

Isolation and Characterization of Protease producing *Bacillus* strain FS-1

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ABSTRACT

A bacterium was isolated from dog dung. Preliminary characterization of the organism according to Burgey's manual suggests that the organism may belong to *Bacillus* species. It has been provisionally named as *Bacillus* FS-1. It has grown at high temperature and pH. It secretes an exoprotease in the growth medium. It forms spores during adverse condition in the growth medium. The enzyme seems to be alkaline metallo protease and is capable of dehairing of skin and hides. The enzyme has commercial importance.

Keywords: Proteases, dog dung, *Bacillus*, alkaline, metallo, dehairing.

1. INTRODUCTION

Protease the enzyme which is catalyzes the total hydrolysis of protein. Protease is applied in pharmaceutical, food and detergent industries, waste treatment and others. Biotechnological importance of these enzymes has been realized by the leather industries for the purpose of dehairing and bating hides as a substitute toxic chemical (Bhosale et al. 1995). In food industry proteases are used as crude preparation. In pharmaceutical industry they are used as ingredients of ointments for debridement of wards and in medicine preparation (Jany et al. 1986). The Aim and objective was undertaken to isolate thermostable alkaline protease producing *Bacillus* sp from dog dung .The organism which produce large quantities of enzyme would be studied for over production. The objective of the studies was as: Identification of the bacterial organism, Growth in different media, Morphology under the microscope, Partial purification and Application on cow skin.

2. MATERIALS AND METHODS

Samples were collected from dog dung. Dog dung sample was collected from four dogs separately. The dog was fed raw meat at night before the collection of the dog dung. For collection of samples, spatula and bottles were used. Undesired bacteria were removed by heating from the sample at 80°C in a water bath for 30 minutes for isolation at heat stable protease producing *Bacillus* species. After heat treatment, samples were included in tripticase Soya broth medium 0.1 ml sample were inoculated in 5 ml broth media. The required ingredients of medium were weighed by an electronic balance and dissolved in distilled water. The medium was sterilized by autoclaving at 121°C for 15 min. The medium was then cooled and kept in

several tubes about 5 ml. Tubes were sterilized at 180°C for 1 hr in a hot air oven. Serial dilution was done for only single colony isolation. Sterilized test tube arranged in a rack. The over night nutrient broth grown culture was diluted up to 10^{-6} times in a series of test tubes containing 5ml sterile Soya broth. 0.1 ml of diluted culture from each tube was generously spread on nutrient agar plates. Inoculated at 37°C and plates containing well separated colonies were stocked for further use. From nutrient agar medium colonies were transferred in to blood agar media. Few colonies from nutrient agar media were collected and transferred to blood agar plates. Those colonies around which clear zone were formed were preserved for further studied. In order to identify the selected isolate, the following characteristics were studied according to Bergey's Manual of Systematic Bacteriology.

2.1 Microscopic Examination

2.1.1 Gram staining for the Bacteria:

A drop of sterilized distilled water was taken on the middle of the clear slide. Then a loopful bacterial suspension (young culture) was transferred to the sterilized drop of water and a very thin film was prepared on the slide by spreading uniformly. The film was fixed by passing it over the gentle flame for two or three times. The slide was flooded with crystal violet solution and allowed to stand for 30 sec and then washed thoroughly with gentle stream of tap water. The slide was then immersed in iodine solution for 1 minute and washed thoroughly with 95% alcohol for 10 sec. Alcohol was drained off and washed thoroughly with gentle stream of tap water. The slide was then covered with safranin for 1 minute. After washing with tap water and blotted dry it and examined under microscope.

2.1.2 Spore staining:

One drop of sterile saline water was taken on a clean glass slide for spore staining. A loopful bacterial old slant culture was taken in the drop and smear was made on the slide. The film was dried over flame gentle heating. The slide was then placed over a beaker and 5% malachite green was added drop wise on the slide. Boiling of the malachite green was avoided by adding more malachite green. The slide was taken out of the stream and washed gently with tap water. The preparation was needed with safranin solution for 1 min. and washed with gentle stream of tap water, and placed under immersion lens with immersion oil.

2.2 Biochemical Test

Tryptone broth was used as a basal medium for fermentation test. A 0.01 % or phenol red was used as an indicator. Fermentation tubes with 1.0 ml of basal medium provided with indicator were made and pH of the medium was adjusted at 7.5 with NaOH, the medium was sterilized at 121°C for 15 minutes. 1.0 ml of filter sterilized glucose, arabinose, xylose and manitol was taken in each tube. The tubes were then inoculated in duplicate with fresh culture of the bacterial isolates and allowed to incubate at 37° C for 72 hrs. The change of color of the indicator to yellow indicated the production of acid.

2.2.2 Catalase Test

Catalase Test carried out of one drop of 30% hydrogen peroxide was placed on a slide. One loopful of the fresh bacterial culture was taken by a sterile needle and placed on the drop of hydrogen peroxide. Bubble production indicated positive result.

2.2.3 Hydrolysis of Starch

Hydrolysis of Starch was carried out of 10 gm soluble starch in 100 ml distilled water was heated in water bath until dissolved. 20 ml of this solution was mixed with 100 ml of melted nutrient agar and poured in the petridish after sterilization. A loopful of fresh bacterial culture was picked up by the sterile needle and stabbed on to the agar plate; After 24 hrs of incubation at 37° C, the plate was flooded with dilute iodine solution. Hydrolysis of starch was indicated by a clear zone around the growth and unchanged starch gave a blue color.

2.2.4 Methyl Red Test

Methyl Red (MR) Test detects acid production to a sufficient degree (below 4.5) from glucose. One ml of fresh bacterial culture grown in glucose phosphate medium was taken in a test tube. Five drops of methyl red reagent was added and read immediately. Positive tests are light red and negative are yellow.

2.2.5 Indole Production Test

For the Indole, one loopful fresh bacterial culture (24 hrs old) was inoculated in peptone broth and incubated at 37° C for 1-3 days, after incubation, Kovac's solution was added and shaken vigorously for one minute. A red color in the reagent layer indicated positive reaction.

2.2.6 Nitrate Reduction Test

Nitrate reduction test was carried out in nitrate broth. The freshly prepared cultures were inoculated in sterile nitrate broth containing tubes and incubated at 37° C for 24 hrs. At the end of incubation 0.1 ml of solutions A was added followed by solution B in equal volume. The appearance of pink deep color showed that bacterial isolates reduced nitrate to nitrite.

2.2.7 Voges Proskauer Test

Voges Proskauer (V.P.) Test carried out of one ml of fresh bacterial culture was grown in phosphate peptone medium. After addition of 0.2 ml of 40% KOH, 0.6 ml of 5% alpha naphthol in absolute ethanol was added. After 10-15 minutes with vigorous shaking bright orange red color developed if acetyl methyl carbinol was present.

2.2.8 Citrate Utilization Test

For the Citrate Utilization Test, slope culture with a 1 inch butt of Simmon's citrate agar was inoculated by streaking over surface with a wire needle and incubated at 37° C for up to 3 days. The color of the medium changed from green to bright blue due to the utilization of citrate and when citrate is not utilized, the color of the medium remain unchanged.

2.3 Preparation of Subculture

A 250 ml conical flask containing 50 ml of sterile nutrient broth was generously inoculated with *Bacillus* strain FS-I. It was grown overnight in a psychrotherm incubated shaker incubator for overnight at 31°C. This was the subculture used for the preparation of batch culture. A set of 10 conical flasks each of 500 ml capacity containing 100 ml sterile nutrient broth was aseptically inoculated with 5 ml subculture in a laminar flow hood. The culture was incubated in a psychrotherm incubated shaker at a temperature of 31°C for 15-20 hours until the absorbance of

growing culture reached 1.5-1.8. The culture was then centrifuged at 10000 rpm in a sorvall RC-26 plus refrigerated super speed centrifuge for 10 minutes at 4°C. The supernatant was collected and used as crude enzyme sample.

2.4 Protease Assay

Quantitative assay of protease activity: Assay of Protease (by Azo-casein digest method): Protease activity was determined with azocasein as substrate by a modified procedure described by Kreger and Lockwood (1981). For the azocasein assay method 800 μ l of the sample and 400 μ l of 1.5% azocasein in 0.05 M Tris HCl (pH 8.5) was taken in a screw cap tube. The mixture was then incubated in a water bath at 37°C for 30 minutes. Then 2.8 ml of 5% TCA was added to stop the reaction and put on ice for 15 minutes. The solution was then centrifuged at 4000 rpm for 5 minutes. In the 2 ml of supernatant 2 ml of 0.5 N NaOH solution was added and mixed well. Absorbance was measured at 440 nm. Appropriate blank was also included. The blank was prepared in the same manner except that 2.8 ml of TCA was added before addition of enzyme. One unit of protease activity is defined as the amount of enzyme that produces an increase in the absorbance of 0.186 under the above assay condition.

2.4.1 Determination of the Effect of pH on Protease Activity

Azocasein was dissolved in TrisHCl buffer solution and the enzyme assay was carried out within pH range (7.0 to 10.0) by azocasein assay method. And of them were used at 0.05M Concentration.

2.4.2 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 CaCl_2 and buffer (0.2 M Tris-HCl buffer, pH 8.0) and incubated at 37°, 40°, 50°, 60°, 65°C temperatures.

2.4.3 Determination of Thermostability of Protease

The thermostability of the protease was determined in the range of 37° to 65°. For this purpose, the crude enzyme was incubated at 37°C, 40°C, 50°C, 60°C, 65°C for 30 minutes in a water bath. After cooling to room temperature the preheated enzyme preparations were added to the reaction mixture and incubated at 37°C for 30 minutes to determine the enzyme activity by the azocasein assay method. Enzyme kept at room temperature was taken as control.

2.4.4 Determination of Stability of Protease

Storage stability of protease was determined at -20°C, 4°C and at room temperature. The enzyme was preserved with sodium azide and kept at room temperature. NaN_3 is a toxic chemical and was added to the enzyme solutions at a final concentration of 0.05%. The activity of the enzyme was checked after regular time intervals (5 days). Two other sets of enzyme preparations were kept at -20°C and 4°C and the activity of the enzyme was checked after five days intervals.

2.4.5 Determination of the Effect of EDTA on Protease Activity

The protease after dialysis was preincubated with different concentration (0.5- 16mM) of EDTA for 10 minutes of 37°C temperature and the residual enzyme activity was determined by the

usual azocasein assay method. Control without EDTA was always used.

2.4.6 Ammonium Sulfate Fractionation of the Enzyme

All proteins are precipitated by ammonium sulfate. This is a technique used in the partial purification of enzyme. About 100 ml of culture filtrate was treated with ammonium Sulfate at of 70% saturation and most of the proteins were precipitated out. The precipitate was collected by centrifugation in a super speed sorval refrigerated centrifuge. The precipitate was redissolved in Tris HCl (0.05 M, P^H 8.0) buffer. The enzyme was dialyzed 3 times against the buffer. The enzyme activity was determined in both the precipitated and supernatant. Most of the enzyme activity lies with the precipitated fraction. This method may be applied for the concentration of the enzyme.

2.4.7 Ultra Filtration by Centricon

Ultra filtration using centricons was used to separate the proteins having molecular weight around 50 KDa. The protein having 50 KDa or above were retained while proteins having molecular weight less than 50 KDa passed through the membrane filtrate after centrifugation for 10 minutes at 5000 rpm in a Sorval super speed centrifuge. Study on some of the preliminary activities of the enzyme suggests that it is active at alkaline pH 8.5 resistant to moderately high temperature, sensitive to EDTA and can be precipitated. The enzyme was stored at -20°C for 50 days. These properties indicate that it might be a metallo protease.

3. RESULTS AND DISCUSSION

Although proteases are wide spread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, limited space required for their cultivation and the ease of separation. The microbes can also be genetically modified to generate new enzymes with altered properties and with very high yield, which are desirable for their various applications (Mala B. Rao et al. 1998). Among the microbes bacterial proteases have been widely studied. Most commercial proteases such as subtilisin and pronase are produced by organisms belonging to the genus *Bacillus*. Protease production, its characterization, genetic manipulation have been well studied in the *Bacillus* strains, *Bacillus Subtilis*, *Bacillus Stearothermophilus* in minute details by a number of workers (Phadatare et al. 1993).

The storage of the enzyme is a precondition for its isolation and purification. Protease enzyme is stable at 0-4°C. The inactivation of the enzyme begins after eight days. The inactivation of the enzyme due to its degradation was achieved by sodium azide was addition in the enzyme solution. Protease may be autolysed Wells & Powers, (1986) that was evidence by subtilisin autolysis. Autolysis of protease enhanced at room temperature occurs with certain conformational changes in structure.

Caseins, gelatins and blood proteins do not enter bacterial cells. For protease production the organism must grow under the condition that permits the solubilized proteins into the bacterial cell. To make the nutrient proteins soluble it needs the secretion of a protease by the cell. If this protease is temperature sensitive it can not digest the proteins at non purposive growth temperature. It is therefore reasonable to expect the bacteria to elaborate a thermostable protease.

The morphological and biochemical data (table 1) on the growth of the organism indicates that

the organism used in the study might belong to the genus of *Bacillus* organisms. But systematic study coupled with molecular study will reveal the true identity of the organism. Since the organism has industrial potentiality it should be identified by ribotyping method. FS-1 is the isolate number rather than any species or strain name.

Table 1: Biochemical Characteristics of the organism

Catalase test	Positive
Indole test	Negative
Nitrate Reduction test	Positive
Urease test	Negative
Methyl Red test	Positive
Lactose fermentation	Negative
Sucrose fermentation	Negative
Gelatin Hydrolysis	Positive
Casein Hydrolysis	Positive
Voges Proskauer test	Positive
Starch Hydrolysis	Positive
Citrate Utilization test	Positive

The organism is grown in liquid media containing tripticase soya protein. The organism secretes exoprotease in this media. Infect blood serum; casein media could also be tried for this work .For time constraint growth of the organism was followed in only one media .It was not followed whether any specific substance induced the synthesis of extracellular protease.

The enzyme was prepared by separating the cells from the culture filtrate. The enzyme activity was checked at 37°C at different time intervals. The enzyme activity was initially increased and then slowed down to become static. The experiment on the time course of enzyme activity clearly indicates that the enzyme is active in the culture filtrate at room temperature. Presumably it has been elaborated secreted by the organism during its growth period. Since the relative enzyme activity was used in the study enzyme units in term of proteins were not always used in the study .Changes in absorbance due to the growth of the organism. under a defined growth condition were measured with increase in enzyme activity.

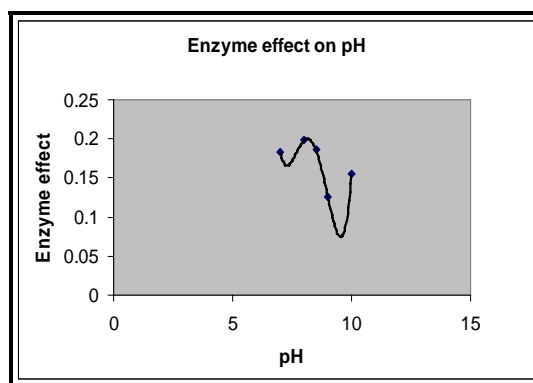


Figure 1: Enzyme effect on different pH

The important aspect of enzyme catalyzed reaction is the effect of pH, temperature and inhibitors on the enzyme. For any purpose use of an enzyme the effect these parameters have to be determined. The study of pH on the activity profile of the enzyme shows that the enzyme is active at the lower side of the alkaline pH. The enzyme has an optimum pH of 8.5 shows in figure 1.

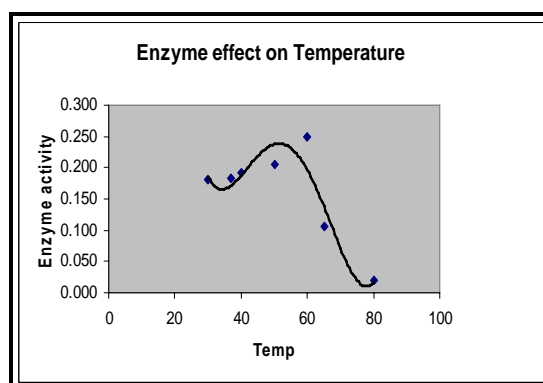


Figure 2: Enzyme effect on different temperature

The effect of temperature on the enzyme shows that its activity increases with increase in temperature and this result was reproducible by repeated testing showed in figure 2.

The effect of EDTA on the enzyme suggests that the enzyme is inhibited by the reagent. The inhibitory activity or EDTA increases with the increasing concentration of this reagent. The enzyme might require a metal ion to show its activity shows on figure 3. EDTA removes the metal the vicinity of the active site of the enzyme by chelation. It may be predicted from this result that the enzyme might be an alkaline, thermostable metallo protease.

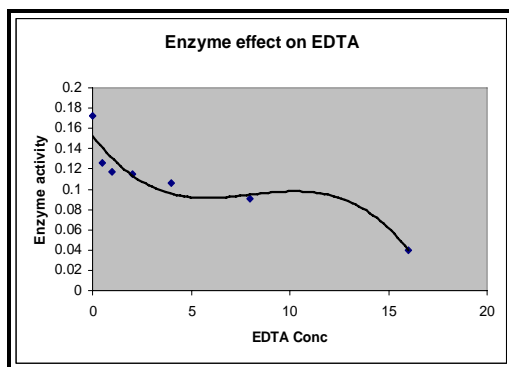


Figure 3: Enzyme effect on different concentration of EDTA

The result of ammonium sulphamate fractionated enzyme showed 0.325 unit/mg and supernatant enzyme activity was 0.082 unit/mg. Result of Ultra filtration by centricon (with 50 kDA cutoff) showed enzyme activity was 0.241 units/mg.

The work was carried out with crude enzyme and it is difficult to predict the behavior of an enzyme under such a condition. However, presence of impurities can not bring about drastic change in the activity profile of the enzyme. It may be said that temperature brings about certain conformational change in the active site of the enzyme, so that it better fits with the substrate. However, the major concern is not the mechanism of action of the enzyme rather achieving the persistence activity of the enzyme at high temperature.

Leather may be processed by detergents at higher temperature. So the temperature is resistance of the enzyme for its enormous potentiality in ternary and detergent industry.

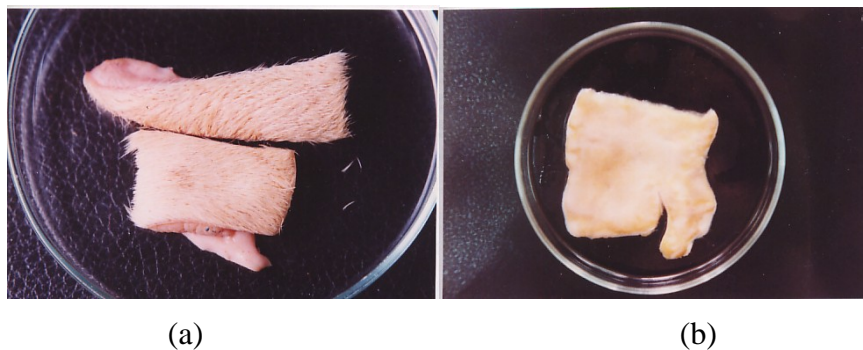


Figure 4: Effect of the Protease enzyme (a) Before applying on the cow skin
(b) After applying of the enzyme

Today enzymes find huge application in various industries. In tanneries alkaline protease has been involved in soaking, dehairing and bating of skin. There are about 175 tanneries in Bangladesh. As leather is one of the major exportable items it deserves quality processing by adoption techniques. The effluent discharging system of the tanneries causes severe pollution to the water bodies. The enzymatic dehairing and bating of hides have been widely accepted as a alternative to the chemical process (Manachini et al. 1988).

There are many tannery industries in Bangladesh. However, use of commercial enzyme (a

mixture of alkaline protease and lipase) in bating step needs import of about 400 MT of bating powder at a cost of BDT 25 million annually. Moreover foreign exchanges will be saved if Bangladesh tanneries become modernized and use enzymes in other steps of leather processing.

Duplication trial showed that alkaline protease obtained in the present study can be applied to leather tanning. This experimental protease removed 100% hairs of cow skin compared to lime-sulphide method. It can be successfully for keratin digestion at high pH. The new enzyme technology would make it possible to obtain good dehairing without using sulphide and other chemicals showed in figure 4. (Malathi and Dhar 1987) reported that enzymatic process is favorable compared to that of lime-sulphide method. Therefore, enzymatic dehairing is an efficient method to produce quality leather without causing pollution to the environment. The action of the enzyme on raw hides and skin shows that the enzyme can remove the hair of the skin within 24 hours. The enzyme might have gone to the hair follicle and digest action filament which attaches the hair to the skin.

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