margin, elevation, opacity, pigment and shape was investigated microscopically and direct observation of the 24h old colony on the agar plate. Gram test was carried out both using general procedure of gram test (Duncan, 2005) and 3% KOH. Biochemical characteristics such as catalase, oxidase, aerobic test, casein hydrolysis test were undertaken as follows. Oxidase test was performed using *N, N, N', N'*-tetramethyl-*p*-phenylenediamine (TMPD). One large colony was taken with a loop and tamped lightly onto the wet filter paper found in a petridish and the result was examined by adding 3-5 drops of this oxidase reagent on the filter paper (Tarrand *et al*., 1982). Catalase test was investigated using 3% H_2O_2 by dropping on the slide with 24 h grown fresh colony and observing whether air bubble was detected or not whereas anaerobic test was carried out using anaerobic jar for 24h incubation. Casein hydrolysis test was examined on milk agar plate (Sellami-Kamoun *et al.,* 2008). Nitrate reduction test was performed in a medium containing 0.2% potassium nitrate with the reagents sulfanilic acid (Nitrate reagent A: 0.8% in 5N acetic acid) and alpha-naphthylamine (Nitrate reagent B: 0.6% in 5N acetic acid). The medium inoculated with a pure culture of the suspect organism (R11) and incubated at 30°C for 24h. After 24h incubation 5 drops of Reagent A followed by 5 drops of Reagent B was added to each tube. Complete reduction of nitrate was confirmed by adding small amount (4 to 5 mg) of zinc dust powder in each tube (Schreckenberger and Blazevic, 1974). Furthermore, the isolate designated as R11, which has the ability to degrade keratin effectively was identified by 16S rRNA amplification at University of Bergen, Norway.

4.7. Effect of medium supplements on growth of the strain

Growth of *Vibrio* sp. strain R11 on basal medium with hair or casein media with different supplements was investigated by modifying the method described by Amare Gessesse (2003) (Table 1). The basal medium contained (g/l) $CaCl_2$. $2H_2O$ (0.013), $MgSO_4$. $7H_2O$ (0.02) and K_2HPO_4 (0.1).

Table 1: Screening of media components for hair degradation with *Vibrio* sp. R11

Basal Medium plus	Medium A	Medium B	Medium C	Medium D	Medium E
Hair (1%)	+	+	+	+	-
Casein (1%)	-	-	-	-	+
Peptone (0.5%)	-	-	-	+	+
Yeast extract (0.05%)	-	+	+	+	+
Glucose (0.5%)	-	-	+	-	-

Key: + = present
- = absent

4.8. Time course of cell growth and enzyme activity

Medium C (Table 1) was used for this experiment because it was suitable for the growth of the selected isolate. It was the standard working medium for succeeding work in this study. The medium was inoculated with 1% starter culture grown for 24h (Gouda, 2006) and incubated at room temperature on rotary shaker at 120 rpm for 252 h. Determination of cell biomass was carried out by measuring the turbidity of broth medium using UV-7804C spectrophotometer with 12h intervals. The date at which the strain attained its maximal enzyme activity was examined by measuring absorbance at 660nm using the same spectrophotometer used in cell biomass assay. The experiment was also conducted at each 12h intervals.

4.9. Preparation of Tyrosine standard curve

To prepare the standard curve 0.5M of Na_2CO_3, 50mM of Glycine buffer, pH 10.00, 1:10 diluted 2N Folin reagent and 200 µg/ml of Tyrosine stock solution with dilution 1:2 were used. A required amount of buffer and Tyrosine were added in each test tube except blank. Then 2.5 ml of 0.5M Na_2CO_3 was added in each test tube including blank and the mixtures were keet at room temperature for 10min. After 500µl of 2N 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 UV-7804C spectrophotometer and the standard curve was plotted (Fig. 4).

Based on the above procedures and experimental results (data not shown), the following standard curve was obtained. So, to determine the keratinolytic activity of *Vibrio* sp. R11, the following calibration curve was used with the regression coefficient of R^2=0.996.

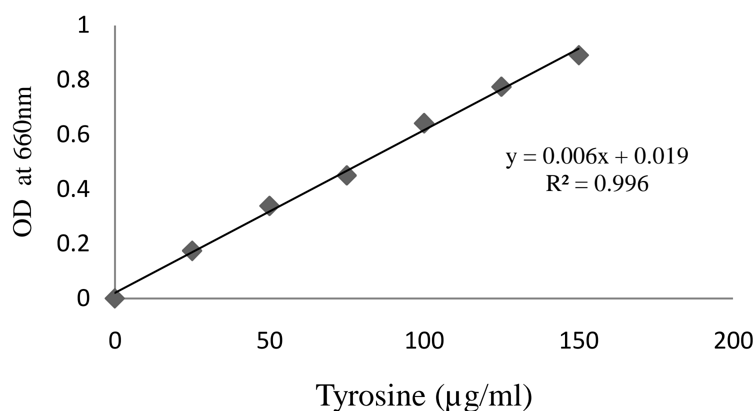

Figure 4: Tyrosine standard curve for determination of keratinolytic activity

$$\textbf{Enzyme Unit} = \frac{(167.07 \times OD \times \text{Dilution factor}) - 3}{\text{Time of incubation}}$$

NB: The equation was generated after rearranging of the equation Y = 0.006X + 0.019 (By exchanging OD value at the x-axis and concentration of Tyrosine at y-axis)

4.10. Keratinolytic protease production and culture conditions

The following broth medium (Medium C) was used throughout the study for keratinolytic protease production. The medium components were: 1% Hair (w/v), 0.5%(w/v) glucose, 0.05% (w/v) yeast extract, 0.013% (w/v) $CaCl_2.2H_2O$, 0.02% (w/v) of $MgSO_4.7H_2O$ (w/v), 0.1% (w/v) $K_2HPO_4.xH_2O$ and 1% Na_2CO_3 as described by (Amare *et al.,* 2003) with a slight modification. Broth culture was grown in 500ml flasks with 1% refreshed cells from starter culture. The culture was incubated on a rotary shaker at 120 rpm for 8 days (192 h) at room temperature, the time at which maximum enzyme activity was observed. Crude enzyme preparation was accomplished by centrifuging the broth at 10,000 rpm for 5 min. The clear supernatant was used as the crude enzyme. The crude enzymes harvested at 8th day and end of fermentation period were used further enzyme characterization and total soluble protein

determination, respectively. Hence, 8th day was considered as optimized period for keratinolytic protease production.

4.11. Determination of total soluble protein from degraded hair

Total soluble protein content of the samples (degraded hair) obtained from six different media was determined according to the method described by Lowry (Lowry *et al.*, 1951). Media combinations were clearly put in Table 2. Concentrations of mineral salts described as in (section 4.7) were similar for all media. Bovine Serum Albumin (Sigma) was used as protein standard with 400 µg/ml BSA stock solution. Working protein standard solution was prepared in the range of 0-360 µg/mL to obtain a standard curve (Fig. 5). The blank was prepared using the same procedure without sample (protein source). Soluble protein content was estimated by calibration with the standard graph. Samples (cell-free culture supernatant) from six different media were diluted to five times with distilled water. Absorbance of the sample was measured spectrophotometrically at 660nm using UV-7804C spectrophotometer.

Table 2: Medium compositions for determination of total soluble protein from degraded hair

Basal medium plus	Supplement	cell
Hair	None	-
	None	+
	Yeast extract	+
	Yeast extract+ glucose	+
	yeast extract + peptone	+
Casein	yeast extract + peptone	+

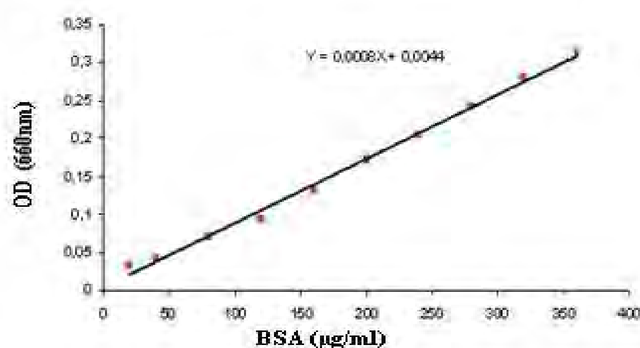

Figure 5: BSA standard curve

4.12. Enzyme assay

Keratinolytic protease activity was determined using casein as a substrate according to the method reported by Amare Gessesse *et al*. (2003). The reaction mixture (450 µl of 1% casein in 50mM Glycine/NaOH, pH 10.00 and 50µl of crude enzyme extract) was incubated at 40°C for 30 min. After 30 min of incubation, 500µl of 10% Trichloroaceticacid (TCA) was added to stop the reaction. Enzyme blank (control) was processed by adding the same amount of enzyme after TCA was immediately added and the tubes were allowed to stand for 20 min at room temperature to facilitate the un-reacted casein precipitation. The precipitated protein was removed by centrifugation at 10000 rpm for 5 min. From the clear supernatant, 500µl was transferred to clean test tube. Then 2.5 ml of 0.5M Na_2CO_3 and 500µl 2N folin Ciocalteau's phenol reagent (1:10 diluted) were added to the solution. Then the solution was immediately mixed with vortex and kept at room temperature for 20 min. Finally, absorbance reading was measured using UV-7804C spectrophotometer at 660nm. One unit of keratinolytic protease was defined as the amount of enzyme that librates trichloroacetic acid soluble fragments from casein, equivalent to 1µg of tyrosine in 1 min under standard conditions at 40°C.

4.13. Characterization of keratinolytic protease produced by *Vibrio* sp. R11

4.13.1. The effect of temperature on enzyme activity and stability

4.13.1.1. Effect of temperature on enzyme activity

Optimal temperature of the enzyme was investigated by following protease assay method described by Amare Gessesse *et al*. (2003) using 1% Casein (w/v) as substrate in 50mM glycine/NaOH pH 10.0. Experiment was conducted by incubating the reaction mixture at temperatures ranging from 30°C to 80°C in the presence and absence of 5mM of Ca^{2+}.

4.13.1.2. Effect of temperature on thermal stability

Temperature stability of the enzyme was determined by measuring the residual activities by following the the modified procedure (Gupta and Khare, 2007). After pre-incubation of the crude enzyme solution in 50mM glycine-NaOH buffer, pH 10.0 at 45, 50, 55 and 60°C, an aliquot of 1ml enzyme solution was incubated in the water bath at the each temperature. Aliquots were withdrawn at desired time intervals (10min) to determine the remaining activity following the standard protease assay procedures. Enzyme incubated at room

temperature (aliquots that was not heat treated) was considered a control and assumed to have 100% activity. At temperatures > 55°C, all the assays were carried out with 5mM of Ca^{2+} because the enzyme was calcium dependent.

4.13.2. The effect of pH on enzyme activity and stability

4.13.2.1. Effect of pH on enzyme activity

The effect of pH on activity of the enzyme was determined by assaying its activity at different pH values within the range of 6.0-12.0 with increment of 0.5 pH units (Gouda, 2006). The experiment was conducted using 1% (w/v) casein as a substrate in the presence of 5mM of Ca^{2+} and 50mM concentration of the following buffers. Phosphate buffer (Na_2HPO4/NaH_2PO4), Tris/HCl, Glycine/NaOH and dibasic sodium phosphate/NaOH used for pH of 6- 7.5, 8 & 8.5, 9-10.5 and 11-12, respectively. The enzyme activity was measured following the standard protease assay procedure and the activity of the pH at which the highest activity was taken as 100%.

4.13.2.2. Effect of pH on enzyme stability

The effect of pH on enzyme stability was determined according to Amare Gessesse *et al.* (2003) described earlier, which was carried out by pre-incubating the enzyme for 1h at room temperature with each buffer. After 1h pre-incubation, the residual enzyme activity was determined following the standard protease assay in glycine/NaOH, pH 10.0 at 40°C for 30 min incubation.

4.14. Effect of NaCl concentration on enzyme stability

The effect of NaCl on enzyme stability was studied by following the method (Sana *et al.,* 2006) with some modification. The crude enzyme was mixed with 50mM Glycine-NaOH buffer pH 10.00 contained different NaCl concentrations ranging from 0M to 3M with 0.5 unit intervals. The reaction mixture was pre-incubated at room temperature for 1h. To determine the residual activity, 50µl of the enzyme solution was taken from pre-incubated solutions for standard protease assay.

4.15. Effect of protease inhibitors

The effect of different inhibitors on crude enzyme activity was determined using two specific metalloprotease inhibitors (Ethylenediaminetetraacetic acid and 1, 10 Phenanthroline) and

one serine protease inhibitor (phenylmethylsulfonyl fluoride) by modified the method described by Amare Gessesse (1997). Aliquots were pre-incubated at room temperature for 1h before normal protease assay was carried out. Residual protease activity was determined by standard protease assay procedure and compared with a control (without inhibitors) but incubated under similar conditions. The control was taken as 100%.

4.16. Effect of detergents, oxidizing and bleaching agents on enzyme activity

Stability of the enzyme in the presence of detergents, oxidizing and bleaching agents was examined by slight modification of the methods described by Sellami-Kamoun *et al.* (2008) and (Haddar *et al.,* 2009). The experiment was carried out by pre-incubating the enzyme for 1h at room temperature with various chemical preparations. The residual activities were determined by applying standard protease assay.

To investigate the effect of oxidizing agents on enzyme stability, hydrogen peroxide was used with different concentrations ranging from 0-30% in the reaction mixture. Pre-incubation of the reaction mixtures was carried out by mixing equal amount of enzyme and H_2O_2. Similarly, stability of the crude enzyme in the presence of surfactants was determined by incubating it with Triton x100 (nonionic detergent), Tween-80 (nonionic detergent) and SDS (ionic detergent). Bleach stability of the enzyme was also tested in presence of sodium Perborate ($NaBO_3$). Enzyme activity of a control sample (without detergent) incubated under similar condition was taken as 100%. Residual activities were measured using standard protease assay.

4.17. Effect of methanol on enzyme stability

Effect of methanol on enzyme stability was tested by following the slight modification of the method described by Xu *et al.* (2009). The concentration range used in this experiment was in the range of 0-30%. Aliquots of the crude enzyme were mixed with methanol in equal proportion and incubated for about 1h at room temperature. Then by taking 50µl of pre-incubated enzyme from each concentration, the standard protease assay was carried out at 40°C for 30 min and compared with control considered as 100% activity.

4.18. Stain removal efficiency of the crude enzyme and commercial powder detergent

The commercial powder detergent, which is known as Aerial contained unspecified enzyme, was taken as a commercial detergent. The mixing proportion recommended by the detergent with dH_2O is 0.3% (w/v), which is written on the pack of the detergent. Therefore, here in this study also used this standard. The detergent was heated at 100^oC for 1h to denature the endogenous enzyme found in the detergent. Non heated detergent was dissolved with standard mixing and assayed to confirm whether the detergent contains protease or not. Heated (denatured) detergent and enzyme (5%) produced by R11 was mixed and assayed to check the enzyme could withstand (compatbble) all the surfactants found in the detergent or not.

Wash performance of the crude enzyme and commercial powder detergent was evaluated by using them on blood and egg yolk stains stained on cotton fabrics following the method with slight modification of Kumar and Bhalla (2004). A clean and white cotton test fabric pieces (15cm x 15cm) were stained with blood and egg yolk. The stained pieces were allowed to dry. Then they were added in to 250ml Erlenmeyer flasks contained 5.8 U/ml and 11.6 U/ml of crude enzyme in 100ml reaction mixture, which was adjusted by 1M of NaOH pH 10.00. Finally, some of the flasks were incubated at room temperature while the rest at 37^oC. Controls were prepared under similar conditions except no enzyme or detergent was added. Stain removal was visually monitored by washing the cloths with tap water.

4.19. Testing crude enzyme for dehairing activity

This experiment was carried out according to (Riffel *et al.,* 2003) with some modification. The excess flesh and fatty tissue under the fresh sheep skins was removed before they were mixed with enzyme. Then the skin pieces were washed gently with tap water and rinsed with distilled water to remove chemicals from the skin, which may hinder enzyme activity during dehairing activity. The skin was dried and cut in to 10 cm x 15cm pieces, each weighing 24g. Cell-free supernatant with 58U/ml enzyme was used as source of crude enzyme for the experiment. Reaction mixture (50 ml) contained equal volume of crude enzyme and 50mM glycine-NaOH buffer pH 10.00. One molar of NaOH buffer pH 10.5 was also used for dehairing activity. A control was prepared under similar conditions except contained a crude enzyme denatured by boiling at 100^oC for 1h. The reaction mixture was incubated both at 37^oC and at room temperatures for 12h and 24h, respectively. Dehairing activity of the

enzyme was accomplished by pulling the hairs with fingers. Finally, the skin pieces were visually examined for complete dehairing activity and any change on the skin.

4.20. Removal of gelatinous coating from used x-ray film

Used x-ray films were washed with distilled water and wiped with cotton impregnated with ethanol. Then they were cut into 4cm *x* 4cm pieces, each weighing 0.423g (Nakiboglu *et al.,* 2003). After dried them in an oven at 40°C for 30 min, each of the films were immersed in 50mM glycine buffer pH 10.00. Enzyme extract with 11.6U/ml concentration was used for experimental unlike control prepared using buffer alone. Removal of gelatin from the film was also measured by Lowry *et al.* (1951) by quantifying the gelatin content in the stripped solution with bovine serum albumin (BSA) as the standard. Furthermore, turbidity of the removed solution was also measured at 660nm.

4.20.1. Effect of temperature and pH on enzyme activity to remove gelatin from used x-ray film

Optimal temperature of the enzyme for gelatin removal was investigated by incubating it from room temperature to 85°C. Similarly, to determine the optimal pH of the enzyme reaction mixtures were also treated in buffers ranging from 7-12. The above two independent experiments were carried out according to the method described by Nakiboglu *et al.* (2001) and Shankar *et al.* (2010) with a slight modifications.

4.20.2. Reuseability of the enzyme for gelatin removal from used x-ray film

To determine the reuseability of enzyme (how many times reused) for removal of gelatin layer from the film, experiment was conducted using 11.6Uml^{-1} of enzyme at temperature ranges from room temperature to 85°C in the presence and absence of 5mM of $CaCl_2$. After complete removal of gelatin, enzyme treated x-ray film was removed from the reaction mixture. Then the untreated one was added to the same enzyme solution and incubation continued until complete removal of gelatin was observed. The process was repeated until gelatin hydrolysis stopped. Time required for complete gelatin removal in each case was noted (Shankar *et al.,* 2010). Considering the cost of enzyme, effect of enzyme concentration with time course of gelatin layer removal from used x-ray film was also studied from 0.29U/ml-58U/ml concentration ranges at 50°C using 1M NaOH pH 10.00.

4.20.3. The effect of Glycine buffer, $CaCl_2$ and incubation period for removal of gelatin

To confirm either removal of gelatin obtained really by enzyme or $CaCl_2$, experiment was conducted in the presence of 5mM of $CaCl_2$ associated with buffer only at 70°C for 30 minutes. Likewise, to determine the effect of incubation period on enzyme activity the experiment was also carried out by incubating the reaction mixture contained enzyme and buffer alone (i.e. without inclusion of the film) for 5 min at 85°C. After 5 min, the film was added to the reaction mixture and kept on the continuous shaking water bath for 30 min.

4.20.4. Weight loss of used x-ray film during silver recovery process

Weight loss of the film was analyzed by measuring the weight of used x-ray film before and after complete removal of gelatin layer from fixed size (4cm x 4cm) of the film.

4.21. Methods of data analysis

All data presented here in this study were the average of at least two measurements and all graphical and numerical data values generated by using Microsoft Excel 2007.

5. Results

5.1. Screening of keratinolytic microorganisms

Out of the ten isolates tested, one isolate, designated as R11, was selected for further study depends on its hair degradation capability. The isolate showed remarkable hair degradation efficiency within 5 days of incubation at room temperature (Fig. 6). The rest of the isolates showed moderate or no complete hair degradation even after long period of incubation.

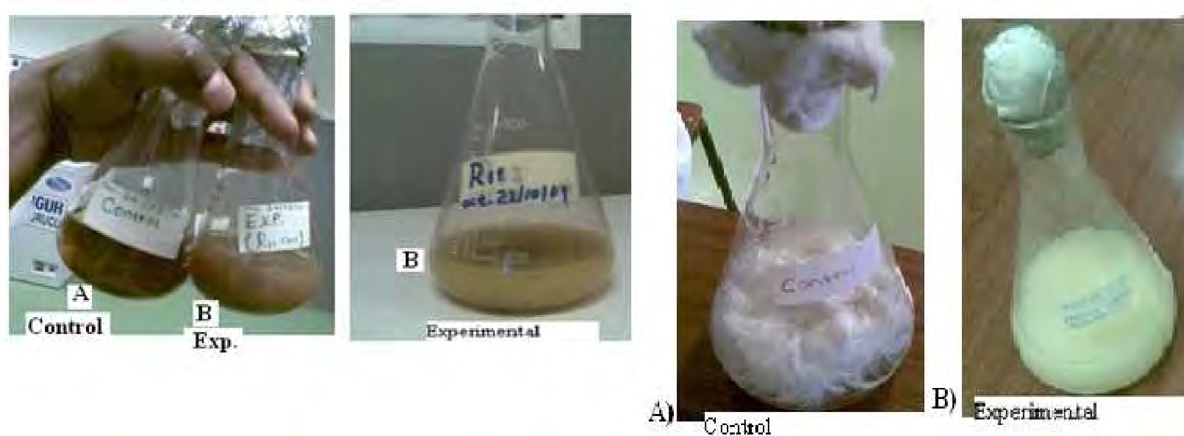

Figure 6: Hair degradation efficiency of bacterial strain *Vibrio* sp. R11 isolated from Lake Arenguade (One of Ethiopian soda lakes) in submerged cultivation at room temperature. (A) Hair control without the bacterial strain (B) Hair after 144 hrs of incubation with the bacterial strain showed complete degradation

5.2. Characterization and identification of *Vibrio* sp. strain R11

Table 3 shows results of morphological and biochemical tests of isolate R11. Based on its morphological and physiological characteristics the isolate was characterized as gram negative, oxidase negative and catalase positive. The colonies were characterized as transparent, mucoid, circular configuration, smooth margin, immerged and non-pigmented. A motile short curved and rod shaped cells were observed under light microscope with 1000X magnification power. The strain was also facultative in nature and has the ability to hydrolyse casein and gelatin. The 16S rRNA sequencing data confirmed that the strain was *Vibrio* sp. and having 99% similar with *Vibrio metschnikovii,* and its sequence has been deposited in GenBank database with accession number AY837747.

Table 3: Morphological and Biochemical tests of *Vibrio* sp. R11

Test	Inference on *Vibrio* sp. R11
Colony morphology	
Configuration	Circular
Margin	Smooth
Elevation	Emerged and Slightly raised
Opacity	Transparent
Pigment	No
Cell shape and arrangement	Curved rod and singly arranged
Gram test	Negative
Physiological and Biochemical tests	
Catalase	Positive
Oxidase	Negative
Motility	Positive
Oxygen requirement	Facultative
Nitrate Reduction	Negative
Gelatin Hydrolysis	Positive
Casein hydrolysis	Positive

5.3. Effect of medium supplements and growth profile of *Vibrio* sp. strain R11 on different media

The growth of *Vibrio* sp. strain R11 was investigated on hair and casein media in the presence of different supplements (Table 1). As shown in Fig. 7, *Vibrio* sp. strain R11 showed appreciable rate of growth when 0.5% glucose was added in the medium. Growth of this organism was relatively fast on casein medium supplemented with peptone and yeast extract (medium E). Meaning that, R11 cells attain their logarithm phase within short period as compare to other media. On the other hand, cell biomass of the strain decreased immediately as compared to the medium contained glucose. Interestingly, media A, B and C showed almost similar rate of bacterial growth until R11 cells enter to logarithm phase. On the contrary, R11 cells grown in media D and E needed relatively less time to attain their logarithm phase.

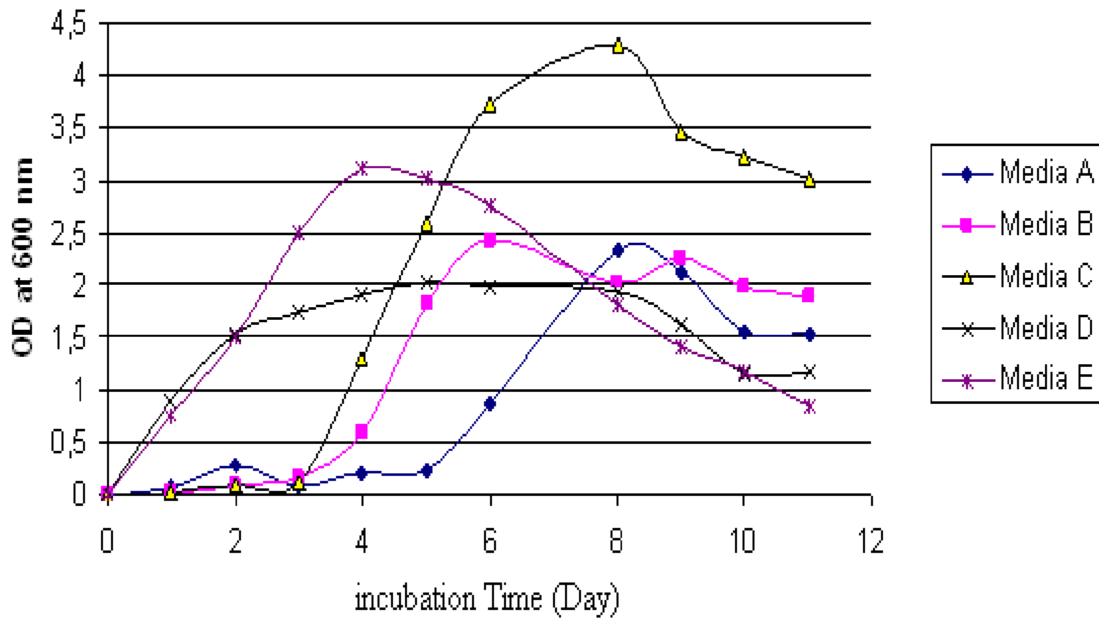

Figure 7: Effect of media composition on growth of *Vibrio* sp. strain R11. Samples were withdrawn at 24 hours interval to measure OD. Basal medium (mineral salts) supplemented with hair (Media A); hair + yeast extract (Media B); hair + glucose + yeast extract (Media C); hair + yeast extract + peptone (Media D) and casein + yeast extract + peptone (Media E). The concentration of each component was described as in (Table 1).

5.4. Time course of cell growth and enzyme activity profile

As shown in Fig. 8, even though turbidity value (OD) was used for measuring both alive and dead cells (total cell biomass) exist in the sample, its value was decreased after 168h of incubation. Keratinolytic protease production was started in the early exponential phase and showed almost linear relationship with cell biomass until late stationary phase. Even though relatively low enzyme production was observed at early exponential phase, maximum keratinolytic protease production was observed at stationary phase after 8 days (192h) of incubation. Hair degradation was started at 84h by *Vibrio* sp. strain R11. This was the exponential phase of the strain. Enzyme activity remained more or less stable until 252h and decreased with increasing incubation time. After 252h of incubation, enzyme production was decreased.

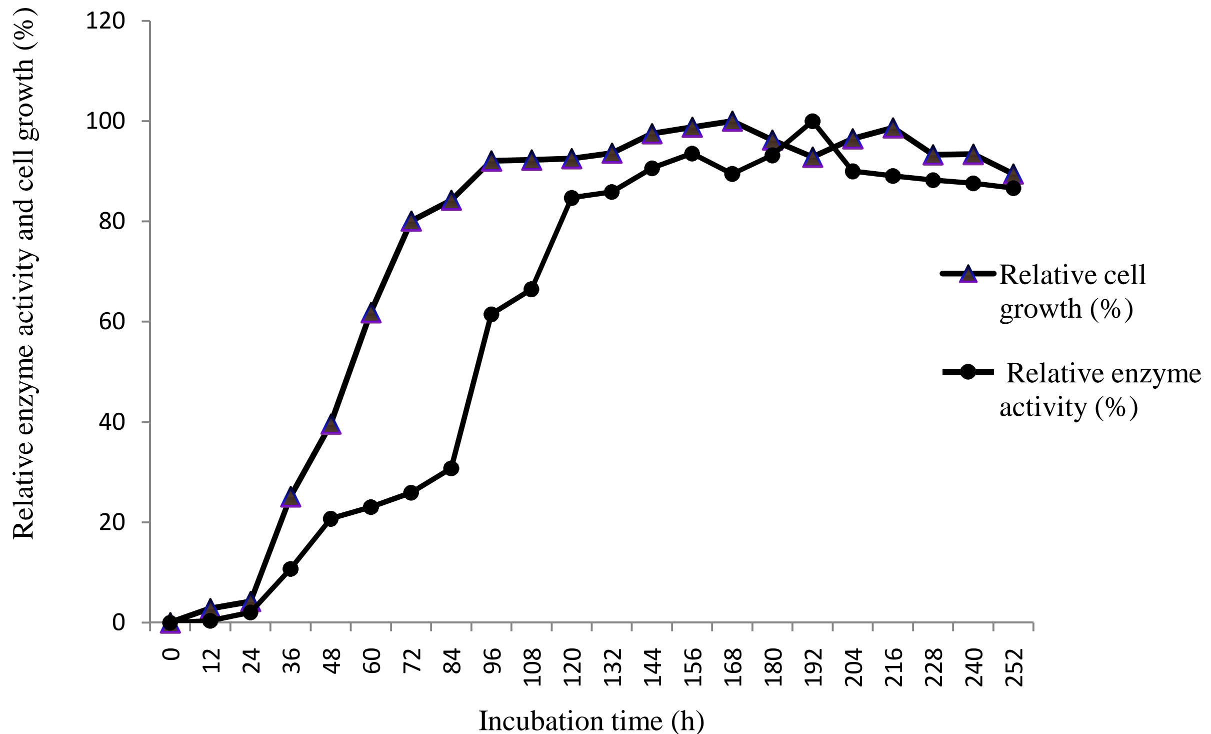

Figure 8: Time course of cell growth and enzyme activity profile at 12 h intervals

5.5. Amount of total soluble protein from degraded hair

Total soluble protein in the culture filtrate obtained from degraded hair from different media compositions was quantified according to the method described by Lowry (Lowry *et al*., 1951). As shown in Table 4, 3.17gm of soluble protein was harvested from 1L broth medium containing 10g of hair and 0.5% of glucose. The total amount of soluble protein obtained from degraded hair was almost equivalent in the basal medium, containing only hair (3.06mg/ml), to same medium supplemented with yeast extract and glucose (3.17mg/ml). On the other hand, the total soluble protein found from degraded hair from media contains basal mediumm assocated with yeast extrat (2.77mg/ml) and yeast extrat plus peptone (2.67mg/ml). Although there was no inclusion of cell in control (Medium without cell), 1mg/ml soluble protein was detected.

Table 4: Total soluble protein from degraded hair by *Vibrio* sp. strain R11

Basal medium plus	Supplemented with	Cell	Concentration of protein (mg/ml)
Hair	None	-	1.25
	None	+	3.06
	Yeast extract	+	2.77
	Yeast extract + glucose	+	3.17
	Yeast extract + peptone	+	2.67
Casein	Yeast extract + peptone	+	2.90

5.6. Characterization of alkaline keratinolytic protease produced by *Vibrio* sp. R11

5.6.1. Effect of temperature on enzyme activity and stability

Protease of R11 was active in the absence of Ca^{2+} particularly at temperature below 55°C (Fig. 9). But thermal activity of the enzyme was Ca^{2+} dependant. The enzyme became active with relative to 0mM Ca^{2+} particularly at higher temperature values (>55° C). The maximal activity of protease R11 was observed with an optimum temperature of 50°C (Fig.9) and retained 50% of its maximal activity after 3h of incubation at this temperature. Inactivation of the enzyme (80.57 %) was observed at 80°C. A further rise in temperature showed a decrease in protease activity.

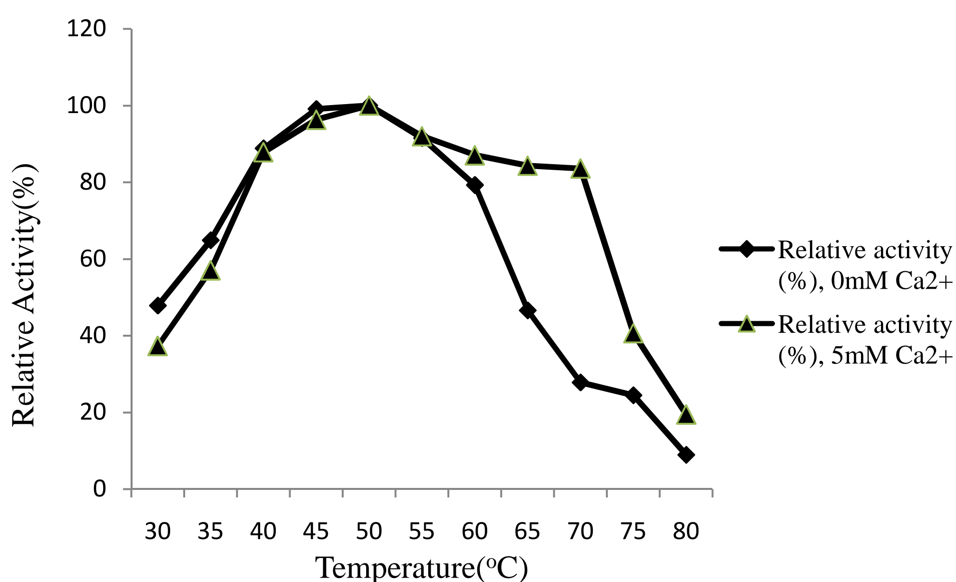

Figure 9: Effect of temperature on keratinolytic protease activity

As shown in Fig. 10, the enzyme was stable at 45 and 50ºC. At 50ºC, the enzyme was stable with a residual activity ranging from 99% to 84% in 10 and 60 minutes of incubation, respectively. At 55ºC, 76% and 13% residual activities were retained.

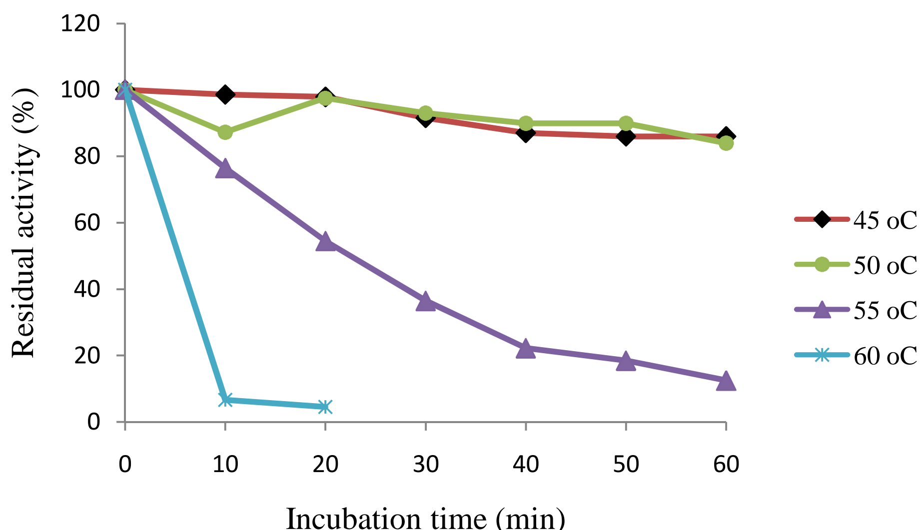

Figure 10: Thermal stability of keratinolytic protease with 5mM of Ca2+ at 45°C, 50°C 55°C and 60°C. Residual enzyme activity was determined from 0-60 min at 10 min intervals.

5.6.2. Effect of pH on enzyme activity and stability

The enzyme was active in a broad pH range and retained greater than 50% of its maximum activity in the pH range of 7.5-11.5 (Fig. 11). However, the activity was decreased to 57% of its maximal value under slightly acidic conditions (below pH 7). Optimum enzyme activity was observed at pH 11.0. An increase in pH beyond 11.0 brought about a rapid decline in keratinolytic protease activity resulting in 30% relative activity at pH 12.0.

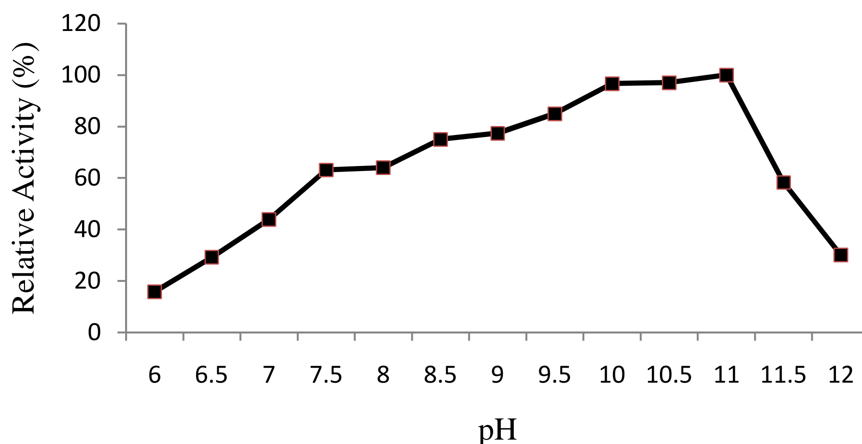

Figure 11: Effect of pH on keratinolytic protease activity at 40°C for 30 min incubation.

Investigations on pH stability shows, the enzyme was stable in the pH range of 7.0 to 11.0 with maximum stability at pH 10.0. As shown in Fig. 12, the enzyme was very stable in a broad pH range, maintaining over 85-87% of its original activity between pH 7.0 and 11.0. The enzyme was stable at both neutral and alkaline pH. But enzyme stability was declined sharply and being retained 21% of the original activity at pH 12.0.

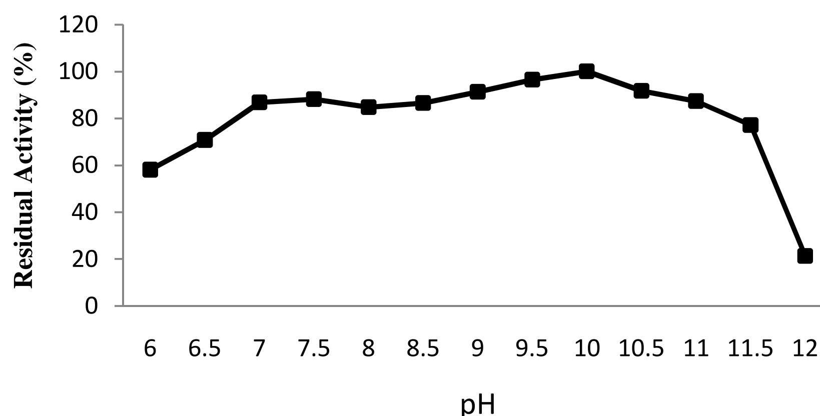

Figure 12: Effect of pH on stability of the alkaline keratinolytic protease was determined by pre incubating the enzyme in different buffers for 1 h at room temperature and the residual activity was measured at pH 10.0 and 40°C. Activity of the enzyme pre-incubated at pH 10.0 and assayed at same pH is considered as 100%.

5.7. Effect of sodium chloride concentration on stability of the enzyme

Salt tolerance of alkaline keratinolytic protease was measured in the presence of NaCl with the concentration range of 0-3M. Maximum enzyme activity was recorded at 0.5 M NaCl (99%). The enzyme exhibits appreciable activity even after 1h pre-incubation with 22% of enzyme activity in presence of 3M NaCl.

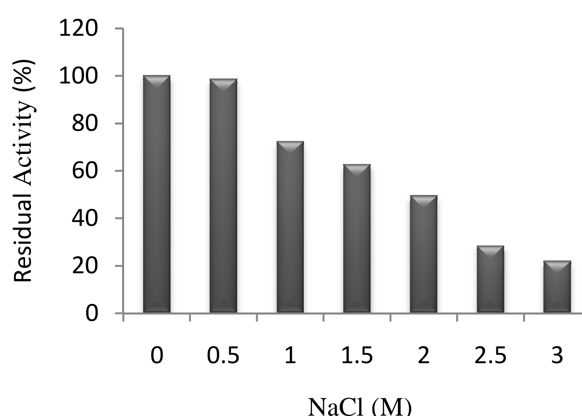

Figure 13: Effect of NaCl on stability of the crude enzyme and percentage residual activities were calculated on the basis that the activity of sample without NaCl was taken as 100%.

5.8. Effect of inhibitors and protease type

As shown in Table 5, the enzyme was moderately inhibited with metalloprotease inhibitors (EDTA and 1,10 Phenanthroline) and significantly with serine protease inhibitor (PMSF). The enzyme was inhibited almost 57.8%, 19.8% and 87.4% of its maximal activity in the presence of 10mM EDTA, 1, 10 Phenanthroline and phenylmethylsulfonyl fluoride (PMSF), respectively.

Table 5: The effect of A) EDTA, B) 1, 10 phenanthroline and C) PMSF on alkaline keratinolytic protease activity

Protease inhibitors	Concentrations (mM)	Residual enzyme activity (%)	% of inhibition
Control without any additives	0	100	0
EDTA	1	-	-
	2	43.0	57.0
	10	42.2	57.8
1, 10 Phenanthroline	1	98.9	1.1
	2	83.3	16.7
	10	80.2	19.8
PMSF	1	86.0	14.0
	2	-	-
	10	12.6	87.4

5.9. Effect of surfactants, detergents and oxidizing or bleaching agents on enzyme stability

After the enzyme was pre-incubated at room temperature for 1h with a concentrations of hydrogen peroxide in the range of 0-30%, the enzyme was very stable and retained >52% of its maximal activity at 20% of H_2O_2 (Fig.14). Whereas, only 17% of its activity was retained at maximum concentration of H_2O_2 (30%).

As shown in Fig. 15, the crude enzyme retained 83% of its maximum activity in the presence of 20% non-ionic surfactant (Triton X-100). Interestingly, the enzyme was enhanced up to 134% by Tween 80 at the concentration of 20%. The strong an ionic surfactant (SDS) at 20% caused a moderate inhibition of the activity, maintained 44% of its original activity.

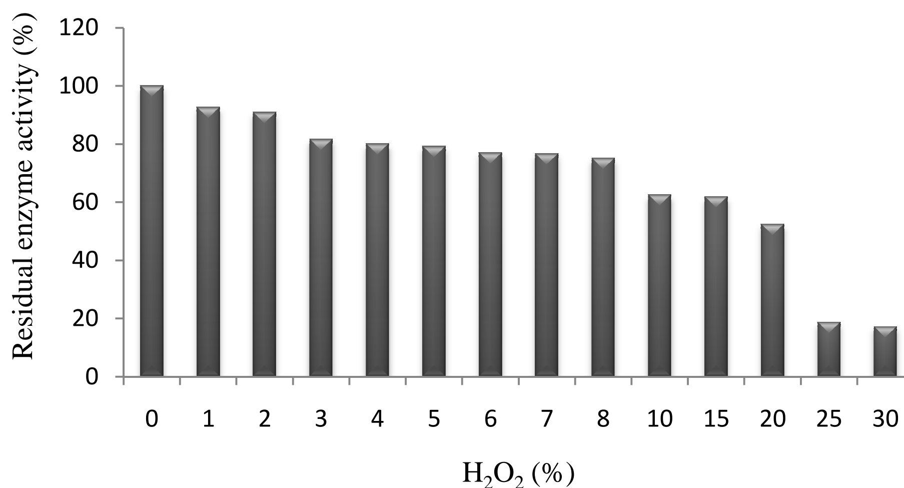

Figure 14: Effect of hydrogen peroxide on enzyme stability at 40°C and pH 10.00 for 30 min after pre-incubation of 1h at room temperature.

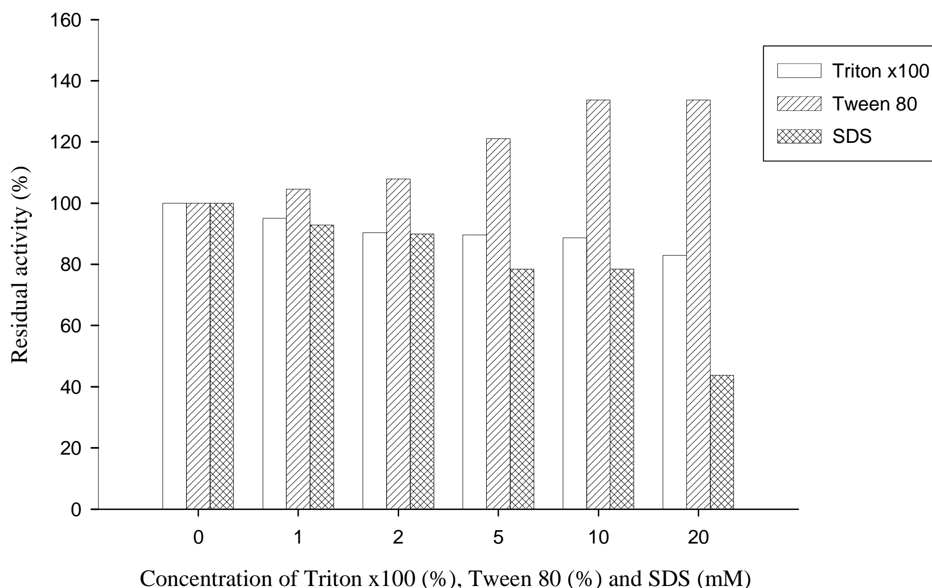

Figure 15: Effect of anionic (SDS) and non-ionic detergents (Tween 80 and Triton X-100) on enzyme stability

5.10. Effect of sodium perborate on enzyme activity

As shown in Fig. 16, $NaBO_3$, which is a bleaching agent, enhances enzyme activity until its concentration reached to 15mM with maximum activity (106%). Beyond 15mM concentration of sodium perborate, the enzyme activity was decreased by 14% from its maximum activity at a concentration of 20mM.

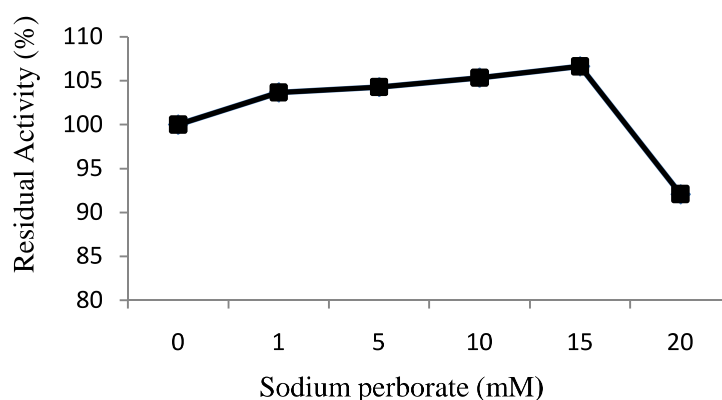

Figure 16: Effect of sodium perborate on enzyme activity

5.11. Effect of methanol on enzyme stability

As shown in Fig. 17, the enzyme was enhanced by the addition of organic solvent (methanol) up to 121% at the concentration of 30%.

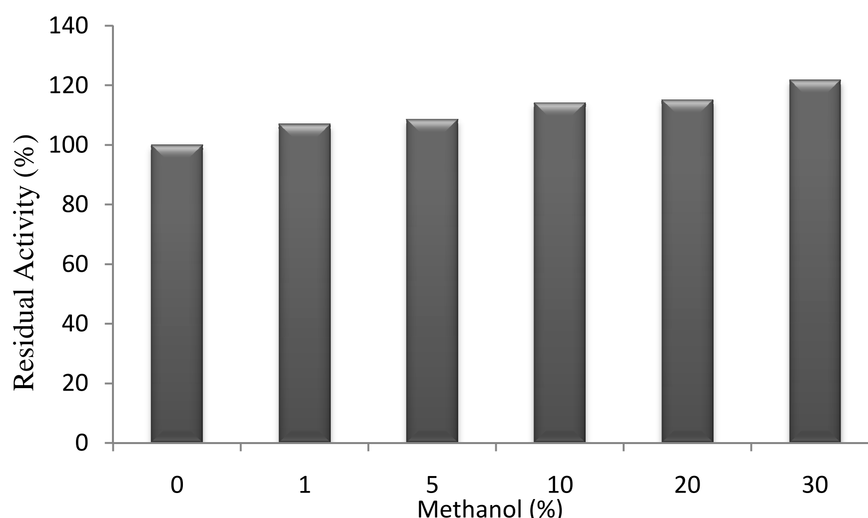

Figure 17: Effect of methanol on enzyme stability after preincubation of 1h at room temperature

5.12. Stain removal efficiency of commercial detergent and keratinolytic protease produced by R11

Although the stained test fabrics were incubated with non heated commercial detergent (Aerial) at 37°C for 30min to accomplish complete removal of both stain, there was remaining stain on the the cotton fabrics (no clear cotton fabrics were obtained) (Fig. 18 & 19 upper pictures) when compare to cotton clothes treated with enzyme. After the standard protease assay was performed, the existence of protease enzyme was not detected in the

detergent. Test fabrics treated with a solution containing detergent (heated) and enzyme was obtained clearly after 30 min of incubation at 37°C.

Potential application of keratinolytic protease for washing purpose was tested without any detergent additives using a cotton fabric stained with egg yolk and animal blood (Fig. 18 & 19 lower pictures). Enzyme with 5.8U/ml took 2h for complete removal of both egg yolk and blood stain at room temperature whereas at 37°C the stains were removed within 1h. But when the concentration was raised to 11.6U/ml, complete stain removal was observed within 1h and 30 min at room temperature and 37°C, respectively.

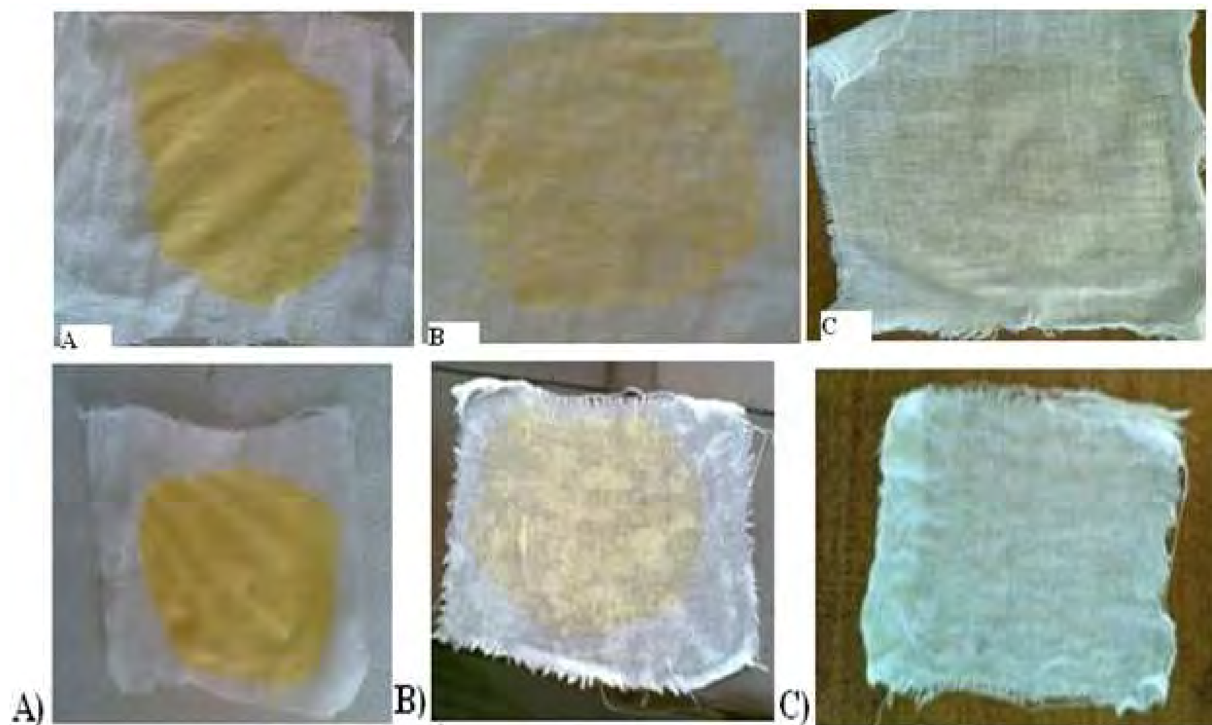

Figure 18: Egg yolk stain removal efficiency of commercial detergent (upper pictures) and alkaline keratinolytic protease produced by *Vibrio* sp.R11(lower pictures): A) Stained cotton fabric before reaction mix; B) control (0% of enzyme or detergent); C) with 11.6Uml^{-1} of enzyme or detergent; incubation time 30 min at 37°C.

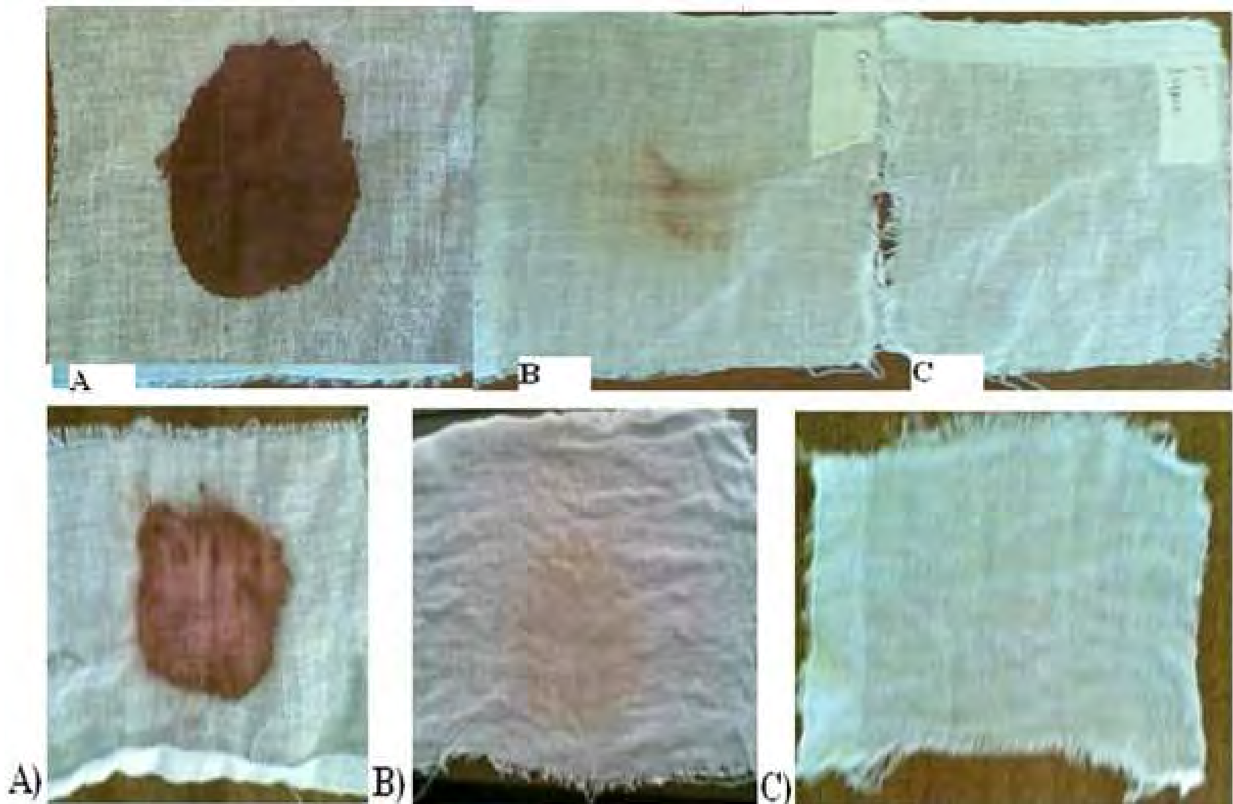

Figure 19: Blood stain removal efficiency of commercial detergent (upper pictures) and alkaline keratinolytic protease produced by *Vibrio* sp.R11(lower pictures): A) stained cotton fabric before reaction mix; B) control (0% of enzyme or detergent); C) with 11.6U/ml of enzyme or detergent; incubation time 30 min at 37°C

5.13. Testing crude enzyme for dehairing activity

Intact hairs of sheep skin pieces treated with enzyme were completely dehaired easily with mechanical action by simple scraping with fingers to remove loose hairs. This was achieved after incubation of 12h at 37°C and at 24h at room temperature. Complete dehairing was achieved using both Glycine and NaOH buffer without any time difference. Hair loosening was not observed in control even by mechanical action of fingers. Pieces of sheep skins treated with enzyme and control showed visible differences. Although the presence of depilated area was noticed on the skins treated with enzyme, no deterioration and color alteration was observed on the dehaired skin as compared to the control (Fig. 20). The skins treated with enzyme showed slightly an increment of surface area as well as they were also softened.

Figure 20: Complete dehairing activity using 58U/ml alkaline keratinolytic protease produced by *Vibrio* sp. without any addition of lime and sodium sulfide in the reaction mixture at 37°C for 12h incubation A) Sheep skin with heat-denatured alkaline enzyme at 100°C for 1h, with buffer (control) and B) Enzymatically dehaired sheep skin incubated with non-denatured enzyme.

5.14. Removal of gelatinous coating from x-ray film

5.14.1. Effect of temperature on hydrolysis of gelatin from used x-ray film

Hydrolysis of gelatin was observed at temperatures ranges from 30-80°C. The appropriate temperature for removal of gelatin layer from used x-ray film (Fig. 21) was 55°C (Fig. 22). Gelatin removal was completed within 3 min at 55°C. Removal of gelatin bellow 55°C was no significant difference in both the presence and absence of 5mM of $CaCl_2$ (Table 6). At higher temperature (>65°C) gelatin removal efficiency of the enzyme was slightly decreased until the temperature reaches 80°C. But temperatures >80°C, no complete removal of gelatin was observed even after 30 min of incubation in the absence of 5mM of $CaCl_2$. In the presence of 5mM of $CaCl_2$, the enzyme was highly active in the first 5 min of incubation even at the temperature of 85°C, and partial gelatin removal was observed. But after 5 min of incubation, the enzyme was totally inactivated and no gelatin removal was observed.

Table 6: Time course and reuse of enzyme for complete removal of gelatin from used x-ray film at a temperature ranging from room temperature to 85°C with 11.6U/ml enzyme in the presence and absence of 5mM of $CaCl_2$

Temperature (°C)	Time (min) in the absence of 5mM of $CaCl_2$			Time (min) in the presence of 5mM of $CaCl_2$		
	1st Cycle	2nd Cycle	3rd Cycle	1st Cycle	2nd Cycle	3rd Cycle
Room	56.8	121	240	57	120	240
30	18.4	30	61	17.56	29.41	61
35	12.6	15	20.6	12.06	15.08	19.43
40	9	9.3	9.44	9	9.21	9.48
45	7	8.3	9	7.05	8.23	8.98
50	4.1	5.84	7.13	4.19	5.83	6.99
55	3.2	4.4	5.03	3.06	4.4	5
60	3.97	5.72	7.03	3.19	4.03	5.5
65	4.56	7.28	12.01	4.24	6.36	6.95
70	5.21	9	Nr	4.4	6.59	7.2
75	6.74	Pr at 12	Nr	5	7.21	9
80	Pr at 8	Nr	Nr	5.50	Pr at 15	Nr
85	Nr	Nr	Nr	Pr at 5	Nr	Nr

Pr = partial removal
Nr = no removal

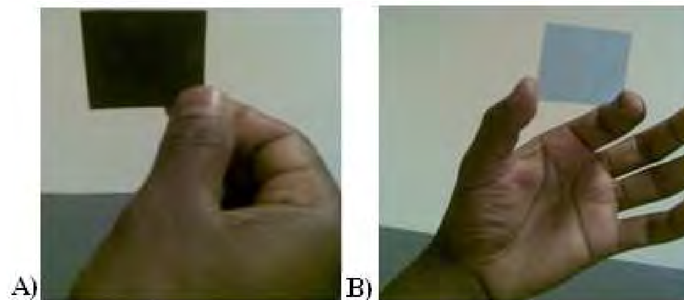

Figure 21: Sample pictures: (A) Before and (B) After gelatin layer removal from the film after 3 min incubation at 55°C with 11.6 U/ml enzyme concentrations.

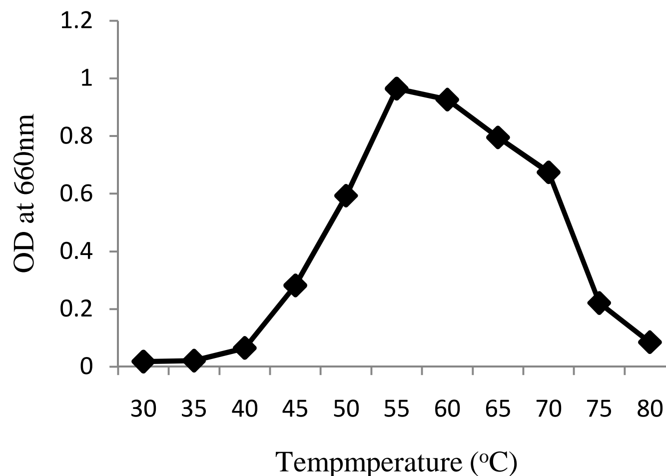

Figure 22: Effect of temperature on hydrolysis of gelatin from used x-ray film by measuring intensity of turbidity of the stripped solution, enzyme concentration 11.6 U/ ml, sample withdrawn 3 min interval, pH 10.00

5.14.2. Reuse of enzyme for gelatin hydrolysis from used x-ray film in the presence and absence of $CaCl_2$

As shown in Table 6, enzyme has a potential to remove gelatin three times within 7h at room temperature, and 14 min at 55°C. As shown Fig. 23, the alkaline keratinolytic protease was incubated for about 145 min (total time incubation) at 50°C. Even though the enzyme was incubated for long time, it has the capability to remove gelatin layer from used x-ray film until 2h and 8min of incubation without increasing the time of removal. This means, removal was observed at a constant time (7 min) for long time within the range of 4-18 cycles. But after 18 cycles, removal was observed with increasing of time without stopping gelatin hydrolysis.

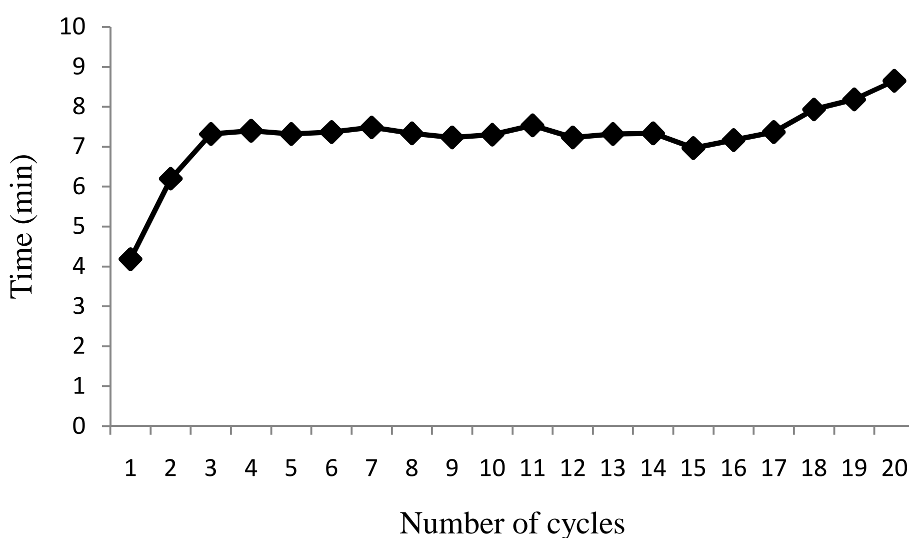

Figure 23: Reuse of enzyme at 50°C with 11.6U/ml of enzyme in the absence of 5mM CaCl2

5.14.3. Effect of pH on hydrolysis of gelatin from used x-ray film

Gelatin removal was observed over a wide pH range of 7 to 11.5. Complete removal of gelatin was observed within 3 min at pH 10.5 (Fig. 25), which was the optimum pH (Fig. 24). But no complete removal was observed at pH 12.

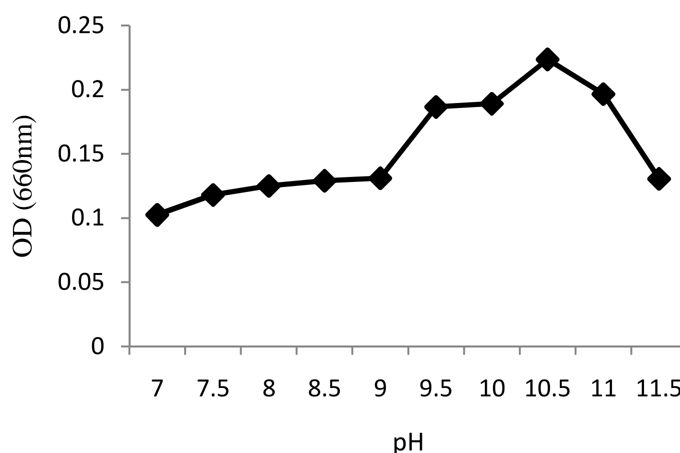

Figure 24: Effect of pH on stripping of gelatin layer from used x-ray film using 11.6U/ml enzyme extract, sample withdrawn 3 min interval, incubation at 50°C.

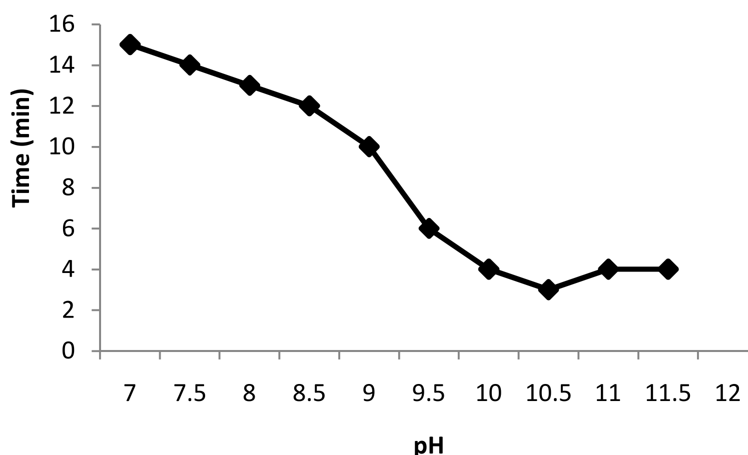

Figure 25: Time course (min) of complete removal of gelatin from used x-ray film in the absence of 5mM CaCl2 with pH value from 7-12 at 50°C.

5.14.4. Enzyme concentration and time course of gelatin hydrolysis from x-ray film

As shown in Fig. 26, rate of gelatin hydrolysis was increased as the amount of enzyme increased. But when the enzyme concentration was decreased, time of gelatin hydrolysis from

the film was increased. Complete removal of gelatin from the film was observed at 2 min and 25 min using 58U ml^{-1} and 0.29Uml^{-1} enzyme concentrations, respectively.

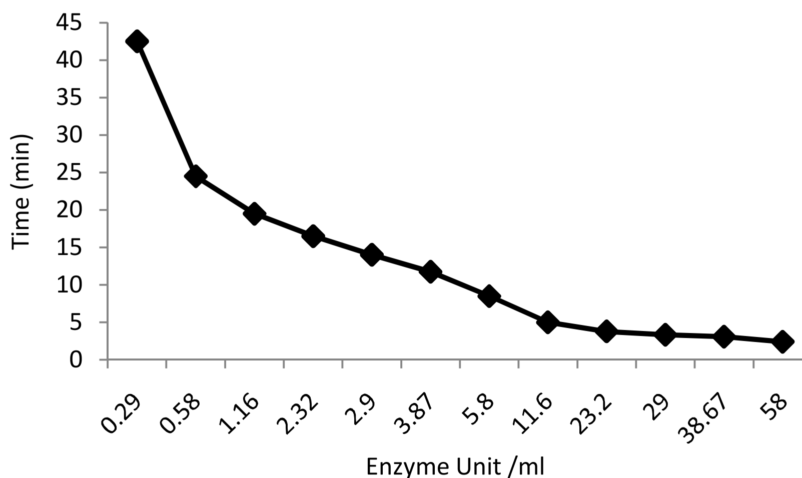

Figure 26: Effect of enzyme concentration and time course of gelatin hydrolysis from used x-ray film at 50°C and pH 10.00.

5.14.5. The effect of Glycine buffer, $CaCl_2$ and incubation period for removal of gelatin

Although the film was added after the enzyme was pre-incubated with buffer alone for 5 min at 85°C, no gelatin removal was observed. Similarly, no gelatin removal was observed at all when the experiment was conducted without incorporation of enzyme in the reaction mixture (5mM of $CaCl_2$ and buffer only) at 70°C for 30 min.

5.14.6. Weight loss of the film during gelatin removal from used x-ray film

Treatment of x-ray films with enzyme resulted in stripping off the sliver bound with gelatin in to the reaction mixture and clean plastic film was recovered. Therefore, weight loss of the film was noticed after clean used x-ray film was obtained. The initial weight of the film was 0.423 gm (4cm x 4cm pieces of the film) before incubation. After gelatin and other components of the film were removed from the film with enzyme having 11.6Uml^{-1} activity in the reaction mixture, the weight of the film becomes 0.4064. Therefore, value of weight loss was calculated as 0.017 gm (0.423 gm – 0.4064 gm). The value indicates that 4% of the film weight was lost after the film was treated with enzyme.

6. Discussion

A total of ten alkaliphilic protease producing bacteria isolated from mud sample collected at the shore of different alkaline soda lakes in the Rift Valley of Ethiopia (Gizachew, 2009) were screened for hair degrading properties. Of these, five isolates showed good growth and keratinolytic protease production using hair medium. Based on their hair degrading efficiency and the level of enzyme production, only one strain designated as R11 was selected for this study because it has the capability of growing and degrading hair at room temperature within short period of incubation when compared to others. This implies that R11 can be cultivated easily using cheap resources at room temperature without any culture environment adjustment (heat energy input) during fermentation process.

As shown Fig. 7, the growth of R11 cells in media A, B and C was relatively took more time at lag phase. This is because the cells need longer time to establish themselves to utilize the hair as a sole carbon and nitrogen source. Whereas, R11 cells grown in media D and E need less time to enter their log phase. Because they can utilize the available peptone and yeast extract easily for their growth. However, once R11 cells establish their lag phase, grown on media A, B and C, they started to utilize hair as a sole source of carbon and nitrogen and spent more time until they attain their decline phase as compare to R11 cells grown on media D and E.

The appreciable growth of R11 in the medium containing hair indicates that the isolate has a potential to utilize hair as a carbon and nitrogen sources. Previous studies also indicated that *Bacillus licheniformis* (Vigneshwaran *et al.,* 2010) and *Chryseobacterium* sp. (Lv *et al*., 2010) have also the potential to degrade hair, and they can use it as a sole carbon and nitrogen sources for their growth. Growth and production of substantial amount of keratinolytic protease by utilizing hair as a cheap fermentation substrate by R11 offers a feasible microbial technology for obtaining keratinolytic enzymes. This is indirectly eexpected to minimize enzyme production cost.

Based on its morphological and physiological characteristics as well as 16S rRNA sequence analysis, the isolate designated as R11 was identified as *Vibrio* sp. having 99% identity with *Vibrio metschnikovii*. Meanwhile, there was still no any report on the topic of potential applications of *Vibrio* sp. and their existence in Ethiopian Soda Lakes.

Vibrio sp. strain R11 was found to be extremely efficient for degradation of hair within 5 days of incubation at room temperature. Unlike the studies conducted by Saber *et al.,* (2010) and Lv *et al.* (2010), in this study no milling or other prior mechanical treatment of the hair was required for the growth of this organism, which greatly reduces processing costs. The isolate could also be used for waste management program because of its keratinous waste removal ability from leather industries, thereby protecting the environment by minimizing wastage.

The highest enzyme production was observed at stationary phase, specifically at 192h (Fig. 8). Therefore, periods before and after 192h were not the exact harvesting times for keratinolytic protease produced by *Vibrio* sp. strain R11. For example, after 252h of incubation a decrease of enzyme production was observed. Other protease by *Bacillus* sp. (Oberoi *et al.,* 2001; Gouda, 2006) and *Salinivibrio* sp. strain AF-2004 (Karbalaei-Heidari *et al.,* 2007) were also produced maximally at stationary phase. A similar trend was also observed for extracellular keratinolytic protease production by *Vibrio* sp. strain kr2 (Sangali and Brandelli, 2000). Furthermore, as Gupta and Ramnani (2006) reported that maximum keratinolytic protease activity is achieved in the late exponential or stationary growth phase.

A decrease in enzyme production was observed at late stationary phase (Fig.8), which is similar to previous reports (Oberoi *et al.,* 2001; Gouda, 2006; Gupta and Ramnani, 2006). This may occur due to the increment of cell death (decline of microbial growth) associated with the depletion of available nutrient, required for the growth of R11 cells. Consequently, there was no more production of the enzyme and those that have been previously produced might have undergone enzymatic autolysis and were inhibited by other end-product in the medium (Syed *et al.,* 2009). Since incubation was continued after cell population reaches at stationary phase, a death phase follows, in which the viable cell population of R11 declined, which was also another factor for the decline of enzyme production at this growth phase. Therefore, understanding of cell growth associated with maximum enzyme production period could be very important for identifying the fermentation period for maximal enzyme productivity. It is also advantageous to use the enzyme for several biotechnological applications.

A new low-cost media formulation was developed in this study using a cheap and readily available keratinous waste (unused animal hair) for keratinolytic protease production. This could result in a substantial reduction in the cost of enzyme production and can led to the production of this enzyme in industrial scale. Although organisms have a potential to produce enzymes, according to Amare Gessesse (1997), production cost of enzyme is the critical issue and the study is preferable to conduct using low-cost substrate.

Although R11 keratinolytic protease was inhibited by serine and metalloprotease inhibitors, the best preference to deduce the enzyme, used in this study, was very likely to be a serine protease because the enzyme was inactivated by PMSF and its optimum pH was found in a basic range. This finding is in line with several earlier reports showing that serine proteases may slightly be affected by metalloprotease inhibitors (Bockle *et al.,* 1995; Manachini *et al.,* 1998; Bressollier *et al.,* 1999; Lin *et al.,* 1999; Singh *et al.,* 2001). Studies have also shown that proteases produced by *Vibrio* strains of environmental origin were usually serine proteases (Kwon *et al.,* 1995; Marcello *et al*., 1996). Lin *et al*. (1992) reported that most of the microbial keratinolytic proteases belonged to serine protease family. Based on all the above mentioned reports, partial inhibition of EDTA on keratinolytic protease produced by *Vibrio sp.* strain R11 does not indicate that the enzyme is necessarily a metalloprotease.

Eventhough unmeasurable hair protein hydrolysate was utilized as nutrient for bacterial growth, 3172.5 mg/L of net soluble protein was obtained from 10 gm of hair. This indicates that 31.73% protein was recovered. A similar study was also conducted and 1440 mg/L of soluble protein was obtained from degraded feather (Deivasigamani and Alagappan, 2008). Therefore, the amount of protein obtained from hair in this study was higher than that of the above study, with 1732.5 mg. As Williams *et al.* (1991) reported, bacterial-treated keratin hydrolysates have similar nutritional features of soybean meal, and have been considered as an excellent source of metabolizable protein. Hence, production of soluble protein from keratinous wastes may be a good alternative to obtain one of the most expensive ingredients of diets. For this reason, the capability to degrade keratinous waste by keratinolytic protease obtained from *Vibrio* sp. strain R11 to soluble proteins offers a tremendous potential for the improvement of animal feedstuffs. Therefore, besides environmental considerations the use of keratinolytic enzymes in the production of soluble proteins and peptides becomes attractive for biotechnological applications.

Although there were no cells in medium (control), 1mg/ml soluble protein was detected probably due to the nature of media (alkaline), which could contribute the crumble of some peptide bonds from the hair. Similarly, some studies infer that amino acids fall to pieces by alkali or thermal processing of feathers (Papadopoulos *et al.,* 1986; Kim and Patterson, 2000). As Coward-Kelly *et al.* (2006) reported, a liquid product rich in amino acids and polypeptides has been obtained from chicken feather treated with lime (calcium hydroxide). Autoclaving process and unpredictable reactions in the reaction mixture during incubation period could also be other factors for the existence of this little amount of protein.

No significant difference of enzyme activity was observed in the absence and presence of Ca^{2+} specially at temperature $<55^oC$. Probably extracellular enzymes from microorganisms adapted in alkaline environments are expected optimally active in the presence of low Ca^{2+} concentration or in the absence of Ca^{2+} at low temperatures. Therefore, the effect of Ca^{2+} was not significant for stability of the enzyme at lower temperature below 55^oC. However, a further rise in temperature ($>55^oC$) resulted in a decrease in keratinolytic protease activity in the absence of Ca^{2+}. But the enzyme was slightly affected positively (become active) at a temperature above 55^oC in the presence of Ca^{2+}. Requirement of Ca^{2+} for enzyme stability at higher temperatures has also been reported by Amare Gessesse (1997). The improvement in protease thermo-stability against thermal inactivation in the presence of Ca^{2+} may be explained by the strengthening of interactions inside the tertiary structure conformation of protein molecules and by binding of Ca^{2+} to autolysis sites (Secades and Guijarro, 2001; Beg and Gupta, 2003).

In the process of detergent formulation, alkaline proteases and chelating agents (that are effective to overcome the problem of water hardness) are commonly added. In the presence of chelating agents, Ca^{2+} can easily be stripped off from the weak binding site of the alkaline protease that requires Ca^{2+} for its stability. Thus, thermal stability of the enzyme could be greatly affected under application conditions. But enzyme produced by *Vibrio* sp. strain R11, which does not require Ca^{2+} for the maintenance of its activity and stability especially at temperatures below 55^oC, could offer tremendous potential for detergent application.

Enzyme activity was highly stable at 45^oC and 50^oC because its catalytic site was well protected by a substrate. Meaning that, the substrate served as stabilizing agent before total conversion of substrate to products was occurred at the above mentioned low temperature

values. After 30 min of incubation, complete inactivation of the enzyme was observed at 70°C and higher temperature values. This could happen due to rapid conformational change of the enzyme, caused by higher temperatures. Similarly, Ghorbel *et al*. (2003) reported that alkaline proteases could be denatured at high temperatures. Another factor for complete inactivation of the enzyme was long incubation period at those temperatures.

Keratinolytic protease was less active under slightly acidic conditions (below pH 7) and there was a rapid decline in its activity at relatively alkaline pH (12.0). The enzyme was active in a range of pH 7.5-11.5, and greater than 50% of its activity was retained in this range. The enzyme showed broad stability to pH range of 7.0 to 11.0. Therefore, the enzyme is said to be typical alkaline protease. This finding is similar to earlier reports which showed pH optima of 11.0 for protease produced by *Bacillus* sp. NCDC 180 (Kumar *et al*., 1999), *Bacillus* sp. RGR-14 (Oberoi *et al*., 2001), *Bacillus* sp. KSM-K16 (Kobayashi *et al*., 1996) and *Bacillus pseudofirmus* AL-89 (Amare Gessesse *et al*., 2003). The result was also similar to Singh *et al*. (1999) who found that a protease produced by *Bacillus sphaericus* was stable in the pH range of 7.5 to 11.0, but at pH 12.0 the enzyme lost its activity. According to the specified nature of this enzyme, it is suitable for applications that require high and wide pH stability range such as in dehairing and preparation of detergents. The enzyme, used in this study, was found to have similar pH stability to commercially important detergent enzymes such as Subtilisin Carlsberg and Subtilisin Novo or BPN (Rao *et al.,* 1998).

The alkaline keratinolytic protease from *Vibrio* sp. R11 was very stable in the presence of well known oxygen bleaching agent (sodium perborate), oxidizing agent (Hydrogen peroxide), non-ionic and ionic detergents (SDS). Therefore, stability of this enzyme is an important characteristic for its eventual use in detergent formulations. Similarly, as Oberoi *et al.* (2001) reported, a good proteolytic enzyme incorporated into detergent formulations must be active as well as stable at high pH range (9-12) and high temperatures (40-50°C). Proteolytic enzyme must also be compatible and stable with all commonly used detergent compounds such as surfactants, bleaches, oxidizing agents and other additives (Kumar and Takagi, 1999).

Proteases containing Methionine (Met) residue in the surrounding area of the catalytic site readily inactivated by chemical oxidants like sodium perborate, one of the chemical oxidants (Siezen *et al.,* 1991). Therefore, the result obtained in this study leads to deduce the enzyme

produced by *Vbrio* sp. R11 has no or a few Met residues in its catalytic site because it has a great potential to withstand the inhibition effect of this bleaching agent up to 15mM concentration.

Non-ionic surfactant (Tween 80), bleach-based detergent ($NaBO_3$) and organic solvent (methanol) were showed important enhancement of enzyme activity (Fig. 15). Specially, the enzyme was highly enhanced and stabled against methanol so that denaturation or unfolding of enzyme was not observed. This capability may be due to the presence of disulfide bond(s) in this protease, which is found to be essential for its activity. This indicates that no reduction process was occurred on the intact disulfide bonds exist in the polypeptides of the enzyme. The result infers that intact disulfide bonds might be important to maintain the molecular folding required for keratinolytic protease to be fully active. Another report also confirmed the importance of disulfide bonds for stability of the protein against solvents (Ogino *et al.,* 2001).

As shown in Fig. 18 & 19, the enzyme is promising for removal of proteinaceous stains such as keratin, blood, egg and other body secretions on fabrics in highly alkaline detergent solution matrices. Interestingly, stain removal process was accomplished without any commercial detergent additives even at room temperature with a minimum concentration of enzyme (5.8U/ml).Therefore, keratinolytic proteases produced by *Vibrio* sp. strain R11 could be one of the most appropriate detergent additives.

Unlike the enzyme used in this study (11.6U ml^{-1}), which has the ability to remove blood and egg yolk stain completely within 1h at room temperature and 30 min at 37^oC, the protease (100U/ml) that was used to remove egg yolk stain from test fabric needs 2h for complete egg stain removal even at 40^oC (Kumar and Bhalla, 2004). The accomplishment of stain removal process even at room temperature with a minimum concentration of enzyme (5.8U/ml) within 2h indicates that this study was highly cost effective and will be needs a little amount of enzyme incorporation during detergent formulation.

Although the skins were treated with crude denatured enzyme (control), no dehairing was observed. This result indicates that the alkaline medium alone has no contribution for dehairing of the skins. On the contrary, R11 keratinolytic protease causes loosening of the hair without deteriorating the leather. This is evident that alkaline keratinolytic proteases

enables the swelling of hair roots and the subsequent attack of protease on the hair follicle protein allowing easy removal of the hair without damaging collagen (Gupta *et al*. 2002; Macedo *et al.,* 2008).

R11 keratinolytic protease could fulfill the required optimum conditions in leather industry. Because it was active at alkaline pH and has a potential to remove hair completely within short incubation time (12h) at 37°C. Choudhary *et al*. (2004) also reported that protease enzymes used in bating are most active in a pH range of 8-9. Apart from pH, temperature also plays significant role in enzymatic dehairing process and accomplishment of dehairing is preferable at temperatures ranging from 32-37°C with time interval <24h. All the previous studies and the result obtained in this study confirmed that R11 keratinolytic proteas is a suitable enzyme for leather industry.

Despite complete dehairing was achieved within 12h when the skin pieces were treated with the combination of crude enzyme preparation, sodium sulfide and lime (Pillai and Archana, 2008), in the present study dehairing was achieved within 12h in the absence of hazardous chemicals like sodium sulfide. Hence, the enzyme used in this study has the potential to replace sodium sulfide in the dehairing process of leather industry. Therefore, considering environmental concern, the present study has highly advantageous for minimizing environmental polution caused by tannery process. Similarly, keratinolytic protease produced by *Bacillus subtilis* also showed a complete deharing activity in the absence of sodium sulfide (Macedo *et al*., 2005; Arunachalam and Saritha, 2009) within 7h-9h interval.

The achievement of complete dehairing using only 1M NaOH buffer indicates that it was cost effective when compared with dehairing experiment designed using Glycine buffer. Achievement of complete dehairing without energy assisted process was also another interesting cost effective investigation of this study. All the above properties make R11 keratinolytic protease become a tremendous candidate for dehairing process in leather industry.

Enzymatic peptide synthesis offers several advantages over chemical methods. However, the major limitation for the use of protease in synthetic chemistry is the strongly reduced activity of the enzyme under anhydrous conditions (Gupta *et al*., 2002). Meanwhile, peptide synthesis can be enhanced by the addition of organic solvents in the reaction mixture including

methanol (Gupta, 1992). Therefore, solvent resistant property of this enzyme could be used for peptide synthesis process and eradication of microbial contamination during fermentation process in the presence of methanol.

In the absence of 5 mM $CaCl_2$, complete gelatin removal was observed only in the 1st cycle at 80°C with non-denatured enzymes (Table 6). After 8 minutes of incubation, no complete gelatin removal was achieved at this temperature because of the enzyme was totally deactivated. However, in the presence of 5 mM $CaCl_2$, 2nd cycle complete removal was achieved. This is evident that the requirement of Ca^{2+} for enzyme stability at higher temperature.

As shown in Fig. 23, gelatin removal efficiency of the enzyme requires more time (>7 min) after 18 cycles reuse. Because the enzyme was incubated continuously for about 2h and 8 min at 50°C to remove gelatin layer from used x-ray film. But the enzyme was highly stable from 10-128 min of incubation. This also confirmed that thermal stability of the enzyme at 50°C. Similar study also conducted by Shankar *et al.* (2010) at 50°C for removal of gelatin from used x-ray film using enzyme more than once, which have a concentration of 5U/ml, and the protease spent 60 min and >120 min in the 1st and 2nd cycles, respectively. Hence, comparing the experiment conducted in this study, which requires 4.18 min and 6.2 min to remove gelatin in the 1st cycle and in 2nd cycle, respectively (Fig. 23), the previous study was highly time consuming. This means that approximately 15 times and 20 times fold increasing of time, respectively. The above relations infer that the enzyme produced by *Vibrio* sp. strain R11 is an excellent candidate for silver recovery process from used x-ray film. In addition to silver recovery pocess the enzyme could also used for gelatin hydrolysis in the food processing industry.

In addition to accomplishing gelatin removal within short time (18 min/three cycles at 50°C) (Fig. 23), it is possible to remove gelatin layer from used x-ray film with a minimum concentration of enzyme (0.29U/ml) with compare to the previous study (Masui *et al.,* 1999). As they reported, 60 min was required for complete removal of gelatin using 5U/ml. Nevertheless, in this study 9.5 min was required using 5.8U/ml of enzyme concentration. This indicates that the time required for the previous study is around 5 times fold increase when compared to this study.

Although the film was added after the enzyme was pre-incubated with buffer alone for 5 min at 85°C, no gelatin removal was observed due to inactivation of enzyme before 5 min pre-incubation at this temperature. On the other hand, no gelatin removal was also observed at all when the experiment was conducted without incorporation of enzyme in the reaction mixture (5mM of $CaCl_2$ and buffer only) at 70°C for 30 min, Therefore, this result confirmed that buffer alone and $CaCl_2$ have no role for removal of gelatin from used x-ray film.

The result obtained in this study indicates that x-ray film could loss its weight by 4% when it is treated with enzyme, having capability of removing gelatin layer from the film. The same study also conducted by Shankar *et al.* (2010) and loss in weight after enzyme treatment was around 5% (w/w) based on initial weight of the film.

Generally, the method developed in this study for removal of gelatin is cost effective as well as it has a minimal or zero level impact on environment as compared to conventional methods. These are due to incorporation of minimum concentration of enzyme and without any additive of hazards chemicals during gelatin removal process. A recovery of pure polyester film obtained after gelatin removal can be reused. This could help to minimize production of large number of films, which is also another cost effective technology.

7. Conclusion and Recommendations

7.1. Conclusion

The media formulated using keratinous waste for keratinolytic protease production was low cost and applicable to cultivate bacterial isolates having keratinolytic activities for the production of the enzyme in industrial scale. Keratinolytic property of the enzyme helps to convert keratinous wastes in to more nutritionally available animal feedstuff. The enzyme is highly active at optimum temperature of $50^{o}C$ and pH 11. It is also active in broad pH and temperature ranges. The enzyme is highly efficient for stain removal and stable in the presence of detergents, bleaching agent and surfactants. All these properties infer that its suitable application as additive in detergent formulations.

The enzyme has a great potential for leather industry because of its dehairing potential and environmental friendly approach.The enzyme also has a potential to remove gelatin layer from used x-ray film so that applicable in silver recovery.

7.2. Recommendations

Depend on the results obtained from this study, the following recommendations should be considered for future work:

1. Optimize and scale up enzyme production
2. Stabilize the enzyme using different stabilizers for commercial scale application
3. Evaluate the potential applications of the enzyme for dehairing, detergent formulation and silver recovery process at a pilot scale
4. Study the feasibility of large scale production for commercial purpose

References

Aboushwareb, T., Eberli, D., Ward, C., Broda, C., Holcomb, J., Atala, A. and Van Dyke, M. (2009). A keratin biomaterial gel hemostat derived from human hair: Evaluation in a rabbit model of lethal liver injury. *J. Biomed. Mater. Res.* **90:**45-54.

Agrawa, D., Patidar, P., Banerjee, T., Patil, S. (2004). Production of alkaline protease by *Penicillum* sp. under SSF conditions and its application to soy protein hydrolysis. *Process Biochemistry*. **39:**977-981.

Amany, L., Kansoh, Ebtsam, N., Hossiny Eman, K. and EL-Hameed, Abd. (2009). Keratinase Production from Feathers Wastes Using Some Local Streptomyces Isolates. *Australian Journal of Basic and Applied Sciences*. **3:**561-571.

Amare Gessesse (1997). The use of Nug meal as a low-cost substrate for the production of alkaline protease by the alkalipilic *Bacillus sp.* AR-009 and some properties of the enzyme. *Bioresource Technology.* **62:**59-61.

Amare Gessesse (1998). Purification and properties of two thermostable alkaline xylanase from an alkaliphilic *Bacillus* sp. *Appl. Environ .Microbiol*. **64:**3533-3535.

Amare Gessesse and Berhanu Gashe (1997). Production of alkaline protease by alkaliphiic bacteria isolated from Soda Lake. *Biotechnol. Lett*. **19:**479-481.

Amare Gessesse, Hatti-Kaul, R., Berhanu Gashe and Mattiasson, B. (2003). Novel alkaline proteases from alkaliphilic bacteria grown on chicken feather. *Enzyme and Microbial Technology*. **32:**519-524.

Anwar, A. and Saleemuddin, M. (1998). Alkaline proteases: a review. *Bioresources Technology.* **64:**175-183.

Apel, P.J., Garrett, J.P., Sierpinski, P., Ma, J., Atala, A., Smith, T.L., Koman, L.A. and Van Dyke, M. (2008). Peripheral nerve regeneration using a keratin-based scaffold: Long-term functional and histological outcomes in a mouse model. *J. Hand Surg. Am.* **33:**1541-1547.

Arunachalam, C. and Saritha K. (2009). Protease enzyme: an eco-friendly alternative for leather industry. *Ind. J.Sci. and Technol.* **2:**29-32.

Balint, B., Bagi, Z., Toth, A., Rakhely, G., Perei, K. and Kovacs, K.L. (2005). Utilization of keratin-containing biowaste to produce biohydrogen. *Appl Microbiol Biotechnol,* **69:**404-410.

Banik, R.M., Prakash, M. (2004). Laundry detergent compatibility of the alkaline protease from Bacillus cereus. *Microbiological Research.* **159:** 135-140.

Beg, Q.K. and Gupta, R. (2003). Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from Bacillus mojavensis. *Enzyme Microb. Technol.* **32:**294-304.

Beg, Q.K., Sahai, V. and Gupta, R. (2003). Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. *Process Biochemistry*. **39:**203-209.

Bernal, C., Cairo, J. and Coello, N. (2006). Purification and characterization of a novel exocellular keratinase from *Kocuria rosea*. *Enzyme Microb. Technol.* **38:**49-54.

Bockle, B., Galunsky, B. and Muller, R. (1995). Characterization of a keratinolytic serine proteinase from *Streptomyces pactum* DSM 40530. *Appl. Environ. Microbiol.* **61:**3705-3710.

Brandelli, A. (2008). Bacterial keratinases: useful enzymes for bioprocessing agroindustrial wastes and beyond. *Food Bioprocess Technol.* **1:**105-116.

Brandelli, A., Daroit, D.J. and Riffel, A. (2010). Biochemical features of microbial keratinases and their production and applications, Mini Review. *Appl. Microbial. Biotechnol.* **85:**1735-1750.

Bressolier, P., Letourneau, F., Urdaci, M. and Nerneuil, B. (1999). Purification and characterization of keratinolytic serine proteinases from *Streptomyces albidoflavus*. *Appl. Environ. Microbiol*. **65:**2570-2576.

Cheng, S.W., Hu, H.M., Shen, S.W., Takagi, H., Asano, M., and Tsai, Y.C. (1995). Production and characterization of keratinase of a feather-degrading *Bacillus licheniformis* PWD-1. *Biosci. Biotechnol. and Biochem..* **59:**2239-2243.

Chikura, T., Izumi, N. and Matsumoto, S. (1994). Manufacture of amino acid containing fertilizers. *Jpn. Kokai Tokyo Koho JP 06, 40, 786.*

Choudhary, R.B., Jana, A.K. and Jha, M.K. (2004). Enzyme technology applications in leather processing. *Indian Journal of chemical technology.* **11:**659-671.

Coward-Kelly, G., Chang, V. S., Agbogbo, F. K., Holtzapple, M.T. (2006). Lime treatment of keratinous materials for the generation of highly digestible animal feed from chicken feathers. *Bioresource Technology*. **97:** 1337-1343.

Crewther, W.G., Fraser, R.D.B., Lennox, F.G. and Lindley, H. (1965). The Chemistry of Keratins. **In:** Advances in Protein Chemistry. (Anfinsen, C.B., Anson, M.L., Edsall, J.T. and Richards, F.M., eds). Academic Press. New York. Volume 20; pp. 191-347.

Datta, M.S. (1993). Role of keratin in fire fighting. *J. Ind. Leath. Technol. Assoc.* **43:**297-299.

Deivasigamani, B. and Alagappan, K.M. (2008). Industrial application of keratinase and soluble proteins from feather keratins. *J. Environ. Biol.* **29:** 933-936.

Duncan, F. (2005). Applied Microbiology Laboratory Manual. 4th edn. pp.1-70.

Eggum, B.O. (1970). Evaluation of protein quality of feather meal under different treatments. *Acta Agricul Scand.* **20:**230-234.

Enshaasy, E.H., Abuol-Enein, A., Helmy, S. and Azaly, E. (2008). Optimization of The industrial production of alkaline protease by *Bacillus licheniformis* in different production scales. *Australian Journal of Basic and Applied Sciences*. **2:**583-593.

Esawy, M.A. (2007). Isolation and partial characterization of extracellular keratinase from a novel mesophilic *Streptomyces albus* AZA. *Research Journal of Agriculture and Biological Sciences*. **3:**808-817.

Friedrich, J., Gradisar, H., Mandin, D. and Chaumont, J.P. (1999). Screening fungi for synthesis of keratinolytic enzymes. Lett. *Appl. Microbiol.* **28**:127-130.

Fujiwara, N., Masui, A. and Imanaka, T. (1993). Purification and properties of the highly thermostable alkaline protease from an alkaliphilic and thermophilic *Bacillus sp. J. Biotechnol.* **30:**245-256.

Gashaw Mamo, Hatti-Kaul, R. and Mattiasson, B. (2006). A thermostable alkaline active endo-β-1-4-xylanase from *Bacillus halodurans* S7: Purification and characterization. *Enz. Microbol. Technol.* **39:**1492-1498.

Gashaw Mamoa, Thunnissen, M., Hatti-Kaul, R. and Mattiasson, B. (2009). An alkaline active xylanase: Insights into mechanisms of high pH catalytic adaptation. *Biochimie* **91:**1187-1196.

Ghorbel, B., Sellami-Kamoun, A. and Nasri, M. (2003). Stability studies of protease from *Bacillus cereus* BG1. *Enzyme and Microbial Technology*. **32:**513-518.

Gioppo , N.M.R., Moreira-Gasparin, F.g., Costa, A.M., Alexanddrio, A.M., Souza, C.G.M. and Peralta, R.M. (2009). Influence of the carbon and nitrogen sources on keratinase production by *Myrothecium verrucaria* in submerged and solid state cultures. *J. Ind. Microbial. Biotech.* **36:**705-711.

Gizachew Haile. (2009). Alkaline protease production by new alkaliphilic microbial isolate under solid state fermentation. MSc. Thesis. Addis Ababa University.

Godfrey, T. and West, S. (1996). Application of Enzymes. **In**: Industrial enzymology, 2nd edn. Macmillan Publishers Inc., New York, pp. 285-291.

Gouda, M.K. (2006). Optimization and purification of alkaline proteases produced by marine *Bacillus* sp. MIG newly isolated from Eastern Harbour of Alexandria. *Polish Journal of microbiology*. **55:**119-126.

Gousterova, A., Nustorova, M., Goshev, I., Christov, P., Braikova, D., Tishinov, K., Heartle, T. and Nedkov,P. (2003). Alkaline hydrolysate of waste sheep wool aimed as fertilizer. *Biotechnol. Equip*. **17:**1140-1145.

Grant, W. D. and Tindall, B. J. (1980). The isolation of alkalophilic bacteria. **In:** Microbial Growth and Survival in Extremes of Environment. (Gould, G. W. and Corry, J.C.L. eds). Academic Press. London. pp. 27-36.

Grant, W.D. and Jones B.E. (2000). Alkaline environments. **In:** Encyclopaedia of Microbiology, 2nd edn. (Lederberg, J. ed). Academic Press. Vol. 1; pp. 126-133.

Grant, W.D., Jones, B.E. and Mwatha, W.E. (1990) Alkaliphiles: ecology, diversity and applications. *FEMS Microbiol Review.* **75:**255-270.

Grazziotin, A., Pimentel, F.A., de Jong, E.V. and Brandelli, A. (2006). Nutritional improvement of feather protein by treatment with microbial keratinase. *Animal Feed Science and Technology.* **126:**135-144.

Gupta, A. and Khare, S.K. (2007). Enhanced production and characterization of a solvent stable protease from solvent tolerant *Pseudomonas aeruginosa* PseA. *Enzyme and Microbial Technology.* **42:**11-16.

Gupta, M.N. (1992). Enzyme functions in organic solvents. *Eur. J. Biochem.* **203:**25-32.

Gupta, R. and Ramnani, P. (2006). Microbial keratinases and their prospective applications: an overview. *Appl. Microbiol. Biotechnol.* **70:** 21-33.

Gupta, R., Beg, Q.K. and Lorenz, P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl. Microbiol. and Biotechnol.* **59:**15-32.

Haddar, A., Agrebi, R., Bougatef, A., Hmidet, N., Sellami-Kamoun, A. and Nasri, M. (2009). Two detergent stable alkaline serine-proteases from *Bacillus mojavensis* A21: Purification, characterization and potential application as a laundry detergent Additive. *Bioresource Technology.* **100:** 3366-3373.

Horikoshi, K. (1971). Production of alkaline enzymes by alkalophilic microorganisms. Part I. Alkaline protease produced by *Bacillus* No. 221. *Agric. Biol. Chem.* **35:**1407-1414.

Horikoshi, K. (1999). Alkaliphiles: some applications of their products for biotechnology. *Microbiology and Molecular Biology, Reviews.* **63:**735-750.

Ito, M., Guffanti, A.A., Zemsky, J., Ivey, D.M. and Krulwich, T.A. (1997). Role of the *nhaC*-encoded Na^+/H^+ antiporter of alkaliphilic *Bacillus firmus* OF4. *J. Bacteriol.* **179:**3851-3857.

Jones, B.E. and Grant, W.D. (1999). Microbial diversity and ecology of the Soda Lakes of East Africa. *Ecology and Diversity of Extremophiles. Proceedings of the 8th International Symposium on Microbial Ecology*, Canada.

Jones, B.E., Grant, W.D., Duckworth, A.W. and Owenson, G.G. (1998). Microbial diversity of soda lakes. *Extremophiles.* **2:**191-200.

Joo, H.S., Kumar, C.G., Park, G.C., Paik, S.R. and Changa C.S. (2004). Bleach-resistant alkaline protease produced by a *Bacillus* sp. isolated from the Korean polychaete, *Periserrula leucophryn. Process Biochemistry*. **39:**1441-1447.

Kaluzewska, M., Wawrzkiewicz, K. and Lobarzewski, J. (1991). Microscopic examination of keratin substrates subjected to the action of the enzymes of *Streptomyces fradiae*. *Int. Biodeterior.* **27:**11-26.

Kansoh, A. L., Hossiny E.N. and Abd El-hameed, E.K. (2009). Keratinase production from feathers wastes using some local *Streptomyces* isolates. *Journal of Basic and Applied Science.* **3:**561-571.

Kansoh, A.L., Hossiny E.N. and Abd EL-Hameed E.K. (2009). Keratinase production from feathers wastes using some local *Streptomyces* isolates. *Australian Journal of Basic and Applied Sciences*. **3:**561-571.

Karbalaei-Heidari, R.H., Ziaee, A.A., Schaller, J., Amoozegar, M.A. (2007). Purification and characterization of an extracellular haloalkaline protease produced by the moderately halophilic bacterium, *Salinivibrio* sp. strain AF-2004. *Enzyme and Microbial Technology.* **40:**266-272.

Karthikeyan, R., Balaji, S. and Sehgal, P.K. (2007). Industrial applications of keratins: a review. *J. scientific and industrial res.* **66:**710-715.

Kazan, D., Denizci, A.A., Kerimak Oner, M.N. and Erarslan, A. (2005). Purification and characterization of a serine alkaline protease from *Bacillus clausii* GMBAE 42. *J. Ind. Microbiol. and Biotechnol.* **32:** 335-344.

Kim, J.M., Lim, W.J. and Suh, H.J. (2001). Feather-degrading *Bacillus* species from poultry waste. *Process Biochem.* **37:** 287-291.

Kim, W.K. and Patterson, P.H. (2000). Nutritional value of enzyme or sodium hydroxide-treated feathers from dead hens. *Poult. Sci.* **79:**528-534.

Kobayashi, T., Hakamada, Y., Adachi, S., Hitomi, J., Yoshimatsu, T., Koike, K., Kawai, S. and Ito, S. (1995). Purification and properties of an alkaline protease from alkalophilic *Bacillus sp.* KSM-K16. *Appl. Microbiol. Biotechnol*. **43:** 473-481.

Kobayashi, T., Hakamada, Y., Hitomi, J., Koike, K. and Ito, S. (1996). Purification of alkaline proteases from a *Bacillus* strain and their possible interrelationship. *Applied Microbiology and Biotechnology*. **45:** 63-71.

Korkmaz, H., Dincer, H. and Hur, S. (2004). Characterization of alkaline keratinase of *Bacillus licheniformis* strain HK-1 from poultry waste. *Annals of Microbiology*. **54:** 201-211.

Krulwich, T.A. and Guffanti, A.A. (1983). Physiology of acidophilic and alkalophilic bacteria. *Adv. Microb. Physiol*. **24:** 173-214.

Krulwich, T.A., Ito, M., Hicks, D.B., Gilmour, R. and Guffanti, A.A. (1998). pH Homeostasis and ATP synthesis: studies of two processes that necessitate inward

proton translocation in extremely alkaliphilic *Bacillus* species. *Extremophiles* **2:** 217-222.

Kumar, A.G., Nagesh, N., Prabhakar, T.G. and Sekaran, G. (2008). Purification of extracellular acid protease and analysis of fermentation metabolites by Synergistes sp. utilizing proteinaceous solid waste from tanneries. *Bioresource Technology*. **99:** 2364-2372.

Kumar, C.G. and Takagi, H. (1999). Microbial alkaline proteases: from a Bioindustrial viewpoint. *Biotechnology Advances, Research review paper.* **17:**561-594.

Kumar, C.G., Joo, H.S., Koo, Y.M., Paik, S.R. and Chang, C.S. (2004). Thermostable alkaline pprotease from a novel marine haloalkalophilic *Bacillus Clausii* isolate. *World J.Microbial. Biotechnol.* **20:** 351-357.

Kumar, C.G., Tiwari, M.P. and Jany, K.D. (1999). Novel alkaline serine proteases from alkalophilic *Bacillus sp*. purification and some properties. *Process Biochemistry*. **34:**441-449.

Kumar, D. and Bhalla, T.C. (2004). *Bacillus sp.* APR-4 protease as a laundry additive. *Indian J. Biotechnol.* **3:**563-567.

Kumar, D., Savitri, Thakur, N., Verma, R. and Bhalla, T.C. (2008). Microbial Proteases and application as laundary detergent additive. *Research Journal of Microbiology*, **3:**661-672.

Kumar. C.G. (1997). Studies on microbial alkaline proteases for use in dairy detergents. PhD. Thesis. National Dairy Research Institute, Deemed University, Karnal, India.

Kunert, J. (1973). Keratin decomposition by dermatophytes: evidence of sulphitolysis of the protein. *Experientia.* **33:**489-498.

Kwon, Y.T., Kim, J.O., Moon, S.Y., Lee, H.H. and Rho, H.M. (1994). Extracellular alkaline protease from alkalophilic *Vibrio metschnikovii* strain RH530. *Biotechnol. Lett.* **16:**413-418.

Kwon, Y.T., Kim, J.O., Moon, S.Y., Yoo, Y.D. and Rho, H.M. (1995). Cloning and characterization of the gene encoding an extracellular serine protease from *Vibrio metschnikovii* strain RH530. *Gene*. **152:**59-63.

Langeveld, J.P., Wang, J.J., Van de Wiel, D.F., Shih, G.C., Garssen, G.J., Bossers, A. and Shih, J.C. (2003). Enzymatic degradation of prion protein in brain stem from infected cattle and sheep. *J. Infect. Dis.* **188:**1782-1789.

Lee, W.J., Son, G.M. and Choi, C. (1991). Production and purification of alkaline protease from *Bacillus* sp. CW-1121. *J. Korean Soc. Food Nutr*. **20:**388-94.

Leon, N.H**.** (1972). Structural aspects of keratin fibres. *J. Soc.Cosmet. Chem*. **23**:427-445.

Letourneau, F., Soussotte, V., Bressollier, P., Branland, P. and Verneuil, B. (1998). Keratinolytic activity of *Streptomyces* sp. SK1-02: a new isolated strain. *Lett. Appl. Microbiol.* **26:**77-80.

Lin, X., Inglis, G. D., Yanke, L. J. and Cheng, K. J. (1999). Selection and characterization of feather degrading bacteria from canola meal compost. *J. Indust. Microbiol. and Biotechnol.* **23:**149-153.

Lin, X., Lee, C.G., Casale, E.S. and Shih, J.C.H. (1992). Purification and characterization of a keratinase from a feather-degrading *Bacillus licheniformis* strain. *Appl. Environ. Microbiol.* **58:**3271-3275.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemsitry*. **193**:265-275.

Lv, L.X., Sim, M.H., Li, Y.D., Min, J., Feng, W.H., Guan, W.J. and Li, Y.Q. (2010). Production, characterization and application of a keratinase from *Chryseobacterium L99* sp. nov. *Process Biochemistry.* **45:**1236-1244.

Macedo A.J., Beys da Silva W.O., Gava R., Driemeier D., Antonio Pegas Henriques, J. and Termignoni, C. (2005). Novel Keratinase from *Bacillus subtilis* S14 Exhibiting Remarkable Dehairing Capabilities. *Appl. and Envir. Microbiol.* **71:**594-5

Macedo, A.J., Silva, W.O.B and Termignoni, C. (2008). Properties of a non collagen-degrading *Bacillus subtilis* keratinase. *Can. J. Microbiol.* **54:**180-188.

Manachini, P.L., Fortina, M.G. and Parini, C. (1998). Thermostable alkaline protease produced by *Bacillus thermoruber* a new *sp.* of *Bacillus*. *Appl. Microbiol. and Biotechnol*. **28:**409-413.

Marcello, A., Loregian, A., Defilippis, V., Fontana, A., Hirst, T.R. and Palu, G. (1996). Identifcation and characterization of an extracellular protease activity produced by the marine *Vibrio* sp. 60. *FEMS Microbiology Letters*. **136:**39-44.

Masui, A., Fujiwara, N., Takagi, M.and Imanaka, T. (1999). Feasibility study for decomposition of gelatin layers on X-ray films by thermostable alkaline protease from alkaliphilic *Bacillus sp*. *Biotechnological Techniques*. **13:**813-815.

Nakiboglu, N., Toscali, D. and Nisli, G. (2003). Novel silver recovery method from waste photographic films with NaOH stripping. *Turk J. Chem.* **27:**127-133.

Nakiboglu, N., Toscali, D. and Yasa, I. (2001). Silver Recovery from Waste Photographic Films by an Enzymatic Method. *Turkey Journal of Chemistry.***25:** 349-353.

Nam, G.W., Lee, D.W., Lee, H.S., Lee, N.J., Kim, B.J., Choe, E.A., Hwang, J.K., Suhartono, M.T. and Pyun, Y.R. (2002). Native feather degradation by *Fervidobacterium islandicum* AW-1, a newly isolating keratinase-producing thermophilic anaerobe. *Archives of Microbiology.* **178:** 538-547.

Oberoi, R., Beg, Q.K., Puri, S., Saxena, R.K. and Gupta, R. (2001). Characterization and wash performance analysis of an SDS-stable alkaline protease from a *Bacillus* sp. *World Journal of Microbiology and Biotechnology*. **17:**493-497.

Odetallah, N.H., Wang, J.J., Garlich, J.D. and Shih, J.C.H. (2003). Keratinase in starter diets improves growth of broiler chicks. *Poultry Sci*. **82:** 664-670.

Ogino, H., Uchiho, T., Yokoo, J., Kobayashi, R., Ichise, R. and Ishikawa, H. (2001). Role of intermolecular disulfide bonds of the organic solvent-stable PST-01 protease in its organic solvent stability. *Appl. and Envir. Microbiol.* **67:**942-947.

Onifade, A.A., Al-Sane, N.A., Al-Musallam, A.A. and Al-Zarban, S. (1998). A review: potentials for biotechnological applications of keratin degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. *Bioresour. Technol.* **66:** 1-11.

Papadopoulos, M.C., Boushy, E.I., Roodbeen, A.R. and Ketelaars, E.H. (1986). Effect of processing time and moisture content on amino acid composition and nitrogen characteristics of feather meal. *Anim. Feed Sci. Technol.* **14:**279-290.

Pillai, P. and Archana, G. (2008). Hide depilation and feather disintegration studies with keratinolytic serine protease from a novel *Bacillus subtilis* isolate. *Appl. Microbiol. Biotechnol.* **78:**643-650.

Poopathi, S. and Abidha, S. (2007). Use of feather-based culture media for the production of mosquitocidal bacteria. *Biological Control*. **43:**49-55.

Priola, S.A. (2001). Prion protein diversity and disease in the transmissible spongiform encephalopathies. *Adv. Protein Chem*. **57:**1-27.

Ramnani, P. and Gupta, R. (2004). Optimization of medium composition for keratinase production on feather by *Bacillus licheniformis* RG1 using statistical methods involving response surface methodology. *Biotechnol. and Appl. Biochem.*. **40:**491-496.

Rao, M.B., Tanksale, A.M., Mohini, S.G. and Deshpande, V.V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews*. **62:**597-635.

Riffel, A. and Brandelli, A. (2006). Keratinolytic bacteria isolated from feather waste. *Brazilian J. Microbiol.* **37:**395-399.

Riffel, A., Brandelli, A., Bellato, C. M., Souza, G. H. M. F., Eberlin, M. N. and Tavares, F. C. A. (2007). Purification and characterization of a keratinolytic metalloprotease from *Chryseobacterium sp.* kr6. *J. Biotechnol*. **128:** 693-703.

Riffel, A., Lucas, F., Heeb, F. and Brandelli A. (2003). Characterization of a new keratinolytic bacterium that completely degrades native feather keratin. *Arch. Microbiol*. **179:**258-265.

Riffel, A., Ortolan, S. and Brandelli, A. (2003). De-hairing activity of extracellular proteases produced by keratinolytic bacteria. *J. Chem. Technol. Biotechnol.* **78:**855-859.

Rouse, J.G. and Van Dyke, M.E. (2010). A review of keratin-based biomaterials for biomedical applications. *Materials.* **3:**999-1014.

Saber, W.I.A., El-Metwally, M.M. and El-Hersh, M.S. (2010). Keratinase production and biodegradation of some keratinous wastes by *Alternaria tenuissima* and *Aspergillus nidulans. Research J. Microbiol.* **5:**21-35.

Saeki, K., Ozaki, K., Kobayashi, T. and Ito, S. (2007). Detergent alkaline proteases: enzymatic properties, genes, and crystal structures. J. *Biosci. and Bioeng.* **103:**501-508.

Sana, B., Ghosh, D., Saha, M. and Mukherjee, J. (2006). Purification and characterization of a salt, solvent, detergent and bleach tolerant protease from a new gamma-Proteobacterium isolated from the marine environment of the *Sundarbans*. *Process Biochemistry.* **41:** 208-215.

Sangali, S. and Brandelli, A. (2000). Feather keratin hydrolysis by a *Vibrio* sp. strain kr2. *J. Appl. Microbiol.* **89:**735-743.

Sayed, S.A., Saleh, S.M. and Hasan, E.E. (2005). Removal of some polluting metals from industrial water using chicken feathers. *Desalination.* **181:**243-255.

Schreckenberger, P.C. and Blazevic, M.J. (1974). Rapid Methods for Biochemical Testing of Anaerobic Bacteria. *Appl. Microbiol.* **28:**759-762.

Secades, P., Guijarro, J.A. (2001). Purification and characterization of a psychrophilic calcium-induced growth-phase-dependent metalloprotease from the fish pathogen *Flavobacterium psychrophilum*. *Appl. Environ. Microbiol*. **67:**2436-2444.

Sellami-Kamoun, A., Haddar, A., Ali, N. E.H., Ghorbel-Frikha, B., Kanoun, S. and Nasri, M. (2008). Stability of thermostable alkaline protease from *Bacillus licheniformis* RP1 in commercial solid laundry detergent formulations. *Microbiological Research.* **163:** 299-306.

Shankar, S., More, S.V. and Laxman, R.S. (2010). Recovery of silver from waste x-ray film by alkaline protease from *conidiobolus coronatus. J. sci.* **6:**60-69.

Sharma, R. and Gupta, R. (2010). Thermostable, thiol activated keratinase from *pseudomonas aeruginosa* KS-1 for prospective application in Prion decontamination. *Research J. Microbiol.* **5:**954-965.

Shih, J.C.H. (1993). Recent development in poultry waste digestion and feather utilization. *A review of Poultry Sci*. **72:**1617-1620.

Sierpinski, P., Garrett, J., Ma, J., Apel, P., Klorig, D., Smith, T., Koman, L.A., Atala, A. and Van Dyke, M. (2008).The use of keratin biomaterials derived from human hair for the promotion of rapid regeneration of peripheral nerves. *Biomaterials*, **29:**118-128.

Siezen, R. J., de Vos, W.M., Leunissen, J. A. M. and Dijukstra, B.W. (1991). Homology modeling and protein engineering strategy of subtilases, the family of subtilisin-like serine proteinases. *Protein Eng*. **4:**719-737

Singh, J., Vohra, R.M. and Sahoo, D.K. (1999). Alkaline protease from a new obligate alkalophilic isolate of *Bacillus sphaericus*. *Biotechnology Letters*. **21:**921-924.

Singh, J., Vohra, R.M. and Sahoo, D.K. (2001). Purification and characterization of two extracellular alkaline proteases from a newly isolated obligate alkalophilic *Bacillus sphaericus*. *Journal of Industrial Microbiology and Biotechnology*. **26:** 387-393.

Sivasubramanian, S., Murali Manohar, B. and Puvanakrishnan, R. (2008). Mechanism of enzymatic dehairing of skins using a bacterial alkaline protease. *Chemosphere.* **70:**1025-1034.

Suh, H. J. and Lee, H. K. (2001). Characterization of a keratinolytic serine protease from *Bacillus subtilis* KS-1. *Journal of Protein Chemistry*. **20:**165-169.

Sundararajan, S., Kannan, C.N. and Chittibabu, S. (2010). Alkaline protease from *Bacillus cereus* VITSN04: Potential application as a dehairing agent. *J. of Biosci. and Bioeng*. doi:10.1016/j.jbiosc.2010.09.009

Syed, D.G., Lee, J.C., Li, W.J., Kim, C.J. and Agasar, D. (2009). Production, characterization and application of keratinase from *Streptomyces gulbargensis*. *Bioresource Technology.* **100:**1868-1871.

Takami, H., Akiba, T. and Horikoshi, K. (1990). Characterization of an alkaline protease from *Bacillus* sp. no. AH-101. *Appl. Microbiol. Biotechnol*. **33:**519-523.

Takami, H., Nakamura, S., Aono, R. and Horikoshi, K. (1992). Degradation of human hair by a thermostable alkaline protease by alkalophilic *Bacillus* sp. AH-101. *Biosci. Biotechnol. Bioche.* **56:**1667-69.

Tanksale, A. M. (2001). Molecular aspects of a fungal alkaline protease. Thesis for the Degree of Doctor of Philosophy in Biotechnology, *National chemical laboratory,* University of Pune.

Tatineni, R., Doddapaneni, K.K., Potumarthi, R.C., Vellanki, R.N., Kandathil, M.T., Kolli, N. and Mangamoori, L.N. (2008). Purification and characterization of an alkaline keratinase from *stryptomyces* sp. *Bioresour. Teechnol.* **99:**1596-1602.

Thanikaivelan, P., Rao, J.R., Nair, B.U. and Ramasami, T. (2004). Progress and recent trends in biotechnological methods for leather processing. *Trends in Biotechnology*. **22:**181-188.

Tobe, S., Takami, T., Hirose, Y. and Mitsugi, K. (1975). Purification and some properties of alkaline proteinases from *Bacillus sp. Agric. Biol. Chem*. **39:**1749-1755.

Ulfig, K., Płaza, G., Worsztynowicz, A. Mańko, T., Tien, A.J. and Brigmon, R. L. (2003). Keratinolytic fungi as indicators of hydrocarbon contamination and bioremediation progress in a petroleum refinery. *Polish Journal of Environmental Studies.* **12:**245-250.

Ulukanli, Z. (2002). Alkaliphilic micro-organisms and habitats. *Turk. J. Biol.* **26:**181-191.

Van Dyke, M.S., Blanchard, C.R., Timmons, S.F., Siller-Jackson, A.J. and Smith, R.A. (2001). Soluble Keratin peptide, *US Pat 6, 270, 791 B1.*

Vigneshwaran, C., Shanmugam, S. and Kumar, T.S. (2010). Screening and characterization of keratinase from *Bacillus licheniformis* isolated from namakkal poultry farm. *Researcher.* **2:**89-96.

Voet, D. and Voet, J. (1995). Proteins:Three-dimensional structure. **In:** Biochemistry, 2nd ed. John Wiley and Sons. New York, pp. 132-134.

Wang, X. and Parsons, C.M. (1997). Effect of processing systems on protein quality of feather meal and hog hair meals. *Poultry Science.* **76:**491-496.

Ward, H.M. (1899). *Onygena equina*, Willd. a horn-destroying fungus. *Philosophical Transactions of the Royal Society of London.* **191:**269-291.

Waterhouse, D. F. (1957). Digestion in insects. *Ann. Rev. Entomol.* **2:**1-18.

Wawrzkiewicz, K., Wolski, T. and Lobarzewski, J. (1991). Screening of the keratinolytic activity of dermatophytes in vitro. *Mycopathologia.* **114:**1-8.

Williams, C.M., Lee, C.G., Garlich, J.D. and Shih, J.C.H. (1991). Evaluation of a bacterial feather fermentation product, feather lysate, as a feed protein. *Poult. Sci.* **70:** 85-90.

Williams, C.M., Richter, C.S., MacKenzie, J.M. and Shih, J.C.H. (1990). Isolation, Identification and characterization of a feather-degrading bacterium. *Appl. Environ. Microbiol*. **56:** 1509-1515.

Xu, B., Zhong, Q., Tang, X., Yang, Y. and Huang, Z. (2009). Isolation and characterization of a new keratinolytic bacterium that exhibits significant feather-degrading capability. *Afr. J. Biotechnol.* **8:**4590-4596.

Yamamura, S., Morita, Y., Hasan, Q., Yokoyama, K. and Tamiya E. (2002). Keratin degradation: a cooperative action of two enzymes from *Stenotrophomonas* sp. *Biochemical and Biophysical Research Communications*. **294:**1138-1143.