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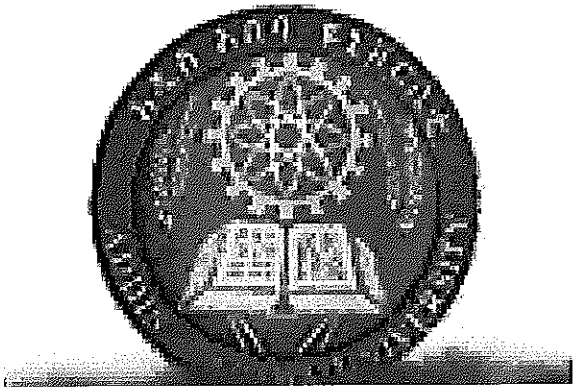

## BIODEGRADATION OF FEATHER KERATIN

**BY**

**ZENEBE TEKA**

**A Thesis submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements of the Degree of Master of Science In Applied Microbiology**

**JULY 2006**

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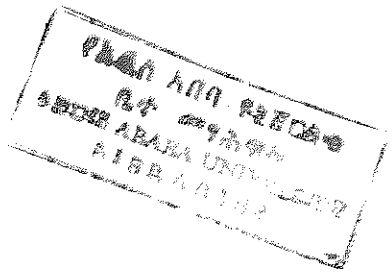

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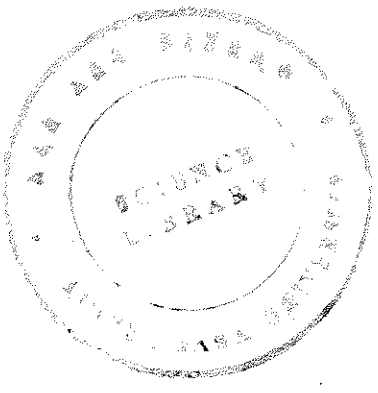

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# Abbreviations and Acronyms

| | |
|---|---|
| FMB | Feather Meal Broth |
| fmb | fish meal broth |
| HMB | Horn Meal Broth |
| PMSF | Phenyl Methyl Sulphonyl Fluoride |
| EDTA | Ethylene Diamine Tetra Acetic Acid |
| Beta-ME | Beta Mercaptoethanol |
| TN | Total Nitrogen |
| DM | Dry Matter |
| BSA | Bovine Serum Albumin |
| rpm | revolution per minute |

# Abstract

A total of 165 bacteria were isolated from samples collected from Mojo Tannery and Abijata Lake, and 17% were found to possess feather-degrading capacity. Two best keratinolytic strains were selected from the 28 feather degrading isolates and tentatively identified as *Bacillus lentus* strain Kr-07 and *Streptomyces sp.* strain Kr-43. The optimal growth period, pH and temperature for the two strains were 96h, 7.5 and 37°C respectively. The proteases of *Bacillus lentus* strain Kr-07 and *Streptomyces sp.* strain Kr-43 were inhibited by PMSF and by EDTA indicating that they are serine-type and metallo-type proteases respectively. Protease kr-07 displayed its optimal activity at a temperature of 35°C and at a pH of 7.5, while protease kr-43 at a temperature of 40°C and at a pH of 7.5. In the presence of 5mM $CaCl_2$ protease Kr-07 showed optimal activity at 45°C at the optimal pH, while protease Kr-43 exhibited optimal activity at 55 °C at its optimal pH. Protease Kr-43 displayed 85.3% relative activity at 4°C indicating that it is cold active protease and hence it could have potential application in the detergent industries, especially in cold areas. The strains can utilize feather, fish wastes, and horn wastes. Therefore, they could be potentially important in the development of waste treatment methods in the biotechnological industries involved in the processing of keratinacious wastes.

Key words: *Keratin, Keratinolytic bacteria, Protease, Keratinase, and Degradation.*

# I. Introduction

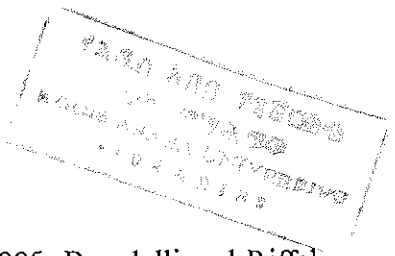
## 1. Background Information on Keratins

Keratins and keratinacious materials are structural proteins. They are the largest and most complex family of cytoskeleton intermediate filament proteins commonly found in the epithelial cells, hair, skin, hides, nails, horns, hooves, feathers, wool, scales, claws, beaks, etc, of vertebrate animals. They are common wastes in commercial animal products processing plants like abattoirs, tanneries, poultry processing, fur and leather industries, etc.

Unlike the linear polymers, which possess only long sequential strands, keratins have long linkage sequences in two or three dimensions and hence are distinguished as cross-linked polymers (Hendrickson *et al.*, 1970, Giradisar *et al.*, 2005).

The main distinguishing feature of keratins is that they contain very high amount of the amino acid cystine, which is responsible for the crosslinking in the protein and the insoluble properties that are fundamental to the structural role that keratins play in nature (anonymous, 2004, http://www.keratec.co.nz).

Their high degree of cross-linking by disulfide bonds, hydrophobic interactions, the tight packing of the keratin chain in alpha-helix (alpha-keratin) or β-sheet (β -keratin) into a supercoiled polypeptide chain and the hydrogen bonds stabilize the keratin filament structure (Sangali *et al.*, 2000; Giradisar *et al.*, 2005). Hendrickson *et al.* (1970) and Scott and Untereiner (2004) reported that such cross-linkages enable the polymers to become hard, durable and made them not melt, soften or dissolve in most cases and have resilience property. Because of their insolubility and multiple disulfide bonds, keratins in their native state are not easily degradable by common proteolytic enzymes such as trypsin, pepsin and papain (Lin *et al.*, 1999;Sangali *et al*, 2000; Scott and Untereiner, 2004; Giradisar *et al.*, 2005). However, both pure keratins and keratinacious proteins can be hydrolyzed by special proteolytic enzymes (keratinases) derived from keratinolytic bacteria and fungi



(Burtt and Ichida, 1999; Chitte *et al.*, 1999; Giradisar *et al.*, 2005; Brandelli and Riffel, 2005).

Feather, which is almost pure keratin protein, containing β-keratin as a major component, occur as pleated sheets twisted into micro-fibrils, and together with other factors this makes them resistant to biological degradation by plant, animal and many known microbial proteases (Amare Gessesse *et al.*, 2003; Scott and Untereiner, 2004). They are composed of about 90% protein and are produced in several millions of tons in the world as waste products of poultry processing (McGovern, 2000; Sangali *et al.* 2000; Amare Gessesse *et al.*, 2003).

In the year 2000 an estimated 8.5 billion chickens were commercially grown and processed in the United States alone (McGovern, 2000) and left behind more than 2.3 billion pounds of feather.

An average chicken processing plant produces 4,000 pounds of feathers an hour and has a low profit margin per bird, so feathers must be moved or processed quickly and very inexpensively (McGovern, 2000). From these figures one can imagine how huge the potential resource is or how critical the potential risk could be if the wastes are not processed into useful products.

When the cost of processing of the wastes becomes unaffordable large, volumes of keratins and keratinacious wastes are disposed off to the environment from the processing plants and thus are becoming critical environmental issues requiring sustainable remedy.

Currently the only means of getting rid off such wastes is by incinerating them at elevated temperatures (Gousterova, *et al.*, 2005). Incineration of such wastes is however costly. As a result keratinacious wastes are thrown away over controlled dunghills, and have created significant ecological and sanitary problems in many parts of the world where such wastes are produced in bulk (Gousterova, *et al.*, 2005). Hence, environmentally friendly, economically sound and safe methods of decontamination and disposal systems are badly needed.

The biological degradation of such wastes is literally reported to contribute much to the ongoing efforts to alleviate this waste-based, recently emerged and rapidly expanding problem.

## 2. Biological Digestion of Keratins and Keratinacious Wastes

Although keratins and keratinacious wastes are not readily digestible by animal and plant proteases and common known microbial proteases, they are proved to be digestible by special proteases produced by keratinolytic microorganisms isolated from different sources such as poultry compost, soil, mud of lake shores and, plumage of living birds (Hubalek, 1978; Burtt and Ichida, 1999, Amare Gessesse *et al.*, 2003).

Prior to 1990,only a few species of fungi (Hubalek, 1978) and a single bacterium, *Streptomyces fradiae* (Noval and Nickerson, 1959), were known to degrade feathers and the term 'keratinolytic' was designated for fungi and bacteria exhibiting the enzymatic ability to attack and utilize keratin. Starting from 1990 keratin utilization has been reported in a wide variety of organisms including non-filamentous and filamentous bacteria, and filamentous fungi (Zaghoul *et al.*, 1998).

Keratinolytic microorganisms may therefore have important applications in biotechnological and industrial processes for the bioconversion of keratin-containing wastes from poultry, fur, slaughterhouses, tannery and leather industries.

### *2.1. Bacterial Degradation of Keratins*

Bacterial keratinases are special proteases that can hydrolyze both native and denatured keratins (Onifade *et al.* 1998) and have wide potential application in the chemical, medical and animal feed manufacturing industries and basic biological sciences (Ichida *et al.*, 2001) and in the detergent industry (Amare Gessesse *et al.*, 2003).

Some of the commonly studied bacteria, that are capable of producing such enzymes include *S. fradiae (*Noval and Nickerson, 1959), *B. licheniformis* and *B. subtilis (*Williams *et al.*, 1990*), S. pactum* (Bockle *et al.*, 1995), *B. pumilis* (Burtt and Ichida, 1999); *Vibrio sp.* (Sangali *et al.*, 2000), *Flavobacterium spp.* (Riffel and Brandelli, 2002), *Bacillus pseudofirmus* and *Nesternkonia aethropica (*Amare Gessesse *et al.*, 2003*), Chryseobacterium spp.* (Riffel and Brandelli, 2005), *Thermoactinomyces spp.* (Gousterova *et al.*, 2005), etc. Zerdani *et al.* (2004) isolated *B.licheniformis* and *B. subtilis* which are very efficient keratin digesters and reported that *B. licheniformis* is very convenient for feather degradation because of its thermophilic properties, and its ability to grow at high temperatures (50 -65 °C).

## *2.2. Fungal Digestion of Keratins*

Some fungal species are able to degrade keratins and keratinacious wastes (Hubalek, 1978). Some infectious fungi, such as those, which cause athlete's foot and ringworm, degrade keratin wastes. The enzymatic ability of some fungal groups to decompose keratin wastes has been interpreted as a key discovery in the evolution of animal dermatopathogenicity (Scott and Untereiner, 2004). Fungi that are capable of degrading these extremely recalcitrant animal polypeptides belong to the filamentous ascomycetes (Scott and Untereiner, 2004).

*Paecilomyces marquandii* and *Doratomyces microsporus* were screened as nonpathogenic keratinolytic fungi that are capable of producing potent keratinases (Giradisar *et al.*, 2005). The enzymes were purified and studies on substrate specificity revealed that skin constituents, such as the stratum corneum, and appendages such as nail were efficiently hydrolyzed by the *P.marquandii* keratinase and about 40% less by the *D. microsporus* keratinase (Giradisar *et al.*, 2005).

## 3. Potential Biotechnological Applications of Keratins and Keratinacious Wastes

Because of their high protein content and special structures, keratins and keratinacious wastes could be considered as potential resources. They can serve as sources of protein and amino acids in animal feed additives. They can also be used as microbial media supplements, as ingredients for cosmetic products for skin and hair care (Uhlig, 1998), for medical aids and for fiber (Stoltz and Tate, 2004), and organic fertilizer production, etc., after being processed using different methods depending on the nature of the expected products.

### *3.1. Use of Keratins as Substrate for Industrial Enzyme Production*

Proteases constitute an important fraction of the global enzyme sales, and a significant portion of this market is accounted for by bacterial proteases (Zaghoul *et al.*, 1998). The production of these enzymes is, however, constrained by a number of factors, among which the cost of growth substrates is the most significant. Therefore, if proteolytic microorganisms are able to grow on these cheap and easily available bulky protein sources, it will be possible to produce least cost enzymes that can have applications in the different biotechnological industries including the detergent, food, pharmaceutical, tanneries, etc, industries.

Keratinolytic bacteria, isolated from feather waste which have depilatory activity, were reported to grow in mineral medium containing bovine hair as sole carbon, nitrogen and energy source (Macedo *et al.*, 2005). This indicates that proteases of keratinolytic bacteria could be efficient in promoting depilation (de-hairing) of bovine pelts, thus avoiding environmental pollution through the use of sulfides could be replaced by protease enxyme.

One such potentially interesting bacterium reported by Macedo *et al.* (2005) is *Bacillus subtilis*, which produces a subtilisin-like keratinase. It is reported that this enzyme can be

chicken feather is reported to be a potential candidate for the enzymatic and/or microbiological hydrolysis of feather to be used as animal feed supplement (Amare Gessesse *et al.*, 2003).

Kurbanoglu *et al.* (2003) demonstrated that use of ram horn hydrolysate as a substrate for the growth of *Aspergillus niger* NRRL330 in submerged fermentation resulted in a significant increase in biomass. The biomass contained about 48.1 % protein, 5.2 % fat, and 9.4 % ash (on dry weight basis) and the amino acid content of the biomass protein contained all the essential amino acids important for animal feed. Thus, it could be concluded that ram horn hydrolysate and other keratin and keratinacious hydrolysates could be used as substrates in the production of fungal protein for use as animal feed (Kurbanoglu *et al.*, 2003).

Hydrolysates obtained from ram horns were also used as substrates to grow bacteria such as *Bacillus cereus* NRRL B-3711, *B.subtilis* NRRL NRS-744 and *E. coli* (Kurbanoglu *et al.*, 2002). The bacterial cells produced in such hydrolysates were found to be rich in total protein (66 %, 68 %, and 71 % for *E.coli, B.cereus* and *B.subtilis,* respectively) and the protein produced contained all the essential amino acids for ruminant feeds. This indicates the potential for the production of animal feed additives from keratin wastes to enhance the productivity of the livestock sector.

### *3.3. Organic Fertilizer Production*

The keratin and keratinacious wastes together with other agricultural by-products can also be converted into organic fertilizers which may have significant contribution in the production of crops, vegetables, fruit trees, etc. These natural organic fertilizers are expected to mitigate the problems arising from the utilization of synthetic fertilizers both economically and ecologically. Fertilizer in the U.S. is commonly made from scraps from the manufacturers of leather products or from hide trimmings from the tanning factory (McGovern, 2000). It is also reported by McGovern (2000) that solid waste residues from effluent of tanning factories rich in keratin might be used for fertilizer production.

Therefore, one potential area of application of the keratins and keratinacious wastes is to use them for the fertilization of agricultural lands by converting them into organic fertilizers biologically.

### *3.4. Single Cell Protein Production (SCP)*

Single-cell proteins (SCP) can be produced from the biomass of different microorganisms and may be used as protein sources for human food or for animal feed. Many raw materials have been considered as carbon, nitrogen and energy sources for SCP production using microorganisms. In many cases, these raw materials have been hydrolysed by physical, chemical and enzymatic methods before use (Kurbanoglu, 2002). Since wastes are cheap, the produced SCPs will also be cheaper than other products that may have similar functions in the human or animal nutrition. For example *Candida utilis* NRRL Y-900 was grown on horn hydrolysate for single-cell protein production (Kurbanoglu, 2002). It was also reported by Kurbanoglu (2002) that the biomass contained all of the essential amino acids for ruminant feeding.

Like feathers, horns consist of keratin, which has very high cystine content (up to 22%) and they also contain most of the common amino acids (Kurbanoglu, 2002). Besides, they contain the components of bone and blood tissues and are rich in some growth factors required by microorganisms and thus, the hydrolysates of these wastes can be considered as potential microbial substrate for SCP production.

The hydrolysates of these wastes are reported to be rich in both organic and inorganic material. Particularly, they contain the substances required for microbial growth such as carbon, nitrogen and minerals. For instance, horn hydrolysate is rich in the essential amino acids and especially arginine and glutamic acid are very high (Kurbanoglu, 2002).

### *3.5. Enhancement of Organic Acid Production by Microbial Fermentation*

The production of organic acids can be enhanced by using keratinacious waste hydrolysates as a supplement for the fermentative activities of the producer microorganisms. For instance, the production of citric acid by *Aspergillus niger* was shown to be improved by using ram horn hydrolysate as a supplement (Kurbanoglu, 2004). The use of ram horn hydrolysate as a substrate for lactic acid production was also investigated by Kurbanoglu *et al.* (2003) using *Lactobacillus casei.*

Therefore, keratinolytic microorganisms and their enzymes may have important applications in biotechnological and industrial processes involving keratin-containing wastes from different sources to develop ecologically safe and economically feasible waste treatment methods. This in turn could make the development of enzymatic and/or microbiological methods for conversion of such wastes into useful products to be a more attractive business.

## 4. Present Knowledge Gap in the Area

Although studies in the isolation of keratinolytic bacteria begun decades ago, only very few microorganisms are reported to be efficient digesters of keratins and keratinacious wastes. Little is also known about the enzymatic applications of the isolated strains. Therefore, in order to develop an efficient biotechnological process for the utilization of keratin wastes, there is a need to look into the isolation of more efficient keratin degrading bacterial strains.

## 5. Potential Sites for Isolation of Keratinolytic Bacteria in Ethiopia.

In Ethiopia there are potential sites where samples could be collected for the isolation of keratinolytic bacteria. The soda lakes which are situated in the Rift Valley of the country, Mojo tannery, Addis Ababa abattoir etc are some examples. Keratinolytic bacteria were successfully isolated by Amare Gessesse *et al.* (2003) from soil samples of Abijata Soda Lake. These places are potentially rich for the isolation of keratin degrading microorganisms due to the presence of keratins and keratinacious wastes serving as nitrogen sources for the wild bacteria dewelling in the areas.

## 6. Objectives of the Study

Having the foregoing background information, this research was initiated to study the biological degradation of keratins by keratinolytic bacteria with special emphasis on the following objectives:

- To isolate keratin degrading bacteria, and
- To characterize the enzymes and evaluate their potential for application.

# II. Materials and Methods

## 1. Feathers, Horns, Fish Wastes and Hair Collection

Feather was collected from freshly slaughtered indigenous chicken and washed with tap water, and dried at 200°C in an oven until constant weight was achieved.

Fresh horn was collected from a household after the slaughter of a buck. The horns were thoroughly washed with tap water and air-dried after which it was oven dried at 200°C till a constant weight was obtained. After crushing and grinding, the powder was sieved using a wire mesh.

Fish waste was obtained from a restaurant where fish dish was commonly served. The collected fish waste was washed with tap water, defatted with acetone and oven dried at 200 °C to constant weight. The dried fish waste was ground and sieved and the resulting fish meal used for further work.

Other chemicals, reagents, and laboratory equipment, were obtained from standard sources.

## 2. Chemical Composition of Feather

### *2.1. Dry Matter Determination*

The dry matter of feather sample was determined by first washing the feather with tap water and drying it in an oven set to 105°C for 72h. Dry weight was then calculated by weight difference.

### *2.2. Ash Determination*

Ash content was determined by weighing 1g of oven-dried feather in triplicate. Samples were placed on preweighed crucibles and were inserted into a furnace adjusted to 550 °C. After 6 h of incineration at this temperature the sample was cooled in a desiccator

containing calcium chloride and then weighed to determine the ash content of the feather samples by weight difference.

## *2.3. Total Nitrogen (TN) Determination*

The total nitrogen content of feather samples was determined using the modified Kjeldhal method described by Sahlemedhin Sertsu and Taye Bekele (2000) in which samples of dried feathers were first mechanically chopped down and then ground using mortar and pistil. The finely ground feather was then sieved using a 500 μm (0.5mm) sieve size. Then, 0.3 gm (300 mg) of the sample was mixed with digestion mixture in Kjeldhal digestion tubes and was let to stand for 2 h. The digestion mixture was composed of sulphuric acid, selenium powder and salicylic acid and 2.5 ml of this was added to each tube and swirled carefully so as to moisten the sample. After 2 h the tubes were heated to 100 °C for 2 h. The tubes were then cooled and 3 ml $H_2O_2$ added to facilitate the oxidation process.

The tubes were then heated to 300 °C until the digest turns to colourless or light yellow color, which took about two hours. After cooling to room temperature 48.3 ml distilled water was added to each tube and thoroughly mixed. The digestion tubes were then let stand over-night. After mixing by shaking the digest was filtered on a 100 ml volumetric flask and its volume adjusted with distilled water.

The core principle is that the TN was determined by distillation of an aliquot from the digest with NaOH, collecting the distillate in boric acid and titrating with 0.1N HCl to the end point of the mixed indicator.

The Kjeldhal procedure is based on the principle that by treating nitrogen-containing materials such as plant, feather etc., with concentrated sulphuric acid it is oxidized and the nitrogen in the sample is being converted into ammonium sulphate, i.e., from organic nitrogen to inorganic nitrogen, during the oxidation process. The acid digest was transferred to the macro-Kjeldhal flask. Twenty millilitres (20 ml) of boric acid solution

was measured into receiver Erlenmeyer flasks corresponding to the number of samples. Two drops of mixed indicator solution were added to each flask. After adding 75 ml of 40 % NaOH solution to the digestion flask containing the digests, it was placed under the condenser and fit to the corresponding holder and distillation commenced. When distillation was complete i.e., when about 80 ml of distillate has been collected to boric acid the flask was removed and distillation process for another sample continued. Titration was then performed using 0.1N $H_2SO_4$. At the end, the volume of $H_2SO_4$ utilized for titration was recorded and finally the TN content of the samples determined. Then the ammonia liberated in the distillation process with NaOH was trapped by the boric acid. The ammonia was adsorbed in the form of $NH_4^+$ ion in boric acid and back titrated with standard $H_2SO_4$. The TN was then determined using the following formula:

$$\%N = (a-b)/s \times N \times 0.014 \times 100$$

where; a = ml of $H_2SO_4$ required for titration of the sample

b = ml of $H_2SO_4$ required to titrate the sample

S =air dry sample weight in grams

N=normality of $H_2SO_4$ (0.1N)

0.014 = meq weight of nitrogen in grams

## *2.4. Fat Determination*

The fat content of the feather sample was determined using the Soxhlet method of fat extraction. Four grams of feather samples were taken in triplicate and dried in dry oven for 1.5 h at 125 °C. The fat was then extracted using diethyl ether after which the extract was dried to constant weight at 100 °C, cooled and then weighed to get the amount of crude fat content in the sample by difference.

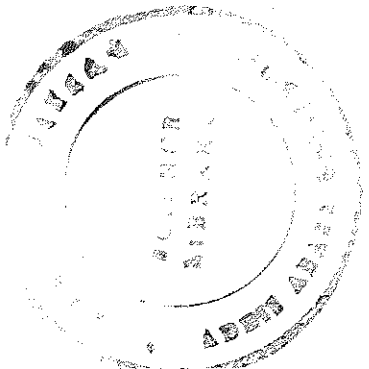
## 3. Isolation of Microorganisms

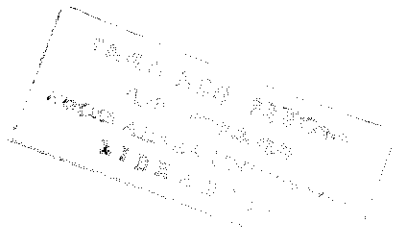
Tannery waste (hair) compost, soil and mud samples were collected from Mojo Tannery and Lake Abijata, respectively. The samples were serially diluted by adding 1g of each sample into 9 ml of sterilized distilled water. All dilutions were streaked onto casein-yeast extract agar plates and incubated for 24 to 48 h at 37 $^0$C(Appendix 1). Dilutions of $10^{-4}$ and $10^{-5}$ were found to form countable colonies and were purified by further streaking and restreaking into agar plates until pure isolates were confirmed morphologically from the growing colonies. The media composition used was (g/l) casein, 5; yeast extract, 1; $K_2HPO_4$, 1; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$ 0.1 and agar 15.The medium was autoclaved for 15minutes at 121°C at 1 atmospheric pressure and the pH adjusted to 7.5. Sodium carbonate ($Na_2CO_3$) was prepared and autoclaved separately and added into the medium after cooling before the agar medium was poured onto petri dishes to a final concentration of 1 % (Amare Gessesse and Berhanu Gashe, 1997).

## 4. Screening of Isolates for Feather Degradation

The isolates were screened for keratin degrading capacity on native feathers in such a way that feather meal broth (FMB) was prepared in minimal basal medium containing the same composition as the agar plate growth medium except for the absence of agar and the substitution of casein by 1 % native feather and the addition of 0.3 % glucose. Inoculants were prepared in broths of the same media composition as the FMB except for the substitution of feather by casein.

The broth for the cultivation of inoculum for the FMB was inoculated from actively growing agar plate cultures and were incubated at 37 $^0$C for 48 h in a Gallenkamp orbital shaker (Appendix 4) at 140 rpm. After 48 h of incubation, 2 ml of the broth cultures were taken and aseptically inoculated into the FMBs in 500 ml Erlenmeyer flasks containing 100 ml broth. The flasks were incubated in an orbital shaker at 37 $^0$C at 140 rpm agitation.





The inoculant cultures were estimated to have an average absorbance of 0.4, which was equivalent to a bacterial population of $10^6$ CFU/ml.

The cultures were periodically supervised visually for the degradation of the feather. Those cultures that showed clear digestion were assayed for protease activity and production using standard enzyme assay procedures described in section 7.2 of this thesis.

Cells and undigested proteins were separated by centrifugation at 10,000rpm for 10 minutes and the clear supernatants were used as enzyme sources. Spore staining was carried out for both isolates to examine the presence, absence, shape, location, size and number of spores per cell using the Wirtz-Conklin method, as described by Tiwari *et al.* (2004).

## 5. Selection of Best-Feather Degrading Isolates

To select the best feather degrading isolates from the already recorded feather degrading isolates some factors that could discriminate some of the strains were set and employed in the selection process. The main factors considered during the selection process included the following:

Dimensions of clear zones formed on casein-yeast extract-agar plates and amount of casein hydrolyzed (0.5-2.5).

- Growth period (fast or slow grower)
- Level of enzyme production in liquid culture.
- Ability of the enzymes to show activity on barbs as substrate instead of casein (keratinolytic activity).
- Growth ability of isolates in FRM in the absence of glucose, yeast extract and other nitrogen supplements, and
- Ability of isolates to utilize protinaciuos wastes (horn and fish meal) as nitrogen sources (Appendix 3).

Based on these criteria all isolates (35 % of the total) were scrutinized for feather degrading capacity and two isolates were selected for further works.

## 6. Cultural Conditions for Optimum Enzyme production

### *6.1. Effect of Incubation Temperature on Enzyme production.*

The isolates were grown on feather meal broth at different temperatures ranging from 30 °C to 55 °C (30, 37, 40, 45, 50, 55 °C) on an orbital shaker (Appendix 4) at 140 rpm to see the effect of incubation temperature on enzyme production. The enzyme production was measured using standard assay procedures.

### *6.2. Effect of Initial pH of the FMB (in Basal Salts Medium) on Enzyme production.*

To investigate the effect of initial pH of the FMB on enzyme production, the medium was adjusted to different pH. Enzyme production was measured following standard assay procedure.

## 7. Characterization of Proteases Kr-07 and Kr-43.

### *7.1. Enzyme Production*

Feather meal broth (100 ml) prepared in 500ml Erlenmeyer flasks was inoculated with 2 ml of a 48 h culture. After 96 h of incubation at 37 °C on an orbital shaker at 140 rpm the cultures were centrifuged at 10000 rpm for 10 minutes to remove cells and insoluble residues, and then the clear supernatants were used as enzyme sources (Amare Gessesse and Berhanu Gashe, 1997).

### *7.2. Enzyme Assay*

Protease activity was assayed according to the method described by Amare Gessesse and Berhanu Gashe (1997) with little modification in media composition.

Casein substrate (0.5%) was prepared in 50 mM glycine NaOH pH 10 buffer. The reaction mixture contained 500 µl crude enzyme and 1.5 ml casein and was incubated for 30 minutes at 50 °C in water bath (SUB36 Water Bath). The reaction was stopped by adding 2 ml of 10 % TCA and then centrifuged for 10 minutes at 10,000 rpm. Then 500 µl of the clear supernatant was mixed with 2.5 ml of 0.5 M $Na_2CO_3$ after which 500 µl 1N Ciocalteus-Folin Phenol reagent was added and immediately vortexed to ensure thorough mixing of the component. This is very important because the Ciocalteus- Folin Phenol reagent remains active for very short period of time under alkaline conditions. The test tubes with their contents were incubated under darkness for 20 minutes. The absorbances of each reaction mixture were measured at a wavelength of 660 nm after the spectrophotometer (JENWAY, 6405 UV/Vis) was calibrated using reagent blank. The enzyme blank contained all the components of the enzyme reaction except for the fact that the enzyme was added after the reaction was stopped by adding 2ml 10 % TCA. While the reagent blank contained substrate, reagents and 500 µl distilled water in place of the 500 µl enzymes. Following these procedures the screening process for keratin degrading organisms was accomplished successfully and ended up with ample keratin degrading isolates for the subsequent works of the research.

### *7.3. Effect of Temperature on Protease Activity*

To study the effect of temperature on the activity of proteases, the reaction mixtures were incubated for 30 minutes at different temperatures ranging from 4 °C to 80 °C in the absence and presence of 5 mM of the divalent cation $CaCl_2$. Activities were measured using the standard assay procedure.

### *7.4. Effect of Temperature on Protease Stability*

The thermal stability of the enzymes was studied by preincubating the enzymes at different temperatures (35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 °C) in the presence and absence of 5 mM $CaCl_2$ for an hour. The residual enzyme activities were then measured based on the standard assay procedures mentioned above.

### *7.5. Effect of pH on Protease Activity*

The isolates were grown in 100 ml FMB in 500 ml Erlenmeyer flask at 37 °C for 96 h with an agitation of 140 rpm. Cells and other undigested proteins were pelleted by centrifugation at 10,000 rpm for 10 minutes and supernatants recovered.

To investigate the effect of pH on protease, activity was assayed using azocasein as substrate. The reaction mixture contained 800 µl of 0.5 % azocasein as substrate in 50 mM of different buffers of different pH (Phosphate, 4-7.5; Tris-HCl, 8.0 and 8.5; glycine-NaOH buffer, 9-12.0), and 200 µl enzyme. After 1 h incubation at 30 °C the reaction was stopped by adding 500 µl of 15 % TCA. After standing at room temperature for 30 minutes undigested substrate was separated by centrifugation at 10,000 rpm for 10 minutes. Then 800 µl of the clear supernatants was mixed with 200 µl of 2M NaOH. To measure the effect of different pH on the activities of the enzymes, absorbance of the resulting coloured compound was measured at 440 nm against a reagent blank.

One unit of protease activity was defined as the amount of enzyme, which results an absorbance increase of 0.01 at 440 nm after 1 h incubation at 30 °C. This was carried out according to the method described by Amare Gessesse *et al.* (2003).

### *7.6. Effect of pH on Protease Stability*

The pH stability was determined by preincubating the enzymes in various buffers (50mM) of different pH values. The enzymes were preincubated at 50 °C for an hour. The residual enzyme activities were then measured using standard assay procedures described above for azocasein hydrolysis.

### *7.7. Effect of Different Nitrogen Sources on the Activity of Protease kr-07 and kr-43*

Different nitrogen sources including some wastes (fish, horn and human hair) were incorporated into the FMB to investigate their effect on enzyme production. The commercially available nitrogen sources utilized included casein, peptone and yeast extract. All the nitrogen supplements were added at a rate of 0.1 % in to the FMBs. Controls were prepared without any nitrogen supplement. The FMBs were prepared in duplicate in 500 ml Erlenmeyer flasks and each containing 100 ml medium incubated on an orbital incubator shaker at 37 °C at a revolution of 140 rpm.

### *7.8. Effect of Different Carbon Sources on Proteases activity*

To study the effect of different carbon sources on protease production glucose, sucrose, maltose, lactose and fructose were incorporated into the FMBs containing minimal basal media to a final concentration of 0.3 % (w/v). The preparations were then incubated in an orbital shaker at 37 °C at 140 rpm in duplicate. Controls were prepared without any external carbon source. The enzyme activities were measured after 96 h.

### *7.9. Effect of Fish Meal and Horn Meal Broths on Proteases Production*

To study the effect of fish meal and horn meal broths the isolates were cultured in basal liquid media containing 0.1% fishmeal and horn meal. Feather meal broth was used as control and results were measured following the standard assay procedures.

### *7.10. Effect of Storage Temperature on Protease stability*

The effect of storage temperature on protease activity was studied by storing the enzymes at 4 °C and -20 °C (deep freeze) for 24, 48, 72, 96 and 120 h. Freshly harvested enzymes were stored at the above temperatures for the predetermined periods in ependorphs tubes. The residual enzyme activities were then measured using the standard assay procedures and results compared with the original activities.

### *7.11. Effect of Feather Concentration on Enzyme Production*

To study the effect of feather concentration on protease production the isolates were grown using ten different concentrations (0.5, 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4, 4.5 and 5 %) of feather. Each experiment was run in duplicate. After 96 h the cultures were harvested by centrifugation.

### *7.12. Effect of Protease Inhibitors on Enzyme Activity*

The effects of three protease inhibitors, namely Phenyl methyl sulphonyl fluoride, PMSF (a serine protease inhibitor), a chelator of divalent cations (Ethylene diamine tetra acetic acid, EDTA) and cysteine protease inhibitor (β-mercaptoethanol) were determined by preincubating with the enzymes for 1 h at room temperature before the addition of substrate. The residual protease activities were then determined using standard assay procedure.

# III. Results

## 1. Chemical Composition of Feather

Feather used in this study had a dry weight of 87.4 %, an ash content of 1.26 %, fat content of 1.24 % and a total protein of 84.4 % (Table1).

The total protein content of the feathers was also measured by the Lowry *et al.* (1951) method using BSA as standard protein and the result was found to be almost similar with the result determined indirectly using the modified Kjeldhal method. To determine the total protein, 96 h feather culture broths were centrifuged and the total protein measured following the standard protein assay procedure. It was taken after digestion and found to be 88 % at a wavelength of 280 nm.

Table 1. Chemical composition of the feather wastes used in the experiment.

| Component | Content in percent (%) |
|---|---|
| Dry matter | 87.40 |
| Total protein | 84.44 |
| Fat | 1.24 |
| Ash | 1.26 |

## 2. Isolation and Screening of Feather Degrading Bacterial Strain

Out of the 165 bacteria isolated 80 were found to be positive for protease production, with 15 of them rated as very good on the basis of clear zone formed on the casein agar plates (Table 2). All protease positive isolates were purified through repeated streaking. Pure colonies were maintained in agar slants.

Table 2. Protease positive strains out of the total isolates in the study.

| Parameter measured | *Enzyme activity | Number of Isolates |
|---|---|---|
| Clear zone formation | ++++ | 6 |
| | +++ | 9 |
| | ++ | 13 |
| | + | 52 |
| No clear zone formation | - | 85 |
| **Total** | | 165 |

*Enzyme activity was qualitatively measured by observing the clear zone formed around the colonies on casein-yeast extract-agar plates.

All protease positive isolates were screened for feather degradation in liquid culture using FMB.

Results indicted that 28 strains (17 %) degrade feather (Table 3).

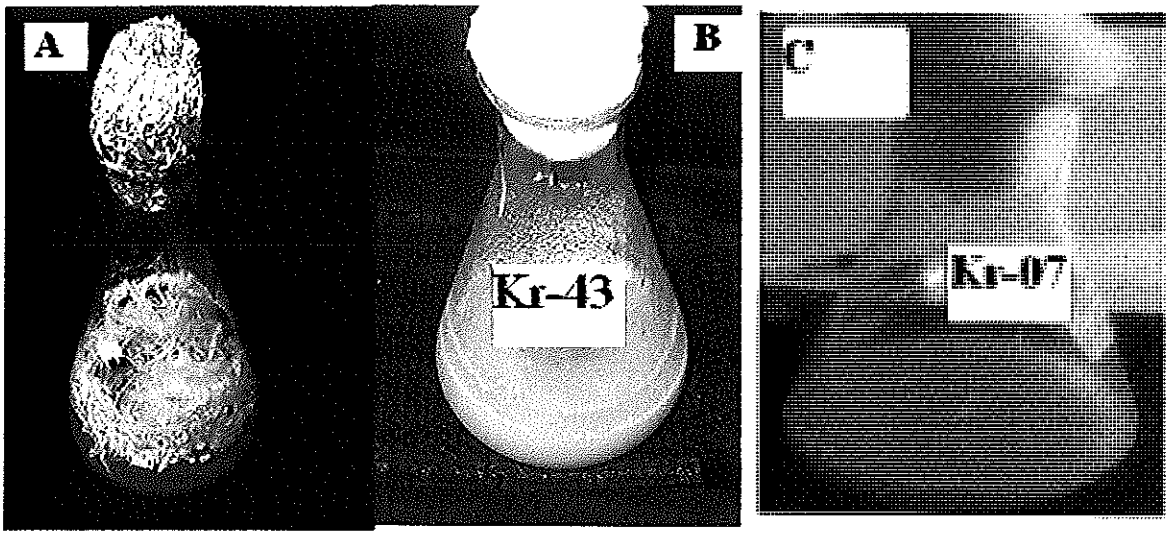



Figure1. Effect of proteases on feather (A) before digestion and (B) Kr-43 after 96h of incubation (C) Kr-07 after 96h of incubation.

Table.3. Isolates screened on feather meal broth for feather degradation.

| Parameter measured | No. of isolates capable to degrade feather | No. of isolates incapable to degrade feather | Total |
|---|---|---|---|
| Feather Degradation | 28 | 52 | 80 |

## 3. Selections and Identification of Two Top-Feather Degrading Isolates

### *3.1. Selection*

Two isolates with very high keratinolytic capacities were selected. The selection was carried out in two phases. In the first phase five isolates viz, kr-01, kr-02, kr-07, kr-16 and kr-43 were found to be eligible. In the second phase these five isolates were tested on two additional criteria i.e. ability to grow on solid substrate (solid-state fermentation) and ability of enzymes to digest feather barbs instead of casein. When these criteria were included all isolates, except kr-07 and kr-43 failed to be candidates. Therefore, kr-07 and kr-43 were selected as top feather digesters for further study.

### *3.2. Identification*

The two keratinolytic isolates were preliminarily identified using the Gram staining reaction, spore staining, some biochemical tests and the CHB-50 kit method. Colonies of both strains were observed to produce bubbles after the addition of 3% (v/v) $H_2O_2$ indicating that they are catalase positive.

The API CHB-50 medium is intended for the identification of *Bacillus* and related genera. It is a ready to use medium, which allows the fermentation of 49 carbohydrates on the API

50 CH strip. The principle is that a suspension is made in the medium with the microorganism to be tested and each tube of the strip is then inoculated with the suspension (anonymous, bioM'erieux sa http://www.biomerieux.com). During incubation the carbohydrates are fermented to acids, which produce a decrease in the pH, detected by the change in color of the indicator. The results make up the biochemical profile, which is used by the identification software to identify the strain.

The cultural, morphological, and physiological characteristics of the isolates were also studied and the micromorphologies microscopically examined.

Kr-07 formed translucent colonies with shiny or refractive fluid on cells after the attainment of full growth. The growing cells on agar plates were easily pickable by loop. Whitish nasal mucus type substance remained on the agar plates when large amount of the colonies were lifted at a time. The cells were motile and grew from 30 °C to 50 °C, with optimal growth at 37 °C. Cells start growth after 15 h and attain full growth afler 24hrs of incubation on agar plates.

Kr-43 starts growth with whitish, rough and dry colonies. It can grow in the temperature range of 30 °C to 50 °C with optimal growth at 37 °C. After 48 h of incubation at 37 °C it started to develop powdery substance, which was proved to be the spore of the bacterium. Before sporulation it was very difficult to lift colonies from the agar plates due to the growing behavior of the cells into the agar media as undergrowth. But once the spores were formed they were easily picked by the inoculating loop. Heaps of spores can be lifted at a time using the loop. When due care was not there, the spores easily dispersed into vicinity areas and grew as contaminants. These cells are also motile.

The Gram staining reaction revealed that the isolates are Gram positive, straight rod-shaped cells with somehow rounded ends. The cells of both isolates occur in singles, in doubles and some in chain forms.

Spore staining results indicated that Kr-07 possessed rounded single spores per cell, situated at the centre, while Kr-43 had subterminal spores, which were oval in shape and single per bacterial cell with filaments.

Kr-43 showed typical earthy (freshly ploughed soil) odour both on the agar plates and culture broths, which is a characteristic feature of the actinomycetes with special reference to the Genus *Streptomyces* (Tortora *et al.*, 1989). After complete removal of the colonies from the agar plates clear undergrowths were recognized in the media. When the agar plates with such remainants were incubated for 24 h, the undergrowths were observed to grow into colonies.

The morphological, cultural, and physiological characteristics of the isolates were compared with data from Bergey's Manual of Determinative Bacteriology (1986) and then were tentatively identified as *Bacillus lentus* strain Kr-07 and *Streptomyces* sp. strain Kr-43 with the assistance of the CHB-50 kit test.

## 4. Cultural Conditions for Optimal Proteolytic Activity

### *4.1. Effect of Incubation Temperature and Incubation Period on the Production of Proteases of kr-07 and kr-43*

The study on the effect of incubation temperature on the proteolytic activities of the proteases indicated that growth, and hence production of enzymes continued up to 50 °C. But at 55 °C there was neither growth nor enzyme activity. The failure of the isolates to grow at this temperature was confirmed by corresponding agar plate culturing from broths of respective flasks. The optimum temperature for growth and enzyme production for both isolates was at 37 °C (Figure 2).

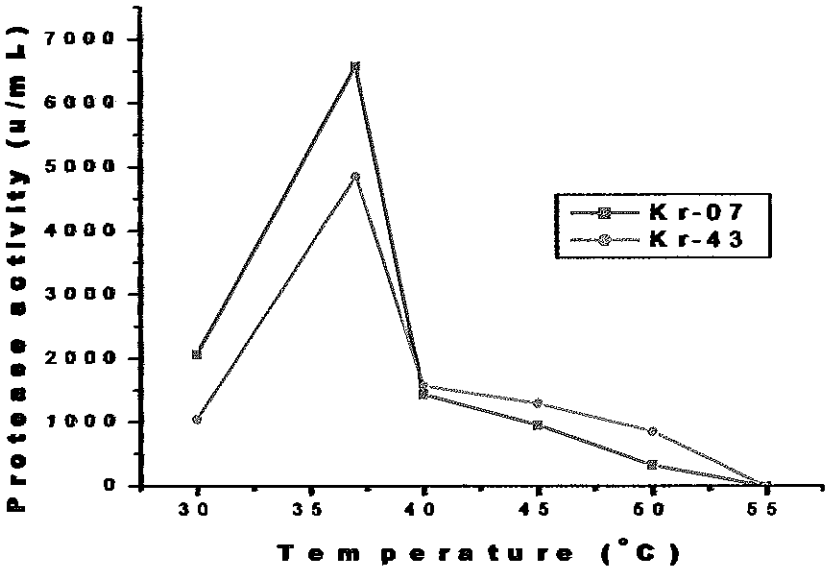



Figure 2. Effect of incubation temperature on the production of proteases by Kr- 07 and of Kr-43.

* Results are averages of two independent experiments.

Maximum protease production by both isolates was measured after 96 h of incubation at 37 °C (Figure 3).

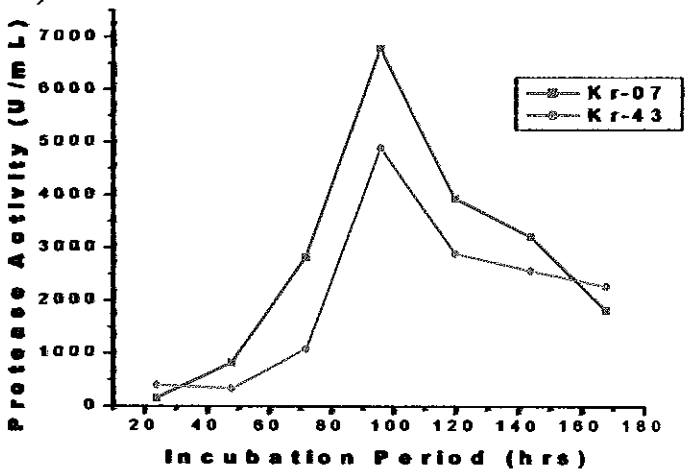



Figure 3. Time course of protease production by Kr-07 and of Kr-43 at 37°C.

* Results are averages of two independent experiments.

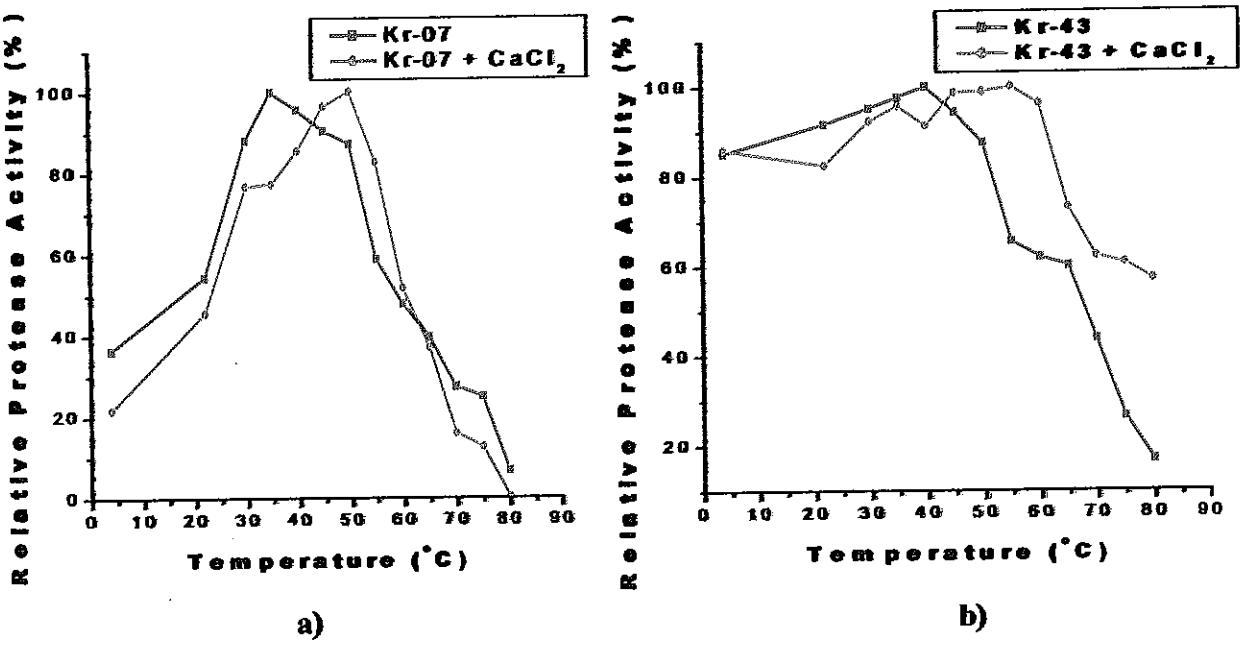



Figure 5. Effect of temperature on the activity of proteases (a) Kr-07 and (b) Kr-43 in the absence and presence of 5mM $CaCl_2$.

### *5.2. Effect of Temperature on Protease Stability*

The stabilities of proteases of Kr-07 and Kr-43 were studied by pre-incubating the enzymes at different temperatures ranging from 35 °C to 80 °C, both in the absence and presence of 5mM $CaCl_2$.

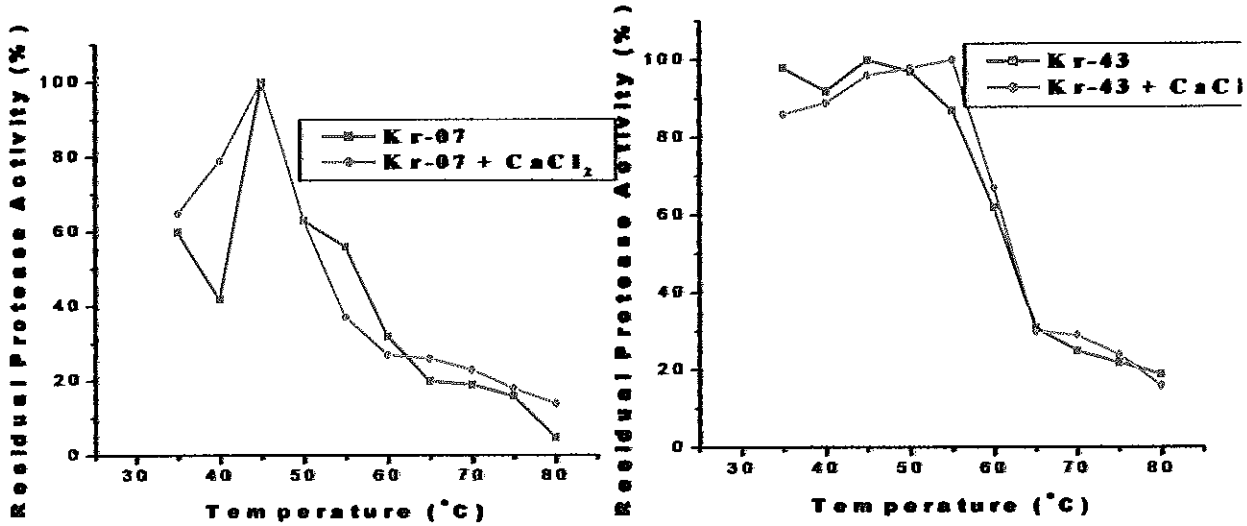



Figure 6. Effect of temperature on stability of proteases Kr-07 (a) and Kr-43 (b) in the presence and absence of 5mM $CaCl_2$.

*Results are averages of two independent experiments.

Proteases kr-07 and kr-43 were found to be stable for an hour at temperatures of 45 °C and 55 °C in the presence of 5mM $CaCl_2$, retaining 71 % and 97 % of their original activities respectively (Figure 6). For both proteases addition of $Ca^{2+}$ did not show any effect on stability (Figure 6).

### *5.3. Effect of pH on the Activities of Proteases kr-07 and kr-43*

Protease kr-07 was active in the pH range of 5.0 to 11.5 with an optimum activity at pH 7.5. Protease Kr-43 also exhibited optimum activity at pH 7.5 and showed low activity at pH values lower than 5.5 and higher than 11.0 (Figure 7).

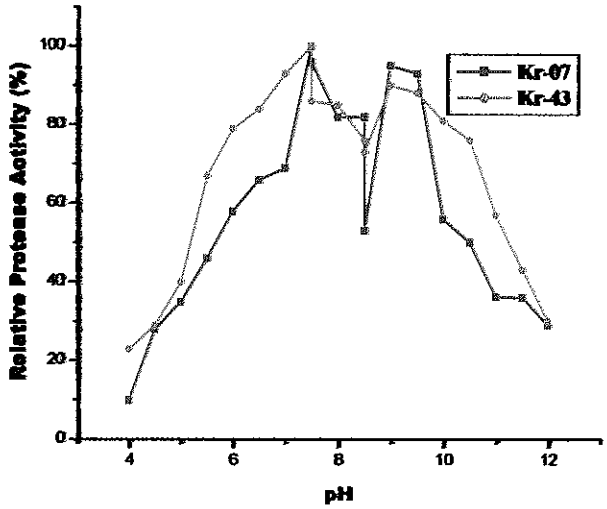
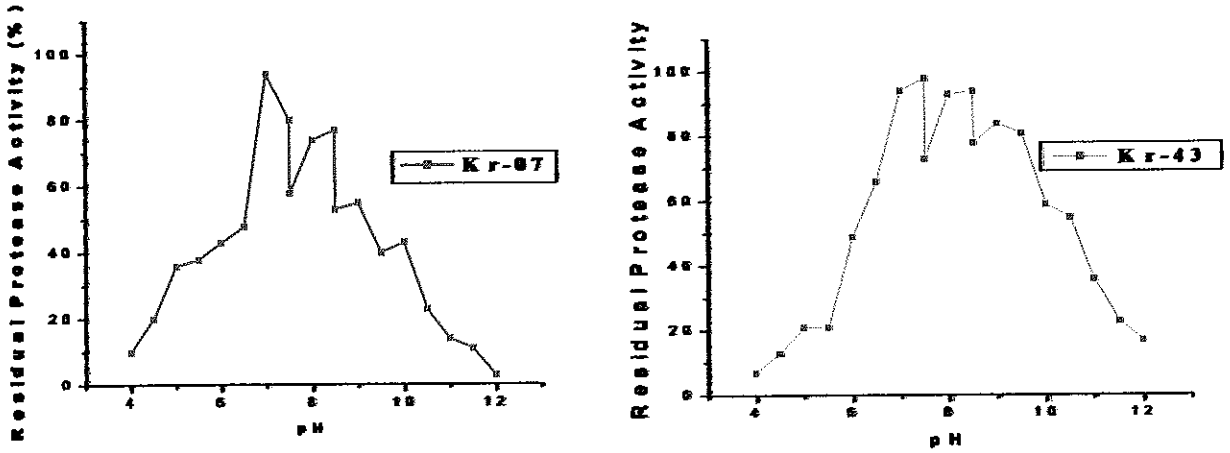



Figure 7. Effect of pH on protease activities of kr-07 and kr-43.

- Buffers used were phosphate (pH 4.0-7.5); Tris-HCl (pH 7.5-8.5) and Glycine-NaOH (pH 8.5-12.0) each at a concentration of 50mM.The substrate used was 0.5 % azocasein.

### *5.4. Effect of pH on Protease Stability*

Proteases kr-07 and kr-43 were found to be stable for an hour at pH values of 7.0 and 7.5 displaying 94% and 98% of their original activities respectively. For the same time interval, protease kr-07 retained 80% of its original activity at pH 7.5, while protease Kr-43 retained 94% of its original activity at pH 7.0 (Figure 8).




a) b)

Figure.8. Effect of pH on protease stability (a) Kr-07 and (b) Kr-43.

### *5.5 Effect of Different Nitrogen Sources on Protease Production*

The effect of different commercially available nitrogen sources, including casein, peptone, and yeast extract and some wastes (fish meal, horn meal and human hair) was studied by adding each to FMB at a rate of 0.1% each. The results of the experimentation are presented in Figure 9.

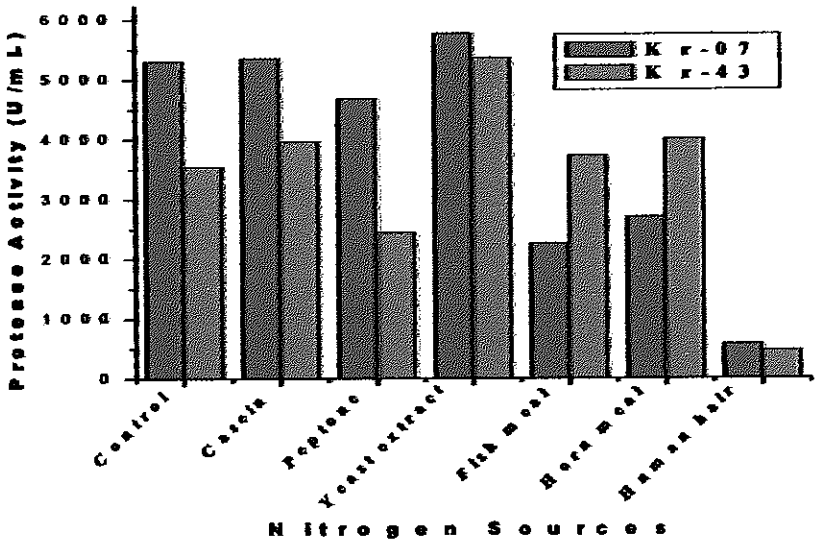



Figure 9. Effect of different nitrogen sources on the production and activities of proteases kr-07 and kr-43.

The results displayed in Figure 9 indicate that the two proteases exhibited maximum activity when yeast extract was used as supplement. This most probably implies that they need some growth factors for their optimal enzyme production. Protease production by Kr-07 was also high and similar with that obtained in FMB when supplemented by casein.
On the other hand protease Kr-43 showed similar enzyme production with that of the FMB when supplemented with casein, fmb and HMB indicating great flexibility in substrate utilization.

### *5.6. Effect of Different Carbon Sources on Proteases Production*

The effect of different carbon sources on enzyme production was investigated by supplementing the FMB with different sugars.

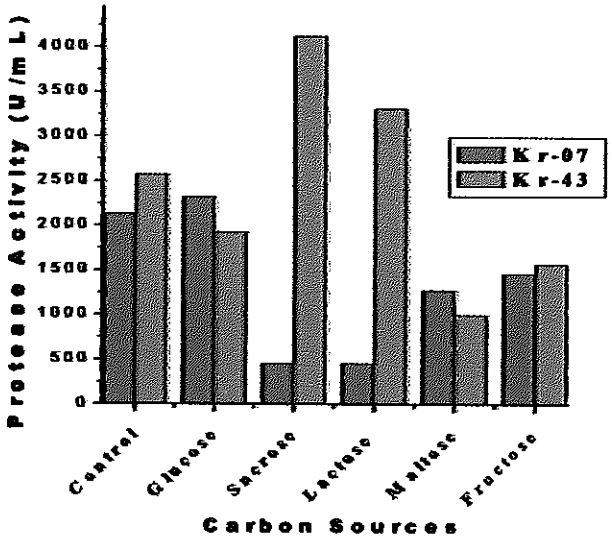



Figure.10. Effect of different carbon sources on the production and activities of the proteases of kr-07 and kr-43

### *5.7. Effect of Fish meal and Horn Meal Broths Proteases Production*

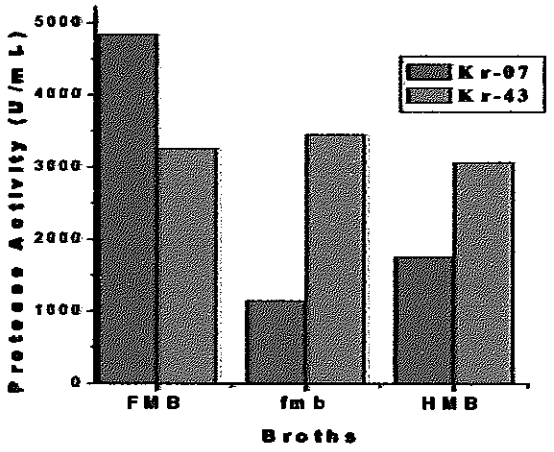



Figure.11. Effect of fish meal broth, and horn meal broth on the production and activities of proteases of kr-07 and Kr-43.

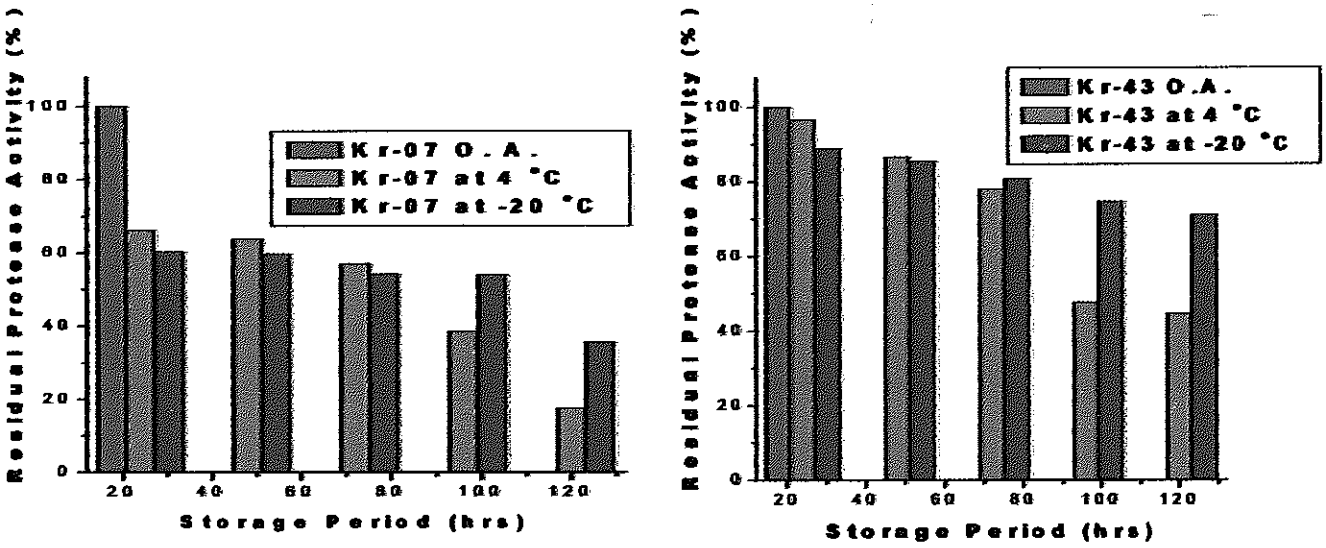
*FMB indicates feather meal broth; fmb, fishmeal broth, and HMB, horn meal broth.

Both isolates produced protease actively when grown using fish meal and horn meal as sole C and N sources. *Bacillus lentus* sp. Kr-07 produced more protease using feather than other substrates while *Streptomyces* sp. Kr-43 produced equal level of protease in all substrates tested.

### *5.8. Storage Stability*

The effect of storage temperature on the activities of proteases through time course was studied by storing the enzymes at 4 °C and at –20 °C (deep freeze) for 24, 48, 72, 96, and 120 h in ependorphs of 1.5 ml after which the residual enzyme activities were measured by running the standard assay procedures. The results displayed in Figure 12 indicate that the enzymes retained some percentage of their original activities after being stored for the specified periods at the predetermined storage temperatures. To put this another way protease kr-07 retained 66 %, 64 %, 57 %, 39 % and 17 % of its original activity at 4 °C after 24, 48, 72, 96 and 120 h of storage respectively. At –20°C it retained 60 % of its original activity after a storage period of 24, and 48 h, and 54 % of its original activity after

a storage period of 72 and 96 h and 36 % activity after a storage period of 120 h respectively. While protease kr-43 retained 97 %, 87 %, 78 %, 48 % and 48 % of its original activity after 24, 48, 72, 96 and 120 h of storage at 4 °C. At -20 °C it retained 89 %, 85 %, 81 %, 75 % and 71 % of its original activity after a storage period of 24, 48, 72, 96 and 120 h, respectively.




a) b)

Figure.12. Effect of storage temperature on protease activity through time course (a) Kr-07 and (b) Kr-43.

* O.A. indicates Original Activity.

### *5.9 Effect of Feather Concentration on Protease Production*

The effect of feather concentration on proteases production by both isolates was studied using ten-concentration levels (0.5 %, 1.0 %, 1.5 %, 2.0 %, 2.5 %, 3.0 %, 3.5 %, 4.0 %, 4.5 % and 5.0 %) (Figure13). Enzyme production by the two strains increased when the feather concentration was increased upto1% and 1.5% for Kr-07 and Kr-43 respectively, and then remains more or less constant in other higher amounts indicating that the bacteria can only

utilize upto the level they need for maximum protease production. It also indicated that the addition of extra feather had not
displayed any substrate inhibition effect.

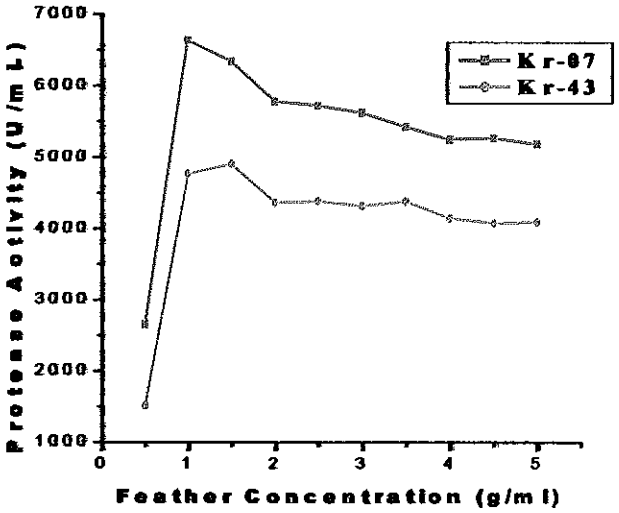



Figure 13. Effect of feather concentration on the production and activity of the proteases of Kr-07 and Kr-43 after 96 h of incubation at 37°C.

### *5.10. Effect of Protease Inhibitors on Enzyme Activity*

The effects of Phenyl methyl sulphonyl fluoride (PMSF), a chelator of divalent cations, Ethylene diamine tetra acetic acid (EDTA) and β-mercaptoethanol (β-ME) were studied by preincubating with the respective enzymes for 1hr at room temperature before the addition of substrate and the results of the experiment are presented in Table 4.

Results of the experiment indicated that protease Kr-07 was inhibited by PMSF but not by EDTA and β-ME showing that it is a serine type protease. On the other hand protease Kr-43 was inhibited by EDTA indicating that it is a metalloprotease. The two proteases regained 100 % of their activity when assayed with EDTA in the presence of 5mM $CaCl_2$.

Table 4. Effect of protease inhibitors on the activity of protease of Kr-07 Kr-43.

| Inhibitor | Concentration (mM) | Remaining activity (%) Kr-07 | Kr-43 |
|---|---|---|---|
| None | --- | 100 | 100 |
| PMSF | 5 | 12 | 52 |
| PMSF | 10 | 17 | 53 |
| PMSF | 50 | 17 | 52 |
| PMSF | 100 | 20 | 52 |
| PMSF | 150 | 17 | 51 |
| EDTA | 5 | 83 | 13 |
| EDTA | 10 | 64 | 34 |
| EDTA | 50 | 64 | 18 |
| EDTA | 100 | 63 | 14 |
| EDTA | 150 | 64 | 12 |
| β-ME | 5 | 100 | 100 |
| β-ME | 10 | 104 | 101 |
| β-ME | 50 | 102 | 100 |
| β-ME | | | |
| EDTA + 5mM$CaCl_2$ | 5 | 100 | 100 |
| EDTA + 5mM$CaCl_2$ | 10 | 117 | 101 |
| EDTA + 5mM$CaCl_2$ | 50 | 95 | 104 |
| EDTA + 5mM$CaCl_2$ | 100 | 120 | 102 |
| EDTA + 5mM$CaCl_2$ | 150 | 118 | 100 |

* PMSF indicates phenylmethylsulphonyl fluoride; EDTA, ethylene diamine tetra acetic acid; β-ME, mercaptoethanol.

# IV. Discussion

Feathers and other keratins are largely composed of protein comprising about 90 % (Amare Gessesse *et al.*, 2003), 81 % (Zerdani *et al.*, 2004) and 84.44 % results of this study. The protein in keratins contains more than ten amino acids including the essential ones for ruminant feeding. Ram horn hydrolysates and native wool were reported to contain 17 amino acids (Kurbanoglu, and 2004; Gousterova *et al.*, 2005) the dominant amino acids being glutamic acid, arginine, leucine, aspartic acid, alanine, serine and valine.

Feather accounts for 4.5-6 % of the live weight of mature indigenous chicken. Currently these potentially important resources are dumped off to the environment and are creating sanitation problem. Therefore, the biological conversion of these wastes into economically useful products could help in two perspectives: first in producing useful products and second in cleaning the environment.

At the Mojo Tannery plant there is bovine hair compost accumulated over years. Sample was collected from this site and 72 strains of bacteria were isolated 12 of which were keratinolytic ones. Among these isolates one strain was found to be highly keratinolytic and identified to species level as *Bacillus lentus* strain Kr-07. The protease of this bacterium was active in a wide range of temperature and pH.

Ninety-three strains of bacteria were isolated from soil and mud samples collected from Abijata Lake. In this area there are numerous birds, mostly Pelicans, of which feathers serve as nitrogen, carbon and energy sources for the decomposing organisms (Amare Gessesse *et al.*, 2003). A strain identified as *Streptomyces* sp. Kr-43 exhibited very high keratinolytic character. It grew optimally at 37°C and at a pH of 7.5 and its protease was active in wide ranges of temperature and pH.

In this study 17% of the isolates were found to be keratinolytic bacteria, and thus it can be seen that it is a common phenomenon to find keratin degrading microorganisms in natural habitats where keratins are expected to be available as dumped wastes or as fallen materials from animals possessing keratins.

The alkaline protease of Kr-43 exhibited unique property in that it is cold active enzyme displaying over 85% of its optimum activity at 4°C both in the absence and presence of 5mM $CaCl_2$. The enzyme was optimally active in the temperature range of 35 °C to 40 °C and in the pH range of 6.0 to 10.0 (Figures 7 and 8). These properties could make the enzyme a promising candidate for application in the detergent industries. Addition of 5mM $CaCl_2$ raised its temperature optimum from 40 °C to 55 °C indicating that $Ca^{2+}$ is required for activity at higher temperature. Most detergent proteases are optimally active above 40 °C, usually 60 to 70 °C. Washing temperature in different countries differs. In some countries it is 50 to 60 °C while in others like Japan it is carried out at ambient temperature, which in turn varies with season, usually being around 4 °C in winter and 20 to 30 °C in summer. In most developing countries washing is performed at ambient temperature. Therefore, cold active proteases could attract the attention of detergent industries for application. Furthermore, the fact that protease Kr-43 is produced using cheap substrates, like feather, horn meal and fishmeal, makes it more attractive for application in the detergent industries.

Proteases kr-07 and kr-43 were found to be stable for an hour at temperatures of 45 °C and 55 °C in the presence of 5mM $CaCl_2$, retaining 71 % and 97 % of their original activities respectively. In the absence of the divalent cation both proteases were found to be stable for an hour at 45 °C retaining 70 % and 90 % of their original activities in that order (Figure 6). This indicated that protease kr-07 was stable for an hour at 45 °C regardless of the presence or absence of 5mM $CaCl_2$, whereas protease kr-43 needs 5mM CaCl2 to be stable at 55 °C for an hour.

Both isolates were efficient feather utilizers as nitrogen source for protease production. This shows that feather can provide all the nitrogen requirements of the organisms and thus there is no need to supplement it with other nitrogen sources. This is an interesting property of the organisms because it reduces the production cost of the enzymes. The study also indicated that the isolates were able to utilize fish and horn wastes. On the contrary, human hair supplied to the isolates as nitrogen supplement was digested poorly.

The effects of different carbon sources on the activities of the proteases kr-07 and kr-43 were studied. The results of the experiment (Figure10) indicate that Kr-43 had high enzyme production in the presence of sucrose and lactose. It was very interesting that Kr-07 showed high protease production without any external carbon source. This indicates that the isolate is capable of utilizing the carbon in feather efficiently and this agrees with the reports of Mohamedin (1999), Amare Gessesse, *et al.* (2003), Zerdani *et al.* (2004), Thys *et al.* (2004), and Gousterova *et al.* (2004).

Given the potential industrial application of protease Kr-43, using sucrose and lactose is extremely interesting, because sucrose and lactose could be replaced by molasses and whey lactose, which are industrial by-products of the sugar and dairy industries respectively. The use of these by-products could reduce the cost of enzyme production with a significant increase in enzyme level by Kr-43 when it uses sucrose as carbon source.

Protease production by Kr-43 was almost identical in all the three substrates indicating that the organism has no preference for feather. This flexibility in substrate utilization could offer a good chance to use substrates depending on their availability and cost for least cost enzyme production.

The production of industrial enzymes in general and proteases in particular is constrained by the cost of microbial growth substrate. In the production of industrial enzymes, upto 30-40 % of the production cost goes to the growth substrate Kalisz (1988) and Hinman (1994) cited in Amare Gessesse *et al.* (2003). Therefore, the use of protease producing microorganisms which at the same time are capable of utilizing feathers and other keratins



and keratinacious wastes as substrate could substantially reduce the cost of enzyme production at industry level. The ability of Kr-43 to utilize different wastes as substrate non-selectively has good implication for potential application in the production of industrial protease by using the cheap, abundant and readily available wastes as substrate.

The results displayed in Figure 12 indicate that protease kr-07 retained 66 %, 64 %, 57 %, 39 % and 17 % of its original activity at 4 °C after 24, 48, 72, 96 and 120 h of storage respectively. At –20°C it retained 60% of its original activity when stored for 24 and 48 h, while 54 % and 36 % of its original activity was retained after storage periods of 72, 96 and 120 h at the same temperature (Figure 12). While protease kr-43 maintained 97 %, 87 %, 78 %, 48 % and 48 % of its original activity after 24, 48, 72, 96,and 120 h of storage at 4 °C and 89 %, 85 %, 81 %, 75 % and 71 % at -20°C for the same storage periods in that order. Although proteases are liable to autolysis, both proteases showed good storage stability at 4 °C and at -20°C.However, better keeping qualities were observed when stored in deep freeze. Since there was some decline in activity, there is still a need to investigate methods for enzyme stabilization during storage.

The isolates were grown on FMBs containing ten-concentration levels of feather. Proteolytic activity increased as the amount of feather increased upto 1 % and 1.5 % for Kr-07 and Kr-43 respectively (Figure 13). Then after, enzyme production remained more or less constant indicating that the organisms can not utilize feathers added beyond these levels. Therefore addition of feather beyond the maximum amounts expressed above does not bring about any change on enzyme production.

The effects of serine protease inhibitor (Phenyl methyl sulponyl fluoride, PMSF), a chelator of divalent cations (Ethylene diamine tetra acetic acid, EDTA) and cysteine inhibitor (β-mercaptoethanol) were studied and the results indicated that Kr-07 produced a serine type protease because its activity was inhibited by PMSF to the extent of 88 % at a concentration of 5mM (Table 4). On the other hand it retained 83 % and 100 % of its activity when assayed with EDTA and β-ME at a concentration of 5mM each. Therefore,

# V. Conclusion

The results of this study showed the possibility to isolate keratinolytic bacteria from samples collected from areas expected to harbor such organisms. It also indicated that there are great possibilities to convert keratins and keratinacious wastes into economically important products using microbiological and/or enzymatic methods.

Two bacterial isolates, preliminarily identified as *Bacillus lentus* strain Kr-07 and *Streptomyces sp.* (Kr-43) were found to be digesters of feathers, horn and fish wastes indicating their potential applicability in the biotechnological industries involved in the conversion of keratins and keratinacious wastes into economically useful products like animal feed additives, fertilizers, microbial media and others.

The two proteases showed activities in wide ranges of temperature and pH. These properties could qualify the enzymes to have potential applications in the detergent and animal feed manufacturing industries.

Besides the above properties, protease Kr-43 is a cold active enzyme and this could make it an interesting candidate in the detergent industries.

# VI. References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## Appendix 4.Gallenkamp Orbital Incubator (Shaker)