Journal of Environmental Studies [JES] https://jesj.journals.ekb.eg/
An International Journal doi: 10.21608/jesj.2025.413330.1147

Production of proteolytic enzymes from a newly isolated *Bacillus subtilis* strain via submerged fermentation for agricultural applications

Alsancak, Erdal[1], Atlı, Burcu[2]*, Dayankaç Öztürk[1], İdil, İstanbullu Çobanoğlu Havva Betül[1], Ulaş, Gökçe[1]

[1] Hektas High Technology Center

[2]Hektas Ankara High Technology Center

Citation: *Alsancak, E., Atlı, B., Dayankaç Ö.İ., İstanbullu, Ç.H,B., Ulaş, G. (2025). Production of proteolytic enzymes from a newly isolated Bacillus subtilis strain via submerged fermentation for agricultural applications . Journal of Environmental Studies, Vol. 40(1): 17-*22.

Article Information
Received 14 Aug. 2025,
Revised 12 Nov. 2025,
Accepted 13 Nov. 2025.
Published online
1 Des. 2025

Abstract: This study aimed to evaluate the extracellular protease production potential of *Bacillus* isolates obtained from different soil samples from various regions of Türkiye. The overarching goal was to identify a high-yielding local isolate that could be further developed for agricultural applications, particularly in soil remediation. Eighty-six *Bacillus* isolates (B1–B86) were screened for extracellular protease activity using casein-milk agar plates. The five most active isolates were selected for further analysis based on the size of the inhibition zones. Among these, isolate B64 was identified as *Bacillus subtilis* using the MALDI-TOF identification method. The proteolytic activities of B64 were optimized under submerged fermentation conditions in a 5 L lab-scale bioreactor. SDS-PAGE analysis was used to determine the molecular weight of the crude enzyme. Isolate B64, identified as *Bacillus subtilis*, demonstrated the highest protease (9.8 U/mL) and milk clotting (738 SU/mL) activities, under the specified conditions. Molecular weight of the crude protease enzyme was approximately 44 kDa. The crude protease from *Bacillus subtilis* B64 isolate exhibits promising properties for potential use in agricultural applications, especially in soil remediation processes.

Keywords: *Bacillus* species, proteolytic enzymes, MALDI-TOF, fermentation, sustainable agriculture

Introduction

Microorganisms play a key role in the production of various enzymes on an industrial scale (Gupta *et al.*, 2002). Both intracellular and extracellular enzymes are utilized in multiple industries, including food processing, leather manufacturing, and detergents. Bacillus species found in soil provide a wide range of enzymes and metabolites (Mahapatra *et al.*, 2022). The primary source of nitrogen in soil organic matter is obtained through the extracellular enzymes secreted by Bacillus, which transform protein-rich wastes such as dairy proteins, seafood, leather, and agricultural residues (Caballero *et al.*, 2020). Although these wastes have negative environmental impacts, they also have the potential to be significant sources of protein-derived biostimulants (Moreno-Hernández *et al.*, 2020). Moreover, microbial enzymes have potential applications as biofertilizers and/or biopesticides for the biological control of phytopathogens and pests (Saxena *et al.*, 2020; Akinsemolu *et al.*, 2024; Liao *et al.*, 2025).

Proteases are a significant group of industrial enzymes and are commercially produced by well-known protease-producing microorganisms such as Bacillus, Lactobacillus, Mucor, and Aspergillus. Among these, Bacillus has remained a prolific producer of various enzymes from past to present (Danilova & Sharipova, 2020; Wu *et al.*, 2024). Fermentation technologies provide a cost-effective and high productivity way for production of marketable-value enzymes used in agricultural sectors (Lee *et al.*, 2023). In this study, to obtain industrially valuable protease-producing Bacillus species, soil samples from different locations in Türkiye were collected and the obtained isolates were screened for proteolytic activity. A locally isolated Bacillus strain exhibiting the highest

* Corresponding author E-mail: burcuatli2006@gmail.com

proteolytic activity was selected for protease production under submerged fermentation in a lab-scale bioreactor.

Materials and Methods

Sample collection and isolation

Soil samples were collected from different locations in Türkiye. The isolates were then purified using the streaking method, and pure colonies were selected for the MALDI identification. Pure colonies were stored at -80 °C for long-term preservation.

Screening

A total of 86 *Bacillus* species were screened for proteolytic activity using well diffusion assay using 6 mm wells on milk-casein agar (Hashmi *et al*., 2022). The inoculated plates were incubated at 30 °C for 48 hours. Their activities were detected based on the appearance of clear zones around the colonies. The most promising strain was chosen for further protease production studies in lab-scale bioreactor.

Identification of the selected isolate

MALDI-TOF analysis of the B64 isolate was performed according to the method reported by Starostin *et al*., (2015). The results were evaluated in accordance with the spectra score (high reliability, 2.300–3.000, possible species, 2.000–2.299, possible genus, 1.700–1.999 and unreliable, 0.000–1.699) of standard strain and the isolated strain in the MS library (Kiehntopf *et al*., 2011).

Submerged Fermentation in lab scale bioreactor

Among the *Bacillus* isolates, the B64 isolate which exhibited a prominent clear zone, was selected for the study of extracellular protease production. The isolate was maintained on Tryptic Soy Agar (TSA) plates and stored at 4 °C. Tryptic Soy Broth (TSB) was used as a seed medium. Skim milk was sterilized separately (110 °C, 15 min), before being aseptically added to the fermentation medium (5% casein, 2.5% yeast extract, 1% skim milk, 1% glucose and 1% antifoam). The lab scale bioreactor (Minifors 2) was inoculated with 300 ml of a 24-hour-old seed culture in 500 ml Erlenmayer flask and incubated at 30 °C with 600 rpm rotation speed and 1 vvm airflow rate for 48 hours. The samples were collected, and after centrifugation (10,000 rpm for 15 min at 4 °C), the supernatant was used for all analyses.

Analytical Methods

The microbial growth was measured at 600 nm using a UV-visible spectrophotometer. Protein concentration was determined according to the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as the standard. The milk clotting activity (MCA) was measured according to Li *et al*. (2019) using commercial rennet standard and was expressed as Soxhlet unit (SU). The proteolytic activity (PA) was calculated using the tyrosine method (Zhao *et al*., 2019) with commercial protease from *Bacillus* (Sigma). The amount of enzyme necessary to release 1 g of tyrosine per mL in minute was considered to represent 1U of enzyme activity. All the experiments were performed in triplicate. The approximate molecular weight of the obtained protease enzyme was determined by standard SDS-PAGE procedure followed by Coomassie Blue staining (Schneider *et al*., 2012) and subsequently imaged using the Bio-Rad Gel Doc XR+ system.

Results

Sample collection and isolation

Soil samples were collected from different regions of Türkiye. The collected samples were diluted with physiological water and spread onto Nutrient Agar (NA) plates. Pure colonies with different morphologies were selected and transferred to a new NA plate using a sterile loop. The plates were incubated at 37 °C for 24-48 hours. Eighty-six pure bacterial colonies, designated B1 to B86, were obtained.

Screening

Semi-quantitative protease activities in Bacillus isolates were determined using clear zone agar plate assay on different protein (casein and skim milk) substrates. In this study, to assess enzyme efficiency was tested on casein and skim milk; casein is a pure protein, whereas skim milk contains mixed proteins, allowing evaluation of enzyme specificity and substrate preference. The obtained data are presented in Table 1. Extracellular protease activity was detected in the majority of the screened Bacillus isolates. However, it was determined that 10 of the screened 86 isolates did not have protease activity. Five of the 86 screened Bacillus isolates showed a significantly higher zone inhibition for protease production in comparison to other isolates. Isolate B64 showed the largest hydrolysis zone (4.40 mm), indicating high protease production potential. B64 isolate showed a zone inhibition (mm) of 4.40 which is considered good potential producer for protease production. The Bacillus isolate B64 with the highest zone for protease production were selected for submerged fermentation.

Identification of the selected isolate

Based on the MALDI-TOF results, the selected isolate B64, which was discovered in the current study, was identified as Bacillus subtilis. The data obtained were used to construct a mass spectrogram, which was arranged according to the levels of similarity (Green Score 2.05) in protein expression among the standard strains in the MALDI library. (Figure 1).

Submerged Fermentation in lab scale bioreactor

The newly isolated Bacillus subtilis B64 was conducted in a 5 L lab scale bioreactor (Minifors 2, Infors) at 3 L working volume with temperature and foam control. The fermentation medium inoculated with B. subtilis B64 was incubated at 30 °C for 2 days. The submerged fermentation was performed at constant 1 vvm airflow rate and 600 rpm agitation speed. Samples were taken at regular intervals throughout the incubation period (47 hrs). The results of total protein, total sugar and optical density (OD600) are given in Figure 2. The maximum optical density (OD600) was observed at 20 h. Total protein amount has increased during incubation. The concentration of total sugar has gradually decreased over time. Additionally, decreasing glucose concentration partially suppressed protease production. However, the obtained results showed no significant correlation between optical density and proteolytic activities.

The results of extracellular protease, milk clotting activity and specific protease activities of the newly *Bacillus subtilis* B64 isolated from Türkiye are presented in Figure 3. It was determined that proteolytic enzyme production started from the 16th hour of fermentation. *B. subtilis* B64 reached its maximum protease activity (9.8 U/mL) after 33 h of fermentation. At the same time, the highest milk clotting activity was 738 SU/ml. At the end of fermentation, loss of proteolytic activities was observed. Therefore, it was determined that the 33rd hour of submerged fermentation was optimal incubation time for the highest extracellular enzyme activities.

The produced crude protease enzyme was partially characterized using SDS-PAGE and a single band was observed. When the results of the bands are evaluated together, molecular weight of the produced crude protease was found as 44 kDa (Figure 4). This result is consistent with the literature reports that provides information that *Bacillus* genus is mostly protease producers less than 50 kDa (Sonuc Karaboga and Logoglu, 2019).

Discussion

Protease enzymes break down proteins by hydrolyzing peptide bonds and have a wide range of commercial applications. The use of proteases in sustainable agricultural practices increases agricultural productivity by providing eco-friendly and effective solutions (Banerjee & Ray, 2017). Foremost, recycling and evaluation of plant waste is of great importance in the agricultural sector (Janeeshma *et al*., 2023). Morever, protease enzymes accelerate the breakdown of organic matter in the soil, allowing the release of nutrients needed by plants. This process supports soil microbial activity and positively affects plant growth. In addition, proteases are known to improve plant nutrition by contributing to the nitrogen cycle in the soil (Acir & Günal, 2019). The integration of proteases into agricultural processes contributes to the creation of a more sustainable and efficient agricultural system (Noreen *et al*., 2024).

Proteases are produced by various microorganisms, especially Bacillus, Lactobacillus, Mucor, and Aspergillus. However, the continuous search for novel, high-yielding strains through isolation and characterization remains crucial in industrial enzyme production. With this perspective, microorganisms isolated from soil samples collected from different regions of Türkiye were screened for their ability to produce proteolytic enzymes on selective media. Protease enzymes produced by bacteria especially Bacillus species are selected for industrial-scale production due to fast production, high stability and its easy genetic manipulation properties (Genckal Demir & Tari, 2006; Ortiz & Sansinenea, 2023). Because of these advantages, this study focused on extracellular proteolytic activity of Bacillus species. Moreover, since it is aimed for application in soil, the isolated Bacillus species from soil were particularly preferred in this study. According to results, 86 Bacillus isolates were screened and 76 of them were found to have proteolytic activity. However, the first five of these strains have the highest proteolytic activity compared to the others. These five isolates were identified by MALDI TOF as Bacillus subtilis (B6), Bacillus subtilis (B47), Bacillus subtilis (B64), Bacillus mojavensis (B67), and Bacillus amyloliquefaciens (B77). Bacillus subtilis is a preferred microorganism in different industries due to its high efficiency and stable protease production. In this study B. subtilis strains stood out in terms of proteolytic activity. Bacillus subtilis B64 isolate was selected for the submerged fermentation at 5L lab scale bioreactor. Casein and skim milk as a protein source are significantly better inducers of protease pathway for highest protease production (Akkale, 2023). In this study, the submerged fermentation media containing combination casein with skim milk was selected for extracellular protease production from Bacillus subtilis

* Corresponding author E-mail: ahmedwanas@du.edu.eg

B64. The highest protease activity (9.8 U/ml) was obtained after 33 hours of the fermentation (at constant air flow and agitation speed). Akkale (2023) reported that protease activity (96.42 U/mL) of Bacillus subtilis NRRL B-3384 was achieved in fermentation conditions (at 30% via a cascaded control of agitation speed (from 300 to 1000 rpm) and 30°C for 30 hours). B. subtilis as a GRAS (Generally Recognized As Safe) microorganism is known to produce several extracellular enzymes during the submerged fermentation, including a milk-clotting enzyme, lipase, amylase etc. (Zhang *et al*. 2021). Therefore, the milk clotting activity of the Bacillus subtilis B64 in the presence of casein was also examined in the study and the maximum milk clotting activity (738 SU/ml) was reached. Wu *et al*. (2013) reported that milk clotting activity (600 SU/mL) of Bacillus subtilis natto was produced under optimized conditions by response surface methodology. The molecular weight of extracellular proteases produced by Bacillus species is a critical parameter determining the functionality and application areas of enzymes. Especially subtilizine-like proteases (27-45 kDa) have significant potential for industrial and agricultural biotechnology (Gupta *et al*. 2002). A varieties of molecular weight for proteases from other Bacillus species had been reported as 42-45kDa (termostable) Bacillus sp. (Banerjee and Bhattacharyya, 1993), 49 kDa Bacillus sp. HUTBS71 (Akel *et al*. 2009). The molecular weight of extracellular protease produced by Bacillus subtilis B64 is consistent with other Bacillus subtilis enzymes in the literature. This study shows that the local Bacillus subtilis B64 isolated from Türkiye is a valuable microbial source for marketable-value protease production and that these enzymes have significant potential for agricultural and industrial applications.

Table 1: Screening of proteolytic activities of the local *Bacillus* isolates.

Code	Zone (mm)	Code	Zone (mm)	Code	Zone (mm)	Code	Zone (mm)	Code	Zone (mm)	Code	Zone (mm)
B1	0	B16	1.64	B31	2.24	B46	3.12	B61	2.11	B76	0.74
B2	1.86	B17	1.15	B32	2.14	B47	3.66	B62	2.43	B77	3.53
B3	2.24	B18	2.03	B33	2.00	B48	0.84	B63	2.64	B78	0.76
B4	1.83	B19	1.85	B34	2.50	B49	3.26	B64	4.40	B79	2.35
B5	2.79	B20	0	B35	1.98	B50	0	B65	2.00	B80	1.02
B6	3.52	B21	2.26	B36	1.98	B51	2.72	B66	2.46	B81	1.51
B7	2.59	B22	2.52	B37	1.44	B52	2.29	B67	4.30	B82	0.57
B8	0	B23	2.84	B38	2.44	B53	1.74	B68	2.13	B83	2.87
B9	2.58	B24	2.63	B39	2.08	B54	2.06	B69	2.18	B84	0
B10	2.12	B25	2.31	B40	1.28	B55	2.32	B70	3.05	B85	2.02
B11	1.61	B26	2.51	B41	0	B56	0	B71	0	B86	1.10
B12	1.80	B27	2.84	B42	1.43	B57	2.08	B72	2.35		
B 13	2.95	B28	1.39	B43	2.79	B58	2.55	B73	2.02		
B14	1.39	B29	1.93	B44	1.44	B59	2.74	B74	3.13		
B15	0	B30	2.78	B45	3.22	B60	0	B75	2.37		

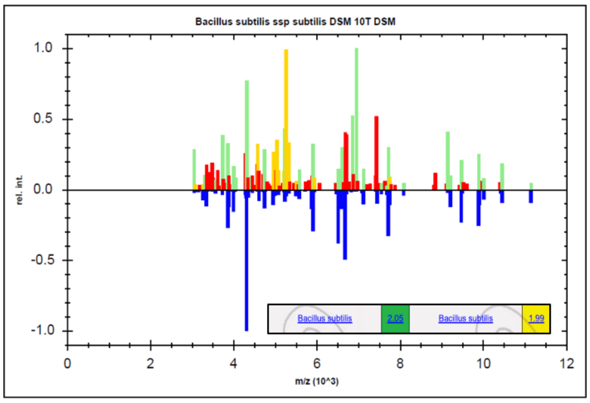

Figure 1: Mass spectrogram of B64 isolate

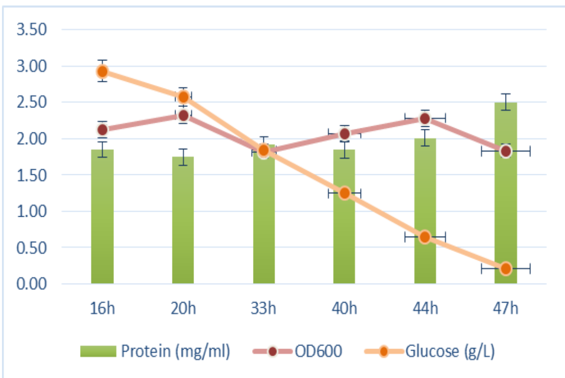

Figure 2: The results of total protein, total sugar and OD_{600} of *Bacillus subtilis* B64

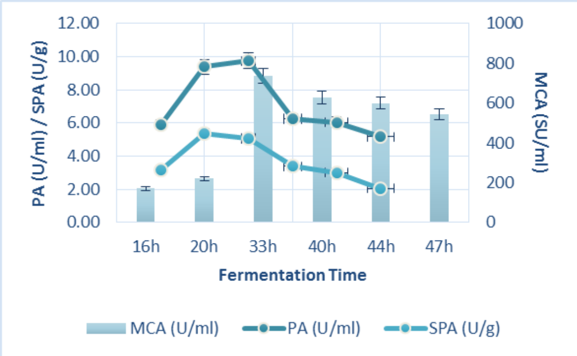

Figure 3: Proteolytic enzyme activities of *Bacillus subtilis* B64

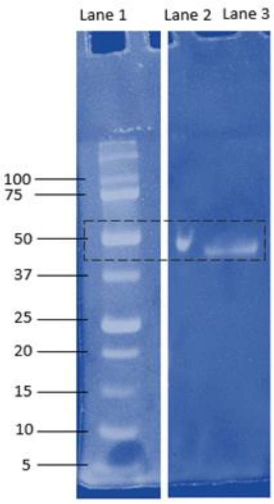

Figure 4: Molecular weight of extracellular protease from *Bacillus subtilis* B64. Lane 1: protein MW markers (Precision Plus Protein, Dual Xtra Standards, BioRad), lane 2: positive control (Protease from *Bacillus* sp., Sigma) (MW ~ 48 kDa), lane 3: the crude *Bacillus subtilis* B64 enzyme (MW ~ 44 kDa).

References

Acir, N., Günal, H. (2019). Tarım arazilerinin toprak kalitesi değerlendirmelerinde biyokimyasal özelliklerinin kullanımı. *Science and Technique in the 21st Century* 1 (11): 17–28.

Akel, H., Quadan, F., Yousef, T. (2009). Characterization of a purified thermostable protease from hyperthermophilic Bacillus strain HUTBS71. European. *Journal of Scientific Research*, 31: 1450.

Akinsemolu, A., Onyeaka, H., Odion, S., Adebanjo, I. (2024). Exploring Bacillus subtilis: Ecology, biotechnological applications, and future prospects. *Journal of Basic Microbiology*, 64.

Akkale, C. (2023). Production, optimization and partial characterization of alkaline protease from Bacillus subtilis spp. subtilis NRRL B-3384 and B-3387. *Hittite Journal of Science and Engineering*, 10 (2): 135–144.

Banerjee, U.C., Bhattacharyya, B.C. (1993). Extracellular alkaline protease of a thermophilic Bacillus. *Indian Journal of Experimental Biology* 31 (5): 418–420.

Banerjee, G., Ray, A.K. (2017). Impact of microbial proteases on biotechnological industries. *Biotechnology and Genetic Engineering Reviews* 33 (2): 119–143.

Bradford, M.M. (1976). Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72 (1–2): 248–254.

Caballero, P., Macías, S., Revilla, E., Tejada, M., Parrado, J., Castaño, A. (2020). Effect of subtilisin, a protease from Bacillus sp., on soil biochemical parameters and microbial biodiversity. *European Journal of Soil Biology*, 101.

Danilova, I.V., Sharipova, M. (2020). The practical potential of bacilli and their enzymes for industrial production. *Frontiers in Microbiology*, 11.

Hashmi, S., Iqbal, S., Ahmed, I., Janjua, H.A. (2022). Production, optimization, and partial purification of alkali-thermotolerant proteases from newly isolated Bacillus subtilis S1 and Bacillus amyloliquefaciens KSM12. *Processes*, 10 (6): 1050.

Herrmann, L.W., Letti, L.A.J., Penha, R.O., Soccol, V.T., Rodrigues, C., Soccol, C.R. (2023). Bacillus genus industrial applications and innovation: First steps towards a circular bioeconomy. *Biotechnology Advances*, 70: 108300.

Janeeshma, E., Habeeb, H., Sinha, S., Arora, P., Chattaraj, S., Das Mohapatra, P., Panneerselvam, P., Mitra, D. (2023). Enzymes-mediated solid waste management: A sustainable practice for recycling. *Waste Management Bulletin*. https://doi.org/10.1016/j.wmb.

Genckal Demir, H., Tari, C. (2006). Alkaline protease production from alkalophilic Bacillus sp. isolated from natural habitats. *Enzyme and Microbial Technology*, 39: 703–710.

Gupta, R., Beg, Q.K., Lorenz, P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. *Applied Microbiology and Biotechnology*, 59: 15–32.

Kiehntopf, M., Melcher, F., Hänel, I., Eladawy, H., Tomaso, H. (2011). Differentiation of Campylobacter species by surface-enhanced laser desorption/ionization-time-of-flight mass spectrometry. *Foodborne Pathogens and Disease*, 8 (8): 875–885.

* Corresponding author E-mail: ahmedwanas@du.edu.eg

Lee, J.A., Kim, H.U., Na, J.G., Ko, Y.S., Cho, J.S., Lee, S. (2023). Factors affecting the competitiveness of bacterial fermentation. *Trends in Biotechnology*, 41 (6): 798–816. https://doi.org/10.1016/j.tibtech.2022.10.005

Li, L., Zheng, W.Z.F., Hao, Y.J., Zhao, X., Cao, Y.Q., Yu, Z.J., Chen, C., Yang, Z.N. (2019). Recombinant expression and structural properties of milk-clotting enzyme from Bacillus methanolicus. *Food Science*, 40: 39–46.

Liao, Z., Boubakri, H., Chen, B., Farooq, M., Lai, Z., Kou, H., Fan, J. (2025). Biofertilizers as an eco-friendly approach to combat drought stress in plants. *Biocatalysis and Agricultural Biotechnology*, 64: 103510. https://doi.org/10.1016/j.bcab.2025.103510

Mahapatra, S., Yadav, R., Wusirika, R. (2022). Bacillus subtilis impact on plant growth, soil health and environment: Dr. Jekyll and Mr. Hyde. *Journal of Applied Microbiology*, 132. https://doi.org/10.1111/jam.15480

Moreno-Hernández, J., Benitez-García, I., Mazorra-Manzano, M., Ramírez-Suárez, J., Sánchez, E. (2020). Strategies for production, characterization and application of protein-based biostimulants in agriculture: A review. *Chilean Journal of Agricultural Research*, 80.

Noreen, S., Hamzah Saleem, M., Ali, B., Iqbal, J.A., Anas, M. (2024). Bacillus subtilis Apr-Ibl04: A promising candidate for hyper-production of alkaline protease – insights into screening, selection, and development strategies. https://doi.org/10.2139/ssrn.4941963

Ortiz, A., Sansinenea E. (2023). Genetically modified plants based on Bacillus genes and commercial Bacillus-based biopesticides for sustainable agriculture. *Horticulturae*, 9 (9): 963.

Saxena, A.K., Kumar, M., Chakdar, H., Anuroopa, N., Bagyaraj, D.J. (2020). Bacillus species in soil as a natural resource for plant health and nutrition. *Journal of Applied Microbiology*, 128 (6): 1583–1594.

Schneider, E.L., Lee, M.S., Baharuddin, A., Goetz, D.H., Farady, C.J., Ward, M., Wang, C.I., Craik, C.S. (2012). A reverse binding motif that contributes to specific protease inhibition by antibodies. *Journal of Molecular Biology*, 415 (4): 699–715.

Sonuc Karaboga, M.N., Logoglu, E. (2019). Purification of alkaline serine protease from local Bacillus subtilis M33 by two steps: A novel organic solvent and detergent tolerant enzyme. *Gazi University Journal of Science*, 32 (1): 116–129.

Starostin, K., Demidov, E., Bryanskaya, A., Efimov, V.M., Rozanov, A.S., Peltek, S.E. (2015). Identification of Bacillus strains by MALDI-TOF MS using geometric approach. *Scientific Reports*, 5: 16989.

Wu, M., Cao, L., Tang, W., Liu, Z., Feng, S. (2024). Improving the anti-autolytic ability of alkaline protease from Bacillus alcalophilus by a rationally combined strategy. *Enzyme and Microbial Technology*, 184: 110561.

Wu, F.C., Chang, C.W., Shih, I.L. (2013). Optimization of the production and characterization of milk clotting enzymes by Bacillus subtilis natto. *Springer Plus*, 2 (1): 33.

Zhang, X., Tong, Y., Wang, J., Lyu X., Yang, R. (2021). Screening of a Bacillus subtilis strain producing both nattokinase and milk-clotting enzyme and its application in fermented milk with thrombolytic activity. *Journal of Dairy Science*, 104 (9): 9437–9449.

Zhao, X., Cai, M., Yang, Z.J., *et al*. (2019). Purification and characterization of a novel milk-clotting enzyme produced by Bacillus amyloliquefaciens GSBa-1. *European Food Research and Technology*, 245: 2447–2457.

* Corresponding author E-mail: burcuatli2006@gmail.com