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Isolation and characterization of proteases enzyme from locally isolated *Bacillus sp.*

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Abstract: A 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 sp in Bergey's Manual of Systematic Bacteriology [24]. It was also identified as Bacillus sp with 99.9% identity by API 50 CHB. 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. This microbe has grown at high temperature and pressure. Its optimum pH and temperature were 8.5 and 60°C. It secretes an extracellular protease in the growth medium. The enzyme hydrolyses a number of proteins including azocasein which suggests that that it is an extracellular protease. The enzyme seems to be alkaline protease which is capable of De-Hairing from skin and hides. 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: Identification, Characterization of Protease, *Bacillus sp.*, Isolation, Alkaline Protease

1. Introduction

Leather is the third largest economic community of Bangladesh. Its industrial production is very beneficial to the country in terms of employment generation as well as foreign exchange earner. Though leather industrial has been set up sporadically house and there in the country it has not yet developed scientifically in an organized way. Recently government of People's Republic of Bangladesh has taken initiative to develop the industry from outside the city and modernize it. Enzymatic De-Hairing in tanneries has been envisaged as an alternative to sulfides [3, 5, 9, 26, 27]. Over the last few decades leather industry is based on large scale chemicals treatment which created worldwide environmental hazards. The chemicals which were used in pre-tanning stage were soda-lime, salts, solvent flashy and biological waste from leather itself. In fact, the raw hides had to undergo a series of chemical treatment before it turned into flattering leather. The chemicals used in this process were mostly toxic. Thus due to these pre-tanning operations, the leather processing industry is one of the worst offenders of the environment. Enzymatic De-Hairing is suggested as an environment friendly alternative to the conventional chemical process [18]. The use of proteolytic enzymes as an alternative to De-Hairing skins has been investigated [20]. 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. Many countries including India has started tannery industries based on enzymatic process. This industry needs a lot of enzymes for soaking, digressing and De-Hairing. Besides enzyme are used for depollution, effluents treatment and by product utilization. In this context 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 [6, 12]. These enzymes could be applied for waste water

treatment, textile, medicine, cosmetic leather and poultry processing industry as well as in the leather industry [17].

2. Materials and Methods

2.1. Isolation and Identification of Bacteria from Local Soil Sample

The soil sample was collected from the poultry wastes in local area, 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. 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, was used to identify species of bacteria. Probabilistic identification of bacterial strains (PIB) were conducted by using a bioinformatics tool, were used. In this tool, different morphological and biochemical tests were given as input data and the program utilize a pre-existing database and compare with the input data. This bioinformatics toll gives a index (ID) value as output. This index value represents the probability of the strain.

2.2. Azocasein Test

Azocasein test, described by Kreger and Lockwood (1981) was done to measure the proteolytic activity of the feather degrading Bacteria. Here azocasein was used as substrate. Optical density was measured at 440 nm.

2.3. Biochemical & Microbiological Tests for bacteria Characterization

To identify the bacteria biochemical properties, of different tests were performed. For correct interpretation of the results in every test Escherichia coli was taken as control. Carbohydrate tests were performed using Glucose, Lactose, Ribose, Sucrose, Mannitol, Adonitol, Arabinose, Sorbitol and Maltose.

Others biochemical tests were performed such as 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.

2.4. Determination of Temperature effect on Protease Activity

For the determination of the effect of temperature, the reaction medium was incubated at 37°, 40°, 50°,60°,65°C temperatures 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-HCI buffer, pH 8.0).

2.5. Determination of pH effect on Protease Activity

For determining the effect of pH on protease activity different buffer systems with different pH were used. Azocasein was dissolved in different buffer solution and the enzyme assay was carried out within a pH range of 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 pH ranges*

Buffer	pH 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

2.6. 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.

2.7. Determination of salts effect on Protease Activity

The protease activity was measured with adding different salts like $ZnSO_4$, $MgSO_4$, $CuSO_4$, NaCl, KCl at different concentration and then azocasein assay was performed.

2.8. Determination of Temperature effect on Bacterial Growth and Protease Activity

The bacterial culture was grown in nutrient broth at various temperatures (25ºC, 30ºC, 35ºC, 40ºC, 50ºC, 60ºC) 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.

2.9. Direct dehairing activity of the enzyme

For De-Hairing 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 De-Hairing capability of the organism. Sodium azide was used at 1% so that no organism can grow. Nutrient broth was used as control.

3. Result and Observation

3.1. Bacteria Isolation and Characterization

The main object of this work was to isolate and characterize the thermophilic enzyme which could specifically be used for De-Hairing 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 [24]. 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 in table-2

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	Red	Negative for acid and gas
Arabinose	Yellow	positive for acid only
Sorbitol	Red	Negative for acid and gas
Maltose	Red	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 4º	positive for gelatinase production
Starch test	bright zone	positive for starch hydrolysis

3.2. Azocasein Test

The proteolytic activity is 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.

3.3. Temperature effect 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 Table-3 and Figure-1

Table3. *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

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.It should be demonstrated that the enzyme has its activity after a second round of temperature.

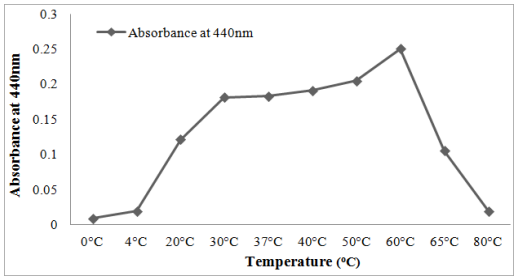

Fig 1.*Graphical presentation of protease activities at different temperature*

Fig-1 shows that the protease was active over a temperature range of 4°C ~80 °C, with an optimum at 60°C.

3.4. pH effect on Protease Activity

Reaction media pH can affect the protease activity. To evaluate this information the enzyme activity over a pH range between 4 and 11 was assayed. The maximum enzyme activity was observed at pH 8.5. The activity declines 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 [22]. 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 [19], B. pumilus FH9 of pH 8.0 [7], Fervidobacterium islandicum AW-1 of pH 9.0 [18].

Table 4. *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

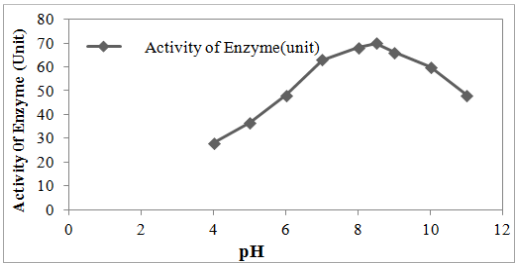

Fig 2. *Graphical presentation of effect of pH on protease activity*

In fig- 2 showed that the enzyme activity increases with the increase of pH of the media. 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.

3.5. Effect of Salts and other Effectors on the Protease Activity

The effect of different salts ($MgSO_4$, $ZnSO_4$, $CuSO_4$, NaCl, KCl) and other effectors (EDTA, 2-mercaptoethanol, sodium thiosulfate) at different concentration was measured. $MgSO_4$ increased the activity and β-Mercaptoethanol decreased the activity of the enzyme. NaCl didn't change the protease activity. Others had little deactivating effect.

Table 5. *Effects of salts and other chemicals on the activity of the protease*

Compound(concentration in mM)	Caseinolytic activity (%)
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 permanganate	96.7

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

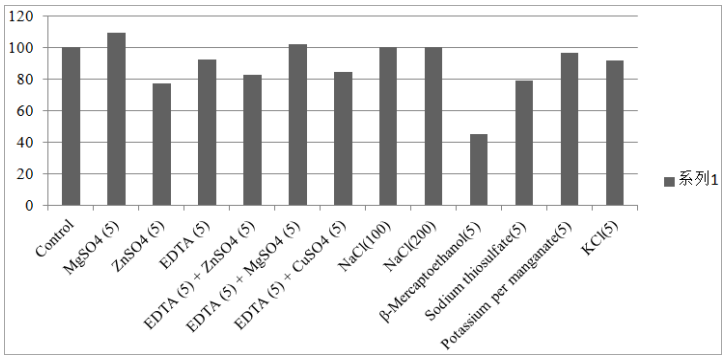

Fig 3. *Graphical presentation of effects of salts and other chemicals on the activity of the protease*

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 [15].

3.6. Determination of Bacteria Growth Profile and Protease Activity at 37ºC

The bacterium 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 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.

3.7. Direct Dehairing Activity of the Enzyme

The hair removing activity of the culture fitrated was tested on 8″×8″ leather for different time interval. Culture supernatant containing enzymes had direct effect to remove hair from leather. Hair removing efficiency increase with incubation time and amount of enzyme. So the use of microbial enzymes as an alternate technology to the conventional methods, and highlights the importance of these enzymes in minimizing the pollution loads.

Table 6. *Bacterial Growth profile and protease activity 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

Fig 4. *Direct dehairing activity of the enzyme - A (Control), B (10% Hair removed), C (90% Hair removed), D (100% Hair removed)*

Table 7. *Direct De-Hairing activity of the enzyme with different incubation priod*

Incubation priod (hrs)	Culture suprenatant (mL)	Observation	Control
		Enzyme effects	
6	10	No hair removed from the skin.	No hair removed
	20	No hair removed but folicles become softerr.	
	30	5% hair were removed by gentlerubbing.	
	40	More than 5% hair were removed by gentle rubbing.	
7	10	5% hair were removed by gentle hand. After few huors incubation with tap water almost 60% were removed.	
	20	30% hair were removed by gentle rubbing.	
	30	More than 50% hair were removed with gentle rubbing.	
	40	More than 70% hair were removed with gentle rubbing.	
8	10	30% hair were removed by gentle rubbing.	
	20	70% hair were removed by gentle rubbing.	
	30	100% hair were removed by gentle rubbing.	
12	10	More than 30% hair were removed by gentle rubbing.	
	20	More than 80% hair were removed by gentle rubbing.	
	30	100% hair were removed by gentle rubbing.	

3.8. Comparison of De-Hairing Ability of Bacillus Subtilis with other Bacteria

De-hairing ability of the protease produced by our strain and other bacterial protease showed that our bacterial protease is very fast in de-hearing compared to other three.

Table8. *Comparison of De-Hairing ability of B. subtilis with other bacteria (1)*

	Time of incubation for de-hairing	Change of color of leather
Bacillus sp.	9h	no change
Vibrio sp kr2	24h	no change
Flavobacterium sp kr6	24h	no change
Bacillus sp kr10	24h	no change

4. Discussion

A bacterium isolated from local soil sample showing de-hearing activity of cattle hides and skins both qualitatively and quantitatively. After various tests it was suggested and the features agreed with the description of Bacillus sp in Bergey's Manual of Systematic Bacteriology [24].

It was also identified as Bacillus sp 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)*. Biochemical characteristics, morphological tests indicate that the organisms might be *Bacillus. B. pumilis* [2], *B. licheniformis* [8, 12]. Phylogenetic characterization using 16S rRNA gene primer could correctly classify the bacteria species.

Assocasein assay developed by Krege 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 [13, 25, and 27]. Therefore, it may be called a very good method for the large scale screening of bacterial protease [14, 11]

The characteristics of the culture filtrate 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 [4]. The assay condition was set up and the optimum temperature and pH of the enzyme was determined. The enzyme seems to have an optimum activity temperature of 60ºC and pH 8.5. This suggests that the enzyme might be an alkaline protease. A number of workers reported the isolation and characterization of alkaline protease from bacillus strain. This result is quite consistent with the work of other works. The enzyme seems to have an optimum temperature of 60ºC. Most proteases possess an optimum activity in the range of 30~80 °C, for example, protease from *B. pseudofirmus* AL-89 is of 60~70 °C [10], *Nocardiopsis* sp. TOA-1 is of 60 °C and a few have exceptionally high temperature optimum of 100 °C (18).

The gram staining showed that the bacteria were Gram-positive rod because the cells had a purple color under the microscope using a 100X (oil immersion) lens. The cellular arrangements of the bacteria were in chains. The spore staining gave green color that means it forms spore. 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 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 [23]. No effect of EDTA was detected on enzyme activity suggesting that the metal might not be involved in enzyme activity [15].

Goat's and cow's skin was qualitatively dehaired by overnight grown bacterial culture. The skin could be dehaired at room temperature within 8-12 hours. This was followed by dehairing with cell free extracts or culture filtrates. The culture filtrate could dehair the skin in specified time. Quantitative estimation has shown that 30mL of culture supernatant could dehair 8×8 cm of leather completely in 8 hours. This shows that the bacterial isolate produce moderate to high amount of enzyme for dehairing. Enzymatic dehairing in tanneries has been envisaged as an alternative to sulfides [16].

5. Conclusion

The culture characteristics and biochemical tests of the organism suggest that it is a thermophilic, Gram positive, spore forming and aerobic bacteria. Probabilistic identification of bacteria (PIB) is a bioinformatics tool that identified the bacteria was *Bacillus sp*. The characterization of protease so far showed that it is an alkaline protease, highly active at temperature near 60ºC. It may contain disulfide bonds and may be halo tolerant. 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 cattle hides and skins, 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.

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