Studies On Animal Skin Dehairing By Alkaline Protease Produced From Bacteria, Isolated From Soil

Temam Abrar Hamza

Abstract: Microbes are living organisms that are too small to be seen by the unaided eye, however, they carry out extremely useful processes that cannot be achieved by other physical and chemical means. Alkaline proteases from those microbes are one of the most important hydrolytic group of enzymes that find varied application in various industrial sectors. The aim of this study was focused on isolation of alkaline protease producing bacteria from the study area which have potential application in dehairing of animal skin. Accordingly, 38 alkaliphilic proteolytic bacteria were isolated from the study area (Arba Minch University, Abay campus). Five isolates were screened which had relatively higher clear zone and further examined for dehairing efficiency of their crude enzymes. Hair removal test was undertaken by incubating skin with 15ml of respective crude enzyme at 40°C. Regarding this, one potential isolate was selected and designated as Bacillus sp.AMUa38. Maximum cell growth were observed from this strain at pH 8, 1% salt (NaCl) concentration and 2.5% of inoculum size. Lactose and peptone are potential carbon and nitrogen source for optimum growth of AMUa38. The optimum activity was reached at pH 9 and 50^{0}C. The enzyme was stable in the pH range of 7 to 10. The crude enzyme from Bacillus sp. AMUa38 removed hair from goat skin after 6hrs at pH 9 and 40^{0}C. These properties suggest that protease from Bacillus sp. AMUa38 could find potential application in leather processing industries which have an economic implication.

Keywords: Alkaline, Bacillus sp. AMUa38, Dehairing, Protease, Skin

1. Introduction

Microorganisms (or microbes) are living organisms that are too small to be seen by the unaided eye which include bacteria fungi, protozoa, microalgae, viruses. Microbes are ubiquitous and live in familiar setting such as Soil, water, food, and animal intestines. They are an essential component They are an essential component of the biodiversity and they have a profound impact in every facet of human life **[1]**.The beneficial microbes are, fascinating, versatile and capable of growing on a wide range of substrates and carry out extremely useful processes that can't be achieved by other physical and chemical means. Moreover, microorganisms are able to degrade many substances to a remarkable spectrum of products that support the growth of living organisms and fulfill numerous human requirements **[2], [3]**. Nowadays, microbial enzymes are highly efficient and environmental-friendly protein catalysts. Alkaline proteases are one of the most important groups of enzymes, used in various industrial products/processes as food, silver recovery, pharmaceutical and detergent industries, as well as in the preparation of leather, textile and wool, among others (Saha et al., 2011;Younes et al., 2011) **[4], [5]**.. Another area of promising application in medical usage and management of industrial and household waste. Microbial Proteases are the most important group of industrial enzymes which account about 60% of total enzymes in the market **[6], [7], [8].** Proteases are useful in dehairing of animal skin for the purpose of leather manufacture. Since, the beginning of human civilization the conventional method of dehairing involves the use of lime and sodium sulphide as the lack of technology.

- *Temam AH: Department of Biotechnology, College of Natural Sciences, Arba Minch University: Email:-temam2abrar2@gmail.com, Arba Minch, Ethiopia*

But currently it is possible to replace chemical dehairing with enzyme based dehairing processes using proteases. This avoiding the use of lime and sulphide are being developed because of their environmental benefits. Thus, enzyme based dehairing processes using proteases help to reduce or even avoid those chemical and offer enormous environmental benefits **[6], [9].** In addition, to improve the quality of leather produced **[10], [11].** The only alternative to manage and control the pollution caused by leather processing is substitution of the used chemicals with enzymes especially proteases. Degradation of unwanted protein by simple eco-friendly and inexpensive method is one of the central requirments in several industries especially leather industries **[6], [9]**. Proteases active in the pH range of 8-12 and stable at alkaline pH are known as potential candidates for dehairing of hides **[8], [12].** Ethiopia is one of the top ten countries in the world that are endowed with abundant livestock resource. In line of this, leather processing has been core of Ethiopia's economy since many centuries. Many of leather processing industries which is found in different region of Ethiopia uses those harsh chemicals to remove hair from animal skin. However, Ethiopia is the cenrer and origin of biodiversity. Despite the broad microbial diversity of Ethiopia, alkaline protease producing bacteria have not yet been explored. Therefore a research project has been initiated to investigate alkaline protease from bacteria isolated from indigenous habitat and examine their potential to be used as dehairing of animal skin. So, investigations on such microorganisms could provide baseline information which help to shift Ethiopian leather industry from chemical to biological dehairing technology.

2. Materials and Methods

2.1. Sample Collection

Soil samples were collected from Arba Minch University, Abaya campus. Each sample was kept in clean sterile sample bottles sealed and transferred to the laboratory and stored at 7^{0}C.

2.2. Isolation of Alkalophilic Proteolytic Bacteria

The soil and wastewater samples were suspended in water by vigorous vortexing and serial dilutions were made and appropriate dilution were added to petri plate on skim milk agar plate. Alkaliphilic proteolytic bacteria were screened. A clear zone formation around the colonies due to skim milk hydrolysis indicated alkaline protease production by the microbes **[13]**.

2.3. Screening and characterization of potential isolate

The bacterial strains were screened for the quality of enzyme produced under submerged condition. The supernatant from each isolate was used as crude enzyme for observing individual dehairing performance. Single isolate which have high potential on removal of hair from the skin was selected for further study. Finally various morphological and biochemical studies were undertaken according to Bergey's manual of determinative bacteriology for identification of the selected potential isolate.

2.4. Optimization of culture condition

Various parameters influencing culture growth: pH (7-11), inoculum size (1-10%), salt concentration (0-20%), various carbon sources (5.0% w/v) and nitrogen sources (0.5% (w/v) were optimized.

2.5. Enzyme Activity

Protease production media containing (g/l): Glucose 10, Peptone 7.5, $MgSO_47H_2O$ 5, K_2HPO_4 2, CaCl 1 **[14]** was prepared for production. The bacterial isolate was inoculated with 1% of inoculum into production medium followed by incubation at 30°C for 48 hours. Culture filtrates were separated by centrifugation at 5000 rpm for 15 min and the supernatants were used as crude enzyme source for quantitative enzyme assay. Protease activity in the culture supernatant was determined using casein as a substrate with some modification of the method described by Gaur et al. **[15]**.

2.6. Enzyme Unit (U)

The enzyme activity was expressed in units (U) and calculated by using tyrosine standard calibration curve. One unit of enzyme was defined as the amount of enzyme that releases 1 µmol of tyrosine per ml of crude extract per minute under standard conditions at 40°C. The tyrosine standard curve was used to generate the enzyme unit determination formula in U/ml.

2.7. Skin dehairing test with crude enzyme

Dehairing activity was performed by some modification of the method described by Pravin et al. **[16]**. Fresh goat skin was cut into 5x5cm 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 15.0 ml of crude enzyme for 6-10 hrs at 40°C. Goat skin treated with only buffer was taken as a control. The skin pieces were virtually analyzed for dehairing activity.

2.8. Data analysis

Average values of duplicate experiments were taken. Microsoft office Excel worksheet 2010 was used for data analysis and presentation.

3. Result

3.1. Isolation of alkalophilic proteolytic bacteria

Fourity two (42) protease positive alkalophilic isolates were obtained. Hence the strains were identified as a protease producer. Out of 42 protease positive alkalophilic bacteria, five potential isolate with relatively higher clear zone was selected for further investigation.

3.2 Screening of best alkaline protease producer for dehairing purpose

The five isolates were compared for hair removal efficiency with respect to time and isolate AMUa38 was selected for further study. The results are summarized in Table 1.

Table 1: *Enzymatic dehairing of fresh goat skins by alkaline protease of selected isolates at 40°C for 6 h.*

Bacterial Isolates	Colony color	Morphology and Gram Reaction	Strict Anaerobe	Probable Identity	Hair removal efficiency	Remark
AMUa8	White	Gram positive rod	Negative	Bacillus Sp	*	
AMUa12	White	Gram positive rod	Negative	Bacillus Sp	++	
AMUa13	White	Gram positive rod	Negative	Bacillus Sp	++	
AMUa25	White	Gram positive rod	Negative	Bacillus Sp	*	
AMUa38	White	Gram positive rod	Negative	Bacillus Sp	++++	**Selected**

(Key:--Not dehairing;++-Partial dehairing;++++ - Complete deharing within 6hrs at 40°C)*

3.3. Morphological and biochemical characteristics of isolate AMUa38

Morphological and biochemical characteristics of isolate AMUa38 Results of morphological and biochemical tests of the selected isolate (AMUa38) are; the isolate was characterized as Gram positive and catalase and motility positive. The colonies were characterized as opaque, irregular and spready configuration and irregular margin. Motile, long irregular, elliptical endospore and rod shaped cells were observed under light microscope with 1000X magnification. The strain was able to hydrolyse casein and indole positive. The result of nitrate reduction, citrate utilization and MR-VP test were been negative. Based on this the isolate was designated as Bacillus sp. AMUa38.

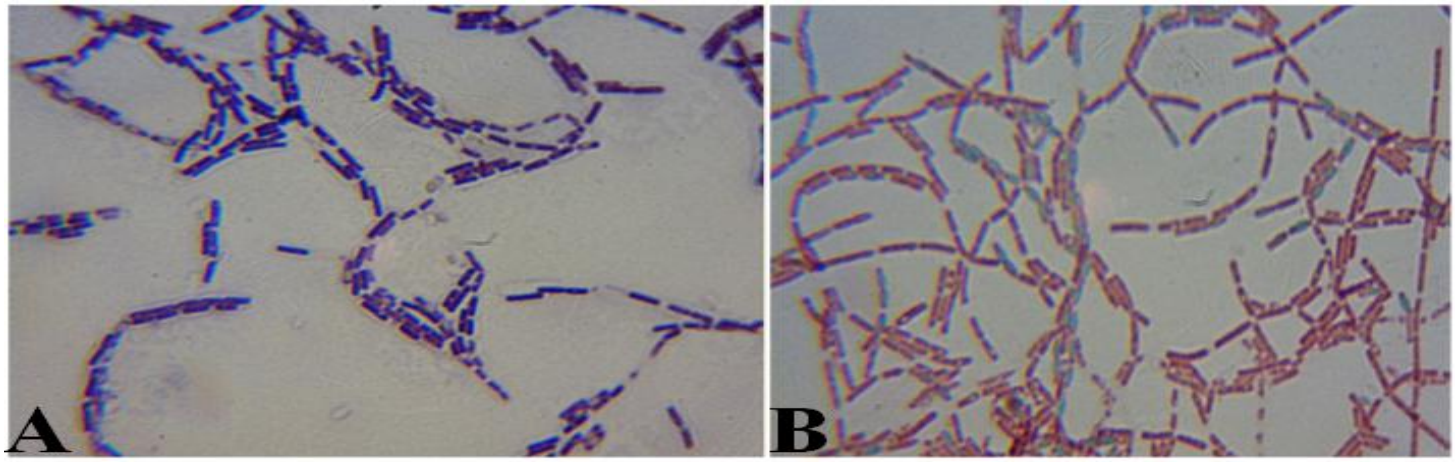

Figure 1: *Gram reaction result (+ve) (a) and Endospore of Bacillus sp. AMUa38 (b) at 40^{0}C and 24 h on milk agar.*

Table 2: *Results of Culture optimization of AMUa38 isolate*

S.no	pH		Salt(NaCl)		inoculums size		Carbon source		Nitrogen source	
	at pH	aOD	Conc.(%)	aOD	Size (%)	aOD	Type	aOD	Type	aOD
1	7	0.413	1	1.25	1	1.029	Glucose	0.88	Peptone	1.034
2	8	0.868	2.5	0.992	2.5	1.085	Mannito	0.668	Yeast extract	1.013
3	9	0.554	5	0.795	5	0.867	Starch	0.761	Urea	0.885
4	10	0.449	7.5	0.357	7.5	0.78	Maltose	0.913	NH4CO3	0.637
5	11	0.22	10	0.155	10	0.313	Lactose	1.023	NH4NO3	0.881
6							Sucrose	0.834		

(N.B: a represent average value of the duplex musearment)

Table 2 describe about culture optimization results of AMUa38 isolate. accordingly, it shows maximum cell growth at pH 8, 1% salt (NaCl) concentration and 2.5% of inoculum size. Lactose and peptone are potential carbon and nitrogen source for optimum growth of AMUa38.

3.4 Dehairing activity of crude enzyme from AMUa38

Alkaline protease from Bacillus sp.AMUa38 was took 6hrs at 40°C for complete removal of hair from goat skin (fig 2). Beside complete removal of hair from the skin (dehairing), also quality skin were produced after treatment with crude enzyme.

3.3. Crude Enzyme Characterization

3.3.1. Effect of pH on enzyme activity and stability

The enzyme was active in the range of pH 7-11 as shown in fig 3. The optimum activity pH was 9.0. The enzyme showed 89% and 82.1% of its maximum activity at pH 8.0 and 10.0 respectively.

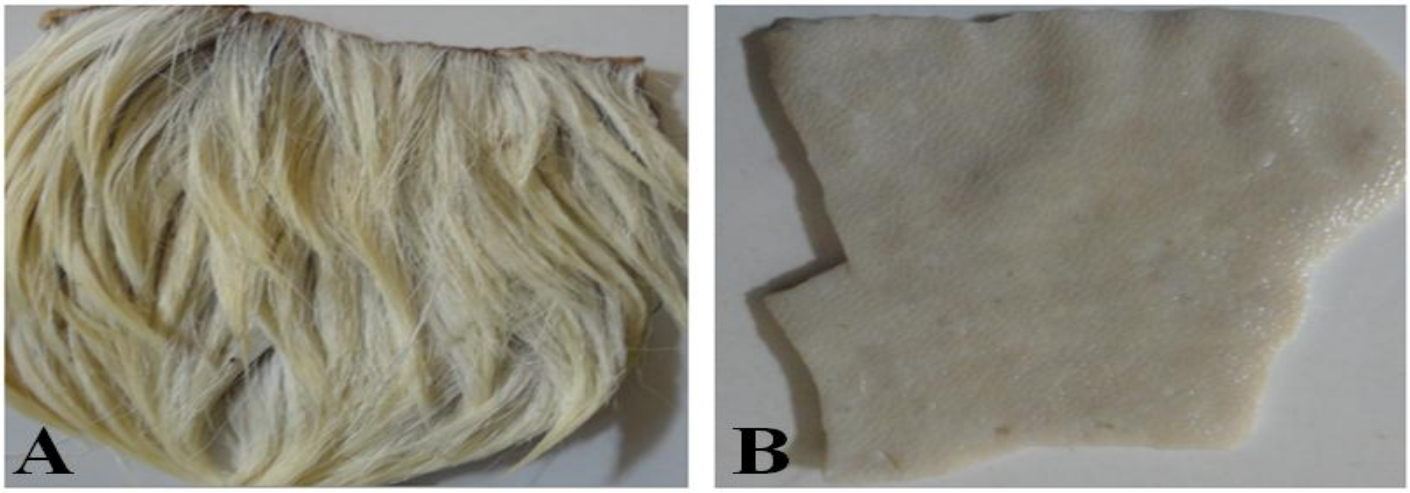

Figure 2*: Hair removal efficiency of enzyme produced by Bacillus sp. AMUa38 fron fresh goat skin at pH 9, 40°C for 6 hrs (A) Control (Skin treated with 15ml buffer with 0% of enzyme); (B) Enzymatically dehaired skin (with 15ml of crude enzyme).*

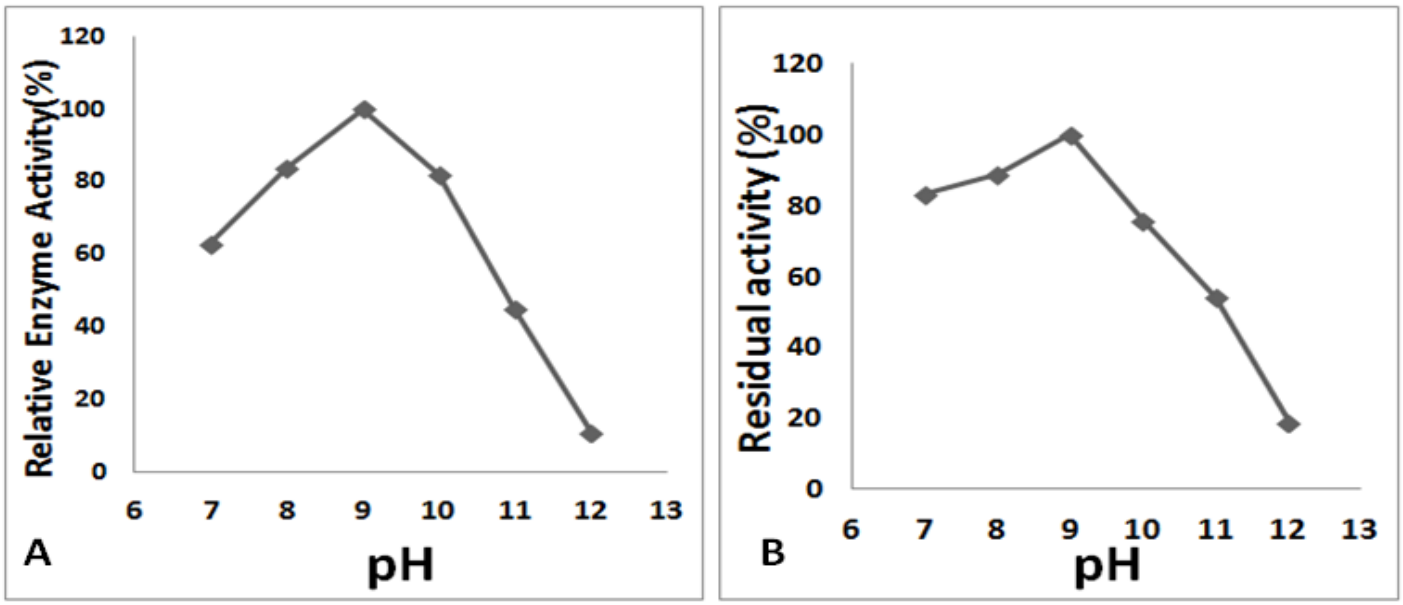

Figure 3: *Effect of pH on enzyme(A) activity (at 40 °C for 30 min incubation) and (B) stability (after 60 min pre-incubation in different pH buffers at 30°C) then assay was performed at 40 °C and pH 9. Activity of the enzyme pre-incubated at pH 9 and assayed at same pH was considered as 100%.*

The enzyme was stable in a broad pH range, maintaining over 83 and 76% of its maximum activity at pH 7 and 10 respectively (fig 3). However, only 19% of the maximum enzyme activity was retained at pH 12.

3.3.2. Effect of temperature on enzyme activity and stability

The protease produced from Bacillus sp. AMUa38 was found to be active at a range of temperatures, between 30 and 60 °C. Fig 4 shows that maximum enzyme activity was recorded at 50°C and the enzyme retained 16% of its maximum activity at 70°C. Around 80% of its maximum activity was expressed at 40 and 55°C.

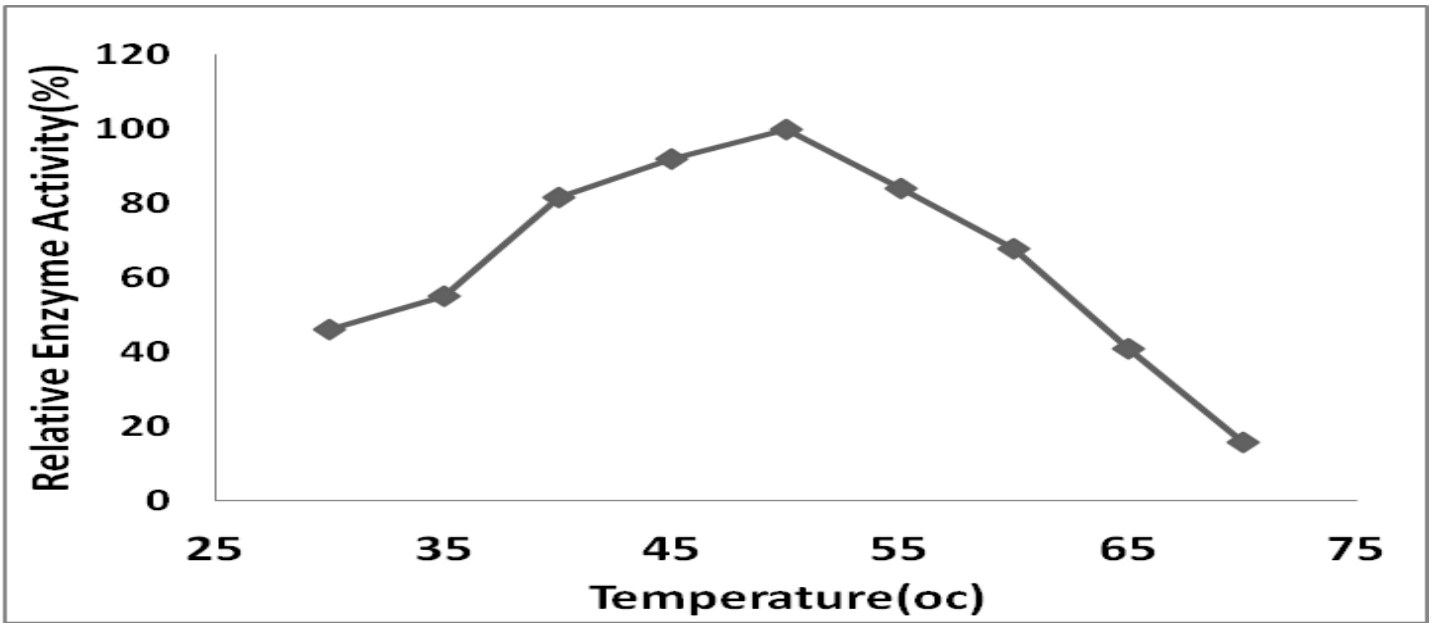

Figure 4: *Effect of temperature on enzyme activity after 30 min incubation at different temperature.*

The enzyme was stable at 45 and 50°C retaining more than 90% and 75% of its maximum activity for 60 min of pre-incubation respectively.

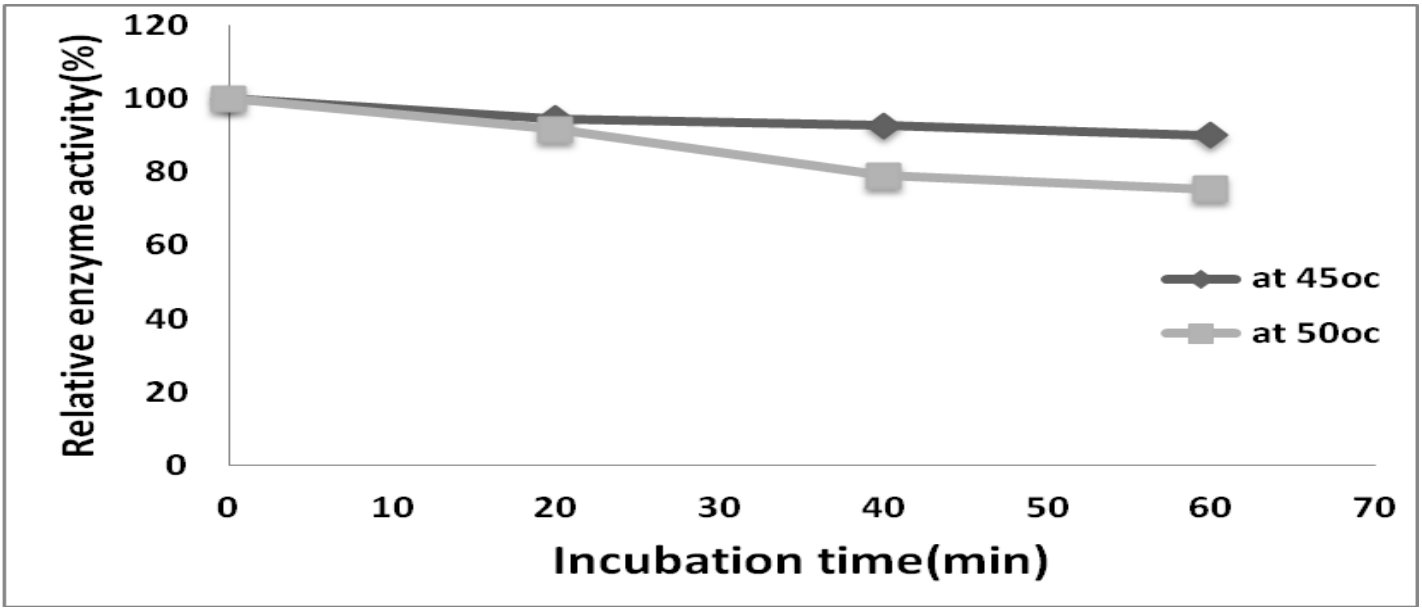

Figure 5: *Effect of temperature on enzyme stability after 60 min incubation at 45 and 50 ^{o}C for 20 min interval regularly.*

4. Discussion

Isolation and identification of bacteria which have vital ability to secret extracellular alkaline protease to be used for removal of hair from animal skin is one of the main concerns of this study. Accordingly, 42 bacterial strains were isolated from study area and single isolate called Bacillus sp.AMUa38 was selected as candidate for alkaline protease production for dehairing purpose . Formation of clear zone around the bacterial colony indicated that protease positive isolates hydrolysed the skim milk present in the media. The use of skim milk agar medium for the isolation of protease producing bacteria has earlier been reported by some workers **[8]** , **[13].** The selected isolate was characterized morphologically and biochemically and identified as Bacillus sp.AMUa38 based on Bergey's Manual of Determinative Bacteriology. Quality of dehaired skin by crude enzymes were studied by different parameters. The morphology and anatomy of dehaired skins were studied by observing the physical structure of the skin with naked eye and under microscope. This was because some strains of bacteria have affect the quality of dehaired skin. The dehaired skin with high quality indicated clear hair pore, clear grain structure and no collagen damage **[9]**. The physiochemical characterization of protease indicates its suitability as a potential application in leather industries. Regarding this, the crude enzyme produced from Bacillus sp. AMUa38 was further characterized to evaluate its potential applications for removal hair from animal skin. It was optimally active between pH 7-10 and stable at alkaline pH. Proteases active in the pH range of 8-12 and stable at alkaline pH are known as potential candidates for dehairing application **[6], [17]**. Similar result was recorded by Sai Smita et al. **[18]** from study done on alkaline protease of Serratia liquefaciens. Another interesting property of the protease produced from Bacillus sp. AMUa38 was its ability to maintain more than 75% of its maximum activity after pre-incubation at its optimum temperature (50°C) for 60 min. The studies on temperature profile of the enzyme showed an optimum temperature range between 40-60°C with maximal activity at 50°C (Fig 4). These findings were comparable to that described by Boominadhan **[13]**, Verma and Baiswar **[11]**, Vijayaraghavan et al. **[19]** and Pravin et al. **[16]**. Maximum activity of protease produced from Bacillus sp. AMUa38 at alkaline pH and temperature of 50°C indicates its high suitability of industrial employment in harsh condition of leather industries. As the method described in this investigation for removal of hair from goat skin undergoes the process without any additive of hazardous chemicals, it would have minimum impact on the environment as compared to conventional chemical methods. With increasing industrial demands for the biocatalysts that can cope with industrial processes at harsh conditions is very crucial. The result seems to be very interesting as the broad optimal temperature and pH range of the alkaline protease produced from Bacillus sp.AMUa38 for leather procesing industries.

5. Conclusions

Alkaline protease produced from Bacillus sp. AMUa38 in the present study has high potential in removal of hair from animal skin with improved product quality. Therefore, alkaline protease produced from Bacillus sp.AMUa38 have potential industrial application for dehairing purpose in leather industries which will also have implications on environmental protection, product quality and shortened processing time.

Acknowledgment

The author wish to thank Ana Sufian for his financial support for publication of this research paper. Also the author would like to thank to Haniza Redwan, Omer Abrar, Nurdin Nassir and Awel Abdella for their supports.

References

[1]. C.N. Aguilar, G. Gutiérrez, P.A. Lilia, R. H.R. Rado, J. L. Martínez, and J. C. Contreras (2008). Perspectives of solid state fermentation for production of food enzymes. American Journal of Biochemistry and Biotechnology, **4**:354-366.

[2]. F. Alemu (2015). Isolation and screening of protease producing bacteria from cheese**.** International Journal of Nutrition and Food Science, **4**:234-239.

[3]. B. Mala, Rao, M. Aparna, Tanksale, S. Mohini, Ghatge, and V. Vasanti (1998). Microbiology and Molecular Biology:Reviews. Microbial and Molecular Biology Reviews, **62**: 597-635.

[4]. M.L. Saha, K.J.M.H. Begum, M.R. Khan, and D.J. Gomes (2011). Bacteria associated with the tannery effluent and their alkaline protease activities. Plant Tissue Culture Biotechnology, **21**:53-61.

[5]. G. Younes, R. Sara, E. Alireza, K. Aboozar, S. Maryam, and T. Najme (2011). Screening and isolation of extracellular protease producing bacteria from the Maharloo Salt Lake. Iranian Journal of Pharmaceutical Sciences, **7**:175-180.

[6]. R. Gupta, Q.K. Beg, and P. Lorenz (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. Applied Microbial Biotechnology, **59**:15-32.

[7]. S. Vadlamani, and S.R. Parcha (2011). Studies on industrially important alkaline protease production from locally isolated superior microbial strain from soil microorganisms. International Journal of Biotechnology Applications, **3**:102-105.

[8]. G. Haile, and A. Gessesse (2012). Properties of alkaline protease C45 produced by alkaliphilic Bacillus sp. isolated from Chitu, Ethiopian Soda Lake. Journal of Biotechnology and Biomatter, **2**:1-4.

[9]. F. Khan (2013). New microbial proteases in leather and detergent industries. Innovative Research Chemistry, **1**:1-6.

[10]. J. Madhavi, J. Srilakshmi, V.M. Raghavendr, and K.R.S.S. Rao (2011). Efficient leather dehairing by bacterial thermostable protease. International Journal of Bio-Science and Bio-Technology, **3**:11-26.

[11]. T. Verma, and V. Baiswar (2013). Isolation and characterization of extracellular thermoalkaline protease producing Bacillus cereus isolated from tannery effluent. The International Journal of Engineering and Science, **2**:23-29.

[12]. R. Rajkumar, R.K. Jayappriyan, and R. Rengasamy (2011). Purification and characterization of a protease produced by Bacillus megaterium RRM2: application in detergent and dehairing industries. Journal of Basic Microbiology**, 51:**614-624.

[13]. U. Boominadhan, R. Rajakumar, P.V.S. Karrpaga, and M.M. Joe (2009). Optimization of protease enzyme production using Bacillus sp. Isolated from different wastes. Botany Research International, **2**:83-87.

[14]. G. Das, and M.P. Prasad (2010). Isolation, purification & mass production of protease enzyme from Bacillus subtilis. International Research Journals of Microbiology, **1**:26-31.

[15]. S. Gaur, S. Agrahari, and N. Wadhwa (2010). Purification of protease from Pseudomonas thermaerum GW1 isolated from poultry waste site. The Open Microbiology Journal, **4**:67-74.

[16]. D. Pravin, B. Sunil, G. Anjana, and S. Bhatt (2014). Isolation, characterization and investigating the industrial applications of thermostable and solvent tolerant serine protease from hot spring isolated thermophililic Bacillus licheniformis U1. International Journal of Applied Science and Biotechnology, **2**:75-82.

[17]. M.B. Rao, A.M. Tanksale, M.S. Ghatge, and V.V. Deshpande (1998). Molecular and biotechnological aspects of microbial proteases. Microbiology and Molecular Biology Research, **62**:597-635.

[18]. P. Sai Smita, Ray and S. Mohapatra (2012). Quantification and optimization of bacterial isolates for production of alkaline protease. Asian Journal of Experimental and Biological Science, **3**: 180-186.

[19]. P. Vijayaraghavan , S. Lazarus, and S.P.V. Gnana (2013). De-hairing protease production by an isolated Bacillus cereus strain AT under solid-state fermentation using cow dung: biosynthesis and properties. Saudi Journal of Biological Sciences, **1**:1-8.