Global Journal of Science Frontier Research: C
Biological Science
Volume 15 Issue 1 Version 1.0 Year 2015
Type : Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals Inc. (USA)
Online ISSN: 2249-4626 & Print ISSN: 0975-5896

Isolation, Identification and Partial Characterization of Protease Producing Bacteria that Exhibiting Remarkable Dehairing Capabilities

By Md. Ekhlas Uddin, Dr. Mustafizur Rahman, Hossain Md. Faruquee, Md. Rezaul Islam Khan, Md. Feroz Mortuza, Mohammad Hafizur Rahman & Pulak Maitra

Islamic University, Bangladesh

Abstract- A novel protease producing bacterium was isolated from the natural source. Gram staining & spore staining showed that the organism is gram positive and forms spore during adverse condition in the growth medium. After various tests it was suggested and the features agreed with the description of Bacillus subtilis in Bergey's Manual of Systematic Bacteriology [20]. It was also identified as B. subtilis with 99.9% identity by API 50 CHB and was also characterized and identified by using a bioinformatics tool PIB (Probabilistic Identification of Bacteria) that suggests the organism was B. subtilis (ID=0.9760).

Keywords: protease, identification, characterization, leather dehairing bacteria, isolation.

GJSFR-C Classification : FOR Code: 279999p

Strictly as per the compliance and regulations of :

Isolation, Identification and Partial Characterization of Protease Producing Bacteria that Exhibiting Remarkable Dehairing Capabilities

Md. Ekhlas Uddin [α], Dr. Mustafizur Rahman [σ], Hossain Md. Faruquee [ρ], Md. Rezaul Islam Khan [ω], Md. Feroz Mortuza[¥], Mohammad Hafizur Rahman[§] & Pulak Maitra [χ]

Abstract- A novel protease producing bacterium was isolated from the natural source. Gram staining & spore staining showed that the organism is gram positive and forms spore during adverse condition in the growth medium. After various tests it was suggested and the features agreed with the description of *Bacillus subtilis* in Bergey's Manual of Systematic Bacteriology [20]. It was also identified as *B. subtilis* with 99.9% identity by API 50 CHB and was also characterized and identified by using a bioinformatics tool PIB (Probabilistic Identification of Bacteria) that suggests the organism was *B. subtilis* (ID=0.9760). In growth curve determination showed that the growth of the organism is increased with the increase of incubation period and the growth reached maximum at around 24 hours of incubation and the protease activity was the maximum of the 26 hours culture. Its optimum pH and temperature were 8.5 and 60°C. The enzyme hydrolyses a number of proteins including azocasein which suggests that it is an extracellular protease. Quantitative estimation has shown that 40mL of culture supernatant could dehair 2×1 cm of leather completely in 9 hours. In future the tanneries will use a combination of chemical and enzymatic processes. A number of companies such as NOVO chemicals started to produce NOVOzymes for tannery industries. The potential for use of microbial enzymes in leather processing lies mainly in areas in which pollution-causing chemicals are being used.

Keywords: protease, identification, characterization, leather dehairing bacteria, isolation.

Author α: Dept. of Biotechnology and Genetic Engineering Islamic University, Kushtia, Bangladesh. e-mail: dipubtge03@gmail.com
Author σ: Professor Dept. of Biochemistry & Molecular Biology Dhaka University, Dhaka. e-mail: m22_rahman@yahoo.com
Author ρ: Assistant Professor Dept. of Biotechnology and Genetic Engineering Islamic University, Kushtia, Bangladesh. e-mail: faruquee@btge.iu.ac.bd
Author ω: Lecturer Dept. of Biochemistry Gano Bishwabidyalaya, Mirzanogor, Savar, Dhaka. e-mail: rezaulbiox@yahoo.com
Author ¥: Senior Scientific Officer Institute of Food and Radiation Biology (Gamma Source Division), Bangladesh Atomic Energy Commission, Ganakbari, Savar, Dhaka. e-mail: firozmortuza@gmail.com
Author §: Dept. of Biochemistry & Molecular Biology Dhaka University, Dhaka. e-mail: hafizbmb51@gmail.com
Author χ: Dept. of Biotechnology and Genetic Engineering Islamic University, Kushtia-7003, Bangladesh. e-mail: pulak.bge22@gmail.com

I. Introduction

Leather industries are one of the most promising fields for export to earn foreign currency in Bangladesh. Most of the tannery industries in Bangladesh use chemicals for Dehairing that led great environmental and health problem. Leather industry has contributes heavily to environmental degradation. The tannery pollutants are causing heavily damage to water resources, agriculture, fisheries and finally to avoid the deleterious effects of chemical agents in tannery industries [15].

Over the last few decades leather industry is based on large scale chemicals treatment which created worldwide environmental hazards. Recently government of People's Republic of Bangladesh has taken initiative to develop the industry from outside the city and modernize it. In the back drop of this scenario enzymes started replacing poisonous chemicals from tannery industries. A number of industries such as NOVO chemicals started producing NOVOzymes for the tannery industries. With the advent of enzymes leather processing in various countries has become environment friendly. Enzymatic dehairing is suggested as an environment friendly alternative to the conventional chemical process [13]. The use of proteolytic enzymes as an alternative to de-hairing skins has been investigated [14].

Cleaner leather processing biotechnology has been used in the tanning industry for several years. Enzymes can be used at all stages in the leather-making industry, with the exception, perhaps, of the actual tanning process. At present, biological methods are being used with relative success in soaking, de-hairing, BATING and, in part, degreasing. Enzymatic dehairing in tanneries has been envisaged as an alternative to sulfides [14]. Proteases could play an important part in biotechnological applications like enzymatic improvement of feather meal and production of amino acids or peptides from high-molecular weight substrates or in the leather industry [3, 12]. These enzymes could be applied for waste water treatment, textile, medicine,

cosmetic leather, feed and poultry processing industry as well as in the leather industry [12].

II. Materials and Methods

a) Isolation of bacteria from soil sample

The soil sample was collected from the poultry wastes in Savar, after serial dilution, culture were given in LB broth media from the sample for 16 h at 37°C. At the next day single colony was found. Among them few colonies were identified on the basis of different colony morphology. Each colony was inoculated into screw capped test tubes containing autoclaved feather with liquid broth media and incubated overnight at 37°C with shaking at 160 rpm. One media was used as negative control.

b) Bacterial identification

Gram's staining; morphological studies, physiological and biochemical characteristics of the isolate were investigated according to Bergey's Manuals [20]. A rapid bacterial identification test kit for Bacillus, API 50 CHB (BioMerieux, France), was used to identify species of bacteria.

c) Different Biochemical & Microbiological tests for the characterization of the organism:

To identify the biochemical properties of the organism different tests were performed. For correct interpretation of the results in every test Escherichia coli was taken as control. Fermentative capabilities of the isolated organisms were tested under anaerobic conditions in Durham tube. The carbohydrate tests that were performed are the Glucose, Lactose, Ribose, Sucrose, Mannitol, Adonitol, Arabinose, Sorbitol, and Maltose.

Others Biochemical tests that were performed are the Hydrogen sulfide test, Motility Test, Indole Production Test, Citrate Utilization Test, Nitrate Reduction Test, Oxidase test (young culture), Catalase Test, Urease test, Indole (SIM) test, Methyl Red (MR) Voges- Proskauer (VP) Test, Starch Hydrolysis Test and Gelatin Liquefaction Test. Some Microbiological tests that were performed are the Gram staining for the Bacteria, Spore staining, colony morphology and growth curve determination.

d) Isolation of Protease Enzyme and Determination of its Proteolytic activity

The microorganism was cultivated in sterile nutrient broth medium. The culture was grown overnight on a rotary shaker at 150 rpm and incubated at 37°C for 15-20 hours. The culture was then centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant was collected and used as crude enzyme sample. Proteolytic activities were assayed by Azocasein test, described by Kreger and Lockwood (1981) was done. Here azocasein is used as a substrate. Optical density was measured at 440 nm

e) Determination of effect of temperature on bacterial growth

The bacterial culture was grown in nutrient broth at various temperatures (25°C, 30°C, 35°C, 40°C, 50°C, 60°C) and was incubated for 48 hours to measure its growth profile.

f) Determination of the Effect of Temperature on bacterial growth and Protease Activity

The bacterial culture was grown in nutrient broth at various temperatures (25°C, 30°C, 35°C, 40°C, 50°C, 60°C) and was incubated for 48 hours to measure its growth profile.

For the determination of the effect of temperature, the culture medium was incubated at temperature ranging from 25-60˚C and the protease activity was determined at 37˚C using the usual methods.

g) Determination of the Effect of pH on Protease Activity

For determining the effect of pH on protease activity different buffer system with different pH were used. Azocasein was dissolved in different buffer solution and the enzyme assay was carried out within pH range (4.0 to 10.5) by azocasein assay method. All of them were used at 0.05M concentration.

Table 1 : Different buffer used and their p^H ranges

Buffer	P^H range
Acetate buffer	4.0-5.6
Sodium phosphate buffer	5.6-8.0
Tris HCl buffer	7.5-8.9
Glycine-NaOH buffer	8.6-10.5

h) Determination of the Effect of Temperature on Protease Activity

For the determination of the effect of temperature, the reaction medium was incubated at varied temperature and the protease activity was determined. For this purpose the enzyme preparation was added to a mixture of 1 mg 1 % azocasein solution, 0.1 ml of 0.06 M CaCl2 and buffer (0.2 M Tris-HCl buffer, pH 8.0) and incubated at 37°, 40°, 50°, 60°, 65°C temperatures.

i) Determination of effect of other effectors on Protease Activity

The activity of the isolated protease was tested in the presence of various known protease effectors (all

obtained from Sigma Chemical Co.), EDTA, 2-mercaptoethanol, potassium di-chromate, sodium thiosulfate. The azocasein assay was used with the addition of these effectors solution to achieve a final desired effectors concentration of 5mM. Control was taken where azocasein assay without these effectors was carried out.

j) Determination of effect of salts on Protease Activity

The protease activity was measured with adding different salts like ZnSO4, MgSO4 , CuSO4, NaCl, KCl at different concentration and then azocasein assay was performed.

k) Direct dehairing activity of the enzyme

For de-hairing studies, the organism was grown in nutrient broth at 37°C for around 20 hours. Then it was centrifuged at 4000 rpm for 8 minutes. The cell free supernatant was added on detergent washed goat skin to observed enzymatic dehairing capability of the organism. Sodium azide was used at 1% so that no organism can grow. Nutrient broth was used as control.

III. Results

a) Isolation and characterization of the organism

The main object of this work was to isolate and characterize thermophilic enzyme which could specifically be used for dehairing the hides and skins of cattle in the tannery industries. In this connection three ways were planned. One was to isolate thermophilic organism from different natural sources. The others is to characterize & identification of the isolated organism. The growth phenotype and some of the biochemical characteristics of the organism was determined. This organism was characterized and identified as a member of gram positive Bacillus family by several test. The features agreed with the description of *Bacillus subtilis* in Bergey's Manual of Systematic Bacteriology [20]. It was also identified as *B. subtilis* with 99.9% identity by API 50 CHB and was also characterized and identified by using a bioinformatics tool PIB (Probabilistic Identification of Bacteria) that suggests the organism was *B. subtilis* (ID=0.9760). So this bacteria is named here as a *Bacillus subtilis*. The results are presented here

Table 2 : Different morphological and biochemical test for the identification of *B. subtilis*

Test performed	Observations	Results
Streak plate isolation:		
NA at 37°C	milky colonies	positive
Gram stain	Small violate colonies singly	Gram positive rods
Spore stain	green color appeared	spore forms
Cultural characteristics:		
Nutrient Agar plates	growth on NA plates	small, non-pigmented, circular
Nutrient Broth	growth on NB	uniform fine turbidity
Nutrient agar slants	Growth on NA slant	moderate, non pigmented
Catalase test	bubbles formed	Positive for catalase production
Oxidase test	Black color formed	positive for oxidase production
Acid & gas production:		
Glucose	yellow	positive for acid and negative for gas
Sucrose	yellow	positive for acid only
Mannitol	red	Negative for acid and gas
Adonitol Arabinose Sorbitol Maltose	red yellow red red	Negative for acid and gas positive for acid only Negative for acid and gas Negative for acid and gas
IMViC test:		
Indole (SIM) test	bright red ring, growth away	Positive for indole and motility
H_2S test	from stab, black color	Positive for H2S production
Methyl red test	deep red ring formed	positive for mixed acid production
Voges-Proskauer test	weak red ring formed	positive for acetoin production
Citrate test	change in color	positive for citrate utilization
Urease test	no bright pink color	negative for urea catabolism
Nitrate test	no color change after zinc dust addition	positive for nitrate reduction
Gelatin test	remain liquefied at 4°	positive for gelatinase production
Starch test	bright zone	positive for starch hydrolysis

b) Determination of Proteolytic Activity of the Enzyme

Proteolytic activities were assayed by Azocasein test, described by Kreger and Lockwood (1981) was done. Here azocasein is used as a substrate. The proteolytic activity was found as 21.13 units for the sample. One unit of proteolytic activity is defined as the

amount of enzyme that produces an increase in the absorbance of 0.01 at 440nm.

c) Effect of temperature on bacterial growth

The aim of this experiment was to monitor the effect of temperature on the bacterial growth. For this purpose this organism was grown in nutrient agar medium at various temperatures (25°C, 30°C, 35°C, 40°C, 45°C, 50°C) for 48 hours and observed the growth profile of the bacteria.

Table 3 : Growth profile of the organism at different temperature

Temperature(°C)			
Time of incubation	Results	Absorbance at 600nm	
25°C	24	+	0.50
	48	+	Not done
30°C	24	++	0.745
	48	++	Not done
35°C	24	+++	0.950
	48	+++	Not done
40°C	24	+++	0.820
	48	+++	Not done
50°C	24	+	0.630
	48	+	Not done

- (NO growth) ± (Some colonies can be seen) + (Moderate growth) ++ (Good growth) +++ (Very good growth)

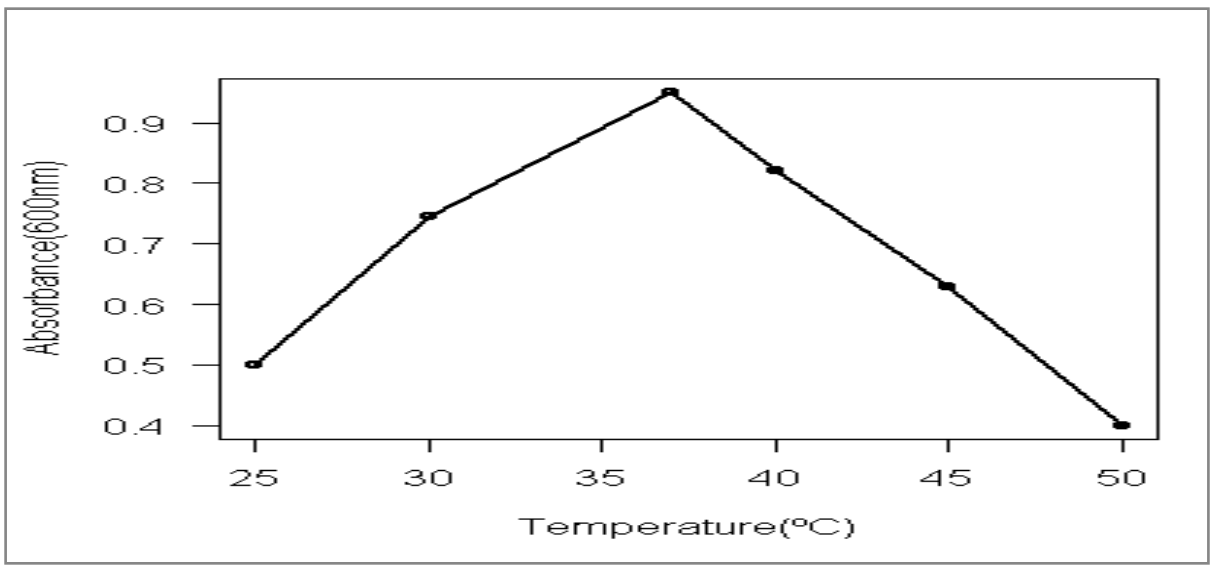

Figure 1 : Graphical presentation of effect of temperature on the organism growth

d) Growth profile and protease activity of the organism at 37°C

The organism was grown in nutrient broth at 37°C. Samples were taken at different time interval and absorbance was taken at 600nm to measure the growth profile. The growth profile of the organism showed that the organism showed optimum growth after about 24 hours and the protease activity was the maximum after 26 hours of incubation.

Table 4 : Growth profile and protease activity of the organism at 37°C

Time at hours	Absorbance at 600nm	Absorbance at 440 nm
4	0.562	0.030
6	0.756	0.052
8	0.864	0.141
10	0.978	0.185
12	1.132	0.212
13	1.197	0.403
14	1.257	0.569
15	1.357	0.578
16	1.393	0.844
18	1.432	1.108
20	1.604	1.497
22	1.731	1.612
24	**1.826**	1.836
26	1.75	**1.924**
28	1.728	1.735

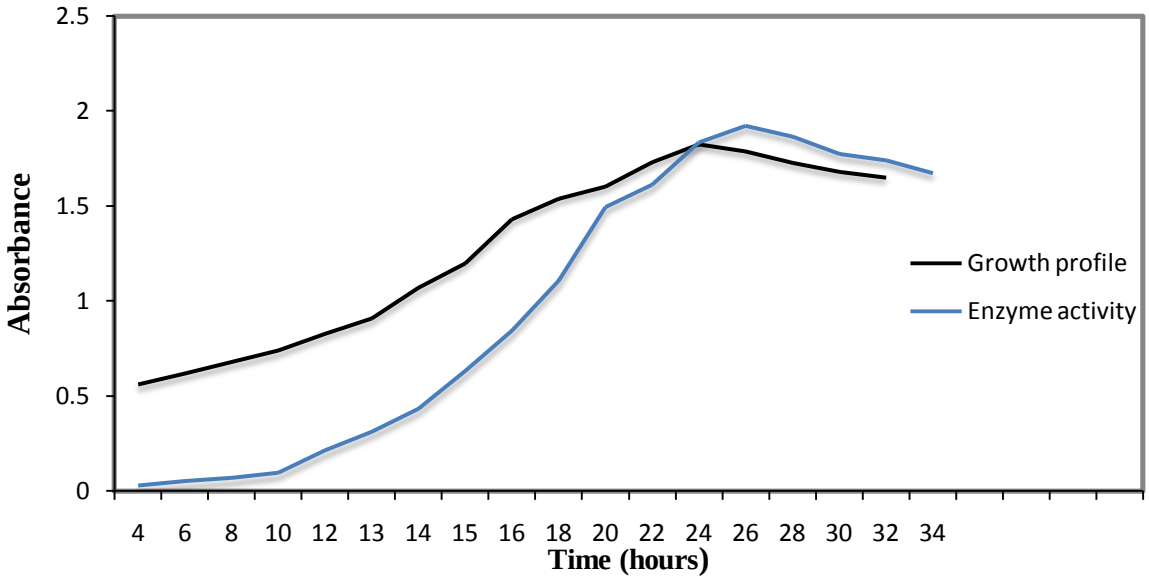

Figure 2 : Graphical presentation of growth of bacteria and protease activity at different time interval at 37° C

In the initial stage of growth there was basal level of extracellular protease which increased with the increase of time. The result showed that there was differential synthesis of enzyme with growth time.

e) Effect of temperature on enzyme activity

The activity of the enzyme was measured over a range of temperature (0°C, 4°C, 20°C, 30°C, 37°C, 40°C, 50°C, 60°C, 65°C, 80°C) and the result is presented in Table-5 and Figure-3.

Table 5 : Protease activity at different temperature (by Kreger and Lockwood method)

Temperature	Absorbance at 440nm
0°C	0.009
4°C	0.019
20°C	0.121
30°C	0.181
37°C	0.183
40°C	0.191

50°C	0.205
60°C	**0.250**
65°C	0.105
80°C	0.019

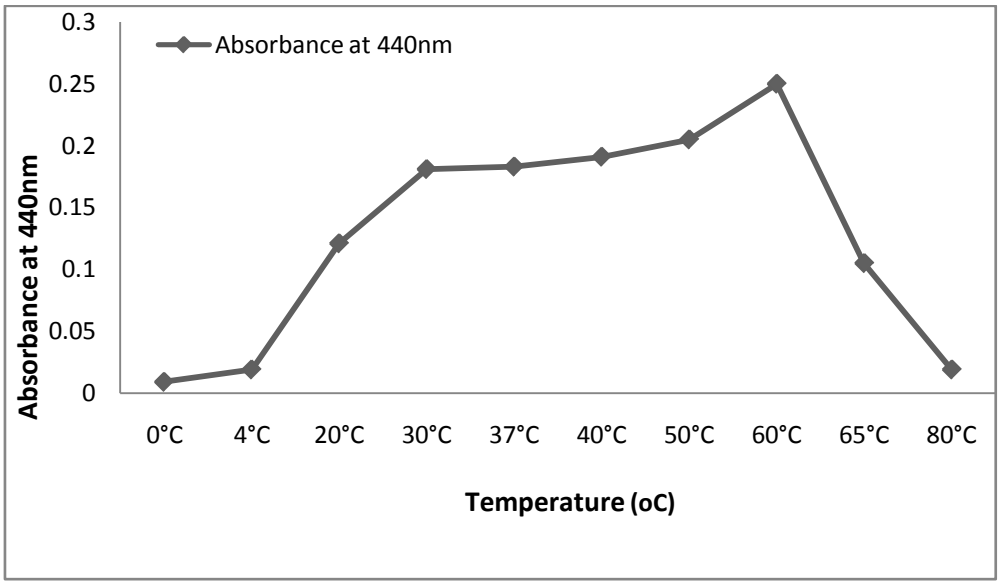

Figure 3 : Graphical presentation of protease activities at different temperature

The enzyme activity is increased with the increase of temperature. The experiment was reported 2 times and the result is reproducible. There was a significant increase in enzyme activity between 20°C to 55°C. The enzyme seems to be active at 60°C and its activity declines as the temperature increase beyond 60°c. At 80°c the enzyme has very little activity. This suggests that the enzyme might be a thermos table enzyme.

Fig-3 shows that the protease was active over a temperature range of 4°C ~80 °C, with an optimum at 60°C. Most proteases possess an activity optimum in the range of 30~80 °C, for example, protease from *B. pseudofirmus* AL-89 is of 60~70 °C [5] and a few have exceptionally high temperature optimum of 100 °C [13].

f) Effect of pH on protease activity from the organism

The pH of the reaction media can affect the protease activity. For this purpose the enzyme activity over a pH range between 4 and 11 was studied.

Table 6 : Effect of pH on Protease Activity

pH	Activity of Enzyme(unit)
4.0	28
5.0	36.5
6.0	48
7.0	63
8.0	68
8.5	**70**
9.0	66
10	60
11	48

The enzyme shows its maximum activity at pH 8.5. The activity decline at pH 8.0 or above 8.5. Therefore pH 8.5 might be the optimum pH for enzyme activity. Additionally, its optimum pH was similar to that of previous reports [18]. Most proteases are active in neutral to alkali conditions, from pH 7.0 to pH 9.5. For example, the activity optimum of protease from *Mycobacterium* kr10 is pH 7.0 [22], *B. pumilus* FH9 of pH 8.0 [4], *Fervidobacterium islandicum* AW-1 of pH 9.0 [13].

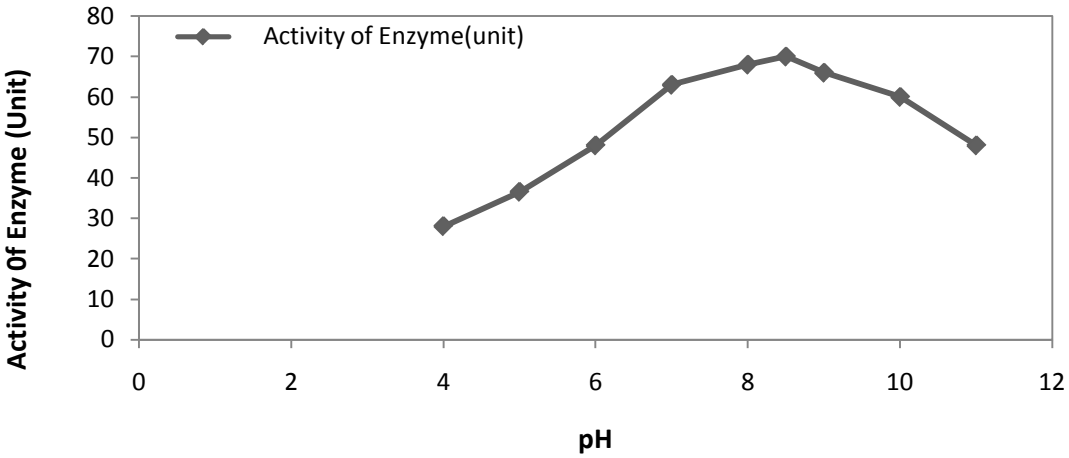

Figure 4 : Graphical presentation of effect of pH on protease activity

The fig-4 shows that the enzyme activity increase with the increase of pH of the media and the optimum pH is 8.5 for the activity of protease enzyme in Tris-HCL buffers. The results showed that the optimum pH of the protease enzyme was 8.5. Studies on growth temperature and pH suggest that the organism might be alkaline and thermophilic Bacillus.

g) Effect of salts and other effectors on the protease activity

The effect of different salts (MgSO4, ZnSO4, CuSO4, NaCl, KCl) and other effectors (EDTA, 2-mercaptoethanol, sodium thiosulfate) at different concentration was measured. MgSO4 increased the activity and β-Mercaptoethanol decreased the activity of the enzyme. NaCl didn't change the activity. Others had little deactivating effect.

Table 7 : Effects of salts and other chemicals on the activity of the protease

Compound (concentration in mM)	Caseinolytic activity (%)a
Control	100
$MgSO_4$ (5)	109
$ZnSO_4$ (5)	77.2
EDTA (5)	92.5
EDTA (5) + $ZnSO_4$ (5)	82.5
EDTA (5) + $MgSO_4$ (5)	102
EDTA (5) + $CuSO_4$ (5)	84.2
NaCl(100)	100
NaCl(200)	100
β-Mercaptoethanol(5)	44.9
Sodium thiosulfate(5)	78.9
Potassium per manganate(5)	96.7

a Caseinolytic activity is expressed as the percentage of the control value (with no addition).

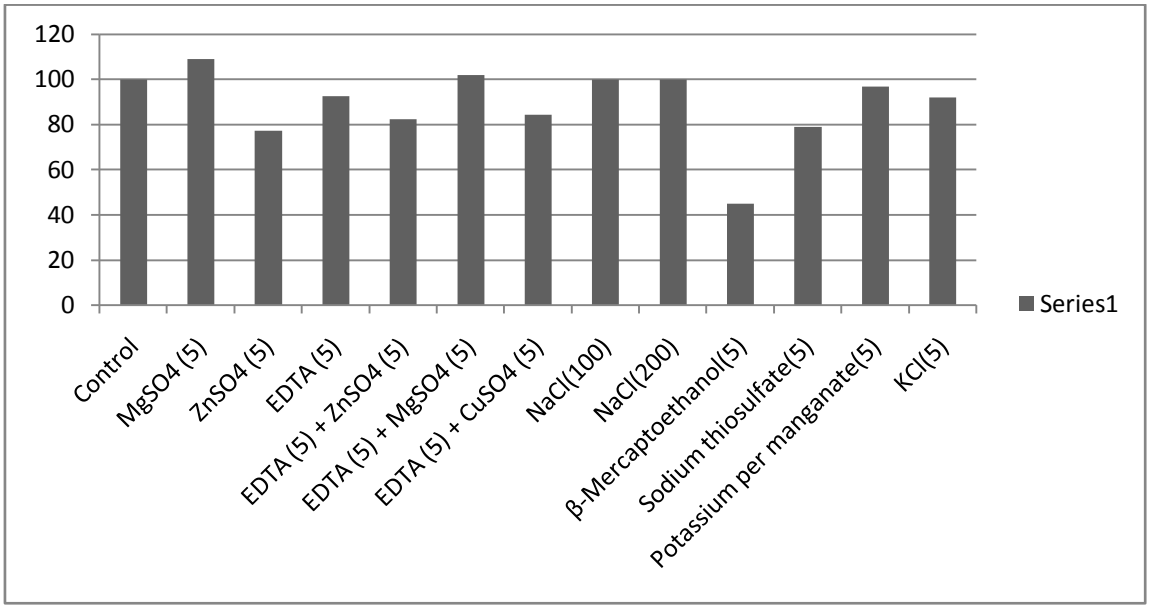

Figure 5 : Graphical presentation of effects of salts and other chemicals on the activity of the protease

The result shows that 5mM Mg^{++} ion slightly increased the activity of the enzyme while Zn^{++} showed slightly decrease. Other elements Na^{+}, K^{+} had no effect on the enzyme. EDTA showed no effect on the protease activity which suggested that the enzyme might not be metallo protease. The enzyme activity was significantly reduced by β- Merceptoethanol. β-Mercaptoethanol has been reported to stabilize cystein proteases by protecting the oxidation of sufhydral group in proteins [19]. No effect of EDTA was detected on enzyme activity suggesting that the metal might not be involved in enzyme activity [11].

h) Determination and Observation dehairing activity of the enzyme

For dehairing studies, the cell-free supernatants were used as sources of crude enzyme. The treated skins and controls showed visible differences after 9 h incubation. No color alteration was observed, although the presence of depilated areas was noticed in the skins treated with enzymes. When hairs were pulled with a forceps, they were very easily released after enzyme treatment.

After 9 h incubation intact hairs could be taken out of the skins easily by simple scraping. In controls, hair loosening was not observed, even by the mechanical action of a forceps. This result was much better than other different bacteria that also caused dehairing. Proteases have been used in the hide dehairing process, where dehairing is carried out at pH values between 8 - 10 [7].

Figure 6 : Enzymatic dehairing by *B. subtilis* on left, control is on the right (without enzyme)

Comparison of dehairing ability of Bacillus subtilis with other bacteria

Dehairing ability of the protease produced by our strain and other bacterial protease showed that our bacterial protease is very fast in dehairing compared to other three.

Table 8 : Comparison of dehairing ability of *B. subtilis* with other bacteria [1]

	Time of incubation for dehairing	Change of color of leather
Bacillus subtilis	9h	no change
Vibrio sp kr2	24h	no change
Flavobacterium sp kr6	24h	no change
Bacillus sp kr10	24h	no change

IV. Discussion

A protease producing bacterium isolated from local soil sample showing de-hairing activity of cow hides and skins both qualitatively and quantitatively. Gram staining & spore staining showed that the organism is gram positive and forms spore during adverse condition in the growth medium. After various tests it was suggested and was also characterized and identified by using a bioinformatics tool PIB (Probabilistic Identification of Bacteria) that suggests the organism was *Bacillus subtilis*. Biochemical characteristics, morphological tests indicate that the organisms might be *Bacillus. B. pumilis*, *B. licheniformis* [8]. The feathers agreed with the description of *Bacillus subtilis*. In Bergey's Manual of Systematic Bacteriology [20].

Azocasein assay developed by Kreger and Lockout is a well accepted method for the assay of wide variety of protease having overlapping specificity. This assay method is simple, easy and quick. A number of protease from different bacteria can be assayed at a time by using this method. Bacillus species have been reported to produce proteases [9, 21, 23]. Therefore, it may be called a very good method for the large scale screening of bacterial protease [6].The characteristics of the culture supernatant suggest that it contain an extracellular enzyme secreted by the bacterium. The enzyme hydrolyses a number of proteins including azocasein which suggest that it is an extracellular protease [2].

In growth curve determination showed that the growth of the organism is increased with the increase of incubation period and the growth reached maximum at around 24 hours of incubation and the protease activity was the maximum of the 26 hours culture. The enzyme seems to have an optimum temperature of 60°C. Most proteases possess an activity optimum in the range of 30~80 °C, for example, protease from *B. pseudofirmus* AL-89 is of 60~70 °C [5], *Nocardiopsis* sp. TOA-1 is of 60 °C and a few have exceptionally high temperature optimum of 100 °C [13].

The effect of different pH ranges on the proteolytic activity of the crude enzymes produced by *B. subtilis* was studied. The enzyme seems to have an optimum temperature of 8.5. Additionally, its optimum pH was similar to that of previous reports[17].The pH value of culture increased to about 8.5. *B. subtilis* strains had been widely utilized for enzyme production, including the proteases [9, 10]. Studies on growth temperature and pH suggest that the organism might be alkaline and thermophilic Bacillus. Preliminary studies on enzyme activity show that the enzyme might be thermophilic alkaline protease.

The effect of a number of ions on the activity of the enzyme was observed. Mg^{++} at 5-10mM level slightly enhances the enzyme activity while Zn^{++} ions slightly decrease the activity of the enzyme. β- Mercepto ethanol is an inhibitor of protease. β-Mercaptoethanol has been reported to stabilize cystein proteases by protecting the oxidation of sufhydral group in proteins [19]. No effect of EDTA was detected on enzyme activity suggesting that the metal might not be involved in enzyme activity [11].

Enzymatic de-hairing may be the ideal de-hairing process. Cow skin was qualitatively dehaired by overnight grown bacterial culture. The skin could be dehaired at room temperature within 8-12 hours. After 9h incubation intact hairs could be taken out of the skins easily by simple scraping. Quantitative estimation has shown that 40mL of culture supernatant could dehair 2×1 cm of leather completely in a 9 hours. This shows that the bacterial isolate moderate to high amount of enzyme for dehairing. Enzymatic dehairing in tanneries has been envisaged as an alternative to sulfides [15]. A significant feature of the enzymatic de-hairing process is complete hair removal and minimal usage of sulfide and the decomposition products formed from the tannery wastewater, with great improvement in wastewater quality as a result.

V. Conclusion

The results presented in this work indicate the bacterial isolates might belong to *Bacillus subtilis*. The

enzyme produced by the bacteria can be utilized in enzymatic dehairing of cow skin in tannery industry to control the environment from pollution. Circumstantial evidences are there to suggest that the enzymes might be proteases. The culture characteristics and biochemical tests of the organism suggest that it is a thermophilic, Gram positive, spore forming and aerobic bacteria. The characterization of protease so far showed that it is an alkaline protease, highly active at temperature near 60°C. The sequencing of the protein and identification of the gene is the future plan of the research work.

As the bacterial protease showed high activity in dehairing of cow skin and our next target is to introduce it to the tannery industries, so that they can use it instead of hazardous chemicals for better leather quality and most importantly for a better environment. Both the isolation, partial purification procedure set up and the characterization study of the protease were important to foresee potential production and uses of this enzyme.

References Références Referencias

1. Alessandro Riffel and Adriano Brandelli. (2003). Dehairing activity of extracellular proteases produced by keratinolytic bacteria. *J. of Chem. Technol. & Biotech.* 78 (8), 855-859
2. Cappuccino, J.G. and Sherman, N. (2001). Microbiology: A Laboratory Manual. 6th Edition. Benjamin Cummings. CA.
3. Dhar S.C., Sreenivasulu S. (1984). Studies on the use of dehairing enzyme for ıts suitibilty in the preparation of improved animal feed. *Leather Sci.*, 31: 261-267.
4. El-Refai, M.H.; Fatah,A.F.A. (2005). Improvement of the newly isolated *Bacillus pumilus* FH9 Keratinolytic activity. *Process Biochem.*, 40, 2325-2332.
5. Gessesse, A., Rajni, H.K., Gashe, B.A. (2003). Novel alkaline proteases from alkaliphilic bacteria grown on chicken feather. *Enzyme Microb. Technol.*, 32 (5):519-524.
6. Hartree E.E. (1972). This modification makes the assay linear over a larger range than the original assay. *Anal. Biochem*. 48:422
7. Ishikawa and Fujiwara, N.(1993). Kinetics and mechanism of enzymatic hydrolysis of gelatin layers of X-ray film and release of silver particles. *J. Ferm. Bioeng.,* 76:300-305.
8. Ichida J & E. H. Burtt, *Journal of Microbiology Methods*, 47, 199, 2001
9. Kim J.D. (2004). Purification and Characterization of a Keratinase from a Feather-Degrading Fungus, *Aspergillus flavus* Strain K-03. *Microbiology*, No. 35 (4), 219-225.
10. Macedo A.J., Henriques J.A. (2005). Termignoni C. Novel keratinase from *Bacillus subtilis S14* exhibiting remarkable dehairing capabilities. *App. and Env. Micro.,*71 (1), 594-596.
11. Madern, D. and Zaccai, G(2000).Halophilic adaptation of enzymes. *Extremophiles*, 4,91-98.
12. Mukhopadyay R.P., Chandra A.L. (1993). Protease of keratinolytic *Streptomycetes* to unhair goat skin. *Indian J. Exp. Biol.*, 31: 557-558.
13. Nam and Y. R. Pyun(2002). Native-feather degradation by *Fervidobacterium islandicum,* a newly isolated keratinase-producing thermophilic anaerobe. *Arch. microbiol*. 178:538–547.
14. Puvanakrishnan, R. and Dhar, S. C., (1986). *Leather Sci.*, 33, 177–191.
15. Raju AA, Chandrababu and Rao NM. (1996). Eco-friendly enzymatic dehairing using extracellular proteases from a *Bacillus* species. *J Am Leather Chem. Assoc* 91: 115-119
16. Ramasami, T. et al. (1999). Beam house and tanning operations: process chemistry revisited. *J. Soc. Leather Technol. Chem.* 83, 39–45
17. Riffel, A., (2003a). Production of an extracellular keratinase from *Chryseobacterium* sp. growing on raw feathers. *Electronic J. Biotechnol.*, 8: 35-42.
18. [Rozs M, Manczinger L, Vágvölgyi C, Kevei F. (2001). Secretion of a trypsin- like thiol protease by a new keratinolytic strain of *Bacillus licheniformis*. 205: 221-224.
19. Scopes, R.K., 1982. "Protein Purification: Principle and practice," Springer-Verlag, New York, pp 195-196.
20. Sneath, P.H.A., Mair, N.S., Sharpe, M.E. & Holt, J.G., (1986). Bergey's Manual of Systematic Bacteriology, Vol. 2 Baltimore: Williams and Wilkins. ISBN 0-683-07893-3
21. Suntornsuk W and Oda K (2005).Purification and characterization of keratinase from a thermotolerant feather- degrading bacterium. *J. Ind. Microbiol. Biotechnol.* 21: 1111-1117
22. THYS and BRANDELLI, A. (2004). Characterization of a protease of a feather-degrading. *Microbacterium* species. *Letters in Applied Microbiology*, February, 39 (2) 181-186.
23. Zerdani I, Faid M, Malki A. (2004). Feather wastes digestion by new isolated strains *Bacillus* sp. in Morocco. Afr. *J. Biotech.* 3: 67-70.