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Characterization and Toxicity of Bacillus Thuringiensis Strain Kfr-24, And Commercial Formulations against Spodoptera Littolaris

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Abstract: *Under these studies Bacillus thuringiensis wettable powder comparable to standard strain, there are only one isolate which confirmed by molecular characterization. Scanning electron microscopy observed that the presence of cuboidal crystals in Bacillus thuringiensis Alex-13, and observed bipyramidal crystals in case of Bacillus thuringiensis HD-1, as standard strain. Polymerase chain reactions (PCR) revealed the presence of Cry1-like sequences in addition to standard strain, which confirmed by gel electrophoresis. Also, during these studies.Bioassay recorded that the highest mortality rate by determination of LC50 for 5 days against Spodoptera littolaris third instars larvae were 69, 86, and 100(%) in case of Bt Alex-13, Bt-HD-1 standard strain and Bt commercial wettable powder, respectively.*

Introduction

Bacillus thuringiensis

Bacillus thuringiensis is a gram-positive, facultative anaerobic bacterium, which produces intracellular and distinctively shaped crystal proteins during its spore-forming period. The crystal proteins, also called insecticidal crystal protein (ICP) [(12)]**.** Two methods used for isolation of *Bacillus thuringiensis* from environmental samples. The first, developed by the World Health Organization (WHO) [(9)]**,** consists on a thermal shock treatment of a soil solution to kill vegetative bacteria and the isolation of bacterial spores. The second, developed by **Travers *et al.*,**[(25)]**,** based on, inhibition of Bt spores germination by sodium acetate and elimination of other bacteria in vegetative form by heat treatment. Novel varieties of Bt were isolated, in North Chicago (USA), from the phylloplane of deciduous and confer tree as well as of other plants.

Materials and Methods

Isolation of *Bacillus thuringiensis* with insecticidal activity from Egyptian soil
Collection of soil samples: Soil samples were collected randomly from different locations in Egypt governorates: Assiut Al_Azhar university, Elwasta; El-minia Abokurkas-Mantout, Mallawy; Alexandria Awaied; Kfrel-sheikh Sedi-salem city; Elgharbia Tanta city, Karsa; Cairo Elmarg; Souhag Balasfoura, Dar-Elsalam; Qena Naga Hammady; and Aswan Draow city, The samples were collected in clean plastic bags (about 500 g each) at a depth of 5-10 cm.

Isolation method: Ten grams of soil sample was added to 100 ml of acetate buffer L-broth in 500 ml Erlenmeyer flask, the mixture was shaken in rotary shaker at 240 rpm, for 4 hours at 30 °C. At the end of the shaking time, samples were heat treated at 80°C for 3 minutes; a serial dilution was made from the heat-treated soil suspension. Under aseptic conditions 0.2 ml of diluted samples (10^{-1} to 10^{-8}) was pipetted onto prepared isolated media (Mannitol-Egg Yolk-Polymyxin B sulfate Agar) using sterile glass spreader, and incubate overnight at 30 °C.The microbial growth was characterized and purified by streaking several times on the isolating medium, and then subcultured on the slants of the nutrient agar (NA) medium.

Molecular characterization of bacterial strains: Scanning Electron Microscope (SEM): Isolation of Genomic DNA from cell cultures: The locally isolated *Bacillus thuringiensis* strains were grown for 24 hrs. On LB broth medium at 30°C and carried out is as follow:

1- Prepare the cell suspension, for cells grown in suspension, pellet 5 x 10^6 cells by spinning at 1200 x g in a centrifuge tube. Discard the supernatant, and wash the cells once with PBS, and resuspended cells with 200 µl cold (4°C) PBS.

2- Add 25 µl of OB Protease (D3496) or Proteinase K (D3495) at 20 mg/ml solution, vortex to mix well, and incubate at 65 °C in a water bath for 5 minutes to effect complete lysis.

3- Add 220 µl Buffer BL and vortex to mix, incubate at 70 °C for 10 minutes, a wispy precipitate may form on addition of Buffer BL, but dose not interfere with DNA recovery, and adjust the volume of Buffer BL required based on amount of starting material.

4- Place the column into a second 2 ml tube and wash by pipetting 750 µl of wash Buffer diluted with ethanol, centrifuge at 8,000 x g for 1 min, and again dispose of collection tube and flow-through liquid.

5- Using a new collection tube, wash the column with a second 750 µl of wash Buffer and centrifuge as above. Discard flow-through.

6- Using the same 2 ml collection tube, centrifuge at maximum speed (10,000 x g) for 2 min, to dry the column. This step is crucial for ensuring optimal elution in the following step.

7- Place the column into a sterile 1.5 ml microfuge tube and add 200 µl of preheated (70°C) Elution Buffer. Allow tubes to sit for 3 min, at room temperature. Incubate at 70°C rather than at room temperature will give a modest increase in DNA yield per elution. Alternatively, the second elution may be performed using the first eluate. The expected yield from a 30 mg sample is 8-35 µg genomic DNA, depending on type of tissue, this technique has determined in Molecular Biology unit at Assiut University.

Polymerase Chain Reaction (PCR)

PCR amplification of the Cry gene of the local *Bacillus thuringiensis* strains were conducted using two primers, StrepF;5'-CCGGTGCTGGATTTGTGTTA-3', Strep R; 5'- AATCCCGTATTGTATCAGCG-3'PCR performed for 100 ml reaction volume contained, PCR amplification buffer (MBI Fermentas Inc, Amherst, NY 14226), 200 mmol of each deoxynucleotides, 100 pmol of each oligonucleotide primers and template DNA (0.25 mg of purified DNA from 1 mg/ml of stock solution) 2 different Bt isolates. Standard Bt strain was also used as positive control. Amplification was carried out in a Thermoblock Uno II, Thermalcycler (Biometra, Made in Germany) with heated lid (104 °C) facility and was run with block temperature control (thermal regulation by 3 °C/s). Denaturation of template DNA was done for 5 min at 94 °C. After hot start, 2U of Taq DNA polymerase (MBI Fermentas, Amherst, NY 14226) was added. PCR was performed for amplification of Cry 1A (b), Cry III and Cry 1V genes under specific thermal profile as follows: Denaturation at 94 °C for 60 s, annealing at 45 °C for 2 min and polymerization at 72 °C for 3 min for 45 cycles followed by final extension at 72 °C for 10 min. The thermal profile for amplification of Cry 1A (a) and Cry 1C gene were carried out as follows; amplification was initiated with hot start and enzyme was added as mentioned above, denaturation at 94 °C for 60 s, annealing at 56 °C for 2 min and polymerization at 72 °C for 3 min for 45 cycles followed by final extension at 72 °C for 10 min. Three ml of amplified PCR product was loaded on agarose gel (Bio Rad Laboratories, CA. 94547) and analyzed after electrophoresis [(14)]. The remaining PCR product was stored at -20 °C for further work. Amplification of Cry gene was

determined at the Molecular Biology unit, at Assiut University.

Agarose gel electrophoresis

The previous applications were determined at the Molecular Biology unit, at Assiut University.
Laboratory studies: Tested insects - Cotton leaf worm strain *S. littoralis* (Bosid) The Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisd.) obtained as egg masses from the cotton fields at Assiut University, plant protection Department, faculty of agriculture, and transferred directly to laboratory of the botany and microbiology department, faculty of science, AL-Azahr University Assiut branch. During 2007 and 2008 cotton seasons. Egg-masses were placed on leaves of castor bean, *Ricinus communis* (L.) on cylindrical glass jars (1Kg). The jars were covered with muslin cloth held with a rubber band. Following eggs hatching, the first instar larvae were transferred into larger rearing jars (2Kg) provided with filter paper at the bottom of the jar to absorb any excess moisture.
The accumulative faces and debris were cleaned out daily. Towards the end of the sixth instars larvae, moist saw-dust was placed on the bottom of the rearing jars so as to provide a pupation sites. The formed pupae were eventually collected and placed in clean jars until adult emergence. The newly emerged moths were sexed and kept in pairs in mating cages. Each cage was provided with 10 % honey solution soaked in cotton wool which was placed in plastic cup for moths feeding. The honey solution was renewed daily to avoid fermentation and growth of microorganisms. As an ovipositon site, fresh green leaves of *Nerium oleander* (L.) were daily introduced into the breeding cages. The newly laid egg-masses were collected daily and transferred into the rearing jars (5 egg-masses/jars). All rearing cages and jars were placed under laboratory conditions of 28±2Cº and 60 ±5 R.H [8]**.** Great care had been taken to prevent fungal and virus infestation which appeared to be the main factors responsible for high mortality in the laboratory. All jars were wished up with 0.10 % formalin solution and sterilized at 150 ºC for 10 minutes to minimize polyhydrosis virus and fungal infestations.

Preparation of *Bacillus thuringiensis* spore-crystal mixtures: *Bacillus thuringiensis* was grown on 400 ml CBI medium in 2 liter Erlenmeyer flasks on a rotary shaker (100 rpm) at 30 °C for 3 days. Upon lysis, vegetative cells, spores and crystals were harvested by centrifugation (3000 rpm) for 15 min, the pelleted particles, mainly consisting of spores and crystal, were resuspended in phosphate buffered saline (PBS).

Purification of crystals: A 200 ml of bacterial suspension precipitate was washed with distilled H_2O three times and once normal saline and resuspended in distilled water. The suspension was shaken vigorously by hand for 5-15 sec. the froth which appears on the surface was held back on filtration with Whatman No. 1 filter paper. Crystals were prepared from the suspension as follows: to 35 ml of the suspension, 30 ml of 1% sodium sulphate and 35 ml of carbon tetrachloride were added and the whole was mixed at 7.000 rpm for 2.5 min, the mixture was allowed to stand for 15 min, and the aqueous phase which contains pure crystals was separated.

Bioassay Experiments: Determination of the toxicity of *Bacillus thuringiensis* commercial wettable powder (W.P) against 3rd instar larvae of *Spodoptera littoralis*. *Bacillus thuringiensis* (Trade name: ECOTECBIO 10%W.P.) Biological insecticide from *Bacillus thuringiensis* (Bt) 10% Wettable powder. This compound was produced under licens from Agricultural Genetic Engineering Research institute ARC. EGYPT. Rate / fed.200gm/fed. The leaf dipping technique was used to test the efficiency of test

compounds (W.P) against the 3rd instar larvae of *S. littoralis*, using avegerage weight of 40 mg. Eight concentrations were prepared from *Bacillus thuringiensis* of (W.P) ranged from (5000, 4000, 2000, 1000, 500, 250, 100 and 50 ppm). Fresh castor bean leaves were dipped in water with 0.1% of Triton x100 for 10 seconds, as standard methods to make distribution of *B. thuringiensis* and Talk (used as carrier). Three replicates of each concentration were used, in addition ten larvae comparable to control, the treated leaves were left to dry for 30 minutes before offering to the larvae, the larvae were allowed to feed on treated leaves of each concentration for 48 hrs, and then transported to untreated leaves until the end of larval stage (six instar larvae) under laboratory conditions. Castor bean leaves were dipped in water mixed with 0.1% of Triton x100 only used as control. Mortality percentages were calculated after treated times of (24, 48, 72, 96 and 120 hrs). The averages mortality obtained were corrected according to **Abbott's formula** [(1)]**.**

The corrected mortalities at different concentrations were subjected to probit analysis according to **(Finney, 1971)**. To determine LC_{50}

$$\% \text{ corrected mortality} = \frac{(\% \text{ mortality in treatment} - \% \text{ mortality in control})}{100 - \text{Mortality \% in control}} \times 100$$

Determination of the toxicity of *Bacillus thuringiensis* Kfr-24. Against 3rd instar larvae of *Spodoptera littoralis*. The bacterial strain was grown until sporulation was completed in LB liquid medium on a rotary shaker (200 rpm) at 30°C for 5 days. The culture was centrifuged at 12,000 rpm for 15 min, at 4°C. The pellet (toxins and spores) was washed once after sporulation with 1M NaCl and twice with sterile distilled water, then the pellet was dried, and used for bioassay. The bioassay methods were done as previously mentioned.

Larval dissection and gut culture: This experiment has been carried out to confirm that the mortality of larvae caused by the used strain. Caterpillars were killed by placing them separately for 15-30 min at –20ºC. The external surface was disinfected through sequential washes with 0.2 µm filter-sterilized 70% Ethanol (2 min), a mix of 5.25% sodium hypochloride and 0.1% Tween 20 (2 min) and several washes with sterile distilled water. As a control for this process, each disinfected caterpillar was rolled on the surface of a LB agar plate, which was later incubated at 30°C for five days. Afterwards, the gut from each caterpillar was dissected aseptically in a laminar flow hood, macerated and inoculated along with its contents in 10 ml of LB broth according to [(6)]**.**

Results

Localities and collection of soil samples: The number of bacterial isolates were ranged from (1-15) per one sample. Three hundred and thirty five (334) strains were isolated from fifty nine soil samples carrying the No. of (1, 2, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 54, 56, 57, 58, 59, 60, 62, 63, 65, 66, and 67). A relatively higher value recorded (i.e. 15 isolates) were obtained from soil sample No. 14. Which were collected from Assiut Governorate, Elwasta city. While, the lowest value recorded (i.e. 1 isolate) was obtained from soil samples Nos. (31 and 59). However, there are no isolate strains of bacteria were obtained from soil samples Nos. (3, 6, 19, 40, 53, 61 and 64). The first objective of this study was to search for unique toxic activity; therefore, the use of sodium acetate as selective medium was developed by **Travers *et al.*,** [(25)]**,** to eliminate most spore forming bacteria and

all non-spore forming organisms in soil samples. This method allows the spore of unwanted bacterial species to germinate, the unwanted bacteria, which enter the vegetative state, are eliminated by controlled heat treatment.

Identification of *Bacillus thuringiensis* strains. Morphological and Physiological Identification of the isolated Bacilli. Microscope examinations of the *Bacillus thuringiensis* strains indicated that the bacteria were gram positive, rod-shape cells containing only one endospore. All the isolates were characterized by the presence of crystals with different sizes (big, medium, and small), and different shapes (irregular, spherical, flat square, and bipyramidal shape). The spore and crystals were present in free or encapsulated form (both inside the sporangium). The sporangium contained only one spore plus one crystal. The presence or absence of crystals is the major criterion for distinguishing between *Bacillus thuringiensis* and closely related *Bacillus cereus*, therefore, it is very important to be able to detect crystals and to determine their morphology which in some cases, may reflect a kind of specificity. Different biochemical tests to identify the bacterial isolates. Were performed as described in Bergey's Manual of Bacteriology (1986) [(16)]**.** The isolates of this study were able to tolerate high salt concentration (7%), produced acid only from glucose, hydrolyze starch and gelatine, utilize citrate, positive voges proskauer test, gave positive catalase reaction, reduced nitrate, did not produce acids from mannose or xylose, and negative with indol production. These morphological and biochemical results suggested that the selected isolates could be classified as *B. thuringiensis* Table (1).

Molecular characterization of *Bacillus thuringiensis* Kfr-24. Classical identification of bacteria is really well established by bacteriologists. However, it is not 100% accurate and some confusion may be found due to the close similarities or even identical results of the subspecies belonging to the same species, as the case of Bt Therefore the molecular biology approach was considered as a trail to resolve this dilemma about the identify of any isolate at the gene level. These include the Cry gene banding, and the PCR technique.

Characterization of Cry gene of local Bt isolate: The isolation and identification of novel strains of Bt by bioassay can be a long and involved process. The polymerase chain reaction (PCR) is a procedure that allows rapid determination of the presence or absence of a target DNA sequence. PCR technology and primers specific for Bt endotoxin genes were used to screen of new Bt isolates.One isolated spore forming strain *Bacillus thuringiensis* Kfr-24, was analyzed using two sets of primers specific for each of these insecticidal groups for lepidopterans. The products 543 bp. for Cry1A and Cry1A in case of *Bacillus thuringiensis* Kfr-24.

Preliminary screening of *Bacillus thuringiensis* strains: Among the collected soil samples, 16 strains (As 20, As 9, Alex 17, Alex 13, Alex 23, Alex 29, Kfr 12, Kfr 3, Az 32, Az 18, Az 22, S 11, S 17, Mm 9, Mm 6 and Tan 7) found to contain *Bacillus thurngiensis* crystal producing strains as judged by microscopic observations. These isolates were bioassayed for insecticidal activity at (4000 ppm) against *Spodoptera littoralis* larvae, comparable to standard strain. The highest insecticidal potencies against *S. littoralis* larvae were obtained with spore-crystal complex of *Bacillus thuringiesis* Kfr-24., where mortality percentage reached 80%, Three strains (As- 20, As- 9 and Az- 22) has shown a middle mortality rates ranged from 26-36%, the other strains has shown less mortality rates ranged from zero-20%. The difference in mortality percentage of *Bacillus thuringiesis* isolates could be attributed to the type of delta-endotoxin crystal protein itself present in each Bt isolate, or could be due to the sensitivity

of the digestive system of the insect (epithelial cell membrane of the mid gut) to reach with or to be damage by endotoxin [13].

Electron Microscope

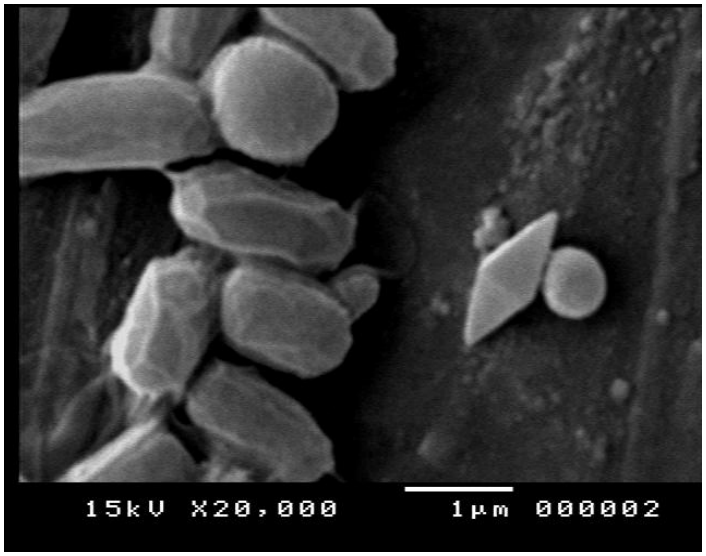
Some characteristics (shape and size) of crystals of *Bacillus thuringiensis* isolated strain recovered from local soil samples. The crystal shape and size of these isolate was different according to comparable strain. The isolated strain of *Bacillus thuringiensis* Kfr-24. Has two shapes of crystal (cuboidal, and bipyramidal, plate 1).

Table 1. Biochemical characteristics of *Bacillus thuringiensis* **detalosi** strain:

Biochemical reaction	Isolated strain
	Kfr-24
Gram stain	Bacilli +Ve
Spore stain	+Ve
Anaerobic growth	+Ve
Parasporal crystals	+Ve
Gelatin liquefication	+Ve
Catalase activity	+Ve
Acid production from. Glucose	+Ve
Galactose	+Ve
Mannitol	–Ve
D-xylose	–Ve
Hydrolysis of. Casein	+Ve
Starch	+Ve
Growth in. 3% NaCl	+Ve
5% NaCl 7% NaCl 10 % NaCL	+Ve +Ve - Ve
Temperature. 30 °C	+Ve
40 °C	+Ve
50 °C	- Ve
Nitrate reduction	+Ve
Indole production	–Ve
Motility	+Ve
VP reaction	+Ve
Urea Hydrolysis	–Ve
Triple Sugar Iron Agar	K/A
Citrate utilization	+Ve
Sugar fermentation (lactose)	- AG

-Ve = Negative, +Ve = Positive, K = alkaline, A = Acid, AG = Acid and gas.

Fig. 1. Agarose gel electrophoresis and the presence of amplified PCR products of CryIA gene from local isolates, Lane 1: DNA marker. Lane 2: gene amplified from *Bacillus thuringiensis* Kfr-24.strain (543 bp), Plate 1: Scanning electron micrograph of crystal and spores of Bacillus thuringiensis isolate Kfr-24 (X20, 000).



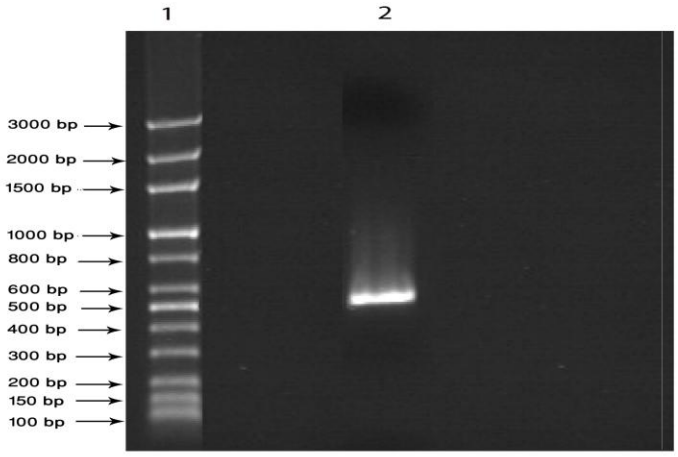
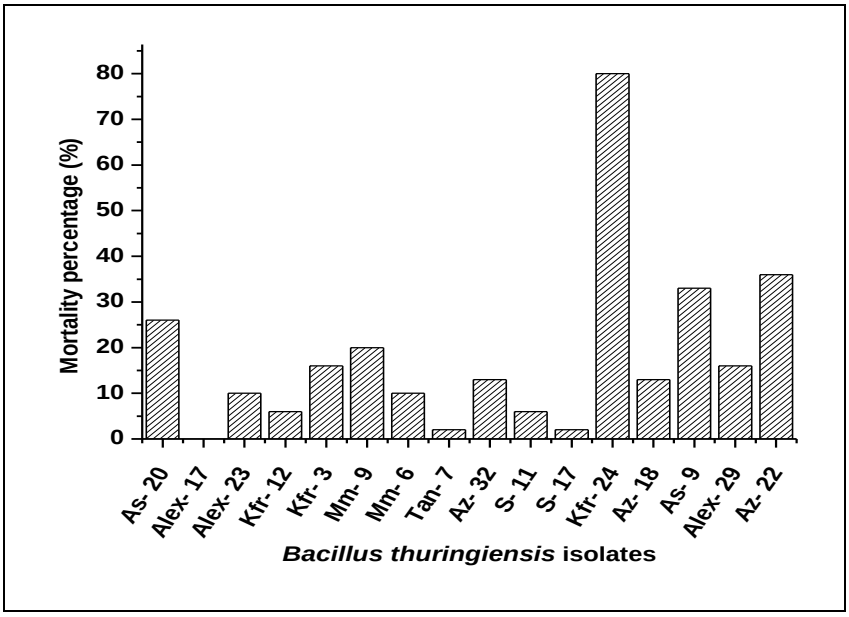


Fig. 2: Toxicity of Bacillus thuringiensis isolates against Spodoptera littolaris 3rd instar larvae



Discussion

Isolation of *Bacillus thuringiensis* from soil: In this study, we collected 59 soil samples from 13 local areas in Egypt. 334 bacterial isolates, 16 isolates identified as *Bacillus thuringiensis*. Four isolates from Alexandria governorate, two isolates from Kfrel-sheikh governorate, one isolate from Tanta governorate, two isolates from Minia governorate, six isolates from Assiut governorate, and two isolates from Sohage governorate.The present study explained characters of soil *Bacillus thuringiensis* strains of different agricultural ecosystems in Egypt governorates. The great size of Egypt, its different climatic regions, and diversity of insects provide the opportunity of isolating novel entomopathogenic bacteria.This study reported that one isolate was identifying as *Bacillus thuringiensis* strain Kfr-24. In this respect, the serovar *kurstaki* as the most common type in Asia, whereas the serovar *israelensis* was most common in Europe and the United States. Similarly[18], **t**he serovar *kurstaki* to be the predominant on the phylloplanes in Japan[19]. Another study from Asia also reported *israelensis* and *kurstaki* as the most frequent types in Jordanian habitats [2]**.**

Characterization of *Bacillus thuringiensis* strains: Phenotypic characterization for isolated strains: Phase contrast Microscope: The identification of *Bacillus thuringiensis* is difficult because of the close genetic relationship between *B. thuringiensis, B. cereus, B. anthracis,* and *B. mycoides.* The main characteristics separating *B. thuringiensis* is the formation of insecticidal crystal proteins [11]**.**

Biochemical characterization of the isolated strains: The present results show that the isolated strains of Bacillus spp. Have ability to hydrolyse gelatin. It could degrade glucose, sucrose and maltose. No degradation of xylose, starch, mannitol and lactose was observed. These result similar to [21]. Who found that the new strain, which designated *B. thuringiensis* serovar *jegathesan,* has the ability to degrade glucose, starch and gelatin; no degradation of lactose, mannitol and xylose. And no production

of arginine di-hydrolase. These properties are similar to those reported for *B. thuringiensis* serovar. *israelensis* except that (Bti) produced arginine dihydrolase [7].

Molecular characterization and Identification of isolated strains: Characterization of Cry genes of local Bt isolates: The analysis of toxic genes cloned from different strains in the same subspecies *kurstaki* by [17] and [26] showed the existence of different toxin genes located on different plasmid species in a single subspecies strains. Also, [24] pointed out that the considerable DNA homology in the similarity among Cry1 and Cry1V genes. Similar to our result [24] identified the most common Cry1 (lepidopteran-active) and Cry111 (coleopteran-active) gene profile. They described a PCR screening method to determine which genes are present in a particular strain. Four general PCR primers, which amplify DNA fragments from the known Cry1 or CryIII genes, were selected from conserved regions. Once a strain was identified as an organism containing a particular type of Cry gene, it could be easily characterized by performing additional PCR with specific Cry1 and Cry111 primers selected from variable regions. In addition, the Cry gene content of the *B. thuringiensis* isolates from China exhibited a wide diversity. According to the PCR analysis, all the Cry1 gene subfamilies, as well as Cry2 and Cry9 genes were observed in our collection. Among them, the cry1-type genes were the most abundant in every region and source. This trend has also been found in other studies [4,5,14].

Electron Microscope: The present study showed that the crystal shape and size of the isolated strain different according to the strain; the isolate having two shapes of crystal (bipyramidal and cuboidal), these results are nearly similar to [20] he found that total of 189 isolates of Bt producing parasporal inclusions, isolated from soil of Japan, were examined for their oral toxicity against larvae of silk worm and the mosquito and adults of a chrysomlid Coleopteran. Of these isolates, 68 (33%) were toxic to insect tested: 48 were Lepidoptera toxic and 20 were mosquito toxic, unlike parasporal inclusions of known Lepidoptera toxic Bt strains. Parasporal inclusions produced by some of these isolates were spherical in shape. About 121 isolates (64%) produced parasporal inclusions with various morphologies including typically bipyramidal ones. Also these results are nearly similar to [27] demonstrated that identification of novel crystal protein genes toxic to lepidopteran insects, 52 *B. thuringiensis* isolates, which were isolated from Korean soil samples, were selected. All of 52 isolates produced bipyramidal-shaped parasporal inclusions with various sizes, and it was reported that these bipyramidal-shaped inclusions were produced by Cry1-type crystal proteins, which have toxicity against lepidopteran insects.

Conclusion

The present data demonstrated the novel isolated strain belonging to the genus *Bacillus thuringiensis* produced insecticidal crystal proteins, which are highly effective against Egyptian cotton leaf worm (*Spodoptera littollaris*), that is have biological economic importance, many of chemical compounds are used for agriculture treatment which major problem in the ecosystem Therefore, it is very important to isolate this strain in large scale to reduce the using of chemical substances in agriculture field.

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