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RESEARCH ARTICLE - BIOLOGICAL SCIENCES

Purification and Characterization of Extracellular Protease and Amylase Produced by the Bacterial Strain, *Corynebacterium alkanolyticum* ATH3 Isolated from Fish Gut

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Abstract The use of enzymes in different industrial sectors increased significantly due to huge industrialization. Different types of protease and amylase are randomly used in industries including food, textile, and paper. For this purpose, purification of extracellular protease and amylase produced by the bacterium, *Corynebacterium alkanolyticum* ATH3 (Acc. No. JX656749) isolated from the distal intestine of a freshwater fish, *Anabas testudineus*, was carried out using column chromatography. The specific activity of protease and amylase significantly increased with each step of purification and finally became 93.73 and 88.1 U/mg protein with a purification fold 26.03 and 44.94, respectively. The sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis showed molecular weight of purified protease and amylase was ∼17 and ∼28 kDa, respectively. To the authors' knowledge, this is the first report of ∼17 kDa protease and ∼28 kDa amylase from the bacterial strain *C. alkanolyticum* ATH3. Further, enzyme activity was also evidenced by zymography analysis. The enzymes acted optimally at pH 7.5–8.0 and temperature 35–45 °C, respectively. So, due to cheapest source, these two enzymes are very important for various purposes in industrial sectors.

Keywords Fish gut bacteria · Protease and amylase purification · SDS-PAGE · Zymography analysis · pH and temperature sensitivity

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1 Introduction

The use of enzymes in industrial sector significantly has increased due to exhaustive industrialization such as food, beverage, textile, leather, and paper industries [1]. Amongst these enzymes, protease and amylase received special attention. Bacterial extracellular enzymes are usually capable of digesting a wide range of insoluble nutrient such as protein, cellulose, and starch and provide energy to the cell for its own function [2]. Among these industrial enzymes, proteases are one of the most important classes of industrial enzymes which contribute about 60 % of the total enzyme market [3]. Beg and Gupta [4] reported that microbial protease has highest commercial value over enzyme obtained from animals and plants. Wide ranges of microbiota (bacteria and fungi) have been reported for protease production, but only few are considered to be commercial producer [5]. The significance of amylase in industrial sector is well understood, and it constitutes nearly 25 % of the world enzyme market [6]. Furthermore, due to high stability (pH and temperature), microbial amylases can be used for a broad range of industrial applications as compared to amylase obtained from animal and plant sources [7]. The use of amylase obtained from fungal and bacterial sources have dominated over the enzyme derived from synthetic sources in different industrial sectors [8]. There are number of published reports on purification of protease and amylase from different bacterial isolates, such as *Bacillus*, *Chromohalobacter*, *Halobacillus*, *Rhodothermus*, *Halomonas*, and *Xenorhabdus* [9–15]. The remarkable differences among these purified protease and amylase lie in their molecular weight, thermal and pH sensitivity and stability, which make them suitable for different industrial purpose. Thus, isolation and characterization of new promising strain is a continuous process for the production of enzymes for industrial use [16].

Gastrointestinal (GI) tract microbiota, especially bacteria, produce different types of extracellular enzymes that break down a wide range of substrates such as protein, cellulose, starch, phytate, and chitin [17]. Bacterial enzymes are the cheapest sources that are widely used in different industries such as food, textile, and beverage, as well as in waste treatment [1,5,18]. Therefore, production, purification, and characterization of different bacterial enzymes are very important in relation to cost management in industries. The GI tract microbiota of fish are characterized by high population density, wide diversity, and complexity of interactions.

To the authors' knowledge, information on purification and characterization of enzymes from fish gut bacteria are scarce. The bacterial strain, *Corynebacterium alkanolyticum* ATH3 (GenBank accession no. JX656749) isolated from the gut of a freshwater fish, *Anabas testudineus*, was selected for the present study mainly because of high protease and amylase producing ability and dearth of information on the characteristics of protease and amylase produced by this particular bacterial isolate. The present study, therefore, aims to (i) purify the extracellular protease and amylase produced by this fish gut bacterial strain, (ii) characterize the purified enzymes by SDS-PAGE and zymography analysis, and (iii) determine their pH and thermal sensitivity.

2 Experimental Methods

2.1 Microorganism

The bacterial strain, *Corynebacterium alkanolyticum* ATH3 (GenBank accession no. JX656749), isolated from the distal intestine of a freshwater fish, *Anabas testudineus*, was selected for the present study [19].

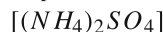
2.2 Production of Crude Enzyme

The bacterial isolate was grown in optimized condition in peptone-gelatin and starch broth (72 h, 37 ± 1 °C) for production of protease and amylase, respectively. The broth was collected and centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant (crude enzyme) was used for enzyme and protein estimation. Extracellular protease and amylase activities were estimated according to the method of Walter [20] and Bernfeld [21], respectively. Protein content was measured according to the method of Lowry et al. (1951).

2.3 Purification of Enzymes

Bacterial protease and amylase were purified according to the methods described by Mohamed [14] and Yandri et al. [1], respectively, with modifications.

2.3.1 Step 1: Ammonium Sulfate Precipitation



The bacterial culture supernatant containing the enzymes was precipitated by using solid ammonium sulfate at different saturation level (40–80 % for protease and 30–70 % for amylase) at 4 °C. Precipitated proteins containing protease and amylase fraction were resuspended in Tris HCl buffer (50 mM, pH 7.0) and phosphate buffer (50 mM, pH 7.0), respectively, followed by determination of enzyme activities of each fraction.

2.3.2 Step 2a: Separation and Purification of Protease

Fraction showing highest activity was dialyzed and subjected to gel filtration chromatography using Sephadex G-50 beads pre-equilibrated with 50 mM Tris HCl buffer (pH 7.5) at room temperature. The protein content in each eluted fraction was determined at 280 nm, and active fractions were lyophilized. The lyophilized fractions were further purified by DEAE cellulose column chromatography using increasing gradient of NaCl (0.2–0.6 M). Protease activity and protein content for each sample were determined carefully and active fractions were stored at 4 °C for further use.

2.3.3 Step 2b: Separation and Purification of Amylase

The precipitated fractions supposed to contain amylase were loaded on DEAE cellulose column (Sigma, 10 × 0.6 cm) pre-equilibrated with phosphate buffer (50 mM, pH 7.0) followed by collection of elution of these enzymes by applying increasing NaCl gradients (0.2–0.6 M). These active fractions were further lyophilized and loaded on Sepahdex G-100 column pre-equilibrated with phosphate buffer (50 mM, pH 7.0). Finally, enzyme activity of active fractions was noted and stored for further use.

2.4 Determination of Molecular Weight by SDS-PAGE

The homogeneity of the purified protease and amylase was examined in NATIVE PAGE according to the method described earlier [1]. Briefly, these enzymes were resolved in 12 % NATIVE PAGE at a constant voltage (60 V) for 2 h 30 min, and molecular weight was determined by using molecular weight marker (Fermentas Cat. No. SM-0671) as standard. The data presented here are representatives of five independent experiments.

2.5 SDS Zymography Analysis

Protease and amylase zymography was done according to Mohamed [14] with some modifications. Briefly, protease

was first loaded on 12 % polyacrylamide gel prepared by mixing casein (0.1 %) and run at a constant voltage (110 mA) for 3 h followed by a thorough wash using 2.5 % triton X-100 for 1 h to remove SDS and incubated in 50 mM Tris HCl buffer (pH 7.5) at 37.4 °C for 24 h. Next day gel was washed with distilled water and incubated in coomassie blue R 250 for 3 h and destained in water.

Purified amylase was loaded on a 12 % polyacrylamide gel prepared by mixing starch (0.15 %) and thoroughly washed with 2.5 % triton X-100 for 1 h to remove the SDS and incubated in 0.1 mM sodium acetate buffer (pH 4.8) at 27. 4 °C for 12 h. Finally, gel washed with distilled water and incubated in Lugal's iodine solution (2 %) for 20 min and destained in water.

2.6 Optimization of pH and Temperature on Activity of Purified Enzymes

The optimum pH of the purified enzymes (protease and amylase) was recorded between 4.0 and 9.0 at a constant temperature (37 °C). Similarly, the optimum temperature of these enzymes was recorded at different temperature ranges (20–60 °C) at constant pH (7.0).

3 Results and Discussion

3.1 Purification of Extracellular Protease and Amylase

The ammonium sulfate precipitation of protease and amylase was maximum at 60 and 50 % saturation levels, respectively (Fig. 1a, b).

Details of protease purification is depicted in Fig. 2. Sephadex G-50 elution profile of protease is presented in Fig. 2a. Two fractions, number 9 and 10 which showed the highest activity, were collected for further purification on DEAE cellulose (Fig. 2b).

Fraction number 20 (at 0.2 M NaCl) showing highest activity was collected. DEAE cellulose profile of amylase showed highest activity of fraction number 19 collected using 0.4 M NaCl gradient (Fig. 3a). Partially purified amylase was again passed through Sephadex G-100, and the elution is shown in Fig. 3b.

The purification summary of both protease and amylase is depicted in Tables 1 and 2, respectively. Table 1 demonstrates that with each step of purification fold, protein content decreased (152.28–0.89 mg), but specific activity increased (from supernatant, 3.6 U/mg protein ml^{-1} to purified protease, 93.73 U/mg protein ml^{-1}). Finally, the purified protease showed significant increase in specific activity from 3.6 (U/mg protein ml^{-1}) to 93.73 (U/mg protein ml^{-1}) with a purification fold 26.03 and yield 15.21 %. Similarly, gradual decrease in protein content (163.87–0.64 mg) enhanced

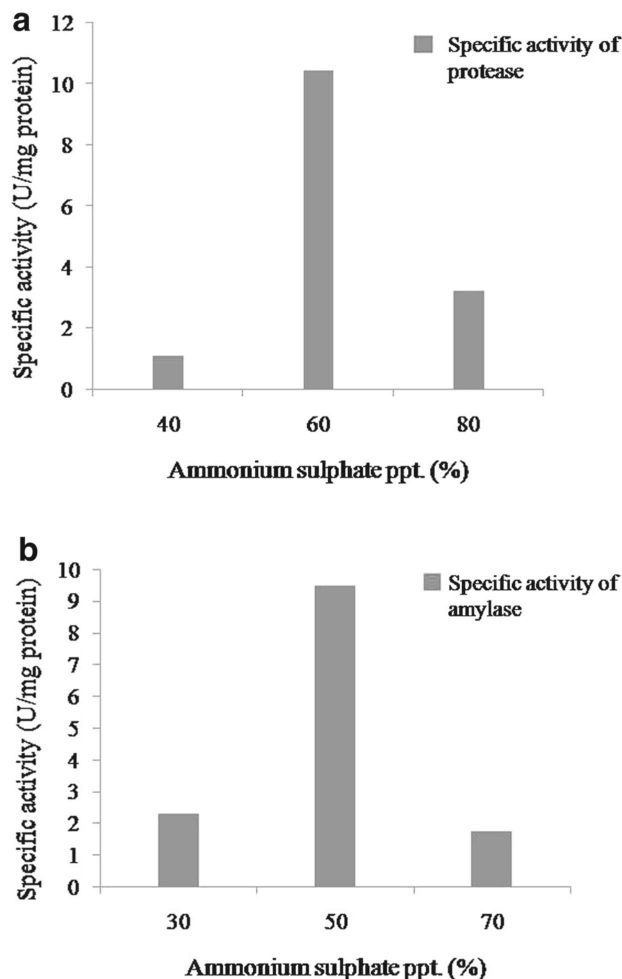


Fig. 1 Ammonium sulfate saturation graph. **a**, **b** Protease and amylase precipitation, respectively. $U = \mu$g of product liberated/min

the amylase activity in each step of purification (Table 2). Purified amylase also showed significant increase in specific activity from 1.96 to 88.1 U/mg protein ml^{-1} with a purification fold 44.9 and recovery 17.55 %. Mukesh kumar et al. [22] purified an amylase from *Bacillus* sp. MNJ23 isolated from cassava waste and reported the specific activity as 2358 U/mg protein with a purification fold and recovery 3.12 and 27.41, respectively. Recently, Asker et al. [23] purified two proteases from *Bacillus megaterium* which showed a specific activity of 561.27 and 317.23 U/mg protein with a purification fold 13.63 and 7.72, respectively. It has been reported that bacterial enzymes are the cheapest source for random use in different industries such as food, textile, and beverage [1,5]. Proteases are used in many countries as milk- clotting agent as well as for cheese preparation, whereas amylase has great importance in starch processing industries. Bacterial enzymes are also used in waste treatment industries, and therefore, production, purification, and characterization of different bacterial enzymes are necessary in relation to cost management in industries.

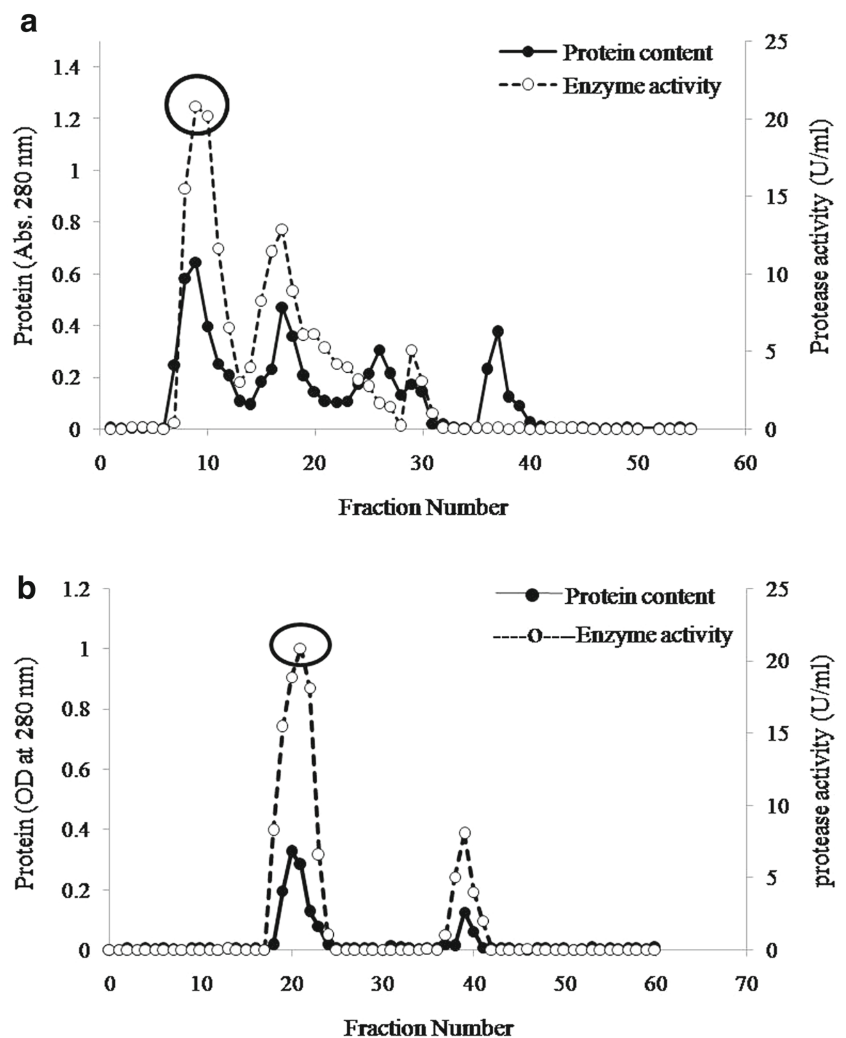


Fig. 2 Demonstrated the purification of protease. **a** Sephadex G-50 column chromatography. The *circle* indicates the active fractions which showed the highest activity. Total 55 fractions each of 4 ml were collected. Flow rate 1.4 ml/min. **b** DEAE cellulose column chromatography. Total 60 fractions each of 4 ml were collected. The *circle* indicates the active fraction (number 20) which showed the highest activity flow rate 0.6 ml/min. $U = \mu$g of tyrosine liberated/min

3.2 Characterization of Purified Protease and Amylase

In the present study, molecular weight of the purified protease and amylase was recorded to be ∼17 and ∼28 kDa, respectively, on SDS-PAGE and the corresponding zymographic spot proved their enzymatic properties (Figs. 4, 5).

Bacterial proteases have several advantages such as stability, substrate specificity, and low fermentation cost and vary greatly in molecular weight [24]. Asker et al. [23] purified two proteases, namely P1 and P2 from the bacterial strain *Bacillus megaterium*, and determined their molecular weight as 28 and 25 KDa, respectively. Vidyasagar et al. [25] purified an extreme thermophilic protease (66 kDa) from *Chromohalobacter* sp. TVSP101. Amylase is very useful in different industries which produce glucose, crystalline dextrose, dextrose syrup, maltose, and maltodextrins [13]. *Bacillus licheniformis, Bacillus subtilis*, *Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens* have been reported to be good source of α-amylase [15]. Gessesse et al. [26] purified a protease of 24 kDa from *Bacillus pseudofirmus*. Thermal and alkalinity sensitivity of enzyme is very important for its proper function. The present investigation demonstrated the effect of temperature and pH on the purified protease and amylase activity. It was observed that the protease activity gradually increased up to pH 8.0 followed by sudden depletion. For the purified amylase, pH 7.5 was observed to be the most suitable environment, indicating its alkaline nature (Fig. 6).

The bacterium *Corynebacterium alkanolyticum* ATH3 which produced these enzymes was isolated from fish intestine. Due to carbonate salts, the pH of the intestine is alkaline in nature, creating a pH of about 8.5 which supports our findings [27]. Alkaline nature of extracellular protease and amylase produced by different bacterial strains has also been reported by many authors [13,28]. Similarly, pH sensitivity of amylase produced by different bacterial strains was reported by de Souza and de Oliveira e Magalhães [29]. Like pH, temperature is one of the most important factors

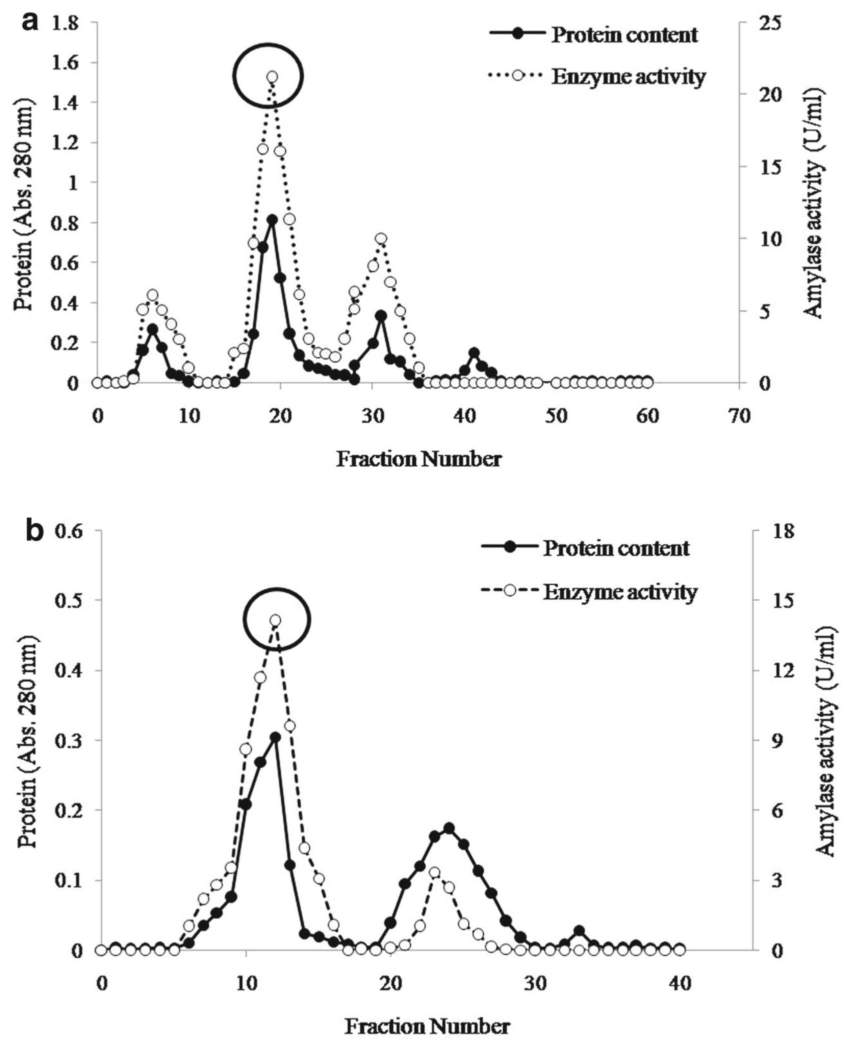


Fig. 3 Demonstrated the amylase purification. **a** Amylase purification by DEAE cellulose column chromatography. Total 60 fractions (4 ml each) were taken. The *circle* indicates the active fraction (number 19) which showed the highest activity. Flow rate 0.7 ml/min. **b** Sephadex G-100 column chromatography. Total 40 fractions each of 4 ml were taken. The *circle* indicates the active fraction (number 11) which showed the highest activity. Flow rate 1.2 ml/min. $U = \mu$g of maltose liberated/min

Table 1 Summary of protease purification

Purification steps	Volume (ml)	Total protein content (mg)	Total enzyme activity (U/ml)	Specific activity (U/mg protein ml^{-1})	Purification (Fold)	Yield (%)
Supernatant	70	152.28	548.21	3.6	1.0	100
$(NH_4)_2SO_4$	20	36.16	377.23	10.43	2.89	68.8
Dialysis	10.2	12.4	225.27	18.16	5.04	41.1
Sephadex G-50	8	2.41	164.34	68.2	18.94	29.97
DEAE cellulose	4	0.89	83.42	93.73	26.03	15.21

$U = \mu$g of tyrosine liberated/min

Table 2 Summary of amylase purification

Purification steps	Volume (ml)	Total protein content (mg)	Total enzyme activity (U/ml)	Specific activity (U/mg protein ml^{-1})	Purification (Fold)	Yield (%)
Supernatant	75.0	163.87	321.18	1.96	1.0	100
$(NH_4)_2SO_4$	15.0	24.16	228.95	9.47	4.83	71.28
Dialysis	8.2	8.11	136.21	16.8	8.57	42.4
DEAE cellulose	4.0	1.98	84.48	42.66	21.8	26.3
Sephadex G-100	4.0	0.64	56.39	88.1	44.94	17.55

$U = \mu$g of maltose liberated/min

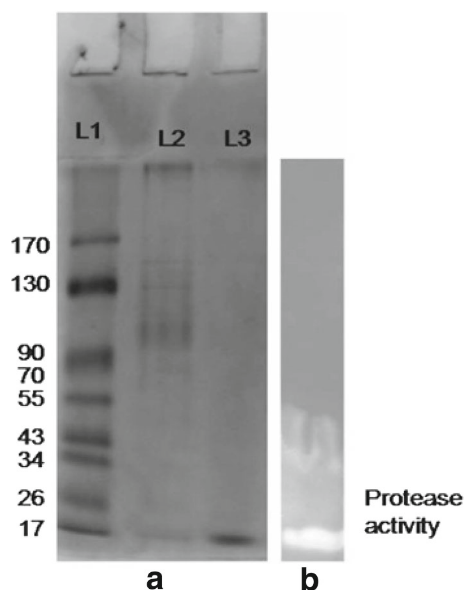


Fig. 4 **a** SDS-PAGE of purified protease. *Lane 1* marker; *lane 2* raw extract; *lane 3* enzyme. **b** the zymography analysis of purified protease

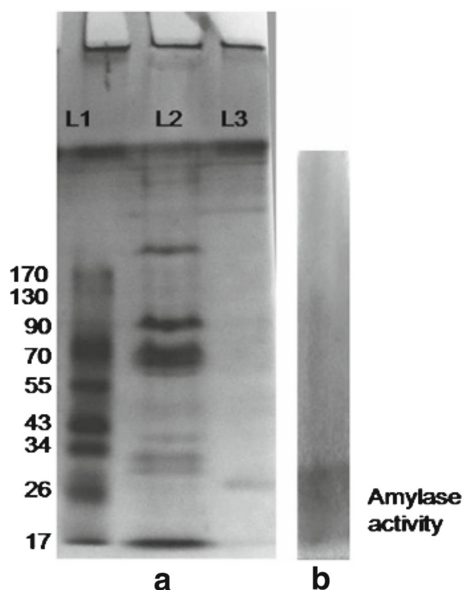


Fig. 5 **a** SDS-PAGE of purified amylase. *Lane 1* marker; *lane 2* raw extract; *lane 3* enzyme. **b** the zymography analysis of purified amylase

for enzyme activity. Our results demonstrated that the activities of protease and amylase were highest at 45 and 35 °C, respectively. It was also observed that the activities of purified protease and amylase were stable up to 50 and 45 °C, respectively, followed by quick degradation (Fig. 7).

Padmapriya et al. [30] purified a protease and reported the highest activity at pH 7.0 and 70 °C temperature. Kunamneni et al. [31] isolated, purified, and partially characterized a protease produced by *Bacillus subtilis* PE ll which was optimally active at 60 °C at pH 10.0. Sareen and Mishra [32] reported a thermoalkaline protease from *B. licheniformis*, which showed maximum activity at 50 °C. Yandri et al. [1] determined the optimum pH (6.0) and temperature (60 °C) of the purified α-amylase, which was 67 kDa. Mukesh Kumar et al. [16] purified amylase from *Bacillus* Sp. MNJ23 which showed stability between pH 8.0–10.0 and temperature 50 °C. Abusham et al. [32] also reported an alkaline protease from *B. subtilis* with highest activity in the temperature range of 35–55 °C. On the other hand, Manachini et al. [33] reported an alkaline protease produced by the bacterium *Bacillus thermoruber* that showed optimum activity at 45 °C. Al-Shehri et al. [34] have isolated, purified, and characterized a protease produced by *Bacillus licheniformis* and recorded the optimum activity at pH 9 and temp 55 °C. Similarly, Kim et al. [35] have purified and characterized a 33 kDa protease from *Acanthamoeba lugdunensis* KA/E2 and determined the optimum pH and temperature at 8.5 and 55 °C, respectively. The results of these studies indicate that bacterial enzymes vary greatly in stability, activity, substrate

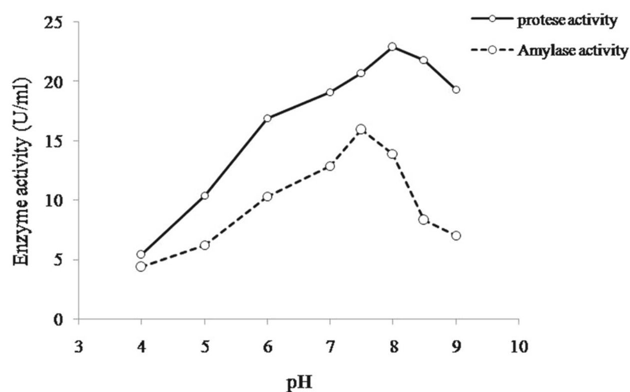


Fig. 6 Effect of pH on enzyme activity. $U = \mu$g of product liberated/min

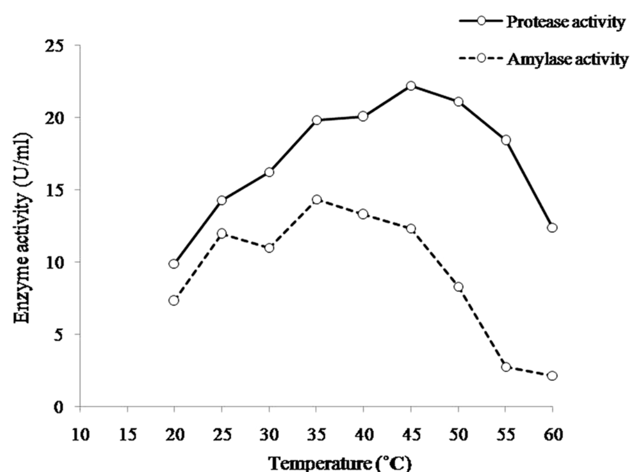


Fig. 7 Effect of temperature on enzyme activity. $U = \mu$g of product liberated/min

specificity, and molecular weight, and thus, suitable enzyme should be selected according to their application.

In recent year, the demand of microbial enzymes in different industries is increasing rapidly. Animal and plant sources of enzymes are unable to meet the industrial demand, and therefore, screening and purification of microbial enzymes are important. Generally, bulk production of enzymes in industries requires little downstream processing and thus is crude in nature, while enzymes that are used in pharmaceutical and clinical sectors require high purity [36]. Detergent industries are the primary consumers of enzymes, especially amylase that is used in all liquid detergent [37,38]. Laboratory-based purification of protease and amylase includes different combinations of gel filtration, ion exchange, hydrophobicity, and reverse-phase chromatography [29,30]. In the present study, we have used the combination of gel filtration and ion-exchange chromatography for the purification of protease and amylase produced by the bacterial strain, *Corynebacterium alkanolyticum* ATH3. Due to high activity of these enzymes (protease 93.73 U/mg protein and amylase 88.1 U/mg protein), they can be efficiently used in different industries. However, the cost for their industrial production needs to be evaluated.

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