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Full Length Research Paper

Ecology and diversity of *Bacillus thuringiensis* in soil environment

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Bacillus thuringiensis **populations ranged between 4.23 x 10^5, 6.52 x 10^5 cfu/g soil and consist of 11 types of isolates with 3 polymorphic, 7 spherical and 1 bipyramidal type of crystals. Polymorphic crystal containing isolates were further characterized.** ***B. thuringiensis*** **isolates were circular, white, flat and undulate or entire. These were gram-positive. These tolerated 5% NaCl and failed to grow anaerobically. None of these were positive for indole production. All were sensitive to streptomycin, vancomycin, polymyxin B and norfloxacin but resistant to penicillin G and ampicillin/sulbactum. All of the isolates were crystal forming rods, width of rods was >0.9 um, catalase positive and sporangia were not swollen. All of the isolates hydrolyzed protein but only BTc 175 produced arginine dihydrolase, BTc 152 and BTc 175 produced urease.**

Key words: *Bacillus thuringiensis*, soil, isolates, polymorphic crystal.

INTRODUCTION

Research of almost 85 years reveals that *Bacillus* spp., especially *B. thuringiensis* and *Bacillus sphaericus* are the most potent biopesticides (Boucias and Pendland, 1998). Available information depict that *B. thuringiensis* is a versatile pathogen capable of infecting protozoa, nematodes, flatworms, mites and insects that are either plant pests or human and animal health hazards (Feitelson, 1993). *B. thuringiensis* has been obtained from soil, phyllosphere, diseased insects, stored products, dumping pits, excreta of vegetarian animals etc. and about 30-100% spore formers of phyllosphere were found to be *B. thuringiensis* (Martin and Travers, 1989; Boucias and Pendland, 1998). An analysis of 27,000 isolates collected from ,100 soil samples all over the world demonstrated that *B. thuringiensis* could be isolated every where, including desert, beach and tundra habitats (Martin and Travers 1989; Attathom et al., 1995). *B. thuringiensis* accounts for about 5-8% of *Bacillus* spp. population in the environment (Hastowo et al., 1992). Till date more than 130 species of lepidopteran, dipteran and coleopteran insects are found to be controlled by *B. thuringiensis* (Dean, 1984). So far, 68 serotypes (81 serovars/varieties) of *B. thuringiensis* having wide array of host range have been isolated and characterized and some of them have already been commercially exploited directly as native form or indirectly as transgenic microbes or plants (Krattiger, 1997).

Insect pests of crops and forest plants and vectors of disease of human beings and other animals are serious threat for agriculture and public health. Worldwide, about US $8000 billion is spent for insecticides and estimates reveal that US $2700 can be substituted by the biopesticide *B. thuringiensis* (Krattiger, 1997). Besides exorbitant cost, and resistance and resurgence of the different pests, the chemical pesticides are the single main cause of health and environmental hazard (Krattiger, 1997). The situation demands the safer pesticides and biopesticides are the most desired alternatives. Bacteria, especially *B. thuringiensis* and *B. sphaericus* are the most potent and successful group of organisms for effective control of insect pests and vectors of diseases (Krattiger, 1997). Potentiality of *B. thuringiensis* as larvicide of *Culex* in India has been demonstrated by Ghosh et al. (1988). *B. thuringiensis* has certain advantages for exploitation as

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Table 1. Types of *Bacillus thuringiensis* isolated from different soils.

Soil no.	Types of Bt	Isolate number	Type of crystal
S1.	5	BTc 152	Polymorphic
		BTc 170, 171,172, 173	Spherical
S2.	6	BTc 61, 175	Polymorphic
		BTc 179, 176, 177	Spherical
		BTc 178	Bipyramidal

Table 2. Colony characters of *B. thuringiensis* isolates and standard culture on nutrient agar plates.

Bacteria no.	Form	Colour	Elevation	Margin
BTc 152.	Circular	White	Flat	Undulate
BTc 61	Circular	White	Flat	Entire
BTc 175	Circular	White	Flat	Entire

biopesticide viz. *B. thuringiensis* can be used directly and as transgenic microbes and plants, being a prokaryote there is no dominant or recessive allele, highly vulnerable to genetic manipulation and the toxin gene is coded by single gene (monocistronic), *B. thuringiensis* is fermentation friendly and therefore commercially exploitable and it is host specific or has narrow host range. These advantages favoured development of about 100 formulations (Federici, 1993) and commercialization of 40 *B. thuringiensis* products internationally and eight products in India exclusively by the multinational organizations (Saxena, 2000). However, none of the formulations marketed in India is an indigenous strain.

Present study was envisaged to isolate and identify the *B. thuringiensis* of indigenous soils of Tarakeswar, Hooghly, West Bengal, India and characterize the polymerphic crystal producing strains, which may be exploited for biological control of a wide range of insect-pests and disease vectors in the long run. The investigation was under-taken with two main objectives; assessment of diversity of *B. thuringiensis* population producing different types of crystals, especially the polymorphic crystal producing strains, and morphological, physiological and biochemical characterization of the polymorphic crystal producing strains.

MATERIALS AND METHOD

Soil samples were collected from the rice fields at two locations (site 1 with clay soil and site 2 with sandy soil) of Tarakeswar of India. Top layer of soil (about 1 cm) was removed. From each location, five samples each of about 10 g were collected from five spots. Samples were mixed thoroughly and put in polythene packets with proper levels. The soils were air-dried up to 20% moisture level, powdered and sieved through a fine mesh and stored in sealed polythene bags within desiccators. The soil was checked time to time to maintain a moisture level of 20%.

Immediately after collection, the soil pH was checked in the laboratory. Detailed characters of the soil were recorded. *B. thuringiensis* were isolated from the soil. One-gram soil was suspended in 10 ml sterile distilled water, logarithmic dilutions were made up to 10^{-2} level, pasteurized at 60°C for 30 min and 10 µl suspension was added to 100 ml nutrient agar (NA) medium (≈ 45°C) and plated on 5 plates (Holt, 1984, Lacey, 1997). The plates were incubated at 30 ± 0.1°C for 72 h. After incubation, a portion of each colony was mounted on a slide with water and observed under a phase contrast microscope. The colonies depicting spores and crystals were marked and streaked on NA plates. Isolated colonies were reconfirmed to be *B. thuringiensis* by checking the spore and crystal production. One single and isolated colony was sub cultured on NA slant, incubated for 72 h at 30 ± 0.1°C and after adequate growth the slants were numbered and preserved at 4 ± 0.1°C. Cultures on NA stabs were preserved at 4 ± 0.1°C for long-term storage. The slant cultures were sub cultured periodically and used for different experiments. Morphological characters of the colonies and the bacteria were studied following the standard microbiological methods (Pelczar et al., 1957; Collee and Miles, 1989; Lacey, 1997). The isolates were streaked on NA plates, incubated for 72 h at 30 ± 0.1°C. The shape, size, colour, margin and opacity were recorded from isolated colonies. The isolates were streaked on NA slants and stab cultured with a straight needle pierced through the centre of the NA stab tubes, incubated at 30 ± 0.1°C for 72 h and growth of the organism were recorded. Morphology of the vegetative cells, spores and crystals were observed under a phase-contrast microscope. Staining characters of the organism were studied for vegetative, reproductive and crystal structure determination. Physiological and biochemical characters of the organisms were checked following the standard methods for identification of the isolates (Pelczar et al., 1957; Collee and Miles, 1989; Lacey, 1997).

RESULTS AND DISCUSSION

B. thuringiensis population ranged between 4.23×10^5 - 6.52×10^5 cfu/g soil. Different types of *B. thuringiensis* isolated from the soils are presented in Table 1. Soil sample from site-1 resulted in 5 types of *B. thuringiensis* of which one produced polymorphic and others produced spherical crystals, site 2 produced 6 types of *B. thuringiensis* of which 2 produced polymorphic, 3 spherical and one bipyramidal crystals (Table 1). Thus in total, there were 11 isolates of which 3 (*B. thuringiensis c* 152, *B. thuringiensis c* 61 and *B. thuringiensis c* 175) isolates were with polymorphic crystals. These were further studied. The colony characters of the *B. thuringiensis* isolates under study were circular, white, flat and undulate or entire (Table 2). The characteristics of vegetative cells,

Table 3. Characters of vegetative cells of *B. thuringiensis* isolates and standard culture.

Bacteria no.	Shape	Length (μm)*		Breadth (μm)*		Motility	Gram stain
		Range	Mean	Range	Mean		
BTc 152.	Rods with rounded ends	4- 5.06	4.70	1-2.1	1.45	Non motile	+
BTc 61	Rods with rounded ends	4.1-6.8	4.30	1.5-2.5	1.80	Motile	+
BTc 175	Rods with rounded ends	4-6	4.2	1.0-2.5	1.7	Non motile	+

* Results of 10 observations.

Table 4. Characters of spores of *B. thuringiensis* isolates and standard culture.

Bacteria number	Shape	Length (μm)*		Breath (μm)*		Spore stain
		Range	Mean	Range	Mean	
BTc 152.	Oval	1-2	1.58	0.5-1.35	0.98	+
BTc 61.	Oval	1-2	1.83	0.5-2.01	1.25	+
BTc 175.	Oval	1.25-2.0	1.85	1-1.50	1.20	+

* Results of 10 observations.

Table 5. Characters of crystals of *B. thuringiensis* isolates and standard culture.

Bacteria number	Shape	Length (μm)		Breadth (μm)		Crystal stain
		Range	Mean	Range	Mean	
BTc 152.	Polymorphic	1.25-2.8	2.20	1.0-2.0	1.34	+
BTc 61.	Polymorphic	1-2.60	1.66	0.5-1.80	1.25	+
BTc 175.	Polymorphic	1.0-2.0	1.60	0.5-2.00	0.78	+

. * Results of 10 observations.

Table 6. Growth characteristics of *B. thuringiensis* isolates and standard culture.

Medium	Bacteria number		
	BTc 152	BTc 61	BTc 175
NA	+	+	+
NA + Sodium chloride (%)			
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	+	+	+
6	-	-	-
7	-	-	-
8	-	-	-

Table 7. Enzymatic activities of different *B. thuringiensis* isolates and standard cultures.

Tests		Bacteria number (BTc)		
		152	61	175
Protease:				
Gelatinase	Growth	+	+	+
	Result	+	+	+
	Clear zone (mm)	32	34 35	35
Casein hydrolysis	Growth	+	+	+
	Result	+	+	+
	Clear zone (mm)	2	1	2

spores and crystals of *B. thuringiensis* isolates are given in Tables 3 - 5. All the bacteria were positive for gram stain, spore and crystal staining (Tables 3 - 5).Organisms tole-rated 5% NaCl (Table 6). The organisms failed to grow anaerobically. Enzymatic activities of different *B. thuringiensis* isolates are shown in Table 7. The physio-logical and biochemical properties of the *B. thuringiensis* cultures are presented in Table 8. None of the organisms were positive for indole production. Response of the org-anisms to the recommended doses of different antibiotics (Table 9) shows that all of them were sensitive to streptomycin, vancomycin, polymyxin B and norfloxacin but resistant to penicillin G and ampicillin/sulbactum. Results of biochemical and other characters (Tables 8 - 10) of the isolates were important for the identification of the isolates. All of the isolates were gram positive, spore and crystal forming rods, width of rods was >0.9 um, catalase positive sporangium was not swollen. All the organisms did not grow anaerobically. The crystals prod-

Table 8. Some physiological and biochemical properties of *B. thuringiensis* isolates and standard culture.

Tests	Bacteria number		
	BTc 152	BTc 61	BTc 175
Catalase	+	+	+
Arginine dihydrolase production	-	-	+
Indole production	-	-	-
Vogues Proskauer test	+	+	+
Nitrate reduction test	+	+	+
Urease production test	+	-	+

+ = Positive result.
- = Negative result.

Table 9. Antibiotic assay of different *B. thuringiensis* isolates and standard cultures.

Antibiotics	Bacteria number					
	BTc 152		BTc 61		BTc 175	
	Re	C	Re	C	Re	C
Vancomycin (30 ug)	S	16	S	21	S	19
Penicillin G (10 units)	R	0	R	0	R	0
Norfloxacin (10 ug)	S	15	S	17	S	16
Bacitracin (10 units)	S	11	S	12	S	11
Ampicillin/Sulbactum (10/10 ug)	R	0	R	0	R	0
Erythromycin (15 ug)	S	25	S	27	S	27
Streptomycin (10 ug)	S	23	S	21	S	33

Re = Reaction. C = Clear zone diameter in mm. S = Sensitive. R = Resistant.

Table 10. Identification scheme of the isolates/cultures**.**

Characters	Bacteria number		
	BTc 152	BTc 61	BTc 175
Shape	Rod	Rod	Rod
Length (µm)	4.70	4.30	4.20
Width (µm)	1.45	1.80	1.70
Sporangium	Not swollen	Not swollen	Not swollen
Spore	Oval	Oval	Oval
Crystal	+ Polymorphic	+ Polymorphic	+ Polymorphic
Gram stain	+	+	+
Catalase activity	+	+	+
Anaerobic growth	-	-	-
Genus	*Bacillus*	*Bacillus*	*Bacillus*
Species	*thuringiensis*	*thuringiensis*	*thuringiensis*

uced by all of the tested isolates were polymorphic.

All of the isolates hydrolyzed protein and only *B. thuringiensisc* 175 produced arginine dihydrolase, *B. thuringiensis* 152 and *B. thuringiensis* 175 produced urease. The results conclusively prove that the organisms are *Bacillus* sp. (Holt, 1984; Sneath, 1986; Garrity, 2001).

Besides the characters of *Bacillus* sp. which are mentioned above, the organisms produced galvanized metallic colonies, polymorphic crystals along with the spores, non-swollen sporangia, oval spores, positive for catalase and VP tests confirmed that the organisms belong to Group I of *Bacillus* sp. (Sneath, 1986; Smibert and Krieg, 1994; Garrity, 2001). Production of crystals and non-swollen sporangia identified the organisms as *B. thuringiensis* (Sneath, 1986; Smibert and Krieg, 1994; Thiery and Frachon, 1997; Garrity, 2001). Occurrence of *B. thuringiensis* in soil and population size is relatively higher than earlier reports (Martin and Travers, 1989; Hastowo and Ohba, 1992; Theunis et al., 1998; Kaur and Singh, 2000). Although there are several reports that all soils may or may not harbour *B. thuringiensis* (Martin and Travers, 1989; Hastowo et al., 1992), higher *B. thuringiensis* population containing different types of crystals reveal that the soils of Tarakeswar are rich in *B. thuringiensis* population and diversity.

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