

Full Length Research Paper

Purification and characterization of a novel protease from *Bacillus* strain SAL1

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The purification and characterization of alkaline protease from a *Bacillus* strain SAL1, isolated from tannery waste have been reported in this paper. This protease was purified to homogeneity by the combination of ammonium sulfate precipitation, DEAE sephacryl ion exchange and phenyl sepharose hydrophobic interaction chromatography. The protease was purified up to 11.18 fold and had a specific activity of 4250 PU/mg. The enzyme was a monomeric protease with a relative molecular mass of 27 kDa by SDS-PAGE. Proteolytic activity of the enzyme was detected by gelatin zymography, which gave a very clear protease activity zone on gel. Molecular mass of the purified protease was also determined by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) that corresponded to the mass determined by SDS-PAGE. The enzyme exhibited its optimal activity at 60°C and at pH 9. The enzyme was stable in the pH range of 7.0 – 10.0 and was able to maintain its stability at 50°C for 1 h.

Key words: Alkaline protease, *Bacillus subtilis*, purification, characterization, tannery waste, DEAE sephacryl, chromatography.

INTRODUCTION

Proteases represent one of the 3 largest groups of Industrial enzymes and find application in detergents, leather industry, food industry; pharmaceutical industry and bio-remediation processes (Anwar and Saleemuddin, 1998; Gupta et al., 2002). Proteases execute a large variety of complex physiological functions. Their importance in conducting the essential metabolic and regulatory functions is evident from their occurrence in all forms of living organisms (Wandersman, 1989). Extracellular proteases are important for the hydrolysis of external proteins and enable the cell to absorb and utilize the hydrolytic products (Kalisz, 1988). They are widely distributed in nature. Microorganisms are the most preferred source of these enzymes in fermentation bioprocesses not only because of their fast growth rate but also for their ability to engineer genetically to generate new enzymes with desirable abilities or simply for enzyme overproduction (North, 1982; Rao et al., 1998).

Microbial proteases play an important role in biotechno-

logical processes and they account for approximately 59% of the total enzymes used (Spinosa, 2000). Alkaline proteases are produced by a wide range of microorganisms including bacteria, moulds, yeasts and also by mammalian tissues. Among bacteria, the *Bacillus* sp. is specific producers of extracellular proteases (Spinosa, 2000; Godfrey and Reichelt, 1985).

Bacillus subtilis proteases have wider specificity than trypsin and chymotrypsin. They possess a number of industrially valuable properties including their ability to excrete several different hydrolytic enzymes into culture medium. The lack of pathogenicity and the ability to grow in simple culture medium can also be accounted for their application in industry (Daniel et al., 1984). With increasing industrial demands for the biocatalysts that can cope with the industrial processes at harsh conditions, the isolation and the characterization of new promising strains are possible ways to increase the yield of such enzymes (Gupta et al., 2002).

Keeping in view the importance of protease in different industries, in this paper, we report the isolation, production, purification and the characterization of an alkaline protease from *Bacillus subtilis* isolated from tannery

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waste which may have useful industrial applications.

MATERIALS AND METHODS

Isolation of microorganism

Samples were collected from different sources (soil, tannery waste) in and around Islamabad, Pakistan. Small amounts of sample (1-5 g) were suspended in sterilized distilled water and incubated at 80°C for 10 min. 100 µl of this suspension was spread on a nutrient agar plate and incubated at 37°C for 24 h. Microorganisms, which formed colonies on the plates, were examined microscopically. Well-isolated rod shaped colonies were picked and further purified by repeated streaking on nutrient agar plates.

Screening for proteolytic activity and identification

The purified bacterial isolates were plated on 10% casein-agar plates and were incubated at 37°C for 24 h. The microbes showing a clear zone of casein hydrolysis were identified as protease producers. Depending upon the maximum relative diameter of the zones, one strain was selected for further experimental studies. The isolated bacteria were identified based on cellular morphology, gram staining, motility and the biochemical profile tests.

Protease production

The culture medium used in this work for the protease production contained 3% nutrient gelatin, 0.8% nutrient broth, 0.5% casein, 0.01% MnCl₂ and 1.2 ml of 20% glycerol, maintained at 37°C for 24 - 72 h in a shaking incubator (150 rpm). After 72 h of growth, the cells were harvested at 10,000 rpm for 15 min and the supernatant thus obtained was used as crude enzyme.

Protease assay

The proteolytic activity was determined by caseinolytic method of (Kunitz, 1947) with some modifications, using azocasein as a substrate. 1 ml of crude enzyme was mixed with 1 ml of 1% azocasein solution, dissolved in 0.02 M Tris-HCl (trihydroxymethane-hydrochloride) buffer (pH 8.5) and was incubated in a water bath at 55°C for 10 min. The reaction was then quenched by the addition of 1 ml of 5% Trichloroacetic acid (TCA). The mixture was kept at 4°C for 15 min followed by the centrifugation at 4000 rpm for 20 min. 1 ml of supernatant was mixed with 1 ml of 0.4 N NaOH and the absorbance was read at 440 nm. The blank was prepared by adding TCA before incubation at 55°C. One unit of protease activity is defined as the amount of enzyme that produces an increase in absorbance of 0.01 under assay conditions.

Protein estimation

Protein was estimated by bicinchoninic acid protein assay kit, with bovine serum albumin (BSA) as standard.

Protease purification

1) Ammonium sulfate precipitation: Ammonium sulfate was added to the cell free culture suspension up to 70% of its saturation. These suspensions were centrifuged at 16000 rpm at 4°C for 15min. The pellets were resuspended in 20 mM Tris-HCl buffer (pH = 8.5) and dialyzed overnight against the same buffer with buffer

changes at least 3 times.

2) DEAE sephacryl chromatographic purification: The dialyzed supernatant was added to DEAE sephacryl matrix (2.0 ml) which was equilibrated with 30 ml of 20 mM Tris-HCl buffer (pH = 8.5). They were shaken in an orbital shaker for 1 h at room temperature. The supernatant and the matrix were separated after the filtration under vacuum. The matrix was washed with 5 aliquots of 2 ml of 20 mM Tris-HCl buffer (pH = 8.5). The proteolytic fractions were combined and were subjected to hydrophobic interaction chromatography.

3) Phenyl sepharose hydrophobic interaction chromatography: The pooled fractions from DEAE sephacryl fractionation were applied to a phenyl sepharose column which was equilibrated with 20 mM Tris-HCl buffer (pH = 8.5) containing 2.5 M sodium chloride. The liquid was collected in a receiving tube. The column was washed with 20 mM Tris-HCl buffer (pH = 8.5) and then bound proteins were eluted with 50% ethylene glycol in 20 mM Tris-HCl buffer (pH = 8.5). Fractions were collected and then analyzed for proteolytic activity. Fractions with highest enzyme activity were pooled and used for further characterization.

Polyacrylamide gel electrophoresis and zymography

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970) with slight modification using a 10% cross linked polyacrylamide gel. Silver staining was performed to visualize protein bands on gel. SDS-PAGE zymograms were performed as described by (Schmidt et al., 1988) with some modification. 10% polyacrylamide gels were copolymerized with 0.05% gelatin. Samples were dissolved in non-reducing sample buffer without heat denaturation and run at 100 V. Following electrophoresis, the gels were washed for 30 min in 50 mM Tris-HCl buffer (pH 7.6) containing 2.5% Triton X-100, with gentle agitation, in order to remove the excess of SDS. Then, the gels were incubated for an additional 4 h with several changes in a solution of 50 mM Tris-HCl (pH 7.6) containing 0.2 M NaCl and 5 mM CaCl₂. The zones of proteolysis were detected by overnight Coomassie blue staining.

Mass spectrometry (MALDI-TOF MS)

MALDI-TOF mass spectrometer, equipped with a nitrogen laser was used for acquiring mass spectra. A saturated solution of sinapinic acid in 30% acetonitrile was used as a matrix. The sample and matrix were mixed in appropriate ratio and 1 µl was applied as a spot on MALDI sample plate. The sample spot was allowed to dry at room temperature. Upon drying, the matrix forms crystals incorporating the sample. The experiment was performed in the positive ion mode. The accelerating voltage was 25 kv. The laser power and delay time were adjusted to generate reasonable signals.

Effect of temperature on the activity and the stability of protease

The effect of temperature on pure enzyme was studied by assaying enzyme at different temperatures in the range of 30-80°C, at pH 8 using azocasein as substrate. The thermo-stability of enzyme was studied by incubating the enzyme preparation at varying temperatures ranging from 25-75°C for 1 h. Subsequently, the enzyme activity was assessed as above.

Effect of pH on the activity and the stability of protease

The effect of pH on the protease activity was determined by

Table 1. Morphological and biochemical characteristics of the organism according to Bergey's manual of Determinative Bacteriology 8th edition.

Characteristic/Assay	Type
Morphology	Rods
Gram staining	Positive
Motility	Motile
Catalase test	Positive
Gelatin hydrolysis	Positive
Nitrate reduction test	Positive
Methyl red test	Positive
Casein hydrolysis	Positive
Starch hydrolysis	Positive
Citrate utilization test	Positive
Indole test	Negative
Urease test	Negative
Sucrose fermentation	Negative

incubating the reaction mixture at pH values ranging from 5.0 to 12.0, in the following buffer systems: 0.1 M sodium acetate (pH 4.0 - 5.5); 0.1 M sodium phosphate (pH 6.0-7.5); 0.1 M Tris-HCl (pH 8.0- 9.0); 0.1 M glycine-NaOH (pH 9.5-12.0). To check the effect of pH on the stability, the enzyme solution (50 μ l) was mixed with 150 μ l different buffer solution and was incubated at room temperature for 1 h after which the proteolytic activity was measured under standard assay conditions.

RESULTS AND DISCUSSION

Characterization of strain SAL1

In our present work, several strains of bacteria were isolated from various areas in Pakistan and were screened for maximum protease production. Among these, the strain SAL1 isolated from the tannery waste showed maximum zone of hydrolysis (diameter, 4.1 cm) on casein agar plates and was selected for further studies. The clear zone of hydrolysis around each bacterial colony was due to the hydrolysis of casein by proteolytic enzyme. It was a gram-positive, motile, rod-shaped bacterium. It is mesophilic, exhibiting an optimum growth temperature of 37°C, with optimal pH of 8.0. On the basis of morphological and biochemical tests (Table 1), it was identified as *Bacillus subtilis*.

Purification of the protease

The supernatant of 72 h grown culture was used as crude source of enzyme. Protease from the culture media was purified by the combination of ammonium sulfate precipitation, DEAE sephacryl anion exchange chromatography and phenyl sepharose hydrophobic interaction chromatography. The results of purification procedure are summarized in Table 2. Ammonium sulfate precipitation increas-

ed the protease activity by concentrating the enzyme. The increase in protease activity by using ammonium sulfate is consistent with available precedence (McKevitt et al., 1989; Sexton et al., 1994). The precipitate so obtained was subjected to dialysis for further removal of excessive salts and impurities.

The dialysis was followed by DEAE sephacryl. Sephacryl played a selective permeable role for protease. All the proteins other than protease were bound to the matrix and the protease was recovered in washing. Similar kind of behavior was also observed in the case of *Bacillus* sp. protease (Shimogaki et al., 1991) and *Pseudomonas* sp. protease (Gupta et al., 2005). During the last step of purification, the specific activity was further enhanced. In this last step of purification, phenyl sepharose role was just opposite to the role of DEAE-sephacryl. The protease was completely retained by the matrix and all other impurities went into washing. The resultant bound protease was further eluted with 50% ethylene glycol. Almost similar behavior was reported for protease from *Pseudomonas* sp. (Gupta et al., 2005).

SDS-PAGE of purified enzyme is shown in Figure 1. A single band with molecular mass of 26000 + 1000 DA was observed. In literature, the alkaline proteases with molecular weight ranging from 16-32 KDa are reported from *Bacillus* sp. (Adinarayana et al., 2004; Kaur et al., 1998; Jaswal and Kocher (2007). The zymogram activity staining indicated one clear zone of proteolytic activity. This zone was due to the degradation of gelatin used as a substrate in zymography (Figure 2).

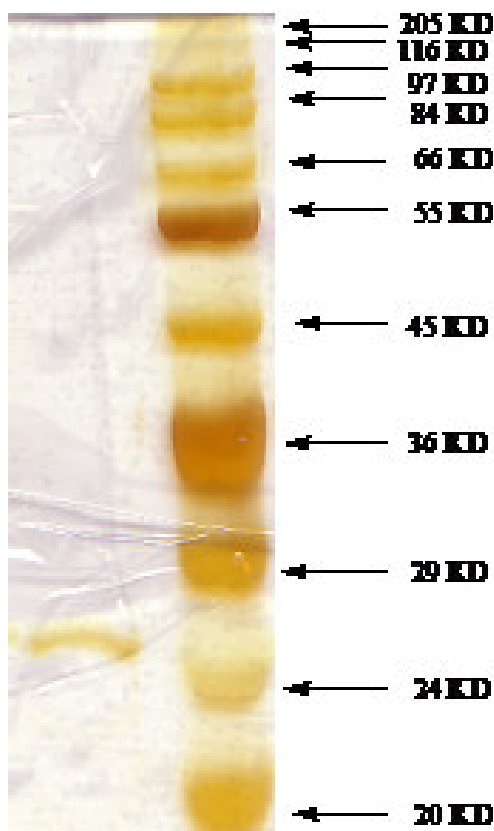
Molecular mass of the purified protease was also determined by mass spectrometer (MALDI-TOF). A single peak of protease activity was eluted which corresponded to a molecular mass of 27168 DA as shown in Figure 3. All these results indicate that the enzyme is a monomeric protein.

Effect of temperature and pH on the activity and the stability of protease

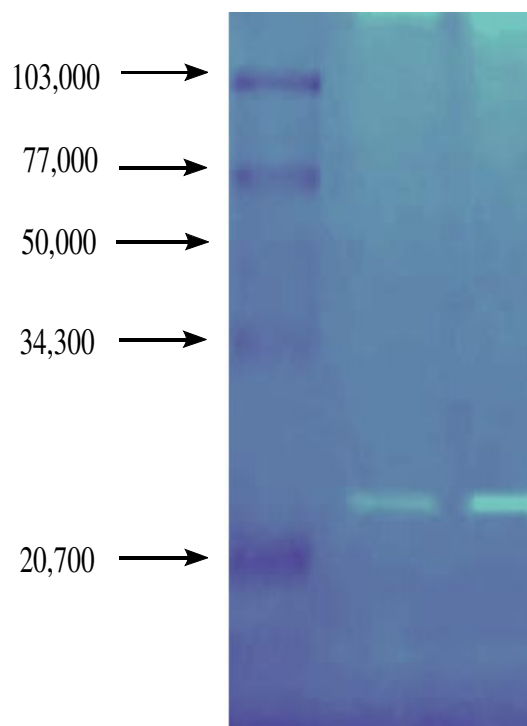
The optimum temperature for protease enzyme activity was found to be 60°C (Figure 5). However, it was not stable under its optimum temperature. Alkaline proteases of *Bacillus* sp. with similar temperature optima have been reported by Adinarayana et al. (2003), Dhandapani and Vijayaragavan (1994), Fujiwara and Yamamoto (1987), Takii et al., (1990) and Ferrero et al. (1996). The thermal stability studies have indicated that the protease retained 88% of its activity when exposed to 55°C for 1 h (Figure 7). However, the percentage stability of enzyme declined to 74% at 60°C. There was a sharp decrease in the proteolytic activity with further increase in temperature. This inactivation of enzyme shows the destruction of enzyme at higher temperature incubation. The results showed that the protease appeared to be heat labile at temperatures between 40-60°C. The optimum pH for protease activity was 9 although the enzyme was active

Table 2. A summary of purification steps of protease produced by *B. subtilis*.

Purification stage	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Activity (PU/ml)	Total activity (PU)	Specific activity (PU/mg Protein)
Culture supernatant	150	0.66	99	251	37650	380
Ammonium sulfate Precipitation	45	0.42	18.9	283	12735	673
DEAE- Sephacryl chromatography	32	0.17	5.44	310	9920	1823
Phenyl sepharose chromatography	12	0.08	0.96	340	4080	4250

**Figure 1.** Polyacrylamide gel electrophoresis of purified sample: PAGE analysis was conducted on 10% polyacrylamide gel.

in the pH range of 7-10 (Figure 4). From a survey of literature it can be seen that the optimum pH range of alkaline proteases is generally between pH 9 to 11. Alkaline proteases of *Bacillus subtilis* PE-11 with similar properties have been reported by Adinarayana et al. (2003). These findings are in accordance with several earlier reports showing pH optima of 9.0-10.5 for protease from *Bacillus* sp. by Durham (1987), from *Xanthomonas maltophilia* by Debette, (1991) and *Vibrio metschnikovii* by Kwon et al. (1994). The effect of pH on the stability of protease was studied. The protease was found to be stable in the pH range 7-10 (Figure 6). Such

**Figure 2.** 10% Gelatin polyacrylamide zymogram showing band of protein cleavage.

highly alkali stable protease obtained from *Bacillus* sp. has been reported by Moon et al. (1994).

Conclusion

A thermophilic alkaline protease from *Bacillus* strain was purified by DEAE sephacryl anion exchange chromatography and phenyl sepharose hydrophobic interaction chromatography. The molecular weight was found to be around 27 kDa by SDS-PAGE and mass spectrometer (MALDI-TOF). The optimal pH and optimal temperature of the protease were 9 and 60°C respectively. The protease was found stable during the 1 h incubation at 50°C and in the pH range of 7-10.

Due to possession of desirable properties such as

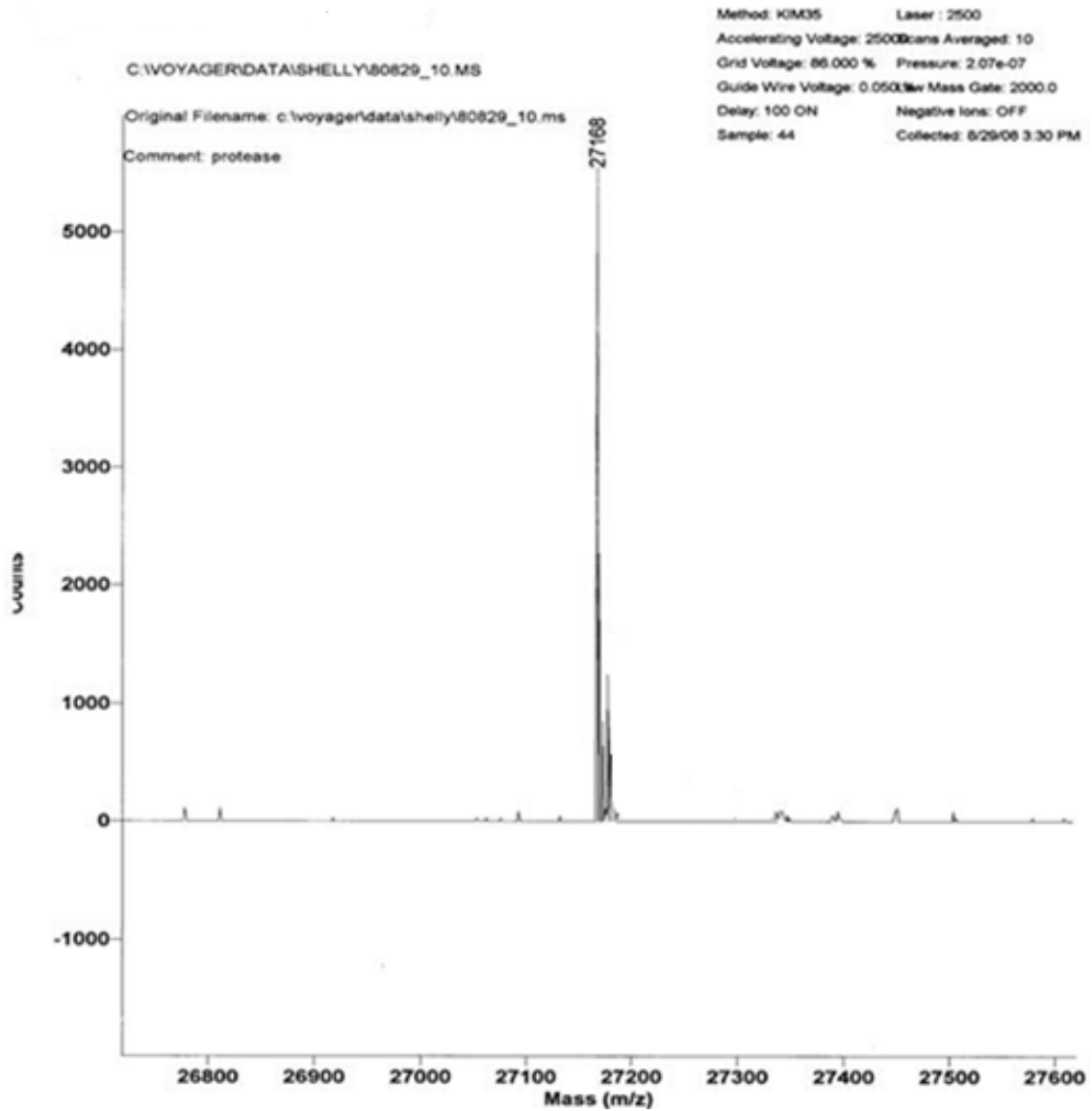


Figure 3. MALDI spectrum of purified enzyme with sinapinic acid as matrix.

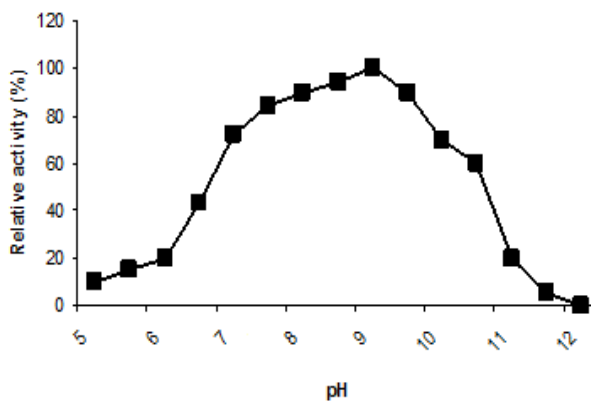


Figure 4. pH optimum of purified protease. pH optima was measured by incubating the enzyme with the substrate at different pH values.

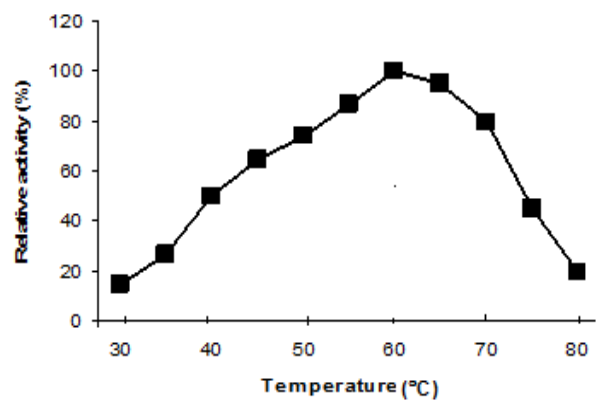


Figure 5. Temperature optimum of protease. The purified enzyme was incubated with the substrate at different temperatures.

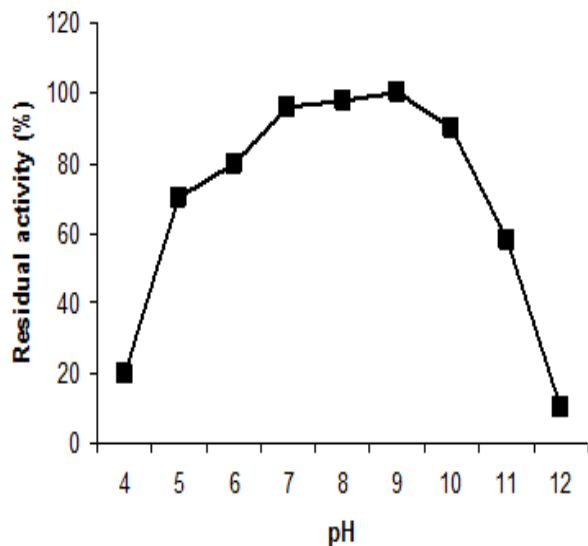


Figure 6. pH stability of protease. Purified enzyme was incubated in buffers of different pH values at 25°C for 1 h and remaining activity was measured under standard assay conditions.

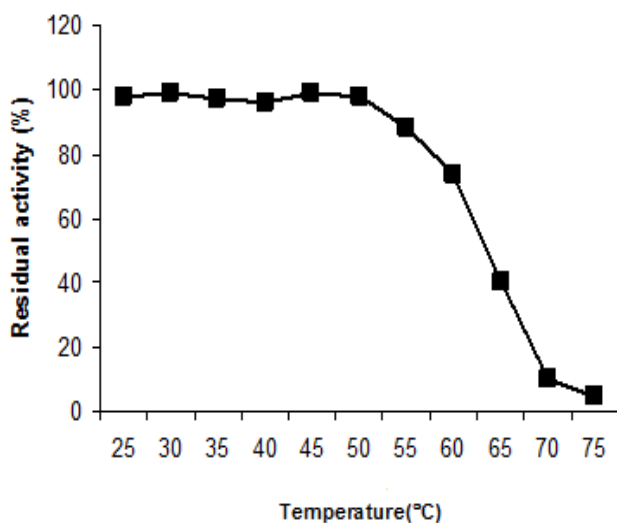


Figure 7. Thermal stability of the purified protease at various temperatures (25 - 75 °C). The enzyme solution was incubated in 20 mM Tris-HCl (pH 8.5) at different temperatures for one hour and remaining activity was measured under standard assay conditions.

activity at high pH and temperature, high pH stability and thermo stability, this protease can be suitable for the commercial applications.

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