GIDA
THE JOURNAL OF FOOD
E-ISSN 1309-6273, ISSN 1300-3070

Research / Araştırma
GIDA (2024) 49 (3) 466-477
doi: 10.15237/gida.GD24017

PRODUCTION OF ALKALINE PROTEASE BY A NOVEL ANAEROBIC BACTERIUM ISOLATED FROM A MUNICIPAL ANAEROBIC TREATMENT SYSTEM

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Received/ Geliş: 28.01.2024; *Accepted* Kabul: 06.04.2024; *Published online*/ Online baskı: 13.05.2024

Börekçi, S. B., Dönmez, S., Avcı, A. (2024). Production of alkaline protease by a novel anaerobic bacterium isolated from a municipal anaerobic treatment system. GIDA (2024) 49 (3) 466-477 doi: 10.15237/ gida.GD4017

Börekçi, S. B., Dönmez, S., Avcı, A. (2024). Belediye anaerobik arıtma sisteminden izole edilen yeni bir anaerobik bakteri ile alkali proteaz üretimi. GIDA (2024) 49 (3) 466-477 doi: 10.15237/ gida.GD24017

ABSTRACT

In this study, alkaline protease enzyme production by a bacterial strain isolated from sludge samples collected from an anaerobic treatment system was investigated. According to the 16S rDNA sequence analysis, the isolate was identified as *Thermoanaerobacter thermohydrosulfuricus* (98.52%). Enzyme activity analyses revealed an optimum pH value of 10, an incubation time of 64 h, and a temperature of 35°C. Arabinose and casein hydrolysates were found to be the best carbon and nitrogen sources, respectively. Maximum protease activity was recorded (864.68 U/mL) when arabinose was used instead of glucose. Moreover, the addition of 1 g/L $MgSO_4.7H_2O$ and 0.25 g/L Tween-80 to the medium increased the enzyme activity. Therefore, it can be concluded that *T. thermohydrosulfuricus* is a significant producer of alkaline protease enzymes in the culture medium. To the best of our knowledge, this is the first study to investigate the optimization of alkaline protease production by *T. thermohydrosulfuricus*.

Keywords: Protease, novel strain, *Thermoanaerobacter thermohydrosulfuricus*, anaerobic

BELEDİYE ANAEROBİK ARITMA SİSTEMİNDEN İZOLE EDİLEN YENİ BİR ANAEROBİK BAKTERİ İLE ALKALİ PROTEAZ ÜRETİMİ

ÖZ

Bu çalışmada, anaerobik arıtma sisteminden toplanan çamur örneklerinden izole edilen bir bakteri suşunun alkali proteaz enzim üretimi araştırılmıştır. 16S rDNA dizi analizine göre, izolatın *Thermoanaerobacter thermohidrosulfuricus* (%98.52) olduğu tespit edilmiştir. Enzim aktivitesi analizleri, optimum pH değerinin 10, inkübasyon süresinin 64 saat ve sıcaklığın 35°C olduğunu ortaya çıkarmıştır. Arabinoz ve kazein hidrolizatlarının sırasıyla en iyi karbon ve nitrojen kaynakları olduğu bulunmuştur. Maksimum proteaz aktivitesi (864.68 U/mL) glikoz yerine arabinoz kullanıldığında kaydedilmiştir. Ayrıca, besiyerine 1 g/L $MgSO_4.7H_2O$ ve 0.25 g/L Tween-80 ilavesi enzim aktivitesini artırmıştır. Bu nedenle, *T. thermohidrosulfuricus*'un kültür ortamında önemli bir alkali proteaz enzimi

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üreticisi olduğu sonucuna varılabilmektedir. Bildiğimiz kadarıyla bu çalışma, *T. thermohidrosulfuricus*'un alkali proteaz üretiminin optimizasyonunu araştıran ilk çalışmadır.
Anahtar kelimeler: Proteaz, yeni tür, *Thermoanaerobacter thermohydrosulfuricus*, anaerobik

INTRODUCTION

Enzymes are biological macromolecules that have a wide range of analytical, scientific, and industrial applications. Microbial enzymes are superior to inorganic catalysts because of their economic production, convenient handling, easy recovery from reaction media, and repeated reusability in industrial processes (Bashir et al., 2018). Proteases are a group of enzymes that catalyze the hydrolysis of proteins into peptides and amino acids (Sharma et al., 2017). Proteases are used in the food, leather, pharmaceutical, cosmetic, silk degumming, silver recovery, chemical, and wastewater treatment industries (Naveed et al., 2021). Microbial proteases are of significant interest, as they represent approximately 60% of the total enzyme market. These enzymes play a role in the synthesis of small peptides, the production of food ingredients by the hydrolysis of food proteins, and the improvement of the digestibility of proteins or amino acids (Dorra et al., 2018).

Most characteristics, including pH, temperature, active-site specificity, substrate specificity, catalytic activity, and stability profiles, tend to vary depending on the protease source. Proteases can be classified based on their optimal pH range, which includes acidic (pH 2-6), neutral (pH 7) and alkaline (pH 8-13) (Arya et al., 2021). Alkaline proteases have attracted considerable attention, accounting for approximately 89% of the protease market (Akhter et al., 2024). Alkaline proteases are important components in the detergent industry. They enhance cleaning efficacy by eliminating proteins such as milk, blood, and food stains (Mahakhan et al., 2023). Several alkaline proteases are produced by yeast, bacteria, actinomycetes, fungi, and plants. On the other hand, commercially produced microbial proteases of extracellular origin are resistant to physical and chemical environmental changes (Al-Dhabi et al., 2020).

Recently, there has been an increased demand for alkaline and thermostable proteases as industrial biocatalysts for biotechnological applications. In addition to their tolerance to pH and temperature, alkaline proteases exhibit high catalytic activity, substrate specificity, environmentally friendly byproduct formation, and cost-effective large-scale production (Datta et al., 2017). *Bacillu*s species are preferred for producing thermostable alkaline proteases because of their stability at high temperatures and pH values. These bacterial enzymes have higher activity in the pH range of 8-12 and temperature range of 50-70°C, which are ideal for producing enzymes on an industrial scale (Thakur et al., 2018). A wide variety of soil microorganisms (Aftab et al., 2006; Palsaniya et al., 2012; Sinha et al., 2013; Chauhan et al., 2020; Jadhav et al., 2020; Farooq et al., 2021; Hashmi et al., 2022) and strains isolated from mangrove ecosystems can produce proteases. Microbes isolated from mangrove ecosystems have a rapid growth rate in a limited space and are easy to genetically manipulate to produce new modified protease enzymes (Kharadi et al., 2020). Moreover, strains isolated from soda lakes and deserts can grow at an extremely alkaline pH and produce naturally stable alkaline enzymes. In addition, investigating the effects of certain parameters, such as cost-effective carbon and nitrogen sources, pH, temperature, agitation, and incubation time, is necessary for designing an effective process (Rathod and Pathak, 2016).

Bacillus, *Aspergillus*, and *Streptomyces* have been counted as predominant and prolific sources of alkaline proteases; however, new strain discovery studies are still important. Working with thermophiles offers some advantages, such as a reduced risk of contamination, owing to their ability to be used at high temperatures during the process. Although there is a study using *Thermoanaerobacter thermohydrosulfuricus* to produce heat-active lipase (Royter et al., 2009), there are no reports on the production of alkaline protease from *T. thermohydrosulfuricus*. In the present study, the optimum conditions (pH, incubation time, temperature, carbon and nitrogen sources, and addition of various compounds) were determined

for the alkaline protease produced by *T. thermohydrosulfuricus* G12 isolated from the sludge samples.

MATERIAL and METHODS

Source of sample collection

In this study, sludge samples collected from an anaerobic treatment system at the Ankara Metropolitan Municipality Tatlar Wastewater Plant were used. The sludge samples were stored in sterile bottles and transported to the laboratory at Ankara University, Turkey. The samples were stored at 4°C until subsequent analysis.

Isolation of bacteria and culture conditions

Skim milk (SM) medium had the following composition (g/L): nutrient broth 8, SM 100, and resazurin 1. The medium was prepared by adding resazurin, a colorimetric pH indicator based on redox potential, to SM and boiling until the resazurin-induced blue color turned pink. The medium distributed in the Hungate anaerobic culture tubes was cooled under an N_2 atmosphere to remove all dissolved oxygen, sealed with a butyl rubber stopper, and sterilized for 15 min. at 121°C. The SM medium was inoculated with 1% sludge sample and incubated at 65°C for 24 h. The cultures were then transferred to the SMNB medium.

The microorganisms were incubated for 24 h at 65°C in SMNB medium and prepared for bacterial isolation. The cultures were then transferred to Petri dishes containing skim milk nutrient agar (SMNA) in an anaerobic environment (Glove Box-Labconco) using the spreading method. After 48-72 h of incubation at 65°C, colonies forming the largest diameter transparent zone were identified and transferred to the SMNB medium (Ibrahim et al., 2007).

Genomic DNA was isolated using DNA isolation kits (Macherey Nagel), and DNA amplification by polymerase chain reaction (PCR) and sequence analysis were performed by a commercial company (REFGEN, Ankara, Turkey). The sequences of the microorganisms that were most similar to the isolate were aligned using CLUSTAL W 1.8 (multiple sequence alignment method) to perform molecular phylogenetic analysis of the isolate. Thus, the genetic diversity and degree of differentiation between species were determined. Evolutionary relationships were detected using the neighbor-joining method (Saitou and Nei, 1987). The evolutionary distance was calculated using the Maximum Composite Likelihood method and the results were obtained in the form of the number of base changes in each region (Tamura et al., 2004). Ten nucleotide sequences were used in the analysis, and gaps and positions containing missing data were eliminated. Phylogenetic analysis was performed using the MEGA5 (Molecular Evolutionary Genetics Analysis software version 5.0) package program (Tamura et al., 2011).

Medium for alkaline protease enzyme production

Horikoshi-I medium had the following composition (g/L): glucose 10, yeast extract 5, peptone 5, KH_2PO_4 1, $MgSO_4.7H_2O$ 0.2, and Na_2CO_3 5 (Horikoshi, 1996) and was used for protease enzyme production. The pH values of both media were adjusted to 9 using 5 M Na_2CO_3.

Alkaline protease activity assay

The modified Cupp-Enyard (2008) method was performed for the protease activity assay, and casein was used as the substrate. Five milliliters of 0.65% (w/v) casein solution were added to each test tube. The solutions were placed in a water bath at 37°C for 5 min. Cell-free supernatants were obtained by centrifugation at 15.000 rpm at 4°C for 5 min. One milliliter of the supernatant was added to the casein solutions, and the solutions were incubated in a water bath at 37°C for 10 min (1 mL of distilled water was used for the blank sample). The reaction was stopped by adding 5 mL of 110 mM trichloroacetic acid, and the solutions were incubated at 37°C for 30 min in a water bath. After incubation, each test solution was filtered using a 0.45 μm filter. Subsequently, 5 mL of 500 mM Na_2CO_3 and 1 mL of Folin's Phenol Reagent (1:4 Folin-distilled water) were added to 2 mL of the test filtrate and incubated at 37°C for 30 min. The absorbance of the samples was measured at 660 nm and extrapolated against a tyrosine standard curve.

One unit of protease activity was defined as the amount of enzyme liberating 1 μg of tyrosine per minute under assay conditions.

Units/ml enzyme = (μmole tyrosine equivalents released) x (A)/ ((B) x (C) x (D))
A= Total volume (mL) of assay
B= Time of assay (minutes) as per the Unit definition
C= Volume of Enzyme (mL) of enzyme used
D= Volume (mL) used in colorimetric determination

To prepare the standards, a solution containing 1.1 mM tyrosine was prepared as a stock solution and then transferred to tubes in certain volumes.

The enzyme activity of the control sample was calculated using samples collected from cultures incubated at 65°C for 24 h in Horikoshi-I medium. In this study, all inoculations were performed at a rate of 1%.

Optimization of cultural conditions for alkaline protease production

Enzyme activity was measured in samples collected at 16 h and every 8 h thereafter to determine the optimum incubation time. To determine the optimum pH for enzyme production, the pH value of the medium was adjusted to values between 6.5 and 10.5 using 5 M Na_2CO_3. The optimum temperature was determined in the samples that were obtained by incubating the cultures at 30-65°C. To select the carbon source for maximum enzyme activity, instead of 10 g/L glucose in Horikoshi-I medium, the medium was prepared by adding different carbon sources at the same concentration. To find the best nitrogen source for maximum enzyme activity, instead of a total of 10 g/L peptone, and yeast extract in Horikoshi-I medium, casein hydrolysate, soy flour (defatted), peptone, and casein mixture (5 g/L + 5 g/L), gelatin, yeast extract and peptone were used. $(NH_4)_2SO_4$ was used as an inorganic nitrogen source and added at a rate of 5 g/L. $CaCl_2$ (0.1 g/L and 1 g/L), KH_2PO_4 (0.5 g/L, 2 g/L), $MgSO_4.7H_2O$ (0.1 g/L, 1 g/L), and Tween-80 (0.25 g/L) were added to the Horikoshi-I medium to determine the effects of different minerals and surfactant on enzyme production.

Statistical analysis

Significant differences between factor levels were determined according to the least significant differences (LSD) at the $P \leq 0.05$ level of probability, and standard deviations (±SD) were shown as column bars in the figures.

RESULTS AND DISCUSSION

Molecular identification of the isolate

The cultures in NBSM were spread on the NASM medium under anaerobic conditions. Thus, a bacterial isolate was obtained and was coded as G-12. This isolate was determined to be a Gram-negative rod-shaped facultative anaerobic bacterium.

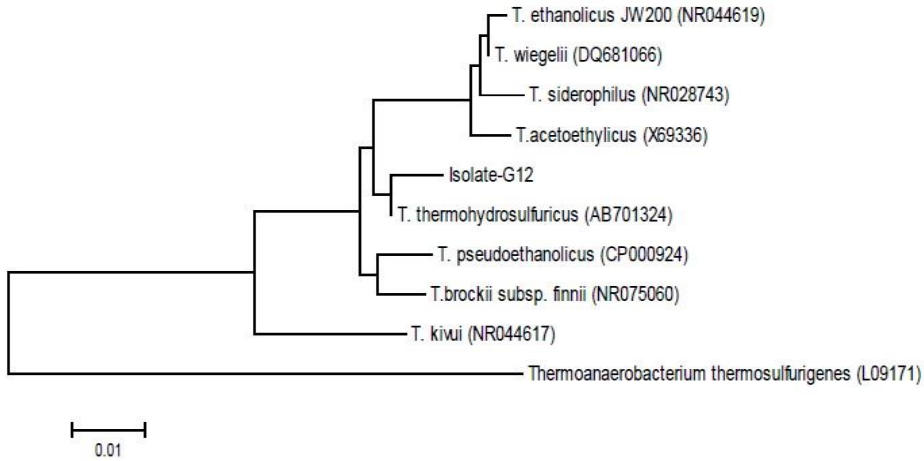

Figure 1. A phylogenetic tree created using MEGA5 with the G-12 isolate and its closest species.

DNA sequence analysis revealed 879 bases in the 16S rDNA region of the G-12 isolate. The DNA base sequences were compared with all DNA sequences from the GenBank BLAST database (www.ncbi.nlm.nih.gov/blast). The results obtained from the database showed that G-12 isolate belonged to *Thermoanaerobacter thermohydrosulfuricus* at a rate of 98.52%. Figure 1 shows the phylogenetic tree created using MEGA5.

Factors affecting enzyme production

Time course of protease production

The enzyme activity was calculated to be 641.58 U/mL in the samples collected at 64 h. However, the differences in enzyme activity between 64 h and 18, 24, and 32 h were statistically significant ($P \leq .05$). There was no statistical difference between the enzyme activity measured in samples taken at 64 h and the samples taken at 72 h. There was also no statistically significant difference in enzyme activity between samples taken at 40, 48, and 56 h and those taken at 64 h ($P \geq .05$). Therefore, 40 h to reach maximum enzyme activity may be sufficient, as prolonging the production time would only result in increased costs, particularly on an industrial scale (Figure 2). Prakasham et al. (2006) investigated the properties of alkaline proteases produced by *Bacillus* sp. and calculated the maximum enzyme activity after 60 h. Dhandapani and Vijayaragavan (1994) examined the thermophilic *Bacillus stearothermophilus* AP-4 to produce thermostable alkaline protease, and after 36 h of incubation, they achieved the maximum protease activity (250 U/mL).

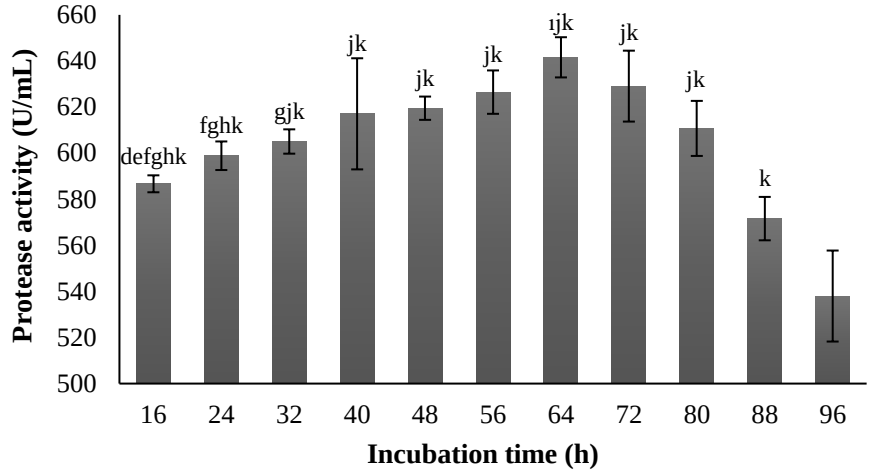

Figure 2. The time course of alkaline protease production (incubations were performed at an initial pH of 9 and at 65°C)

Vertical bars indicate standard deviations of the means and values are the means of two replicates
(a) 16, (b) 24, (c) 32, (d) 40, (e) 48, (f) 56, (g) 64, (h) 72, (ı) 80, (j) 88 and (k) 96 h
Different letters indicate significant differences according to the least significant difference (LSD) test at $P \leq .05$.

Effects of initial pH and incubation temperature on enzyme production

As shown in Figure 3, alkaline protease activity increased until it reached pH 10 and then decreased at pH 10.5. The highest enzyme activity (863.69 U/mL) was observed at pH 10. The differences between enzyme activities at pH 10 and all tested pH values were statistically significant ($P \leq .05$). Studies have shown that the optimum pH value for alkaline proteases ranges from 9 to 11 (Banerjee et al., 1999; Denizci et al., 2004; Gençkal and Tari, 2006; Patel et al., 2006; Sellami-Kamoun et al., 2008; Wilson and Remigio, 2012; Asha and Palaniswamy, 2018). In contrast, other studies have shown that the optimum pH value range is 8-9 (Mohamedin, 1999; Hutadilok-Towatana et al., 1999; Chi et al., 2007; Silva et al., 2007; Elbanna et al., 2015).

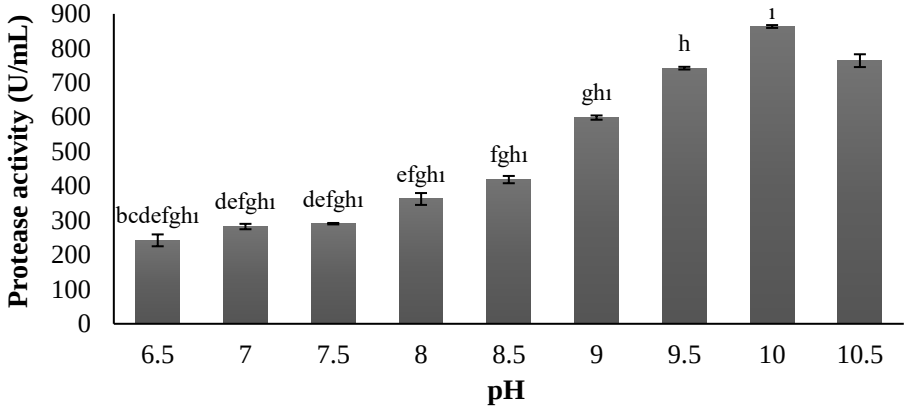

Figure 3. Effect of initial pH on alkaline protease production

Vertical bars indicate standard deviations of the means and values are the means of two replications
(a) 6.5, (b) 7.0, (c) 7.5, (d) 8.0 (e) 8.5, (f) 9.0, (g) 9.5, (h) 10.0, and (ı) 10.5
Different letters show significant differences according to least significant difference (LSD) test at $P \leq .05$.

The highest enzyme activity was determined to be 713.51 U/mL for samples incubated at 35°C (Figure 4). However, there was no statistically significant difference between the enzyme activities calculated at 35 and 40°C ($P \geq .05$). Although some researchers have reported similar results (Asha and Palaniswamy, 2018; Charles et al., 2008), others have found an optimum temperature of 37°C (Gençkal and Tari, 2006; Patel et al., 2006; Olajuyigbe and Ehiosun, 2013). However, many studies have reported optimum temperatures between 50 and 80°C (Denizci et al., 2004; Mohamedin, 1999; Hutadilok-Towatana et al., 1999; Silva et al., 2007; Aqel et al., 2012).

In a previous study, *Bacillus* sp. isolated from saline-alkali soils was selected for its ability to produce alkaline proteases in a milk agar medium. Maximum enzyme activity was obtained in the presence of glucose (1% w/v) and NH_4Cl (1% w/v) at pH 10.5, at a temperature of 40°C, and after 20 h (Mehrotra et al., 1999). In another study, the highest enzyme activity was observed in *Bacillus* sp. B18 under extreme conditions, with optimum pH and temperature values of 12-13°C and 85°C, respectively (Fujiwara et al., 1993).

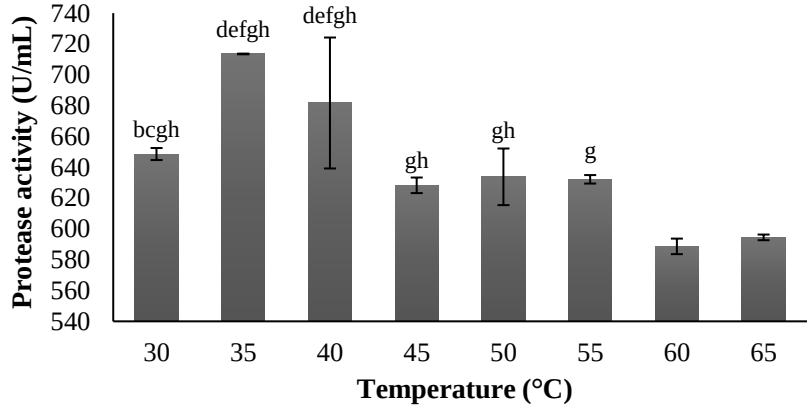

Figure 4. Effect of incubation temperature on alkaline protease production

Vertical bars indicate standard deviations of the means, and values are the means of two replications
(a) 30, (b) 35, (c) 40, (d) 45, (e) 50, (f) 55, (g) 60, and (h) 65°C
Different letters show significant differences according to least significant difference (LSD) test at $P \leq .05$.

Effect of carbon and nitrogen sources on enzyme production
Enzyme production could not be achieved because of the inability of microorganisms to grow well when inulin, sorbitol, starch, sucrose, and raffinose were added to the medium. Various carbon sources were used to determine the maximum enzyme production, as shown in Table 1. The highest enzyme activity (864.68 U/mL) was observed in the presence of arabinose. Following arabinose, the addition of xylose, sorbose, galactose, and fructose also increased enzyme activity. The difference between the enzyme activities obtained when arabinose and xylose were used in the medium was not statistically significant ($P \geq .05$). Maltose resulted in the lowest enzyme production. Kanekar et al. (2002) obtained the second highest enzyme activity using *Arthrobacter ramosus* (89.73 U/mL) in the presence of xylose. The activity of protease produced by the thermophilic *Bacillus* sp. SMIA-2 was 0.530 U/mg protein when glucose was used (9 h of incubation at 50°C and an initial pH of 7) (Nascimento et al., 2004). Prakasham et al. (2006) investigated certain properties of the alkaline protease that was produced by *Bacillus* species and reported that the enzyme activity increased with the addition of xylose and maltose to the medium at a rate of 1%. Akcan and Uyar (2011) reported that the maximum alkaline protease activity produced by *Bacillus subtilis* RSKK96 was 4688.2 U/mg, which was obtained by the addition of 1% arabinose.

Examination of the effects of different nitrogen sources on enzyme production revealed that the highest enzyme activity was 656.03 U/mL when casein hydrolysate was present in the medium. The difference between enzyme activities obtained when casein hydrolysate and yeast extract were used in the medium was not statistically significant ($P \geq .05$). Compared with the control, the addition of soy flour caused a large decrease in enzyme activity, followed by gelatin and $(NH_4)_2SO_4$ (Table 1). Johnvesly and Naik (2001) investigated the effects of various inorganic and organic nitrogen sources on alkaline protease production by thermophilic and alkalophilic *Bacillus* sp. JB-99. The researchers found that the highest enzyme activity (12780 U/mL) was obtained in the presence of $NaNO_3$, followed by samples containing yeast extract (10850 U/mL). Asha and Palaniswamy (2018) reported that casein was the best nitrogen source for producing alkaline proteases from *Bacillus cereus* FT 1. Lazim et al. (2009) found that $(NH_4)_2SO_4$ and yeast extract increased protease activity compared with the control. In contrast, malt extract, peptone, NH_4NO_3, $NaNO_3$, and casein decreased enzyme production. Some studies have reported that yeast extract is the best nitrogen source for enzyme production (Nadeem et al., 2008; Salihi et al., 2017; Rekik et al., 2019). Joo et al. (2002) isolated *Bacillus horikoshii* to investigate extracellular alkaline protease production and obtained the highest enzyme activity (115.3 U/mL) using soybean meal (1.5%, w/v) and casein (1%, w/v) at pH 9 and 34°C after 18 h.

Effects of minerals and Tween-80 on enzyme production
The medium components most likely to affect alkaline protease production were K_2HPO_4, $MgSO_4.7H_2O$, and $CaCl_2$ (Jadhav et al., 2020). The addition of 1 g/L $MgSO_4.7H_2O$ to the medium yielded the highest increase in enzyme activity compared to the control sample, and the activity was calculated to be 726.50 U/mL ($P \leq .05$). Enzyme activity increased when the concentration of KH_2PO_4 was increased from 0.5 to 2 g/L. Compared with the control sample, protease activity decreased with the addition of 0.1 g/L and 1 g/L $CaCl_2$, respectively. Moreover, using 2 g/L KH_2PO_4 and 0.1 g/L $MgSO_4.7H_2O$ in the medium did not create a statistically significant effect compared to the control sample ($P \geq .05$).

To ensure optimal performance during the washing process, alkaline proteases must demonstrate compatibility and stability with various components commonly found in detergents, including surfactants, oxidizing agents, and other additives (Hammami et al., 2017). Therefore, the efficacy of the enzyme was evaluated using Tween-80, which is a potential constituent of the formulation. The addition of Tween-80 to the production medium caused a statistically significant increase in enzyme activity compared with that of the control sample ($P \leq .05$) (Table 2).

Table 1. Effects of carbon and nitrogen sources on alkaline protease production

Carbon sources (g/L)	Protease activity (U/mL)	Relative activity (%)	Nitrogen sources (g/L)	Protease activity (U/mL)	Relative activity (%)
Glucose (Control)^{cdfghij}	598.88±6.20	100	Peptone+yeast extract (Control)^{bcdefg}	598.88±6.20	100
Mannose^{cdfij}	553.57±8.27	92.43	$(NH_4)_2SO_4$^{cdefgh}	379.83±11.02	63.42
Galactose^{deghij}	722.77±11.48	120.69	Casein hydrolysate^{defh}	656.03±11.25	109.54
Xylose^{efgh}	830.91±4.59	138.74	Soy flour^{efgh}	106.06±1.84	17.71
Lactose^{fhij}	583.77±7.81	97.48	Peptone+casein hydrolysate^{fh}	625.83±9.42	104.50
Fructose^{ij}	723.42±16.07	120.79	Gelatine^{gh}	227.36±4.82	37.96
Rhamnose^{je}	542.69±5.74	90.62	Yeast extract^{h}	641.74±6.2	107.16
Maltose^{ij}	518.66±48.91	86.60	Peptone	591.73±1.61	98.81
Arabinose^{j}	864.68±32.15	144.38			
Sorbose	792.59±14.24	132.35			

Carbon sources: (a) Control, (b) Mannose, (c) Galactose, (d) Xylose, (e) Lactose, (f) Fructose, (g) Rhamnose, (h) Maltose, (ı) Arabinose and (j) Sorbose.
Nitrogen sources: (a) Control, (b) $(NH_4)_2SO_4$, (c) Casein hydrolysate, (d) Soybean flour (e) Peptone+casein hydrolysate, (f) Gelatine, (g) Yeast extract, and (h) Peptone.
Different letters show significant differences according to least significant difference (LSD) test at $P \leq .05$.

Table 2. Effects of minerals and surfactant on alkaline protease production

Minerals and surfactant (g/L)	Protease activity (U/mL)	Relative activity (%)
Horikoshi-I (Control) ^{bcdgh}	598.88±6.20	100
$CaCl_2$ (0.1 g/L)^{cefgh}	576.47±1.15	96.26
$CaCl_2$ (1 g/L)^{defgh}	517.52±1.84	86.41
KH_2PO_4 (0.5 g/L)^{efgh}	564.29±5.97	94.22
KH_2PO_4 (2 g/L)^{g}	613.16±12.63	102.38
$MgSO_4.7H_2O$ (0.1 g/L)^{gh}	596.76±16.07	99.65
$MgSO_4.7H_2O$ (1g/L)^{h}	726.50±3.90	121.31
Tween 80 (0.25)	628.27±0.46	104.91

(a) Control, (b) $CaCl_2$ (0.1 g/L), (c) $CaCl_2$ (1 g/L), (d) KH_2PO_4 (0.5 g/L), (e) KH_2PO_4 (2 g/L), (f) $MgSO_4.7H_2O$ (0.1 g/L), (g) $MgSO_4.7H_2O$ (1g/L), (h) Tween 80 (0.25)
Different letters show significant differences according to least significant difference (LSD) test at $P \leq .05$.

Datta et al. (2017) obtained the highest protease production (0.128 U/mL) by *Aeromonas caviae* P-1-1 at pH 8 and 37°C after 42 h of incubation in a medium containing Tween-40. Zanphorlin et al. (2011) examined a new alkaline serine protease from the thermophilic fungus *Myceliophthora* sp. The researchers reported that the addition of Tween-80 caused an 80% decrease in enzyme activity. In another study investigating the effect of surfactants on the activity of proteases produced by *Bacillus cereus*; Tween-20, Tween-40, Tween-60, Tween-80, and Triton X-100 caused an increase in enzyme activity compared with the control sample (Esakkiraj et al., 2009).

CONCLUSION

Our results revealed that the concentration of alkaline protease produced by *T. thermohydrosulfuricus* isolated from sludge samples was greater than that of enzymes produced by many *Bacillus* and thermoalkaliphilic bacteria species. However, control and optimization of other process parameters are required to increase enzyme activity during large-scale production.

ACKNOWLEDGEMENT

The authors received no financial support for this work.

CONFLICT of INTEREST
There is no content conflict of interest.

AUTHOR CONTRIBUTIONS
Bilge Sayın Börekçi: Formal analysis, Writing-original draft, Sedat Dönmez: Supervisor, Writing-review & editing, Conceptualization. Ayşe Avcı: Formal analysis, Writing-review & editing.

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