

CHARACTERIZATION AND STABILITY PROFILE OF THERMOSTABLE ALKALINE PROTEASE PRODUCED FROM *BACILLUS* SP. AI-5

Aliya Riaz^{1*}, Isma Idrees¹, Samina Khan¹, Sana Ahmad², Shahana Rasheed Qureshi¹ and Shah Ali Ul Qader³

¹Department of Biochemistry, Jinnah University for Women, Karachi-74600, Pakistan

²Bahria Medical College and University, Karachi, Pakistan, Karachi-74600, Pakistan

³Department of Biochemistry, University of Karachi, Karachi- 75270, Pakistan

*Corresponding author: E.mail: aliyariaz@hotmail.com; Contact: 03362148470

ABSTRACT

Proteases are hydrolytic enzymes that catalyze the cleavage of peptide bonds within proteins. They represent one of the most commercially significant class of technical enzymes, with wide-ranging applications across various industries, including food, detergent, leather textile, and pharmaceutical industries. Therefore, the present study focused on the kinetics and stability profile of partially purified proteases produced by *Bacillus* sp. AI-5, isolated from the coastal soil of Karachi. The partially purified protease required 25 minutes for the enzyme–substrate reaction. The K_m and V_{max} values calculated from Line-weaver Burk plot were found to be 2.5 mg/mL and 85.21 U/ml/minutes respectively. The enzyme exhibited thermostability, with optimal activity at 55 °C, retaining 71% activity at 50 °C after 30 minutes and 61% activity at 40 °C for up to 90 minutes. The enzyme was classified as a neutral protease based on its optimal activity at pH 7.5. The proteolytic efficiency of enzyme was enhanced in the presence of 1mM Ca^{+2} , while $CdCl_2$ and $HgCl_2$ strongly inhibited its activity. Protease showed moderate stability in the presence of organic solvents particularly hexane and DMSO retaining 76% and 68% residual activity respectively, while xylene, methanol and ethanol strongly inhibited its activity. The protease from *Bacillus* sp. AI-5 showed compatibility towards H_2O_2 , Tween-80 and SDS and retained about 72% activity when incubated with these agents for 30 minutes. Whereas, Triton X-100 was found to be the strong inhibitor of proteases. The protease exhibited storage stability at -18 °C retaining about 55% activity after thirty days. In conclusion, the study revealed the kinetics and stability profile of partially purified protease from *Bacillus* sp. AI-5, highlighting its potential for industrial applications.

Keywords: Proteases, *Bacillus*, kinetics, thermal stability, surfactants, metal ions, organic solvents.

INTRODUCTION

Proteases are enzymes that catalyze the hydrolysis of peptide bonds in proteins, playing a critical role in various biological processes such as digestion, immune response, and cell signaling (Natalia *et al.*, 2022). Proteases are a crucial class of enzymes that regulate physiological homeostasis and support overall health (Rani *et al.*, 2012). Apart from their role in growth, healing, and defense against infections, they also play a significant part in variety of industrial processes, offering significant economic benefits such as in meat tenderization and solubilization, cheese manufacturing, feed processing, blood decolorization, leather processing and waste recycling (Olusola *et al.*, 2024). These enzymes are categorized based on their active sites and substrate specificities, with serine, cysteine, aspartic, and metalloproteases being the most common classes (Danny *et al.*, 2013). All living things, including plants, animals, and microorganisms such as moulds, fungus, algae, and bacteria especially *Staphylococcus* species, *Bacillus*, *Pseudomonas*, *Helomonas* and *Arthrobacter* produce proteases (Sadia *et al.*, 2024). Microbial proteases have gained significant attention due to their stability, broad substrate specificity, and potential industrial applications, making them a preferred choice over plant and animal-derived proteases (Natalia *et al.*, 2022). Due to its broad substrate specificity, high activity, and stability across a range of physiological conditions, the genus *Bacillus* is currently ruling the industry. Proteases from *Bacillus* species, such as *Bacillus subtilis* and *Bacillus licheniformis*, have been extensively studied for their stability under extreme pH and temperature conditions, which enhances their utility in detergents, food, and pharmaceutical industries (Iuliia and Sharipova, 2020). The genetic adaptability of *Bacillus* species also facilitates overproduction of proteases, further expanding their industrial applicability (Tanveer *et al.*, 2024). Characterization of proteases involves studying their molecular properties, including optimum pH, temperature, kinetic parameters, and stability against inhibitors and denaturants (David *et al.*, 2021). These attributes are critical in determining the enzyme's suitability for specific industrial applications. Proteases with high thermostability are preferred in industrial settings that require prolonged operation at elevated temperatures, ensuring consistent performance (Rawat and Gandhary, 2024). Studies on *Bacillus*-derived proteases

have demonstrated remarkable stability, attributed to their robust tertiary structures and the presence of stabilizing disulfide bonds (Lars *et al.*, 2003). Furthermore, the addition of stabilizing agents or chemical modifications has been shown to enhance the stability and functionality of proteases in industrial environments (Haichuan *et al.*, 2024). Among the diverse categories of proteases, including acidic, neutral, and alkaline types, alkaline proteases contribute the largest share of commercial enzymes market. Characterized by their stability and activity at elevated pH levels, these enzymes typically operate within a neutral to basic pH range (Mayuri *et al.*, 2019). Alkaline proteases can be further classified into metallo-type or serine-type enzymes, and their extensive use in various industries, such as detergents, food, leather, and pharmaceuticals, has sparked considerable research interest (Hina *et al.*, 2021). Therefore, the present study was designed to fully exploit the industrial potential of alkaline proteases through a comprehensive evaluation of their kinetics and detailed characterization, with particular emphasis on stability parameters. This assessment aims to enable industries to utilize proteases effectively while minimizing activity loss.

MATERIALS AND METHODS

Preparation of culture media and protease production

After the selection of medium, protease was produced in medium containing (g/L): 1.0 glucose, 10.0 peptone, 0.2 yeast extract, 0.1 CaCl₂, 0.5 K₂HPO₄ and 0.1 MgSO₄. pH of the medium was kept at 7.0 before autoclaving at 121 °C for 15 minutes. After sterilization, 10 ml inoculum was seeded with a loop full of overnight culture of *Bacillus* sp. collected from the slant. Following incubation at 45 °C for 24 hours, the inoculum was transferred into 90 ml of sterilized medium for fermentation (Aliya *et al.*, 2023).

Collection of crude enzyme from fermented medium

The fermented medium was centrifuged at 10,000 rpm for 10 minutes at 0 °C. The resulting supernatant, designated as the cell-free filtrate (CFF), was collected and stored at -18 °C for subsequent estimation of protease activity.

Partial Purification of Proteases

Protease purification was carried out using gradient ammonium sulfate precipitation, followed by dialysis and gel filtration chromatography (Aliya *et al.*, 2023).

Protease activity assay

Protease activity was assayed based on the method described by Anson (1938), using casein as the substrate and L-tyrosine as the standard. 5 mL of casein solution (0.45 g%) was mixed with 1 mL of enzyme solution and incubated at 55 °C for 10 minutes. The reaction was terminated by adding 5 mL of TCA, followed by incubation at 37 °C for 30 minutes and centrifugation at 10,000 rpm for 25 minutes at 0 °C. The supernatant was treated with 500 mM Na₂CO₃, followed by diluted Folin–Ciocalteu reagent, and incubated at 37 °C for 30 minutes. Absorbance was measured at 660 nm to calculate the amount of tyrosine amino acid liberated by the action of proteases (Anwar *et al.*, 2009). One unit of protease activity was defined as “the amount of enzyme required to hydrolyze casein, releasing 1 μmol of tyrosine, per unit time under standard assay conditions.”

Characterization of partially purified proteases

Effect of time on enzyme-substrate reaction

The optimal reaction time of protease with its substrate casein was investigated by incubating 1 mL partially purified enzyme with 5 mL casein solution (0.45 g %) for different time intervals ranging from 5-30 minutes. After incubation the protease activity was estimated under standard assay conditions.

Effect of substrate concentration on protease activity

The effect of different substrate concentrations on protease activity was investigated by reacting partially purified enzyme with different concentrations of casein ranging from 2.0 - 6.0 mg/mL under standard assay procedure. The K_m and V_{max} values were determined by plotting Lineweaver Burk plot and Michaelis-Menten plot, respectively.

Effect of reaction temperature on protease activity

The optimum temperature for maximum protease activity was investigated by conducting the enzymatic reaction at temperature range of 40 to 70 °C with an increment of 5 °C.

Effect of pH on protease activity

The pH at which proteases showed maximum activity was also examined. For this purpose, enzyme was incubated separately with 0.45 g % substrate prepared in 50 mM Tris-HCl buffer of different pH ranging from 6 to 9.

Effect of metal ions on protease activity

The inhibitory or stimulatory effect of MgCl₂, CaCl₂, MnCl₂, NiCl₂, KCl, AlCl₃, NaCl, HgCl₂ and BaCl₂ was determined by mixing and incubating equal volumes of partially purified enzyme and 1 mM metal ion solutions at room temperature for 30 minutes. Thereafter, 1 ml of enzyme-metal solution was used as Test for protease activity. Activity of untreated enzyme was taken as Control.

Thermal stability of proteases with respect to time

Thermal stability of proteases was also noticed at different temperatures ranging from 40 °C to 70 °C with respect to time in minutes i.e. 30, 45, 60, 90 and 120 minutes. For this experiment, enzyme aliquots were placed at the above mentioned temperatures for specified time intervals. After each time interval, enzyme aliquots were taken out from each of the incubation temperature and evaluated for their activities under standard assay conditions. The residual activity was calculated by considering the sample without incubation as control.

Compatibility of proteases with organic solvents

In order to check the compatibility of proteases in different organic solvents, 2 mL partially purified protease and 2 mL (5 %; v/v) organic solvents (hexane, DMSO, xylene methanol, and ethanol) were left together at 37 °C for 30 minutes. 2 mL of that mixture was then used for estimation of residual enzyme activity, using the activity of untreated enzyme as Control.

Compatibility of proteases with surfactants

Different surfactants were analyzed for their effects on stability of protease. For this purpose, partially purified enzyme was mixed with equal volume of 1% (v/v) surfactants (Triton X-100, Tween-80, SDS and H₂O₂) separately and then incubated at 37 °C for 30 minutes. The enzyme-surfactant solutions were then used as Tests for protease activity. Activity of untreated enzyme was taken as Control.

Storage Stability of Proteases

The storage stability of partially purified proteases was evaluated by incubating several aliquots of enzyme at -18 °C, 4 °C and 37 °C for one month. The protease activity was measured by using aliquot of each temperature, after every 2-4 days under standard assay conditions.

RESULTS

Characterization of Partially Purified Proteases

Effect of time on enzyme-substrate reaction

The effect of reaction time on protease activity was carried out by incubating reaction mixture for different time intervals ranging from 5-30 minutes with the interval of 5 minutes (Fig. 1). Initially a linear relationship was observed between reaction time and protease activity. The partially purified enzyme showed maximum activity when incubated with 0.5 g % casein for 25 minutes at 55 °C beyond which the enzyme activity was found to be declined.

Effect of substrate concentration on partially purified protease activity

In the present study, casein was used as a substrate and protease activity was estimated at different concentrations of casein (2.0-6.0 mg/mL). It was observed that initially as the substrate concentration was increased enzyme activity was also found to be increased. At 4.5 mg/mL concentration, the enzyme activity was observed to be maximal as shown in Fig. 2. Lineweaver-Burk plot was plotted using graph pad prism in order to calculate Michealis-Menten constant (Fig. 3). The Km and V_{max} values of Proteases from *Bacillus* sp. AI-5 was 2.5 mg/mL and 85.2 U/mL/minute, respectively

Effect of reaction temperature on partially purified protease activity

The effect of temperature on protease activity was examined by measuring enzyme activity at different temperatures (37 °C to 65 °C). It was observed that enzyme activity was gradually increased up to 55 °C; thereafter the activity was found to be declined (Fig. 4). Maximum activity of partially purified proteases at 55 °C proved the enzyme to be thermostable.

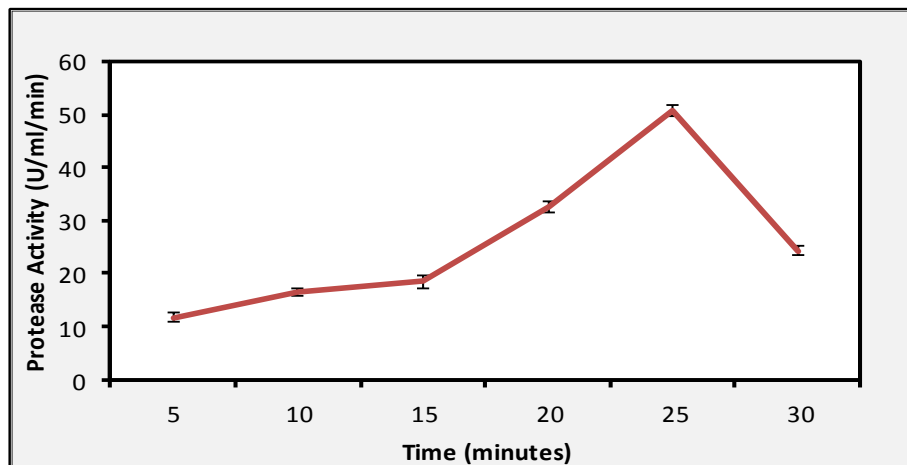


Fig. 1. Effect of reaction time on partially purified protease activity with casein as a substrate

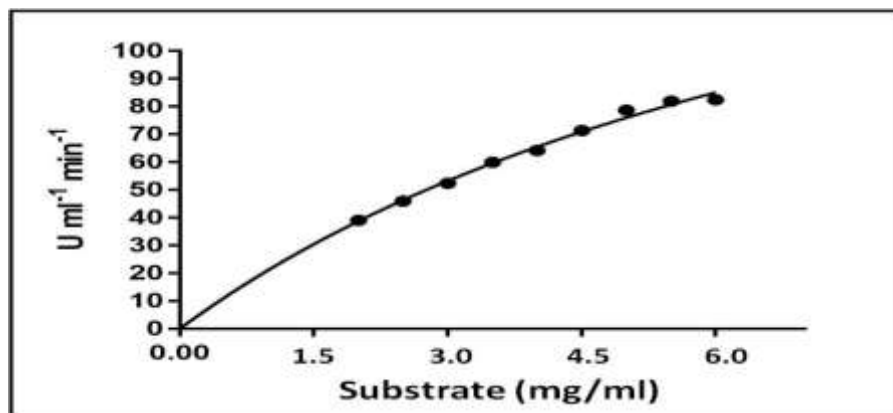


Fig. 2. Michealis-Menten plot for partially purified protease from *Bacillus* sp. AI-5

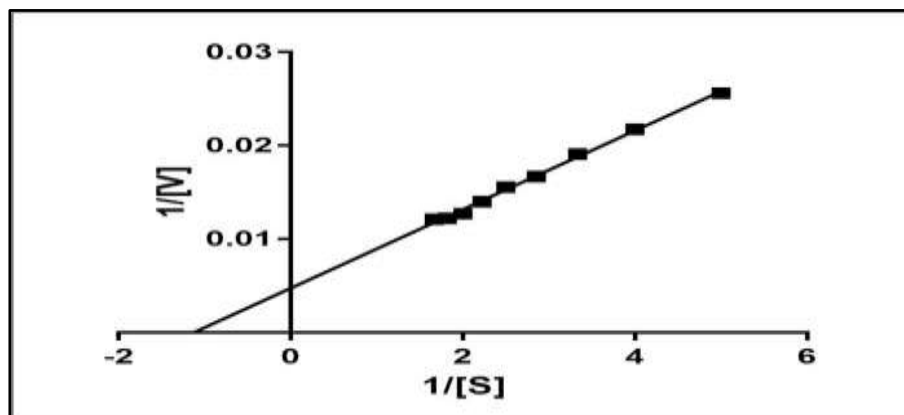


Fig. 3. Lineweaver-Burk plot for partially purified protease from *Bacillus* sp. AI-5. (Standard error of K_m and V_{max} were 0.033 mg/mL and 7.67 U/mL/min, respectively)

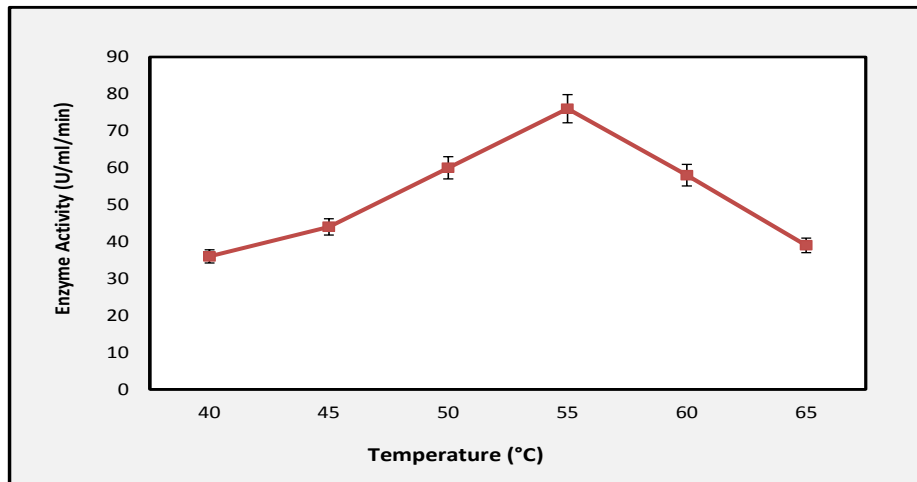


Fig. 4. Effect of enzyme-substrate reaction temperature on partially purified protease activity

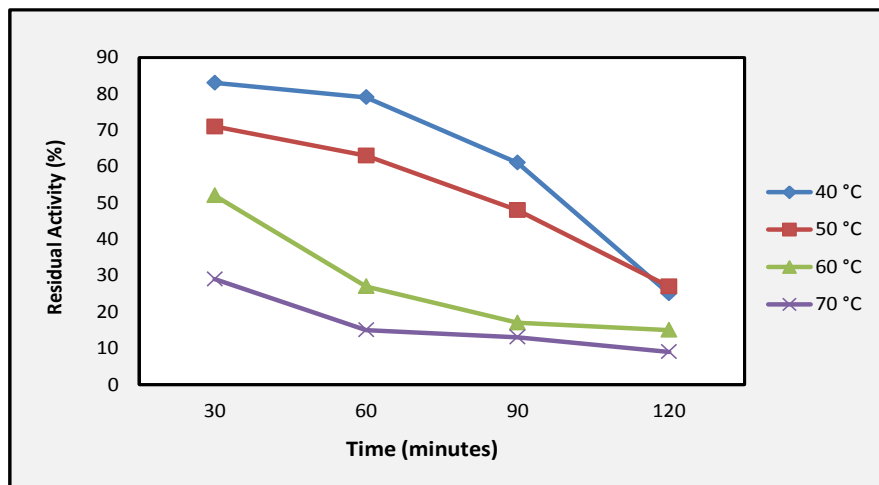


Fig. 5. Thermal stability of partially purified protease with respect to time

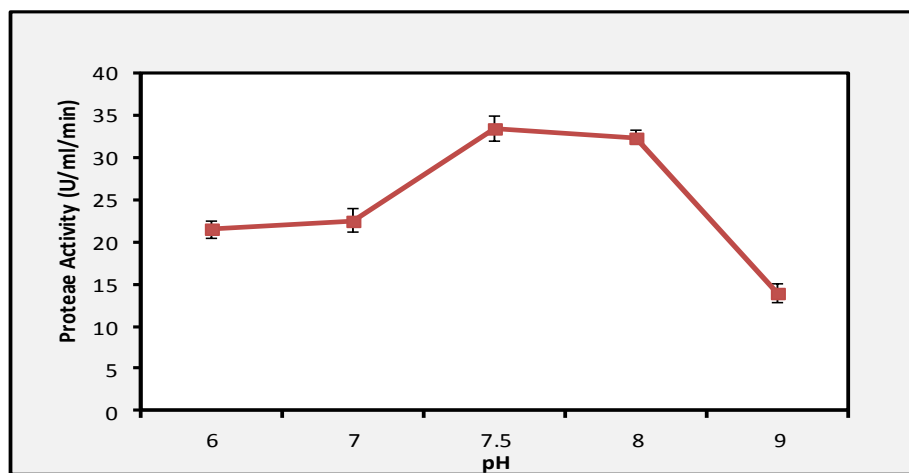


Fig. 6. Effect of pH on partially purified protease activity

Thermal stability of proteases with respect to time

The thermal stability of partially purified proteases was investigated by incubating the reaction mixture at different temperatures ranging from 40 °C to 70 °C, for different time intervals (30, 60, 90 and 120 minutes). It was observed that proteases from *Bacillus* sp. AI-5 was thermostable as the protease retained about 71 % activity at 50 °C after 30 minutes. Similarly, at 60 °C enzyme retained 52 % of its activity after 30 minutes. Enzyme showed more stability at 40 °C and retained 61 % activity even after 90 minutes of incubation. Whereas, at 70 °C, protease was found to be inhibited as it showed only 29 % activity after 30 minutes (Fig. 5).

Effect of pH on partially purified proteases activity

The activity of the partially purified enzyme was measured by using phosphate buffer of different pH ranging from 6–9. The optimum pH of the proteases was pH 7.5, thereafter the enzyme activity was observed to be decreased (Fig. 6).

Effect of metal ions on partially purified proteases activity

The effect of various metal ions in concentration of 1mM on partially purified proteases was observed by incubating the enzyme with the metal ion solution for 30 minutes at 37 °C. It was observed that proteases activity was enhanced in the presence of Ca^{+2} as it showed 5% increased activity as compared to control (Fig. 7). However some metal ions including Al^{+3} and Mn^{+2} showed a little inhibitory effect on protease activity as the enzyme showed 11 and 35 % activity loss respectively. Moreover a strong inhibitory effect of CdCl_2 and HgCl_2 on protease activity was observed at 1mM concentration and enzyme was observed to retain only 26 % and 25 % activity respectively.

Compatibility of proteases with organic solvents

Stability of proteases activity against different organic solvents including hexane, DMSO, xylene, methanol and ethanol was checked by incubating enzyme with organic solvent at 37 °C for 30 minutes before adding the substrate. The residual enzyme activities were estimated according to the method described in materials and methods section.

According to results showed in Fig. 8, it was observed that hexane was the best organic solvent to attain highest percentage of residual protease activity of 76 % as compared to control. DMSO also retained 68% residual activity whereas xylene, methanol and ethanol were found to be the strong inhibitors of protease activity.

Compatibility of proteases with surfactants

Compatibility of proteases in the presence of different surfactants was checked and the residual activity is represented in Fig. 9. Triton X-100 was found to be the strong inhibitor of proteases whereas the enzyme retained about 72 % activity in the presence of H_2O_2 , Tween-80 and SDS as compared to control.

Storage stability of partially purified proteases

The protease enzyme was stored at three different temperatures including 37 °C, 4 °C and -18 °C to determine the shelf life of enzyme for thirty days and the activity was measured at 5 days of interval. It has been perceived from the results that the enzyme was quite stable at all the three storage temperatures for about 15 days. Furthermore the proteases exhibited better stability at -18 °C as 55 % activity was retained even after thirty days (Fig. 10).

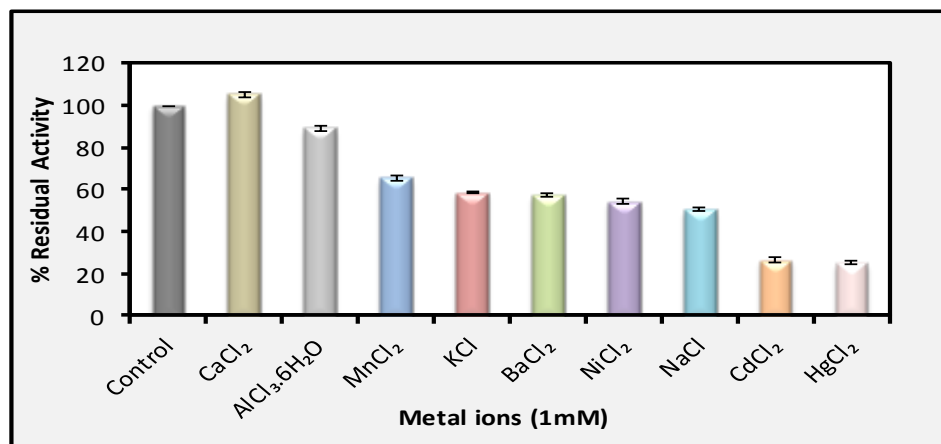


Fig. 7. Effect of metal ions on partially purified protease activity

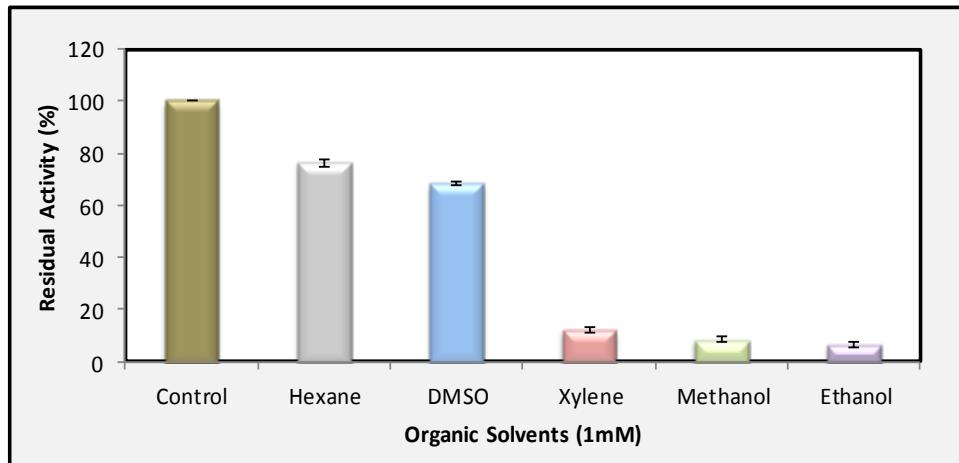


Fig. 8. Effect of organic solvents on partially purified protease activity

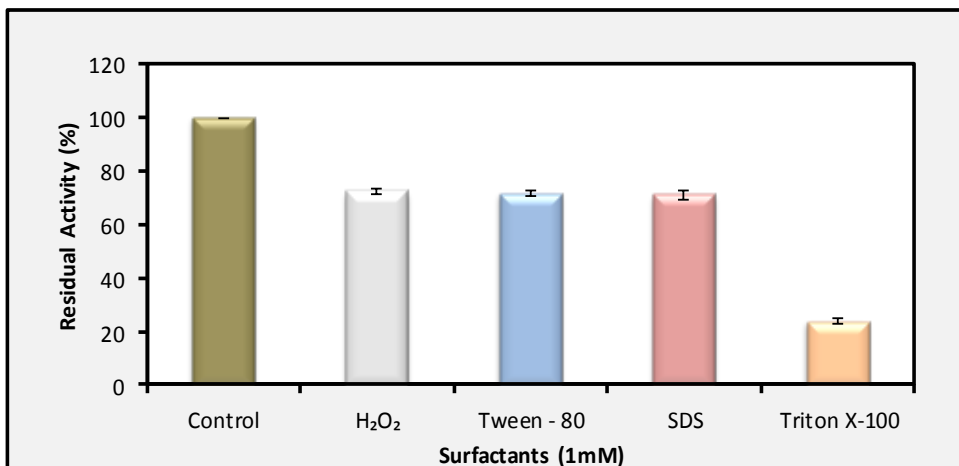


Fig. 9. Effect of surfactants on partially purified protease activity

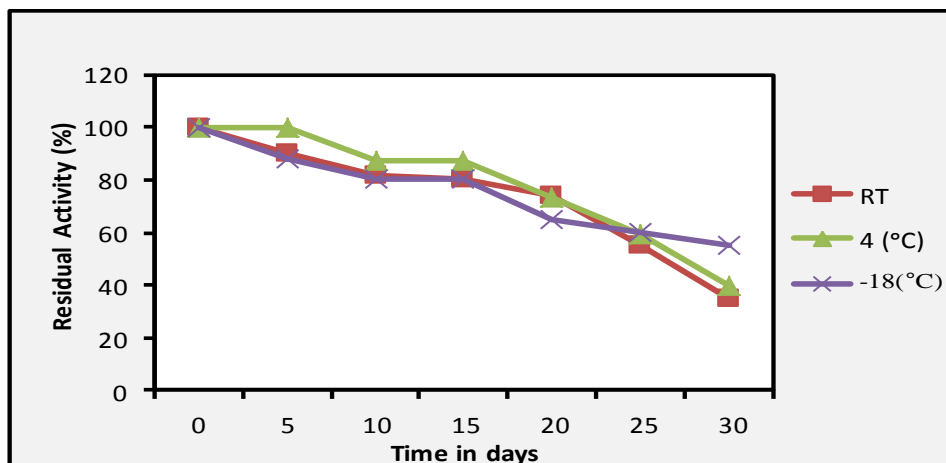


Fig. 10. Storage stability of partially purified proteases

DISCUSSION

The characterization studies of proteases were performed in order to define its application in industrial processes. Every enzyme of industrial use should possess some properties which make the enzyme active and stable under hostile conditions. Therefore, in order to get maximum catalytic activity of partially purified enzyme, the reaction time of proteases with casein was optimized. The results showed that proteases exhibited its maximum activity at 25 minutes of reaction (Fig. 1). Palash *et al.* (2013) reported that alkaline protease from *Bacillus licheniformis* P003 was maximally active at 70 minutes of reaction. Ambreen *et al.* (2012) reported 20 minutes for the optimum activity of cysteine protease. A reaction time of 10 minutes has also been reported previously (Zareena *et al.*, 2015).

The kinetic parameters of proteases from *Bacillus* sp. AI-5 has been investigated using casein as a substrate. K_m values represents the dissociation constant of enzyme-substrate complex, lower the K_m value greater will be the affinity between enzyme and substrate. Therefore K_m values are used to determine the catalytic efficiency of enzyme. In the present study, Line-weaver Burk plot between different concentrations of casein and reaction velocity revealed the K_m and V_{max} values of proteases from *Bacillus* sp. AI-5 as 2.5 mg/ml and 85.2 U/mL/minute, respectively (Fig. 3). The V_{max} and K_m values of protease from thermophilic *Bacillus* species have been reported as 473 U/minute and 2.3 mg/ml respectively using casein as a substrate (Aysha *et al.*, 2015). Muhammad *et al.* (2013) reported 5 mg/mL as the K_m value and 61.58 μ M/ml/min as V_{max} of the reaction. A K_m of 11.6 mg/mL with wheat bran as a substrate has also been reported for serine proteases from *Aspergillus heteromorphus* URM0269 (Lígia *et al.*, 2024).

Temperature is one of the most important physical parameter that affects the activity of enzyme (Oyeleke *et al.*, 2011). In the present study the protease activity was determined over temperatures ranging from 40 to 65 °C. Optimum temperature of proteases was found to be 55 °C therefore the enzyme was considered as thermo stable (Fig. 4). As the temperature was increased beyond 55 °C, a decline in protease activity was observed which might be due to the breaking of the weak hydrogen and hydrophobic bonds that maintain the structure of the enzyme (Hamid and Haq, 2012). In accordance to the results of our study, Najmeh *et al.* (2024) reported 50 °C as an optimum temperature for protease activity from *Aeromonas* sp. An optimum temperature of 40 °C has been reported by Dambar *et al.* (2024). Whereas Tanveer *et al.* (2024) reported an optimum temperature of 60 °C for proteases produced from thermophilic *Bacillus subtilis* BSP strain.

Stability of enzyme at higher temperature determines its role in different industrial processes especially in detergent formulation and leather processing (Ahmetoglu *et al.*, 2015) as the enzyme lost its three dimensional structure due to breakage of non-covalent interactions in enzyme protein. Thermal stability of proteases from *Bacillus* sp. AI-5 was also investigated by incubating the partially purified proteases at different temperatures ranging from 40-70 °C for 30, 45, 60, 90 and 120 minutes and the residual activity at each temperature was recorded. The thermo stability of the partially purified protease showed that the enzyme was stable at 50 °C and retained 71 % activity after 30 minutes (Fig. 5). Whereas, when the enzyme was incubated at 60 °C for 30 minutes, it retained only 54 % activity. The partially purified proteases showed more stability at 40 °C as it showed 61 % residual activity even after 90 minutes of incubation. However, at 70 °C enzyme was found to be unstable as it showed only 29 % activity after 30 minutes. Similar results have been shown by Biswanath *et al.* (2011) who worked on alkaline protease from *Bacillus licheniformis* NCIM-2042 and reported maximum stability of proteases at temperature ranging from 40 to 50 °C. Another study reported the higher stability of proteases at 50 °C with the half-life of 50 minutes whereas enzyme lost its activity at 60 and 70 °C (Sayem *et al.*, 2006).

pH is the second most important factor which play an important role in many enzymatic process, enzyme substrate reaction and ionic groups stability present on the active site of enzymes (Walid *et al.*, 2017). In the current study, proteases from *Bacillus* sp. AI-5 showed optimum pH 7.5 for its maximum activity. The enzyme remained active between pH 7-8. Based on the results, the enzyme from *Bacillus* sp. AI-5 could be classified as neutral proteases (Fig. 6). The neutral proteases have been reported to have applications in food industry as they hydrolyzed the hydrophobic amino acids in proteins present in food hydrolysate thereby reduced the bitterness of food (Chandran *et al.* 2005). The reduction in the protease activity due to change in the pH of the reaction medium below or above the optimum was might be due to the altered charge distribution on substrate and enzyme molecules that may cause conformational alteration in the three-dimensional structure of the enzyme leading to decreased association between enzyme and substrate (Vidushi *et al.*, 2015; Shruti *et al.*, 2017). Various studies reported similar results of optimum protease activity at pH 7.5 (Guangrong *et al.*, 2006; Sang-Chul *et al.*, 2007), whereas, protease from *B. licheniformis* MZK03 showed optimum activity at pH 8.5 (Sayem *et al.*, 2006).

The metal ions have stimulatory or inhibitory effect on the activity of proteases and for this purpose; the effect of several metal ions on protease activity was tested. In the current study activity of proteases was found to be

strongly stimulated by Ca^{2+} at 1mM concentration as it showed about 5 % increase in activity as compared to control (Fig. 7). A stronger inhibitory effect on protease activity was noticed in the presence of CdCl_2 and HgCl_2 at 1mM concentration. The results correlate with the findings of Sang-Chul *et al.* (2007) who reported Ca^{2+} (1mM) as stimulator of proteases produced by *Bacillus* sp. S17110. An increase in activity in the presence of Ca^{2+} was might be due to the presence of calcium ion in the enzyme's active conformation that also protected the enzyme from thermal denaturation and enhanced its stability (Tracey *et al.* 1997). According to Abou-Elela *et al.*, (2011) Cd^{2+} exerted negative effect on protease activity produced by *Bacillus cereus*. Inhibitory effect of Cu^{2+} , Hg^{2+} and Na^+ has also been reported at 1mM concentration for alkaline protease (Veerapandian *et al.*, 2016). On the other hand, Palash *et al.* (2013) observed that calcium ions did not produce any significant effect on protease activity.

Stability of enzymes in organic solvents, not just in aqueous environment, is a very important characteristic of enzyme regarding its industrial applications. Most of the compounds used in industrial processes require organic solvents for their solubility therefore the enzyme used in that processes should remain stable in this environment for the better results. In the present study maximum residual activity of 76 % has shown in the presence of hexane as compared to control (Fig. 8). DMSO also retained 68 % residual protease activity. Xylene, methanol and ethanol strongly inhibited the protease activity. This inhibition of proteolytic activity was might be due to destabilization of reaction transition state as reported earlier (Qing *et al.*, 2013). Enzyme stability in the presence of solvent was might be due to the formation of solvent-water boundary around protein so that the active confirmation of enzyme molecule was maintained (Munishwar, 1992). Additionally, the increased solubility of substrate and product made the recovery of product easy (Hiroyasu *et al.*, 1995). Similar finding has been stated in case of alkaline serine protease from the thermophilic fungus *Myceliophthora* sp. that exhibited more than 50 % activity loss in presence of methanol and ethanol (Zanphorlin *et al.*, 2011). In contrast, Shaghayegh *et al.* (2015) reported the stimulatory effect of ethanol and methanol at all tested concentrations.

Stability and compatibility of proteases in the presence of detergents, oxidizing agents and surfactants determines its utilization in the detergent formulation as all of these compounds are used in detergent making (Rani *et al.*, 1999). Different surfactants and oxidizing agents in concentration of 1 mM were tested for their effects on proteolytic activity of enzyme produced from *Bacillus* sp. AI-5. Among the tested surfactants (H_2O_2 , Tween-80, SDS, Triton X-100) H_2O_2 , Tween-80 and SDS showed considerable stability of proteases as the enzyme retained about 72 % catalytic efficiency in the presence of these surfactants. Whereas Triton X-100 exhibited a marked decrease in protease activity as only 23 % residual activity was observed (Fig. 9). In contrast to the results of present study, Kezia *et al.*, (2011) stated high stability of proteases in the presence of 1% H_2O_2 , Triton X-100 and Tween-20 whereas the enzyme was slightly stable after treatment with 1 % SDS. Muhammad *et al.*, (2013) reported the inhibition of alkaline protease from *Bacillus licheniformis* UV-9 by 1% of SDS and showed residual activity of 45.83%. H_2O_2 (1%) was also found to decrease the protease activity by 22 % being an oxidizing agent (Samiha *et al.*, 2017). Hafsa *et al.* (2017) reported that proteases from *Aspergillus niger* KIBGE-IB36 was strongly inhibited by SDS.

Storage stability of enzyme protein is another important characteristic that should be evaluated before starting the commercialization of enzyme-aided processes because of the high cost of commercially available purified enzymes Storage temperature has a significant effect on the shelf life of enzyme. Most of the enzymes should be stored at low temperature might be due to maximum protein stability or minimum biochemical reactions as the enzyme activities are also declined (Richard, 2010). In the present study stability of proteases from *Bacillus* sp. AI-5 was observed at 4 °C and -18 °C and room temperature (30 °C) as a function of time. It was observed that enzyme remained stable at -18 °C up to 30 days and retained 55% residual activity (Fig. 10). The enzyme was remained stable at 4 °C and room temperature and showed about 72 % residual activity at both the temperatures. Hamid and Haq (2012) reported that alkaline protease showed only 20 % residual activity after 3 weeks of incubation at room temperature. The results are in accordance with Palash *et al.* (2013) who reported about 66% residual activity when enzyme was stored at 4 °C and 46 % residual activity at room temperature (30 °C) after 28 days.

Conclusion

Much of the work regarding protease production from microbial strains has been ended with the discovery of alkaline proteases but in the present study the enzyme was classified as neutral proteases that possess great application in food industry such as the processing of soy sauce and reduction of unwanted bitterness during cheese ripening. Another sparking feature of the enzyme was its thermostability that made the enzyme a suitable candidate to be used in laundry detergent and leather processing. Furthermore the enzyme exhibited considerable stability in the presence of Ca^{+2} , Al^{+3} , H_2O_2 , Tween-80, SDS and hexane that indicated its potential application in industrial processes.

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