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Exploration of Natural Habitats of Vidarbha Region for the Presence of Native *Bacillus thuringiensis* Isolates

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ABSTRACT: The purpose of the current research was to isolate and characterize indigenous *Bacillus thuringiensis* isolates from the Vidarbha region's various habitats in order to ascertain whether *Bt* is widely distributed throughout the ecosystem. In total 80 sample of soil not having *Bt* history, phylloplane and insect cadaver was collected from Akola, Amravati, Bhandara, Gadhchiroli, Gondia, Nagpur and Yavatmal districts of Vidarbha region of Maharashtra state. Maximum amount of probable *Bt* isolates were obtained from soil samples (57), followed by phylloplane (6) and insect cadaver (5) with the help of Travers Sodium acetate selective isolation method. Similarities were observed in the colony morphology characters of indigenous *Bt* isolates and the that of standard strain *Bacillus thuringiensis* sub sp. *kurstaki* HD -1 (*Btk* HD-1). Biochemical characterization of these isolates with the help of IMViC test suggested that more than 50% of the total native isolates showed significant resemblance with standard strain. Microscopic observations with the help of different staining techniques indicated presence of endospores and crystals in 33 local isolates confirming these isolates as *Bt*. Further calculation of *Bt* isolation index suggested that soil consist of highest population of *Bt* followed by insect cadaver and phylloplane. This study thus gives an indication of the abundance of *Bt* in a natural ecosystem and promotes further research into other potential habitats for *Bt* bacteria.

Keywords: *Bacillus thuringiensis*, isolation, Gram staining, spore staining, IMViC, insect cadaver.

INTRODUCTION

Bacillus thuringiensis is naturally occurring, gram positive, facultative anaerobic, motile, endospore forming bacteria with rod shaped vegetative cells. This bacterium is available everywhere in the environment. It can be used as a biological control agent against many insect pests due to its entomopathogenic potential. Being omnipresent, it can be isolated from different natural habitats such as soil, water, plant surfaces dead insects, and insect cadaver (Yammamoto *et al.,* 2014; Padole *et al.,* 2017). It is a member of the morphological group of *Bacilli* named as *Bacillus cerus* group along with the other bacteria such as *Bacillus cereus, B. anthracis, B. mycoides and B. laterosporous*. *Bt* can be distinguished from other members of *the Bc* group by its defining feature which is ability to produce proteinaceous insecticidal crystal during the sporulation phase of its lifecycle. The bacteria occasionally lose their ability to form crystals and become indistinguishable from *B. cereus* itself. (Yammamoto and Powell 1993; Sanahuja *et al*., 2011).

Over the recent decades, the uncontrolled and imprudent use of chemical insecticides has produced a number of environmental risks, many of which are toxic to both humans and beneficial fauna. This has caused a variety of issues, such as chemical residues, the emergence of insect pests that are resistant to treatment, resurgence, and secondary pest outbreaks (Singh and Mandal 2013). There is need of looking for better environmentally friendly control methods as a result of these undesirable side effects.

The superior alternative to synthetically produced pesticides is the use of ecologically sound and target-specific pest management techniques such as use of microbial biopesticides (Majeed *et al.,* 2017). These microbes have the potential to reduce the use of dangerous chemical pesticides because they are specific to their target and are natural enemies of insects. Hence microbial biopesticides can be used against a wide variety of agricultural insect pests in many agroecosystems (Ruiu 2018). Among all the microbes used for the purpose of pest control, *Bacillus thuringiensis* (*Bt*) is most widely used and important entomopathogen as it produces insecticidal crystal (Cry) and cytolytic (Cyt) proteins named δ-endotoxins encoded by *cry* and *cy*t genes (Crickmore *et al.,* 2020) along with newly identified Vip protein (Yu *et al.,* 2010). Out of the total 10% of bio pesticides used globally, approximately 90% of the microbial insecticides are derived from *Bt* (Osman *et al.,* 2015). Continuously excessive application of *Bt* and use of a same *cry* gene for insect control can causes development of resistance in insect pests after few generations (Zago *et al.* 2014). Thus it is of much importance to search for the highly virulent and more effective indigenous *Bt* isolates from different unexplored natural habitat with the possibility to find with intended insecticidal genes.

MATERIAL AND METHODS

Collection of samples. For the purpose of this work, samples were collected from different natural habitat of *Bacillus thuringiensis,* such as soil, phylloplane *i.e.* surface of leaves and insect cadaver.

Collection of soil samples. Soil samples were collected by scraping off soil surface with sterile spatula and then 10 g sample 2-5 cm below the surface and stored in sterile aluminum foil bag at 4°C from areas with no previous *Bt* history, neither sown nor spread (Martin and Travers 1989).

Collection of leaf samples. Leaves of some important agronomical as well as horticultural crops from different locations of the university fields, of Dr. PDKV, Akola were collected. Three to five leaves from the lower middle and upper part of the canopy were collected and stored in sterile aluminum foil bags at 4 °C until further use (Asokan and Puttaswamy 2007).

Collection of insect cadaver. Regular field visits were made to the university fields, of Dr. PDKV, Akola as well as farmers' fields to check the presence of dead/diseased/ moribund larvae of insects; such insect cadavers were collected each in separate sterile micro centrifuge tube and stored at 4°C until further use (Padole *et al.*, 2017).

Isolation of *Bacillus thuringiensis* from the collected samples. Isolation of *Bt* from these sample collected from natural habitat was done by the sodium acetate selective isolation and heat shock treatment (Travers *et al.*, 1987).

Isolation from soil samples. For the purpose of isolation, one gram soilwas added to 10 mL of LB broth buffered with 0.25 M sodium acetate in falcon tubes. This mixture was shaken for 4 hours at 250 rpm at 30°C and heat shocked at 80 °C for three min. Serial dilutions (10^{-1} to 10^{-6}) were made in sterile saline solution. 100 µL of each dilution was spread on petri plate containing Luria agar and incubated at 30°C overnight. Chalky white colonies were picked up and plated on T_3 medium and incubated at 30°C for 72 hours. The colonies grown on T_3 medium were further purified using single colony isolation technique and maintained on Luria Agar at 4°C until further use (Asokan and Puttaswamy 2007).

Isolation from Phylloplane. To remove the superficially adhering micro flora, the 3-5 gram of leaves were dipped in sterile distilled water and then placed in 100 mL of sterile double distilled water and rotated at 250 rpm 30°C for 4 hours. This suspension was then poured in polypropylene falcon tubes and then centrifuged at 10,000 rpm at 4°C for 5 min. and supernatant was discarded. Further 5 mL of Luria broth buffered with 0.25 M sodium acetate was added to the pellet. This mixture was shaken for 4 hours at 250 rpm at 30°C and heat shocked at 80°C for three minutes. Serial dilutions (10^{-1} to 10^{-6}) were made in sterile distilled water and 100 µL of each dilution was spread on Luria agar and incubated at 30°C overnight. Chalky white colonies were picked up and plated on T_3 medium and incubated at 30°C for 72 hours. Colonies showing typical characters were selected and further purified using single colony isolation technique and maintained on Luria agar at 4°C until further use.

Isolation from insect cadaver. The dead larvae were surface sterilized using rectified sprit and individual larva was homogenized in a microfuge tube in 1 mL of LB buffered with 0.25 M sodium acetate. This mixture was shaken for 4 hours at 250 rpm at 30°C and heat shocked at 80°C for three minutes. Serial dilutions (10^{-1} to 10^{-6}) were made in sterile distilled water and 100 µL of each dilution was spread on Luria agar and incubated at 30°C overnight. Three replications were maintained for each dilution and chalky white colonies were picked up, plated on T_3 medium and incubated at 30°C for 72 hours. Colonies which showed typical characters were selected and further purified using single colony isolation technique and maintained on Luria agar at 4°C until further use (Asokan and Puttaswamy 2007).

Characterization of local isolates

Characterization based on colony morphology. Single colonies were obtained by using single colony isolation technique and observations were recorded regarding colony morphology parameters namely, colony size, colony shape, colony elevation, colony margin, colony color and opacity of the bacterial colony. Strain *Btk* HD-1 was used as standard for the comparison with local probable *Bt* strains.

Microscopic characterization. Microscopic characterization was carried out by different staining

techniques which include, gram staining, spore staining with Malachite green and Amido black. Crystal staining was carried out by using coomassie brilliant blue.

Biochemical Characterization. Morphologically characterized colonies were further be confirmed by the biochemical characterization with the help of IMVIC test, which comprises of indole production, methyl red, Voges-Proskauer, citrate utilization test (Agrahari *et al.* 2008).

Indole Test. A loopful culture of probable *Bacillus thuringiensis* isolates were inoculated in tryptone broth containing NaCl. pH of culture was maintained at 7.2 and kept for incubation at 37°C in environmental shaker for 24 hours. Kovac's reagent was added after 24 hours of incubation to the bacterial culture. Absence of pink ring at the surface of the culture indicated negative test.

Methyl Red Test. MRVP medium was inoculated with a loopful of twenty four hours old cultures of probable *Bacillus thuringiensis* isolates. The culture was incubated at 30°C on rotary shaker at 100 rpm for 48 hours. A drop of methyl red indicator was added to the test tube, red color resulted positive test indicating presence of acid while yellow color of media indicated negative reaction to the test.

Voges- Proskauer test. MVRP medium was inoculated with a loopful of twenty four hours old cultures of probable *Bacillus thuringiensis* isolates. Then culture was incubated at 30°C on environmental rotary shaker at 100 rpm for 48 hours. 1-2 drops of α-naphthol reagent and 4-5 drops of 40% KOH were added in culture tube. Opened tubes then were placed in slanting position in order to increase contact with air. Change of surface color to pink in 10-15 min. indicated the positive test with acetyl methyl carbinol production.

Citrate Utilization test. This test was conducted to test the ability of bacterial culture to utilize citrate as a sole source of energy for its growth. For this, Simmon's citrate agar media was autoclaved, poured into sterile test tube kept at slanting position and allowed to solidify. Solidified slants were inoculated with loopful of bacterial culture and incubated for 24 hours. Growth of bacterial culture accompanied with change in the color of media from green to blue indicates positive test whereas, no growth with no change in media color indicated negative test.

RESULTS AND DISCUSSION

In order to explore the different habitats for the presence of *Bt* isolates, samples of native ecological niche, were collected from various locations of Vidarbha region. As *Bt* seems to be found in wide variety of niches, samples of soil, phylloplane, and insect cadavers were collected. In order to search for more efficient *Bt* isolates, the soil sample were collected from the areas where there is no previous history of *Bt* neither sown nor sprayed, phylloplane of various crops from university fields and insect cadavers from different locations were collected for the purpose of isolation of *Bt* (Asokan and Puttaswamy 2007). Total 80 samples were collected from 80 different locations with coordinates mentioned in Table 1. Out of these, 67, 7, 6 samples were collected from soil, phylloplane, insect cadaver respectively. For the purpose of *Bt* isolation, sodium acetate selective method was used (Travers *et al.,* 1987). With the help of Travers selective isolation method 57 probable *Bt* were isolated from soil, 6 from insect and 5 probable *Bt* were isolated. The results represent in Table 2 indicate that, out of the total samples collected maximum number of probable *Bt* isolates were obtained from soil (57), followed by phylloplane (6) and insect cadaver (5). The number of probable *Bt* isolates presented in Table 6 it can be estimated that *Bt* is abundantly present in the ecology of the collected samples (Table 2). Previous Travers *et al.* (1987) suggested the ubiquitous nature of *Bt* and isolated 85 of *Bt* out of 1,115 soil samples collected from United States and 29 other countries. *Bt* can be considered as part of the common leaf microflora of many plants (Smith and Couche 1991). Three common hypothetical niches of *B. thuringiensis* in the environment which include insect cadaver, phylloplane inhabitant, and soil are the important habitats for isolation of *Bt* (Meadows, 1993). Similar studies were conducted previously by Agrahari *et al.* (2008); Shishir *et al.* (2012); Padole *et al.* (2017); Amha *et al.* (2021) further confirmed that *Bt* can be isolated successfully from soil, phylloplane and insect cadaver with the help of Travers' acetate selective isolation method.

The morphological characterization of all the isolates obtained were carried by considering various important characters including colony size, colony shape, colony elevation, colony margin, colony color an opacity is reported in Table 3. The strain from NCBI, *Bacillus thuringiensis* sub sp *kurstaki* HD-1 (NCIM Accession No. 5118) was used as positive standard for the purpose of morphological characterization. The colony characters such as circular shape, flat elevation, opaque colony and creamy white colony color with irregular margin and wavy surface with fried egg like appearance was observed in the standard strain of *Btk* HD-1. Colony characters similar to the standard strain were reported from the 51 isolates out of total 68 probable isolate and remaining 17 isolates showed slight difference in colony color (off white and dirty white colony color) and slightly elevated colony. Amongst the total 51 isolates showing exactly similar characters as standard strain 44 out of 57, 4 out of 6 and 3 out of 5 isolates from soil, phylloplane and insect cadaver respectively were classified as probable *Bt* isolates. The different colony morphology predominantly of flat, circular, creamy white color colony with irregular margin and wavy surface was recorded (El-kersh *et al.*, 2016; Padole *et al.,* 2017) whereas creamish to off white color colonies with mucoid or glistening surfaces

having entire edges and density ranging between translucent to opaque was recorded from native isolates from Punjab (Kaur *et al.*, 2006).

Microscopic observations of the total 68 probable isolates were taken on the basis of various staining techniques including gram staining spore staining with malachite green and amido black and crystal staining with coomassie brilliant blue (CBB G-250). In addition to the staining methods, the shape and ends of vegetative bacterial cells were also observed. Positive gram staining test, rod shaped vegetative cells having terminal spore, positive spore staining with amino black and malachite green and positive crystal staining with coomassie brilliant blue G-250 was observed in standard strain *Btk* HD-1. All 68 possible isolates were stained using these methods and the results were recorded in Table 4, where 55, 6 and 5 isolates from soil, phylloplane and insect cadaver respectively resulted in positive gram staining and having rod shaped vegetative cells. Positive spore staining was observed for both malachite green and amido black staining in the 52, 6 and 5 isolates from soil, phylloplane and insect cadaver respectively. With the help of spore staining it was also observed that 43, 3 and 4 isolates showed terminal spore position and 9, 2 and 1 isolates from soil, phylloplane and insect cadaver respectively showed middle spore position in vegetative cell. However crystal protein staining with CBB G–250 was positive for 28, 2 and 3 isolates from soil, leaf, and insect cadaver, respectively. Similar results were recorded that *Bt* is gram positive bacteria and have terminal or median endospore (Baig *et al.,* 2010; Padole *et al.,* 2017). Presence of crystals were recorded with help of CBB staining (Kati *et al.,* 2007; Shishir *et al.,* 2012).

Biochemical characterization of the total 68 isolates was carried out by using indole, methyl red, Voges-Proskauer, and citrate test (IMViC). Out of 57 isolates from the soil, 6 from phylloplane, and 5 from insect cadaver, 42, 4 and 4 isolates showed Voges-Proskauer test positive, 14, 2, and 1 showed positive reaction to methyl red test whereas 55, 6, and 4 showed negative reaction to citrate utilization test. However, all the 57, 6, and 5 isolates showed negative reactions to the indole test as indicated in Table 5. Standard strain *Btk* HD-1 showed positive reaction Voges-Proskauer test while negative for methyl red, indole and citrate utilization test. The results regarding biochemical studies were in accordance with Eswarapriya *et al.* (2010), reported native *Bt* isolates positive to Voges- Proskauer (VP) test and negative reaction to Methyl Red (MR) test. It was recorded that *Bt* produces acetylmethyl carbinol from glucose fermentation as it was positive for VP test among IMViC test (Deepak *et al.,* 2011; Ghosh *et al.,* 2017; Purohit, 2019). Negative reaction for citrate utilization test was recorded from *B. thuringiensis* subsp. *kurstaki* (De Barjac and Frachon 1990; Abirami *et al.*, 2016; Padole *et al.,* 2017). It was previously recorded that isolates do not have the ability decompose the amino acid tryptophan to indole which is in accordance with present study (Yoo *et al.*, 1996; Deepak *et al.*, 2011; Abirami *et al.*, 2016).

The Bt isolation index was calculated by dividing the population of crystalliferous *Bt* isolates by the total population of *Bacillus* for each sample collected from different sources. The result present in Table 6 indicates that the highest *Bt* isolation index was obtained from soil samples of Bhandara district (0.75) followed by soil from Gadhchiroli (0.66), Gondia (0.60), Amravati (0.52), Yavatmal (0.50) and Akola (0.36) and the lowest *Bt* isolation index was reported in soil sample from Nagpur district. Whereas, 0.60 and 0.33 *Bt* index was reported for insect cadaver and phylloplane respectively. Similarly, highest *Bt* index of 0.67 was from soil samples and minimum 0.40 from leaf samples (Shishir *et al.,* 2012) whereas, higher *Bt* index recorded from forest soils (0.60) as compare to agricultural soils (0.33) (Lone *et al.,* 2017). Hence this study provides the idea about abundance of and diversity of *Bt* isolates present in the habitat of Vidarbha region.

Table 1: Samples collected for isolation and their location.

Sr. No.	Sample no.	Place	Location (Co-ordinates)
Habitat-Soil			
Amravati district			
1.	SA-1	Chikhaldara Forest	21.396152, 77.328419
2.	SA-2	Salona	21.427576, 77.406223
3.	SA-3	Salona	21.422672, 77.406577
4.	SA-4	Chikhaldara Forest	21.406717, 77.306599
5.	SA-5	Chikhaldara Forest	21.403484, 77.306188
6.	SA-6	Semadoha, tiger reserve	21.498055, 77.337455
7.	SA-7	Semadoha,	21.468479, 77.305540
8.	SA-8	Amzari, Chikhaldara	21.419671, 77.342286
9.	SA-9	Bhawai	21.487579, 77.371157
10.	SA-10	Bihali	21.396471, 77.456710
11.	SA-11	Bihali	21.400733, 77.465307
12.	SA-12	Bihali	21.395561, 77.455545
13.	SA-13	Kolkas	21.504071, 77.200986
14.	SA-14	Kolkas	21.507204, 77.200888
15.	SA-15	Talegaon	21.224659, 77.883384

16.	SA-16	Pohara-sawanga road	20.873610, 77.899837
17.	SA-17	Bhankheda khurd MH SH243	20.916449, 77.819885
18.	SA-18	Bhankheda khurd	20.913498, 77.822583
19.	SA19	Pohara	20.906200, 77.847187
20.	SA-20	Pohara	20.901592, 77.853684
21.	SA-21	Pohara	20.8820038, 77.9051799
		Akola District	
22.	SAk-1	Chinchkhed Patur	20.409682, 76.931117
23.	SAk-2	Malrajura Forest	20.397169, 76.943628
24.	SAk-3	Dr. PDKV, Campus	20.699753,77.034738
25.	SAk-4	Dr. PDKV, Campus	20.700505,77.036289
26.	SAk-5	Dr. PDKV, Campus	20.698429, 77.036238
27.	SAk-6	Dr. PDKV, Campus	20.697408, 77.038743
28.	SAk-7	Dr. PDKV, Campus	20.697898, 77.037970
29.	SAk-8	Dr. PDKV, Campus	20.697844, 77.037041
30.	SAk-9	Dr. PDKV, Campus	20.695430, 77.035654
31.	SAk-10	Dr. PDKV, Campus	20.703286, 77.036298
32.	SAk-11	Dr. PDKV, Campus	20.698173, 77.037090
		Bhandara District	
33.	SBn-1	Chandpur	21.508006, 79.815860
34.	SBn-2	Murli	21.502811, 79.811055
35.	SBn-3	Deulgaon	21.427565, 79.555331
36.	SBn-4	Rajdoh	21.145655, 79.755304
37.	SBn-5	Kakada Gondi	21.150195, 79.797547
		Gadhchiroli District	
38.	SGd-1	Porla	20.2926282, 79.9860286
39.	SGd-2	Wadadha	20.3284764,80.0982295
40.	SGd-3	Illur Defence Ground	19.6581033,79.8043153
41.	SGd-4	Thakari	19.637036, 79.799029
42.	SGd-5	Ashti	19.666342, 79.796268
43.	SGn-1	Navegaon Bandh	20.9146992,80.1074811
44.	SGn-2	Navegaon Bandh	20.9049078, 80.1088933
45.	SGn-3	Kanholi,	20.870940,80.130070
46.	SGn-4	Itiya Doha	20.807041,80.155704
47.	SGn-5	Pangdi	21.4088401, 80.0984209
		Nagpur District	
48.	SN-1	Sillari	21.575827,79.287562
49.	SN-2	Gorewada	21.188762,79.036411
50.	SN-3	Adegaon, Bor Forest	21.1110595,78.9952684
51.	SN-4	Digdoha	21.086889,78.753655
52.	SN-5	Thakurwadi	21.043530,78.735185
53.	SN-6	Khapa khurd	21.047762,78.733595
54.	SN-7	Ramtek	21.398585,79.330329
55.	SN-8	S R P Camp, Nagpur	21.110373, 79.019140
56.	SN-9	S R P Camp, Nagpur	21.113585, 79.018774
57.	SN-10	Seminary Hills, Nagpur	21.161871, 79.066063
		Yavatmal District	
58.	SY-1	Madkona	20.405756,78.225300
59.	SY-2	Chausala	20.418471, 78.078724
60.	SY-3	Chinchghat	20.011124,77.594293
61.	SY-4	Tipeshwar	19.922177, 78.441983
62.	SY-5	Lohara	20.3954105,78.0837393
63.	SY-6	Dhumnapur, Chinchbardi	20.3960513,78.0758747
64.	SY-7	Yeldari, Pusad	19.910918, 77.493313
65.	SY-8	Amrut Nagar, Taluka-Pusad	19.902576, 77.437629
66.	SY-9	Belona	20.411299, 78.256523
67.	SY-10	Bhairam tekdi, Jam road	20.351872,78.110925
		Habitat- Phylloplane	
	Sample no.	Plant and place	Location
68.	L-1	Brinjal, Dr. PDKV Campus	20.697545, 77.039817
69.	L-2	Pigeon pea, Dr. PDKV Campus	20.697585, 77.042671
70.	L-3	Cotton, Dr. PDKV Campus	20.706095, 77.053530
71.	L-4	Chilli, Dr. PDKV Campus	20.708043, 77.044549
72.	L-5	Groundnut, Dr. PDKV Campus	20.707437, 77.044783
73.	L-6	Seasamum, Dr. PDKV Campus	20.698411, 77.042828
74.	L-7	Citrus, Dr. Dr. PDKV Campus	20.697898, 77.039942
		Habitat- Insect Cadaver	
Sr. No.	Sample no.	**Insect and place**	Location
75.	I-1	Diamond Back Moth, Babhulgaon, Akola	20.707317, 77.086514

76.	I-2	Diamond Back Moth, Babhulgaon, Akola	20.707317, 77.086514
77.	I-3	Diamond Back Moth, Babhulgaon, Akola	20.707317, 77.086514
78.	I-4	White Grub, Dr. PDKV Campus	20.705423, 77.053339
79.	I-5	Diamond Back Moth, Dongargao, Akola	20.693726, 77.099906
80.	I-6	Diamond Back Moth, Dongargao, Akola	20.693726, 77.099906

Table 2: Isolation of *Bacillus thuringiensis* from different habitat through Traver's selective isolation method.

Sr. No.	Particulars	Habitat			Total
		Soil	Phylloplane	Insect Cadaver	
1.	Total samples collected	67	07	06	80
2.	No. probable *Bt* isolates	57	06	05	68

Table 3: Morphological characterization of representative isolates of *B. thuringiensis.*

Sr. No.	Sample No.	Colony Size (mm)	Colony Shape	Colony Elevation	Colony Margin	Colony Color	Colony Opacity
Habitat-Soil							
1.	SA-1	4.33	C	F	IR	CW	Opaque
2.	SA-2	4.00	C	F	IR	CW	Opaque
3.	SA-3	2.67	C	F	IR	OW	Opaque
4.	SA-6	4.00	C	F	IR	CW	Opaque
5.	SA13	6.33	C	SE	IR	CW	Opaque
6.	SAk-6	5.67	C	F	IR	CW	Opaque
7.	SAk-7	3.00	C	F	IR	CW	Opaque
8.	SAk-8	3.33	C	F	IR	OW	Opaque
9.	SAk-9	5.00	C	SE	IR	CW	Opaque
10.	SBn-1	5.00	C	F	IR	OW	Opaque
11.	SBn-2	6.33	C	F	IR	CW	Opaque
12.	SGd-1	4.33	C	F	IR	CW	Opaque
13.	SGd-2	5.00	C	F	IR	CW	Opaque
14.	SN-2	3.66	C	F	IR	CW	Opaque
15.	SY-4	4.33	C	F	IR	CW	Opaque
16.	L-1	3.66	C	F	IR	CW	Opaque
17.	L-2	4.67	C	SE	IR	DW	Opaque
18.	I-3	4.00	C	F	IR	CW	Opaque
19.	I-5	2.67	C	SE	R	DW	Opaque
20.	I-6	3.33	C	SE	IR	CW	Opaque

CW - Creamy white color colony, DW - dirty white, OW - Off white, C - Circular colony, IR - Irregular margin, R - Regular margin, F - Flat elevation colony, SE - Slightly elevated colony

Table 4: Microscopic characterization of representative local *Bt* isolates based on staining.

Sr. No.	Sample No.	Gram staining	Ends	Spore staining (Malachite green)	Spore staining (Amido Black)	Spore position	Protein staining with CBB
Habitat-Soil							
1.	SA1	+	R	+	+	T	+
2.	SA2	+	R	+	+	T	+
3.	SA3	+	R	+	+	T	-
4.	SA6	+	R	+	+	T	+
5.	SA13	+	R	-	-	-	-
6.	SAk6	+	R	+	+	T	+
7.	SAk7	+	R	+	+	T	-
8.	SAk8	+	R	+	+	T	+
9.	SAk9	+	R	+	+	M	+
10.	SBn1	+	R	+	+	T	+
11.	SBn2	+	R	+	+	M	+
12.	SGd1	+	R	+	+	T	+
13.	SGd2	+	R	+	+	T	+
14.	SN2	+	R	+	+	T	+
15.	SY4	+	R	+	+	T	+
16.	L1	+	R	+	+	T	+
17.	L2	+	R	+	+	M	-
18.	I3	+	R	+	+	T	+
19.	I5	+	R	+	+	T	-
20.	I6	+	R	+	+	M	-

R - Rounded ends
T - Terminal spore position
M - Middle spore position
+ - Positive reaction
- - Negative reaction

Table 5: IMViC test of representative local *Bacillus thuringiensis* isolates.

Sr. No.	Sample No.	Voges-Proskauer Test	Methyl Red	Indol Test	Citrate Utilization Test
Habitat- Soil					
1.	SA1	+	-	-	-
2.	SA2	+	-	-	-
3.	SA3	-	+	-	+
4.	SA6	+	-	-	-
5.	SA13	-	+	-	-
6.	SAk6	+	-	-	-
7.	SAk7	-	+	-	-
8.	SAk8	+	-	-	-
9.	SAk9	+	-	-	-
10.	SBn1	+	+	-	-
11.	SBn2	+	-	-	-
12.	SGd1	+	-	-	-
13.	SGd2	+	-	-	-
14.	SN2	+	-	-	-
15.	SY4	+	-	-	-
16.	L1	+	-	-	-
17.	L2	+	-	-	-
18.	I3	+	-	-	-
19.	I5	-	+	-	+
20.	I6	+	-	-	-

+ : Positive test - : Negative test

Table 6: Summary of confirmed local *Bt* isolates.

Sr. No.	Habitat	Total samples collected	*Bt* index
1.	Insect Cadaver	07	0.60
2.	Phylloplane	07	0.33
3.	Soil	67	0.49
	Location soil sample		
	Amravati	21	0.52
	Akola	11	0.36
	Bhandara	05	0.75
	Gadhchiroli	05	0.66
	Gondia	05	0.60
	Nagpur	10	0.28
	Yavatmal	10	0.50

Fig. 1. Native *Bt* isolate.

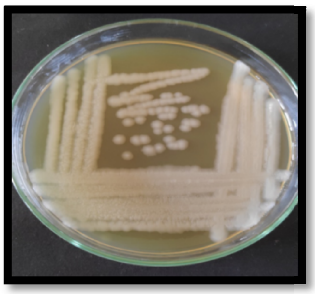
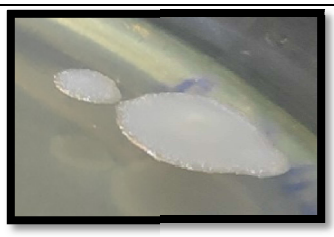


Fig. 2. Standard strain *Btk* HD-1.

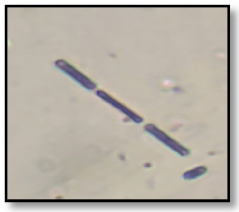
(a) Creamy white colony.

(b) Circular shape with irregular margin.

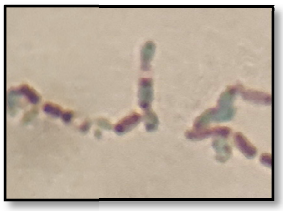


(c) Flat Elevation.

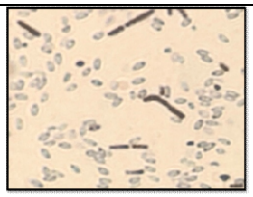
Fig. 3. Colony Morphology.



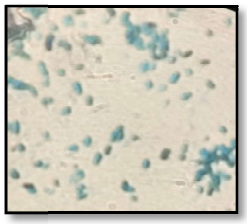
(a) Gram positive, rod shaped



(b) Spore staining with Malachite green

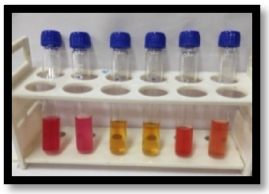


(c) Amido Black Staining, (Dark blue crystals, Pale blue spores)

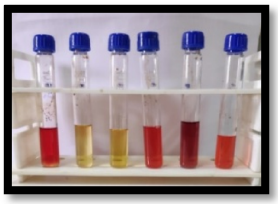


(d) Crystal Staining with CBB

Fig. 4. Microscopic Obsrvations.



(a) Methyl Red Test



(b) Voges-Proskauer Test

(c) Indole Test

(d) Citrate Test

Fig. 5. IMViC Tests.

CONCLUSION

The results of the present study revealed that the *Bacillus thuringiensis* (*Bt*) bacterium is widely distributed throughout the Vidarbha region of Maharashtra state, India, including the districts of Yavatmal, Gadhchiroli, Amravati, Akola, Bhandara, and Gondia. However, the occurrence was predominant in soil samples compared to other sources, such as leaves and insect cadaver. Additional morphological, microscopic, and biochemical analyses revealed the presence of endospore in bacteria, and the presence of parasporal crystalliferous inclusion further distinguished *B. thuringiensis* from other *Bacillus* spp., confirming its presence in the collected samples.

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Conflict of Interest. None.

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