Identification and study of extracellular enzymatic activity of *Bacillus velezensis* isolated from saline soils of the Fergana Valley

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Received: May 06, 2025; Reviewed: May 23, 2025; Accepted: June 05, 2025

This study focuses on the extracellular enzymatic activity of the dominant bacterial species *Bacillus* sp. isolated from saline soils of the Fergana region. Among more than 25 isolates studied, isolate No. 2, obtained from the topsoil layer (0–15 cm), and exhibited the highest hydrolytic activity. Notably high activity was observed for pectinase and amylase, with hydrolysis zones ranging from 38 to 45 mm. Moderate activity was shown by xylanase, cellulase, protease, and lipase, with hydrolysis zones between 24 and 34 mm. Based on molecular genetic analysis of the 16S rRNA gene sequence, the highly active dominant strain was identified as *Bacillus velezensis* TM.

Keywords: *Soil, isolates, bacteria, identification, Bacillus velezensis, hydrolases, pectinase, amylase, xylanase, protease, cellulase, lipase*

INTRODUCTION

The selection of low-cost enzyme production methods and the mismatch between optimal operating temperatures are limiting factors. Microorganisms such as bacteria, filamentous fungi, and yeasts can be used for the production of hydrolases, such as amylases and cellulases. Under appropriate working conditions, the production of these enzymes, for example, using *Bacillus velezensis* – can be a cost-effective alternative to traditional enzyme production methods based on submerged or solid-state fermentation. This is because enzyme biosynthesis directly within microorganisms may help reduce overall process costs, as the use of commercial enzymes is associated with significant economic impact (about 15.7% of the total process cost) (Devos et al., 2024).

Bacillus species have become common industrial microbial chassis, with approximately 60% of enzyme products on the market derived from *Bacillus*. Compared to the complexities of molecular-genetic manipulation in fungi and safety concerns related to endotoxins in *Escherichia coli*, *Bacillus* expression systems offer several distinct advantages. These include short expression cycles, high protein yields, the non-pathogenic nature of the host, absence of endotoxins in expressed proteins, excellent thermostability, lack of significant codon bias, and an efficient secretion signal peptide and chaperone system (Ye et al., 2024).

Recent advances in microbial genomics and plant-microbe interactions have clarified the mechanisms through which *B. velezensis* contributes to plant health, making it a promising candidate for sustainable agricultural practices (Bach et al., 2025; Kang et al., 2025).

Bacillus velezensis is widely found in various fermented products, such as fermented soybean paste (*doenjang*) and fermented vegetables like *kimchi*. The use of *B.velezensis* as a starter culture has increased and has been reported to affect the technological properties of salted seafood during fermentation. It has also been reported that *B.velezensis* SS360–1, isolated from traditional soy sauce, influences the production of aromatic compounds *in vitro* (Na et al., 2023; Peng et al., 2025; Revankar & Bagewadi, 2025).

In addition, *B.velezensis* HF-14,109 was isolated from the intestine of healthy carp (*Cyprinus carpio* L.) and demonstrated tolerance to extreme environmental conditions, such as high temperatures, acidity, and bile salts. It was also sensitive to various antibiotics and capable of secreting amylase, proteases, and other digestive enzymes, as well as xylanase, β-mannanase, and other non-starch polysaccharide-degrading enzymes (Wang et al., 2024; Zhang et al., 2025).

The isolated strain *Bacillus velezensis* NA16, in a submerged fermentation culture of poultry feathers, produced a large number of proteases, thereby overcoming the common obstacle of high substrate costs typically encountered in industrial enzyme production. During a 72-hour cultivation period, NA16 degraded chicken feathers with an efficiency of 99.3% and produced a high

 DOI: https://doi.org/10.62088/timbb/9.1.2

concentration (7,599 mg/L) of amino acids (Ablimit et al., 2024; Esawy et al., 2025).

The aim of this study was to isolate and identify *Bacillus* species from saline soils in the Fergana region and to study their hydrolytic activity.

MATERIALS AND METHODS

Study of enzymatic activity using qualitative colorimetric chemical reactions. Detection of extracellular hydrolytic activities was carried out on solidified (agar-based) media containing various substrates, which allowed for visual identification of hydrolysis zones. Amylase, cellulase, pectinase, xylanase, and lipase activities were assessed using agar media supplemented with 1% starch, 1% carboxymethyl cellulose, 1% pectin, 1% xylan, and 1% olive oil, respectively.

After the appearance of microbial colonies, the plates were stained with iodine solution and Congo red solution. When stained with iodine, the presence of yellow zones on a blue background indicated extracellular amylase activity; light brown zones on a brown background indicated extracellular cellulase activity; and yellow zones on a brown background indicated extracellular lipase activity.

When stained with 1% Congo red solution, the appearance of red zones on a light brown background indicated extracellular pectinase activity, while light yellow zones on an orange background indicated extracellular xylanase activity.

Proteolytic activity was determined by the presence of clear (halo) zones around colonies grown on milk agar (Romanova et al., 2020).

Bacterial identification. To identify the bacterial strains, a nutrient broth was prepared and inoculated with the bacterial culture, which was then incubated at 30° for 12 hours. Genomic DNA was extracted from the bacterial culture grown in 10 mL of nutrient broth using the RIBO-prep reagent kit (InterLabService, Russia), following the manufacturer's protocol. The extracted DNA samples were analyzed using 0.9% agarose gel and a spectrophotometer, and stored at –20°.

Amplification of the 16S rRNA gene was performed using the PCR method. The 16S rRNA gene was selected for molecular-genetic identification of the bacterial cultures. The total reaction volume for amplification was 20 µL, using a ready-to-use lyophilized PCR core kit (Isogene, Russia). Primers (5 pmol/µL) were added in a volume of 2 µL each, along with 2.5 µL of dNTPs and 2 µL of DNA (20 ng/µL). Then, 10 µL of buffer was added, and the final volume was brought to 20 µL with deionized water.

The PCR reaction was carried out using the following thermal cycling program: initial denaturation at 95° for 5 minutes; 35 cycles of denaturation at 95° for 20 seconds, annealing at 57° for 20 seconds, extension at 72° for 40 seconds; and a final extension at 72° for 3 minutes. The PCR product was analyzed by electrophoresis in 2% agarose gel.

The PCR product was purified using the GFX™ PCR DNA and Gel Band Purification Kit, and its concentration was measured with a Qubit 2.0 fluorometer (Invitrogen, USA). The purified PCR product was subjected to sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA). The sequencing product was cleaned using the BigDye XTerminator Purification Kit and loaded into the sequencer.

Sequencing reactions and purification procedures were carried out according to the manufacturer's instructions. The resulting sequence data were processed using CodonCode Aligner software and compared (via BLAST) with the NCBI database (Kosimov et al., 2024).

RESULTS AND DISCUSSION

Isolation of pure bacterial cultures was carried out from moderately saline soil samples (more than 25 isolates) from the Fergana region. At the initial stage of the study, selection of the most effective cultures was based on salt tolerance and biomass formation. Next, we investigated the hydrolytic enzyme activities of the bacterial isolates (amylase, cellulase, xylanase, pectinase, protease, and lipase) using qualitative methods. The presence of enzymatic activity was determined by the degradation of substrate materials, which was indicated by turbidity, clearing, or changes in color.

According to the results, isolate No. 2 demonstrated the ability to produce the corresponding enzymes based on the substrates used (starch, cellulose, pectin, and xylan, as well as casein and fats such as olive oil) (Table 1).

As shown in Table, isolate No 2 exhibits high amylase activity, with a hydrolysis zone measuring 45 mm, followed by pectinase (38 mm), xylanase (34 mm), and cellulase (26 mm). This culture also displays proteolytic and lipase activities, with the hydrolysis zone for oil reaching 25 mm (Table).

In addition to the hydrolysis zones on the respective substrates, the activity of the tested enzymes was confirmed by chromogenic staining of the hydrolysis products (Fig. 1).

Table. Qualitative indicators of enzymatic activity of culture No. 2

Substrates	Diameter of the Hydrolysis Zone of Substrates, mm
Xylan from oat "Spelta"	34± 0.025
Potato starch	45±0.034
Beet pectin	38±0.05
Wood cellulose (CMC - Carboxymethylcellulose)	26±0.05
Milk casein	24±0.03
Olive oil	25±0.025

For microorganisms exhibiting the largest clearance zones around their colonies, species identification was performed using MALDI-TOF MS analysis. It was found that the effective strain No. 2 preliminarily belongs to the genus *Bacillus*, species *velezensis*.

Currently, phylogenetic analysis based on the 16S rRNA nucleotide sequence is widely used as a tool in bacterial taxonomy, and the advantages of this method have been confirmed by numerous studies (Zhang et al., 2025).

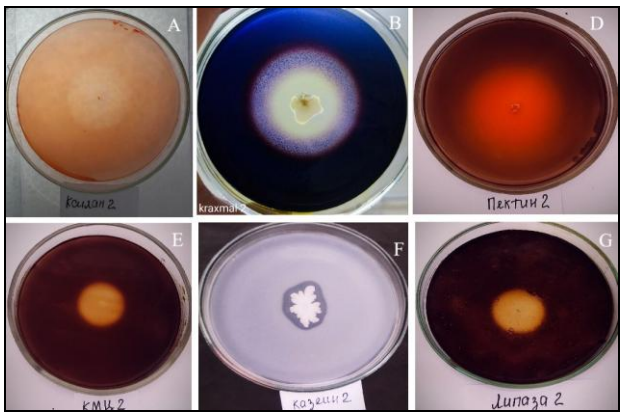

Fig. 1. Tests for the hydrolysis product formation of substrates, according to the enzymatic activity of *Bacillus* sp., strain 2: A – amylase, B – xylanase, D – pectinase, E – cellulase, F – protease, G – lipase.

The functionality of this gene is essential for the cell, which makes it highly conserved; the role of ribosomes has remained unchanged for 3.8 billion years, and due to the gene's considerable length (approximately 1500 nucleotides), the sequence data can be statistically evaluated (Devos et al., 2024).

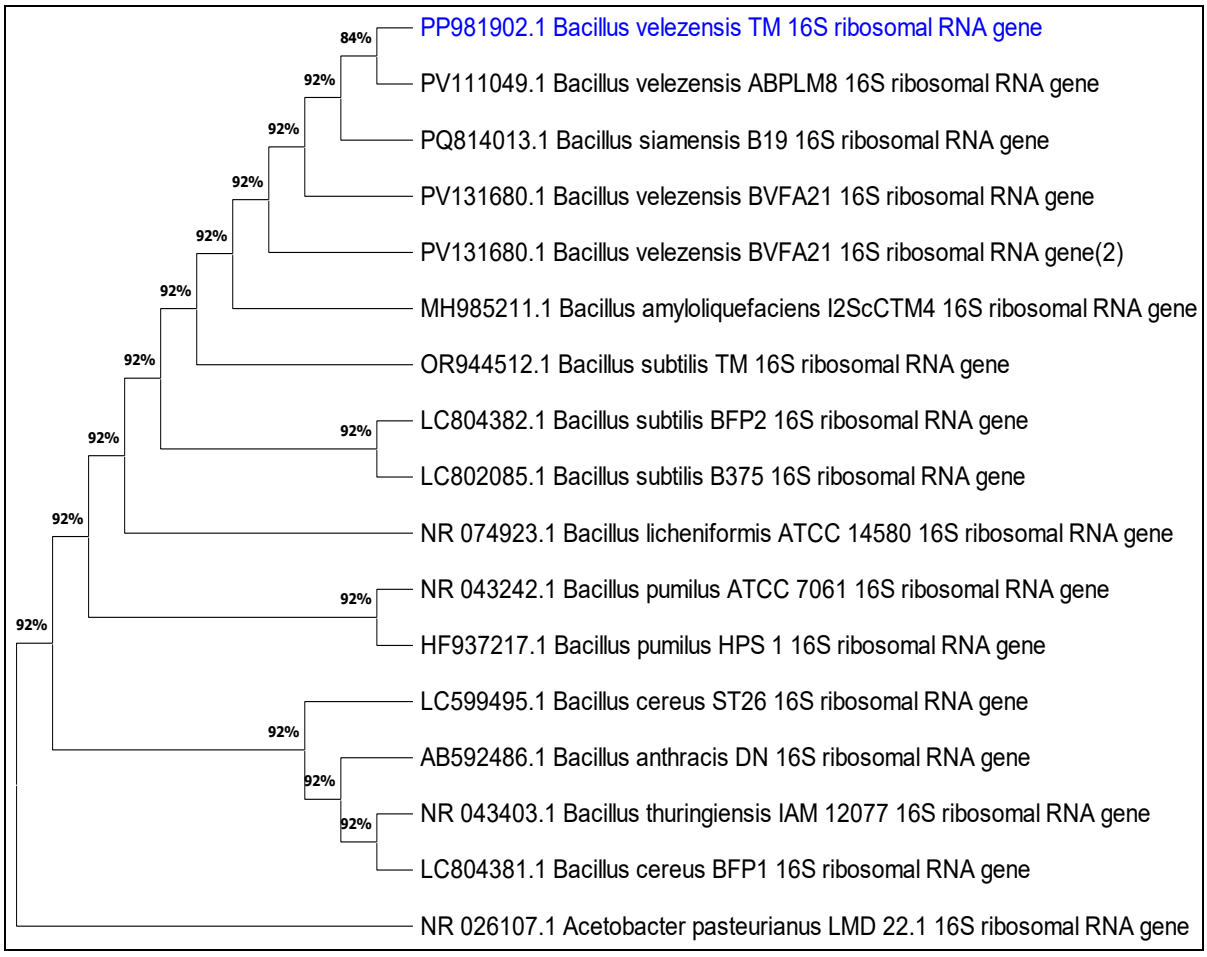

Fig. 2. Phylogenetic tree of the local *Bacillus velezensis* TM strain, isolated from saline soils of the Fergana region (The phylogenetic tree was constructed using the Unigene Ver35 and Chromas software).

Based on the above, further molecular-genetic identification involving the determination of the 16S rRNA nucleotide sequence showed 100% concordance with the results obtained by MALDI-TOF MS analysis. A comparative BLAST analysis revealed that the nucleotide sequence of the 16S rRNA gene from the local *Bacillus velezensis* TM strain shares 92% homology with the sequences of known strains *Bacillus velezensis* PV11049.1, *Bacillus velezensis* PQ814013.1, and *Bacillus velezensis* PV131680.1 (Gene Bank NCBI).

As a result of the conducted research, the local *Bacillus velezensis* TM strain was registered under the number PP981902.1 in the National Center for Biotechnology Information (NCBI) database (Fig. 2).

CONCLUSION

In the course of the study, it was established that the local salt-tolerant strain *Bacillus velezensis* TM has the ability to hydrolyze various polysaccharides (starch, cellulose, xylan, pectin), protein (casein), as well as fats (olive oil). The obtained results indicate the high biotechnological potential of the local *Bacillus velezensis* TM strain, isolated from moderately saline soils of the Fergana region.

The identified properties of this salt-tolerant culture open up new opportunities for the application of *Bacillus velezensis* TM in various fields of agriculture and industry. Accordingly, ongoing research focuses on studying the enzyme activity of this strain under submerged cultivation conditions over time, using inexpensive substrates containing polysaccharides, proteins, and fats. The goal is to explore the potential for producing a variety of hydrolytic enzymes to meet the needs of different industrial and agricultural sectors.

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Fərqanə vadisinin şoran torpaqlarından ayrılmış Bacillus velezensis bakteriyasının hüceyrə xarici fermentativ aktivliyinin identifikasiyası və öyrənilməsi

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Bu tədqiqat Fərqanə regionunun şoran torpaqlarından ayrılmış dominant bakteriya növü *Bacillus* cinsinə aid olan bakteriyaların hüceyrə xarici fermentativ aktivliyinə həsr olunmuşdur. Tədqiq olunan 25-dən çox izolyat arasında 0-15 sm dərinlikdəki üst torpaq qatından əldə olunmuş 2 nömrəli izolyat ən yüksək hidrolitik aktivlik göstərmişdir. Xüsusilə pektinaza və amilaza üçün yüksək aktivlik müşahidə edilmiş, hidroliz zonalarının diametri 38-45 mm arasında olmuşdur. Ksilanaza, sellulaza, proteaza və lipaza üçün orta səviyyəli fermentativ aktivlik qeyd olunmuş, bu fermentlər üçün hidroliz zonaları 24-34 mm arasında dəyişmişdir. 16S rRNA geninin ardıcıllığının molekulyar-genetik analizi əsasında yüksək aktivliyə malik dominant izolyat Bacillus velezensis TM kimi identifikasiya edilmişdir.

Açar sözlər: *Torpaq, izolyatlar, bakteriyalar, identifikasiya, Bacillus velezensis, hidrolazalar, pektinaza, amilaza, ksilanaza, proteaza, sellulaza, lipaza*

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