

## Isolation, Characterization and Crystal Morphology Study of *Bacillus thuringiensis* Isolates from Soils of Punjab

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The bacterium *Bacillus thuringiensis* widely used as bio-pesticide. The specific toxic activity to insects and another microbe(s) regarding the existing of crystals that have different morphologies, sizes, numbers and compositions, as *Bt* strains the crystals have more different proteins, which contain encoded *Cry* distinct genes. In this work we isolated 140 *Bacillus thuringiensis* isolates from 68 soil samples from 19 local areas in Punjab. Colonies examination was done by microscope for toxin production. *Bacillus thuringiensis* isolates showed a typical colony morphology, predominantly off white to creamish with irregular margins. Based on certain biochemical characteristics they were confirmed to be strains of *Bacillus thuringiensis*. *Bt* strains harboring crystalline inclusions that might have potential for use as bioinsecticidal agent was done under phase contrast microscope and Scanning electron microscope. Based on preliminary screening with phase contrast microscopy, its parasporal crystals have unusual shapes. *Bt* isolates were classified into bi-pyramidal crystals, pyramidal, cuboidal, amorphous and round crystal protein inclusions. After preliminary screening with phase contrast microscopy, Scanning electron microscopy confirmed the surface view of rod shaped bacterial cells found in chains and cluster arrangement. *Bt* isolated tended to have sub-terminal spores.

**Keywords:** *bacillus thuringiensis*, crystal, phase contrast, scanning electron microscopy.

One of the few alternatives to chemical compounds for biological control is *Bacillus thuringiensis* (*Bt*). This facultative aerobic, Gram-positive, spore-forming saprophyte soil bacterium, has been successfully used as a biological insecticide over the last 6 decades and constitutes 95% of all commercial bio-insecticides, due to its high specificity, safety and effectiveness in the control of wide spectrum of human disease vectors and agriculture-pests (Schunemann *et al* 2014).

Bacterial spores are presently a subject of intense study, including cytological investigation, which makes it possible to establish connections between the biological and physicochemical properties of the spores and the features of their

specific surface structures. Spores are the best studied of the dormant forms (DF), which are used for the propagation and preservation of a species (Oestergaard *et al* 2007). Spore-formation enables *Bt* to survive in harsh environments resulting in a ubiquitous distribution. *Bt* has been isolated from soil, aquatic environments including sewage, dead insects and their breeding sites, herbivore faeces, stored grains, phylloplane and forest. *Bt* bacteria has a remarkable characteristic that can produce insecticidal crystal proteins killing insects during sporulation phase. During sporulation, *Bt* produces one or more parasporal insecticidal proteinaceous crystals (ICPs), comprised of one or more crystal (*Cry*) and cytolytic (*Cyt*) proteins, recognized as  $\delta$ -endotoxins (Palma *et al* 2014).

Colony morphology can help to distinguish *B. thuringiensis* colonies from other

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*Bacillus* species. The organism forms white, rough colonies, which spread out and can expand over the plate very quickly. *B. thuringiensis* strains have unswollen and ellipsoidal spores that lie in the subterminal position. The morphology, size, and number of parasporal inclusions may vary among *B. thuringiensis* strains. However, four distinct crystal morphologies are apparent: the typical bipyramidal crystal, related to Cry 1 proteins, cuboidal inclusions related to Cry 2 proteins and usually associated with bipyramidal crystals, amorphous and composite crystals related to Cry 4 and Cyt proteins and flat, square crystals, related to Cry 3 protein (Lopez-Meza and Ibarra, 1996). Spherical and irregular pointed crystal morphologies can also be observed in *B. thuringiensis* strains.

Observing parasporal crystal proteins during sporulation of *Bt* strain by SEM is the most direct and effective method to identify *Bt* bacteria. The principle behind scanning electron microscope (SEM) is focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens. The signals which arise from electron-sample interactions reveal information about the sample including external morphology (texture), chemical composition, and crystalline structure and orientation of materials making up the sample.

The present work deals with isolation, biochemical characterization and crystal morphological study of the spore. Spores of bacteria of the *Bacillus cereus*-*B. thuringiensis* group were chosen for the morphological analysis of their surface structures by a variety of electron microscopic techniques. *B. thuringiensis*, the species related to *B. cereus*, is the best studied entomopathogenic bacterium, which is used for production of efficient, environmentally safe preparations against insect pests. In some cases, insecticidal activity of *B. thuringiensis* results from combined action of spores and crystalline proteinaceous toxins. In this paper, we described the observing protocol for identifying the spores and crystals of *Bt* isolates by using scanning electron microscope in our laboratory in detail.

## MATERIALS AND METHODS

### Soil sample collection and *Bt* isolation

One hundred and forty soil samples were

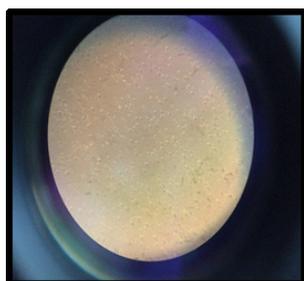
collected from different regions across the Punjab state. The soil surface was scraped with sterile spatula; about 10 g were taken from a depth of 2 to 5 cm, and stored in sterile cups. Samples were kept in the laboratory at 4°C until their processing. *Bt* isolation from soil samples was carried out by the selective sodium acetate heat pasteurization method as previously described (Martin and Travers 1989; Xavier *et al* 2007b). Briefly, 5 g of the sample was added to 10 ml of Luria Bertani (LB) broth containing 0.25 M sodium acetate. Presumably, sodium acetate selectively inhibits the germination of *B. thuringiensis* spores. Inoculated flasks were then incubated on a rotary shaker (200 rpm) for 4 h at 30°C. After incubation, 1 ml aliquot of thoroughly mixed culture broth was transferred to a pre-warmed 6 ml glass tube and heated at 80°C for 10 min, and then serially diluted aliquots were placed on LB agar and incubated overnight at 30°C. *Bt* colonies that were white, spread out and seems to fried egg on plate (Ammons *et al* 2005) were examined by phase contrast microscopy.

### Phase contrast microscopy

*Bt* colonies were suspended in sterile distilled water as a wet mount and examined with phase contrast microscope (100X oil immersion

**Table 1.** Sampling sites and crystal positive isolates

Sr. No.	Location	No. of soil samples withdrawn	Number of crystal forming isolates
1	Abohar	7	14
2	Bathinda	4	4
3	Kotkapura	3	6
4	Patiala	3	6
5	Nawanshehar	5	12
6	Gurdaspur	3	4
7	Ropar	3	2
8	Samrala	2	7
9	Faridkot	3	9
10	Ferozepur	3	8
11	Ludhiana	9	17
12	Muktsar	2	3
13	Khanna	4	8
14	Sangrur	3	9
15	Malout	2	6
16	Nabha	3	2
17	Barnala	3	2
18	Jalandhar	3	3
19	Hoshiarpur	3	4

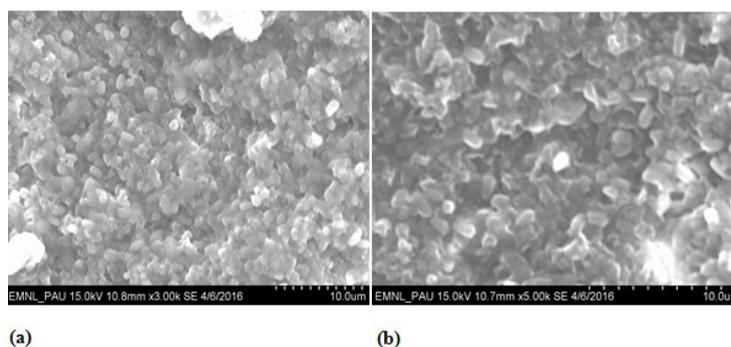


**Fig. 1.** Phase contrast microscopy of crystalline inclusions of *Bacillus thuringiensis*

objective) for the presence of parasporal crystals. *Bt* colonies were then classified into different groups based on their crystal shape (Lopez-Pazos *et al* 2009). Potential *Bt* candidates were subcultured and purified on nutrient agar plates and then stored as stock culture in sterile liquid Nutrient Broth containing 50% glycerol at “20°C (Hernandez *et al* 2005).

**Scanning electron microscopy**

In order to obtain the spore-crystal mixture, *Bt* isolates were grown in nutrient agar



**Fig. 2.** Scanning electron micrograph of the spore (S) and crystal proteins(C) from *Bacillus thuringiensis* isolate (a) parasporal bodies, (b) bipyramidal crystalline inclusions, (c) different crystalline inclusions – cuboidal, spherical, amorphous

**Table 2.** Crystal morphology of *B. thuringiensis* isolates from Punjab

Sr. no.	Type of crystals	Isolates	Total no of isolates
1	Bi-pyramidal	Bt-7, Bt-9, Bt-10, Bt-11, Bt-14,Bt-16, Bt-20, Bt-21, Bt-23, Bt-27, Bt-29, Bt-33, Bt-34, Bt-35, Bt-37, Bt-39, Bt-41, Bt-42, Bt-43, Bt-44, Bt-45, Bt-47, Bt-59, Bt-63, Bt-67, Bt-71, Bt-72, Bt-74, Bt-76, Bt-79, Bt-81, Bt-83, Bt-88, Bt-92, Bt-94, Bt-95, Bt-98, Bt-99, Bt-101, Bt-104, Bt-105, Bt-106, Bt-109, Bt-111, Bt-114, Bt-115, Bt-119, Bt-120, Bt-125, Bt-131, Bt-139, Bt-140	52
2	Pyramidal	Bt-3, Bt-5, Bt-15, Bt-19, Bt-24, Bt-31, Bt-36, Bt-38,Bt-48,Bt-50, Bt-51, Bt-52, Bt-53,Bt-54, Bt-66, Bt-70, Bt-77, Bt-78, Bt-80, Bt-84, Bt-87, Bt-91, Bt-93, Bt-110, Bt-118, Bt-121, Bt-122, Bt-123, Bt-129, Bt-135, Bt-138	31
3	Cuboidal	Bt-1, Bt-6, Bt-12, Bt-17, Bt-18, Bt-25, Bt-49, Bt-55, Bt-56, Bt-57, Bt-64, Bt-82, Bt-85, Bt-96, Bt-97, Bt-112, Bt-117, Bt-124, Bt-128, Bt-132, Bt-136	21
4	Amorphous	Bt-4, Bt-8, Bt-22, Bt-30, Bt-32, Bt-46, Bt-58, Bt-61, Bt-62, Bt-68, Bt-69, Bt-86, Bt-100, Bt-103, Bt-113, Bt-126, Bt-130, Bt-134,	18
5	Round	Bt-2, Bt-13, Bt-26, Bt-28, Bt-40, Bt-60, Bt-65, Bt-73, Bt-75, Bt-89, Bt-90, Bt-102, Bt-107, Bt-108, Bt-116, Bt-127, Bt-133, Bt-137,	18

medium for 5 days at 30°C, until lysis. The samples were immersed in 2.5% glutaraldehyde and kept at 4°C for 24 hrs, rinsed thrice with 0.1 M cacodylate buffer. Then, 1% osmium tetroxide was added and dehydrated by passage through graded aqueous ethyl alcohol series (30, 50, 70, 90 and 95%), placed in 100% ethanol at room temperature for few minutes. It was then dried with a critical point dryer unit mounted on aluminum stubs with silver glue and coated with gold-palladium using anion sputtering unit. The samples were then examined under SEM. Samples were examined and photographed with a FEI-Inspect S50, scanning electron microscope operating at a voltage of 1500 kV at 24,000× magnifications (Kati *et al* 2007), SEM unit at EMN lab, Punjab Agricultural University, Ludhiana.

## RESULT AND DISCUSSION

### Localities and collection of samples

A total of sixty eight (68) soil samples were collected from 19 different regions of Punjab (Table 1). The regions are representative of diverse agro climatic zones and geographical areas of the state. Using selective acetate method, 140 isolates were randomly picked up from agar plates seeded with soil samples obtained from nineteen different locations. This method eliminates most of the spore formers and non-spore formers in the soil samples. The spores of the unwanted bacterial species germinate on acetate buffered Luria broth while preventing the desired spores of *B. thuringiensis* from germinating. The unwanted bacteria that enter the vegetative stage are killed by controlled heat treatment. The inability of Bt species to germinate in the presence of acetate buffer allows the use of this trait to screen for this organism in the environmental samples.

### Morphological and Physiological Identification of the isolated *Bacilli*

Morphological observations with the crystal positive bacilli showed the vegetative cells as native, gram positive rods with dimensions of 1.1-1.3 × 2.5-4.5µm. All the strains tested along with the standard showed the typical colony morphology which was predominantly off-white to creamish in colour with irregular margins. A firm pellicle formation under stationary conditions was observed in these isolates which did not

disperse but sank to the bottom when the tube was gently tilted. This characteristic was observed to differentiate *B. thuringiensis* from other *Bacilli* by Angus (1954).

### Crystal protein morphology of *Bacillus thuringiensis*

After screening the task is to identify strains harboring crystalline inclusions that might have potential for use as bioinsecticidal agent. Cultures were analyzed and viewed under Phase-contrast microscope under 100X magnification to observe the presence of parasporal inclusions. Parasporal cells with shining crystals confirmed the presence of *Bacillus thuringiensis* (Fig.1). Some biotypes of *Bt* have unique crystal shapes which can be observed under phase contrast microscope (Table 3). Although crystal number varied from isolate to isolate, all showed the presence of bipyramidal, cuboidal, round coleopteran specific crystals. These isolates were confirmed as *B. thuringiensis* on Hi-chrome Bacillus agar medium.

### Scanning electron microscopy of crystalline inclusions of *Bacillus thuringiensis* isolate

After preliminary screening with phase contrast microscopy, Scanning electron microscopy confirmed the surface view of rod shaped bacterial cells found in chains and cluster arrangement. *Bt* isolates tended to have sub-terminal spores. Based on the diversity of crystal morphology, *Bt* isolates showed crystal architectures of irregular, bipyramidal, spherical, marginal and rhomboidal (Fig 2). This is similar to a study of El-kersh *et al* 2012 which showed Bt isolates with spherical crystals, while, irregular, bipyramidal, and attached crystal to the spores. The differences in the crystal protein morphology distribution might be due to genetic variation caused by the difference in the environmental conditions or to habitat effects (Al-Momani *et al* 2004).

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