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PURIFICATION AND CHARACTERIZATION OF ALKALINE PROTEASE PRODUCED BY A MUTANT STRAIN OF *BACILLUS SUBTILIS*

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Abstract

The present study describes the production, purification and characterization of alkaline protease from mutant strain of *Bacillus subtilis* EMS-8. The enzyme was purified using ammonium sulphate precipitation which gave 2.64 fold purification with 81.5% yield at 70% saturation. The molecular weight of the enzyme was determined using SDS-PAGE and it was found to be 25 KDa. The optimum pH of enzyme activity was 8.5; however the enzyme remained stable up to pH 10 after 24 hrs of incubation. Similarly, the optimum temperature for enzyme activity was 40ºC, whereas it remained stable up to 90ºC with greatly reduced activity. Alkaline protease showed highest specificity towards casein. Among different inhibitors, Phenylmethylsulphonyl fluoride (PMSF) completely inhibited the enzyme activity indicating the serine nature of protease. Similarly, the protease activity was greatly reduced in the presence of $MnCl_2$, whereas $MgCl_2$ enhanced its activity. The shelf life of the protease was also determined and it was found that the activity of the enzyme came to an end after second week, when the enzyme was stored at room temperature.

Introduction

Proteases belonging to the class of Hydrolases (EC. 3.4) are enzymes that conduct protein catabolism by hydrolysis of the peptide bonds (Sigma and Mooser, 1975). Proteases with their high temperature resistant quality, high specific activities and their superior chemical and physical stability characteristics would seem to be good candidates for current and future biotechnological applications (Woods *et al*., 2001). They are found in wide diversity of sources such as plant, animals and microorganisms. Microbial cells are the usual sources of proteases for industrial use. Microbial proteases account for approximately 40% of the total worldwide enzyme sale (Godfrey & West, 1996).

Among microorganisms, bacteria are of great practical and scientific interest to the biotechnologists. Many bacteria synthesize and excrete large quantities of proteases into surrounding medium and they are the most dominant group of alkaline protease producers (Genckal & Tari, 2006). *Bacillus* species are commercially very important producers of proteases. Many species of *Bacillus* have been reported to produce extracellular proteases which are used in food, brewing and laundry industry (Horikoshi, 1999; Barredo, 2004; ; Ahmad *et al.,* 2011). These include *Bacillus amyloliquefaciens* (George *et al.,* 1995), *B. megaterium* (Joo, 2005), *B. subtilis* (Haq & Mukhtar, 2006; Shaheen *et al*., 2008; Adinarayana *et al*., 2005), *B. stearothermophilus* and *B. thermoproteolyticus* (Rehman *et al*., 2005).

Purification of proteases is carried out using different techniques which include precipitation methods (Arulmani *et al.,* 2007; Dumont, 1994), chromatographic methods (Thumar & Singh, 2007; Gupta *et al*., 2005) and electrophoretic methods (El-Shanshoury *et al*., 1995). Purification by precipitation can be carried out by inorganic salts, organic solvents and high molecular weight polymers. Salting out of proteins by inorganic salts can fulfill dual purpose of both purification and concentration of specified proteins. The most commonly used salt is ammonium sulphate (Towatana *et al*., 1999; Iftikhar *et al.,* 2011). At saturation, it is of sufficiently high molarity that causes precipitation of most proteins. Various organic solvents such as methanol, ethanol, propan-2-ol, acetone and diethyl ether can also be employed for the precipitation of proteins. The addition of organic solvents to aqueous solutions of proteins reduces the solubility of proteins by reducing the dielectric constant of the medium (El-Shanshoury *et al*., 1995). As increasing amounts of organic solvent are added, protein molecules tend to interact more with other protein molecules than with water and get precipitated. However, large scale precipitation of enzymes with organic solvents is not recommended. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is an excellent method which is used to identify and monitor proteins during purification, to obtain the homogeneity of purified fractions and to determine their molecular weights (Laemmli, 1970; Iftikhar *et al.,* 2008). Single band in such a gel is a criterion of purity (Andrews, 1986). The enzyme once isolated and purified, is characterized to determine the effect of pH, temperature, additives, inhibitors and chelators on its activity and stability.

The main goal of present study is the partial purification and characterization of alkaline protease produced by a mutant strain of *Bacillus subtilis* EMS-8 so that its industrial applications can be optimized.

Materials and Methods

Microorganism and maintenance: The microorganism used in the present study was a mutant strain of *Bacillus subtilis* EMS-8 which was obtained from the culture bank of Institute of Industrial Biotechnology, G.C. University, Lahore. Culture was revived and maintained by sub culturing on the nutrient agar slants and was stored in the cold cabinet at 4ºC for further use.

Shake flask experiments: The bacterial inoculum was prepared in 250 ml Erlenmeyer flask containing 50ml nutrient both. The flask after sterilization was aseptically inoculated with *Bacillus subtilis* EMS-8 from a 48 hrs old slant. The flask was then placed in incubator shaker, rotating at a speed of 200 rpm, at 37ºC for 24 hrs. One millimeter of this inoculum was then transferred to each flask containing fermentation medium for protease production.

Fifty ml of fermentation medium (g/l: soybean meal, 20; starch, 10; glucose, 5.0; polypeptone, 10; KH_2PO_4, 1.0; $(NH_4)_2SO_4$, 1.0 and Na_2CO_3, 5.0) contained in 250ml Erlenmeyer flasks was used for the production of extra cellular protease by *Bacillus subtilis*. The flasks containing sterilized production medium were inoculated with 1 ml of inoculum and were incubated in an incubator shaker at 37ºC at 200 rpm for a time period of 48 hrs. After 48 hrs, the contents of the flasks were subjected to centrifugation at 6,000 rpm for 10 minutes. The supernatant was used for enzyme purification and characterization. The fermentation experiments were carried out using already optimized cultural conditions (Mukhtar & Haq, 2008).

Protease assay: The proteolytic activity of the fermentation both was assayed by the method of McDonald & Chen (1965). To 1ml of enzyme, 4ml of 1.0 % casein was added. The reaction was carried out at 37ºC for one hour and was stopped by adding 5 ml of 5% TCA. The precipitates were allowed to settle for 15 minutes. The contents of the tube were centrifuged at 6000 rpm for 10 minutes and one milliliter of the supernatant was mixed with 5ml of alkaline reagent. Then 1ml of 1N NaOH and 1ml of Folin and Ciocalteau reagent were added; as a result blue colour was produced which was read at 700 nm on UV/VIS spectrophotometer.

One unit of protease activity is defined as the amount of enzyme that releases 1 µg of tyrosine per ml under defined conditions.

Protein estimation: The protein content was measured by the method of Bradford (1976), using BSA as standard.

Enzyme isolation and precipitation: The fermentation broth, after fermentation was subjected to centrifugation at 10,000 g for 10 min at 4ºC. The pellets containing cells and other debris were discarded and the supernatant containing alkaline protease and other soluble proteins were subjected to ammonium sulphate precipitation for partial purification. Saturation between 30 to 80% was achieved. Ammonium sulphate was added slowly in the supernatant which was then stirred gently for 1 hr using a magnetic stirrer. After 1 hr, the pellets were collected by centrifugation at 10,000 g for 15 min at 4ºC and the pellets of different fractions were resuspended separately in Tris-HCl buffer (pH 8).

The resuspended pellets were dialyzed against Tris-HCl buffer (pH- 8) by putting them in a visking dialysis membrane with a molecular weight cut off (MWCO) value of 12000-14000. The salt was removed out of dialysis bag by constant stirring for 12 hrs and periodic changeover of buffer. The protease activity and protein content of the dialyzed sample were assayed by respective methods.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): The sample for SDS-PAGE was prepared by mixing the dialyzed fraction of alkaline protease with the sample buffer in a ratio of 1: 1. The mixture was heated at 95ºC for 5 min by suspending the sample tubes in the boiling water. Using micropipette, 10 µl of each sample was loaded into the wells formed in the stacking gel and electrophoresis was carried out. After complete electrophoresis, the gel assembly was removed and the stacking gel was cut off and discarded. All the steps were carried out at room temperature.

The gel was transferred into a fixative or staining solution of Coomassie Brillaint Blue R-250 for about 30 min with gentle agitation at room temperature. After staining, the gel was destained using a destaining solution until the stained background had been satisfactorily removed and protein bands became clear.

Results and Discussion

Production and purification of enzyme: Fermentation experiments for the production of alkaline protease by *Bacillus subtilis* EMS-8 were carried out in shake flasks. The flasks were incubated for 48 hrs and after 48 hrs of incubation, the contents of the flasks were assayed for the proteolytic activity. The results showed that the mutant strain of *Bacillus subtilis* EMS-8 produced 897.72 U/µg/ml.

Fermentation broth after centrifugation was subjected to ammonium sulphate precipitation at levels of 30-80% (w/v) saturation. Ammonium sulphate fractions were separately collected and it was observed that the fractions of ammonium sulphate obtained between 30-50% saturation did not show any protease activity. The enzyme started to precipitate when the concentration of ammonium sulphate was raised upto 60% (679.15 U/µg/ml) and the fraction collected at 70% saturation gave the highest units of protease (730.86 U/µg/ml).

Different buffers including Tris-HCl buffer, Sodium Potassium Phosphate buffer, Potassium Phosphate buffer (0.1 M; pH 7.5) and water were used for resuspending the enzyme pellets. It was observed that the purification results were best (730 U/µg/ml) when the pellets were dissolved in Tris-HCl buffer and dialyzed against the same buffer. The pH of the dialysis buffer was also optimized and it was observed that there was a maximum recovery of alkaline protease when the pH of the buffer was adjusted at 8.0 (734 U/µg/ml). Similar results have been reported by Zhu *et al*., (2007) and Heidari *et al*., (2007) who have also used Tris-HCl as a dialysis buffer (Table 1).

Table 1. Purification summery of alkaline protease produced by *B. subtilis* EMS-8 using ammonium sulphate precipitation.

Ammonium sulphate (%)	Protease units (U/µg/ml)	Protein (µg/ml)	Specific activity (U/µg/ml)	Recovery (%)	Purification fold
Crude	897.72	321	2.79	100	1
20	-	318	-	-	-
30	-	310	-	-	-
40	-	279	-	-	-
50	-	294	-	-	-
60	679.15	131	5.18	75.66	1.85
70	730.86	99	7.37	81.5	2.64
80	-	85	-	-	-

Characterization of alkaline protease

Effect of pH on protease activity and stability: The activity of partially purified protease was measured at different pH values ranging from 7.0-12.0. From the results, it was observed that there was a gradual increase in the activity of protease when the pH of the enzyme was raised from 7.0 (590 U/µg/ml) and reached maximum (735 U/µg/ml) when the pH was adjusted at 9.0 (Fig. 1). As the pH of the reaction mixture was increased beyond 9.0, there was a gradual decrease in the protease activity up to pH 10 (630 U/µg/ml) and the enzyme activity was greatly reduced at pH 12 (421 U/µg/ml). The protease stability at different pH values was also determined and the results showed that the enzyme was stable between pH ranges of 7.0 to 12.0 after 1hr of contact and showed 52 % residual activity at pH 12.0. However after 24 hrs of incubation, it was observed that the enzyme was stable only at a pH range of 8.5 to 10. It becomes unstable beyond pH 11.0 (Fig. 2).

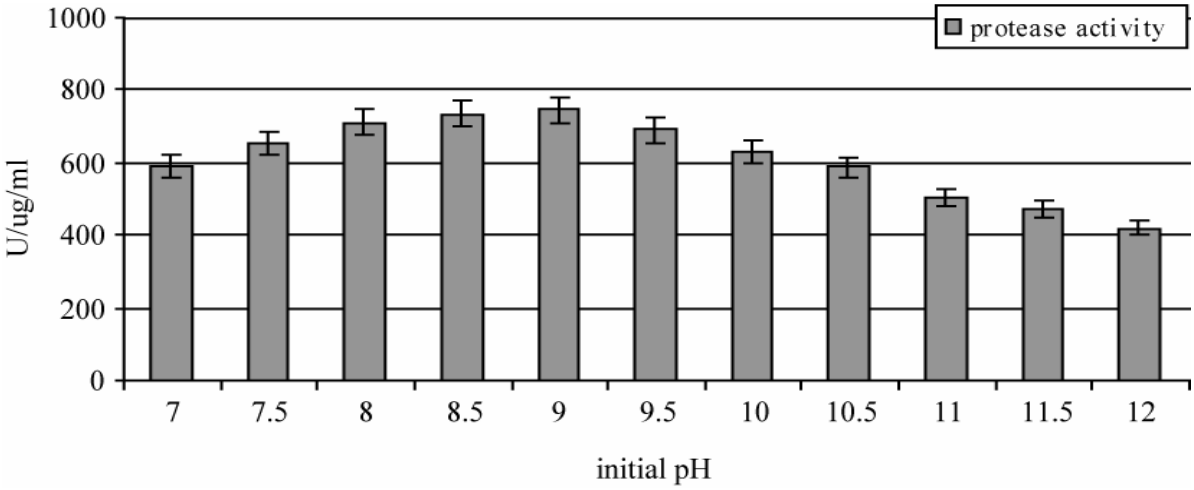

Fig. 1. Effect of pH on the activity of alkaline protease.

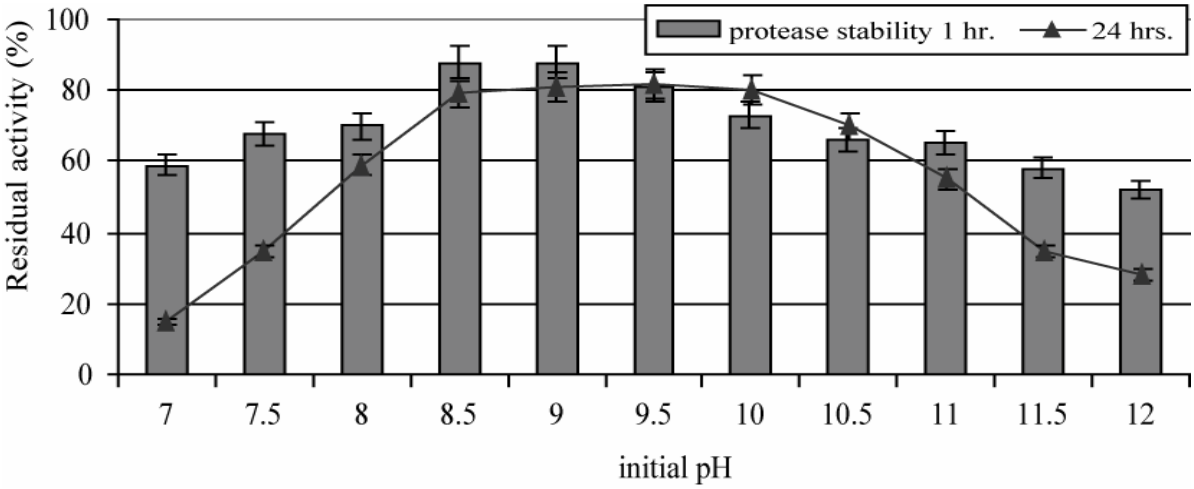

Fig. 2. Effect of pH on the stability of alkaline protease. Y-error bars indicate the standard error from mean.

The activity and stability of enzymes are greatly influenced by surrounding pH. Changes in pH can cause denaturation of enzyme resulting in the loss of catalytic activity. Each enzyme acts best at a certain pH, which is specific to its activity and the activity of the enzyme slows down with any decrease or increase in its optimum pH. pH changes may alter the conformation of the enzyme, the binding of the substrate and the catalytic activity of the groups in the active site of the enzyme. Stability depends on the length of time at which the enzyme has been maintained at unfavorable pH conditions. Most enzymes undergo denaturative changes when exposed to pH values below 6.0 and above pH 10 (Roe, 2001).

Effect of temperature on protease activity and stability: The effect of temperature on protease activity was determined by incubating the enzyme-substrate mixture at different temperatures ranging from 30-100ºC for 30 min and the protease activity was measured as per assay procedure (Fig. 3). The results showed that the activity of the protease was maximum at 40°C (739 U/µg/ml) and the enzyme remained active even at temperature 60°C (650 U/µg/ml). However the activity of protease was very low at 90ºC and beyond 90ºC protease activity became negligible. The protease stability was also observed by incubating the partially purified protease at different temperatures ranging from 30-100ºC for one hour and residual protease activity was measured. It was observed from the results that the residual activity of the enzyme gradually increased from 30°C up to 40ºC and then started to decrease (Fig. 4). It was also found that the enzyme was stable even at temperature 90°C (31%). However the residual activity of protease was left 20% at 100°C.

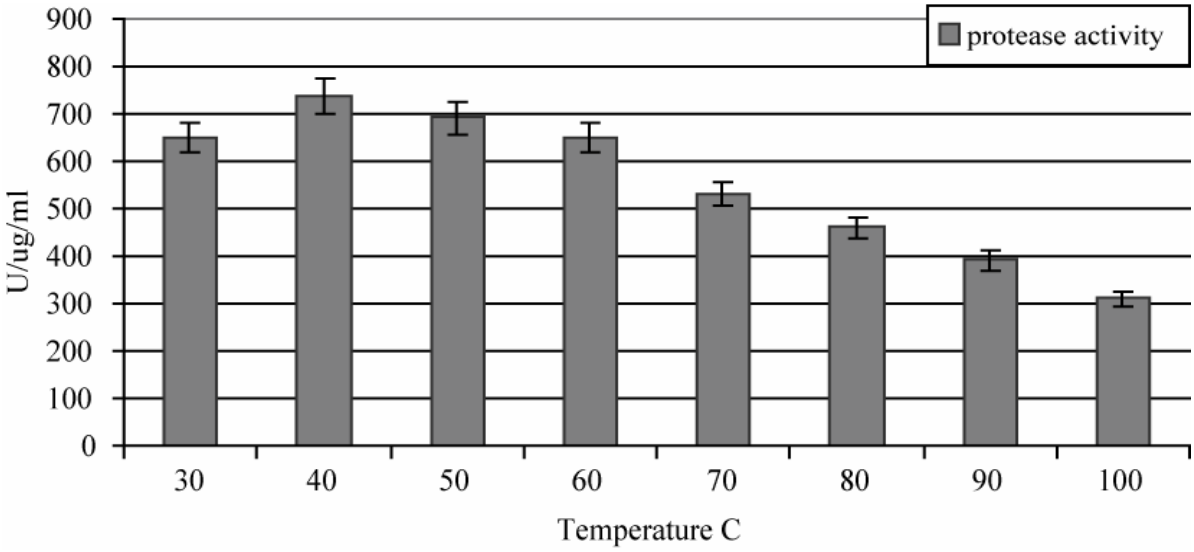

Fig. 3. Effect of temperature on the activity of alkaline Protease.

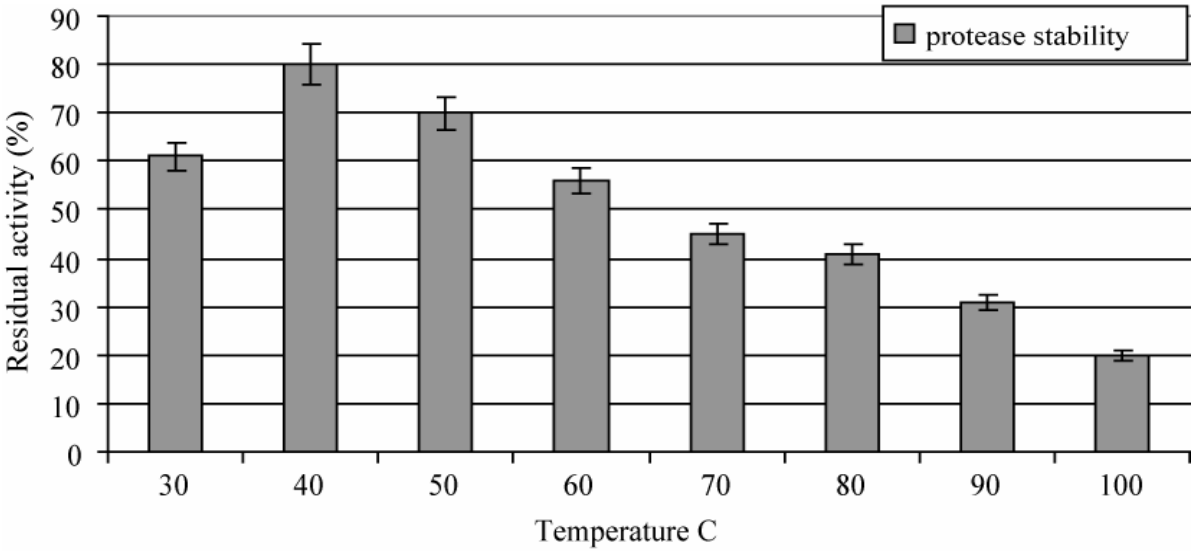

Fig. 4. Effect of temperature on partially purified protease stability. Y-error bars indicate the standard error from mean.

The reaction rate initially increases as temperature rises, due to increased kinetic energy of reacting molecules. However as the temperature is increased the kinetic energy of the enzyme exceeds the energy barrier. It results in the breaking of the weak hydrogen and hydrophobic bonds that maintain the structure of enzyme. At this temperature the enzyme is denatured with a loss in its catalytic activity. Therefore, at a given temperature the actual unit activity of the enzyme is decreased as the total incubation time is increased (Singh, 2007).

Substrate specificity of alkaline protease: Different substrates such as casein, bovine serum albumin, ovalbumin and gelatin were used to evaluate the specificity of alkaline protease for these substrates (Fig. 5). It was observed that the enzyme was highly specific for casein and showed maximum activity against it (893 U/µg/ml). It was followed by BSA (536 U/µg/ml), ovalbumin (379 U/µg/ml) and gelatin (327 U/µg/ml). The results also showed that the enzyme was not highly specific and was active on a number of different substrates which is a promising feature for its diverse applications. Kobayashi *et al*., (1995) and Arulmani *et al*., (2007) had also used different substrates for protease specificity and found casein as a best substrate.

The reason of greater activity with casein may be that the peptide chain of the substrate binds into a surface groove on the enzyme (Turk, 1999). Enzyme act by combining with their specific substrate at unique and confined region. Once this enzyme- substrate complex is formed, catalytic amino acid residues provided by the enzyme, focus their catalytic effect on the substrate and hence convert it to the product. In addition to the perfect fit in a physical sense, the match must be exactly right in a chemical sense as well, if the strength or binding energy of the interaction is to be maximized. This binding energy is a crucial determinant in terms of substrate affinity (Weiner & Williams, 1995).

Effect of inhibitors and additives: Different inhibitors such as Phenylmethylsulphonyl fluoride (PMSF), Sodiumdodecyl sulphate (SDS), Mercaptoethanol (ME), Ethyldiamine tetra acetic acid (EDTA) and Tween 80 were tested for their inhibitory effect on the activity of partially purified protease (Fig. 6). From the results, it was observed that the activity of the protease was completely inhibited (0.05 %) in the presence of PMSF. A slight decrease in the residual activity of the enzyme was observed in the presence of SDS (81.9 %). However, ME (89 %), EDTA (87 %), and Tween (94 %) showed a slight inhibitory effect on the activity of enzyme. Similar results with PMSF as an inhibitor of protease had also been reported by Gupta *et al.,* (2005), Thumar & Singh (2007) and Arulmani *et al*., (2007). According to these workers, the inhibitory effect of PMSF on the enzyme activity showed the presence of serine residues at the active sites. So it can be inferred that the alkaline protease produced by *Bacillus subtilis* EMS-8 is a serine alkaline protease (3:4:21).

The commonly used inhibitors for proteases are sulfonylflourides, of which the most common is PMSF (Phenylmethylsulfonyl flouride). Most of the sulfonyl fluorides are unstable in solution (Whitaker, 1994) so it is necessary that the inhibitor should be stable. Normally they inhibit the protease at lower concentration and may reverse it at higher concentrations (Van, 1994; Cutler, 1996). Most of these inhibitors bring about covalent modification of the active site residues and are thus irreversible (Doonan *et al*., 2005).

The role of different additives as enzyme inhibitors and activators was determined. For this purpose several salts such as $MgCl_2$, $CoCl_2$, $CaCl_2$, NaCl, $MnCl_2$ and EDTA were investigated for their effect on protease activity (Fig. 7). It was observed from the results that the protease activity was greatly reduced in the presence of $MnCl_2$ (125 U/µg/ml) whereas, $MgCl_2$ (901 U/µg/ml) showed increasing effect on the activity of protease. However, $CaCl_2$ showed no effect on the protease activity.

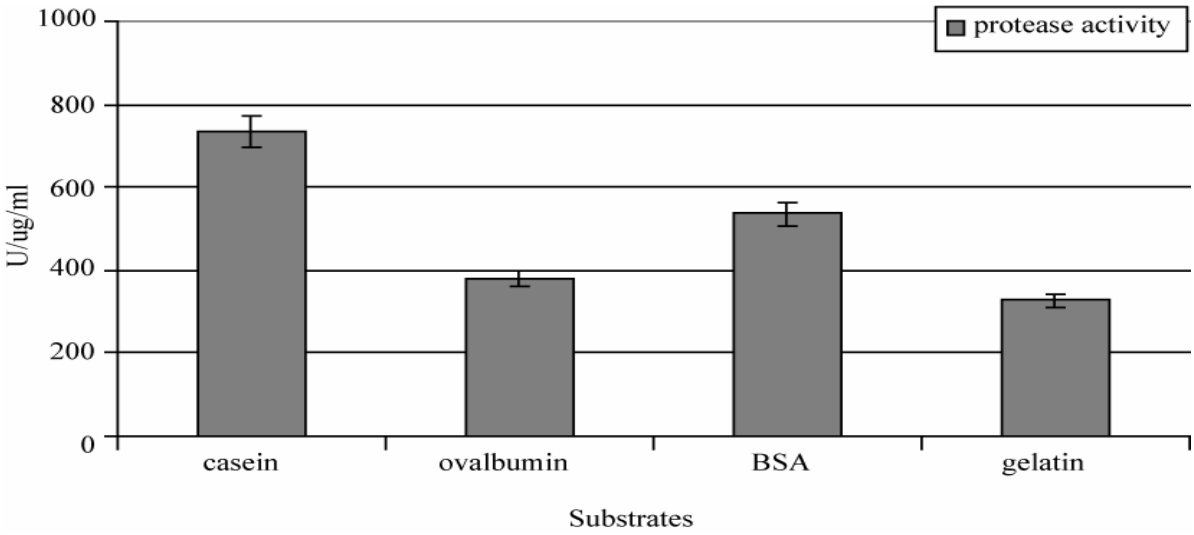

Fig. 5. Substrate specificity of alkaline protease produced by *Bacillus subtilis*. Each value is a mean of triplicates. Y-error bars indicate the standard error from mean.

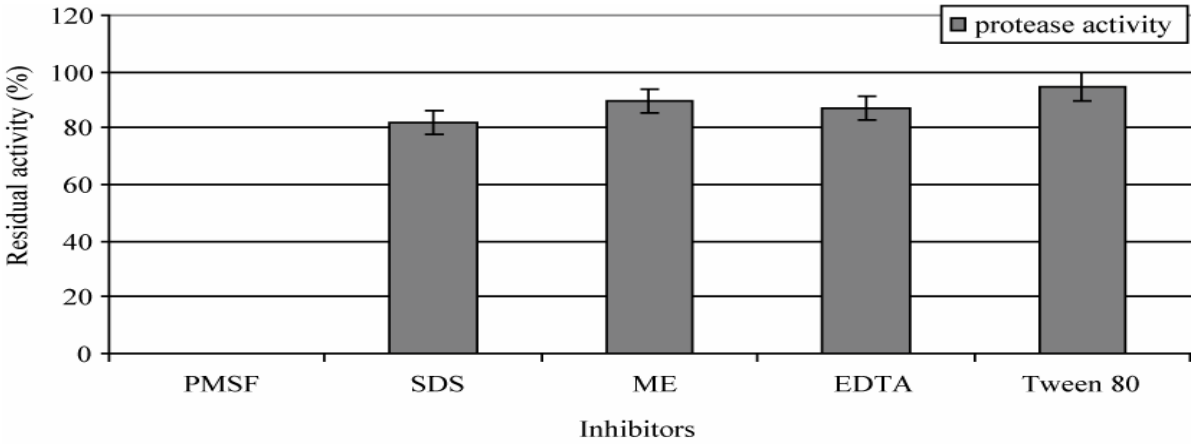

Fig. 6. Effect of inhibitors on the activity of alkaline protease produced by *Bacillus subtilis*.

Abbreviations

PMSF=Phenylmethylsulphonyl fluoride; SDS=Sodiumdodecyl sulphate; ME=Mercaptoethanol; EDTA=Ethyldiamine tetra acetic acid

Y-error bars indicate the standard error from mean

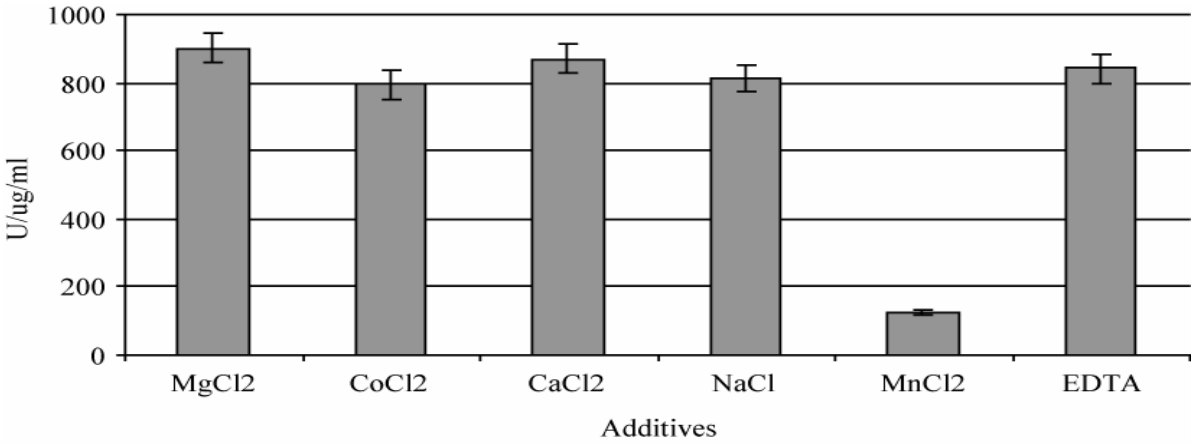

Fig. 7. Effect of additives on the activity of alkaline protease produced by *Bacillus subtilis*.

Metal ions play a vital role in the biological functions of many enzymes. Metals can serve as electron donors or acceptors, Lewis acids or structural regulators. Those participate directly in the catalytic mechanism usually exhibit anomalous physiochemical characteristics. Metal ions may block the essential biological functional group of enzyme by modifying active conformation of biomolecules and by displacing the essential metal ions of the biomolecule itself. Enzyme contains number of metals and displacement or substitution of one of these ions by another metal ion, either with same charge or similar size, results in inhibition in the activity of the enzyme (Prasad, 2004).

Shelf life of enzyme: To determine the shelf life of protease, the enzyme was stored at different temperatures such as 0°C, 4°C, 10°C and room temperature (35°C) for four weeks. It was observed from the results that the protease remained stable at 0°C (20%) upto three weeks, although its activity was greatly reduced (Table 2). Similarly the enzyme remained stable at 4°C (12%) and 10°C (4%) with considerable decrease in its activity. Moreover, the shelf life of the protease comes to an end after second week, when the enzyme was stored at room temperature. However, the enzyme completely lost its activity after fourth week of storage at all temperatures.

Table 2. Shelf life of alkaline protease at different temperatures at different time intervals.

Temperature °C	Residual Protease activity after 1st week (%)	Residual Protease activity after 2nd week (%)	Residual Protease activity after 3rd week (%)	Residual Protease activity after 4th week (%)
0	79	53	20	0
4	61	40	12	0
10	52	31	4	0
25ºC	49	27	0	0

It has been well established that most enzymes should be stored at low temperature due to the fact that at temperature around freezing, enzyme activity is usually minimized and protein stability is maximized (Cuyper and Bulte, 2001). Storage temperature has a significant effect on the shelf life of enzyme and refrigeration promotes a marked improvement in shelf life (Kilcast and Subramaniam, 2000). The low temperature in the process inhibits undesirable chemicals and biochemical reactions and minimizes loss of catalytic activity of the enzymes. For enzymatic reactions, the water activity is very important. Water content decreases at high temperature storage; therefore decrease of water activity causes an inhibition in enzyme activity (Haard & Simpson, 2000).

Molecular weight determination: Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was run to determine the molecular weight of protease. Mixture of marker proteins with known molecular weights was loaded as a standard. Molecular weight marker in the range of 17-150 kDa was used to detect the molecular weight of alkaline protease. The band of partially purified alkaline serine protease was aligned with the marker of protein having molecular weight of 25 KDa (Fig 8). Leigh (1997) had reported an alkaline serine protease with molecular weight of 19 KDa, determined by SDS-PAGE which was also inhibited by PMSF. Durham *et al*., (1987) had reported the molecular weight of serine protesae to be 27 KDa. Similarly, the molecular weight of serine protease was found 30KDa, 28 KDa, and 19 KDa by Koki (1999).

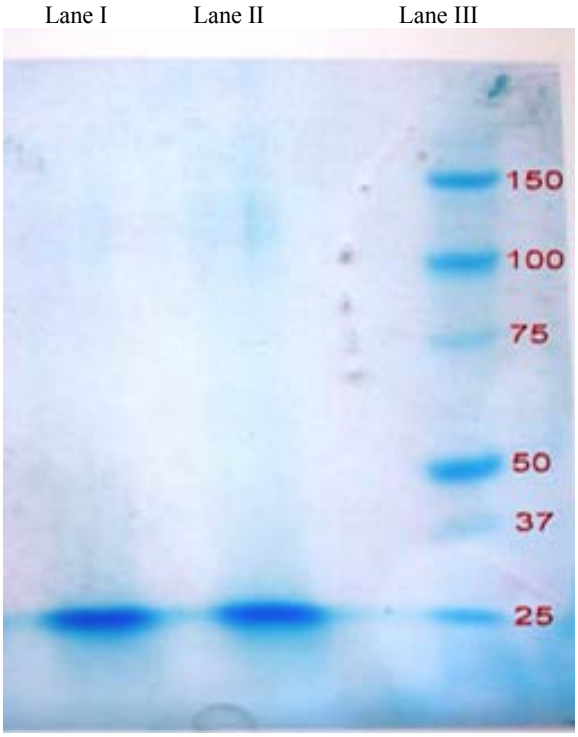

Fig. 8. SDS PAGE of alkaline protease produced by *Bacillus subtilis*
Lane I & II: Purified Enzyme; Lane III: Protein Ladder

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