

Production of Alkaline Enzymes by Marine Actinobacteria Isolated from Black Sea Sediments

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ABSTRACT: Alkaline enzymes obtained from microbial sources find numerous applications in different industrial processes and have commercial value. The aims of the study were to investigate the potential of two actinobacteria (*Streptomyces* sp. K16 and K19) isolated from Black Sea sediments to produce industrial alkaline enzymes and to characterize their activities under different chemical and physical conditions. The optimal incubation time for the production was determined as 96 h, and by using two actinobacteria respectively 7.91 and 7.94 U mL⁻¹ of activities for amylases, 0.55 and 0.82 U mL⁻¹ for lipases and 3.07 and 2.34 U mL⁻¹ for proteases were obtained. The optimal pH and temperature values for the enzymes were found to be pH 8.0 and 37°C. All the enzymes exhibited stability in different quantities after the incubation at pH values ranging from 3.0 and 10.0 for 2 h. On the other hand, the lipases remained stable up to 50°C although the amylases and proteases showed stability in varying ratios after 2 h incubation under the tested temperatures. In the light of the findings, the actinobacteria and their enzymes are thought to have potential for further studies.

Keywords: *Streptomyces* sp., amylase, lipase, protease, production, stability

Karadeniz Deniz Sedimentlerinden İzole Edilen Denizel Aktinobakteriler Tarafından Alkali Enzimlerin Üretimi

ÖZET: Mikrobiyal kaynaklardan elde edilen alkali enzimler, farklı endüstriyel süreçlerde pek çok uygulama bulmaktadırlar ve ticari değere sahiptirler. Bu çalışmada, Karadeniz sedimentlerinden izole edilen iki aktinobakterinin (*Streptomyces* sp. K16 ve K19) endüstriyel alkali enzimleri üretme potansiyellerinin incelenmesi ile farklı kimyasal ve fiziksel koşullar altında aktivitelerinin karakterize edilmesi amaçlanmıştır. Üretim için optimum inkübasyon süresinin 96 saat olduğu bulunmuş ve bu iki aktinobakteri kullanılarak sırası ile amilazlar için 7.91 ve 7.94 U mL⁻¹, lipazlar için 0.55 ve 0.82 U mL⁻¹ ve proteazlar için 3.07 ve 2.34 U mL⁻¹ aktiviteler elde edilmiştir. Enzimler için optimum pH ve sıcaklık değerlerinin pH 8.0 ve 37°C olduğu bulunmuştur. Bütün enzimler, 3.0 ile 10.0 arasında değişen pH değerlerinde 2 saat inkübasyondan sonra değişen miktarlarda stabilite göstermişlerdir. Diğer taraftan, amilazlar ve proteazlar, denenen sıcaklık koşulları altında 2 saatlik inkübasyondan sonra değişen miktarlarda stabilite gösterirken lipazlar 50°C'ye kadar stabil kalabilmişlerdir. Bulgular ışığında, bu aktinobakteriler ve onların enzimlerinin, ileriki çalışmalar için potansiyele sahip oldukları düşünülmektedir.

Anahtar Kelimeler: *Streptomyces* sp., amilaz, lipaz, proteaz, üretim, stabilite

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INTRODUCTION

Enzymes are proteins used in various industries, e.g., medicine, chemistry, biofuel, food, beverage and agriculture. These biological catalysts have gained importance due to their some features such as substrate specificity, minimum by-product formation and low cost. They are also biodegradable and non-toxic, and usually require moderate conditions to catalyze reactions and are alternatives to harmful chemical counterparts (Singh et al., 2016). Therefore, the demand for enzymes is increasing day by day worldwide. The market for industrial enzymes was priced at \$4.75 billion in 2016 and is predicted to rise at \$6.30 billion at the end of 2022 (Industrial Enzymes Market, 2018).

Microorganisms are sources of biotechnologically important metabolites owing to their technological advantages such as fast growth rate and easy availability (Gurung et al., 2013; Singh et al., 2016) and searching novel metabolites produced by these organisms is an existing subject for researchers. Among these metabolites, hydrolytic enzymes, especially alkaline ones capable of operating at alkaline conditions, are outstanding proteins and are used in a variety of applications such as the production of detergents, fuel alcohols, papers, fermented products, beverages, textile products, medicinal drugs and leather products (Gupta et al., 2002; de Souza and Magalhaes, 2010; Cherif et al., 2011).

Enzymes obtained from microbial sources are preferred to those obtained from plants or animals because their production is cheap, controllable and reliable (Arikan et al., 2003). However, in general, microorganisms cannot produce extracellular enzymes in sufficient quantities for industrial applications. Therefore, isolating novel microbial strains having the desired enzyme systems is an important task, and by optimizing their enzyme production

conditions it is possible to obtain commercial quantities of the final products.

Due to their above-mentioned importance, the production of industrial alkaline enzymes at high rates by a single microorganism is very important. Thus, in the present paper, we focused to investigate the production of extracellular alkaline enzymes (amylase, lipase and protease) by two actinobacterial strains previously obtained from sediments from Black Sea.

MATERIALS AND METHODS

Actinobacteria

Actinobacteria used in the present study were *Streptomyces* sp. strains K16 and K19 previously isolated in a study from Black Sea sediments (Özcan, 2013). The bacterial strains were genetically identified by using 16S ribosomal RNA (rRNA) sequencing and their NCBI accession numbers are KX674561.1 and KX674563.1, respectively. The strains were stored at +4°C on stock solutions of 20% glycerol throughout the study.

Growth Kinetics and Crude Enzyme Preparations

To investigate the effect of incubation time on the growth and enzyme production, 2.5 mL of actinobacteria obtained after growing 5 µL of spore stocks separately in production media at 28°C for 4 days on a rotary shaker were inoculated in 250 mL Erlenmeyer flasks containing 50 mL of liquid media buffered with phosphate buffer (pH 8.0). The extracellular production was performed respectively in starch medium [g L⁻¹: starch, 10.0; yeast extract, 3.0; peptone, 5.0; NaCl, 3.0 and MgSO₄, 0.5] (Deljou and Arezi 2016), tributyrin medium [g L⁻¹: tributyrin, 10.0; yeast extract, 5.0; peptone, 5.0, NaCl, 0.5; CaCl₂, 0.05 and Tween 80, 5.0] (Kumar et al., 2005) and protease medium [g L⁻¹: glucose, 10.0; yeast extract, 5.0; peptone, 5.0, KH₂PO₄, 1.0 and MgSO₄, 0.2] (Mehrotra et al., 1999) on a rotary shaker at 120 rpm and 28°C

for nine days. During the incubation, 5 mL of samples were withdrawn daily and centrifuged at 6000 rpm for 15 min. The pellets after the centrifugation were kept at 55°C until dried to constant weight, and dry weight was specified as biomass per liter of culture medium (g L^{-1}). The supernatants were used as crude enzyme sources.

Enzyme Assays

The amylase assay was performed by using the slightly modified method of Rick and Stegbauer (1974). 500 μL of the samples were incubated in Eppendorf tubes containing 500 μL of 1% soluble starch at 37°C for 15 min. Then, one mL of dinitrosalicylic acid reagent (DNS) was added to the tubes and the tubes were incubated in boiling water for 5 min. After cooling at room temperature, the mixtures were diluted up to ten mL with distilled water. The absorbances were determined at 546 nm using a spectrophotometer (Yalçın and Çorbacı, 2013). One unit of amylase was defined as the amount of enzyme that catalyzes the conversion of soluble starch to one μmol of maltose per mL per min under assay conditions.

The lipase activity was determined by using the slightly modified method of Rapp and Backhaus (1992). 750 μL of p-nitrophenyl palmitate (pNPP) solution and 500 μL of the samples were mixed in Eppendorf tubes and incubated at 37°C for 15 min. After the incubation, 100 μL of 1 M sodium carbonate (Na_2CO_3) solution was added, and the tubes were re-incubated at 37°C for 15 min. Then, the absorbances were determined at 415 nm using a spectrophotometer (Özcan and Çorbacı, 2017). One unit of lipase was defined as the amount of enzyme required to the conversion of one μmol of p-nitrophenol from pNPP per mL per min under assay conditions.

The protease activity was assayed according to the Sigma's universal protease assay method with minor modifications. 320 μL of the samples were added to Eppendorf tubes

containing 800 μL of 0.65% casein solution and the tubes were incubated at 37°C for 15 min. After the incubation, 800 μL of 0.11 M trichloroacetic acid (TCA) solution was added to the tubes, and the tubes re-incubated at 37°C for 30 min. The tubes were centrifuged at 8000 rpm for 10 min. 500 μL of filtrate, 1.25 mL of 0.5 M Na_2CO_3 solution and 250 μL of Folin-Ciocalteu reagent (1:4 dilution) were mixed in new tubes and further incubated at 37°C for 30 min. Then, the absorbances were determined at 655 nm using a spectrophotometer (Özcan and Çorbacı, 2017). One unit of protease was defined as the amount of enzyme that catalyzes the hydrolysis of casein to one μmol of tyrosine per mL per min under assay conditions.

In the determination of enzyme activities, blank solutions were incubation media in which actinobacteria were not inoculated.

Effect of pH on Enzyme Activity and Stability

To determine the optimal pH conditions, the substrates were dissolved in buffers at different pH values. For this purpose, 50 mM citrate-phosphate buffer (pH 5.0 and 6.0), 50 mM phosphate buffer (pH 7.0 and 8.0) and 50 mM glycine-NaOH buffer (pH 9.0 and 10.0) were used and the enzyme activities were determined as described above. To investigate the effect of pH on stability, pH values of the crude enzyme solutions were adjusted to values ranging from 3.0 to 10.0 with 0.2 M HCl or 1 M NaOH and the solutions were incubated at 37°C for 2 h.

Effect of Temperature on Enzyme Activity and Stability

To determine the optimal temperature conditions, the enzyme activities were evaluated at different temperatures (30, 37, 45, 50, 55 and 60°C) in substrate solutions dissolved in 50 mM phosphate buffer (pH 8.0) as described above. For the effect of temperature on stability, the crude enzymes were incubated at different temperatures (from 20 to 70°C) for 2 h.

Total Protein

The total protein contents of the samples were determined by Bradford method using bovine serum albumin (BSA) as a standard (Bradford, 1976).

Statistical Analysis

All experiments in the study were performed in triplicate and the results were given as the mean±standard deviation.

RESULTS AND DISCUSSION

Production of Alkaline Enzymes

One of the most successful approaches in the finding of novel enzymes is the screening of extracellular enzymes produced by microorganisms isolated from natural habitats. As mentioned earlier, we aimed to investigate the production of alkaline enzymes by two actinobacteria strains (*Streptomyces* sp. K16 and K19) isolated from Black Sea sediments. First, we surveyed separately the growth kinetics of the strains on the media used (data not shown) and observed that both strains showed similar kinetics in terms of the biomass yielded and the enzyme production on the media. Briefly, they passed to the stationary phase at the end of the 4th day and the enzyme activities reached the highest level. Then, the enzyme yields decreased gradually with increasing incubation time. In many studies, various researchers have reported similar results consistent with our findings (Aly et al., 2012; Akhtar et al., 2013); however, we also encountered different growth and

production kinetics for actinobacteria, which was due to the differences in species or strains studied.

The enzyme activities and the protein contents of the production media incubated with *Streptomyces* sp. K16 and K19 at 28°C for four days were summarized in Table 1. Both actinobacteria showed similar productions close to each other under the assay conditions. Comparing the findings with results from several studies, we observed that our strains are well producers, considering their production abilities of the enzymes studied. For instance, in a research conducted by Kumar et al. (2012), the authors aimed to screen actinomycetes for antimicrobial activity and industrial enzymes, and obtained forty-eight strains from earthworm castings. They screened them for the production of five enzymes including amylase, caseinase and lipase, and reported that only ten isolates produced all the enzymes studied. Thus, isolating organisms capable of producing several extracellular enzymes used for industrial purposes are of great importance.

pH and Temperature Characteristics of Enzymes

The hydrolysis of the substrates by the enzymes was determined at different pH conditions. As seen in figures, the enzymes showed their highest activity at pH 8.0 and apart from the value, the activities gradually decreased (Figure 1-3).

Table 1. Activities and total protein contents of crude enzyme solutions

Enzymes	<i>Streptomyces</i> sp. K16			<i>Streptomyces</i> sp. K19		
	Activity (U mL ⁻¹)	Total protein (mg mL ⁻¹)	Specific activity (U mg ⁻¹)	Activity (U mL ⁻¹)	Total protein (mg mL ⁻¹)	Specific activity (U mg ⁻¹)
Amylase	7.91±0.08	0.12±0.01	65.92±4.57	7.94±0.08	0.13±0.01	61.08±3.91
Lipase	0.55±0.03	0.08±0.00	6.88±0.41	0.82±0.04	0.06±0.00	13.67±0.61
Protease	3.07±0.04	0.11±0.01	27.91±2.12	2.34±0.02	0.11±0.01	21.27±1.61

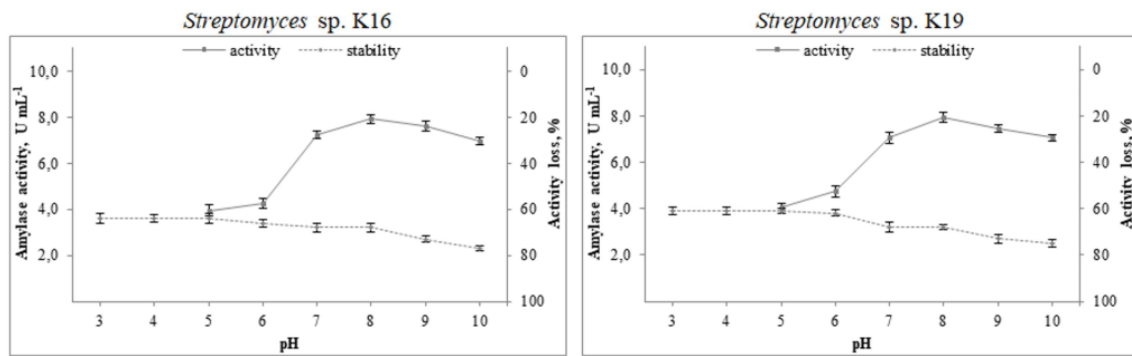


Figure 1. Effect of pH on amylase activity and stability (Bars represent standard errors of means, n=3)

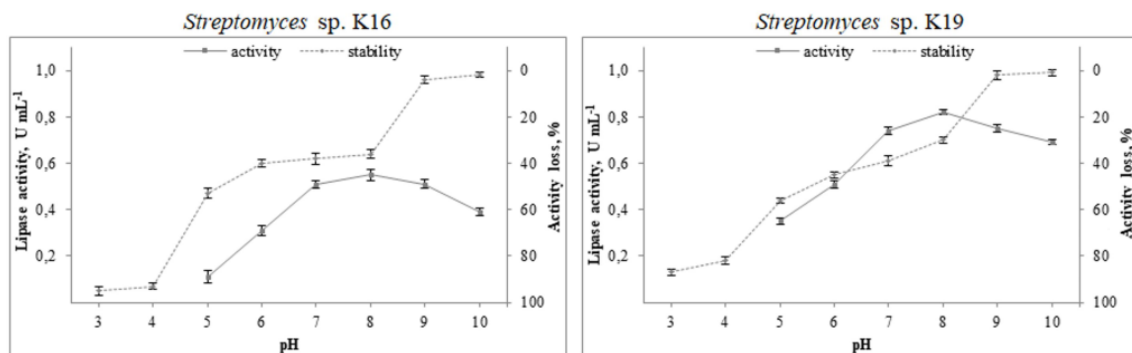


Figure 2. Effect of pH on lipase activity and stability (Bars represent standard errors of means, n=3)

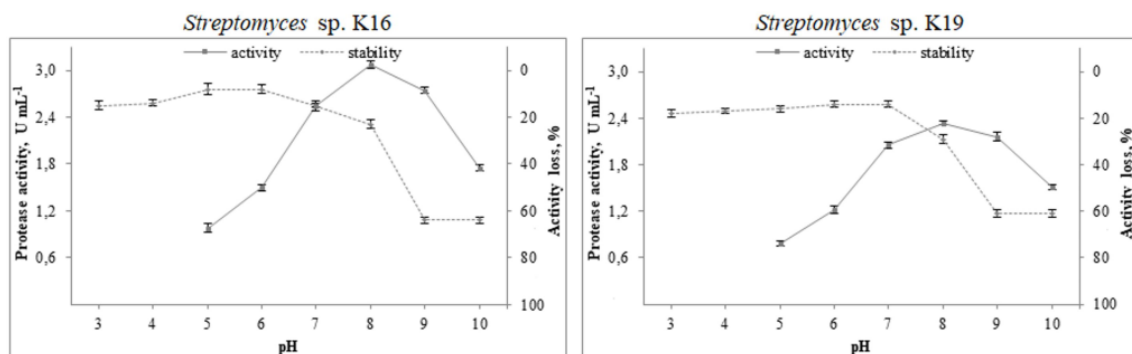


Figure 3. Effect of pH on protease activity and stability (Bars represent standard errors of means, n=3)

For pH stability, we adjusted the pHs of the crude enzyme solutions to values ranging from 3.0 to 10.0 with 0.2 M HCl and 1 M NaOH, and incubated the solutions at 37°C for 2 h. As seen in the figures, we found that the enzymes had different stability over a wide pH range of 3.0-10.0. The amylases showed their maximal stability under acidic and neutral pH values after the incubation for 2 h (Figure 1), and remained stable at approximately 20-30% under alkaline conditions (pH 8.0-10.0). On the other hand, the lipases retained almost all of their

activity (nearly 100% for pH 9.0 and 10.0) under alkaline pH values although they lost 90-95% under acidic conditions (Figure 2). In the proteases, they were stable (nearly 85-90%) at acidic and neutral pH values (Figure 3).

For determining temperature stability, we incubated the crude enzymes at different temperatures for 2 h (Figure 4-6) and found that the enzymes showed different thermostabilities. For the amylases, they gave nearly 100% of their activity at 20 and 30°C. The enzymes retained 20% at above 30°C, and completely inactivated

at 80°C. Thus, the amylase enzymes studied were found to be moderately temperature stable (Figure 4). For the lipases, *Streptomyces* sp. K16 lipase showed better results than that of *Streptomyces* sp. K19. While both lipases remained stable 60 and 100% of their activity at 20 and 30°C, the lipase from *Streptomyces* sp. K19 retained 43% after the incubation at 40°C for 2 h. Otherwise, the lipase from *Streptomyces*

sp. K16 gave 98% of its activity at 40°C. Interestingly, after the incubation at 50°C for 2 h, *Streptomyces* sp. K19 lipase showed a little activity at the rate of 7% (Figure 5). In the proteases, they were found to be moderately temperature stable. The enzymes respectively retained 23 and 29% of their activity after the incubation at 70°C for 2 h (Figure 6).

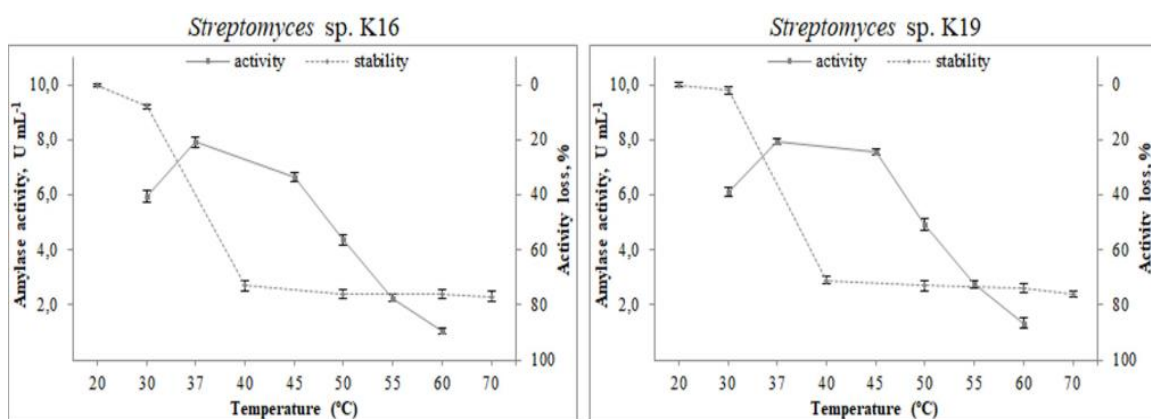


Figure 4. Effect of temperature on amylase activity and stability (Bars represent standard errors of means, n=3)

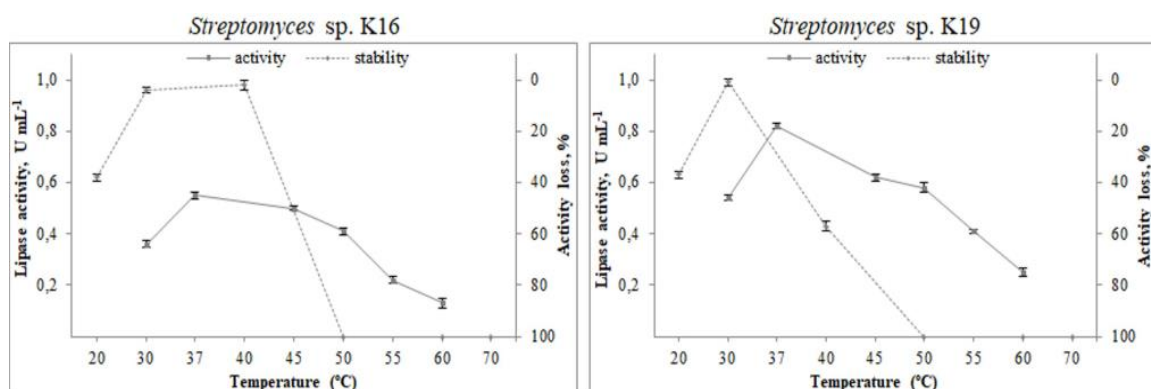


Figure 5. Effect of temperature on lipase activity and stability (Bars represent standard errors of means, n=3)

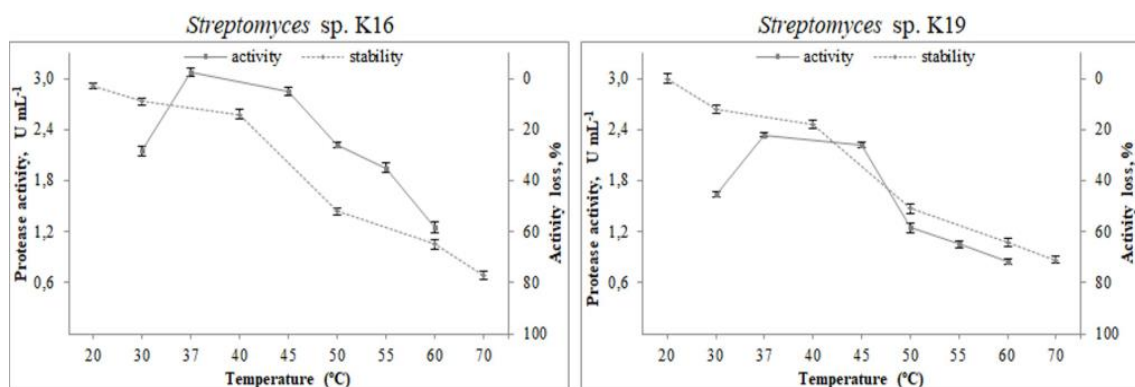


Figure 6. Effect of temperature on protease activity and stability (Bars represent standard errors of means, n=3)

By comparing the stability results with data in literature, we found that the enzymes studied in the study exhibited significant thermostability. For instance, Hoque et al. (2006) investigated the characterization of alpha-amylase activity of *Streptomyces clavifer*, and reported that the enzyme was stable at pH 6.0 to 8.0 and temperature up to 55°C. Apart from these values, the stability of the enzyme decreased sharply, especially for pH. In another study, Sharma et al. (2012) cloned and characterized a gene encoding extracellular lipase from metagenomic DNA extracted from hot spring soil. The authors revealed that the enzyme showed the highest activity at 50°C and pH 9.0. The enzyme remained stable from 20 to 50°C for 30 min, and retained 44% of its activity after the incubation at 55°C for 60 min. The activity also decreased to nearly 50% after 60 min incubation at pH 10.0 and room temperature. Besides, in a study conducted by Ghorbel et al. (2014), the authors studied the isolation and characterization of extracellular protease from *Streptomyces flavogriseus* HS1, and investigated its pH and thermal stability. Researchers indicated that the enzyme was stable more than 70% of its original activity between pH 5.0 and 9.0 at 25°C for 1 h. It was also found that the enzyme retained nearly 70% of its initial activity at 50°C and completely inactivated after the incubation for 1 h at 60°C.

CONCLUSION

In our study, two actinobacteria strains were investigated for the production of three enzymes (amylase, lipase and protease) used intensively in several industries. The enzymes have shown optimal activity at alkaline conditions and stable over a wide range of pH and temperature conditions. In this respect, it is thought that these organisms and their enzyme systems may have potential uses in industrial processes.

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