

# Production optimization and characterization of alkaline protease from bacteria strains for skin dehairing

**Author: Fekede Meshesha Namu**

Debre Berhan University College of Natural and computational Science Department of Biotechnology

Email: [fkdms2008amu@gmail.com](mailto:fkdms2008amu@gmail.com), phone: +251 928631110

P.O.BOX 24 Debre Berhan, Ethiopia

**Abstract-** Enzymes are highly efficient environmental-friendly protein catalysts, synthesized by living systems. Alkaline proteases are one of the most important groups of microbial enzymes that find varied uses in various industrial sectors. The study focused on optimization and characterization of bacterial alkaline protease that has potential application in dehairing of skin. Many strains of alkaliphilic proteolytic bacteria were isolated from samples collected from different habitats around Arba Minch and their diversity studied. The screening of these isolates took place depending on clear zone formation, dehairing application of goat skins and keratinase activity by plate assay method. The selected isolate was identified as *Bacillus badius* by morphological and biochemical characterizations. The dehairing test was done on fresh goat skins with alkaline protease by incubating the skin pieces with crude enzyme at 40°C for 6h and hair successfully removed with improved skin quality. Alkaline protease characterized with different conditions and found that it is more active at 40°C, pH10, 0.5% casein as a substrate and stable at temperature 30-60°C and pH 7-11. Culture conditions were characterized for the optimum growth and enzyme production of selected isolate. Accordingly, temperature 40°C, pH 9-10, 1% inoculum size and incubation time of 36 h was identified as optimum condition for growth and enzyme production by *Bacillus badius*. The maximum production of enzyme 997.36 U/ml was found at these conditions. Production optimization of alkaline protease was done with different cheap substrates, carbon and nitrogen sources. Cow dung was found to be the best substrate for enzyme production by *Bacillus badius*. Also in this study chicken manure was found as the best cheap nitrogen source for alkaline protease production. Alkaline protease produced by *Bacillus badius* in the present study has high potential in skin dehairing with improved skin quality.

**Key words:** Alkaline, *Bacillus badius*, Dehairing, Keratinase, Protease, S35

## 1. Introduction

Enzymes are highly efficient environmental-friendly protein catalysts, synthesized by living systems. They have significant advantages over chemical catalysts, of which the most important is specificity, high catalytic activity, ability to work at moderate as well as extreme temperatures, and the ability to be produced in large quantities. The current demand for better utilization of renewable resources and pressure on industry to operate within environmentally compatible limits stimulated development of new enzyme-catalyze industrial process (Barredo, 2005).

Recent developments in industrial biotechnology has offered an alternative approach for the reduction (or in some cases total elimination) of pollution from many industrial sectors without affecting production efficiency and product quality. At present enzymes find increasing application in many industrial processes. As a result the global industrial market is growing very fast with a current estimated value of US\$7 billion. Although enzymes are found in all living organisms, most industrial enzymes currently in use are obtained from microorganisms (Gessesse *et al.*, 2011). Ethiopia is endowed with unique microbial diversity which could serve as a source of novel enzymes for industrial application. Despite its huge potential for biotechnology innovation, to date the country make no use of this resource (Gessesse *et al.* 2003).

Bacterial proteases are the most preferred group of industrial enzymes as compared to animal and fungal proteases, because of their ability to grow in simple culture medium with minimum space requirement, faster growth rate, higher productivity and low production cost. Alkaline proteases have extensive applications in industries like laundry detergents, pharmaceutical, food industry, leather processing and proteinaceous waste bioremediation (Bayouhd *et al.*, 2000). Recently, bacterial alkaline proteases have received attention as a viable alternative for bioremediation of protein rich tannery waste and their use in treatment of raw hide by replacing the hazardous chemicals to produce quality leather without causing environmental pollution (Ahmad and Ansari, 2013).

Leather processing involves a complex set of steps, from skin to finished product, including soaking, dehairing, bating, and tanning. These operations involve the application of materials that are capable of degrading proteinaceous matter present in the hides and skins. The conventional methods of leather processing involve the application of various hazardous chemicals, notably sodium sulfide, which generates several environmental and waste disposal problems. In order to overcome the hazards caused by these effluents, enzymes have often been proposed as viable alternatives (Gupta *et al.*, 2002).

Keratinases are a group of metallo or serine proteinases that can degrade the insoluble structure forming keratin substrates. In enzymatic catalysis, the

disulfide bonds of keratin are reduced by disulfide reductase followed by the action of keratinases, which simultaneously degrade the keratin into oligo and monomeric products (Gupta and Ramnani, 2006).

Currently, microbial alkaline proteases are used to ensure a faster absorption of water and reduce the time required for soaking (Pillai P and Archana G, 2012). The conventional lime-sulfide process is, however, known to generate large amounts of sulfide, which poses serious health and waste disposal problems (Pandeti *et al*, 2011). Bio treatment of leather using an enzymatic approach is preferable as it offers several advantages such as easy control, speed and waste reduction, thus being eco-friendly (Verma *et al.*, 2011).

In the process of enzyme production up to 40% of the production cost is always accounted for by the growth substrate. The enzymes considered in this study grow using very cheap substrates such as cow dung and wheat bran. For example if glucose, peptone, and yeast extract are to be used for our 300 l fermenter the cost of the substrate (based on current local price) is estimated to be US\$ 350 per batch. If cow dung or wheat bran is used the cost of the growth substrate will be less than US\$50 per batch. However, cost effectiveness a certain enzyme does also depend on the level of enzyme production. Therefore, due attention has been given in selecting high yielding strains and in the optimization of the fermentation condition (Gessesse *et al.*, 2011).

Even though some microorganisms found from certain habitats, until now no proteolytic microorganisms with high

potential for dehairing applications of alkaline protease have been isolated in Ethiopia. Therefore, this study focuses on isolation and diversity study of best alkaline protease producers of bacterial isolates for dehairing application in Ethiopian leather industry.

## 2. Materials and methods

Goat skins were purchased from Arba Minch town. Chicken feathers, animal blood and other cheap substrates were supplied from local sources. Agricultural byproducts of carbon and nitrogen sources were found from local agricultural farm. Other materials and reagents were supplied from Arba Minch University research laboratories and also purchased from chemical suppliers in Addis Ababa.

### 2.1 Sample collection and isolation of bacterial strains

Strains of alkaliphilic proteolytic bacteria were isolated from samples collected from different habitats around Arba Minch such as: Chamo Lake, Abaya Lake, fish processing area, Arba Minch town abattoir, Compost site, Arba Minch University abattoir, Arba Minch University garden soil and Arba Minch University effluents. These samples were suspended in water by vigorous vortexing and serial dilutions were made up to  $10^{-6}$  in sterile water. From the appropriate dilution, 0.1 ml was added to petri plate on milk agar at pH 10 and incubated at 40°C for 24h. A clear zone of milk agar hydrolysis around the colonies indicated alkaline protease production by an organism. Isolates with good zone formation were further analyzed. The colonies were picked and purified by streaking on milk agar. The

purified proteolytic isolates were stored and maintained for further study.

## 2.2 Screening of best alkaline protease producers

Many bacterial isolates with special ability of protease enzyme production (proteolytic activity) were screened depending on different growth parameters such active growth on alkaline medium, active growth at high temperature, clear zone formation, etc. They were screened by sub culturing repeatedly on alkaline media at pH 10 and 40°C. These isolates were screened for further enzyme production and biodiversity studies. The screening took place depending on size of clear zone formed.

## 2.3 Biodiversity study of selected isolates

Bacterial isolates with prominent zone of clearance and showing efficient enzyme production were processed for the determination of colony morphology, Gram staining, motility, biochemical tests and identified in accordance with the Bergey's Manual of Determinative Bacteriology.

## 2.4 Protease production media and culture conditions

The culture was grown in 250 ml of Erlenmeyer flasks containing 100 ml medium consisting of glucose 1.5%, K<sub>2</sub>HPO<sub>4</sub> 0.2%, MgSO<sub>4</sub>.7H<sub>2</sub>O 0.5%, CaCl<sub>2</sub> 0.1%, peptone 0.75% for 48 h on an orbital shaker at 150 rpm at 40°C inoculated with a loop full of 24 h old colony from milk agar plate. The pH of the medium was adjusted to 10 and the supernatant was collected after

centrifugation at 5000 rpm for 20 min as the crude enzyme source.

## 2.5 Testing dehairing activity of an enzyme

Dehairing activity was performed by some modification of the method described by Pravin *et al.* (2014). Fresh goat skin was cut into 5cm<sup>2</sup> pieces and it was washed gently with tap water and rinsed with distilled water to remove chemicals from the skin, which may hinder enzyme activity during dehairing activity. Then it was incubated with 10 ml of crude enzyme for 6 h at 40°C. Goat skin treated with only buffer was taken as a control. The skin pieces were virtually analyzed for dehairing activity. The quality of dehaired goat skin was checked with high quality indicators such as clear hair pore, clear grain structure and no collagen damage.

## 2.6 Further screening of isolates for alkaline protease production

### 2.6.1 Screening depending on dehairing potential of isolates

The screening of an isolates was depending on their ability in dehairing of hide or skin. Different characteristics of an organism were considered during the screening. The morphology and anatomy of dehaired skin was studied by looking up the physical structure of the skin with naked eye and also observing under microscope. The completely dehaired skins with white to grey spots, clear hair pore and clear grain structure were selected and the isolates were further analyzed.

## 2.6.2 Screening of keratinase production by plate assay

The isolates were screened for keratinase activity. This was by inoculating an organisms according to method described by Raju and Divakar (2013), on the feather powder agar plates containing 0.4% feather powder (washed feathers was dried at 50°C in a forced draught oven (ISO 9001 Glass ware drying cabinet). The dried feathers were ground into fine fractions (<90, 90, 150, 300, 425 and 850µM) with test sieves of appropriate diameters) incubated at 40°C for 48 h. A clear zone formation by an isolate indicated keratinase activity. Keratinase positive isolate was selected for further study.

## 2.7 Further identification of the selected bacterium

Further identification of the selected bacterium was carried out by doing biochemical tests such as starch hydrolysis test, indole production test, citrate utilization test, round spore test and Voges Proskauer test (Prabhavathy *et al.*, 2012).

## 2.8 Enzyme characterization

### 2.8.1 Effect of pH and temperature on alkaline protease activity and stability

The protease activity was evaluated using the standard assay method in the following buffer systems at 0.1 mol l<sup>-1</sup> concentrations in the reaction mixture: sodium phosphate buffer, pH 6 to 7; tris-buffer, pH 8; NaHCO<sub>3</sub>-NaOH buffer, pH 9 to 11; NaHPO<sub>4</sub>- NaOH buffer, pH 12 and KCl-NaOH buffer, pH 13. To check the pH-stability, 1 ml of the enzyme solution was mixed with 2 ml of the buffer solutions (pH7-13) and aliquots of the mixture was taken to measure the

protease activity under standard assay conditions after incubation for 1 h. The effect of temperature on enzyme activity was studied according to method described by Chaudhari *et al.* (2013) by conducting the reactions at various temperatures (30, 40, 50, 60, and 70°C) using the standard assay method. To evaluate heat-stability of the protease, enzyme was denatured at various temperatures ranging from 30 to 70°C for 1 h.

### 2.8.2 Effect of metal ions, NaCl and methanol on enzyme stability and substrate concentration on enzyme property

To evaluate the effect of ions (0.01 mol l<sup>-1</sup>) on enzyme activity, the enzyme sample was pre-incubated with various divalent ions (Ca<sup>2+</sup>, Mg<sup>2+</sup>, Cu<sup>2+</sup>, Fe<sup>2+</sup>, Hg<sup>2+</sup>, and Zn<sup>2+</sup>) at 40°C for 1 h and the residual activity was measured (Vijayaraghavan *et al.*, 2014). The effect of NaCl on enzyme stability was studied by following the method described by Suganthi *et al.* (2014) with some modifications. The crude enzyme was mixed with 0.5 M NaHCO<sub>3</sub>-NaOH buffer pH 10 contained different NaCl concentrations ranging from 0M to 3M with 0.5 unit intervals. The reaction mixture was pre-incubated at room temperature for 1 h. To determine the residual activity, 1ml of the enzyme solution was taken from pre-incubated solutions for standard protease assay.

Effect of methanol on enzyme stability was tested by following the slight modification of the method described by Pravin *et al.* (2014). The concentration range used in this experiment was in the range of 0-20%. Aliquots of the crude enzyme were mixed with methanol in equal proportion and incubated for

about 1 h at 40°C. Then by taking 1ml of pre-incubated enzyme from each concentration, the standard protease assay was carried out at 40°C for 1 h and compared with control considered as 100% activity.

Different concentrations of casein according to Lakshmi *et al.* (2014) (0.5%, 1%, 2%, 3%, 4% and 5%) in Carbonate–Bicarbonate buffer pH 10.0 was used as enzyme substrate with the above mentioned parameters to determine optimum concentration of substrate.

## **2.9 Optimization of culture conditions for alkaline protease production**

### **2.9.1 Effect of cheap substrates on alkaline protease production**

Banana peels, cow dung, chicken feather and wheat bran were used as the substrate for the production of alkaline protease in the production medium (glucose 1.5%, peptone 0.75%,  $\text{KH}_2\text{PO}_4$  0.2%,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.1%,  $\text{CaCl}_2$  0.1%, substrates 2% (w/v). All substrates were sun dried for several days and further dried at 60°C for 1 h. Two g of substrate was taken in separate flasks and autoclaved at 121°C and 15 lb pressure for 20 min. After cooling, the flasks were inoculated with equal quantity of inocula. After 36 h of incubation on an orbital shaker at 150 rpm and 40°C, the enzyme was extracted by centrifuging at 5000 rpm for 20 min and enzyme activity was assayed according to Roja *et al.* (2012). The best substrate which allowed the secretion of higher protease was selected for further process.

### **2.9.2 Effect of different carbon and nitrogen sources on alkaline protease production**

The production medium was separately amended with 2g of glucose, fructose, sucrose, maltose, lactose, mannitol and starch in flasks inoculated with equal quantity of inoculum and incubated for 36 h on an orbital shaker at 150 rpm and 40°C. Nitrogen sources such as ammonium nitrate ( $(\text{NH}_4)_2\text{NO}_3$ ), ammonium carbonate  $(\text{NH}_4)_2\text{CO}_3$ , yeast extract, peptone and urea were amended in the production medium separately and incubated on an orbital shaker at 150 rpm at 40°C for 36h.

Cheap carbon sources such as banana peel, wheat bran, corn cob and sugar cane bagasse were prepared in each flask and inoculated with equal size of inoculum. It was incubated for 36 h on an orbital shaker at 150 rpm at 40°C. Cheap nitrogen sources such as chicken manure, ground nut pod, dried blood and chicken feather were studied by following the same production mentioned above.

### **2.9.3 Effect of pH, temperature, incubation time and inoculum sizes on alkaline protease production**

To observe the effect of initial pH on enzyme production, 7 production medias of different pH (7, 8, 9, 10, 11, 12 and 13) was taken in each flask and inoculated with equal size of inoculums and incubated for 36 h at 40°C.

The culture media with pH10 and inoculated with equal inoculums was incubated to find out the effect of different temperatures (30, 40, 50, 60 and 70°C) on protease production and

incubated for 36 h at these temperatures.

The effect of incubation periods on alkaline protease activity by the test isolate was studied. For protease production, culture media was incubated at different time intervals namely 12, 24, 36, 48, 60, 72, 84 and 96 h.

The effect of different inoculum size on alkaline protease production was done by following method described by Smita *et al.*, 2012. Inoculum concentrations of 0.1ml, 0.5 ml, 1ml, 1.5 ml and 2ml of a 24 h old culture was inoculated into the assay medium for protease production at 40°C, pH 10, 150 rpm for 36 h.

### 2.11 Alkaline protease assay

According to Roja, *et al.* (2012), the enzyme was assayed in the reaction mixture containing 2.0 ml of 0.5% casein solution in 0.1M Carbonate–Bicarbonate buffer pH10 and 1ml enzyme solution in a total volume of 3.0ml. Reaction mixture was incubated for 5min at 40°C. The reaction was terminated by adding 3ml of 10% trichloro-acetic acid. The tubes were incubated for 1h at room temperature. Precipitate was filtered through whatman no.1 filter paper and the filtrate collected. For the color development for the assay of tyrosine in the filtrate, 5ml of 0.4 M Sodium carbonate and 0.5 ml of Folin phenol reagent was added to 1ml of filtrate, vortexed immediately, incubated for 20 min at room temperature and optical density (OD) was taken at 660 nm. Concentration of tyrosine in the filtrate was read from a standard curve for tyrosine already prepared.

One unit enzyme activity was taken as the amount of enzyme producing 1µg of

tyrosine under standard assay conditions and expressed as units per ml enzyme.

### 2.12 Method of data analysis

For statistical analysis of data, a standard deviation for each experimental result was calculated using the Excel Spreadsheets available in the Microsoft Excel. Graphs, tables and photographs were prepared and used to present the data.

## 3. Results

### 3.1 Isolation of bacterial strains

From samples collected from different habitats around Arba Minch University 197 strains of proteolytic bacteria were isolated. These isolates were screened from total strains of bacteria grown on each petri plate depending on proteolytic activities. Pre-screening took place according to clear zone of an organism (table 1).

### 3.2 Screening of best alkaline protease producers

From total 197 proteolytic isolates, 53 bacteria with special ability of protease enzyme production (proteolytic activity) were screened depending on different growth parameters such active growth on alkaline medium, active growth at high temperature, proteolytic activities, etc. They were screened by sub-culturing repeatedly on alkaline media with pH10 and incubated at 40°C. These isolates were screened for further enzyme production and biodiversity studies. The first selection was depending on size of zone formed by an organism. Therefore, in this situation

high clear zone formers were suggested as high alkaline protease producers.

**Table 1:** Morphological characterization of selected isolates

C.No	Morph.	Color	Motility	G.stain	C.Z.Size (mm)	C.No	Morph.	Color	Motility	G.Stain	C.Z.Size* (mm)
S35	Rod	White	Motile	+	35	S20	Rod	White	Motile	+	25
S36	Cocci	White	"	+	35	S34	Rod	White	"	+	25
S3	Cocci	Yellow	"	+	35	S33	Cocci	White	"	+	25
S32	Rod	White	"	+	35	S46	Rod	White	"	+	25
S24	Cocci	White	"	+	35	S39	Cocci	White	"	+	25
S12	Rod	White	"	+	35	S1	Cocci	White	"	+	25
S13	Rod	White	"	+	35	S41	Rod	Yellow	"	+	24
S52	Rod	White	"	+	35	S29	Cocci	Yellow	"	+	24
S30	Cocci	White	"	+	34	S43	Rod	White	"	+	24
S44	Rod	White	"	+	34	S16	Cocci	White	"	+	24
S25	Rod	White	"	+	34	S23	Cocci	White	"	-	24
S38	Rod	White	"	+	34	S7	Cocci	White	"	+	24
S11	Cocci	Yellow	"	+	34	S2	Rod	White	"	+	24
S17	Rod	White	"	+	34	S4	Rod	White	"	+	23
S50	Cocci	Yellow	"	+	33	S8	Rod	White	"	+	23
S10	Cocci	Yellow	"	+	33	S18	Cocci	White	"	+	23
S37	Cocci	White	"	+	25	S47	Cocci	White	"	+	23
S40	Rod	White	"	-	25	S5	Rod	White	"	+	22
S49	Rod	Yellow	"	+	25	S21	Rod	White	"	+	15
S53	Cocci	White	"	+	25	S26	Cocci	White	"	-	15
S42	Cocci	Yellow	"	+	25	S27	Rod	Yellow	"	-	15
S45	Rod	White	"	+	25	S19	Cocci	White	"	+	15
S48	Rod	White	"	+	25	S15	Rod	White	"	+	15
S9	Cocci	White	"	+	25	S14	Rod	Yellow	"	+	15
S28	Rod	White	"	+	25	S6	Rod	White	"	+	15
S31	Rod	White	"	+	25	S22	Cocci	White	"	+	14
S51	Cocci	Yellow	"	+	25						

\*C.Z - Clear Zone

### 3.3 Biodiversity study of selected isolates

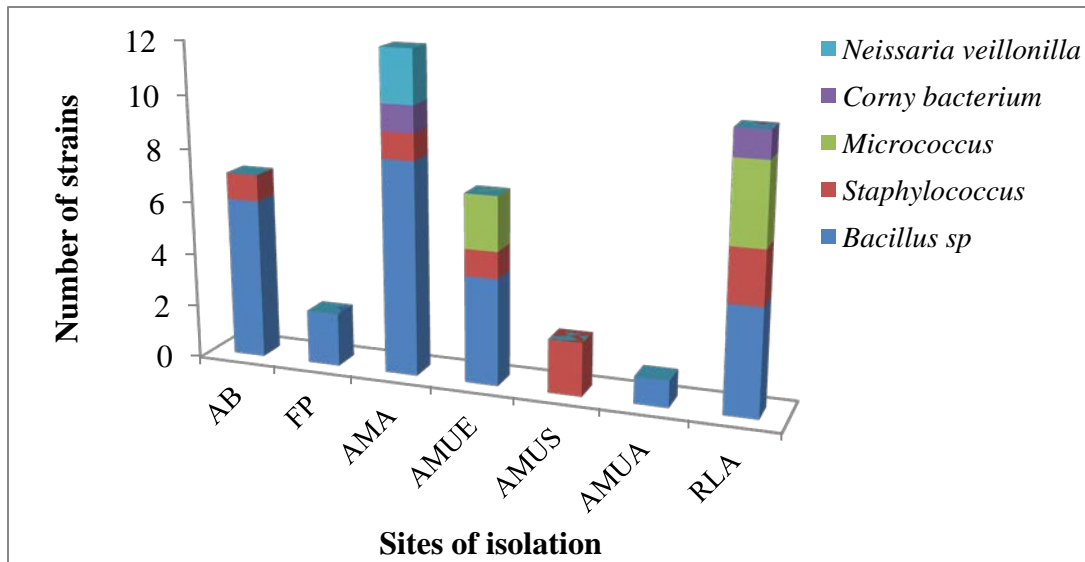
Bacterial isolates with prominent zone of clearance and showing efficient enzyme production were processed for the determination of colony morphology, colony color, Gram staining, endospore staining, motility, biochemical tests and identified in accordance with the Bergey's Manual of Determinative Bacteriology. The morphologies of these 53 isolates were observed as rod and cocci and colony colors white and yellow. Gram reaction indicated that 49 bacteria were gram positive and the others 4 were gram negative. From these isolates, 25 were spore formers and the others non formers.



Table 2: Biochemical characterization of selected isolates

S. no	Biochemical tests	Positive	Negative	Total
1	Catalase test	14	10	24
2	Acid fast	-	2	2
3	Citrate utilization	-	1	1
4	Voges Proskauer	-	1	1
5	Indole production	1	-	1
6	Starch hydrolysis	-	1	1
7	Roundspore test	-	1	1
8	Strict anaerobes	-	25	25

From 41 isolates identified by different biochemical tests, 25 *Bacillus sp.*, 7 *Staphylococcus sp.*, 5 *Micrococcus sp.*, 2 *Corynebacterium sp.* and 2 *Neissaria veillonilla* were found from samples collected from different habitats.



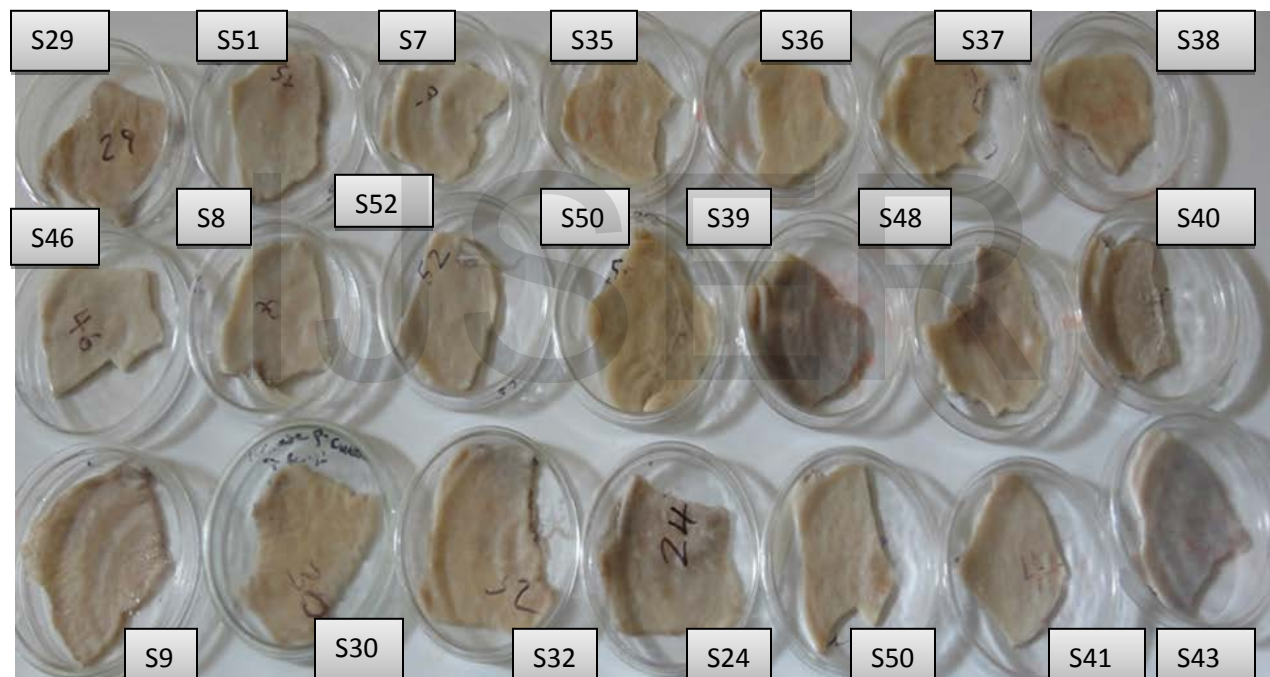
\*AB = Abaya, FP = Fish processing, AMA = Arba Minch Abattoir, AMUE = Arba Minch University Effluent, AMUS = Arba Minch University Soil, AMUA = Arba Minch University Abattoir, RLA = Research Laboratory Air

**Figure 1:** Distribution and taxonomic characteristics of micro-organisms isolated from 7 different habitats around Arba Minch.

### 3.4 Testing dehairing activity of an enzyme

Dehairing of skin tested for each 53 isolates and 51 isolates were observed as good to excellent in dehairing potential. Only 2 isolates were not successful in hair removal from goat skin pieces. More than 56.6% of isolates successfully removed hairs completely (100%) from the skin (figure 2 and 3).

About 37.7% isolates removed hair partially (70-90%) and the skin was not completely dehaired (figure 4). The result of this study did not only focus on complete removal of hair from the skin (dehairing), but also on skin quality improvement and reduction of the duration of incubation time for dehairing. The complete removal of hair from the skin took place within 6 h incubation time.



**Figure 2:** Enzymatically dehaired goat skins by alkaline proteases of 21 different isolates in reaction at 40°C for 6 h with only enzymes.

### 3.5 Further screening of isolates for alkaline protease production

#### 3.5.1 Screening depending on dehairing potential of isolates

The dehaired skin with high quality showed clear hair pore, clear grain structure and no collagen damage. Depending on these parameters 5 isolates with best dehairing characteristics were selected from all 53 bacteria checked for dehairing. These isolates did not only completely dehair hides, but also improved the skin quality. The structure of

dehaired skins observed under microscope and the isolate S35 found without damaged part and showed the highest quality of skin.



**Figure 3:** Completely dehaired goat skins by crude proteases of 5 isolates (S32, S35, S36, S46, and S51) at 40°C for 6 h; (control) skin treated with only buffer of pH 10.

### 3.5.2 Screening of Keratinase production by plate assay

The isolates were screened for Keratinase activity. Depending on this screening technique isolate S35 was selected for further study. This isolate formed large clear zone on feather powder agar plates.

### 3.6 Further identification of the selected bacterium

Further identification of the selected bacterium was carried out by doing biochemical tests such as citrate utilization test, Voges Proskauer test, indole production test, Starch hydrolysis and round spore test (table 2).

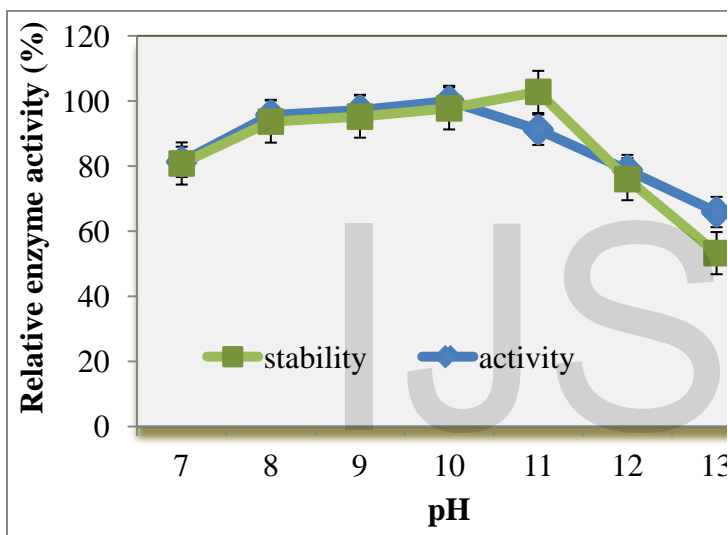
According to Bergey's Manual of Determinative Bacteriology, the isolate was identified as *Bacillus badius*.

### 3.7 Enzyme characterization

#### 3.7.1 Effect of pH on alkaline protease activity and stability

The optimum protease activity (459.24 U/ml) was found at pH 10. The enzyme activity increased from pH 7 to 10 (Figure 11). As indicated on figure 11, the activity of the enzyme was found to be 78.78% at pH 12 and 65.9% at pH 13.

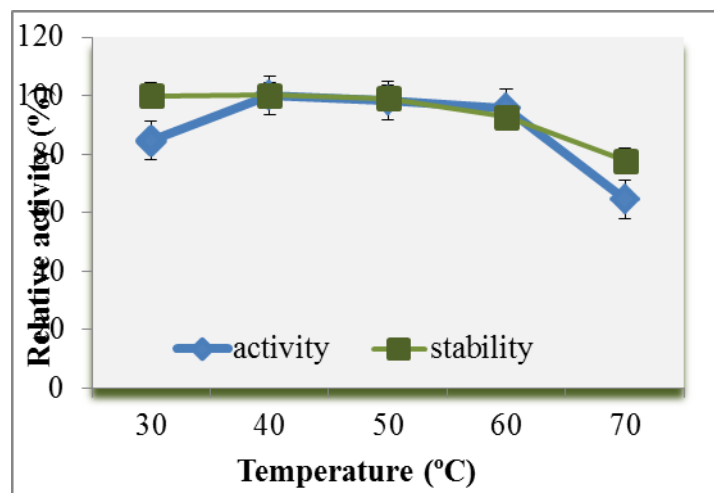
The protease produced by *Bacillus badius* was stable in pH 7- 11 (Figure 11). But the stability decreased above pH 11.



**Figure 4:** Effect of pH on activity and stability of alkaline protease from *Bacillus badius* incubated for 1 h in different buffers.

#### 3.7.2 Effect of temperature on alkaline protease activity and stability

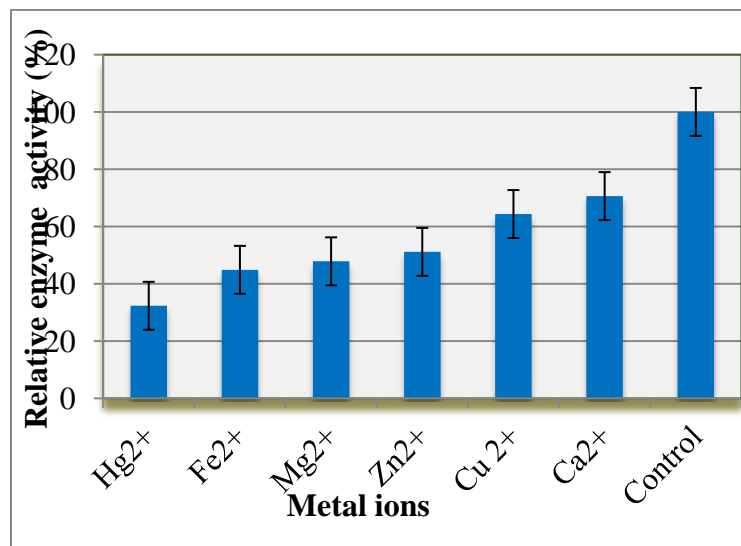
The enzyme produced by *Bacillus badius* was found optimally active at 40°C. The maximum activity of 775.85 U/ml (100%) was found at this temperature after which the activity slightly decreased. The alkaline protease by *Bacillus badius* was stable at moderate temperature range of 30°C to 60°C (Figure 12).



**Figure 5:** Effect of temperature on alkaline protease activity and stability by incubating enzyme at different temperatures for 1h.

#### 3.7.3 Effect of metal ions on enzyme activity

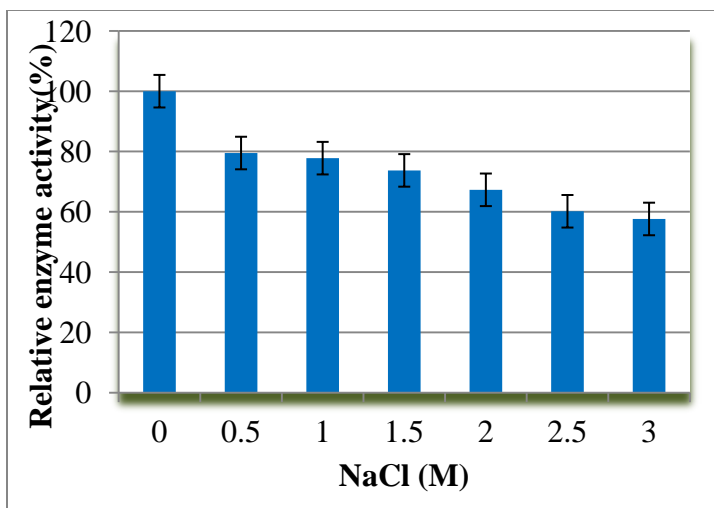
All of the cations tested inhibited the enzyme activity (Figure 13). Ions such as  $Hg^{2+}$ ,  $Fe^{2+}$ ,  $Mg^{2+}$  and  $Zn^{2+}$  strongly inhibited the enzyme activity, and the enzyme activities were 32.34%, 44.88%, 47.85% and 51.15%, respectively.  $Ca^{2+}$  and  $Cu^{2+}$  resulted 70.63%, and 64.36% activities respectively.



**Figure 6:** Effect of different metal ions on alkaline protease activity by incubating an enzyme for 1 h in the solution of metal ions.

### 3.7.4 Effect of NaCl on enzyme stability

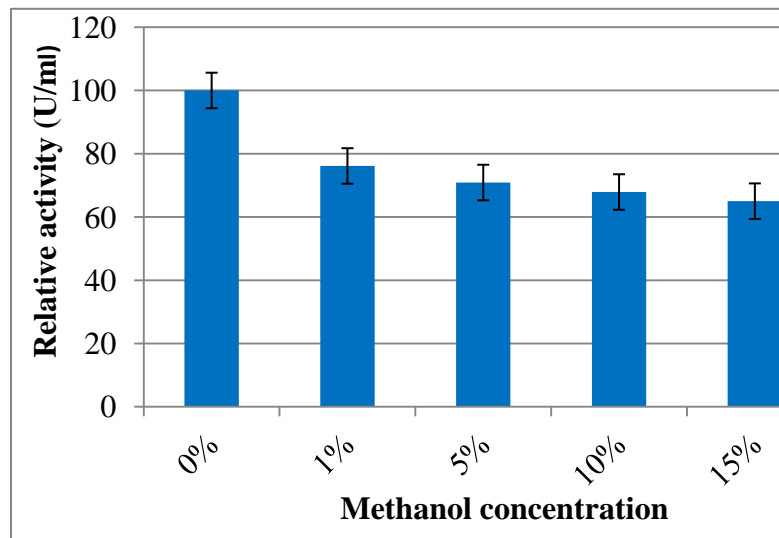
The maximum enzyme activity as compared with others concentrations was recorded at 0.5M NaCl (Figure 14).



**Figure 7:** Effect different concentrations of NaCl on alkaline protease activity by incubating crude enzyme in 0 to 3M NaCl for 1 h.

### 3.7.5 Effect different concentrations of methanol on enzyme stability

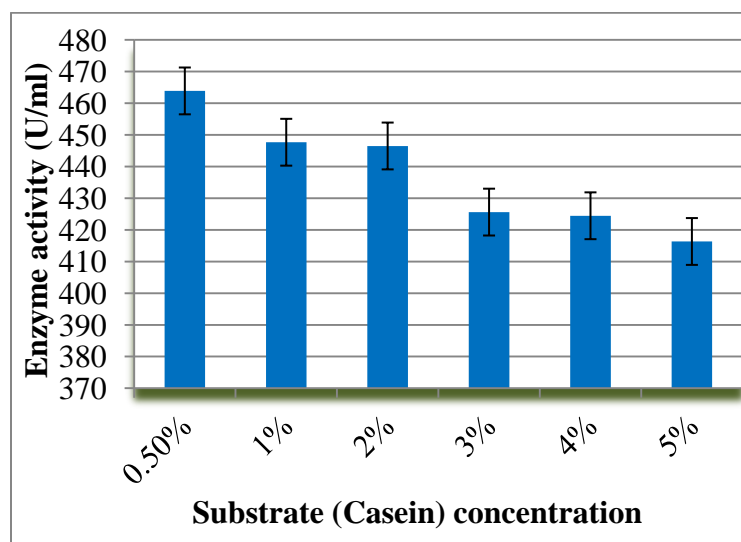
Methanol inhibited the protease activity of *Bacillus badius*. As it can be observed from figure 15, the activity of the enzyme at 1% concentration methanol was 76.13% of the control. The enzyme was more repressed at 20% concentration of methanol (62.25%).



**Figure 8:** Effect of different concentrations of methanol on stability of protease enzyme pre-incubated with methanol for 1 h.

### 3.7.6 Effect of Substrate Concentration on Activity of Protease

The enzyme activity was negatively affected by higher concentrations of casein (figure 16). Maximum activity of protease was found at 0.5% casein concentration (463.88 U/ml) and decreased as concentration increased.

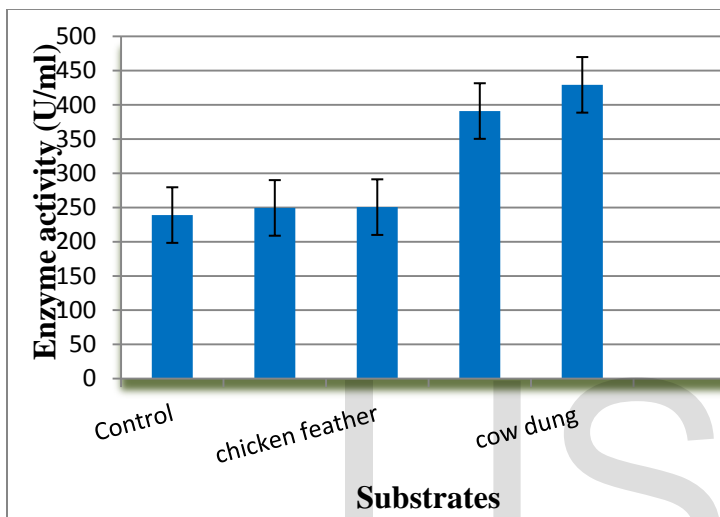


**Figure 9:** Effect of different concentrations of casein on activity of protease produced by *Bacillus badius*.

### 3.8 Optimization of culture conditions for alkaline protease production

#### 3.8.1 Effect of cheap substrates on alkaline protease production

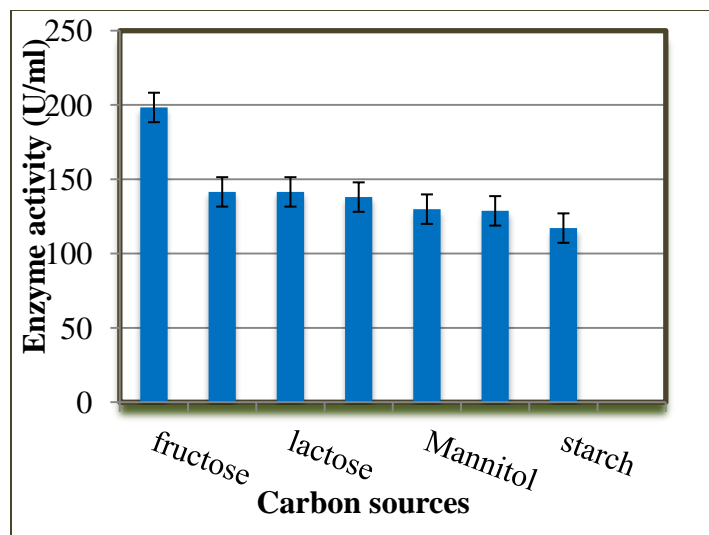
Among cheap substrates evaluated for alkaline protease production by *Bacillus badius*, cow dung showed the highest protease activity (429.09 U/ml) followed by banana peel with enzyme activity of 390.83 U/ml (Figure 17).



**Figure 10:** Effect of cheap substrates on alkaline protease production by inoculating the isolate in production media containing different substrates and incubating at 40°C, pH 10 and 150 rpm for 36 h. Control was production medium with no added substrate.

#### 3.8.2 Effect of different carbon sources on alkaline protease production

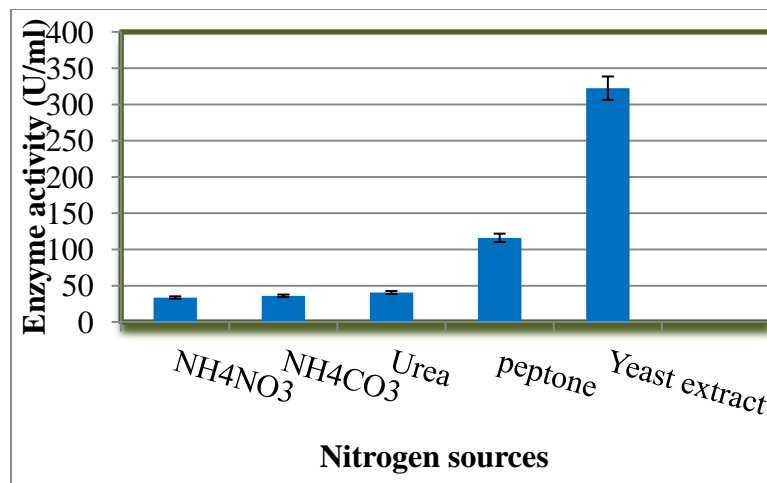
Among different carbon sources evaluated for alkaline protease production by *Bacillus badius*, fructose indicated the highest protease activity of 198.31 U/ml (Figure 18). Glucose and lactose showed equal enzyme activity of 141.48 U/ml while sucrose, mannitol, maltose and starch resulted in 138 U/ml, 129.89 U/ml, 128.73 U/ml and 117.13 U/ml activities respectively.



**Figure 11:** Effect of different carbon sources on alkaline protease production by *Bacillus badius* by incubating the inoculated media at 40°C and 150 rpm for 36 h.

#### 3.8.3 Effect of different nitrogen sources on alkaline protease production

Among different nitrogen sources amended for protease production, yeast extract indicated the maximum protease activity of 322.40 U/ml. Peptone indicated protease activity of 115.97 U/ml (Figure 19). But urea,  $(\text{NH}_4)_2\text{CO}_3$  and  $(\text{NH}_4)_2\text{NO}_3$  showed almost similar lower protease activities of 40.59 U/ml, 35.95 U/ml and 33.63 U/g respectively.

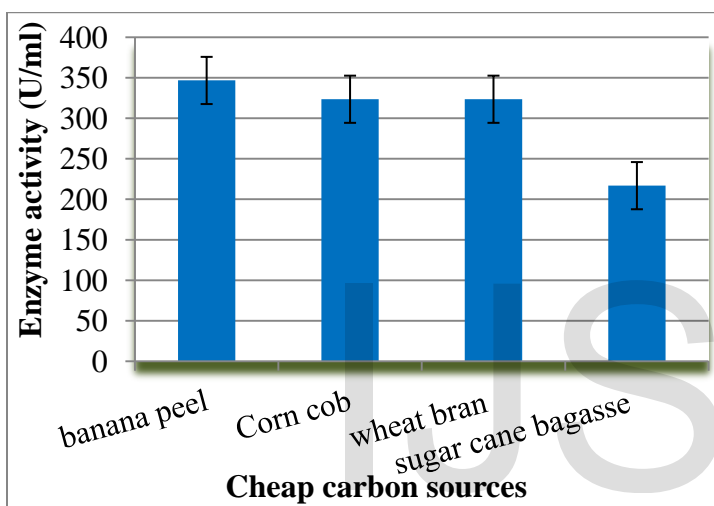


**Figure 12:** Effect of different nitrogen sources on alkaline protease production by *Bacillus*

*badius* by incubating the inoculated media at 40°C and 150 rpm for 36 h.

### 3.8.4 Effect of cheap carbon sources on protease production

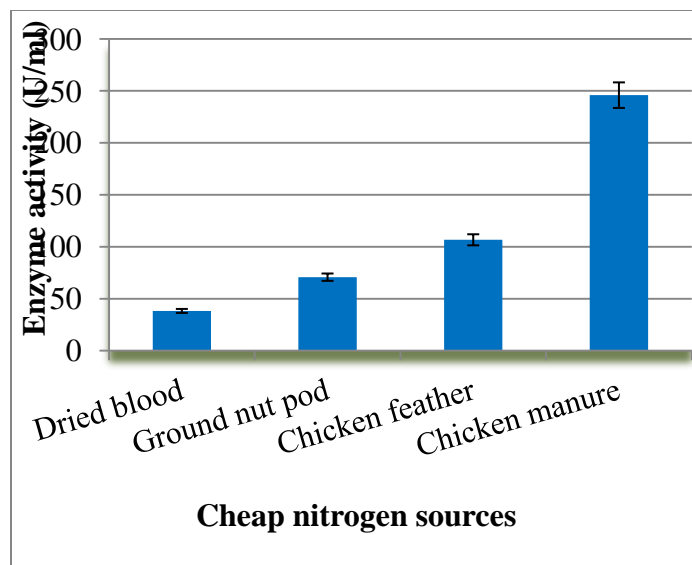
Among cheap carbon sources evaluated for protease production, banana peel showed the highest protease activity of 346.76 U/ml followed by corn cob and wheat bran with equal enzyme activity of 323.56 U/ml (Figure 20). Also sugar cane bagasse indicated 216.87 U/ml activity of alkaline protease by *Bacillus badius*.



**Figure 13:** Effect of cheap carbon sources on alkaline protease production by *Bacillus badius* by incubating the inoculated media at 40°C and 150 rpm for 36 h 150 rpm.

### 3.8.5 Effect of cheap nitrogen sources on protease production

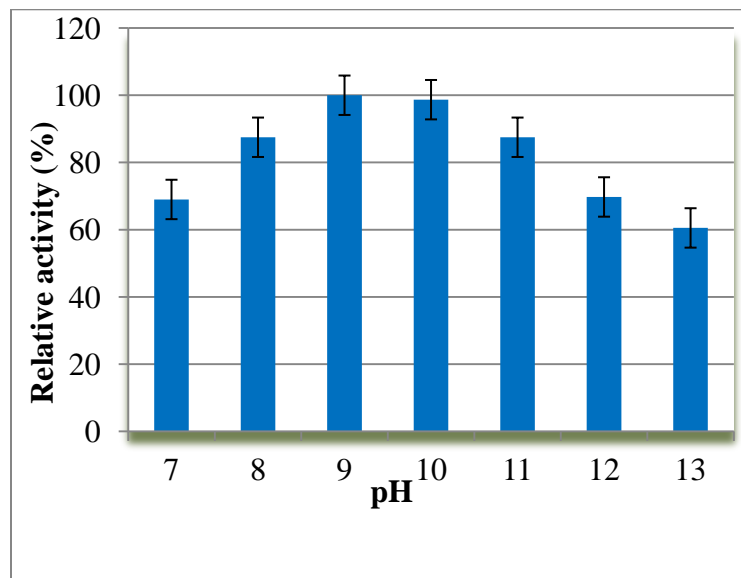
Among cheap nitrogen sources evaluated for production alkaline protease by *Bacillus badius*, chicken manure showed the maximum protease activity of 245.86 U/ml (Figure 21). Chicken feather, ground nut pod and dried blood indicated enzyme activities of 106.69 U/ml, 70.74 U/ml and 38.27 U/ml respectively.



**Figure 14:** Effect different cheap nitrogen sources on alkaline protease production by *Bacillus badius* by incubating the inoculated media at 40°C and 150 rpm for 36 h.

### 3.8.6 Effect of pH on alkaline protease production

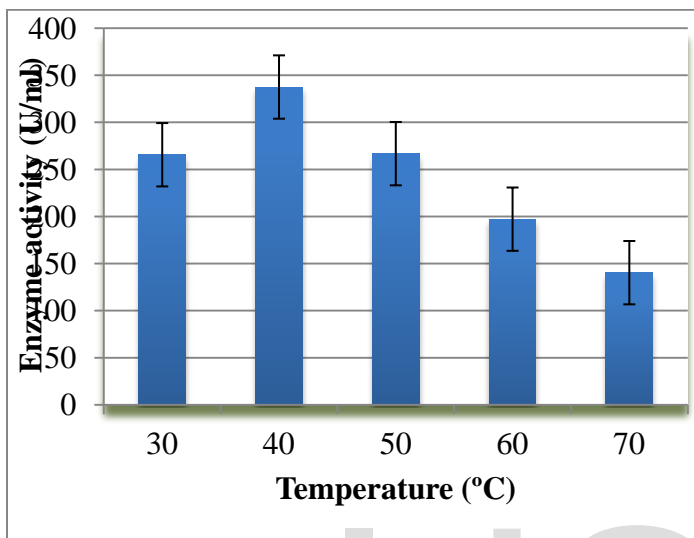
The highest alkaline protease production by *Bacillus badius* was found at pH 9-10 (Figure 22). At pH 7 and 13 the relative activities of the enzyme were 69 and 60.53% respectively.



**Figure 15:** Effect of pH on alkaline protease production by *Bacillus badius* by incubating inoculated media with different pH at 40°C and 150 rpm for 36 h.

### 3.8.7 Effect of temperature on alkaline protease production

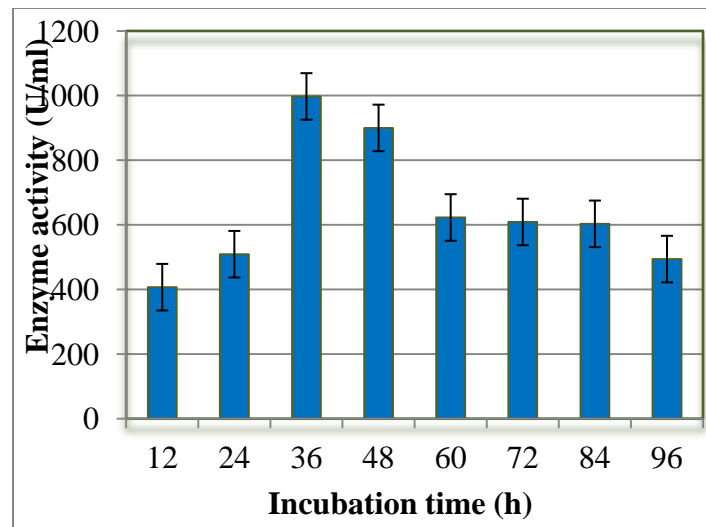
The maximum alkaline protease production by *Bacillus badius* was observed at 40°C (Figure 23). The enzyme production declined after 40°C.



**Figure 16:** Effect of temperature on alkaline protease production by *Bacillus badius* incubating inoculated media at different temperatures and 150 rpm for 36 h.

### 3.8.8 Effect of incubation time on alkaline protease production

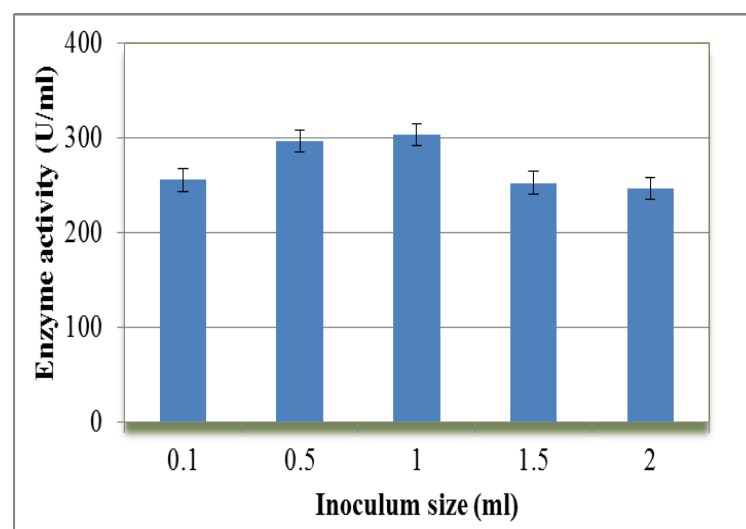
The maximum enzyme activity of 997.36 U/ml was found at 36 h incubation by *Bacillus badius* (Figure 24). After this optimum production time, the enzyme activity was slowly decreased up to 48 h after which it sharply decreased.



**Figure 17:** Effect of different incubation times on alkaline protease production by *Bacillus badius* at 40°C and 150 rpm.

### 3.8.9 Effect of inoculum size on alkaline protease production

Results from the present study showed that optimum inoculum size of *Bacillus badius* for protease production was 1ml from 24 h old culture broth. The enzyme activity at this inoculum size was 303.85 U/ml. As indicated on figure 25, the activities at 0.1 and 2 ml inoculum sizes were 256.29 U/ml and 247.02 U/ml respectively.



**Figure 18:** Effect of inoculum size on production of alkaline protease by *Bacillus badius*



*badius* inoculated with different sizes at 40°C and 150 rpm for 36 h.

#### 4. Discussion

A total of 197 proteolytic bacteria were isolated from different habitats around Arba Minch such as Chamo Lake, Abaya Lake, fish processing area, Arba Minch town abattoir, Arba Minch University abattoir, Arba Minch University garden soil and Arba Minch University effluents. Depending on clear zone formation 53 bacteria were then screened as potential alkaline protease producers. Clear zones were formed because of the hydrolysis of casein by protease produced from the isolates. In fact these bacteria are known for their abilities to secrete large amounts of alkaline proteases having significant proteolytic activity and stability at considerably high pH and temperatures.

As shown on figure 1, 29.3% of these isolates were found from Arba Minch town abattoir, 17% from Lake Abaya and 17% from Arba Minch University effluents. This indicates that these three habitats are rich in proteolytic bacteria for alkaline protease production. It might be due to the fact that, these three sites have more alkaline condition and proteolytic in nature that suits for distribution of proteolytic bacteria in the area. About 36.7% were isolated from Arba Minch University garden soil, Arba Minch University abattoir, fish processing area and also from air around research laboratory. Fish processing area was rich in most proteolytic organisms. This is because the area has much protein residues which were accumulated from fish byproducts. The strain with highest proteolytic activity (selected for this study) was found from the area.

Dehairing test done on goat skin indicated that about 96.23% of tested isolates were with potential ability of dehairing skin and only 3.77% were not at all. This dehairing test is in addition for completely removal of hairs from the skin, it also focused on improvement of the quality of the skin dehaired and the duration of

incubation time. In this case, the successfully dehaired skins were soaked in the enzyme for 6 h. So that, the time required for unhairing in the present study was the minimum. This is because when it is compared with previous works, the incubation period is the shortest. For example, Pravin *et al.*, 2014 reported complete dehairing of goat skin after 8 h incubation time by *Bacillus licheniformis* U1. Prabhavathy *et al.*, 2013 showed complete dehairing of cow skin after 24 h incubation time by isolate *Bacillus subtilis*. Nadeem *et al.*, 2009 reported complete dehaired goat skin after 12 h incubation with alkaline protease from *B. licheniformis* UV-9.

Depending on their dehairing potential, 5 different bacteria with optimum proteolytic activities were screened for further enzyme production and characterization. The morphology and anatomy of dehaired skins were studied by observing the physical structure of the skin with naked eye and under microscope during screening. This was because some strains of bacteria have negative effect on quality of dehaired skin and in this study these were checked by observing the physical structure of the skin to identify the damaged collagen. These isolates were identified as 3 *Bacillus sp.*, 1 *Micrococcus sp.* and 1 *Staphylococcus sp.* When bacterial isolates were identified which yielded prominent zones of clearance on the skim milk agar medium, it was found that about 80% were represented by the genus *Bacillus* (Nadeem *et al.*, 2009).

These 5 selected isolates were further screened based on plate assay techniques to evaluate keratinase activities of organisms. Based on this technique, isolate S35 was with highest clear zone formation on feather agar medium and selected as best producer of alkaline protease (Figure 6).

The selected S35 isolate was characterized morphologically and biochemically and identified as *Bacillus badius* (table 1 & 2). The strain was isolated from fish processing area which is rich in protein sources. Due to the fact

that, this isolate was found with highest proteolytic activity and stability at considerably high pH and temperatures.

The optimum protease activity of 459.24 U/ml was determined at pH 10 (100%). This was suggested that it was an alkaline protease and potential candidate for industrial application. The result was similar with that of Gururaj *et al.*, 2012. He reported the highest protease activity of 184.8 U/ml at pH 10. Generally, commercial proteases from microorganisms have maximum activity in the alkaline pH range of 8-12 (Gupta *et al.*, 2002). Optimum pH of 10 for alkaline proteases from various *Bacillus* species has been reported by some workers (Adinarayana *et al.*, 2003; Gupta *et al.*, 2005). PH stability of alkaline protease produced by *Bacillus badius* was evaluated and the result indicated that the enzyme was stable under various pH ranges 7 to 11 (figure 11). The activity of the protease was 78.78% at pH 12 and 65.9% at pH 13. It shows that as an enzyme has more potential for application in industries at harsh conditions. Similar result was recorded by Smita *et al.*, 2012 from study done on alkaline protease of *Serratia liquefaciens*.

As it indicated on figure 12, the optimum activity of alkaline protease was found at 40°C. At 50°C the enzyme activity was 98.5% and at 60°C it was 95.66%. Also at 70°C the activity of the enzyme was 64.4%. This indicates that the enzyme was thermostable because it has good activity even at higher temperature. Ravindran *et al.*, 2011 and Ahmad, 2011 reported similar results from study done on alkaline protease produced by *Bacillus cereus* and *Streptomyces aurantiogriseus* EGS-5 respectively.

The enzyme was thermally stable through different temperatures from 30°C to 60°C (residual activity 100%) and gradually decreasing at temperature higher than 60°C. Also at 70°C the activity of the enzyme was 77.7%. This indicates the thermostability of the selected isolate and the enzyme. The result seems to be very interesting as the broad

optimal temperature range of the isolate is a very suitable characteristic for its industrial acceptability including tanneries and a common feature for getting the bacterial alkaline protease commercialized. The result suggests elevated temperature required for optimum catalytic activity. The result is in agreement with earlier reports (Pravin *et al.*, 2014).

The influence of various divalent ions on protease activity of *Bacillus badius* was investigated and a significant inhibitory effect was observed with all ions ( $Hg^{2+}$ ,  $Fe^{2+}$ ,  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $Cu^{2+}$  and  $Zn^{2+}$ ) ( figure 13). This demonstrated that the alkaline protease produced by *Bacillus badius* is metal independent and does not require any metal ion for its activity. Likewise, Najafi *et al.*, 2005 and Vijayaraghavan *et al.*, 2014 reported a metal-independent protease from *Pseudomonas aeruginosa* PD100 and *Pseudomonas putida* strain AT respectively.

The effect of NaCl on enzyme stability was evaluated and the maximum enzyme activity as compared with others concentrations was recorded at 0.5M NaCl. The residual activity of the enzyme was 79.49% at this concentration. As shown on Figure 14, the enzyme showed good activity (57.62%) even after incubation for 1 h at 3M NaCl. This indicates that NaCl has a minimum effect on the activity of alkaline protease produced by *Bacillus badius* in concentration less than 3M. Similar result was reported by Pravin *et al.*, 2014 from study done on thermostable and solvent tolerant serine protease from hot spring isolated thermophilic *Bacillus licheniformis* U1.

The effect of different concentrations of methanol on protease activity was tested and the result indicates that methanol inhibits the alkaline protease produced by *Bacillus badius*. Specifically, the activity of the enzyme was more repressed at 20% methanol concentration. Pravin *et al.*, 2014 reported similar result from study done on thermostable and solvent tolerant serine protease from hot spring isolated thermophilic *Bacillus*

*licheniformis* U1. The level of stability towards solvents is the unique properties of enzymes. Biocatalysis in organic media offers several advantages including the higher solubility of hydrophobic substrate enabling their active reactions, reduced microbial contamination and reusability leads to the development of novel application prospects (Gupta and Khare, 2006).

The effect of substrates on the activity of alkaline protease was tested and the result indicates that the activity of the enzyme was negatively affected at higher concentration of substrate. As it can be observed from figure 16, the activity of the enzyme was 416.33 U/ml at 5% concentration of casein. Likewise, Lakshmi *et al.*, 2014 reported similar result from alkaline protease produced by *Halo alkaliphilic Bacillus sp.*

From cheap substrates amended for alkaline protease production by *Bacillus badius*, cow dung showed the highest enzyme activity of 429.09 U/ml. Cow dung was able to provide all the necessary nutrients for the growth of the bacterium and for the synthesis of the enzyme. Therefore, the result indicated that cow dung is the best substrate for alkaline protease production by *Bacillus badius*. Similar result was reported by Vijayaraghavan *et al.*, 2014 from the investigation on alkaline protease produced by *Pseudomonas putida* Strain AT. Likewise, Vijayaraghavan *et al.*, 2013 reported the same result from the study done on alkaline protease by *Bacillus cereus* strain AT.

Among various carbon sources evaluated fructose indicated as the best carbon source for alkaline protease production by *Bacillus badius*. Similar result was reported by Kumar *et al.*, 2012 from study done on protease produced by *Bacillus subtilis*. Likewise, Joshi *et al.*, 2007 reported comparable result from alkaline protease produced by *Bacillus cereus* MTCC 6840.

Among the various nitrogen sources tested, yeast extract exhibited the maximum production of protease (322.40 U/ml) (Figure

19). Likewise, Nayera *et al.*, 2014 reported similar result from alkaline protease produced by *Streptomyces ambofaciens* in free and immobilized form. Also the same result was reported by Raj *et al.*, 2012 from protease produced by *Pseudomonas aeruginosa*. Vijayaraghavan *et al.*, 2014 reported similar result from alkaline protease produced by *Pseudomonas putida* strain AT. Nadeem, 2009 reported the same result from alkaline protease produced by *Bacillus licheniformis* N-2. Among various complex nitrogen sources, yeast extract and casamino acid have been reported as suitable sources for alkaline protease production (Rahman *et al.*, 2005; Prakasham *et al.*, 2006).

Ammonium nitrate ((NH<sub>4</sub>)<sub>2</sub>NO<sub>3</sub>), ammonium carbonate ((NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub>) and urea repressed the growth and yield of alkaline protease by *Bacillus badius*. The repression of growth and protease biosynthesis might be attributed to the fast release of ammonia from these inorganic nitrogen sources. Rahman *et al.* (2003) reported comparable results for thermostable alkaline protease production by *B. stearothermophilus* F1 in the presence of organic and inorganic nitrogen sources. Many other researchers have also reported that organic nitrogen sources are better for enzyme production than inorganic ones (Shikha *et al.*, 2007; Ravishankar *et al.*, 2012 and Vijayaraghavan *et al.*, 2014).

Among different cheap carbon sources evaluated for protease production banana peel showed the highest protease activity of 346.76 U/ml (Figure 20). It indicated that banana peel is the best source of carbon for alkaline protease production by *Bacillus badius*. Among different cheap nitrogen sources evaluated for alkaline protease production, chicken manure showed the maximum protease activity of 245.86 U/ml by *Bacillus badius* (Figure 21). The study result indicates that chicken manure is the best nitrogen source for alkaline protease production by *Bacillus badius*. This result is new and not reported by other researchers.

Among various pH evaluated for alkaline protease production by the isolate, the highest enzyme activity was found at pH 9 (100%) followed by pH 10 (98.68%) (Figure 22). At higher pH, the metabolic action of the bacterium could have been suppressed, thus decreasing the enzyme production. Similar trends have been observed in protease production by *Bacillus sp.* (Prakasham *et al.*, 2006; Okafor and Anosike, 2012). Likewise, Vijayaraghavan *et al.*, 2013 reported the comparable result from the study done on alkaline protease production by *Bacillus cereus strain AT*.

The optimum alkaline protease production was found at 40°C (100%). At 50°C the activity was 79%. At 60 and 70°C the protease retained 58.4% and 41.58% activities respectively (figure 23). This is because the metabolic reaction and biosynthesis of enzyme highly takes place at temperature 40°C and slightly decreased after that. Similar result was reported by Josephine *et al.*, 2012 from a study done on alkaline protease production by *Bacillus SNR01*. This indicates that the result of the present study is similar with previous work by other researchers. The growth and enzyme activity of microorganisms is greatly influenced by different incubation temperatures. Temperature significantly regulates the synthesis and secretion of bacterial extracellular proteinase by changing the physical properties of the cell membrane (Balaji *et al.*, 2012).

The effect of incubation periods on the for alkaline protease production by the test isolate was studied and maximum enzyme activity of 997.36 U/ml found at 36 h incubation time (Figure 24). This indicates that *Bacillus badius* is fast grower and produce the highest protease enzyme during last log phase at 36 h incubation period. The enzyme activity was 494.04 U/ml at 96 h. The growth of the organism is essential for the production of enzyme. Most extra cellular enzymes are produced during log phase of the organisms. Maximum enzyme activity of 410 U/ml at 36 h incubation period was reported by Verma and

Baiswar, 2013 from study done on thermo alkaline protease producing *Bacillus cereus* isolated from tannery effluent. Many workers have reported a broad incubation period ranging from 36 to 96 h for the maximum yield of protease enzyme by *Bacillus* strains (Shafee *et al.*, 2005; Genekal and Tari, 2006; Jaswal and Kocher, 2007; El-Enshasy, *et al.*, 2008; Khosravi-Darani *et al.*, 2008; Raj *et al.*, 2012 and Vijayaraghavan *et al.*, 2014).

Results from the present study showed that optimum inoculum size of *Bacillus badius* for protease production was 1 ml from 24 h old culture broth. The less protease production in small inoculum sizes of 0.1ml and 0.5ml may be due to insufficient number of bacteria, which would have led to reduced amount of secreted protease and the decrease even though luxurious growth was observed in higher inoculum size of 2.0ml, may have resulted due to reduced dissolved oxygen and increased competition towards nutrients. Similar result was reported by Sai Smita *et al.*, 2012 from investigation on quantification and optimization of bacterial isolates for production of alkaline protease.

## 5. Conclusion

About 197 proteolytic bacteria were isolated from different habitats around Arba Minch. From these isolates 53 with enough proteolytic properties were screened for alkaline protease production. Among these one isolate was selected by its enzyme titer, keratinase production and quality of skin after dehairing. The bacterium was identified as *Bacillus badius*. It grew well at pH 10, 40°C for 36 h. The protease produced was maximally active at pH 10 and 40°C. The optimum enzyme production conditions were pH 9-10, 40°C, 1% inoculum and 36 h incubation. The optimum activity was expressed at pH 10, 40°C and 0.5% casein as substrate. It was stable in pH 7-11 and 30-60°C after pre-incubation for one h in respective buffers and temperatures. All metal ions tested ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Hg}^{2+}$  and

$\text{Cu}^{2+}$ ) inhibited the enzyme at 0.01M and 1 h pre-incubation. Methanol also inhibited the enzyme. However, NaCl had minimal effect on the enzyme stability. Among carbon and nitrogen sources tested for the enzyme production fructose and yeast extract resulted in maximum yield. Cow dung, banana peel and chicken manure were identified as the best cheap substrates for potential feasible production of the enzyme. Alkaline protease produced by *Bacillus badius* in the present study has high potential in skin dehairing with improved skin quality. Therefore, its feasible production from cheap substrates and industrial application for dehairing will have implications on environment protection, product quality and shortened processing time.

### Acknowledgements

The author wish to thank Dr. Fantahun Woldesenbet for his contribution assistance to the article. Also the author would like to say thank Dr. Adey Desta for her supportive by giving certain required chemicals during the work. This work was supported in part by a grant from Arba Minch University research directorate.

### References

1. Adinarayana, K., Ellaiah, P. and Prasad, D. (2003). Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. *AAPS Pharm. Sci. Tech.* **4**: 440-448.

2. Ahmad, J. and Ansari, T.A. (2013). Alkaline protease production using proteinaceous tannery solid waste. *J. Pet. Environ. Biotechnol.* **4**: 1-4.
3. Balaji, N., Rajasekaran, K.M., Kanipandian, N., Vignesh, V. and Thirumurugan, R. (2012). Isolation and screening of proteolytic bacteria from fresh water fish *Cyprinus carpio*. *Int. Multidiscipl. Res. J.* **2**: 56-59.
4. Barredo, J.L. (2005). *Microbial enzyme and biotransformation*. Published by Humana press Inc. 999 Riverview Drive, New Jersey 07512 USA. 151-179.
5. Bayouth, A., Gharsallah, N., Chamkha, M., Dhouib, A., Ammar, S. and Nasri, M. (2000). Purification and characterization of an alkaline protease from *Pseudomonas aeruginosa* MN1. *J. Industrial Microbiol. Biotechnol.* **24**: 291-295.
6. Bergey, D.H. and Holt, J.G. (2000). *Bergey's manual of determinative bacteriology*, 9th edn., Philadelphia: Lippincott Williams and Wilkins. PP: 1-8.
7. Bhattacharya, S., Bhardwaj, S., Das, A. and Anand, S. (2011). Utilization of sugarcane bagasse for solid- state fermentation and characterization of  $\alpha$ -amylase from *Aspergillus flavus* isolated from Muthupettai Mangrove, Tamil Nadu, India. *Aust. J. Basic Appl. Sci.* **5**: 1012-1022.
8. Chaudhari, S.G., Chaudhari, S.D., Khobragade, R.M. and Deshmukh, A.M. (2013). Studies on production of alkaline proteases from *Bacillus species* and its application in detergent industry. *DAV Int. J. Sci.* **2**: 44-49.
9. El-Enshasy, H., Abuoul-Enein, A., Helmy, S., and El-Azaly, Y. (2008). Optimization of the industrial production of alkaline protease by *Bacillus lechineforomis* in different production scale. *Aust. J. Basic Appl. Sci.* **2**: 583-593.
10. Gessesse, A., Hatti-kaul, R., Berhanu, A. Gashe and Mattiasson, B. (2003). Novel Alkaline protease from alkaliphilic bacteria grown on chicken feather. *Enz. Microb. Technol.* **32**: 519-527.
11. Gupta, A. and Khare, S.K. (2006). A protease stable in organic solvents from solvent tolerant strain of *Pseudomonas aeruginosa*. *Bio resource. Technol.* **97**:1788-1793.
12. Gupta, R. and Ramnani, P. (2006). Microbial keratinase and their prospective applications: an overview. *Appl. Microbiol. Biotechnol.* **70**: 21–33.
13. Gupta, R., Beg, Q.K. and Lorenz, P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl. Microbiol. Biotechnol.* **59**: 15-32.
14. Gururaj, T., Basavaraj, U. and Praveenkumar, N. (2012). Isolation of Proteolytic Bacteria and Characterization of their Proteolytic Activity. *Int. J. Adv. Eng., Sci. and Technol.* **2**: 185-192.
15. Jaswal, R.K. and Kocher, G.S. (2007). Partial characterization of a crude alkaline protease from *Bacillus cirulans* and its detergent compatability. *Inter. J. Microbiol.* **4**: 1-9.
16. Josephine, F.S., Ramya, V.S., Devi, N., Ganapa, S.B., Siddalingeshwara, K.G., Venugopal, N. and Vishwanatha, T. (2012). Isolation, production and characterization of protease from *Bacillus* sp. isolated from soil sample. *J. Microbiol. Biotechnol. Res.* **2**: 163-168.
17. Joshi, Kumar, S. and Sharma, V. (2007). Production of moderately halotolerant, SDS stable alkaline protease from *bacillus cereus* mtcc 6840 isolated from Lake Nainital, Uttaranchal state, India. *Brazilian J. Microbiol.* **38**: 773-779.

18. Kumar, Rameetha, R., Lincy, L., Priyadarashini, Sandhiyachittiybabu and P.T. Kalaiichelvan. (2012). Destaining and dehairing capability of partially purified *Bacillus subtilis* protease from optimized fermentation medium. *Asian J. Exp. Biol. Sci.* **3**: 613-620.
19. Lakshmi, P.V. Ratna sri, K. Ambika Devi and K.P.J. Hemalatha. (2014). Screening, optimization of production and partial characterization of alkaline protease from *haloalkaliphilic bacillus sp.* *Int. J. Res. Eng. and Technol.* **3**: 435-443.
20. Nadeem, M., Qazi, J.I. and Baig, S. (2009). Effect of aeration and agitation rates on alkaline protease production by *Bacillus licheniformis* UV-9 mutant. *Turk J. Biochem.* **34**: 89-96.
21. Najafi, M.F., Deobagkar, D. and Deobagkar, D. (2005). Potential application of protease isolated from *Pseudomonas aeruginosa* PD100. *Electronic. J. Biotechnol.* **8**: 197-203.
22. Okafor, U.O.G. and Anosike, E.E.M. (2012). Screening and optimal protease production by *Bacillus sp.* Sw-2 using low cost substrate medium. *Res. J. Microbiol.* **7**: 327–336.
23. Pandeeti, E.V, Pitchika, G.K, Jotshi J, Nilegaonkar, S.S. and Kanekar, P.P. (2011). Enzymatic depilation of animal hide: identification of elastase (LasB) from *Pseudomonas aeruginosa* MCM B-327 as a depilating protease. **6**: 1-8.
24. Pillai, P. and Archana, G. (2012). A novel process for biodegradation and effective utilization of chrome shavings, a solid waste generated in tanneries, using chromium resistant *Bacillus subtilis* P13. *Process Biochem.* **47**: 2116–2122.
25. Prabhavathy, M. Rajasekara Pandian and B. Senthilkumar. (2012). Optimization and production of extracellular alkaline protease by solid state fermentation using *Bacillus subtilis*. *J. Acad. Indus. Res.* **1**: 427-430.
26. Prabhavathy, M. Rajasekara Pandian and B. Senthilkumar. (2013). Identification of industrially important alkaline protease producing *Bacillus subtilis* by 16s rRNA sequence analysis and its applications. *Int. J. Res. Pharm and Biomed. Sci.* **4**: 332-338.
27. Prakasham, R.S., Rao, C.S. and Sarma, P.N. (2006). Green gram husk - An inexpensive substrate for alkaline protease production by *Bacillus sp.* in solid-state fermentation, *Bio resource Technol.* **97**: 1449-1454.
28. Pravin, B. Sunil, G. Anjana and S. Bhatt. (2014). isolation, characterization and investing the industrial applications of thermostable and solvent tolerant serine protease from hot spring isolated thermophilic *Bacillus licheniformis* U1. *Int. J. Appl. Sci. Biotechnol.* **2**:75-82.
29. Rahman, R.N.Z.R.A., Basri, M. and Salleh, A.B. (2003). Thermostable alkaline protease from *Bacillus stearothermophilus* F1; nutritional factors affecting protease production. *Ann. Microbiol.* **53**: 199-210.
30. Rahman, R.N.Z.A., Geok, L.P., Basri, M. and Salleh, A.B. (2005). Physical factors affecting the production of solvent-tolerant protease by *Pseudomonas aeruginosa* strain K. *Bioresour. Technol.* **96**: 429-436.
31. Raj, N. Khes, N. Pujari, S. Bhattacharya, A. Das and S.S. Rajan. (2012). Enhancement of protease production by *Pseudomonas aeruginosa* isolated from

- dairy effluent sludge and determination of its fibrinolytic potential. *Asian Pacific J. Trop. Biomedicin.* **5**: 1845-1851.
32. Raju and Divakar, G. (2013). Screening and Isolation of Keratinase Producing Bacteria from Poultry Waste. *Int. J. Pharm. Res. and Appl. Sci.* **2**: 70-74.
  33. Ravishankar, Kumar, P.S, Jacob, B., Saravanan, K., Kumar, M.A. and Jacob, A. (2012). Optimal conditions for production of extracellular alkaline protease from a newly isolated *Bacillus subtilis* strain AKRS3. *Res. Biotechnol.* **3**: 45-54.
  34. Roja, R., Lalitha, K., Hanuma, S. and Siva, P. (2012). Isolation and screening of alkaline protease producing bacteria and induction of overproducing *Bacillus licheniformis* mutants through UV irradiation. *ISSN.* **1**: 2250-3013.
  35. Smita, S.P. Ray and S. Mohapatra. (2012). Quantification and optimization of bacterial isolates for production of alkaline protease. *Asian J. Exp. Biol. Sci.* **3**: 180 – 186.
  36. Shafee, N., Aris, S.N., Rahman, R.N.Z.R.A. and Salleh, A.B. (2005). Optimization of environmental and nutritional conditions for the production of alkaline protease by newly isolated bacterium *Bacillus cereus* strain 146. *J. Appl. Sci. Res.* **1**: 1-8.
  37. Shikha, Sharan, A. and Darmwal, N.S. (2007). Improved production of alkaline protease from a mutant of alkalophilic *Bacillus pantotheneticus* using molasses as a substrate. *Bio resource Technol.* **98**: 881-85.
  38. Suganthi, A. Mageswari, S. Karthikeyan, M. Anbalagan, A. Sivakumar and K.M. Gothandam. (2013). Screening and optimization of protease production from a halotolerant *Bacillus licheniformis* isolated from saltern sediments. *J. Genetic Eng. and Biotechnol.* **11**:47-52
  39. Tari, C., Genckal, H. and Tokatli, F. (2006). Optimization of growth medium using a statistical approach for the production of alkaline protease from a newly isolated *Bacillus sp.* L21. *Process Biochem.* **41**: 659-665.
  40. Verma, A., Pal, H.S., Singh, R. and Agarwal, S. (2011). Potential of alkaline protease isolated from *Thermo actinomyces* sp. RM4 as an alternative to conventional chemicals in leather industry dehairing process. *Int. J. Agric. Environ. Biotechnol.* **2**: 173-178.
  41. Vijayaraghavan, S. Lazarus and Vincent. (2013). De-hairing protease production by an isolated *Bacillus cereus* strain AT under solid-state fermentation using cow dung: Biosynthesis and properties. *Saudi J. Biol. Sci.* **1**: 1-8
  42. Vijayaraghavan, S. Saranya, and S.G.P. Vincent, (2014). Cow Dung Substrate for the Potential Production of Alkaline Proteases by *Pseudomonas putida* Strain AT in Solid-State Fermentation. *Chinese J. Biol.* **7**: 2-