



***Streptomyces* sp. K47 alkaline proteases: partial purification and analysis by zymography**

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ABSTRACT. Microbial enzymes are used as organic catalysts in different industrial processes. In this study, we aimed to produce and investigate alkaline proteases from a novel actinobacterium strain isolated from a Black Sea marine sediment. The optimal production conditions for *Streptomyces* sp. K47 alkaline proteases was 4-days incubation at 28°C in a salt-free medium buffered with 50 mM Tris-HCl buffer (pH 9.0) and containing glucose (1.0%, w v⁻¹) and yeast extract (0.5%, w v⁻¹). The enzyme solution was partially purified using (NH₄)₂SO₄ precipitation (40-70%). After desalting, it was purified 1-84 fold with a recovery of 19.42%. Zymogram analyses revealed the presence of more than one protease enzyme. The enzyme solution exhibited maximum activity at pH 9.0 and 37°C, remaining stable after a 2-hour incubation at all tested conditions. *Streptomyces* sp. K47 has the potential to be used in industrial processes because of its ability to produce multiple protease enzymes displaying stability in a broad pH and temperature range.

Keywords: *Streptomyces* sp.; alkaline protease; partial purification; SDS-PAGE; zymography.

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Introduction

Enzymes are proteins used in medical, agricultural, chemical, biofuel, food and beverage industries. They are important low-cost catalysts because of their specific properties such as substrate specificity and minimum production of by-product arising from their chemo-, regio-, and stereo-selectivity. They are also biodegradable and non-toxic compounds requiring moderate conditions to catalyze reactions and are therefore an alternative to harmful chemical counterparts (Singh, Kumar, Mittal, & Mehta, 2016; Sanchez & Demain, 2017; Prasad & Roy, 2018).

The demand for enzymes throughout the world is increasing day by day and they have been used extensively in an industrial scale over the last few decades. The global enzyme market amounted to \$1.5 billion in 2000, \$3.1 billion in 2009 and \$5.01 billion in 2016 and this value is guessed to be \$6.32 billion in 2021 (Kirk & Borchert, 2002; Chapman, Ismail, & Dinu, 2018).

More than 75% of the industrial enzymes are composed of hydrolytic ones. These catalysts are obtained from animal, plant and microbial sources. Proteases, also termed as proteolytic enzymes or proteinases, are one of the important groups of enzymes that hydrolyze peptide bonds in proteins (Rani, Rana & Datt, 2012), and correspond to about 60% of the market (Laxman et al., 2005). They perform a wide variety of complex physiological functions in protein catabolism, cell growth and migration, tumor growth and proliferation, secretion of secretory proteins to membranes, etc. They are also used in detergent, textile, food, cosmetic, pharmaceutical, waste processing, medical diagnostic and other industries (Sevinc & Demirkan, 2011). The most well-known plant-derived proteases are papain, keratinase, bromelain and ficin whereas proteases from animal origin are renin, pepsin, chymotrypsin and trypsin. Certain inadequacies of animal and plant proteases have increased interest in microbial resources (Rao, Tanksale, Ghatge, & Deshpande, 1998).

Microbial proteolytic enzymes are secreted by numerous species belonging to bacteria, fungi and viruses. Their wide biochemical diversity and potential for genetic manipulations make microorganisms an excellent source for enzymes. Furthermore, almost all possess features such as rapid growth rates and simple nutritional requirements as prerequisites for biotechnological applications (Rao et al., 1998).

The aim of our research was to investigate the production of alkaline proteases from actinobacterium strain K47 from a marine sediment from Black Sea. Partial purification, characterization and zymogram analysis were also studied.

Material and methods

Microorganism and 16S rDNA sequencing

An actinobacterium strain labeled as K47 was isolated from a marine sediment of Black Sea by the method by Özcan et al. (2013) on agar plates of ISP2 medium (in g L⁻¹: dextrose, 4.0; malt extract, 10.0; yeast extract, 4.0; agar, 20.0, pH 7.2). The strain was maintained at +4°C in a spore stock solution containing 20% (v v⁻¹) glycerol throughout the study.

Genomic DNA isolation was carried out as described (Liu, Coloe, Baird, & Pedersen, 2000). The 16S rDNA region was amplified with BioRad Thermal Cycler (Özcan et al., 2013). The sequence of 16S rDNA region was released in GenBank under accession number KX674575. The evolutionary history was inferred using the neighbor-joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches (Felsenstein, 1985). The evolutionary distances were computed using the Tamura-Nei method (Tamura & Nei, 1993). Evolutionary analyses were conducted in MEGA7 (Kumar, Stecher, & Tamura, 2016).

Determination of proteolytic activity and protein content

The proteolytic assay was performed according to the slightly modified universal protease activity method proposed by Sigma. Briefly, 320 µL of samples were mixed with 800 µL of 0.65% (w v⁻¹) casein solution in Eppendorf tubes and the tubes were incubated at 37°C for 15 min. After adding 800 µL of 110 mM trichloroacetic acid (TCA), the tubes were incubated again at 37°C for 30 min and then centrifuged at 6000 g for 10 min. 500 µL of supernatants transferred to fresh Eppendorf tubes were mixed with 1.25 mL of 500 mM sodium carbonate (Na₂CO₃), and 250 µL of Folin-Ciocalteu reagent at 1:4 dilution. The tubes were incubated at 37°C for 30 min and absorbances of the samples were measured at 655 nm in a spectrophotometer. A proteolytic unit is expressed as the quantity of protease catalyzing the conversion of casein to one µmol tyrosine under the conditions studied. Total protein contents of the samples were quantified using Bradford reagent with bovine serum albumin (BSA) as standard (Bradford, 1976).

Determination of optimal production conditions

The production medium used in the study is protease medium consisting of (in g L⁻¹) glucose 10.0, peptone 5.0, yeast extract 5.0, KH₂PO₄ 1.0 and MgSO₄ 0.2 (Mehrotra, Pandey, Gaur, & Darmwal, 1999). For the activation of actinobacteria strain, 50 mL of the protease medium in an Erlenmeyer flask, inoculated with 5 µL of spore stock solution, was incubated at 28°C and 120 rpm for 4 days.

To detect the optimal production conditions for proteases, carbon, nitrogen and salt content of the medium, as well as incubation pH, time and temperature, were considered.

To determine the optimal incubation time, an Erlenmeyer flask containing the protease medium buffered with 50 mL phosphate buffer (pH 7.0) and inoculated with 2.5 mL of the medium obtained after activation of actinobacteria was incubated at 28°C and 120 rpm for 6 days. During incubation, 5 mL of the samples were withdrawn daily and centrifuged at 3500 g for 15 min. The supernatants were used to determine enzyme activity and protein content. The pellets were dried at 55°C until constant weight and dried weight was expressed as g biomass per L of the culture medium (Çorbacı & Özcan, 2019).

The most suitable temperature and pH conditions for the production of proteases were determined first by carrying out the production at 28 and 37°C in the protease medium buffered with 50 mM phosphate buffer (pH 7.0), and then the medium buffered at different pH values ranging from 5.0 to 10.0 [50 mM citrate-phosphate buffer (pH 5.0 and 6.0), 50 mM phosphate buffer (pH 7.0), 50 mM Tris-HCl buffer (pH 8.0 and 9.0) and 50 mM glycine-NaOH buffer (pH 10.0)] were incubated at defined optimal temperature for 4 days (Abdelwahed, Danial, El-Naggar, & Mohamed, 2014).

To determine the influence of several carbon sources, the medium was supplemented with different carbon sources (glycerol, maltose, starch and sucrose) to replace the prescribed carbon source (glucose) of the medium. To investigate the influence of several nitrogen sources, the medium was supplemented with various nitrogen sources (casein, gelatin, malt extract, peptone, tryptone and yeast extract), to replace the prescribed nitrogen sources (peptone and yeast extract). To detect the appropriate NaCl content for protease production, different NaCl concentrations (0.25, 0.50, 1.0 and 2.0%, w v⁻¹) were added to the medium (Thumar & Singh, 2007).

Production and partial purification of proteases

To obtain large amounts of proteases for ammonium sulfate $[(\text{NH}_4)_2\text{SO}_4]$ precipitation, 10 μL of spore stock solution was transferred to each of 100 mL optimized protease medium (in g L^{-1} : glucose, 10.0; yeast extract, 10.0, KH_2PO_4 , 1.0 and MgSO_4 , 0.2) buffered with 50 mM Tris-HCl buffer (pH 9.0) and the flasks (A total of 1800 mL) were incubated at 28°C and 120 rpm for 4 days. To determine the most suitable $(\text{NH}_4)_2\text{SO}_4$ concentration for the precipitation of proteases, $(\text{NH}_4)_2\text{SO}_4$ at concentrations ranging from 40-80% (w v^{-1}) was applied and the samples were mixed slowly with stirring overnight at +4°C. Pellets were collected by centrifugation at 3500 g for 20 min and dissolved in 10 mL of 50 mM Tris-HCl buffer (9.0). After determining the enzyme activities of samples, a large-scale $(\text{NH}_4)_2\text{SO}_4$ precipitation was performed in 1500 mL of the culture-free supernatant. To remove $(\text{NH}_4)_2\text{SO}_4$, dialysis was performed overnight at +4°C in a dialysis bag against 50 mM Tris-HCl (pH 9.0) with several changes of buffer (Winarti et al., 2018).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining

SDS-PAGE analyses of the samples were carried out under denaturing conditions according to the method of Laemmli (1970) using a 12% acrylamide gel. After electrophoresis, the gel was silver stained (Shevchenko, Wilm, Vorm, & Mann, 1996) with Precision Plus Protein All Blue (Bio-Rad) as a marker.

Zymogram analysis and Coomassie blue staining

Casein and gelatin zymograms were performed using the slightly modified method (Goto, Miyazaki, Funabiki, & Yasumitsu, 1999); 0.5 mg mL^{-1} of substrate (casein or gelatin) was added to each loading gel. Zymography was carried out in a similar manner to SDS-PAGE but in the absence of a reducing agent and heating. The gels obtained after electrophoresis were renatured in 50 mM Tris-HCl (pH 7.5) containing 2.5% (v v^{-1}) Triton X-100 and 100 mM NaCl for 2 h at room temperature, and then washed three times at room temperature for 5 min with wash buffer (50 mM Tris-HCl, pH 7.5) followed by two times for 1 min with ultra-pure water. After washing, the gels were allowed to incubate in incubation buffer (50 mM Tris-HCl, pH 7.5 with 10 mM CaCl_2) at 37°C for overnight. The gels stained with 0.1% Coomassie Brilliant Blue R-250 were stored at +4°C in a solution containing 5% (v v^{-1}) glacial acetic acid.

Effects of pH and temperature on enzyme activity and stability

To study the influence of pH on activity, casein was dissolved in different buffers with different pH values [50 mM citrate-phosphate buffer (pH 5.0 and 6.0), 50 mM phosphate buffer (pH 7.0), 50 mM Tris-HCl buffer (pH 8.0 and 9.0), 50 mM glycine-NaOH buffer (pH 10.0) and 50 mM $\text{Na}_2\text{HPO}_4/\text{NaOH}$ buffer (pH 11.0 and 12.0)]. To provide pH stability, the pH of the enzyme solution was adjusted to 3.0-10.0 using 0.2 M HCl and 1 M NaOH, and the solutions were incubated at 37°C for 2h (Çorbacı & Özcan, 2019).

To study the optimum temperature, the assay was evaluated under different temperatures (28, 37, 50, 60 and 70°C) in 50 mM Tris-HCl buffer (pH 9.0). For temperature stability, the enzyme solution was incubated for 2 h at different temperatures ranging from 20-70°C (Çorbacı & Özcan, 2019).

Statistical analysis

All the experiments were carried out in triplicate and the results were presented as the mean \pm standard deviation.

Results

Phylogenetic relationships of actinobacterium

A novel protease-producing isolate labeled as K47 was isolated, and on the basis of 16S rDNA sequencing, the isolate showed > 99% similarity with members of the *Streptomyces* genus. A phylogenetic tree based on the neighbor-joining method confirmed the evolutionary relationship of the strain with the other *Streptomyces* species (Figure 1).

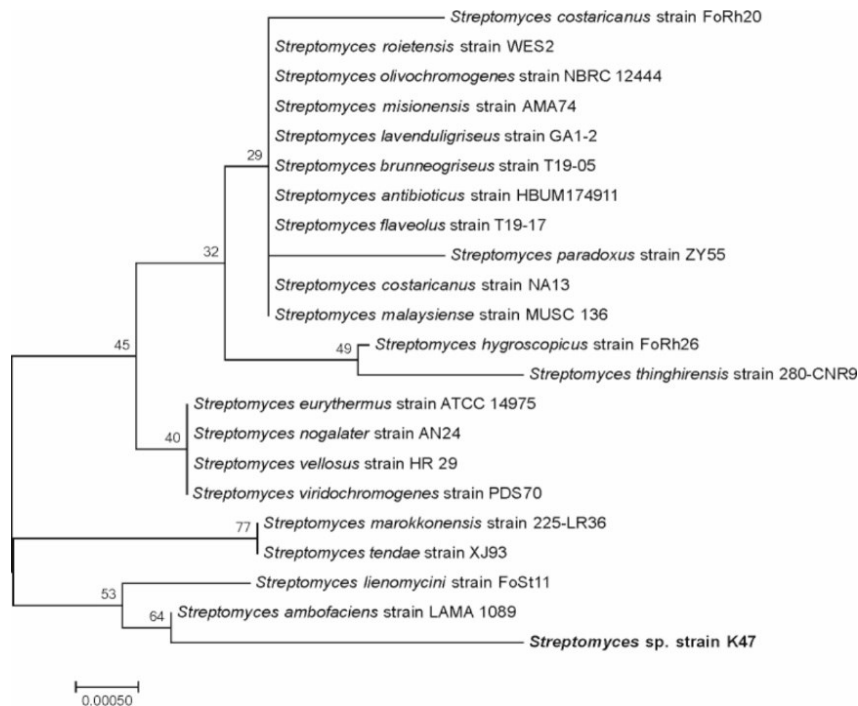


Figure 1. Evolutionary relationships of *Streptomyces* sp. K47. The sequences of other *Streptomyces* species were acquired from the GenBank.

Optimal protease-producing conditions

To study the influence of incubation time on production in submerged fermentation with agitation, the enzyme activities and biomasses of the samples withdrawn daily from the medium were calculated (Figure 2). The actinobacteria strain yielded the highest production rate at 96 h of incubation, entering the stationary phase after this time. The optimal growth temperature was found to be 28°C when both enzyme activity and total protein content were evaluated. After incubation at 28°C for 4 days, enzyme activity and total protein were $1.80 \pm 0.060 \text{ U mL}^{-1}$ and $0.08 \pm 0.003 \text{ mg mL}^{-1}$, respectively, whereas they were found to be $0.51 \pm 0.035 \text{ U mL}^{-1}$ and $0.08 \pm 0.004 \text{ mg mL}^{-1}$ after incubation at 37°C. The actinobacteria strain exhibited the highest protease production rate in Tris-HCl buffer (pH 9.0) after incubation for 4 days at 28°C (Figure 3); a gradual decrease was observed below and above this pH in both enzyme activity and total protein content.

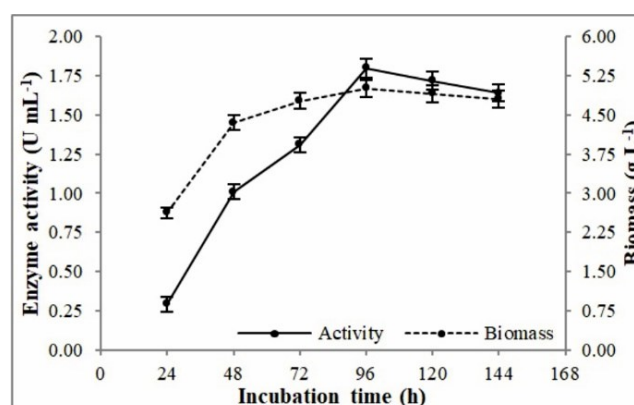


Figure 2. Time-course effect of incubation on protease production and biomass. Bars represent standard errors of mean, $n=3$.

The influence of different carbon and nitrogen sources as well as salt concentration on the production of proteases by *Streptomyces* sp. K47 was also determined. Glucose and yeast extract were found to be the best carbon and nitrogen sources for the production of proteases (Figure 4A and B), yielding the highest values in terms of both enzyme activity and specific activity. Although media containing peptone and yeast extract, gelatin or peptone as nitrogen source promoted slightly better growth of the actinobacteria strain, they resulted in lower yields in terms of protease production (Figure 4B). The effect of different NaCl concentrations on the production of proteases is shown in Figure 5. The maximal yield was obtained in the

salt-free medium after incubation at 28°C for 4 days. The growth and protease production were gradually decreased with increase in NaCl concentration.

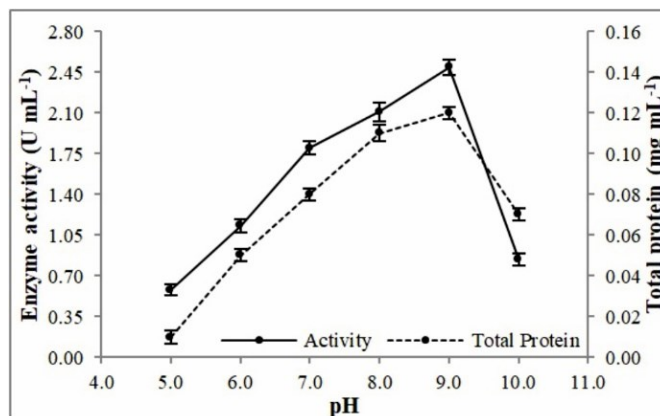


Figure 3. Effect of pH on protease production and total protein. Bars represent standard errors of mean, $n = 3$.

Thus, with the optimized parameters, the highest yield was obtained in the protease medium buffered with 50 mM Tris-HCl (pH 9.0) and containing glucose and yeast extract as carbon and nitrogen sources after 4 days incubation at 28°C. Compared with the initial production, enzyme activity was 1.58-fold increased.

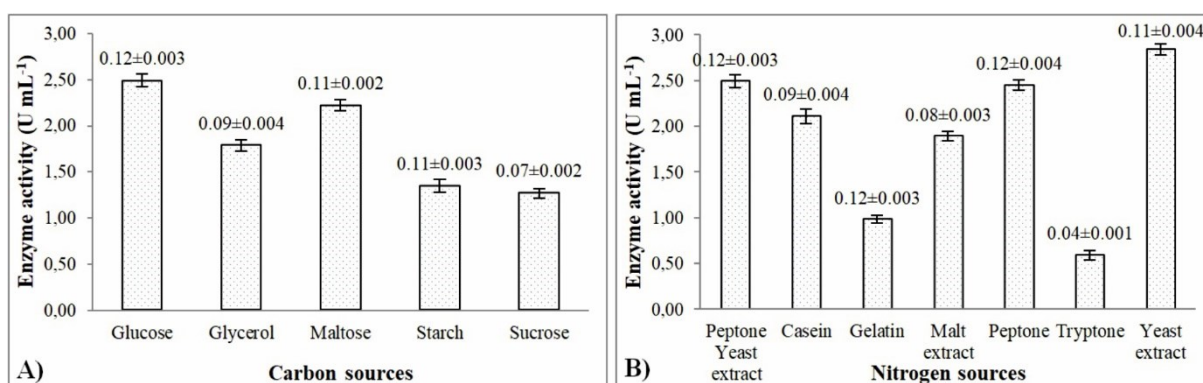


Figure 4. Effects of carbon (A) and nitrogen (B) sources on protease production and total protein. Values above columns are total protein contents of samples in mg mL^{-1} . Bars represent standard errors of mean, $n = 3$.

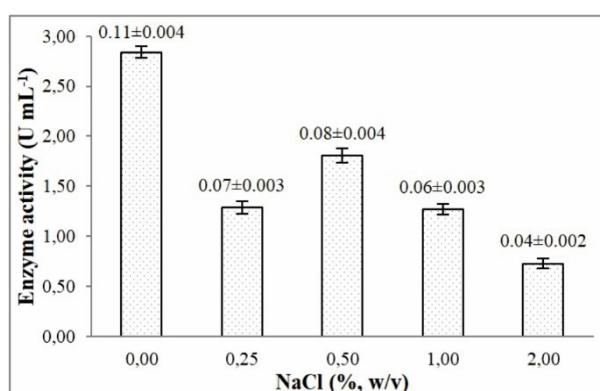


Figure 5. Effect of NaCl on protease production and total protein. Values above columns are total protein contents of samples in mg mL^{-1} . Bars represent standard errors of mean, $n = 3$.

Partial purification and SDS-PAGE and zymogram profiles of proteases

The cell-free supernatant obtained after the large amounts of production of proteases was used as the crude enzyme solution for precipitation and dialysis. The protease solution was precipitated using 40-80% $(\text{NH}_4)_2\text{SO}_4$ fractions; 40-70% of $(\text{NH}_4)_2\text{SO}_4$ fractions yielded higher protease precipitation and the activity was not detected above 80% saturation. After desalting by dialysis at +4°C against 50 mM Tris-HCl (pH 9.0), the protease enzyme was purified 1.84-fold with a recovery of 19.42%. SDS-PAGE profiles of the samples after

silver staining are shown in Fig. 6A. SDS-PAGE analysis revealed that the intensity of some protein bands ranging in size from 20 to 50 kDa increased after the $(\text{NH}_4)_2\text{SO}_4$ precipitation.

The casein and gelatin zymogram profiles of the samples after dialysis are shown in Figure 6B and C. According to the results of both zymograms, *Streptomyces* sp. K47 is capable of producing more than one protease enzyme under the studied conditions. At least five protease bands were detected on the gelatin zymogram while more protease bands were observed on the casein zymogram in a range of 20 to 150 kDa.

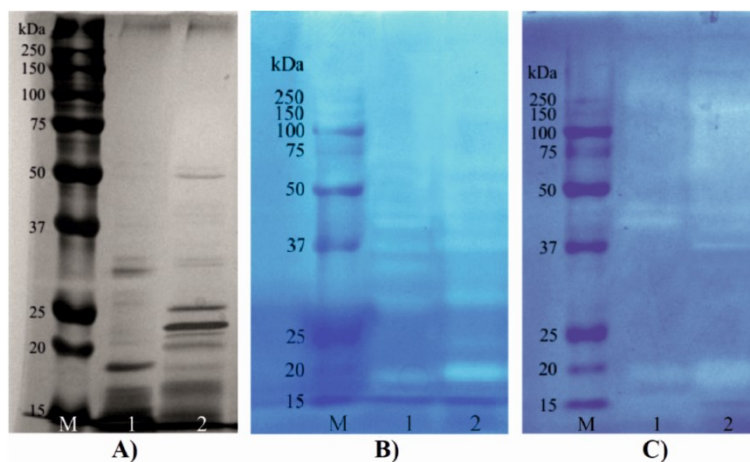


Figure 6. SDS-PAGE (A), casein (B) and gelatin (C) zymogram profiles of samples. M, Marker (Precision Plus Protein All Blue Prestained Protein Standards); Lane 1, Before $(\text{NH}_4)_2\text{SO}_4$ precipitation and dialysis; Lane 2, After $(\text{NH}_4)_2\text{SO}_4$ precipitation and dialysis.

Effect of pH and temperature on enzyme activity and stability

The partially purified protease solution showed optimum activity in Tris-HCl buffer at pH 9.0 (Figure 7A). Although the solution showed activity in varying rates between pH values from 5.0 to 12.0, it was more active at alkaline pH and the activity decreased as the pH approached acidic values. Our protease solution showed maximal stability (100%) at pH 5.0, and 92 and 87% stability, respectively, at pH 6.0 and 7.0 after 2 h incubation. In addition, it remained stable, albeit at different rates, at all tested pH values. The enzyme solution exhibited optimum activity at 37°C, followed by 50 and 28°C, respectively (Figure 7B). After 2 h of incubation, it was stable at a temperature range from 20-70°C, retaining 93 and 86% of residual activity at 20 and 30°C, respectively.

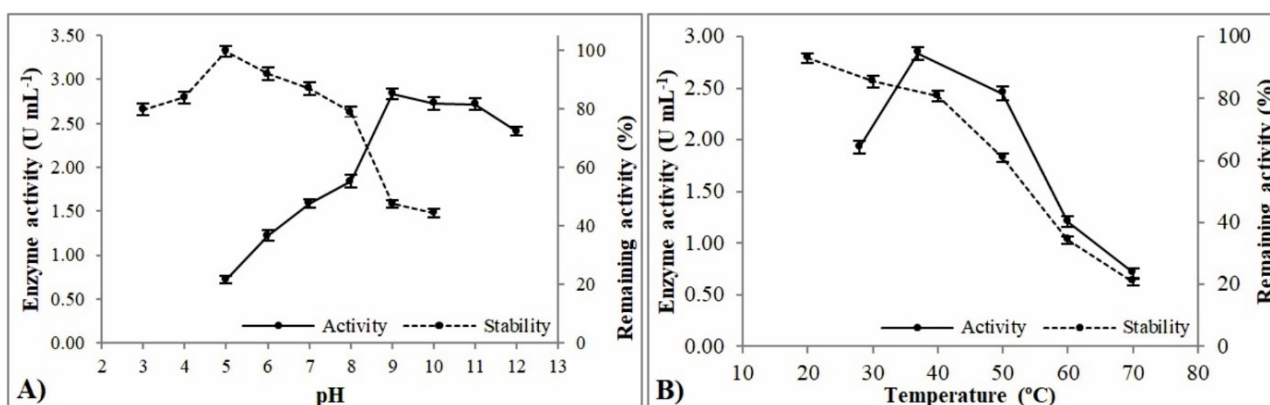


Figure 7. Effect of pH (A) and temperature (B) on enzyme activity and stability. Bars represent standard errors of mean, n = 3.

Discussion

Proteolytic enzymes, which are present in all living organisms, are essential for many biological processes and are also considered as important classes of industrial catalysts (Sevinc & Demirkan, 2011). Therefore, it is important for researchers to find novel enzymes and their sources with potential for use in industrial applications under extreme conditions.

The incubation conditions such as pH and temperature are the most fundamental factors affecting the production of extracellular enzymes by microorganisms since optimal growth ranges of strains belonging to

same microbial species are highly variable. The incubation time is also important due to the proteinaceous nature of enzymes (Abdelwahed et al., 2014; Al-Zahrani, 2018). Our results revealed that *Streptomyces* sp. K47 showed the best production of proteases at pH 9.0 and 28°C, which are consistent with previous findings on the production of proteases by *Streptomyces* species (Vishalakshi, Lingappa, Amena, Prabhakar, & Dayanand, 2009; Hosseini, Saffari, Farhanghi, Atyabi, & Norouzian, 2016).

Since the components of medium play a significant role in producing protease enzymes (Sharma, Kumar, Panwar, & Kumar, 2017; Singh et al., 2017), in our study, the contents of medium were also examined and glucose and yeast extract had a positive impact on the protease production. Our findings are in agreement with Abdelwahed et al. (2014) and Joshi, Kumar and Sharma (2007) who studied protease production by *Streptomyces* sp. strains. In several studies (Thumar & Singh, 2007; Al-Zahrani, 2018), the highest protease activities were observed at NaCl concentrations of 5.0 and 7.0%. We obtained a maximal production yield in the salt-free medium after incubation at 28°C for 4 days. These differences with our results might be due to the nature of actinobacteria and the ingredients of medium used.

The proteases produced by *Streptomyces* sp. K47 were found to have a tendency to precipitate with $(\text{NH}_4)_2\text{SO}_4$ at a concentration around 70%, which is consistent with the literature (Awad, Mostafa, Saad, Selim, & Hassan, 2013; Winarti et al., 2018). SDS-PAGE analysis of the crude and partially purified enzyme solutions revealed that our proteases had molecular weights ranging from 20-50 kDa. In the literature, protease enzymes appear to have highly variable molecular masses in the range of 15-212 kDa. However, it should be noted that most proteases have a molecular weight ranging from 15-50 kDa (Rao et al., 1998). In our study, *Streptomyces* sp. K47 produced more than one protease enzyme under conditions tested by zymography. This is a common phenomenon for *Streptomyces* species. However, there is a little information about the nature and characteristics of proteases obtained from *Streptomyces* sp. strains (El-Shanshoury, El-Sayed, Sammour, & El-Shouny, 1995; Ghorbel, Kammoun, Soltana, Nasri, & Hmidet, 2014).

Compared to literature data, our partially purified proteases showed activity in a wider pH range. They also have higher stability than proteases previously obtained from *Streptomyces* sp. strains. For example, Ghorbel et al. (2014) found that crude enzyme solution obtained from *Streptomyces flavogriseus* HS1 had maximal activity at pH 7.0 and displayed only 40% of its activity at pH 5.0 and 10.0. In another study conducted by Moreira et al. (2001), protease fractions obtained from *Streptomyces clavuligerus* 3585 and *S. clavuligerus* 644 showed their maximal stability at pH 7.0, and their stability significantly decreased below and above this pH. Our proteases appear to be moderately thermotolerant and their thermal stability is much higher than of proteases from other *Streptomyces* sp. strains (Moreira et al., 2001; Ghorbel et al., 2014; Xin et al., 2015).

Conclusion

Due to its ability to produce more than one protease enzyme and their stability in a broad pH and temperature range, *Streptomyces* sp. K47 obtained from a marine sediment of Black Sea is thought to have the potential to be used in many industrial applications requiring protease activity under alkaline conditions.

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