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Research Article

Functional microbial diversity in arid soils of Uzbekistan: discovery of a biotechnologically valuable *Bacillus amyloliquefaciens* strain

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Abstract

In this study, we assessed the functional microbial diversity of arid and anthropogenically impacted soils of Uzbekistan to identify bacterial strains with biotechnologically important enzymatic activities. A total of 20 *Bacillus* isolates obtained from oil-sludge–contaminated areas and intensively irrigated agricultural fields were screened for their ability to produce proteases with milk-clotting activity (MCA). Preliminary screening on skim-milk agar revealed three isolates exhibiting strong proteolytic activity (hydrolysis zones > 20 mm). Quantitative assays performed in a lactose-based fermentation medium identified isolate 6/4/2 as the most active strain. MALDI-TOF mass spectrometry and 16S rRNA gene sequencing confirmed this isolate as *Bacillus amyloliquefaciens* UzRSMMT-413. Under optimal cultivation conditions (35–40 °C; pH 7.5), UzRSMMT-413 reached a maximum MCA of 400 U mL^{-1} after 48–72 h and demonstrated an MCA/protease activity ratio of 5.18, indicating high specificity toward casein with minimal nonspecific proteolysis. These findings highlight the arid soils of Uzbekistan as an ecologically rich yet understudied reservoir of microbial resources with significant biotechnological potential. The functional diversity identified in these soils offers promising enzymatic candidates for cheese making, fermented dairy production, and other low-energy bioprocesses. Overall, the study underscores the conservation value and applied importance of microbial biodiversity within the semi-natural landscapes of the Palearctic–Oriental transition zone.

Keywords: Arid soils; microbial diversity; Bacillus amyloliquefaciens; milk-clotting activity; protease; dairy biotechnology

Introduction

The development of natural and sustainable milk coagulants has become a strategic direction in modern microbial biotechnology. Milk coagulation represents a key step in the manufacture of various dairy products, including cheese, yogurt, and fermented milk, where coagulating enzymes determine the yield, texture, and organoleptic characteristics of the final product (Dobozi et al., 2023). Milk coagulation primarily occurs through three mechanisms—enzymatic hydrolysis, acidification, or a synergistic combination of both. Enzymatic coagulation, in particular, is mediated by milk-clotting enzymes (MCEs) that catalyze the cleavage of specific peptide bonds in κ-casein, leading to destabilization of casein micelles and curd formation (Mohsin et al., 2024).

According to Mohsin et al. (2024), milk-clotting proteases (MCPs) are extensively applied in cheese production, whereas acid or mixed fermentation is used for yogurt manufacture. The enzymatic cleavage between phenylalanine (Phe105) and methionine (Met106) in κ-casein has been identified as the critical reaction initiating milk coagulation. Currently, the predominant natural coagulant in the dairy industry is calf rennet, containing chymosin (EC 3.4.23.4). However, due to the limited supply of animal rennet and growing industrial demand, there is increasing interest in discovering and developing alternative microbial or plant-based coagulants (Liburdi et al., 2018). Microbial proteases exhibiting milk-clotting activity (MCA) are of particular interest because of their advantageous biochemical characteristics, such as high thermostability, broad pH adaptability, and cost-effective production through microbial fermentation. Fungal enzymes, notably those from *Rhizomucor miehei* and *Rhizomucor pusillus*, are the most widely used commercial substitutes for calf rennet, as their three-dimensional conformations closely resemble that of chymosin (Alahmad Aljammas et al., 2022; Yamazaki et al., 1999).

In recent years, *Bacillus* species have attracted attention as efficient producers of milk-clotting proteases. For instance, *Bacillus sp. P45* produces a potent MCP under submerged fermentation on feather-meal substrate, showing activity comparable to chymosin (Lemes et al., 2016). Similarly, *B.subtilis* and *B. velezensis* have been reported to synthesize metalloproteases with significant MCA and specificity toward casein (Dutt et al., 2009; Zhang et al., 2023). These microbial enzymes exhibit promising potential as sustainable alternatives to animal-derived rennet. Therefore, the screening and characterization of novel *Bacillus* strains with enhanced proteolytic and milk-clotting properties are of great importance for biotechnological innovation in enzyme-based dairy processing. The present study aims to isolate and evaluate Bacillus strains capable of producing proteases with high MCA, contributing to the development of efficient microbial coagulants suitable for industrial applications.

Materials and methods

In this study, 20 bacterial isolates belonging to the genus *Bacillus* were investigated. The isolates were obtained from various soil samples collected across different regions of Uzbekistan, including oil sludge-contaminated soils, industrial wastewater, and arid zones of the Kashkadarya, Samarkand, and Tashkent regions. All strains are preserved in the collection of the Enzymology Laboratory at the Institute of Microbiology, Academy of Sciences of the Republic of Uzbekistan. They are maintained in two forms: as lyophilized cultures and as frozen stocks in 10% glycerol at –80 °C. For the primary screening of active *Bacillus* strains producing proteases with milk substrate activity (MSA), both qualitative and quantitative assessments were performed. Qualitative screening was conducted on a solid nutrient agar medium with the following composition (g/L): agar – 20; skim milk – 20. Agar was dissolved in 800 mL of distilled water and sterilized by autoclaving at 121°C for 15 minutes. Skim milk was sterilized separately at a pressure of 0.5 atm for 15 minutes and then aseptically added to the cooled agar (~50 °C) to a final volume of 1 L.

The isolates were inoculated onto the surface of the prepared medium and incubated under appropriate conditions. The formation of clear hydrolysis zones around the colonies was used as an indicator of proteolytic activity (Naveed et al., 2022). Colonies exhibiting hydrolysis zones greater than 20 mm in diameter were considered potential protease producers with MSA activity and were selected for subsequent quantitative analysis. Quantitative screening was carried out using the selected strains from the primary screening stage. The strains were cultured in 250 mL Erlenmeyer flasks containing 70 mL of liquid nutrient medium. Cultivation was performed on a rotary shaker at 150 rpm and 37°C for 96 hours. The composition of the liquid medium was as follows (g/L): lactose – 2.5; peptone – 1.5; yeast extract – 1.0; $MgSO_4 \cdot 7H_2O$ – 1.0; KH_2PO_4 – 1.0; $(NH_4)_2HPO_4$ – 1.0; $CaCl_2$ – 0.01. The initial pH of the medium was adjusted to 6.0–6.5 (Lemes et al., 2016).

Bacterial growth was assessed by measuring the optical density (OD) of the cell suspension at 590 nm using a TP-8X UV/Vis spectrophotometer (Chongqing Top Oil, China), with appropriate sample dilutions taken into account. Measurements were performed in cuvettes with a path length of 10 mm. In addition, cell growth was evaluated based on the dry cell weight. For this, the culture fluid (CF) was centrifuged at 10,000 rpm for 10 minutes, and the cell pellet was collected for drying and weighing. Following centrifugation, the supernatant (culture fluid filtrate) was used for determining total protein content, protease activity, and MSA (milk substrate activity).

The total protein content in the culture filtrate (CFF) was determined using the method of Lowry et al. (1951). Protease activity (PA) was evaluated by the method of Anson (1938) at different pH

values (5.3, 7.5, and 9.0) during the growth of the strains. Milk-clotting activity (MCA) was determined according to the procedure described by Arima, Iwasaki, and Tamura (1967). Briefly, 10 mL of 10% skim milk containing 10 mM $CaCl_2$ was added to 20 mL test tubes and preheated in a water bath at 35 °C for 5 minutes. Subsequently, 0.1, 0.5, or 1.0 mL of the CFF was added to the tubes, which were then incubated at 35 or 40 °C until the first visible signs of coagulation appeared. The tubes were gently shaken at regular intervals during incubation. Control samples were prepared by replacing the active CFF with 1.0 mL of heat-inactivated (pre-boiled) CFF (Meng et al., 2018; Karam et al., 2024). Milk coagulating activity (MCA) was calculated using the following formula:

$$\mathrm{MCA} = \frac{2400 \times V_S \times N}{T \times V_E}$$

where: 2400 is the total number of seconds in 40 minutes; VS is the volume of skim milk (mL); N is the dilution factor of the MCA; TT is the clotting time (seconds), and VE is the volume of the CFF added (mL). One unit of MCA is defined as the amount of enzyme that coagulates 10 mL of 10% reconstituted skim milk in 0.01 M $CaCl_2$ at 35–40°C within a specified time. The results were expressed in Soxhlet units (SU), a standard unit used to quantify milk clotting activity. One Soxhlet unit is defined as the amount of enzyme capable of clotting 1 mL of standard milk in 40 minutes at 35°C.

Results

In this study, bacterial strains were isolated from various soil samples, including those contaminated with oil sludge. The taxonomic affiliation of the isolates was determined based on MALDI-TOF mass spectrometry, as well as morphological and cultural characteristics. All isolates were identified as belonging to the genus *Bacillus.* Primary screening for protease activity (PA) was conducted on solid nutrient agar supplemented with skim milk. According to the results, three out of the twenty isolates formed hydrolysis zones with diameters exceeding 20 mm, indicating significant proteolytic activity. The largest hydrolysis zones were observed in the following strains: *Bacillus spp. 6/4/2* - 33 mm, *Bacillus spp. RP1* - 28 mm, and *Bacillus spp. RP2* - 25 mm (Fig. 1).

A B C

Figure 1. Hydrolysis zone of skim milk: A – *Bacillus spp. 6/4/2, B – Bacillus spp. RP1, C – Bacillus spp. RP2*

Further screening of the active strains was conducted by culturing them in a liquid nutrient medium. Strains that exhibited the largest zones of milk hydrolysis on solid medium were selected for this analysis. Milk coagulating activity (MSA), protease activity (PA), and total protein content were determined in the culture fluid filtrate (CFF) at two incubation temperatures, 35°C and 40°Cthroughout strain growth. Before cultivation, strains stored at –80°C were revived by inoculation into tryptone soy broth (TSB) and incubated at 37°C for 12–14 hours. Subsequently, 4% (v/v) of the preculture was transferred into sterile 250 mL Erlenmeyer flasks containing 70 mL of liquid nutrient medium, with lactose serving as the primary carbon source. Cultivation was carried out at 37 °C for 96 hours on a rotary shaker set at 150 rpm.

As is well known, bacterial strains of the genus *Bacillus* (including *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, and *B. cereus*) exhibit varying capacities to synthesize proteolytic enzymes. These microorganisms are capable of producing both neutral and alkaline proteases, whereas the production of acidic proteases is relatively rare. The spectrum and level of enzymatic activity depend not only on the species of the strain but also on the cultivation conditions. According to the results of this study, quantitative assessment of protease activity revealed that the maximum activity occurred at pH 7.5, 24 hours after inoculation. At this point, the biomass concentration reached 0.796 mg/L, protease activity peaked at 136 U/mL, and the total protein concentration was 0.47 mg/L. At pH 9.0, the protease activity was slightly lower, at 98.85 U/mL, while at pH 5.3, it was significantly reduced to 42.6 U/mL. A subsequent decline in protease activity was observed at later time points. By the 72nd and 96th hours of cultivation, activity decreased to 77.12 U/mL and 90.82 U/mL, respectively, accompanied by protein concentrations of 0.45 mg/L and 0.43 mg/L (Fig. 2).

For the bacterial strains *Bacillus spp. RP1* and *Bacillus spp. RP2,* the protease activity was relatively low. At the 24th hour of cultivation, with protein concentrations of 0.27 mg/L and 0.28 mg/L, respectively, the protease activity at pH 7.5 reached only 33.51 U/mL for RP1 and 44.31

U/mL for RP2. Throughout the cultivation period, the maximum recorded protease activity for *Bacillus spp. RP1* and RP2 did not exceed 39.15 U/mL and 31.07 U/mL, respectively, indicating limited enzymatic potential under the tested conditions.

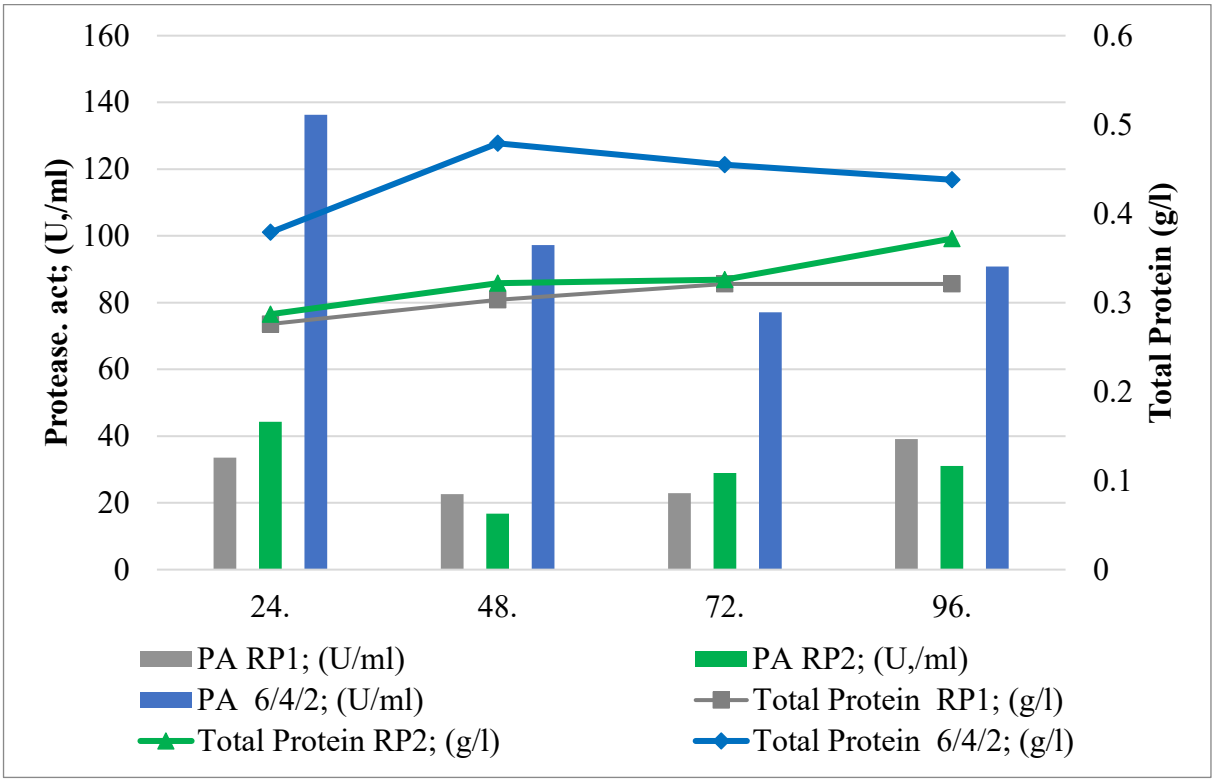

Figure 2. Dynamics of biosynthesis of proteases and the amount of protein in bacteria of the genus *Bacillus* at a temperature of 35 °C

The highest milk coagulating activity (MCA) was recorded for *Bacillus spp. 6/4/2*, reaching 400 U/mL between 48 and 96 hours of cultivation. For *Bacillus spp. RP1*, the maximum MCA was observed at the 24th hour and reached 242.4 U/mL. In the case of *Bacillus spp. RP2,* the MCA peaked at 325.2 U/mL during the same time point. Notably, a decline in milk-clotting activity was observed for both strains starting from the 48th hour of cultivation. For *Bacillus spp. RP1*, MCA decreased to 130.7 U/mL and 45.7 U/mL by the 72nd and 96th hours, respectively. Similarly, in *Bacillus spp. RP2,* the activity declined to 160.0 U/mL at 72 hours and 139.8 U/mL at 96 hours.

According to literature data, the ratio of milk coagulating activity to protease activity (MCA/PA) serves as an important indicator of the quality of milk-clotting factors (MCFs) produced by microorganisms. Strains that produce MCFs with high MCA and low PA are particularly valuable for cheesemaking applications. This is because MCA reflects the enzyme's capacity to induce milk coagulation, thereby supporting efficient curd formation. In contrast, PA indicates the enzyme's proteolytic potential, i.e., its ability to hydrolyze milk proteins. While moderate

proteolysis is essential for flavor development and cheese ripening, excessive protease activity can result in undesirable effects, such as bitterness and deterioration of texture [13, 14].

The results of the study showed that the *Bacillus spp. 6/4/2* strain exhibited a high MSA/PA ratio of 5.18 at the 72nd hour of cultivation at 35 °C. This strain also demonstrated a rapid milk coagulation rate, with clotting times ranging from 48 to 96 hours, not exceeding 60 seconds. In contrast, *Bacillus spp. RP1* and RP2 showed significantly slower coagulation, with clotting times ranging from 750 to 1120 seconds. The maximum MSA/PA ratios for these strains were recorded at the 72nd hour of cultivation, reaching 1.14 for RP1 and 1.029 for RP2 (Fig. 3, 4).

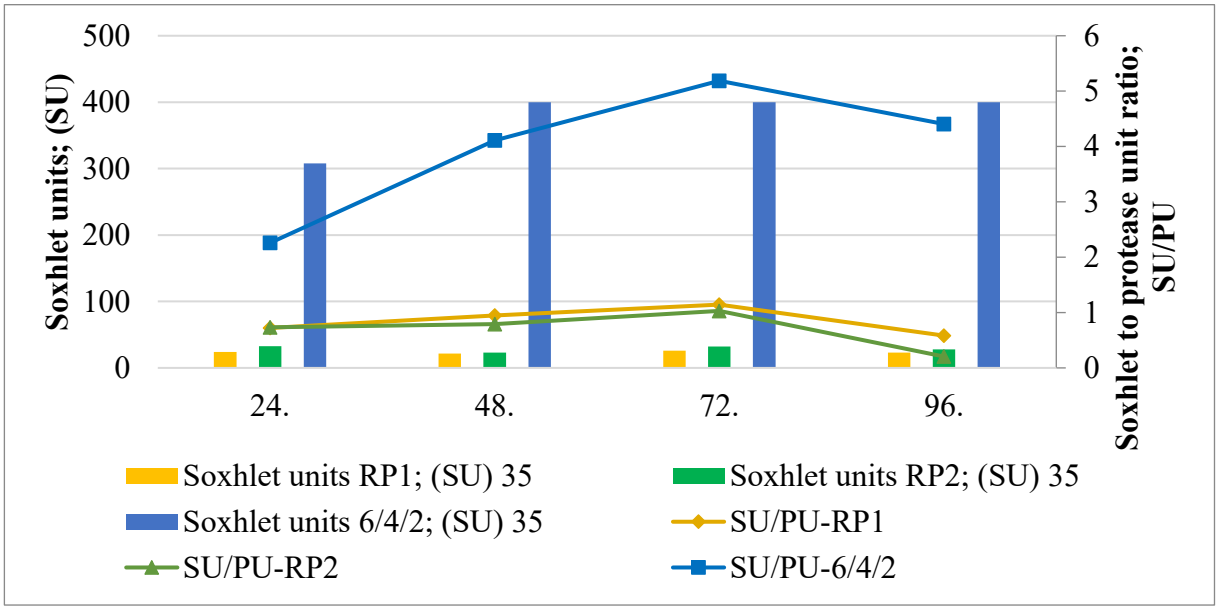

Figure 3. Dynamics of MSA, MSA/PA ratio of strains of bacteria of the genus *Bacillus* at a temperature of 35°C

As a result of the conducted studies, the bacterial strain *Bacillus spp. 6/4/2* was identified as a promising producer of milk-clotting factors (MCF). The strain was taxonomically classified as *B. amyloliquefaciens* based on morphological and cultural characteristics, MALDI-TOF mass spectrometry, and molecular genetic analysis, including 16S rRNA gene sequence homology. The strain has been deposited in the culture collection of the Institute of Microbiology under the accession number UzRSMMT-413 and is also registered in the National Center for Biotechnology Information (NCBI) database under the accession number PV664479.

Bacillus spp. 6/4/2

Bacillus spp. RP1

Figure 4. MSA of strains after 72 hours of cultivation at 35ºC

Thus, the results of this study demonstrated that the *Bacillus spp. 6/4/2* strain exhibits high protease and milk-clotting activity, with peak values observed between 48 and 96 hours of cultivation at 35 °C. The highest milk-clotting to protease activity ratio (MCA/PA), reaching 5.18, was recorded at the 72nd hour of cultivation. These findings highlight the strain's strong potential as a producer of milk-clotting enzymes suitable for use in dairy processing applications.

Discussion

The results of this study demonstrate that among the 20 *Bacillus* isolates examined, *Bacillus amyloliquefaciens UzRSMMT-413* exhibited the most pronounced milk-clotting and proteolytic activity. The strain achieved a maximal milk-clotting activity (MCA) of 400 U/mL after 48–72 h of cultivation, with an optimal pH of 7.5 and an MCA/PA ratio of 5.18, which reflects a high specificity toward κ-casein. These parameters are consistent with the characteristics of other microbial rennet analogs reported in previous studies. For example, *Bacillus sp. P45* produced a comparable enzyme with 320 U/mL MCA (Lemes et al., 2016), while *Bacillus velezensis DB219* secreted a metalloprotease with strong milk-coagulating potential (Zhang et al., 2023).

The high MCA/PA ratio observed in *B. amyloliquefaciens UzRSMMT-413* indicates that the enzyme is suitable for cheese production, as it effectively coagulates milk while minimizing nonspecific proteolysis. According to Fanqiang Meng et al. (2018) and Dutt et al. (2009), an ideal milk-clotting enzyme should possess high MCA but relatively low protease activity to prevent bitterness and textural defects in cheese. The enzyme from *B. amyloliquefaciens UzRSMMT-413* meets these criteria, suggesting that it may be a valuable substitute for calf rennet in industrial dairy applications.

Environmental adaptability also appears to contribute to the strain's efficiency. *B. amyloliquefaciens* species are known to thrive in nutrient-limited and stress conditions, enabling robust enzyme production under moderate fermentation settings (Karam et al., 2024). The optimal activity of *B. amyloliquefaciens UzRSMMT-413* at near-neutral pH (7.5) and moderate

temperature (35–40 °C) supports its potential for industrial-scale processes that require stable operation and low energy input. Comparatively, fungal coagulants such as those from *Rhizomucor miehei* and *Rhizomucor pusillus* have long been used as microbial substitutes for chymosin (Alahmad Aljammas et al., 2022; Yamazaki et al., 1999). However, these enzymes often exhibit excessive proteolytic activity or lower thermostability than bacterial enzymes. In contrast, *Bacillus*-derived proteases are characterized by broader pH tolerance, shorter fermentation cycles, and high thermostability (Mohsin et al., 2024; Liburdi et al., 2018), making them advantageous candidates for rennet replacement. Moreover, the observed decline in enzyme activity beyond 72 h of cultivation may result from protease autolysis or accumulation of inhibitory peptides, a common phenomenon in microbial fermentation (Dobozi et al., 2023). Optimization of medium composition, aeration, and immobilization techniques could therefore enhance enzyme yield and stability, as suggested by Karam et al. (2024). Overall, this study provides strong evidence that *B. amyloliquefaciens UzRSMMT-413* represents a promising microbial source of milk-clotting enzymes. Its high MCA, favorable MCA/PA ratio, and rapid curd formation capacity highlight its potential application as an eco-friendly, cost-effective alternative to animal rennet for sustainable cheese and fermented milk production.

Conclusion

This study successfully identified and characterized *Bacillus* strains capable of producing proteases with significant milk-clotting activity (MCA). Among the 20 isolates screened, *Bacillus amyloliquefaciens UzRSMMT-413* demonstrated the highest potential as a microbial source of milk-clotting enzyme, achieving a maximum MCA of 400 U/mL after 48–72 hours of cultivation. The enzyme exhibited optimal activity at pH 7.5 and 35–40 °C, with a high MCA/protease activity ratio (5.18), indicating strong specificity toward milk casein and limited non-specific proteolysis.

The findings confirm that *B. amyloliquefaciens UzRSMMT-413* produces a protease that fulfills the essential requirements for use as a microbial rennet substitute—namely, high coagulation efficiency, moderate proteolytic action, and stability under near-neutral conditions. Compared to other microbial and fungal coagulants, the enzyme derived from this strain offers advantages in terms of thermal tolerance, fermentation efficiency, and eco-friendly production. These results highlight the potential industrial applicability of *B. amyloliquefaciens UzRSMMT-413* for the development of sustainable, cost-effective milk-coagulating enzymes suitable for large-scale cheese and dairy product manufacturing. Further optimization of cultivation conditions, enzyme purification, and immobilization strategies is recommended to enhance productivity and stability for commercial implementation.

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