# ENZYMATIC CHARACTERISTICS OF BACTERIA ASSOCIATED WITH THE POTATO TUBER MOTH (*PHTHORIMAEA OPERCULELLA*)

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***Abstract.*** *In this study, bacterial isolates were obtained from the gut microbiota of the potato tuber moth (Phthorimaea operculella) and their morphological, biological, and enzymatic characteristics were investigated. A total of nine bacterial isolates were recovered from pest specimens, among which representatives belonging to the genera Bacillus, Enterobacter, and Brevibacillus were identified. The insecticidal activity of the isolated bacteria against the potato tuber moth was evaluated, and isolates N23, K10, and K7 exhibited the highest biological efficacy. In addition, the activities of chitinase, protease, and amylase enzymes were determined, with the highest values recorded for Bacillus thuringiensis isolates N23 and K10. The obtained results demonstrated that these bacterial isolates possess significant entomopathogenic potential and may serve as promising biological control agents against the potato tuber moth.*

***Keywords:*** *phthorimaea operculella, potato tuber moth, entomopathogenic bacteria, Bacillus thuringiensis, Brevibacillus laterosporus, chitinase, protease, amylase, insecticidal activity, biological control.*

**Literature review.** Nowadays, protecting agricultural crops from pests is considered one of the important factors in ensuring food security. According to recent data, despite pesticide and non-chemical control measures, approximately 37% of crops are lost annually due to the impact of pests, of which 13% is attributed to insects, 12% to pathogens, and 12% to weeds. In potato cultivation as well, insect pests and diseases pose a constant threat, leading to a reduction in yield of up to 40% [1, 2].

Potato (*Solanum tuberosum* L.) is of great importance as an important food crop in world and Uzbekistan agriculture, and one of the main factors reducing its productivity is various insect pests. In particular, the potato tuber moth (*Phthorimaea operculella*) and the Colorado potato beetle (*Leptinotarsa decemlineata*) cause significant economic damage to potato crops, resulting in reductions in both yield quantity and quality.

Phthorimaea operculella damages potatoes both in the field and during storage. It reduces product quality and increases the risk of infection by pathogens. Because this pest simultaneously damages both leaves and tubers, crop yield is significantly reduced. Under warm climatic conditions, storage losses may even reach up to 100% [3, 4].

The main method used to control these pests has been the application of chemical insecticides. However, their prolonged use has resulted in several adverse effects, including the development of resistance in pest populations, environmental pollution, a reduction in beneficial organisms, and the accumulation of pesticide residues in food products. Therefore, the

development of environmentally safe and biologically effective control strategies has become an important scientific and practical priority. Modern agriculture faces two major challenges: increasing crop productivity and reducing negative environmental impacts. Pests and diseases are among the principal causes of food losses, leading to annual reductions of 20–40% in the global yields of major crops. Although synthetic chemical pesticides were initially employed as the primary means of insect pest control, their excessive and long-term use has contributed to the emergence of pesticide resistance, ecosystem contamination, biodiversity loss, and adverse effects on human and animal health [5].

In pest management, biopesticides—biological products that include metabolites synthesized by microorganisms, compounds of plant and animal origin, natural minerals, and semiochemicals—are also widely used [5]. Furthermore, microbiological biopesticides account for more than 55% of the total biopesticide market, confirming their important role in sustainable agriculture [6]. Recent technological advances, particularly in the fields of microbial fermentation, metabolomics, genomics, and synthetic biology, have accelerated the discovery and agricultural application of these natural compounds [7–9].

Bacteria used as biological control agents are functionally classified into facultative pathogens (*Pseudomonas aeruginosa*), obligate pathogens (*Bacillus popilliae*), crystal-forming spore-producing bacteria (*Bacillus thuringiensis*), and potential pathogens (*Serratia marcescens*). Among these groups, spore-forming bacteria are widely utilized because of their high efficacy, safety, and specificity [10].

Bacteria belonging to the families *Pseudomonadaceae*, *Streptococcaceae*, *Enterobacteriaceae*, *Bacillaceae*, and *Micrococcaceae* are commonly used in biological control. Among them, certain species of the genus *Bacillus*, particularly *Bacillus subtilis*, have been granted the status of “Generally Recognized as Safe” (GRAS) by the United States Food and Drug Administration (FDA) [11].

Approximately 90% of biopesticide products in the United States are based on *Bacillus thuringiensis* (Bt), which is widely used for pest control in medicine, agriculture, and forestry [12]. During the sporulation process, Bt produces insecticidal proteins in the form of parasporal crystals. These crystals are primarily composed of proteins known as Cry and Cyt toxins and are also referred to as δ-endotoxins [13]. Cry proteins are considered highly safe, selective, and sustainable alternative agents for pest management [14].

The β-exotoxin produced by *Bacillus thuringiensis*, known as thuringiensin, is a heat-stable secondary metabolite that exhibits insecticidal activity against insects belonging to the orders *Diptera*, *Coleoptera*, *Lepidoptera*, *Hymenoptera*, *Orthoptera*, and *Isoptera*, as well as against certain nematodes [15]. Another successful example is Spinosad, which is widely used as an insecticidal metabolite produced by *Saccharopolyspora spinosa* [16].

Furthermore, the insecticidal activity of metabolites produced by *Chromobacterium subtsugae*, identified as a novel insecticidal bacterium, has also been confirmed [13]. Various subspecies of *Bacillus thuringiensis* are effective against a broad spectrum of insect pests, including representatives of the orders *Lepidoptera*, *Coleoptera*, and *Diptera*, and are widely used in commercial biopesticide formulations. In addition, the toxin complex synthesized by *Xenorhabdus nematophilus* possesses insecticidal properties and enhances the pathogenicity of entomopathogenic nematodes through symbiotic interactions [14].

The application of biopreparations based on beneficial microorganisms in potato agroecosystems not only reduces pest populations but also improves soil microbiological activity.

Compared with chemical insecticides, microbial preparations are environmentally friendly and prevent the accumulation of toxic residues in agricultural products. Therefore, the importance of microbiological control methods within integrated pest management systems is continuously increasing [17].

Analysis of the scientific literature presented above indicates that the use of entomopathogenic bacteria is one of the most promising approaches for the control of potato pests. However, the composition of the microbiota associated with pests occurring in potato agroecosystems, as well as the biological characteristics and entomocidal activity of bacteria isolated from them, have not been sufficiently studied under local conditions. Therefore, in the present study, bacterial isolates were obtained from specimens of the potato tuber moth (*Phthorimaea operculella*), and their morphological and biological characteristics were investigated.

**Materials and Methods**

**Isolation of Bacteria from *Phthorimaea operculella* Larvae**

Standard microbiological methods were used for the isolation of bacterial strains. Specimens of *Phthorimaea operculella* collected under field conditions were transported to the laboratory in sterile containers. The external surfaces of the samples were sterilized with 70% ethanol for 1 min and subsequently rinsed several times with sterile distilled water. This procedure enabled the removal of external microflora and facilitated the selective isolation of internal microorganisms.

Under sterile conditions, *P. operculella* larvae were dissected, and gut tissues were aseptically removed. The isolated tissues were homogenized in sterile physiological saline solution containing 0.85% NaCl, and the resulting suspension was serially diluted to $10^{-1}$–$10^{-3}$. Diluted samples were inoculated onto Nutrient Agar (NA), Tryptic Soy Agar (TSA), MacConkey agar, and Eosin Methylene Blue (EMB) agar media. The inoculated plates were incubated at 37°C for 24–48 h. After incubation, morphologically distinct colonies were selected and purified by repeated streaking to obtain pure cultures [18].

**Determination of Hydrolytic Enzyme Activities (Chitinase, Protease, and Amylase) of Bacterial Isolates**

**Protease Activity.** Proteolytic activity was determined using a hemoglobin substrate-based method with slight modifications. A 0.1% hemoglobin solution prepared in citrate buffer (pH 5.5) was used as the substrate. Enzyme activity was evaluated by measuring the amount of leucine released from hemoglobin. One unit (U) of protease activity was defined as the amount of enzyme required to release 1 mg of leucine at 37°C within 30 min. For the assay, 0.5 mL of crude enzyme extract was mixed with 3 mL of 1% hemoglobin solution and incubated at 37°C for 30 min. The reaction was terminated by adding 3 mL of 5% trichloroacetic acid (TCA), and the mixture was subsequently filtered. The filtrate was mixed with 0.5 mL of distilled water and 1 mL of ninhydrin reagent and then boiled for 20 min. After cooling, 5 mL of diluting solution ($H_2O$ = 1:1) was added. Protease activity was determined by measuring the optical density at a wavelength of 570 nm [19].

**Chitinase Activity.** Chitinolytic activity was evaluated using the 3,5-dinitrosalicylic acid (DNS) method with colloidal chitin as the substrate, following the methodology of Shivalee et al. For this assay, 1.0 mL of enzyme solution was mixed with 1.0 mL of 0.5% colloidal chitin prepared in 0.1 M citrate buffer (pH 7.0) and incubated at 37°C in a shaking water bath for 30 min. The reaction was terminated by adding 2 mL of DNS reagent and heating the mixture in a boiling water

bath for 10 min. After cooling, the mixture was centrifuged at 10,000 rpm for 10 min. The optical density of the resulting supernatant was measured at 540 nm against a control sample. One unit (U) of chitinase activity was defined as the amount of enzyme producing reducing sugars in 1 mL of solution per minute under the assay conditions [20].

**Amylase Activity.** Amylolytic activity was determined using the standard 3,5-dinitrosalicylic acid (DNSA) method. To prepare the DNS reagent, 1.4 g of 3,5-dinitrosalicylic acid was dissolved in 70 mL of 0.5 N NaOH solution, followed by the addition of 30 g of sodium potassium tartrate, and the final volume was adjusted to 1000 mL. Standard maltose and test solutions consisting of 0.5 mL of 1% soluble starch solution and 1 mL of DNS reagent were incubated at 40°C for 15 min. Subsequently, 5 mL of distilled water was added, the mixture was cooled, and absorbance was measured at 540 nm. Based on the obtained results, the amount of reducing sugars released as a result of starch hydrolysis was calculated [21].

**Results and Discussion**

During the study, specimens of *Phthorimaea operculella* were collected from potato agroecosystems located in the Piskent, Bekabad, and Boka districts of the Tashkent region. Field observations revealed that the pest was widely distributed throughout the surveyed areas. The collected specimens were transported to the laboratory, where bacterial isolates were obtained from their gut microbiota for further investigation.

Bacterial isolation from the gut microbiota of *Phthorimaea operculella* was successfully performed. Following incubation on culture media, colonies exhibiting diverse morphological characteristics were observed. Morphologically distinct colonies were selected and purified through repeated streaking to obtain pure cultures (Figure 1).

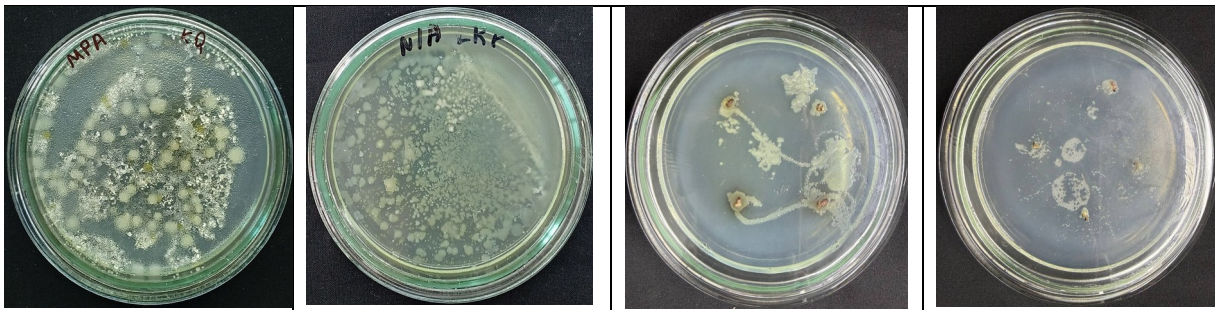


***Figure 2. Isolation and purification of bacterial isolates from the gut microbiota of Phthorimaea operculella.***

As a result, nine bacterial isolates were obtained from *P. operculella* specimens. The morphological and biological characteristics of the isolated strains were investigated, and selected isolates were subjected to further studies and microscopic examination (Figure 2).

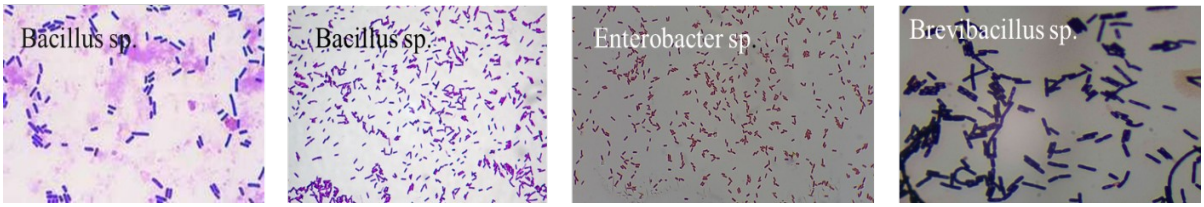



***Figure 2. Morphological and cultural characteristics of bacterial isolates obtained from Phthorimaea operculella.***

Gram staining and microscopic observations revealed the cell morphology and Gram reaction of the isolated bacterial strains (Figure 1). Among the investigated isolates, two representatives of *Bacillus* sp., one *Enterobacter* sp., and one *Brevibacillus* sp. were identified.

The *Bacillus* isolates were Gram-positive and consisted of purple-stained rod-shaped cells. The cells were observed either singly or arranged in short chains. In contrast, the *Enterobacter* sp. isolate exhibited a Gram-negative reaction and appeared as short pink-stained rod-shaped cells. The *Brevibacillus* sp. isolate was characterized by Gram-positive, relatively long rod-shaped cells, which were occasionally arranged in chains.

The obtained results confirmed that the isolates belonged to their respective bacterial groups based on their morphological characteristics and Gram-staining properties. The morphological features revealed through Gram staining were consistent with the results of molecular identification, allowing a preliminary taxonomic characterization of the isolates. These findings provide an important basis for subsequent physiological, biochemical, and molecular studies.

**Disease Suppression Activity**

The antagonistic activity of different bacterial isolates against the phytopathogen increased significantly over time (Figure 3). During the initial 3 days of the experiment, disease suppression levels were relatively low in all treatments, ranging from 4.5% to 20.3%. As the incubation period progressed, the protective efficacy of the isolates gradually increased, and the highest levels of disease suppression were observed on day 15.

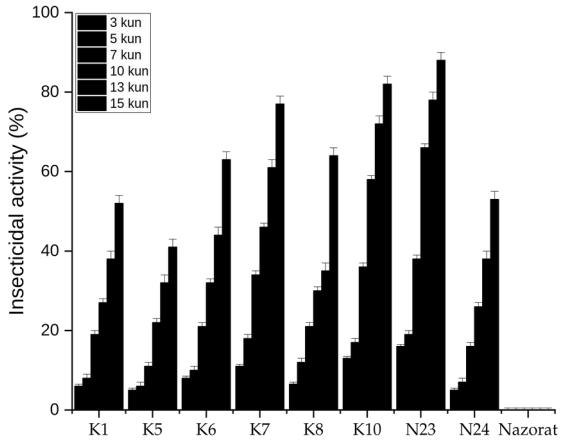



***Figure 3. Insecticidal efficacy of bacterial isolates against the potato tuber moth (Phthorimaea operculella).***

Among the investigated isolates, N23 exhibited the highest antagonistic activity. The disease suppression rate of this isolate was approximately 20% on day 3 and increased to 87–88% by day 15. Similarly, K10 and K7 also demonstrated high efficacy, achieving disease suppression rates of approximately 80% and 77%, respectively, at the end of the experiment. Positive results were also observed for K8 and K6, which successfully restricted disease development by 63–65% on day 15.

The antagonistic activities of isolates N24 and K1 were moderate, with disease suppression rates of approximately 53% and 52%, respectively, recorded at the end of the experiment. The lowest levels of activity were observed in the K5 isolate and the control treatment. While natural disease progression continued in the control treatment, the protective efficacy of K5 remained considerably lower than that of the more active isolates.

Overall, the results demonstrated that the ability of the bacterial isolates to suppress phytopathogen development increased over time and that isolates N23, K10, and K7 possess considerable potential as biological control agents. These isolates represent promising candidates for the development of biocontrol products and for further investigation of their mechanisms of action.

**Hydrolytic Enzyme Activities of Bacterial Isolates**

The hydrolytic enzyme activities of nine bacterial isolates obtained from the gut microbiota of *Phthorimaea operculella* were evaluated. According to the preliminary screening results, *Bacillus thuringiensis* K10, *Bacillus thuringiensis* N23, and *Brevibacillus laterosporus* K7 exhibited high hydrolytic enzyme activities (Figure 4).

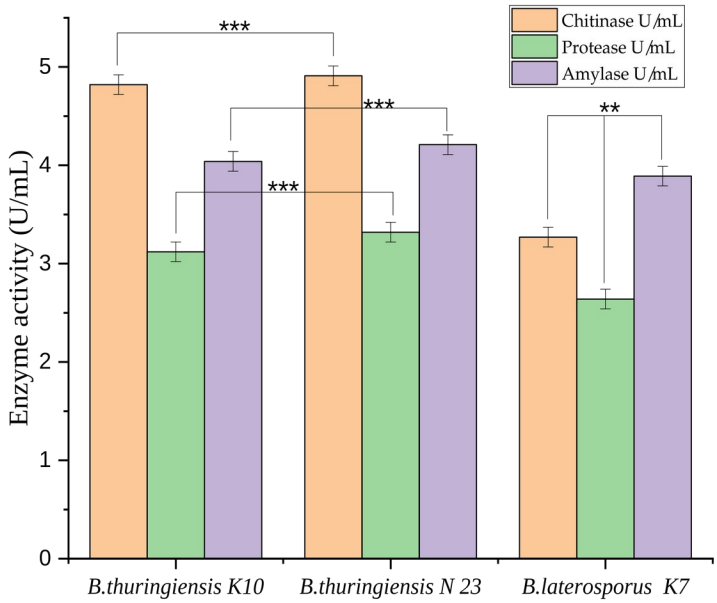



***Figure 4. Activities of hydrolytic enzymes (chitinase, protease, and amylase) produced by bacterial isolates isolated from Phthorimaea operculella.***

According to the obtained results, the highest chitinase activity was recorded in *Bacillus thuringiensis* N23, reaching 4.91 ± 0.12 U/mL. The corresponding values for isolate K10 and *Brevibacillus laterosporus* K7 were 4.82 ± 0.13 U/mL and 3.27 ± 0.21 U/mL, respectively.

Protease activity was also highest in isolate N23, with a value of 3.32 ± 0.08 U/mL. The activities recorded for K10 and K7 were 3.12 ± 0.07 U/mL and 2.64 ± 0.11 U/mL, respectively.

Similarly, isolate N23 exhibited the highest amylase activity, reaching 4.21 ± 0.11 U/mL. The enzyme activities of isolates K10 and K7 were 4.04 ± 0.20 U/mL and 3.89 ± 0.14 U/mL, respectively.

These results indicate that *B. thuringiensis* N23 and K10 possess a strong capacity for the synthesis of hydrolytic enzymes.

The findings of the present study demonstrated that *Bacillus thuringiensis* N23 and K10 isolates, obtained from the gut microbiota of *Phthorimaea operculella*, exhibited high chitinase, protease, and amylase activities. In particular, isolate N23 showed the highest values for all investigated enzymes. This observation suggests that these isolates may possess considerable entomopathogenic potential.

Our results are consistent with those reported by Liu et al. (2002), who demonstrated that chitinase enzymes are widely distributed among different *B. thuringiensis* strains and contribute to enhanced insecticidal activity. According to their findings, chitinase-producing strains increased the larvicidal efficacy of *B. thuringiensis* toxins by up to 2.35-fold. This enhancement was attributed to the ability of chitinase to degrade the insect cuticle and peritrophic membrane, thereby facilitating toxin penetration and activity [22].

The importance of chitinase in entomopathogenicity has also been confirmed in subsequent studies. Qin et al. (2020) reported that chitin-binding proteins of *B. thuringiensis* interact with the peritrophic membrane of the insect gut and play an important role during the infection process [23]. Likewise, Nielsen-Leroux et al. (2012) noted that chitinolytic enzymes contribute to the disruption of gut barriers, thereby facilitating the access of bacteria and toxins to epithelial cells [24].

In the present study, *B. laterosporus* K7 also exhibited chitinase and protease activities, although these values were lower than those observed in *B. thuringiensis* isolates. Nevertheless, these findings support the entomopathogenic potential of *Brevibacillus laterosporus*. Prasanna et al. (2013) identified inducible extracellular chitinases in *B. laterosporus* strains and suggested that these enzymes contribute to the bacterium's biocontrol and insecticidal properties [25].

The protease activity observed in this study is also in agreement with previous reports. Malovichko et al. (2019) emphasized that, in addition to Cry toxins, proteases and chitinases represent important virulence factors of *B. thuringiensis*, contributing to the progression of infection within the insect host [26]. Similarly, Marche et al. (2018) demonstrated that the expression of chitinase and protease genes in *B. laterosporus* strains is associated with insecticidal activity [27].

Overall, the obtained results indicate that *B. thuringiensis* N23 and K10 possess high enzymatic activity, which may enhance their entomopathogenic potential. Comparison with previously published studies suggests that chitinase and protease enzymes play crucial roles in the degradation of insect cuticles and gut barriers. Therefore, these isolates may be considered promising biological agents for the development of biocontrol strategies against the potato tuber moth (*P. operculella*).

## REFERENCES

1. EPA, Biopesticides, Environmental Protection Agency, 2024.
2. Barbara D.J., Clewes E., Plant pathogenic Verticillium species, Mol. Plant Pathol., 2003, pp. 297–305.
3. Brar S.K., Verma M., Tyagi R.D., Valero J.R., Advances in Bacillus thuringiensis biopesticides, Process Biochemistry, 2006, pp. 323–342.
4. Javorekova S., Kovacsova S., Medo J., Makova J., Petrova J., Hleba L., Kostalova D., Artimova R., Soil amended with organic fertilizers as a source of actinomycetes with biocontrol potential, Journal of Microbiology Biotechnology and Food Sciences, 2019, pp. 1352–1359.
5. Waghunde R.R., Shinde C.U., Pandey P., Singh C., Fungal biopesticides for agroenvironmental sustainability, Industrially Important Fungi for Sustainable Development, 2021, pp. 479–508.

6. Ayilara M.S., Akinola S.A., Adebajo M.T., Benefits and Drawbacks of Microbial Inoculant in Terms of Human Health and the Environment, Microbial Inoculants: Applications for Sustainable Agriculture, 2024, pp. 411–435.
7. Messing R., Brodeur J., Current challenges to the implementation of classical biological control, Biocontrol, 2018, pp. 1–9.
8. Kour D., Rana K.L., Yadav A.N., Yadav N., Kumar M., Kumar V., Vyas P., Dhaliwal H.S., Saxena A.K., Microbial biofertilizers: bioresources and eco-friendly technologies for agricultural sustainability, Biocatal. Agric. Biotechnol., 2020.
9. Hernández-Fernández M., Cordero-Bueso G., Ruiz-Muñoz M., Cantoral J.M., Culturable yeasts as biofertilizers and biopesticides for sustainable agriculture, Plants, 2021.
10. Pucheta D.M., Macias A.F., Navarro S.R., Mayra D.L.T., Mechanism of action of entomopathogenic fungi, Microbiol., 2006, pp. 2164–2171.
11. Sharma S., Malik P., Biopesticides: types and applications, 2012.
12. Mora M.A.E., Castilho A.M.C., Fraga M.E., Classification of entomopathogenic fungi, 2017.
13. Copping L.G., Menn J.J., Biopesticides: action and applications, Pest Manag. Sci., 2000, pp. 651–676.
14. Heuskin S., Lorge S., Godin B., Leroy P., Frère I., Verheggen F.J., Haubruge E., Wathelet J.P., Mestdagh M., Hance T., Lognay G., Semiochemical formulation optimization, Pest Manag. Sci., 2012.
15. Sivapragasm A., Biopesticides and their regulation in Malaysia, Asia-Pacific Biofertilizers and Biopesticides Information Platform, 2022.
16. Fenibo E.O., Ijoma G.N., Matambo T., Biopesticides in sustainable agriculture: current status and future prospects, New Developments in Biopesticide Research, 2022.
17. Pineda S., Alatorre R., Schneider M.L., Martinez A.M., Pathogenicity of entomopathogenic fungi, 2007, pp. 43–52.
18. Cappuccino, J. (2018). *Microbiology a laboratory manual*. Pearson.
19. Afrin, S., Tamanna, T., Shahajadi, U. F., Bhowmik, B., Jui, A. H., Miah, M. A. S., & Bhuiyan, M. N. I. (2024). Characterization of protease-producing bacteria from garden soil and antagonistic activity against pathogenic bacteria. *The Microbe*, *4*, 100123.
20. Xie, J., Zhang, L., Yang, K., Zhang, H., Jiang, M., Liao, S., ... & Shen, N. (2025). Enhanced chitinase production by Bacillus paralicheniformis GXMU-J23. 1: Optimization, genomic insights, and chitin degradation mechanism. *Bioresource Technology*, *418*, 131911.
21. Kim, J. A., Kim, M. J., Yim, J. H., Kim, I. C., Rhee, J. S., & Han, S. J. (2025). Isolation and Characterization of Low-Temperature and High-Salinity Amylase from Halomonas sp. KS41843. *Fermentation*, *11*(8), 465.
22. Liu, D., Cai, J., Xie, C., Liu, C., & Chen, Y. (2002). Purification and partial characterization of a 36-kDa chitinase from *Bacillus thuringiensis* subsp. *colmeri* and its biocontrol potential. *Enzyme and Microbial Technology*, *31*(6), 828–834.
23. Qin, Y., Zhang, J., Liu, Y., Zhang, X., & Wang, Q. (2020). Chitin-binding proteins contribute to the insecticidal activity of *Bacillus thuringiensis*. *Toxins*, *12*(4), 252.
24. Nielsen-Leroux, C., Gaudriault, S., Ramarao, N., Lereclus, D., & Givaudan, A. (2012). How the insect pathogen bacteria *Bacillus cereus* and *Bacillus thuringiensis* induce host death. *Current Opinion in Microbiology*, *15*(3), 220–224

25. Prasanna, L., Eijsink, V. G. H., Meadow, R., & Gåseidnes, S. (2013). A novel chitinase from the entomopathogenic bacterium *Brevibacillus laterosporus*. *Microbiological Research, 168*(6), 358–365.
26. Malovichko, Y. V., Nizhnikov, A. A., & Antonets, K. S. (2019). Repertoire of the *Bacillus thuringiensis* virulence factors unrelated to major classes of protein toxins and its role in specificity of host-pathogen interactions. *Toxins, 11*(6), 347.
27. Marche, M. G., Mura, M. E., Ruiu, L., & Delrio, G. (2018). Pathogenicity and virulence factors of *Brevibacillus laterosporus* associated with insecticidal activity. *Toxins, 10*(12),