

Original Research Article

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SCREENING, OPTIMIZATION AND EXPLORATION OF MICROBIAL ENZYMES WITH SPECIAL CHARACTERISTICS FOR BIOTECHNOLOGICAL APPLICATIONS

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ABSTRACT: The aim of this study was to explore a novel microbial enzyme was screened, optimized, characterized and purified from the local soil gram positive and spore forming bacterium and to study their ability to degrade chicken feathers and pretreatment of industrial residues from leather industry. After different biochemical and microbiological tests it was suggested that the organism was *Bacillus subtilis*. It was also characterized and identified by using a bioinformatics tool PIB that suggests the organism was *B. subtilis*. The isoelectric point was 5.1 and the optimum temperature for enzyme activity was at 60°C but they can produce feather degrading enzymes at 53°C and at pH 8.5. Native enzyme preparations typically showed a Km and Vmax of 0.40mM and 12,200 U mg⁻¹, respectively. The proteolytic activity was 21.13 units for the sample. This enzyme was purified by ammonium sulfate fractionation, dialysis, DEAE cellulose chromatography and electrophoretic analysis. The most active enzyme protein preparation could be obtained at the ammonium sulphate level of 60%. In this process the protein were purified to 4.9 fold. Enzymatic dehairing estimation has shown that culture supernatant could dehair of leather completely in a 9 hours of incubation. In future the tanneries will use a combination of chemical and enzymatic processes. The potential use of microbial enzymes in leather and poultry processing industry for promoting the hydrolysis of proteins that showing significant industrial and biotechnological applications.

KEYWORDS: Screening, *Bacillus subtilis*, Optimization, Characteristics, Electrophoretic analysis.

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1. INTRODUCTION

Enzymes are the well-known biocatalysts that perform a multitude function of chemical reactions and are commercially exploited in the detergent, food, pharmaceutical, leather and poultry processing industries. Over the last few decades leather industry is based on large scale chemicals treatment which created worldwide environmental hazards. 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 ^[10]. 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]. Most of the tannery industries in Bangladesh use chemicals for dehairing that led great environmental hazards and health problem. However, leather industries are one of the most promising fields for export to earn foreign currency in Bangladesh. Recently government of People's Republic of Bangladesh has taken initiative to develop the industry from outside the city and modernize it. Enzymatic dehairing is suggested as an environment friendly alternative to the conventional chemical process ^[17]. Enzymes have been pursued as one of the promising alternates to lime and sodium sulfide ^[6]. 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 ^[2]. In recent years proteases find application in leather making among the different industrial proteases the most widely used enzymes in leather manufacturing ^[4]. Alternatively, feather biodegradation processes have been proposed as viable substitutes ^[31]. Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of Enzymology. 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 ^[1]. Genetic engineering could be used to increase the gene copy number as an effective method for improving enzyme productivity ^[12]. Genes coding for microbial proteases have been cloned and expressed in a broad range of microorganisms in order to modify their properties or their expression levels. 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 poultry processing industry and enzymatic dehairing of skin in tannery industry to control the environment from pollution, which is a prerequisite for biotechnological applications.

2. MATERIALS AND METHODS

Sample collection, Isolation of the Bacterial Strain and culture conditions:

The experiment was conducted at the vaccine research laboratory under the department of Biochemistry and Microbiology of the Gono University, Dhaka, Bangladesh, with the objective to Screening, Optimization and Exploration of Microbial Enzymes from local soil samples with Special Characteristics for Biotechnological Applications. 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]. 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.

Screening of soil sample for Identification of Protease producing microbial Strains:

A rapid bacterial identification test kit for Bacillus, API 50 CHB (BioMerieux, France), was used to identify species of bacteria. Gram's staining; morphological studies, physiological and biochemical characteristics of the isolate were investigated according to Bergey's Manuals ^[25]. 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, Catalase Test, Urease test, Indole (SIM) test, MR-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 of the organism.

Assessment of protein content and Evaluation of 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. One unit of proteolytic activity is defined as the amount of enzyme that produces an increase in the absorbance of 0.01 at 440nm.

Exploration of the Effect of Temperature on bacterial growth and Protease Synthesis:

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.

Determination of Effect of pH and Temperature on Protease Activity and Stability:

For determining the effect of pH on protease activity and stability 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. 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.

Observation and Evaluation of Dehairing Capability of the Enzyme:

For the dehairing studies, the organism was grown in TSB 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 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.

Growth of the Organisms and Feather Degradation:

The organisms were allowed to grow at different temperature and its feather degradation capabilities were also observed up to 72 hours. The growth pH profile was seen for each organism. Effect of divalent ions on the growth of these bacteria was tested.

Optimization of cultural conditions for Protease production:

The obtained microorganisms, labeled F1 medium will be supplemented with different carbon sources glucose, sucrose, lactose, maltose, starch and Mannitol in 1% [w/v] concentration to study the effect of different carbon sources on protease production. The sterile F1 media (pH 7.5) with different carbon sources will be inoculated with 5% (v/v) inoculums and then incubated at 35°C in mechanical shaker at 120rpm for 24h. At the end of the incubation period, the samples will be assayed for protease activity. The effect of different nitrogen sources on the protease production will be studied by supplementing the F1 medium with beef extract, tryptone, peptone and casein or soy bean meal in 0.4% (w/v) concentration. The culture media will be inoculated with obtained bacterium and incubated at 35°C in mechanical shaker at 120rpm for 24h. The culture samples will be assayed for protease activity using standard assay. In the prescreening of various elements, magnesium sulfate heptahydrate (0.2g/L) in the sterile F1 medium (pH 7.5) was substituted by magnesium sulfate heptahydrate (0.1g/L and 1g/L). Also F1 medium was enriched with the addition of ammonium nitrate (5g/L), Tween 80 (0.25g/L), CaCl₂ (0.1g/L and 1g/L), individually. In another formulation, F1 medium was modified where the concentration of KH₂PO₄ was changed from 1g/L to 0.5 and 2 g/L respectively.

Purification of the Protease Enzyme:

All subsequent enzyme 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°C for chromatographic analysis. 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. 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 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°C for gel analysis. 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.

Determination of Kinetic Parameters and Isoelectric Point of Protease:

The kinetic parameters K_m and V_{max} were determined in 30 mM Tris-HCl, pH 9.0, at 25°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, with pH values ranging from 3 to 10 and the broad pI calibration kit (Amersham Biosciences) as pI marker.

Determination of Effect of Various Reagents on Protease Activity:

The activity of the isolated protease was tested in the presence of various known protease effectors. 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. The protease activity was measured with adding different salts like ZnSO₄, MgSO₄, CuSO₄, NaCl and HCl at different concentration and then azocasein assay was performed.

Determination of storage stability of protease:

The Storage stability of protease was determined at 4°C, room temperature and room temperature with Sodium benzoate 0.60%. The residual enzyme activity of each line measured under standard assay condition at every 7 days of interval.

3. RESULTS AND DISCUSSION

Characterization and Identification of the Isolated Bacterial Stains:

The main objective of this work was to screened, optimized and characterize thermophilic protease enzyme producing bacterium which could specifically be used for poultry processing industry and dehairing the hides and skins 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 [25]. 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*.

Azocasein Test 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. The enzyme hydrolyses a number of proteins including Azocasein which suggest that it is an extracellular protease [7]. *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 [11].

Evaluation of Effect of Temperature on Bacterial Growth and Enzyme Synthesis:

The aim of this experiment was to monitor the effect of temperature on the bacterial growth.

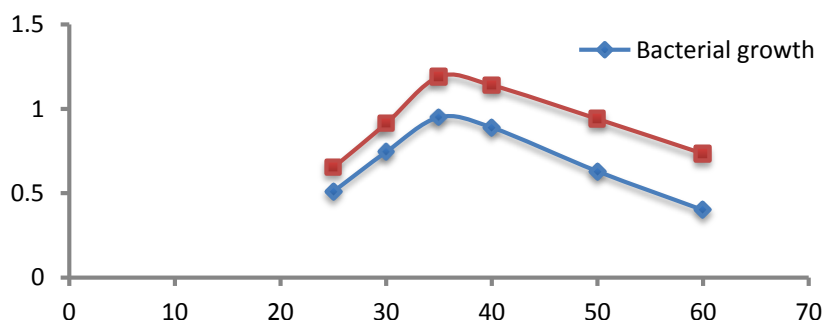


Figure -1: Graphical presentation of bacterial growth and protease activity at different temperature.

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, 60°C) for 48 hours and observed the growth profile of the bacteria. The presented figure-1 shows that the optimum growth temperature is 37°C. Above this temperature the organism grows very slowly. The growth rate also determined by taking absorbance of the bacteria growth culture at 600nm. Enzyme activity of each culture was measured after 24 hours of growth. No substantial change in enzyme activity could be shown after 24 hours. This showed that the enzyme remain stable at 60°C in growth media at least up to 10-12 hours.

Effect of salts and other effectors on the protease activity:

The effect of different salts and other effectors at different concentration was measured. MgSO₄ increased the activity and β-Mercaptoethanol decreased the activity of the enzyme. NaCl didn't change the activity. The effect of a number of ions on the activity of the enzyme was observed. 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- Mercaptoethanol. β-Mercaptoethanol has been reported to stabilize cystein proteases by protecting the oxidation of sufhydral group in proteins [24]. No effect of EDTA was detected on enzyme activity suggesting that the metal might not be involved in enzyme activity [18].

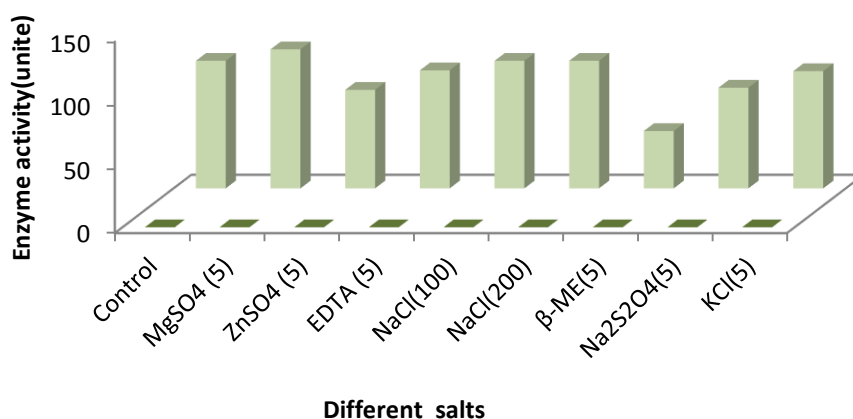


Figure-2: Graphical presentation of effects of salts and other chemicals on the activity of the protease.

Effect of pH and Temperature 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 declined 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]. *B. subtilis* strains had been widely utilized for enzyme production, including the proteases [16]. 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^[8], *Fervidobacterium islandicum* AW-1 of pH 9.0^[19]. 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). 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. 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^[9] and a few have exceptionally high temperature optimum of 100 °C^[19].

Optimization of Media Formulation for Protease Production by *Bacillus subtilis*:

In the preliminary screening studies on the development of the production medium, various industrially low-cost carbon and nitrogen sources and elements were screened and as a result, maltose, soybean meal, Tween 80 and pH of the medium were found to be important factors in enhancing the alkaline protease formation. As it is seen except for whey, molasses and potato starch, the rest of the carbon sources gave satisfactory specific protease activity results if compared with the control. However if total protease enzyme activities were considered Glucose–Fructose (35%) and Maltose (55%) were taken as best carbon sources. These two sources were also very much satisfactory with respect to their specific protease activities. In these experiments the carbon source was taken as Maltose (55%) at a constant level of 10 g/L. According to these results, soybean meal or corn steep liquor could be used as a single nitrogen source, provided that there is Tween 80 at 0.15 g/L and CaCl₂ at 0.7 g/L concentrations. Therefore since soybean meal resulted in slightly higher activity than corn steep liquor, we decided on using soybean meal as the nitrogen source in the experimental design set up for response surface method analysis. In order to study the effects of different elements on the protease activity, Tween 80 was used in these experiments in order to determine if it had a promoting or inhibiting effect on the enzyme synthesis. The positive effect of Tween 80 and CaCl₂ at 0.1g/L concentration was pronounced with respect to the specific protease activities as well. Using Tween 80 at its maximum concentration with CaCl₂ at minimum level decreased the protease activity by at least 17.8 %. Therefore according to this preliminary study, we decided to keep the CaCl₂ concentration constant at the maximum concentration of 0.7 g/L and use Tween 80 as the main variable to be used in the response surface.

Purification of Protease Enzyme:

To remove unwanted proteins from the crude enzyme solution, 40–80% saturation of (NH₄)₂SO₄ had the best effect on enzyme purification. Most of the protein in bacterial culture filtrate precipitated at 60% saturation. This result was in complete accordance with other workers^[15]. 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 enzymatic properties of the protease. 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-3. Figure-3 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. 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 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. 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. 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.

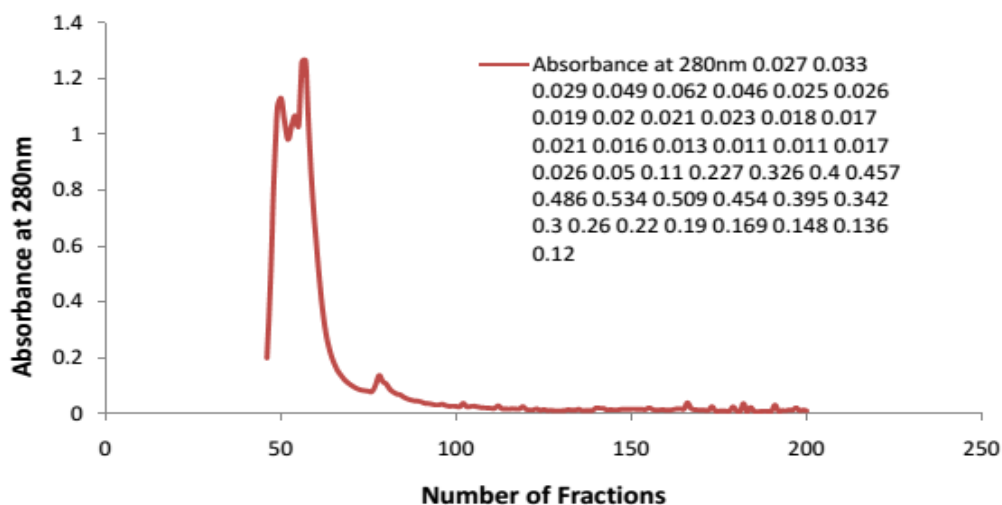


Figure-3: Graphical presentation of OD of collected fractions from DEAE cellulose column chromatography.

Three different protein picks of different molecular weight was found and one of the pick showed considerable enzyme activity [3]. In this process the protein was purified to 11.5 fold. 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. The subunit molecular mass of the protease was estimated by comparing the electrophoretic mobility of the protease with the electrophoretic mobilities of marker proteins. The level of purification is higher than those reported in other similar papers [15].

Assessment of Kinetic Parameters and Isoelectric point of Protease:

The *k_{cat}* kinetic parameter was determined using some common acetylated substrates and the values. The Michaelis constant *K_m* for alkaline proteases was 0.40 € 0.02 mM and the maximal velocity *V_{max}* 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.

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. 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. 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]. 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 [13]. In most cases the enzymes work and bring about efficient dehairing within 6-20h [30].

Screening of Storage Stability of Protease from *Bacillus subtilis*:

The crude extracellular protease was stored under three different condition namely 4°C, room temperature and room temperature with Sodium Benzoate 0.60% for 4 weeks. Enzyme activity was determined from each sample at seven days intervals and the process was continued for 4 weeks. There was no significant decrease in enzyme activity when stored at 4°C within the above mentioned period of investigation. After fourth week the enzyme activity was found for 562U/ml (100%) at 4°C, room temperature 492U/ml (87.5%) and room temperature with chemical 542 U/ml (96.4%) respectively.

Growth of the organisms and feather degradation:

The organism grew almost at similar rate at 60°C but feather degrading capabilities are quite high at 53°C (Figure-4).



Figure-4: Feather degrading activity of the enzyme-A (Control) and B (100% Feather degraded).

This result also indicates that the bacteria secretes an enzyme which is proteolytic and responsible for feather degradation. Visible degradation of feather could be observed after 24 hours. It is apparent from the figures that the feather degradation activity of the organism was reasonably high.

4. CONCLUSION

In this research the results presented the bacterial isolates might belong to *Bacillus subtilis*. Bacterial alkaline protease has got its particular ecofriendly technical applications in leather processing, detergent and feathers digestion to feed in Bangladesh. The culture characteristics and biochemical tests of the organism suggest that it is a thermophilic, Gram positive, spore forming and aerobic bacteria. Circumstantial evidences are there to suggest that the enzymes might be proteases. As the bacterial protease showed that high activity in poultry processing industry and dehairing of animal skin and our next target is to introduce it to the poultry and tannery industries, so that they can use it instead of hazardous chemicals for better quality and most importantly for a better environment. The results showed that the *B. subtilis* proteases enzyme can be utilized in poultry processing industry and enzymatic dehairing of skin in tannery industry to control the environment from pollution, which is a prerequisite for biotechnological applications. The isolation, identification, purification procedure set up and the characterization study of the protease were important to foresee potential production and uses of this enzyme. The sequencing of the protein and identification of the gene is the future plan of the research work. 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 and poultry processing industry.

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CONFLICT OF INTEREST

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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