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Microbial Production Of Alkaline Proteases And Evaluation Of Its Performances For Pretreatment Of Leather Industry

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***Abstract*—A high alkaline protease producing bacterial strain was isolated and identified a local soil sample. The organism was 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. It was also identified as B. subtilis with 99.9% identity by API 50 CHB. The enzyme hydrolyses a number of proteins including azocasein which suggests that it is an extracellular alkaline protease. *The* experimentally determined isoelectric point was 5.1 and the optimal enzyme activity was at *60°C* and at pH *8.5*. The esterase preferentially hydrolyzed short-chain fatty acids. Native enzyme preparations typically showed a Michaelis constant (Km) and Vmax of 0.40mM and 12,200 U mg)$^{-1}$, respectively. This microbial enzyme was partially purified by ammonium sulfate fractionation, dialysis, DEAE cellulose chromatography and electrophoretic analysis. Enzyme purity was tested by SDS-PAGE. 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. In practical applications, protease is a useful enzyme for promoting the hydrolysis of proteins and showing significant industrial applications.**

***Keywords*—Bacillus subtilis, SDS-PAGE, Alkaline proteases, Azocasein test, Leather industry, Electrophoretic analysis**

Introduction

Over the last few decades leather industry is based on large scale chemicals treatment which created worldwide environmental hazards. Leather manufacturing is one of the industrial activities globally wide spread, which involves the use of wide range of chemicals many of which are hazardous, highly toxic and obnoxiously odorous [6, 25]. Enzymatic dehairing in tanneries has been envisaged as an alternative to sulfides [26]. Use of enzymes for industrial processing has received considerable attention in recent years owing mainly to environmental concerns [11]. Proteases help in breakdown of proteins into simpler form that exist between two amino acids of a polypeptide chain by the process of hydrolysis [21].

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. Recently government of People's Republic of Bangladesh has taken initiative to develop the industry from outside the city and modernize it [18]. Enzymatic dehairing is suggested as an environment friendly alternative to the conventional chemical process [18]. Enzymes have been pursued as one of the promising alternates to lime and sodium sulfide [7]. Enzymes display a high capability of degrading insoluble keratin substrates of their several potential uses associated to the hydrolysis of keratinous substrates and other applications [3]. In recent years proteases find application in leather making among the different industrial proteases the most widely used enzymes in leather manufacturing [5]. 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.

Higher cost of enzyme is one of the major factors for the system not being practiced through found environmentally friendly. It is essential to develop a cost effective and eco-friendly technology by screening for efficient enzymes from microbial sources and producing them in large quantities by applying recombinant DNA technology. Enzymes found in nature are quite often not readily available in quantities sufficient for industrial use, so use of gene expression methods to express recombinant proteins in suitable heterologous expression systems is required [2]. Genetic engineering could be used to increase the gene copy number as an effective method for improving enzyme productivity [13]. Secretion of recombinant proteins is a common strategy for heterologous protein expression. The major goal of the research showing that proteases enzyme can be utilized in enzymatic dehairing of cow skin in tannery industry to control the environment from pollution, which is a prerequisite for biotechnological applications.

Materials and Methods

Microorganism, Culture Medium and Growth Conditions: Soil samples were collected from the poultry wastes in Savar, after serial dilution, culture was 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. Chemicals used in the experiment were from Oxoid Ltd. (Basingstoke, UK), Merck AG (Darmstadt, Germany), and Sigma (USA). Azokeratin was synthesized based on the method described in a previous study [20].

Isolation and Identification of Bacteria from Local 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. Gram's staining; morphological studies, physiological and biochemical characteristics of the isolate were investigated according to Bergey's Manuals [24]. A rapid bacterial identification test kit for *Bacillus*, API 50 CHB (BioMerieux, France), was used to identify species of bacteria.

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. 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.

Production of Protease and Proteolytic Activity Evaluation by Azocasein Test: 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.

Evaluation of Growth profile and protease activity of the organism at 37ºC: The organism was grown in nutrient broth at 37ºC. 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. 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.

Effect of pH and temperature on enzymatic activity and stability: 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

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.

Evaluation of Dehairing Capability of the Enzyme: For dehairing studies, the organism was grown in nutrient broth at 37ºC 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.

Determination of Ammonium Sulfate Fractionation and Dialysis of Protein Mixtures: All subsequent purification steps were carried out at 0–4 °C.

For Ammonium sulfate fraction of protein mixtures, 20 hours grown bacterial culture was centrifuged at 8000 rpm for 6 minutes. The cell free supernatant was then saturation with ammonium sulfate slowly but frequently to dissolve in crude culture supernatant. After 60% saturation culture supernatant was kept in freeze for 12 hours. After that time maximum protein was precipitated.

Then centrifuged at 14000 rpm for 7-10 minutes to collect the precipitates and precipitates were redissolved in Trise-HCl (0.1M, pH 7.7) buffer. Dialysis was carried out to remove the ammonium salts in a cellophane bag for 8-12 hours using Trise-HCl buffer. Then the collected sample was stored at -20^{o}C for chromatographic analysis.

Ultra Filtration by Millipore Centricons: Ultra filtration using centricons was used to separate the proteins having molecular weight around 100 KDa. The protein having molecular weight 100 KDa or above were retained in the upper part of the centricon while small proteins were passed through the membrane filtrate after centrifugation for 30 minutes at 5000 rpm in a Sorval super speed centrifuge.

Ion-Exchange Column Chromatography for Protein Purification: The enzyme solution was applied to a DEAE cellulose powder in 0.1M Trise-HCl buffer (pH 7.5) in a beaker and left it to swell for few hours. The gel suspension was packed in a column of desired length. After packing the column was equilibrated with 0.1M Trise-HCl buffer. The proteins were eluted according to their molecular weight from the column with same buffer by linear and the adsorbed proteins were then eluted with a linear gradient of 0.1–5.0 M NaCl in the same buffer at a flow rate of 12 ml/ min). The absorbance was taken at 280nm to measure the OD of collected fractions (200 tubes). The most active fractions were concentrated from 15ml to 3ml by PEG-6000. Then the concentrated sample was stored at -20^{o}C for gel analysis.

Determination of Kinetic Parameters and Isoelectric point: The kinetic parameters Km and Vmax were determined in 30 mMTris–Cl, pH 9.0, at 25^{o}C over the substrate concentration range from 0.01 to 5 mM p-nitrophenyl acetate. Analytical isoelectric focusing of the purified enzyme was performed with an AmpholinePAGplate precast polyacrylamide gel (Amersham Biosciences), with pH values ranging from 3 to 10, and the broad pI calibration kit (Amersham Biosciences) as pI marker.

Examination of Enzyme Purity: As described by Laemmli (1970), the protein purity of the enzyme was evaluated by SDS-PAGE using 1 mm thick slab gels containing 14% (w/v) separating gels and 5% (w/v) stacking gels. After running the gel was fixed overnight in a solution of TCA and stained with Coomassie brilliant blue G-250 using the ultrasensitive method. Alternatively the gels were submitted to silver staining. It was then kept immersed in freshly prepared destaining solution till the gel background became transparent. The electrophoretic migration of the protein was compared with that of low-molecular-mass protein markers (Pharmacia, Sweden). Zymography was determined according to the method described in the study of Riffel et al.

Results

Morphological and Biochemical Characterization of the Isolated Soil Bacterium: 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 and 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* sp. by several test. The features agreed with the description of *Bacillus subtilis* in Bergey's Manual of Systematic Bacteriology [24]. It was also identified as *B. subtilis* with 99.9% identity by API 50 CHB. So this bacteria is named here as a *Bacillus subtilis.* The results are presented here in a table-2.

Assay for 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.

Table-2: Morphological and biochemical test for the characterization 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:		
Catalase Test	Bubbles formed	Positive
Indole (SIM)Test	Bright red ring, growth away	Negative
Nitrate Reduction Test	No color change after zinc dust addition	Positive
Urease Test	no bright pink color	Negative
Methyle Red Test	deep red ring formed	Positive
Sucrose Fermentation	yellow	Negative
Gelatin Hydrolysis	remain liquefied at 4ºC	Positive
Voges Proskauer Test	weak red ring formed	Positive
Starch hydrolysis	bright zone	Positive
Glucose	Yellow color	Positive for acid and negative for gas
Citrate test	change in color	Positive for citrate utilization

Determination of Growth profile and protease activity of the organism at 37ºC: The Bacteria was grown in nutrient broth at 37ºC. Samples were taken at different time interval and absorbance was taken at 600nm to measure the growth profile.

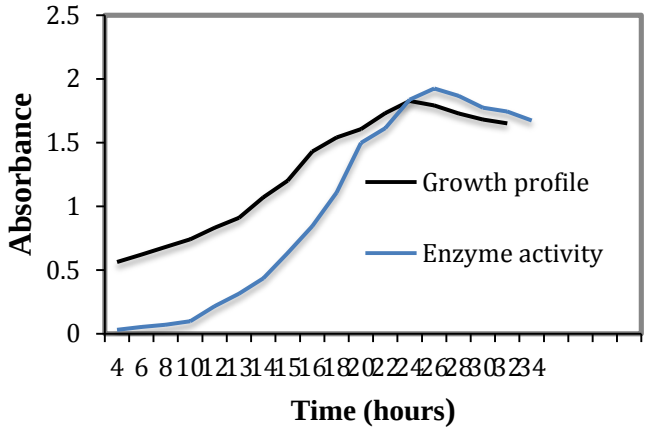

Figure-1: Graphical presentation of growth of bacteria and protease activity at different time interval at 37º C.

The growth profile of the organism showed optimum growth after about 24 hours and the protease activity was the maximum after 26 hours of incubation. In the initial stage of growth there was basal level of extracellular protease which increased with the increase of time. The figure-1 shows that there was differential synthesis of enzyme with growth time.

Effect of p^H 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.

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 [23]. 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, *B. pumilus* FH9 of pH 8.0 [9], *Fervido bacterium islandicum* AW-1 of pH 9.0 [19].

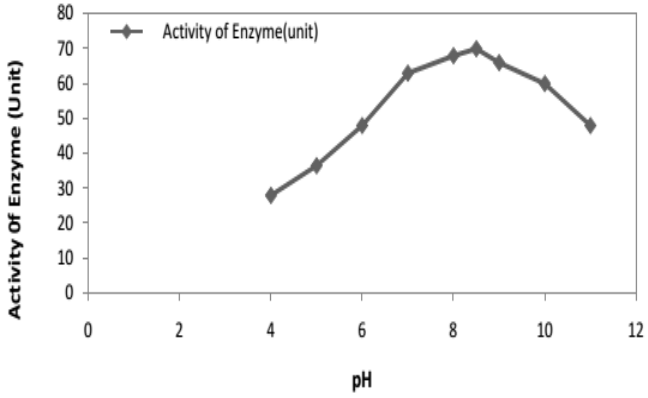

Figure-2: Graphical presentation of effect of pH on protease activity.

The figure-2 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*

Effect of Temperature on Enzyme Activity: The activity of the enzyme was measured over a range of temperature (0°C, 4°C, 20°C, 30ºC, 37ºC, 40ºC, 50ºC, 60ºC, 65ºC, 80°C) and the result is presented in figure-3

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 thermostable enzyme.

Figure-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 [10] and a few have exceptionally high temperature optimum of 100 °C [19].

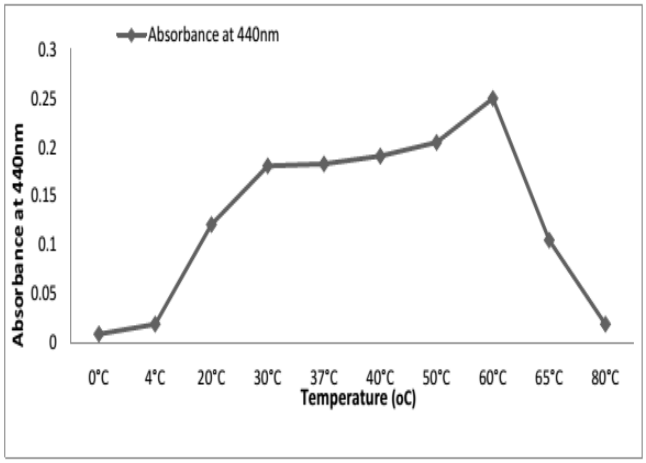

Figure-3: Graphical presentation of protease activities at different temperature.

Evaluation Dehairing Capability of the Isolated Protease Enzyme: 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 [14]. In most cases the enzymes work and bring about efficient dehairing within 6-20h.

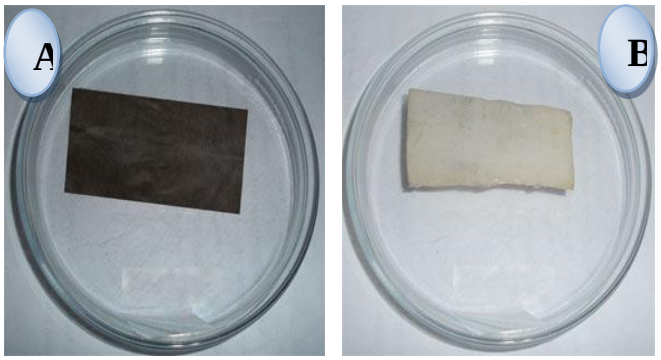

Figure-4: Direct dehairing activity of the enzyme-A (Control) and B (100% Hair removed).

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-3: 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

Partial Purification of Protease Enzyme: To remove unwanted proteins from the crude enzyme solution, 40–80% saturation of $(NH_4)_2SO_4$ had the best effect on enzyme purification. Most of the protein in bacterial culture filtrate precipitated at 60% saturation. The obtained most active enzyme protein preparation could be obtained at the ammonium sulphate level of 60%. This result was in complete accordance with other workers [15].

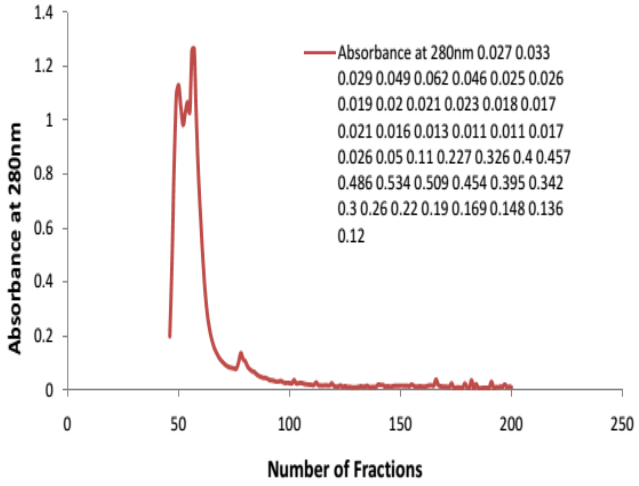

Figure-5: Graphical presentation of OD of collected fractions from DEAE cellulose column chromatography.

The overall purification factor was about 22.6 fold and the final yield was 51%. The final product had a specific activity of about 839.41 U/mg. Protein purification and different enzymetic properties of the protease are presented in a table-4. Ion-exchange DEAE cellulose column chromatography was for protein purification. The desired enzyme was found in 53-55 numbers tube by Azocasein test. The result is presented in figure-5

Figure-5 shows that the desired enzyme was found in 53-55 numbers of tubes/fractions and it was also found that 54 numbers of tube/fraction contains large amount of desired enzyme. Enzyme purity was tested by SDS-PAGE according to Laemmli (1970) and operated at 4°C. It was found that a single band is appeared in the gel. It proves that the enzyme has purified and separated.

Table-4: Protein purification and different enzymatic properties of the protease.

Protein purefication status	Protein conc. mg/mL	Protease activity	Enzyme unites/ mL	Specific activity	Protein purificati -on fold
Crude culture supernatant	0.82	1.159	289.75	350	1
60% saturation with ammonium sulfate	3.52	1.79	447.5	127	2.3
Removal of salt by dialysis	1.96	0.967	242	123	2.9
Ultrafiltration by centricon	1.36	1.01	252.5	185	4.9
Gell filtration chromatography	135µg/ mL	0.217	54.25	54.41	11.5

Kinetic Parameters and Isoelectric point: The *kcat* kinetic parameter was determined using some common acetylated substrates and the values. The Michaelis constant Km for alkaline proteases was 0.40 € 0.02 mM and the maximal velocity Vmax was 12,200 € 500 U mg)$^{-1}$. The pI of the protein was estimated by isoelectric focusing to be approximately 5.1, in agreement with the theoretically predicted pI value of 5.0.

Discussion

Novel protease enzyme was isolated from local soil bacterium showing remarkable dehairing activity of cow hides and skins both qualitatively and quantitatively. After various biochemical characteristics, morphological tests suggested and the features agreed with the description of *B. subtilis* in Bergey's Manual of Systematic Bacteriology [21]. Azocasein assay developed by Kreger and Lockout is a well accepted method for the assay of wide variety of protease having overlapping specificity. The enzyme hydrolyses a number of proteins including Azocasein which suggest that it is an extracellular protease [8]. *Bacillus* species have been reported to produce proteases [28].

Therefore, it may be called a very good method for the large scale screening of bacterial protease [12]. 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 60ºC. 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 [6], *Nocardiopsis* sp. TOA-1 is of 60 °C and a few have exceptionally high temperature optimum of 100 °C [19]. The enzyme seems to have an optimum pH of 8.5. Additionally, its optimum pH was similar to that of previous reports [22]. *B. subtilis* strains had been widely utilized for enzyme production, including the proteases [17].

Enzymatic dehairing may be the ideal process. Quantitative estimation has shown that 40mL of culture supernatant could dehair 2×1 cm of leather completely in a 9 hours. After 9h incubation intact hairs could be taken out of the skins easily by simple scraping. 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 [20]. A significant feature of the enzymatic dehairing 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.

A trial was given to obtain the partially purified proteases from the culture supernatant of *Bacillus* sp. from one hand to create an interesting comparative study of the characteristics of the purified enzyme preparations from the other hand. This microbial enzyme was partially purified by ammonium sulphate fractionation, dialysis, DEAE cellulose chromatography and electrophoretic analysis. The obtained most active enzyme protein preparation could be obtained at the ammonium sulphate level of 60%. This result was complete accordance with other workers [15]. The protease precipitated by the ammonium sulphate had been reported in many previous studies [28]. The precipitates were found to be very active after the dialysis. This gave 2.9 fold purification of the proteins. Ultra filtration is another method for the separation of proteins of different molecular weight [26].Proteins having molecular weight higher than or equal to 100kDa were used. In this process the protein were purified to 4.9 fold.

After ultra filtration protein was further purified by gel filtration chromatography using DEAE cellulose. This method is very laborious and time consuming but separation of protein is very reliable. Three different protein picks of different molecular weight was found and one of the pick showed considerable enzyme activity [4]. In this process the protein was purified to 11.5 fold. The subunit molecular mass of the protease was estimated by comparing the electrophoretic mobility of the protease with the electrophoretic mobilities of marker proteins. It was found that a single band was appeared in the gel indicating the enzyme has fully separated and purified. The level of purification is higher than those reported in other similar papers [16]. 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.

Conclusion

Bacterial alkaline protease has got its particular eco friendly technical applications in leather processing, detergent and feathers digestion to feed in Bangladesh. The results showed that the *B. subtilis* proteases enzyme can be utilized in enzymatic dehairing of cow skin in tannery industry to control the environment from pollution, which is a prerequisite for biotechnological applications. The cultural 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 60ºC. Finally, it plans to clone and over-express the genes encoding enzymes for large scale industrial production and commercial use for pretreatment of industrial residues from leather industry for biogas production.

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