AMERICAN JOURNAL OF BIOSCIENCE AND CLINICAL INTEGRITY
https://biojournals.us/index.php/AJBCI
ISSN: 2997-7347

Article

Molecular Identification and Antibacterial Evaluation of Bacillus thuringiensis Isolated from Soil Samples in Central Iraq

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Citation: Al-Rammahi, A. A. K & Alkhazraji, E. K. Molecular Identification and Antibacterial Evaluation of Bacillus thuringiensis Isolated from Soil Samples in Central Iraq. American Journal Of Bioscience And Clinical Integrity 2026, 3(5), 14-21

Received: 10th Feb 2026
Revised: 21th Mar 2026
Accepted: 18th Apr 2026
Published: 05th May 2026

Abstract: *Bacillus thuringiensis* is an environmental bacteria found in many environments that have the ability to produce highly effective proteotoxins that can be used as an antibacterial pathogen. During this study, 50 soil samples were collected from four governorates of Baghdad, Babylon, Najaf, and Karbala. The soil was treated with 0.85% NaCl and then exposed to a temperature of 80 °C to obtain the spores and kill of other bacteria associated with *Bacillus* in the soil. 0.5-1 ml of suspended broth was taken and incubated with LB broth incubated for 6-16 hours to reduce the growth of bacteria, and then dilution were applied to bacterial growth and cultured in different media to diagnose the bacteria microscopicly and biochemically, including Hichrome Bacillus agar medium, where the bacteria appeared blue and the medium color was yellow, which indicates that they ferment menthol and produce the enzyme glycosidase. The general characteristics of the bacteria were then studied, as it was a mobtle bacillus producing spores, beloved to catalysis, methyl red, negative to oxidase, and to assay indole, degrading starch, and strong positive for lecithinase, fermented for glucose and menthol and hemolysis of Blood. It was genetically diagnosed based on the specific 16sRNA, then BT toxin was extracted after stimulating the bacteria to produce spores on nitrogen poor media, collecting the spores, extracting the cry protein(Bt toxin) with a weight 2μg-0.5mg, and testing its ability as an antibacterial, as it showed high effectiveness against the pathogenic bacteria and a minimum inhibitory concentration was 0.15 μg against Gram-positive pathogenic bacteria and 0. 31 μg against Gram-negative bacteria.

Keywords: *Bacillus thuringiensis*, Hichrome Bacillus agar, Bt toxin, cry protein, sporulation

Introduction

B. thuringiensis is a distinct species of Gram-positive rod-shaped bacteria belonging to the family Bacillaceae within the phylum Firmicutes. This bacterium is widely found in natural environments, particularly in agricultural soil and on plant surfaces, and can also be isolated from insect remains and the digestive tract of some insect species [1][2] B. thuringiensis is characterized by its high capacity to form endospores, which gives it superior resistance to adverse physical and chemical factors such as drought and high temperatures, which contributes to its survival and spread in various environments [3].

B. thuringiensis is a motile bacterium, thanks to the presence of peripheral flagella, and is either obligately aerobic or facultatively anaerobic [4]. Its most important biological characteristic lies in the production of distinctive crystalline protein complexes known as crytoxins (δ-endotoxins), which are formed intracellularly during sporulation [5]. These toxins are highly effective against the larvae of many insect orders, producing a toxic effect after being ingested by the insect. The crystals dissolve in the digestive tract and are proteolytically activated to penetrate the intestinal epithelial cells, causing their collapse and the death of the larva [6].

Thanks to this unique property, B. thuringiensis is one of the most widely used microorganisms in the field of biocontrol of agricultural pests, as it represents a safe and environmentally friendly alternative to traditional chemical pesticides [7]. The genes responsible for producing crystal toxins have also been used in genetic engineering to develop genetically modified insect-resistant crops, commercially known as Bt crops, making these bacteria of significant scientific and applied importance in the fields of bio-agriculture and modern biotechnology [8].

Materials and Methods

Sample Collection

Take 10–20 g of soil from a depth of 1–10 cm (avoiding the visible contaminated surface layer). Place each sample in a sterile tube/bag and label it (location, date). Temporarily store in a refrigerator (4°C) if not immediately processed; processing is preferable within 24–48 hours [9].

Soil Suspension Preparation (For Dilution and Spore Retention)

In a 50 mL tube: Add 10 g of soil + 90 mL of sterile saline solution (0.85% NaCl) → 1:10 suspension. Shake well or shake for 10–15 minutes to dislodge cells from particles. Let the heavy sediment settle for 2–3 minutes; draw the liquid over the sediment for use [10].

Heat shock treatment for spore selection

Place 1–2 mL of the suspension in a taped tube. Heat at 80°C for 10 minutes (or 65°C for 30 minutes—but 80°C/10 min is common for seed selection). The goal is to kill the sporozoites and retain the spores. Cool quickly over ice water or leave at 25°C for a few minutes. Note: The heat-shock step is common for isolating Bacillus sp. because it produces heat-resistant spores [11].

Enrichment (optional but recommended).

Add 0.5–1 mL of the heat treatment to 5–10 mL of sterile LB broth in a tube (or 50 mL in a vial) and incubate with shaking at 30–37°C for 6–16 hours. (B. thuringiensis reproduces well at 28–37°C; 30°C sometimes helps differentiation.) Then use for dilution and sequencing [12].

Serial crystallization and culture on solid media

Grow dilution series (10^-1 to 10^-6) in sterile saline. 100 µL of each dilution is cultured on food agar, LB agar, or Hicrome Bacillus agar, if available, by spread plate or streak for isolation technique. Incubate plates for 24–48 hours at 30–37°C. Colonies may appear after 18–24 hours [13].

Initial Microscopic and Stain Examinations

Gram stain: Bacillus = Gram-positive rods (may appear clumped or single), usually larger than Gram-positive rods. Catalase test: Place a drop of H2O2 on a colony — Furan = Catalase . Spore stain (Schaeffer–Fulton): Looks for intracellular spores. Microscopic observation: To detect parasporal crystals (crystals surrounding the spores) — these crystals are the hallmark of B. thuringiensis. Incubate the isolate on sporulation-inducing medium (see below) and then examine budding/induced colonies. Additional biochemical tests: oxidase, motility. Throghth the study the isolates culture on growth media for diagnosis Nutrient agar, blood agar, Egg yolk Starch agar: Complete starch hydrolysis (translucent zone after iodine addition) [14]

Molecular confirmation

Use universal primers (a common example) for 16S sequencing and submit the sequence for comparison in a BLAST database to identify the species to the species level). Livak, and Schmittgen ,2001)

Common primers: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'(.

Typical PCR setup: 25–50 µL final volume, 1× buffer, 1.5–2.5 mM MgCl2, 0.2 mM dNTPs, 0.2–0.5 µM of each primer, 0.5–1 U Taq, 1–2 µL DNA template, ~30 cycles. (Annealing temperature depends on the primer—typically ~50–55°C for universal primers.)

Bacillus thuringiensis (Bt toxins) extraction

Because proteins are present in crystalline form with spores, you must first:

1-Induce sporulation

Cultivate B. thuringiensis in a nitrogen-poor medium (sporulation agar) at 28–30°C for 48–72 hours until spores and crystals form [15][16].

2- Harvesting cells and crystals

Centrifuge the culture (6,000–8,000 g for 10–15 minutes) to collect the sediment containing spores and crystals. Wash the sediment repeatedly with a buffer solution such as PBSl to remove residual medium [17].

3- Solubilization

The crystals are dissolved in a basic solution (pH 9–10.5) such as:

50 mM Na2CO3 + 10 mM DTT. A suspension of the sediment is prepared in the solution and incubated at 37°C with stirring for several hours until the crystals dissolve [18].

4-Proteolytic activation

Cry protein dissolves in a protoxin form (~130 kDa). It can be treated with trypsin or chymotrypsin to produce the active form (~65 kDa) [19].

Pathogenic bacteria:

Pathogenic isolates(St. pneumonia, S. aureus, E. coli, P. aeruginosa) were obtained from the Advanced Microbiology Laboratory at the College of Science, Department of Biology, University of Babylon.

Minimum Inhibitory Concentrations (MIC)

Minimum inhibitory concentrations (MICs) were determined using the serial dilution technique. MICs, also known as the "gold standard," are the lowest concentrations of antimicrobials that inhibit the growth of microorganisms after 24 hours of incubation (µg/mL) [20][21]. A series of concentrations were prepared as shown in the table.

Table 1. Serial dilution technique to determent MICs.

	Tube No									
	A.C	1	2	3	4	5	6	7	8	G.C
Mueller Hunton Broth (1ml)	1	1	1	1	1	1	1	1	1	1
Extract solution (1ml)	1	1	1	1	1	1	1	1	1	0
Bacteria stuck (1ml)	0	1	1	1	1	1	1	1	1	1
Final volume in each tube: (2ml)	2	2	2	2	2	2	2	2	2	2
v) Final concentration of extract µg/ml	5	5	2.5	1.25	0.62	0.31	0.15	0.078	0.039	0

Results and Discussion

Sample collection

During the study, 50 soil samples were collected from the roots of some field plants from different agricultural areas in different provinces of Iraq, especially from Baghdad, Babaylon, Karbala, and Al-Nahaf. The samples were distributed according to Figure 1 as follows: 20(40%) samples from Baghdad, 12(24%) from Babylon, 8(16%) from Karbala, and 10(20%) from Najaf, so that the number of samples became 50(100%) samples from agriculture soils.

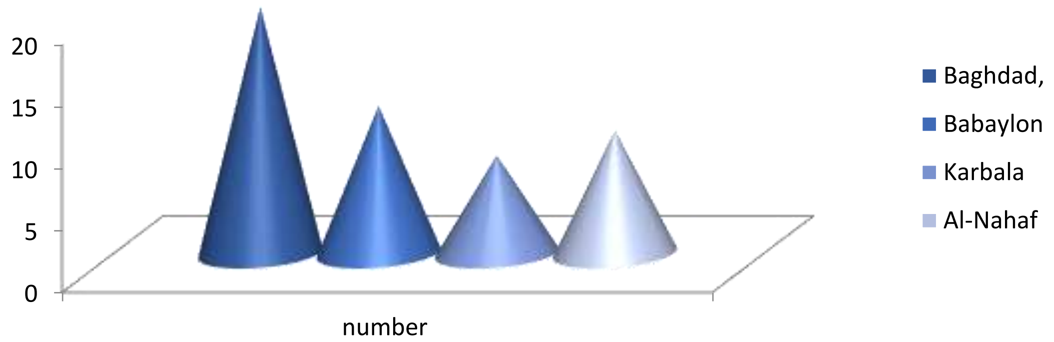

Figure 1. Soil samples distribution.j

Isolation and identification of *B. thuringiensus*

During this study, the samples were cultured on different differential and selective media to isolate and neutralize *Bacillus thuringiensus* bacteria().Ten isolates of *Bacillus thuringiensus* were obtained and purified on the Hicrome bacillus base, where the isolates appeared blue in color as showe in figure2 .

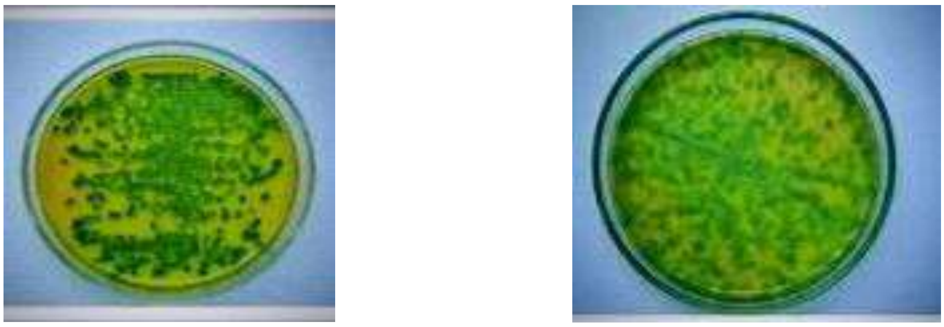

Figure 1. *B. thuringiensis* on Hicrome Bacillus agar

The medium contains chromogenic substrates which are special compounds that are degraded only by enzymes produced by certain species of Bacillus as is the case with *Bacillus thuringiensis*. Other species do not have these enzymes, so the colonies remain white or dull. The medium contains bile salts or mild inhibitors that inhibit the growth of bacteria that are not of the genus Bacillus, Bacillus can grow due to its tolerance to these inhibitors [22].

Colony Screening and Selection

Bacillus colonies typically appear round, slightly rough, and have a matte/stained appearance, sometimes with a prominent center. *B. thuringiensis* may resemble *B. cereus.* Select distinct individual colonies and store them on fresh plates to obtain pure isolation. After getting the pure Culture was diagnosed based on microscopic and biochemical examination As shown in the table 2

Table 2. General characteristics and Biochemical tests specific to *Bacillus thuringiensis*

Characteristics and Biochemical	Results
Colony	rough, matte colonies.

Gram stain	+
shape	Rod-shaped
spores	+
Motility	+
O_2 requirement	Facultative Aerobe
Catalase test	+
Oxidase test	-
Indole test	-
Methyl Red (MR)	+
Voges-Proskauer (VP)	+
Citrate utilization	+
Urease test	±
Nitrate reduction	+
Glucose fermentation	+
Mannitol fermentation	+
lecithinase activity	Strong +
hemolysis	Clear β-hemolysis
starch hydrolysis	+

B. thuringiensis exhibits distinct reactions in laboratory tests. It is positive for the catalase test, indicating its ability to decompose hydrogen peroxide into water and oxygen, but negative for the oxidase test because it does not produce the enzyme oxidase [23][24]. It also has the ability to reduce nitrate to nitrite, making it positive for the nitrate test. They are negative for the indole test because they do not hydrolyze tryptophan to indole. In fermentation tests, they are positive for the methyl red (MR) test, indicating acid production from glucose fermentation, and often also positive for the Voges-Proskauer (VP) test due to acetoin production [25]. These bacteria can use citrate as their sole carbon source, so they give a positive result in the citrate test. They also decompose gelatin by secreting the enzyme gelatinase, and starch by secreting the enzyme amylase, and often test positive for lipase or lecithinase on egg yolk medium. They ferment several sugars, such as glucose, sucrose, and mannitol, producing acids without gas [26].

The diagnosis of Bacillus thuringiensus was genetically confirmed using specific 16sRNA gene as show in figure 3

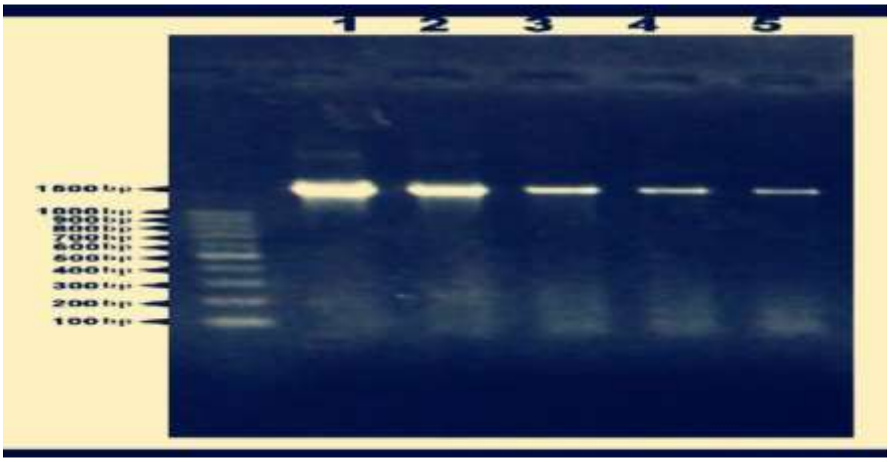

Figure 3. Gel electrophoresis of 16sRNA gene for *Bacillus thuringiensus* bacteria in voltage (85V) time (70 minute)and 5μL of PCR product loaded in well (1-5). Lane M : DNA ladder (100bp). All these sample are positive for the presence of this gene.

Bacillus thuringiensis (Bt toxins) extraction

The bacteria were stimulated to form the spors using a nitrogen poor medium and the formation of crystals containing Bt toxins that were extracted using a basic solution to dissolve the crystals in the form of a protoxin and treated with trypsin and chymotrypsin to produce the active form (~65 kDa).. 6 toxin-producing isolates were obtaned , toxins were collected, and the weight of the product ranged from 2 µg for the isolate 4 to 0. 5 grams for isolation 2.table3

Table 3. Number and weight of Bt toxin

No.of Isolates	Weight of toxin
Bt1	4 µg
Bt2	0.5mg
Bt3	5 µg
Bt4	2 µg
Bt5	6 µg
Bt6	0.2mg

These bacteria are distinguished from other Bacillus species by their ability to produce toxic protein crystals known as Cry or δ-endotoxins during the spore formation stage [26][27]. The presence of toxic protein crystals is the most important factor that distinguishes *Bacillus thuringiensis* from its genetically close cousin Bacillus cereus, which does not produce these crystals and is not toxic to insects [28][29]. *B. thuringiensis,* on the other hand, is commercially exploited as a bioinsecticide and as the basis for the development of genetically modified insect-resistant crops (Bt-crops) such as corn and cotton, making it one of the most useful bacteria in modern organic agriculture [30] .

Minimum Inhibitory Concentrations (MICs) of Bt toxin .

Minimum inhibitory concentrations (MICs) were determined using the serial dilution technique. MICs are defined as the lowest concentration of antimicrobial that inhibits the growth of microorganisms after overnight incubation (Flynn et al., 2015). A series of extract concentrations were used: 5, 2.5, 1.25, 0.62, 0.31, 0.15, 0.078, and 0.039 µg/ml. The results of the current study revealed that the MIC for Gram-positive bacteria was 0.31 µg/ml. The MIC for Gram-positive bacteria was 0.15 µg/ml, as shown in the table 4 and figure 4.

Table 4. Minimum (MICs) for the extract.

Minimum Inhibition Concentration (MIC) (µg/ml)	Test organisms
0.15	*St. pneumonia*
0.15	*S. aureus*
0.31	*E. coli*
0.31	*P. aeruginosa*

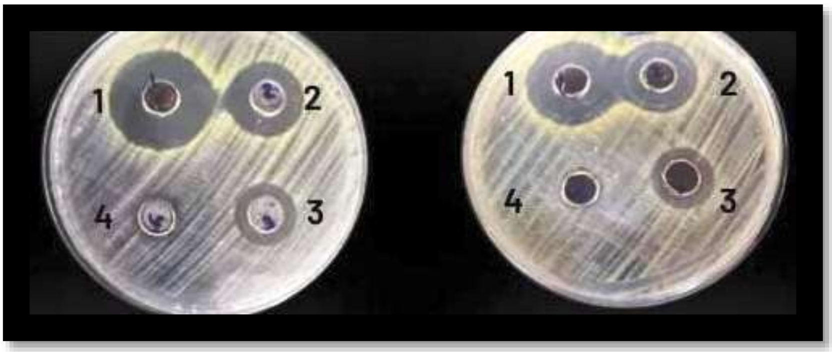

Figure 4. Antibacterial activity of MICs Bt toxin .

By measuring the minimum inhibitory concentration of the extract against the bacteria under study, it was found that Gram-positive bacteria were more sensitive to the extract, as the minimum inhibitory concentration for positive bacteria was 0.15 micrograms/ml, while it was 0.31 micrograms/ml for inhibiting Gram-negative bacteria. The difference in the sensitivity of both types of bacteria to the extract may be due to the difference between bacteria in terms of wall composition and the presence of a peptidoglycan layer, in addition to the nature of the composition of Gram-negative bacteria and their high content of fats, which delay or nullify the absorption or assimilation of substances [31][32]. The nature of the extract and the mechanisms it requires to enter the bacterial cell may require specific concentrations depending on the chemical composition of the wall of Gram-negative and Gram-positive bacteria [33][34][35].

Conclusion

Bacillus thuringiensis is gram positive bacteria ,found in agricultural soils and can be isolated and diagnosed on culture, microscopy and genetics. *B. thuringiensus* produces protein toxins that can be extracted during the spore formation period in different proportions, and these toxins are effective against Gram-negative and Gram-positive bacteria.

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