Isolation, Production, Purification, Assay and Characterization of Alkaline Protease Enzyme from Aspergillus niger and its Compatibility with Commercial Detergents

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Abstract

Aspergillus niger is a highly potent fungus used in the production of alkaline protease. Extra cellular alkaline protease was purified from A. niger in a two-step procedure involving ammonium sulphate precipitation and Sephadex G-100 column chromatography. The molecular mass of the enzyme was determined to be 60 kDa by SDS-PAGE. The enzyme activity was also analyzed by zymogram with gelatin. The enzyme was more stable over a wide range of pH (6–12) and the temperatures up to 37 °C. It showed optimum enzyme activity at pH 8.0 and a temperature of 30°C. If the enzyme concentration is gradually increased then activity increases and if the enzyme concentration is kept constant, and the substrate concentration is gradually increased then also activity increases. The activity of the enzyme on substrate casein also increases with increase in concentration of the enzyme. The protease enzyme was completely inhibited by the serine protease inhibitor of phenylmethylsulfonyl fluoride (PMSF) and Calcium chloride was the most suitable activator. The crystallization of the purified enzyme was performed by hanging drop vapour diffusion method using PEG 6000 as the precipitant. The micro crystals occurred in 40-50% of PEG 6000. The enzyme was most compatible with commercial detergent Tide in absence of substrate and most compatible with sunlight in presence of substrate.

Keywords: Alkaline protease, PMSF, Aspergillus niger, Zymography, purification, characterization, detergent, crystallization.
Introduction
Protease enzyme breaks down proteins. It conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein. Proteases work best in acidic conditions. Proteases, also known as proteinases or proteolytic enzymes occur naturally in all organisms and belong to hydrolase class of enzymes, classified based on the source from which it is extracted, optimum temperature of activity. Proteases precisely act on peptide bonds formed by specific amino acids to hydrolyze them (Muhammad Ayaz Shaikh,, 2010).

An alkaline protease producing strain Aspergillus niger was isolated from local soil samples and enzyme production was optimized under submerged conditions. Maximum enzyme production of the culture occurred in mesophilic temperature 45°C and pH 8.5. Glucose and ammonium sulfate proved to be the best carbon and nitrogen sources respectively. The molecular weight of the enzyme determined by SDS-PAGE was found to be 38 kDa. The enzyme acted optimally at pH 10 and 50°C. It was thermo stable and retained full activity even at the end of 1 hour of incubation at 40°C. It was inhibited by Cu++, Hg++, Zn++, and EDTA and sodium azide. The enzyme retained more than 50% activity after 60 min incubation at 40°C in the presence of detergents such as Tide, Surf, Wheel and Henko indicating its suitability for application in detergent industry (Kalpana Devi et al., 2008).

The production of enzymes by bioprocesses is a good value added to agro industry residues. A comparative study was carried out on the production of protease using different varieties of Rice brokens (PONNI, IR-20, CR-1009, ADT-36 and ADT-66) from Rice mill wastes as substrates in solid-state fermentation (SSF) by Aspergillus niger. Among the all tested varieties of rice broken PONNI produced the highest activity as 67.7 U/g while ADT-66 produced lowest protease as 44.7 U/g/ under solid state fermentation conditions. The optimized conditions for producing maximum yield of protease were incubation at 35°C, 96 h and pH 7.0. The protease production from waste treatment could be commercially used in detergents and leather industry (R. Paranthaman et al., 2009).

A thermo stable alkaline protease was isolated from the Aspergillus niger Z1 strain in a liquid Czapek Dox medium, containing casein (1% w/v) as the sole nitrogen source. Enzyme extract was subjected to electrophoresis in SDS-polyacrylamide gel. Two protein bands were seen on polyacrylamide gel. Active enzyme was visualized in a zymogram and protease activity exhibited a molecular mass of 68 kDa on SDS-polyacrylamide gel. The optimum pH for activity was found to be 9.0. The temperature optima of the enzyme was found to be 40 °C at pH 9.0 and it remained stable up to 90 °C, with 48.4% of the original activity retained after heat treatment at 90 °C for 15 min. Proteolytic activity was inhibited by PMSF, but slightly inhibited by SDS (G. Coral et al .,2003).

In industrial products, the classification relates to the pH optimum of the respective enzymes. There are acidic, neutral, and alkaline proteases with some overlapping in definition (Rao et al., 1988).Proteases are physiologically necessary for living organisms, and are ubiquitous, being found in a wide diversity of sources such as plants, animals and microorganisms (Rao et al., 1988). Most commercial
proteases, mainly neutral and alkaline are produced by organisms belonging to the genus Bacillus. Fungi elaborate a wider variety of enzymes than do bacteria.

Alkaline proteases of microbial origin possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures (Rao et al., 1998; Agarwal et al., 2004; Kalpana Devi et al., 2008). The alkaline proteases find their largest use in house hold laundry with a worldwide annual production of detergents of approximately 13 billion tons (Nehra et al., 2002).

Alkaline proteases were in fact the first enzyme produced in bulk. Plant, animal and microbial sources are employed in enzyme production. Microbial proteases are preferred to plant and animal sources to various advantages (Kalpana Devi et al., 2008). A variety of microorganisms such as bacteria, fungi, yeast and Actinomycetes are known to produce these enzymes (Madan et al., 2002). Molds of the genera Aspergillus, Penicillium and Rhizopus are especially useful for producing proteases, as several species of these genera are generally regarded as safe (Sandhya et al., 2005). Aspergillus clavatus ES1 has been recently identified as a producer of an extracellular bleaching stable alkaline protease (Hajji et al., 2007, 2008).

The main drawback with production of bacterial protease is the requirement of cost intensive procedures for separation of enzymes from cells, on the other hand enzyme from fungal origin offer an advantage of separation of mycelium by simple filtration. Besides, the fungus can be grown on inexpensive substrates (Kalpana Devi et al., 2008).

The use of alkaline protease as active ingredient in laundry detergent is the single largest application of this enzyme (Nehra et al., 2002; Kalpana Devi et al., 2008). Earlier attempt at the production of detergent protease at commercial scale had problems due to the cases of development of allergic reaction of the workers by the enzyme dust. This was solved by the development of enzyme granules of average 0.5mm diameter. In this process enzyme in a mixture of inorganic salt and sugar as preservative, is present in an inner core with a protective colloid. This core is coated with polymers made from polyethylene glycol along with a hydrophilic binder. The granulated enzyme was found to be more resistant toward various additives like peroxide, optical brightener, and detergent present in the formulation (Sengupta et al., 2006).

For the production of enzymes for industrial use, isolation and characterization of new promising strain is a continuous process (Kumar et al., 2002). They are generally produced by using submerged fermentation due to its apparent advantages in downstream in spite of the cost intensiveness for medium components (Prakasam et al., 2005). Reports on bleach stable alkaline protease from fungal sources are scanty (Mulimani et al., 2002). Therefore, a need was felt to explore native fungal isolates, capable of producing alkaline proteases and at the same relatively stable at the operating conditions (Kalpana Devi et al., 2008).

Fungi elaborate a wider variety of enzymes than do bacteria (G.Coral et al., 2003). The application in the leather industry for dehairing and bating of hides to substitute currently used toxic chemicals is a relatively new development and has conferred
added biotechnological importance (Fogarthy, 1996). Thermo stability, especially alkaline properties of an *Aspergillus niger* strain protease should make it an excellent fungal enzyme for biotechnological aims (G.Coral *et al.*, 2003).

**Materials and Methods**

**Isolation of *Aspergillus niger* from soil sample**

*Aspergillus niger* was isolated from soil sample by serial dilution of the soil and a pure culture was prepared in potato dextrose agar media. After inoculating the media, the culture was kept at room temperature in dark for 5-7 days for growth.

**Identification of *Aspergillus niger***

*A. niger* was identified by physical characterization and using Lacto phenol cotton blue mounting of fungi. Lacto phenol cotton blue is a stain commonly used for making semi permanent microscopic preparations of fungi. It stains the fungal cytoplasm, and provides a light blue background, against which the walls of hyphae can readily be seen. It contains four constituents: phenol, which serves as a fungicide; lactic acid, which acts as a clearing agent; cotton blue, which stains the cytoplasm of the fungus; and glycerin; which gives a semi permanent preparation. A permanent preparation may be made by incorporating polyvinyl alcohol in place of glycerin into the mounting medium.

For rapid and routine examination of almost all types of fungi, spores and spore bearing structures are teased out on a clean slide in a drop of mounting fluid (lacto phenol cotton blue) and a cover glass placed over the preparation which is then ready for microscopic examination.

A drop of lacto phenol cotton blue was placed on a cotton slide. A small tuft of fungus, preferably with spores and spore bearing structures is transferred into the drop using the flamed cooled needle. The material is teased using the two mounted needles to mix gently the stain with the mold structures. A cover slip is placed on the top of the preparation. All the air bubbles are removed from the preparation by applying pressure or gently heating or by addition of more lacto phenol cotton blue. The excess mountant was removed from around the cover with 70% alcohol on a cotton swab or with blotting paper. The preparation is left overnight to dry.

**Screening of isolates for protease production**

Screening of protease production was done on gelatin plate assay as per Upadhyay *et al.* (2002). Fungal isolate was inoculated in Petri plate containing NAM with 1% gelatin and inoculated at 28°C for 5 days. The plate was flooded with 15% of HgCl₂ and the clear zone around the colony was observed.

**Isolation and production of alkaline protease from *A. niger***

*Organism and fermentation conditions:* *A. niger* was isolated from heavy metal contaminated soil sample. The culture was maintained on malt extract agar (MEA) and contained (g/L) glucose 20, malt extract 20, peptone 1, and agar 20 at 4°C. Fungal spores were obtained from 5–7 day old culture grown on MEA at 30°C. The spores
were collected in 0.01% (w/v) Tween-80 solution. The basal modified Mandels and Reese production media containing (g/L) defatted soybean 10 and KH2PO4 5, at a pH of 8-8.5. The fungus was grown aerobically in a 250 ml flask containing medium. The suspension of 1108 spores were inoculated into the 100 ml medium, and then incubated on a rotary shaker at 30°C, 250 rev/min for 10 days. The mycelia were separated from the fermentation broth by centrifugation at 6,000 rpm for 15 min and the clear supernatant was used for analysis. The supernatant is used as the crude enzyme extract. The amount of enzyme was expressed as unit per milliliter of the supernatant.

**Purification of alkaline protease**

**Ammonium sulphate fractionation**
The fibrinolytic enzymes were also purified by ammonium sulfate saturation. The protein fraction precipitated with 85% ammonium sulfate. The suspension was centrifuged at 10,000 rpm for 15 minutes. The precipitate was collected and dissolved in 25 mM NaCl; 10Mm Tris base buffer. Ammonium sulfate was found to activate the protease activity after dialysis.

**Dialysis**: The precipitate obtained after ammonium sulphate precipitation was dissolved in 25 mM NaCl; 10Mm Tris base buffer. Then it is put inside a semi permeable membrane bag and kept immersed in Tris base buffer for dialysis at 4°C. After dialysis the enzyme was taken out and centrifuged at 8000 rpm for 15 minutes. The supernatant is collected as the pure enzyme after dialysis.

**Ion exchange chromatography**: Protease enzyme was partially purified by using anion exchange column chromatography (DEAE Cellulose, MERK). Anion exchanger resin DEAE cellulose was used. 1 gm of DEAE cellulose was dissolved in 25 ml of distilled water and it was kept for 1 hour. Then filtration was done. The filtrate was soaked in 25 ml of 1 N HCl for 30 minutes and then washed with distilled water for 30 minutes. The process is again repeated. Then it is washed with 1 N NaOH and again washed with distilled water. The process repeated and then 25 mM NaCl, 10Mm Tris base buffer was added and kept for 30 minutes. Different fractions of (25%, 50%, 75%, 100%) 2ml Tris NaCl buffer was used. And the enzyme was eluted. For further purification, gel filtration with Sephadex G200 (MERK) gel equilibrated with 10mM Glycine-NaOH buffer (pH, 8) was performed. The active fractions were precipitated with acetone and then lyophilized.

**Protein estimation by Lowry method**
Protein can be estimated by different methods as described by Lowry and also by estimating the total nitrogen content. 5 test tubes were taken. In each of them, different volumes of BSA standard solution were poured and the volume was made up to 1ml by adding required amount of distilled water. Then 5 ml of reagent C was added. And the set up was incubated for 10 minutes at room temperature. Then 0.5ml of FC reagent was added in each of the test tubes and was incubated for 30 minutes in dark condition at room temperature. Then OD and concentration was measured at
visible range 660nm by a spectrophotometer. The procedure was repeated for
different volumes of crude enzyme instead of BSA standard and OD and
concentration was measured at 660nm.

Assay of enzyme
Then assay of the different fraction of alkaline protease enzymes was determined by
spectrophotometrically. The reaction mixtures contain 0.5% of casein and 2.95 ml of
0.1 (M) Tris HCl buffer, pH-8.5 and 0.1 ml of each of crude enzyme, pure enzyme
after ion exchange, and all fractions of eluted enzymes were incubated at 50˚C. After
10 minutes, the reaction was stopped by adding 3ml of cold 10% TCA. After 1 hour,
the culture filtrate was centrifuged at 8000 rpm for 5 minutes to remove the
precipitate and absorbance was read spectrophotometrically at 280nm. Enzyme
activity was calculated by measuring mg of tyrosine equivalent released and
compared with the standard. One unit (U) of enzyme activity represents the amount of
enzyme required to liberate 1µg of tyrosine under standard assay condition.

Assay of enzyme when concentration of enzyme was constant
Assay of alkaline protease to determine the activity of enzyme
spectrophotometrically: Reaction mixture containing different volumes of 0.5 % of
casein in 3 ml of 0.1 (M) Tris HCl buffer, pH-8.5 and 0.1 ml of crude enzyme was
incubated at 50˚C. After 10 minutes, the reaction was stopped by adding 3ml of cold
10% TCA. After 1 hour, the culture filtrate was centrifuged at 8000 rpm for 5 minutes
to remove the precipitate and absorbance was read spectrophotometrically at 280nm.

Assay of enzyme when concentration of substrate was constant
Similarly another set of reaction mixture was prepared containing a constant volume
of 0.5ml of 0.5% casein in 3 ml of 0.1 (M) Tris HCl buffer, pH-8.5 and different
volumes of enzyme was incubated at 50˚C . After 10 minutes, the reaction was
stopped by adding 3ml of cold 10% TCA. After 1 hour, the culture filtrate was
centrifuged at 8000 rpm for 5 minutes to remove the precipitate and absorbance was
read spectrophotometrically at 280nm.

Caseinolytic activity of alkaline protease
In each of six test tubes, 0.5ml of 0.5% of casein solution was taken and different
volumes of crude enzyme were taken. The volume was maintained at 1.5 ml by
adding required amount of distilled water. And then 5ml of reagent C was added. Test
tubes were incubated at room temperature for 10 minutes. Then 0.5ml of FC reagent
was added. The set up was incubated for 30 minutes in dark condition. Then
concentration and OD was measured at 660nm.

Enzyme Assay by plate zone method
Activity of alkaline protease was determined by casein and gelatin plate method. The
Casein solution [2.5 ml of 2% (w/v) gelatin (Merck) in 0.1M Sodium Phosphate
buffer, pH 8] was missed with 2ml of skim milk after sterilization agarose solution in
Petri dish (100by15mm). After the dishes were allowed to stand for 30 min at room
Isolation, Production, Purification, Assay

The holes were made on a plate by suction by using steel gel puncture (0.5cm). 50µl and 100µl enzyme solution was dropped into each hole and incubate at 30°C for 18 hours. After measuring the dimension of the clear zone, the number of units was determined. One unit of the enzyme activity was defined as the amount of enzyme in 25µl of enzyme solution that produced a clear zone of 1mm² at pH 8 and 30°C for 18 hours.

Characterization of alkaline protease

Effect of pH on the crude enzyme

Reaction mixture containing 0.5 ml of 0.1M of Tris HCl buffer, 0.5ml of 0.5% casein as substrate, 0.1 ml of crude enzyme and 3ml of reagent C was maintained at different pH (3-14) and incubated for 10 minutes. Then FC reagent 0.5ml was added. And the set up was kept in incubator for 30 minutes 30°C. OD was measured at 660nm.

Effect of temperature on the activity of enzyme

0.1ml of enzyme was treated at different temperatures ranging from 20°C to 100°C. Then 0.5ml of 0.5% of casein, 0.4ml of distilled water, and 3ml of reagent C were added and incubated for 10 minutes at room temperature. Then FC reagent was added and incubated for 30 minutes in dark condition. Then OD was measured at 660nm.

Action of activator in enzyme activity

0.5 ml of 0.5% casein solution was incubated at 30°C for 10 minutes. MnCl₂, CaCl₂, FeSO₄, FeCl₃ (1%) were chosen as activators. 0.5 ml of activator was added. Then 0.1 ml of enzyme solution was added. Then incubated at 30°C for 10 minutes, then 5ml of reagent C and 0.1ml of distilled water were added and incubated for 30 minutes at 37°C. Then filtrated using wattmann’s filter paper. 2ml of the filtrate was taken and to it 0.5 ml of reagent A and 0.5 ml of FC reagent were added and incubated at 30°C for 30 minutes. Then OD was measured at 660nm.

Effect of inhibitor on the activity of enzyme

0.5 ml of 0.5% casein solution was incubated at 30°C for 10 minutes. EDTA, AgNO₃, Potassium Ferrocyanide, PMSFS, hydrogen peroxide, SDS (1%) was chosen as inhibitors. 0.5 ml of inhibitor was added. Then 0.1 ml of enzyme solution was added. Then incubated at 30°C for 10 minutes, then 5ml of reagent C and 0.1ml of distilled water were added and incubated for 30 minutes at 30°C. Then filtrated using wattmann’s filter paper. 2ml of the filtrate was taken and to it 0.5 ml of reagent A and 0.5 ml of FC reagent were added and incubated at 30°C for 30 minutes. Then OD was measured at 660nm.

Polyacrylamide Sodium Dodecyl Sulphate Slab Gel electrophoresis (SDS PAGE)

Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out at 10% (w/v) isolation gel and 5% (w/v) concentration gel according to the method of Laemmli where 2-mercaptopethanol was used as reducing agent (Grebeshova et al., 1999). The molecular weight was determined by interpolation from a linear semi-logarithmic plot of relative molecular mass versus the Rf value (relative mobility). And the following
proteins were used as standards: phosphorylase (97.4 kDa), BSA (66.2 kDa) and trypsin inhibitor (20.1 kDa).

**Immobilization**
For industrial exploitation of enzyme, it was immobilized by Ca alginate and Polyacrylamide gel entrapment methods (Mosbach, 2005). Enzyme leakage was checked by taking 1 ml of water in which immobilized beads were dipped for 24 h at 4°C, in place of enzyme the water in which beads were kept was taken and activity was determined by standard assay procedure.

**Zymogram analysis:** For the activity staining of the protease activity, the polyacrylamide gel (10%), containing 0.1% gelatin, was prepared. The gelatin was incorporated into the separating gel prior to the addition of ammonium per sulfate and polymerization. To prepare a zymogram, enzyme samples were mixed with electrophoresis sample buffer without heat denaturation prior to electrophoresis. The gel was then developed by soaking in staining solution and subsequently rinsed with methanol-acetic acid-water solution (4:1:5, by volume).

**Study of compatibility with commercial detergents**
Detergent solutions (Tide, surf excel, sunlight, Websi, Ariel) at a concentration of 7µg/ml were prepared in double distilled water. This solution were boiled for 10 minutes to destroy any protease already present and cooled. Fixed enzyme concentration was added and incubated for 20 minutes at 30°C. Then 3ml of 10% of TCA solution was added in each test tubes, kept at 30°C for 1 hour. Then OD was measured at 280nm.

A reaction mixture was prepared which contained 1ml of 1% of casein in a 0.1M of phosphate buffer (7.5) and 0.5ml of detergent solution (7 µg/ml) were added to it. After that 10 minutes incubation was done. Then 1 ml of crude enzyme solution was added to it and incubated for 20 minutes at 30°C. Then 3ml of 10% of TCA solution was added in each test tubes, kept at 30°C for 1 hour. Then OD was measured at 280nms.

**Crystallization of alkaline protease by vapor diffusion method**
A purification protocol of alkaline protease purification using crystallization was developed by investigating the effects of pH, temperature, initial enzyme concentration, salt (as crystal inducer) concentration, and the presence of impurity proteins. The crystallization of the purified enzyme after ion exchange was performed by hanging drop vapour diffusion method using PEG 6000 as the precipitant and NaCl was used as a crystal inducing salt. The micro crystals occurred in 40-50% of PEG 6000. Both entail a droplet containing purified protein, buffer (25 mM NaCl; 10Mm Tris base buffer pH 9), and precipitant being allowed to equilibrate with a larger reservoir containing similar buffers and precipitants in higher concentrations. Initially, the droplet of protein solution contains an insufficient concentration of precipitant for crystallization, but as water vaporizes from the drop and transfers to the reservoir, the precipitant concentration increases to a level optimal for
crystallization. Since the system is in equilibrium, these optimum conditions were maintained until the crystallization was completed. The optimum condition for the crystallization was: pH 9.0, 25°C temperature, PEG 53 mg/ml or higher enzyme concentration, and minimum 5 % (w/w) NaCl concentration. (Rhodes, 1993; McRee, 1993).

Results
Isolation of *Aspergillus niger* from soil sample: It was observed that fungal colony was black in colour and spongy in nature. The time course of protease secretion and substrate degradation by *A. niger* in a basal Mandels and Reese salt medium containing defatted soybean was determined by batch culture. After complete incubation extract was prepared from culture supernatant, which acts as crude protease enzyme. The extra cellular protease activity of crude enzyme extract increased during cell growth and reached maximum value 90.50 U/ml at 4th day of incubation, thereafter decrease slowly at periodic incubation. Approximately Similar observation was recorded Haq *et al.* (2006).

![Figure 1.1: 3 day old culture of Aspergillus niger.](image1)

![Figure 1.2: 6 day old culture of Aspergillus niger.](image2)

Identification of *Aspergillus niger*

![Figure 2: Aspergillus niger as seen under microscope.](image3)
The protein estimation of alkaline protease by Lowry method: The graph obtained from this experiment clearly showed the quantity similarity between the protein standard BSA and crude of alkaline protease. The protein content of the working standard BSA was equal to that of the crude up to a concentration of 0.3µg/ml but on increasing the concentration further, it was observed from the graph that the protein content of the crude was higher than that of the BSA.

![Graph showing protein estimation by Lowry method.](image)

**Figure 3:** Protein estimation of alkaline protease by Lowry Method.

Assay of alkaline protease: When substrate concentration was kept constant and enzyme concentration was gradually increased then an increase in activity of the enzyme was observed.

![Graph showing assay of alkaline protease when substrate concentration is constant.](image)

**Figure 4:** Graph showing assay of alkaline protease when substrate concentration is constant.

Assay of alkaline protease: when the enzyme concentration was kept constant and substrate concentration was gradually increased, and again an increase in its activity was observed.
Isolation, Production, Purification, Assay

**Figure 5:** Graph showing assay of alkaline protease when enzyme concentration is constant.

Activity of enzyme eluted by ion exchange chromatography: Enzyme alkaline protease was purified by ion exchange chromatography. The elute obtained by adding 50%, 75%, 100% fraction of the Tris base NaCl buffer showed very less activity in the assay, but the elute obtained by adding 25% of buffer showed 40% activity and the pure enzyme eluted showed 90% activity. The crude enzyme was showed about 67% activity in the assay.

**Figure 6:** Graph showing effect on activity of alkaline protease after ion exchange chromatography.

**Caseinolytic activity of alkaline protease:** The graph showed that with an increase in concentration of enzyme, the enzyme-substrate interaction was increased and the product concentration increased, so, the activity was increased. But after a certain point, even on increasing the concentration of the enzyme, the activity did not increased but remained constant.
The activity of alkaline protease was also measured by casein and gelatin plate technique. One unit of enzyme activity was defined as the amount of enzyme in 25µl of enzyme solution that produced a clear zone of 1mm² at pH 8 and 30°C for 18 hours.

25µl - 1mm²  1U
50µl - 20 mm²  10U
100µl - 30 mm²  15U

The 10 U for 50µl and 15U of activity for 100µl were achieved by alkaline protease.

**Effect of pH on the crude enzyme:** The optimum pH for activity of enzyme protease was 9 and the enzyme activity decreased rapidly at level below pH 5. The enzyme
was very stable in the pH range of 7 to 11 at 30°C for 2 – 3 hours. Above pH 11, enzyme activity abruptly decreased.

Figure 9.1: Column graph showing effect of pH on the activity of alkaline protease.

Figure 9.2: Line graph showing effect of pH on the activity of alkaline protease.

Effect of temperature on the activity of crude enzyme: The effect of temperature on the activity of alkaline protease was examined. The temperatures showing maximal enzyme activity was between 27°C to 37°C. But as the temperature was gradually increased, the activity decreases due to denaturation of the enzyme. It was concluded that the enzyme was most active at an optimum temperature of 27°C.
**Figure 10.1:** Column graph showing effect of temperature on the activity of alkaline protease.

**Figure 10.2:** Line graph showing effect of temperature on the activity of alkaline protease.

**Action of activator in enzyme activity:** When 0.1 ml of the enzyme was incubated at 37°C for 10 min. with 0.1% of manganous chloride, calcium chloride, ferrous sulphate, ferric chloride then it was observed that enzyme activity shows maximum increase with calcium chloride whereas enzyme activity was partially activated by MnCl₂, FeSO₄, and FeCl₃.
Figure 11: Graph showing action of activator on the activity of alkaline protease.

Effect of inhibitor on the activity of crude enzyme: When 0.1 ml of the enzyme was incubated at 37°C for 10 min. with 0.1% of EDTA, AgNO₃, K₄Fe(CN)₆, HgCl₂ and H₂O₂ then PMSF showed maximum inhibition. Enzyme activity was partially inhibited by EDTA, H₂O₂ and SDS but no inhibition was showed by rest.

Figure 12: Graph showing relative activity of alkaline protease due to action of different inhibitors.

SDS PAGE: The protein bands found on SDS PAGE for alkaline protease were approximately 60 kDa and 58 kDa. It was concluded that the molecular weight of purified protein was approximately 60 kDa.
<table>
<thead>
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<th>Sl.No.</th>
<th>Samples</th>
<th>Molecular Weight</th>
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<td>1</td>
<td>Marker</td>
<td>97.4, 66.2, 20.1 KDa</td>
</tr>
<tr>
<td>2</td>
<td>Crude</td>
<td>60 kDa</td>
</tr>
<tr>
<td>3</td>
<td>Pure</td>
<td>60 kDa</td>
</tr>
<tr>
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</tr>
<tr>
<td>7</td>
<td>100% fraction</td>
<td>60 kDa</td>
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</table>

**Figure 11:** SDS PAGE showing the molecular weight of alkaline protease enzyme produced by *A. niger* (60 kDa).

**Zymography:** The activity of alkaline protease was also observed by Zymography. The gel containing gelatin as substrate was partially digested by crude enzyme lane (2) and completely digested by pure enzyme after ion exchange (3), 25% fraction (4), 50% fraction (5) and 75% fraction while 100% fraction was partially digested by alkaline protease at room temperature for 20 hours.

**Figure 12:** Digestion of substrate gelatin was showed by enzyme alkaline protease.
Compatibility with Commercial Detergent

Compatibility of alkaline protease with various commercial detergents in presence of substrate: In presence of substrate, alkaline protease retained 98.7% of its activity in presence of sunlight. Alkaline protease was 85.14%, 83.5%, 78.35% compatible with Ariel, Surf Exel, and Websi respectively and the enzyme was the least compatible (73.71%) with Tide in presence of casein as the substrate.

![Graph showing compatibility of alkaline protease with various detergents (in presence of substrate).](image)

**Figure 13.1:** Graph showing compatibility of alkaline protease with various detergents (in presence of substrate).

Compatibility of alkaline protease with various commercial detergents in absence of substrate: It is observed that Tide was 98% compatible with alkaline protease in absence of the substrate whereas Websi was least compatible (72.05%) with it. The enzyme retained 80-90% of its activity in presence of the rest of the detergents like Sunlight, Surf Exel and Ariel.

![Graph showing compatibility of alkaline protease with various detergents (in absence of substrate).](image)

**Figure 13.2:** Graph showing compatibility of alkaline protease with various detergents (in absence of substrate).
Discussion

The assay of enzyme alkaline protease showed that on increasing the concentration of the enzyme the activity increased but after a certain point the activity became constant in accordance to Michaelis Menten Equation. Crude enzyme showed maximum activity at a temperature of 27°C in contrast to the maximum protease production observed at 45°C according to Kalpana Devi et al., 2008, in contrast to present study enzyme activity was showed maximum activity at 27°C but maximum protease production was observed at 32°C. Another factor affecting the production of protease is the initial pH of the medium. Protease production by Aspergillus niger was observed in the range 7-9 pH by Kalpana Devi et al., 2008. Similarly, the present study also observed that the crude alkaline protease showed maximum activity at pH 8-9. The results clearly indicated alkalinophilic nature of the fungus. Likewise pH 7 has been reported to be optimum for Aspergillus flavus (Sutar et al., 1992). The results of (Sumandeep et al. 1999) showed that alkaline protease appeared to be more stable at alkaline pH 9. Addition of 5mM CaCl₂ enhanced the activity 105.3% of alkaline protease enzyme produced by Aspergillus niger according to the study of Kalpana Devi et al., 2008. Likely, it was observed that calcium chloride was the best activator of the crude alkaline protease; in contrast to our investigation same result was observed that CaCl₂ was the best activator. According to Kalpana Devi et al., 2008, the enzyme was 90% inhibited by metal chelator EDTA. The similar results were observed by (Madan et al. 2002) for Bacillus polymyxa alkaline protease. In contrast to our investigation inhibition of enzyme was observed that PMSF and SDS are better inhibitors than EDTA for the alkaline protease. In the present study molecular weight of the enzyme was 60 kDa as determined by SDS PAGE. But in contrast previous study, the molecular weight of purified enzyme as determined by SDS PAGE was found to be 38 kDa (Kalpana Devi et al., 2008). The molecular weight in the range of 32-33 kDa has also been reported for the enzyme from Malbranchea inlchella (Voordouw et al., 1974). Arial detergents retained only 23% of enzyme activity (Kalpana Devi et al., 2008). However, in the present study, it was observed that in presence of Tide, the crude retained 98% of its activity. The enzyme retained 80-90% of its activity in presence of the rest of the detergents like Sunlight, Surf Exel and Ariel. The activity of enzyme also showed on zymogram in present study, it conclude that about 60 kDa molecular weight of enzyme showed a clear digestion on zymogram.

Conclusion

The alkaline protease isolated from A. niger is a serine protease. It is stable at alkaline pH 8-9 and temperature 27-37°C. The molecular weight of enzyme was found to be around 60 kDa by SDS PAGE. The protease activity was inhibited by PMSF, SDS, and EDTA and was activated by CaCl₂. Substrates specificity tests indicated that casein was the best substrate among three substrates tested (casein, BSA and gelatin). These properties indicate that the possibilities for use of the protease in the detergent industry. This enzyme can be exploited commercially.
Acknowledgements
I would like to thanks my M.D. (Smt.Taposi Char) for their kind inspiration and help. Without their help this research would not have been possible. I would also like to thank my parents for their blessings and support. I am also thankful to all my friends for their help.

Reference


