Mysore J. Agric. Sci., 59 (1) : 172-186 (2025)

Characterization of *Bacillus thuringiensis* Isolated from Central and Southern Dry Zones of Karnataka

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Received : October 2024
Accepted : November 2024

Abstract

The bacterium *Bacillus thuringiensis* (*Bt*) is widely used as a bio-pesticide against insects. The specific toxicity of a *Bt* against insects is due to crystal proteins that comes with different morphologies, sizes, numbers and compositions. These crystal proteins are broadly known as cry proteins encoded by distinct *cry* genes. These Cry proteins and their respective genes shows wider variations which makes it an interesting area of research. In the present study, twenty-five *Bt* isolates were obtained from soil samples collected from South Karnataka, India. These isolates were subjected to morphological and biochemical characterization. The colony morphology of these isolates were found to be white to creamish colour with irregular margins. Further, biochemical tests such as starch hydrolysis, proteolytic activity, catalase activity, malonate test, mannitol test, citrate utilization and Voges Proskauer (VP) activity confirmed their close relation with *Bt*. These isolates were harbouring crystalline inclusions which are potent bioinsecticidal entity were subjected to phase contrast and table top microscopy imaging. A cursory examination with phase contrast microscopy revealed that the parasporal crystals were with varying shapes. The *Bt* isolates were then classified based on their crystal morphologies like bi-pyramidal, pyramidal, cuboidal, amorphous and round crystal proteins. Further, table top microscopy imaging confirmed the presence of clustered bacilli possessing central spores which confirmed that these isolates were indeed *Bt*. The present study can thus be used as a guide for preliminary characterization of *Bt*.

Keywords : *Bacillus thuringiensis*, Biocontrol, Crystals, Table top microscopy imaging

Biological control is the most valuable tool of Integrated Pest Management (IPM) which involves the use of bioagents such as parasitoids, predators and entomopathogens belonging to fungi, bacteria, viruses and nematodes (Singh and Jagadish, 2018). *Bt* is the most commonly used, safe and effective entomopathogenic bacterium (Sarwar, 2015). Target insects do not develop resistance to *Bt* toxins as quickly as they respond to chemical pesticides, thereby increasing their efficacy (Jurat-Fuentes and Jackson, 2012).

Bacillus thuringiensis (*Bt*) is a Gram-positive, rod-shaped, facultative, aerobic, spore-forming saprophytic soil bacterium that produces a crystal protein called δ-endotoxin during the stationary phase of its growth. The most well-known types of toxic proteins produced by *Bt* are Crystal (Cry), Cytotoxic (Cyt), Vegetative Insecticidal Protein (Vip) and Secreted Insecticidal Protein (Sip). The mode of action of these proteins is by binding to specific receptors present in the midgut of coleopteran pests. The efficacy of *Bt* toxin proteins against insects lies in their high specificity. The primary insecticidal activity of *Bt* is mostly brought by the presence of Cry proteins, also known as endotoxins, which are found inside polyhedral polysporal structures (Dominguez *et al*., 2020). Other insecticidal

compounds include proteases, heat-sensitive and heat-tolerant exotoxins, phospholipases, lectinases, Vips and chitinase (Gupta *et al.,* 2021). During sporulation, *Bt* produces parasporal insecticidal proteinaceous crystals (ICPs), comprised of one or more Crystal (Cry) and Cytolytic (Cyt) proteins, recognized as-endotoxins. Hence, *Bt* is considered as an important tool for insect pest management. Many insect species from the orders Coleoptera, Lepidoptera and Diptera, as well as a few Hemiptera are sensitive to certain crystal proteins (Khorramnejad *et al*., 2020).

Bt has been found in a wide range of environments, including compost, soil, plant surfaces (Phylloplane), insect-rich habitats like grain storage areas, stored products, insect cadavers, herbivorous vertebrate feaces and aquatic ecosystems. Due to its ability to thrive in such varied habitats, it is often described as ubiquitous and globally distributed (Garcia *et al.*, 2021). Its capacity to form spores allows *Bt* to endure harsh environmental conditions, further contributing to its widespread presence.

Bt is a aerobic saprophytic bacterium of ubiquitous nature. Because of its high specificity, safety, quick degradation from soil and consistent pest control, it is the most widely accepted commercial biopesticide across the globe (Manjunatha *et al*., 2023). *Bt* is found in a wide variety of environments, including soil, compost, plant surfaces, insect-rich habitats and aquatic ecosystems. Its colony morphology helps differentiate it from other *Bacillus* species, with the organism typically forming white, rough colonies that spread rapidly across agar plates. *B. thuringiensis* is also biochemically positive in tests such as starch hydrolysis, casein hydrolysis, proteolytic activity, malonate, mannitol, citrate utilization, Voges-Proskauer and catalase tests (Sharma and Iyer, 2018). The bacterium is well-known for its ability to produce insecticidal crystal proteins (Cry toxins) during sporulation, which are lethal to insects. *Bt* strains can synthesize crystal and cytolytic toxins called Cry and Cyt toxins widely called as δ-endotoxins. Upon ingestion by insect pests, the activation of these toxins takes place within the midgut, facilitated by midgut proteases. Once activated, the toxins bind to specific receptors on the midgut epithelial cells, ultimately disrupting cell function and causing cell death (Manjunatha *et al.*, 2023).

Bt strains have unswollen and ellipsoidal spores that lie in the subterminal position. The morphology, size and number of parasporal inclusions may vary among different *Bt* strains. The diversity of Cry toxins is significant, with different strains of *Bt* producing various types of parasporal inclusions. Typically four distinct types of crystal protein morphologies have been reported: bipyramidal crystals associated with Cry1 proteins, cuboidal inclusions with Cry2 proteins, amorphous or composite crystals related to Cry4 and Cyt proteins and flat, square crystals related to Cry3 proteins (Loutfi *et al.*, 2020). In addition, spherical and irregularly pointed crystals have been noted in some strains (Mukhija and Khanna, 2018). To explore the presence and diversity of these toxins, observing parasporal crystal proteins during the sporulation phase of *B. thuringiensis* under tabletop microscopy is considered one of the most direct and effective method for morphological identification (Jin *et al*., 2023). This method allows researchers to visualize the distinct crystal shapes produced by different *B. thuringiensis* strains, offering insights into the diversity and potential applications of its toxins.

All reported *Bt* insecticidal proteins do not have any effects on animal, humans, plants and other beneficial bacteria, which can be explained by the *Bt* mode of action (Liu *et al*., 2021). In fact, there are many synthetic pesticides available in the market effective against agricultural pests. However, there are associated risks with the application of these formulations on the environment, human health and non-targets species (Carvalho, 2017). The residues of the compounds can be detected in the food chain and their adverse effects are reported on the human health. Moreover, there are cases of developing resistance in the pests against these compounds due to their persistent and uncontrolled application. The most important concern with the application of these compounds is their ecological effects, by killing non-targets species. In keeping all these limitations, it is always needed to explore safe biological resources

such as *Bt* strains for their potential to control the important pests of agricultural crops with their minimal effect on the environment (Prabhu and Narasimha, 2017). It is a fact that the biological control approaches, particularly based on *Bt* crystals can't be as effective as synthetic pesticides, however several limitations of pesticides could be overcome by the bacterial biopesticides.

The present work emphasizes on isolation, biochemical characterization of *Bt* isolates and crystal morphological study of the spores by microscopic techniques. Also, the study emphasizes on observation protocol for identifying the spores and crystals of *Bt* isolates by using table top microscopy imaging. The study serves as preliminary screening guide for differentiating *Bt* isolates and their spores based on morphology and biochemical tests.

Material and Methods

Soil Sample Collection and Isolation of *Bacillus thuringiensis*

Fifteen soil samples were collected from different regions across South Karnataka (Fig. 1). Ten gram soil was taken from a depth of five cm and stored in sterile cups. The samples were kept in the laboratory at 4°C until further processing. *Bt* isolation from soil samples was carried out by the selective sodium acetate heat pasteurization method as described (Plate 1) (Martin and Travers, 1989). Five gram sample was added to 10 mL Luria Bertani (LB) broth containing 0.25 M sodium acetate. 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, serially diluted on LB agar and incubated overnight at 30°C. *Bt* colonies that were white and seemed like fried egg were examined by phase contrast microscopy (Olympus, Model : CX21iLEDFS1, India) (Plate 2) (Ammons *et al*., 2005).

Morphological Observations of *Bt* Colonies

Morphological characters of the colonies and bacteria were studied under 100X magnification of compound microscope following the standard microbiological methods (Hussey and Zayaitz, 2007).

Morphology of Cells, Spores and Crystals and Motility

Morphology of vegetative cells, spores and crystals were observed using a phase contrast microscope (Olympus, India) under a 100X objective. For observing vegetative cells and motility, less than 18 hours old culture was used, spores were observed in cultures that were older than 5 days.

Staining Characters of Vegetative Cells, Spores and Crystals

Staining characters of the organism were studied for vegetative, reproductive and crystal structure determination. For spore and crystal staining, a small amount of greater than 5 days old bacterial suspensions was smeared on oil free clean slides. The slide was air dried and heat fixed over a flame.

Gram's Staining

A thin smear of fresh bacterial culture was prepared in a clean grease-free slide and heat-fixed. The smear was flooded with crystal violet for one minute and washed with distilled water. The slide was flooded with Gram's iodine for 45 seconds and washed with distilled water. Then it was flooded with 95 per cent ethyl alcohol to decolorize for 10 seconds. The smear was flooded with safranin (counter stain) for one minute and washed with distilled water. Excess water was removed by using blotting paper, air dried and observed under the microscope (Olympus BX41) at 100X using oil immersion (Hussey and Zayaitz, 2007).

Spore Staining

A thin smear of culture was prepared on a clean glass slide. The smear was flooded with malachite green and steamed over a boiling water bath for five

minutes keeping the smear moist by adding dye. The slide was washed with distilled water and counterstained with safranin for 30 seconds. It was washed with tap water air dried and observed under a microscope (Olympus BX41) at 100X using oil immersion (Hussey and Zayaitz, 2007).

Crystal Staining

A thin film of five days culture was taken on a clean grease-free glass slide. The smear was air dried and heat fixed at 110°C for 10 minutes. The smear was stained with 0.25 per cent coomassie brilliant blue for 3 minutes washed with distilled water and observed under a microscope (olympus BX41) at 100X using oil immersion and crystals were stained blue (Hussey and Zayaitz, 2007).

Biochemical Characteristics of *Bt* Isolates

The biochemical tests were carried out according to Aneja, 2007. The *Bt* strains were subjected to crystal staining for presence of parasporal proteinaceous crystals. Further the citrate utilization test resulted in a colour change from green to blue due to alkali production by bacterial growth indicating a positive result for citrate utilization. The motility test was done to confirm that bacteria from young cultures were motile. The VP test resulted in a cherry red ring, confirming glucose fermentation. The starch hydrolysis test showed a clear zone after iodine addition. Casein hydrolysis was evident from the formation of a clear zone around bacterial growth on a milk-supplemented nutrient agar. Catalase activity was confirmed by the presence of effervescence upon adding hydrogen peroxide to nutrient agar slants inoculated with the bacteria. The malonate test showed abundant growth and a colour change to blue, indicated positive utilization of malonate. Finally, carbohydrate fermentation tests with bromocresol purple nutrient agar containing 10 per cent mannitol turned the medium yellow, indicating acid production from carbohydrate utilization (Cappuccino and Sherman, 1992). Endospore forming Gram positive bacteria that were positive for the biochemical tests were expected to be *Bt*.

Table Top Microscopy Imaging based Observations of *Bt*

In order to obtain the spore-crystal mixture, *Bt* isolates were grown in nutrient agar medium for 5 days at 30°C, until lysis. The samples were immersed in 2.5 per cent glutaraldehyde and kept at 4°C for 24 hrs, rinsed thrice with 0.1 M calcium carbonate buffer. Then, 1 per cent osmium tetraoxide was added and dehydrated by passage through graded aqueous ethyl alcohol series (30, 50, 70, 90 and 95%), placed in 100 per cent ethanol at room temperature for few minutes. It was then dried with a critical point dryer unit mounted on aluminium stubs with silver glue and coated with gold-palladium using anion sputtering unit. The samples were examined and photographed with a table top microscopy imaging unit (Hitachi, Model: TM4000 PlusIII, India) operating at a voltage of 15.0 kV and 3000X magnification.

Results and Discussion

Population of *Bt* Isolates in Soil Samples

Bacillus strains were isolated from soil samples collected from different parts of South Karnataka (Table 1 & Fig. 1). A total of fifteen soil samples were used to isolate *Bacillus*. The population of *Bacillus* was enumerated by following selective isolation procedure adapted by Travers *et al*. (1987). It was found that, the population of the *Bacillus* isolates varied considerably among places of soil samples studied. The isolates were then picked and observed on the plates by considering the variability in morphology. As a result the number of colonies picked indicated the extent of variability in the *Bacillus* isolates that could be observed in the soil samples.

Colony morphology of *Bt* on nutrient agar plate was white, mucoid, slightly raised centre, flat with wavy/ edged margin and fried egg appearance (Plate 2a). All the strains tested showed the typical colony morphology which was predominantly off-white to creamish in colour with irregular margins (Hassan *et al*., 2021). A firm pellicle formation under stationary

TABLE 1

Collection of soil samples for isolation of *Bt* isolates

Cropping system	Source	Place	Latitude	Longitude
Sugarcane	Soil	Pandavapura	12.4929° N	76.6643° E
Sugarcane	Soil	Mandya	12.5218° N	76.8951° E
Groundnut	Soil	Hiriyur	13.9452° N	76.6140° E
Groundnut	Soil	Challakere	14.3134° N	76.6528° E
Sugarcane	Soil	Maddur	12.5867° N	77.0453° E
Sugarcane	Soil	Mandya	12.5218° N	76.8951° E
Groundnut	Soil	Madhugiri	13.6643° N	77.2089° E
Groundnut	Soil	Pavagada	14.1031° N	77.2807° E
Sugarcane	Soil	Maddur	12.5867° N	77.0453° E
Sugarcane	Soil	Pandavapura	12.4929° N	76.6643° E

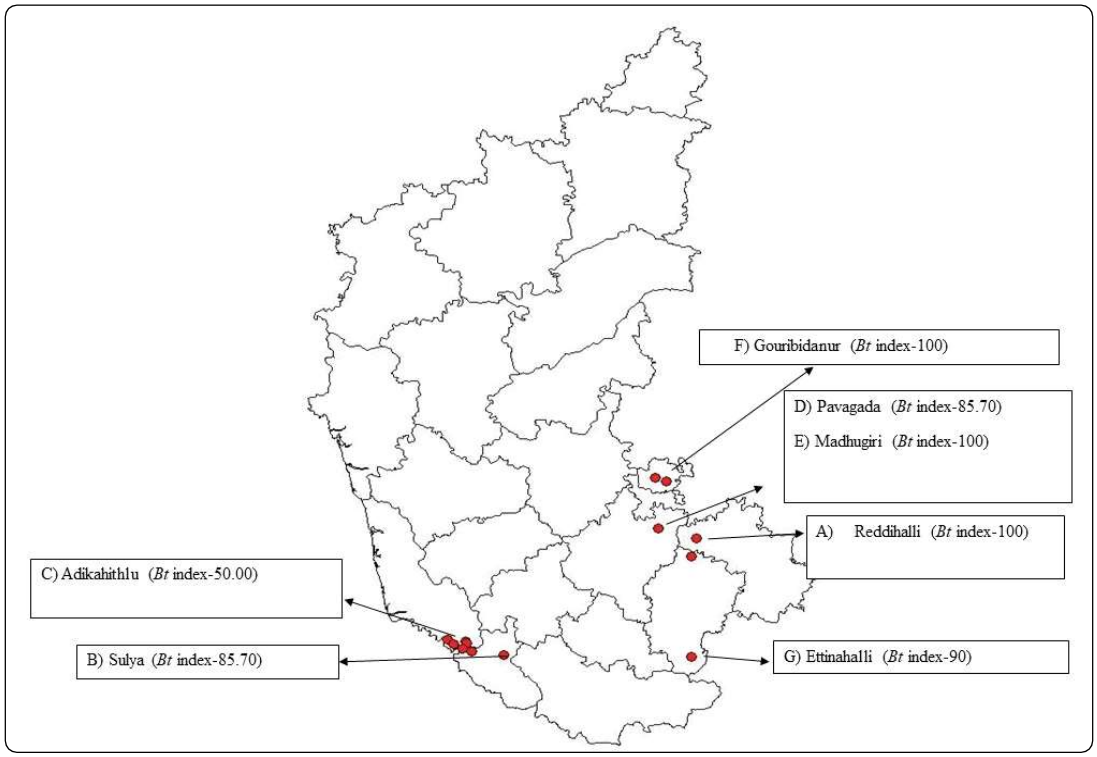


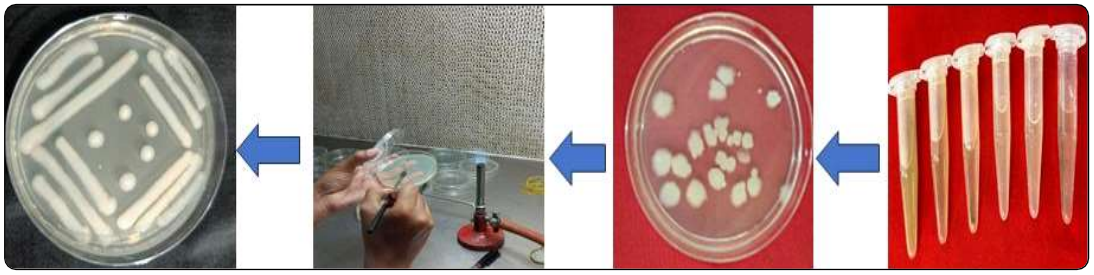
Fig. 1 : Locations of soil samples collected across Central and Southern dry zones of Karnataka for isolation of *Bt* with their respective *Bt* index mentioned in the bracket enclosed

(**Bt* index = The ratio between *Bt* colonies and total number of colonies found on LB agar plates)

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 *Bt* from other *Bacilli* by Sharma and Dangar (2016). Further the colonial morphology of all the native *Bt* isolates were highly in agreement with the colony characteristics of *Bt* described by other authors (Kavitha *et al*., 2011) (Table 3).

Bt shows great amount of genetic diversity in toxicity with different toxic potential mostly due to plasmid exchange between strains (Fayad *et al*., 2021). Each

a. Soil sample | b. Soil suspension in LB borth | c. Incubation for 4hrs at 98 rpm | d. Heat treatment at 80°C for 10 min.



h. Pure culture of *Bt* | g. Aseptic transfer onto LB agar plate | f. Spread plating on LB agar | e. Serial dilution of soil samples

Plate 1 : Isolation of entomopathogenic bacterium *Bt* from soil [A-H]

habitat may contain a novel *Bt* strain awaiting discovery which is toxic to a target insect group. Therefore, *Bt* strains have been collected from different environments and characterized to evaluate their toxic potential against various insect orders (Khorramnejad *et al*., 2020). Various techniques have been used to isolate *Bt* and other entomopathogenic spore-formers. The use of a pasteurization technique is not as selective as acetate pasteurization or an antibiotic isolation method (Martin and Travers, 1989). The acetate and antibiotic methods have mainly been used to isolate *Bt*. Selective isolation methods assist in identification by narrowing down the species of bacteria isolated (Martin and Travers, 1989). Travers *et al*. (1987) developed a chemically defined medium to increase the frequency of isolation of *Bt* from soil that was used in the present study. The above authors observed that *Bt* spores did not germinate in the presence of 0.25 M sodium acetate in the medium and the authors could detect even one spore of *Bt* in a background of 10^9 bacteria/gram of soil. In this method, germination of *Bt* spores is selectively inhibited by sodium acetate, while most of the undesired spore formers germinated.

The vegetative form of the spore formers and the non-spore forming bacteria are eliminated by heat treatment of 80°C for three minutes. Nevertheless, Travers *et al.* (1987) observed that in addition to *Bt*, other closely related *Bacillus* species like *B. cereus*, *B. anthracis*, *B. chitinoporus*, *B. sphaericus* and *B. globisporus* also get co-selected in the above method of isolation. Anticipating this possibility of very closely allied *Bacillus* species to also get mixed up with the *Bt*, it was necessary that the isolates picked be verified again for their identity. Keeping this in mind, a series of studies were carried out to characterize the bacteria picked from among the CFUs observed from each soil sample to pin point *Bt* isolates. The morphological traits (Table 2 & Table 3) of the colonies were recorded after growing the isolated bacteria on the specific growing medium.

A total of twenty soil samples were collected from seven different regions of Karnataka (Fig. 1).

TABLE 2

Morphological traits selected for the colony characterization of *Bt* isolates

Colony morphology	Colony characteristics
Colour	Chalky white/fried egg
Texture	Smooth/rough
Shape	Circular/oval/irregular ellipsoid
Margin	Smooth/perforated

The regions are representative of diverse agro climatic zones and geographical areas of the state. Preliminarily the unwanted non spore forming bacteria that entered vegetative stage were killed by controlled heat treatment, further using selective sodium acetate method twenty five isolates were randomly picked up from agar plates seeded with soil samples obtained from seven different locations. This method eliminated most of the non spore formers from the soil.

TABLE 3

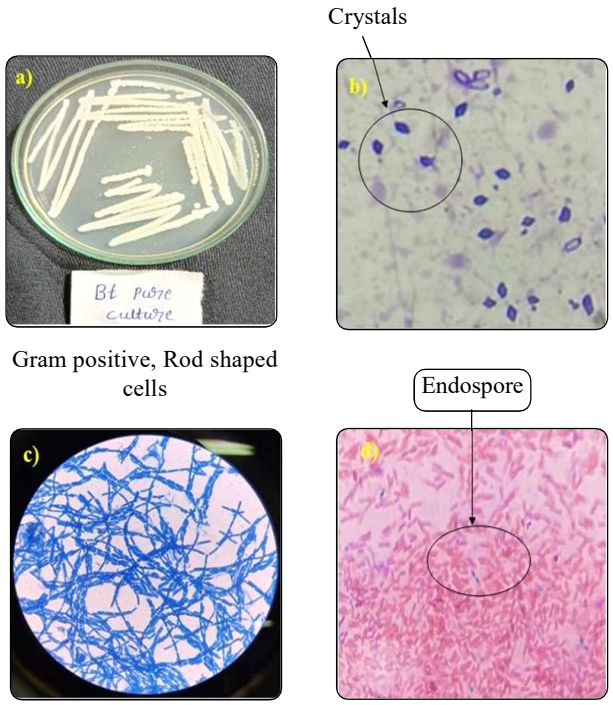
Morphological and biochemical characteristics of *Bt* isolates from different soil samples

Isolate Code	Colony configuration	Margin	Elevation	Colour	Shape	Gram stain	Endospore stain	Starch hydrolysis	Casein hydrolysis	Catalase test
UASBt1	Circular	Entire	Flat	White	Rods	+	+	+	+	+
UASBt2	Irregular	Undulate	Flat	White	Rods	+	+	+	+	+
UASBt3	Circular	Entire	Flat	White	Rods	+	+	+	+	+
UASBt4	Circular	Entire	Flat	White	Rods	+	+	+	+	+
UASBt5	Oval	Entire	Flat	White	Rods	+	+	+	+	+
UASBt6	Circular	Entire	Flat	White	Rods	+	+	+	+	+
UASBt7	Circular	Entire	Flat	White	Rods	+	+	+	+	+
UASBt8	Circular	Serrate	Flat	Cream	Rods	+	+	+	+	+
UASBt9	Circular	Entire	Flat	White	Rods	+	+	+	+	+
UASBt10	Circular	Entire	Flat	White	Rods	+	+	+	+	+
UASBt11	Circular	Entire	Flat	White	Rods	+	+	+	+	+
UASBt12	Circular	Serrate	Flat	White	Rods	+	+	+	+	+
UASBt13	Circular	Serrate	Flat	White	Rods	+	+	+	+	+
UASBt14	Irregular	Entire	Flat	White	Rods	+	+	+	+	+
UASBt15	Circular	Entire	Flat	White	Rods	+	+	+	+	+
UASBt16	Circular	Entire	Flat	White	Rods	+	+	+	+	+
UASBt17	Circular	Entire	Convex	White	Rods	+	+	+	+	+
UASBt18	Irregular	Entire	Flat	White	Rods	+	+	+	+	+
UASBt19	Circular	Entire	Flat	White	Rods	+	+	+	+	+
UASBt20	Circular	Entire	Flat	White	Rods	+	+	+	+	+
UASBt21	Circular	Serrate	Flat	White	Rods	+	+	+	+	+
UASBt22	Circular	Entire	Convex	White	Rods	+	+	+	+	+
UASBt23	Circular	Entire	Flat	White	Rods	+	+	+	+	+
UASBt24	Circular	Entire	Flat	White	Rods	+	+	+	+	+
UASBt25	Circular	Entire	Flat	White	Rods	+	+	+	+	+

Note : '+'-positive

Morphological Identification of the Isolated *Bt*

All the *Bt* isolates were characterized using several morphological and biochemical tests. The cells in all the isolates appeared purple to violet at the end of Gram staining and isolates were endospore positive when stained with malachite green. The exact shapes of the crystals were not clear under light microscopy. All the *Bt* isolates obtained from different regions had almost similar colony morphology, the kinds of colony morphology that have been described by many workers (Travers *et al*., 1987). Majority of the colonies were found to be white circular with entire margin and were flat elevated (Table 3). Observations on the cells stained with crystal violet, malachite green and safranin all *Bt* isolates showed that all the vegetative cells were rod shaped (Plate 2c). Observations made at the sporulation stage revealed that spores were greenish, while the vegetative cells were reddish after staining (Plate 2d). During the lysis phase the crystals stained deeply with crystal violet and appeared bluish and the spores were greenish. A set of biochemical tests was developed for the rapid identification of different biochemical types of *Bt* isolates. This system is based on the biochemical tests that have been published for known varieties for which the serotypes have been identified and have been used for *Bt* classification in many investigations (Hassan *et al*., 2021). The same biochemical tests were also used in the present study. Starch hydrolysis, casein hydrolysis and catalase tests were conducted to know the reaction



a) *Bt* pure culture b) Crystal proteins c) Rod shaped cells d) Microscopic view of *Bt* endospore at 100X

Plate 2 : Phase contrast microscopy images showing crystalline inclusions of *Bt* isolates

TABLE 4

Biochemical characteristics of *Bt* isolates from different soil samples

Isolate Code	Biochemical tests				
	Malonate	VP	Citrate utilization	Catalase	Mannitol
UASBt1	+	+	+	+	+
UASBt2	+	+	+	+	+
UASBt3	+	+	+	+	+
UASBt4	+	+	+	+	+
UASBt5	+	+	+	+	+
UASBt6	+	+	+	+	+
UASBt7	+	+	+	+	+
UASBt8	+	+	+	+	+
UASBt9	+	+	+	+	+
UASBt10	+	+	+	+	+
UASBt11	+	+	+	+	+
UASBt12	+	+	+	+	+
UASBt13	+	+	+	+	+
UASBt14	+	+	+	+	+
UASBt15	+	+	+	+	+
UASBt16	+	+	+	+	+
UASBt17	+	+	+	+	+
UASBt18	+	+	+	+	+
UASBt19	+	+	+	+	+
UASBt20	+	+	+	+	+
UASBt21	+	+	+	+	+
UASBt22	+	+	+	+	+
UASBt23	+	+	+	+	+
UASBt24	+	+	+	+	+
UASBt25	+	+	+	+	+

Note : '+' - positive

of the isolates. *Bt* is positive to all the tests (Plate 3, Table 4).

Crystal Protein Morphology of *Bt* Isolates using Microscopy

The presence of parasporal crystals that are adjacent to the spore in the mother cell is the best criteria to distinguish *Bt* from other closely related *Bacillus* species. The morphology, size and number of parasporal inclusions may vary among *Bt* strains. There is a relationship between toxic activity and crystal shape of *Bt* strains and therefore the observation of crystal morphology by phase contrast microscopy can provide important clues. For instance, among twenty two isolates collected from marine sediments of Japan, two isolates of *Bt* subsp. kurstaki, which are toxic to lepidopteran larvae, formed typically bipyramidal inclusions, whereas spherical crystals were formed by isolate higo, which is toxic to mosquitoes (Maeda *et al*., 2000). The toxin shape is due to disulphide bonds, hydrophobicity and hydrogen bonds.

Phase contrast microscope was used to examine the presence of parasporal crystals. *Bt* colonies were then classified into different groups based on their crystal shape (Lopez-Pazos *et al*., 2009). Parasporal cells with shining crystals confirmed the presence of *Bt* (Plate 2b). Although crystal number varied between isolates, all of them exhibited the presence of bipyramidal, cuboidal, round coleopteran specific crystals.

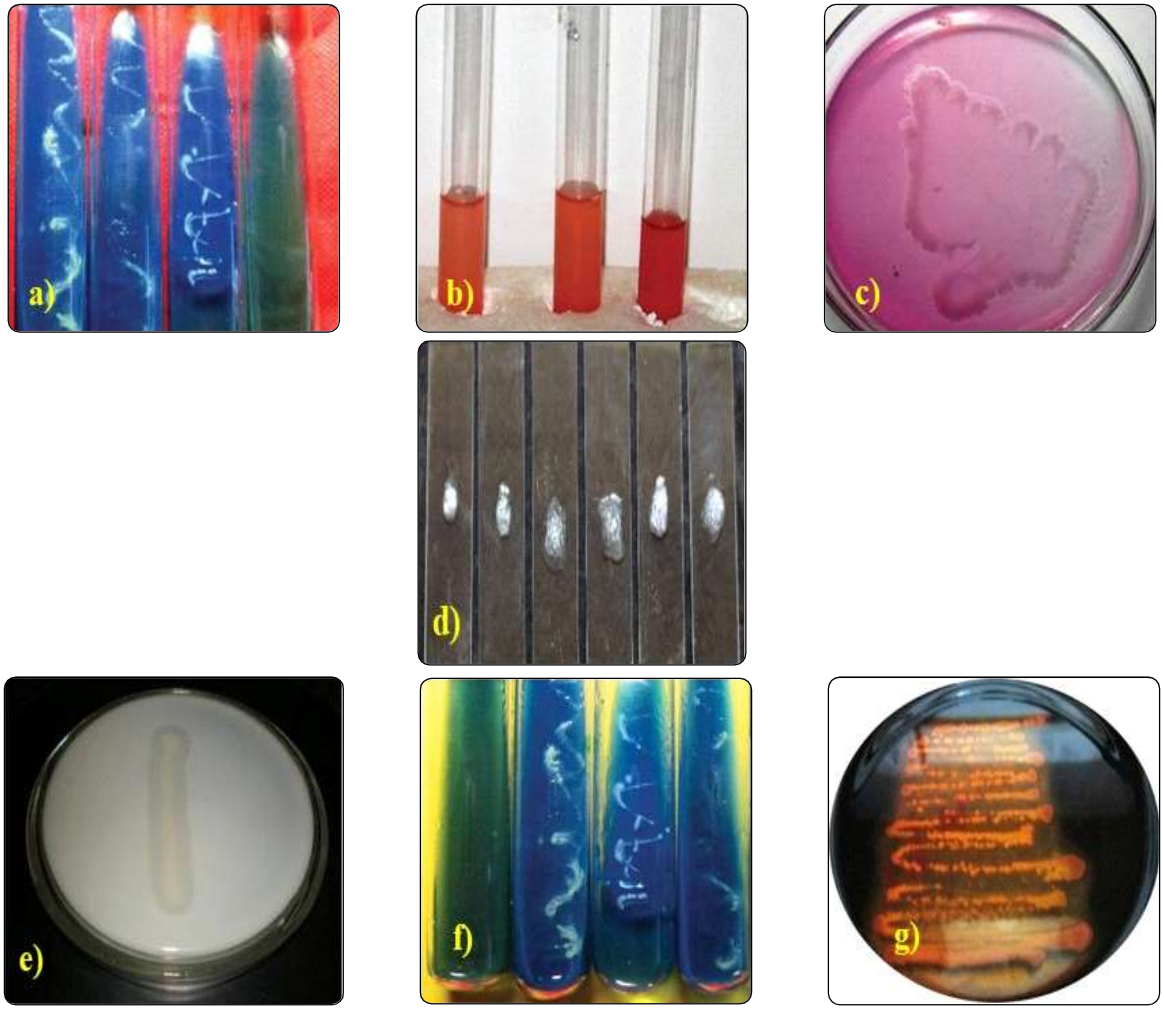
Biochemical Tests for the *Bt* Isolates

The most potential *Bacillus* isolates were selected for additional biochemical characterization using five biochemical tests (Table 4). *Bt* serovars were also characterized based on several biochemical characteristics as previously reported by several studies (Garcia *et al*., 2021). Few of these biochemical characters were used in the present study to characterize the selected *Bacillus* isolates. Those biochemical characters were grouped into three categories. The first consisted of characters positive for all *Bt* serovars. Whereas the second grouped together those characters that are generally negative for *Bt.* Since the target of the present study was not serotyping, these characters were considered for preliminary identification of *Bt*. The third category grouped together characters which are taxonomically useful because they act as discriminant factors between serovars. The main factors in the group were: the presence of utilization of citrate and the ability to ferment sucrose, mannose. Also included in this group is the production of acetyl-methyl-carbinol (VP test) that is negative only in a few cases.

All the isolates tested were positive for malonate, mannitol and citrate production (Plate 3). This was also the feature observed by Park *et al*. (2022), where discriminant characters for the serovar kurstaki appear to be homogeneous. The present study considered these biochemical keys as the preliminary characteristics to see the presence of variability in the different *Bacillus* isolates obtained from the soils of Karnataka. Further confirmation of the identity of the bacteria was extensively carried out using table top microscopy imaging.

Although there are several reports that all soils may or may not harbor *Bt* (Hofte and Whiteley, 1989) reasonable *Bt* index and significant *Bt* population reveal that the soil collected from botanical garden, Ooty was rich in *Bt* population. Furthermore, there is no record of field use of *Bt* at the site *i.e*., in and around the Ooty botanical garden (Sharma and Dangar, 2016). In the present study amongst the soil sample the highest *Bt* index were found to be in soils collected from Reddihalli, Madhugiri and Gouribidanur (*Bt* index:100). *Bt* index were found to be 90.00 in soil samples obtained from Etthinahalli arecanut field followed by Sulya and Pavagada soils (*Bt* index: 85.70), however soil from Adikahithlu was found to contain the least *Bt* index (50.00) (Table 5).

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. Another parameter *Bt* index was calculated (Table 5) for each positive sample as the number of *Bt* isolates per total number of isolates of sporulated bacilli (Thaphan *et al*., 2008). The *Bt* index from Krabi



[a) Malonate test, b) Voges-Proskauer (VP) test, c) Mannitol test, d) Catalase test, e) Casein hydrolysis test, f) Citrate utilization test, g) Starch hydrolysis test]

Plate 3 : Biochemical characteristics of *Bt* isolates [a-g]

TABLE 5

***Bt* index of *Bt* isolates obtained from the soil samples collected from different parts of South Karnataka**

Location Code	Location	Population (CFU/gram)	No. of *Bacillus* colonies picked	No. of *Bt* isolates	*Bt* index
A	Reddihalli	$1.6x10^6$	9.0	9	100.00
B	Sulya	$1.2x10^7$	7.0	6	85.70
C	Adikahithlu	$8.0x10^6$	10	5	50.00
D	Pavagada	$1.8x10^6$	7.0	6	85.70
E	Madhugiri	$7.0x10^6$	6.0	6	100.00
F	Gowribidanuru	$2.0x10^6$	11	11	100.00
G	Etthinahalli Arecanut field	$1.2x10^7$	10	9	90.00

**Bt* index = The ratio between *Bt* colonies and total number of colonies found on LB agar plates

province ranged from 0.009 to 0.380 in the soil samples studied. In various soils values ranged from 0 to 0.2 in United States, 0.2 to 0.5 in New Zealand and 0.75 in Bangladesh (Hossain *et al*., 1997). Vilas-Boas and Manoel (2004) suggested the *Bt* index may be a consequence of biotic environmental factor, *e.g*., microorganism in the soil, the type of insect commonly found in the area or the vegetal top besides, abiotic factors such as the pH, texture, oxygen and nutrient availability, temperature and humidity. It also indicates richness of soil as far as *Bt* is concerned. This index revealed the prevalence of *Bt* in various environments, providing valuable insight into its potential as a bioinsecticidal agent (Priyanka *et al.*, 2022). A higher *Bt* index indicates a greater concentration of *B*t in the sample, which may suggest enhanced insecticidal activity in that environment.

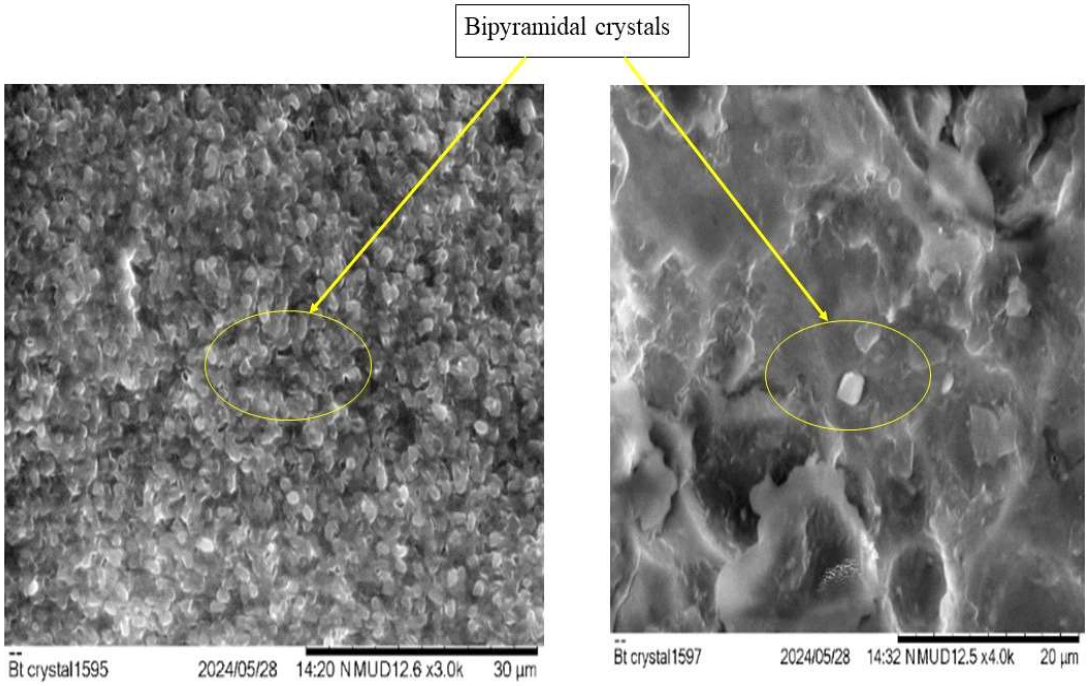
Table Top Microscopy Imaging of Crystalline Inclusions of *Bt* Isolates

Bt produces parasporal crystals proteins that play a major role in their insecticidal activity. These parasporal crystals have different forms depending on the nature of their δ-endotoxins and corresponding genes (Wang *et al*., 2013). The level of crystals synthesized by the bacteria depends on the expression of encoding genes. In the present study, to characterize each strain in terms of their parasporal activity, the morphology of their crystals was studied using table top microscopy imaging (Plate 4) shows the images of crystal morphologies of *Bt* strains.

After preliminary screening with phase contrast microscopy, table top microscopy imaging confirmed the surface view of rod shaped bacterial cells found in chains and cluster arrangement. Based on the diversity of crystal morphology, *Bt* isolates showed crystal architectures of irregular, bipyramidal, spherical, marginal and rhomboidal (Plate 4a) which is similar to a study by Hassan *et al*. (2021). *Bt* strains produce parasporal crystal inclusions with different morphologies, sizes and numbers. Based on literature, some distinct crystal morphologies were apparent, *viz.*, bipyramidal crystals related to Cry 1 proteins, cuboidal inclusions associated to Cry 2 proteins, flat and square crystals associated with Cry 3 proteins and amorphous and composite crystals associated with Cry 4 proteins (Mane *et al.,* 2015).

In the present study *Bt* isolates revealed rectangular, cuboidal and bipyramidal type of crystals. The three-dimensional (3D) structure of 12 different Cry activated toxins has been described by Xu *et al.* (2014). These include Cry1Aa, Cry1Ac, Cry1Da, Cry1B and Cry1A with specificity against lepidopteran insects; Cry3Aa, Cry3Bb and Cry8Ea toxins active against coleopteran; Cry4Aa and Cry4Ba active against dipteran; Cry5Ba active against nematodes and Cry7Ca active against the orthopteran *Locusta migratoria manilensis* (Jing *et al*., 2019). Al Thani *et al*. (2022), six *Bt* strains from Qatar soil were used to explore their crystal morphologies and endotoxins profiles. Bipyramidal crystal producing strains exhibited the genes *vip*3Aa58, *cry*1Aa/Ac, *cry*2Ab, *cry*1Ba and *cry*1Ia, predicting toxicity against Lepidopteran, Dipteran and Hemipteran insects. The table top microscopy imaging showed bipyramidal, cuboidal and spherical crystals among the strains. Thus considering type of inclusions, isolates in the present study can be effective against various insect orders *viz*., bipyramidal crystals related to Cry 1 proteins are toxic to Lepidopteran insects, cuboidal inclusions associated to Cry 2 proteins are toxic to Dipteran insects, flat and square crystals associated with Cry 3 proteins are toxic to Coleopteran insect pests. However, such a hypothesis needs more conclusive evidence through more studies in future.

Noguera and Ibarra (2010) reported spherical, balloon shaped, bipyramidal, cuboidal and flat square shaped crystals from various *Bt* strains. Nair *et al*. (2019) also reported spherical, bipyramidal and cuboidal shape crystal structures harboured by various *Bt* isolates. Lone *et al*. (2017) characterized *Bt* strains from North-western Himalayas under table top microscopy imaging and observed the presence of spherical, bipyramidal, irregular and flat crystal shapes. Martin and Travers (1989) found that *Bt* isolates with bipyramidal and spherical crystals were more common and reported that the majority (44%) showed bipyramidal crystals while 27 per cent and 12 per cent were irregular and attached to the spores



(a) Table top microscopic images of the spores and crystal proteins (b) Magnified view of bipyramidal crystals

Plate 4 : Table top microscopy images of different types of crystal morphologies and the spores produced by *Bt* isolates

respectively. Similarly, Aramideh *et al*. (2010) found that the bipyramidal crystals were present in (58.3%) of isolates. However, it is interesting to notice that while most of the *Bt* strains produce free crystals in the mother cell compartment, it was observed that some *Bt* strains presented the crystal adhered to the spore. It is well known that the spore and crystal are liberated to the environment by the action of lytic enzymes that destroy the membrane (Guerrero, 2023). 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 (Alves *et al*., 2023). The sporulation phase of the *Bt* strains and the existence of cry genes can vary due to the heterogeneity in environmental conditions (Toukabri *et al*., 2023).

Bacillus thuringiensis is a ubiquitous soil bacterium, attempts are being made worldwide to isolate novel *Bt* strains from various sources for successful management of various insect pests. Keeping this in mind, the present investigation was attempted to isolate indigenous *Bt* strains from central and southern dry zones of Karnataka. Twenty five *Bt* strains were isolated from soil samples collected from central and southern dry zones of Karnataka. They were characterized morphologically and biochemically. Table top microscopy images revealed the presence of different types of crystal morphologies (spherical, irregular, cubic and bipyramidal crystals) amongst the isolates. From this study it was found that there was greater diversity in the morphology and type of crystal proteins produced by *Bt* strains. The increase in the number of *Bt* collections has led to an increase in the discovery of new *Bt* isolates with insecticidal activity against a diverse range of insect pests. This could provide a large genetic resource for the utilization of *Bt* as a microbial insecticide or for the incorporation of cry gene into other organisms for insect pest control and hence our study serves as a guide for preliminary characterization of *Bt*.

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