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Characterization of *Bacillus thuringiensis* Isolates from Soils of Rice Ecosystem in UKP Area, Karnataka, India

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An investigation was conducted to study the diversity of entomopathogenic bacteria *Bacillus thuringiensis,* in rice ecosystem in UKP area Karnataka, India. Survey was conducted to collect soils from different places and *Bacillus thuringiensis* were isolated. Totally 72 *B. thuringiensis* isolates were collected from the survey. The cell sizes of the *B.thuringiensis* isolates varies from 1.45x0.83 μm to 3.49x0.63 μm, where as endospore size varies from 0.83-0.66 μm to 1.66-0.66 μm. Among collected bacteria 64 % of the isolates produced bipyramidal crystal shape and 3 % produced triangular shape, 9 % produced bipyramidal and triangular shape, 9% produced spherical, 2 % produced ovoid and 2% produced triangular and bipyramidal shape. The pathogenicity of isolates against 2 nd instars Diamond back moth larvae showed, mortality ranged from 0 to 100 per cent. Bioassay showed that LC_{50} value was minimum in Thanegundi isolate ($1.9x10^{-4}$) followed by Karkalli, Yevur and Gogi isolates.

Key words: *Bacillus thuringensis*, Bioassay, Crystal protein staining and Rice ecosystem.

Pests are one of the major constrains for production and productivity of rice crop in India. There are many pests causing damage to rice crop, so for 1131 insects have been recorded on rice crop. Several approaches are available to control insect pests, but farmers are rely only on chemical method of controlling. First and foremost concern is that farmers are spraying insecticide indiscriminately and at higher than recommended dosage to get quick and complete control of insect pests. This resulted in contamination of environment, ill effects on human health, residue in export quality rice and development of resistance and resurgence in insect pests. Even it is more severe in case of rice, because insecticides are directly sprayed to water. This provoked the use of microorganisms as an alternative and efficient means of pest control (Lacey and Harper, 1986). The bioagents include predator, parasitoid and pathogens, particularly pathogens like bacteria and fungi are giving promising results. Among the microorganisms considered to be natural enemies of insects, *Bacillus thuringiensis* is nowadays the most important. *Bacillus thuringiensis* is a ubiquitous, gram positive, aerobic, spore forming soil bacterium that produces crystalline inclusions containing entomocidal proteins, also referred as Bt toxins or endotoxins during the sporulation process. Crystal proteins or insecticidal crystal

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proteins (ICPs) from the *B. thuringiensis* are toxic to a wide variety of pests (Hofte and Whiteley, 1989).

India is considered as one of the centers of biodiversity. In India, many scientists attempted on isolation of *B. thuringiensis* from soils. There is dearth of research on exploration of entomogenous bacteria (apart from *B. thuringiensis*) from salt affected and anaerobic soil of rice ecosystem. Upper Krishna Project area (UKP) comes under northern part of Karnataka, India. Where temperature is usually high with dry weather and soils are salt affected or sodic soil due to unscientific irrigation of canal water. This area is classified as northern dry zone of Karnataka. Entomogenous bacterial strains isolated from such region could be considered as temperature tolerant, stress tolerant and salt resistant strains, it could be utilized as biocontrol agent in Northern Karnataka. The genetic diversity and toxicity of *B. thuringiensis* strains differ from region to region. There is immense scope to identify and explore indigenous bacterial entomopathogens (spore forming bacteria) in UKP area. This type of research will be the first of its kind particularly in Northern Karnataka. The study presents the isolation, morphological and toxicological characterization of *B. thuringiensis* isolates in the Upper Krishna Project area, Karnataka, India.

MATERIALS AND METHODS

Roving survey for collection of soil

Roving survey was conducted to collect soil samples in rice ecosystem from Upper Krishna Project area Karnataka, India. Totally sixty seven number of villages were surveyed comprising three taluks of UKP area. 100 grams of soil samples were collected from each rice field in each village of Upper Krishna Project area. Sample was collected from rice hills uprooted from paddy field and wet soil was taken from active rhizosphere in a sterilized polythene bags, such five samples were taken from each plot. The soil samples were brought to laboratory, air dried, ground to powder by grinder, mixed well and 100gm of soil was taken from mixed soil. (As a representative sample from each village). The soil was kept at 4 ^{0}C in refrigerator for further analysis.

Selective isolation of *Bacillus thuringiensis* from soil samples

The protocol for isolation of *B. thuringiensis* from the soil was followed as described by Travels *et al* (1989) and Andrezejczak and Lonc (2008) with some modification. In this method the heating was continued from 3 min to 20 min and L-syrine amino acid was used to screen *B. thuringiensis* isolates efficiently. 1.0 gm of soil sample (air dried) was taken in a conical flask containing Luria broth buffered with sodium acetate (0.25mM). The mixer was shaken well for 4hr at 250 rpm at 30C. Further the samples were heated at 80 ^{o}C for 20 min in water bath. Then suspension was serially diluted & spread on Luria agar, incubated at 30 ^{o}C for 48hr. Random samples of colonies were picked on to M9 medium(6 g Na_2HPO_4-$7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 0.5 g NH_4Cl, 0.024 g $MgSO_4$, 0.0001 g $CaCl_{2,20g}$ Agar, 1000ml with distilled H_2O) supplemented with L-syrine (0.2mM) amino acid. Identification of *B. thuringiensis* isolate was done by colony morphology as well as the presence of crystal proteins. The colony showing flat, cream, rough surface and irregular edges was identified and subjected to gram's staining, endospore staining for confirmation of *B thuringiensis* isolates. Further cultures were stained with Comossive Brilliant Blue and observed shape of the parasporal bodies under phase contrast microscope. The colony showing similar morphology and crystal proteins are sub cultured and maintained in the slants. The isolates also subjected to micrometer to determine sizes of cells & endospores.

Staining of parasporal bodies of *B. thuringiensis* isolates

The bacterial cells were heat fixed on to slides and Comossive Brilliant Blue (0.25 %) was used to stain parasporal bodies.

DBM maintenance in laboratory

Diamond back moth (DBM), *Plutella xylostella* larvae was brought from field and maintained in the laboratory on mustard seedling grown on plastic petriplates, which were filled with vermiculite.

Proving Koch's postulates of *B. thuringiensis* isolates

The bacterial calls were suspended in 50 ml Luria broth for 72hrs. The turbid solution was centrifuged at 200 rpm to remove contents of media. The supernatant was taken for conducting bioassay. The cells were counted by haemocytometer and adjusted the cell

concentration accordingly.

The mustard seedlings were maintained in pots, leaves were used for conducting bioassay. The leaves were washed with 0.1 % formaldehyde, transfer the leaves serially to water blanks to remove the traces of formaldehyde, air dried, dipped in the bacterial suspension solution for a period of 3 min, air dried. The air dried leaves were kept in the petriplates, the petiole was moistened with wet cotton, wrapped with aluminum foil over the cotton. Known number of 2nd instar uniform aged DBM larvae were released on the leaves. Care was taken to prevent escape of larvae from petri plates by keeping tissue paper. Observed for the mortality of larvae at 24 hr interval. The dead larvae were again used to isolate *B thuringiensis* isolates and confirmed the pathogencity. The pathogens which gave higher mortality in the initial screening were used for conducting bioassay and LC_{50}, LT_{50} value were established for the virulent isolates.

Bioassay to *B. thuringiensis* isolates against Diamond back moth

Before conducting bioassays in the laboratory, bracketing procedure suggested by Daoust and Roome (1974) for pathogens was followed. Accordingly, serial dilutions were prepared using sterile distilled water to arrive at approximate range of concentrations effecting mortality of larvae between 10 and 90 per cent. Five different concentrations were selected and used for determination of median lethal concentration (LC_{50}) and median lethal time (LT_{50}).

The procedure for conducting bioassay was followed as mentioned earlier. The air dried leaves were dipped in the respective concentrations of *B. thuringiensis* isolates, twenty five uniformly aged second instar larvae were released on to the leaves. Bioassay was done with five replication and one untreated control for each concentration used, incubate the plates at 25°C. Observation was taken at 24 hr interval upto 144 hrs (6 day) and per cent mortality was calculated.

RESULTS

The results on collection of bacteria , their pathogencity and bioassay are presented in this chapter.

Diversity of isolates morphology

Survey was undertaken throughout the UKP area, Karnataka, India from June to December 2010 for the collection of soil samples in the rice ecosystem. Totally 72 soils were collected, among them 56 soils gave *B. thuringiensis* isolates. The *B. thuringiensis* isolates were confirmed by Grams staining, spore staining and parasporal body staining. The cell size of *B. thuringiensis* isolates varied from 1.45x0.83 µm to 3.49x0.63 µm, where as endospore size varied from 0.83-0.66 µm to 1.66-0.66 µm (Table 1). The cell size and spore size was found to be maximum in the isolates viz Naykal, B Gudi, Thanegundi and Guddada Thanda. The cell size and spore size was 3.49x0.63 µm, 1.66x0.83 µm respectively (Table 1). The shape of parasporal bodies also varied viz., bipyramidal, irregular, triangular, spherical and ovoid. Among collected *B. thuringiensis* isolates 64 % were bipyramidal in shape and 3 % were triangular in shape, 9 % were bipyramidal and triangular shape, 9% were spherical, 2 % were ovoid and 2% were triangular and bipyrmidal shape(Fig-1).

Bioassay against DBM to establish LC_{50} and LT_{50}

Pathogencity of *B. thuringiensis* against DBM showed that mortality ranged from 0 to 100 per cent. The isolates which caused more than 80 per cent mortality were chosen for conducting bioassay(Thanegundi, Gogi, Karkalli and Yevur). The details of concentrations used and LC_{50} values were presented in the Table 2. 1

The bioassay of *B. thuringiensis* isolates (Thanegundi, Gogi, Karkalli and Yevur) against DBM were carried out in the laboratory and LC_{50} and $LT_{50\ values}$ were established by leaf dipping method. The result showed that LC_{50} value was minimum in Thanegundi isolate ($1.9x10^{-4}$) (Table 2). This was followed by Karkalli , Yevur and Gogi isolates (6.7×10^{-4} 7.4×10^{-4} and 13.9×10^{-4} respectively).

The LT_{50} values were also established. As the concentration of bacteria cells increase from 1×10^{-1} to 1×10^{-5} cells/ml, LT_{50} values were also increased, this reveals that time taken to kill 50 per cent population increases as concentration decreases. The LT_{50} for Thanedungi isolate were 1.23, 1.48, 2.17, 3.71 and 5.69 days at 1×10^{-1}, 1×10^{-2}, $1x10^{-3}$, 1×10^{-4} and $1x10^{-5}$ cells/ml at respectively (Table 3).Whereas for Karkalii isolate, the LT_{50} values were 1.41, 1.83, 3.31, 5.91 and 6.28 days (Table 4) and for Yevur isolate LT_{50} values were 1.33,1.87,3.51,6.74 and 13.34 days (Table 5), that of

Table 1. The schedule of survey undertaken during summer season 2010

Place visited	Date of collection	*B. t.* isolates	Cell size (µm)	Endospore size	Shape of cry protein	Percent mortality
Yalagi	30-3-2010	-				0
Adanur	2-5-2010	-				0
Hayyal	3-5-2010	-				0
Haikur	4-5-2010	+	3.32×0.83	1.03-0.83	Bipyramidal	60
Naganatagi	7-5-2010	+	2.45×0.83	0.83-0.66	Bipyramidal	36.36
Narayanapur	8-5-2010	+	3.32×0.82	1.25-0.83	Tringular	43.75
Gugadihal	9-5-2010	+	3.32×0.83	1.03-0.83	Bipyramidal	40.9
Naykal	10-5-2010	+	3.49×0.63	1.66-0.83	Bipyramidal	77.27
Hattigudur	26-3-2010	-				0
Devathkal	28-3-2010	+	3.32×0.83	1.03-0.83	Bipyramidal +tringular	26.92
Ganagapur	31-3-2010	+	2.45×0.83	1.25-0.83	Bipyramidal	19
Dhariyapur	3-4-2010	+	2.08×0.62	1.25-0.83	Sperical	0
Chennur-2	3-4-2010	+	1.87×0.83	1.03-0.83	Irregular	30
B gudi	3-4-2010	+	3.49×0.63	1.66-0.83	Sperical	76
Karkalli -1	3-4-2010	+	3.32×0.83	1.45-0.83	tringular+ sperical	90
Thanegundi	3-4-2010	+	3.49×0.63	1.66-0.66	Sperical	100
Gogi	3-4-2010	+	3.32×0.83	1.25-0.83	Bipyramidal	80
Koudimatti	3-4-2010	+	2.45×0.83	1.25-0.83		72
Anaka-1	12-4-2010	-				0
Halesagar	12-4-2010	+	2.45×0.83	1.03-0.83	Bipyramidal	25
Yevur	12-4-2010	+	3.32×0.83	1.03-0.83	Irregular	90
Chennur-3	12-4-2010	+	3.32×0.82	1.03-0.83	Sperical	66
Hebbal-2	12-4-2010	+	3.32×0.83	1.25-0.83	Sperical	66
Hebbal-1	12-4-2010	-				0
Anak-2	15-4-2010	+	2.45×0.83	1.03-0.83	Bipyramidal	72
Karkalli-2	15-4-2010	-				0
Ursgundi	15-4-2010	+	3.32×0.83	1.25-0.83	Bipyramidal	25.92
Yadigiri	15-4-2010	-				0
Shantapur	15-4-2010	+	2.45×0.83	1.25-0.83	Bipyramidal	19.04
Mudnal	15-4-2010	-				0
Rubnallii	15-4-2010	-				0
Budnur	18-4-2010	+	1.66×0.66	1.03-0.83	Bipyramidal	20
Hothpet	18-4-2010	+	2.45×0.83	1.03-0.83	Bipyramidal	16
Yadagiri	18-4-2010	-				0
Kadamegara	18-4-2010	-				30
Hunasagi	18-4-2010	+	2.28×0.83	1.03-0.83	Bipyramidal+ triangular	25
Saidapur	18-4-2010	+	3.32×0.83	1.03-0.83	Bipyramidal+ triangular	36
Hattikuni	26-4-2010	+	3.32×0.83	1.25-0.83	Irregular	66.66
Bandhalli	26-4-2010	+	1.45×0.83	0.83-0.83	Irregular	27
Kakkera	26-4-2010	+	3.32×0.83	1.25-0.83	Bipyramidal	40
Basavanthapur	26-4-2010	+	1.66×0.62	0.83-0.83	triangular	33
Madnal	26-4-2010	-				0
Kanapur	26-4-2010	+	2.91×0.62	1.03-1.02	Bipyramidal	70
Vibhuthihalli	26-4-2010	+	1.45×0.62	1.03-1.03	Bipyramidal	18
Shirval	2-3-2010	+	3.32×0.83	1.25-0.83	Bipyramidal	25
Devapur	3-3-2010	+	3.32×0.83	1.25-0.83	Bipyramidal	20
Rastapur	5-3-2010	+	2.08×0.42	1.45-0.83	Bipyramidal	60.87
Kamanatagi	5-3-2010	+	2.08×0.43	1.45-0.84	Bipyramidal	50

Dornahalli	7-3-2010	+	3.32×0.83	1.25-0.83	Bipyramidal	6.6
Nagarahalli	7-3-2010	+	3.32×0.84	1.25-0.83	Bipyramidal	12.5
Kanchalakavi	7-3-2010	+	3.32×0.66	1.03-0.83	Bipyramidal	9.37
Yaddallli	10-3-2010	+	2.91×0.62	1.45-0.83	Irregular	6.6
Balakall	10-3-210	+	3.32×0.66	1.25-0.83	Bipyramidal	9.6
Nalagera	15-3-2010	+	2.91×0.62	1.03-0.83	Bipyramidal	6.2
Alameshwar	15-3-2010	+	3.32×0.83	1.25-0.83	Bipyramidal	12.5
Gundahalli	17-3-2010	+	2.91×0.62	1.03-0.83	Bipyramidal	3.2
Kanapur	17-3-2010	+	3.32×0.83	1.03-0.83	Bipyramidal	12.5
Jagondabhavi	20-3-2010	+	2.91×0.62	1.03-0.84	Oval	23.52
Vadagera	20-3-2010	+	2.91×0.62	0.83-0.66	Bipyramidal+ triangular	6.6
Managalli	20-3-2010	+	3.32×0.83	0.91-0.83	Bipyramidal	6.4
Malla	22-3-2010	+	2.91×0.62	1.03-0.83	Bipyramidal	38
Gaddada Tanda	22-3-2010	+	3.49×0.62	1.66-0.66	Bipyramidal	37.5
Shattghera	22-3-2010	+	3.49×0.63	1.66-0.66	Bipyramidal	12
Chmanl-1	24-3-2010	+	2.91×0.62	1.25-0.66	Bipyramidal	12
chamnal-2	24-3-2010	+	3.32×0.83	1.25-0.83	Bipyramidal+ triangular	22
Handarhal-1	26-3-2010	+	3.32×0.84	1.03-0.83	Bipyramidal	12
Handaerhall-2	26-3-2010	+	3.32×0.85	1.25-0.66	Bipyramidal	21.87
Sadypur-1	28-3-2010	+	3.49×0.63	1.66-0.66	Triangular	40
sadyapur-2	28-3-2010	+	2.91×0.62	1.25-0.66	Bipyramidal	28.12

Table 2. Probit analysis of spore concentration mortality response of *Plutella xylostella Bacillus thuringiensis* isolates

Isolate name	Regression equation Y=a+bx	$LC_{50}10^{-4}$ spores/ml	Fiducial limit at 95 % CI (10^{-4})	LC_{90} 10^{-6}	Chi square
Thanegundi	y=-2.24+0.6023x	1.9	0.3-7.9	2.56	7.09
Yevur	y=-1.42+0.45x	7.4	3.6-15.1	50.48	3.34
Karkalli-1	Y=-1.50+0.473x	6.7	3.3-13.1	34.03	2.89
Gogi	y=-1.15+0.402x	13.9	6.4-31.3	212.01	0.802

Table 3. Time mortality response of *Plutella xylostella* to different concentrations of *Bacillus thuringiensis* Thanegundi isolate

Conc	Regression equation	LT_{50}	LT_{50} at 95 % CI	Chi-square
1×10^{-1}	y= -1.36365+1.10021x	1.23	1.31-1.91	5.6
1×10^{-2}	y= -0.5105+2.972x	1.48	1.25-1.69	2.208
1×10^{-3}	y=-0.96557+0.44301x	2.17	1.78-2.51	0.134
1×10^{-4}	y=-1.1339+0.30548x	3.71	3.17-4.79	0.021
1×10^{-5}	y=-1.2179+0.2139x	5.69	4.35-11.78	0.224

Gogi isolate the LT_{50} values were 1.39,1.87, 3.7,5.66,11.98 days respectively (Table 6).

There was a direct relationship between mortality and cell concentration and inverse relation between cell concentration and time taken. The plot of regression showed increase in mortality with increase in cell concentration. Conversely, time taken for mortality increase as cell concentration decreases. The Chi-square test showed homogeneity of test population which is reflection of a goodness of fit.

Table 4. Time mortality response of *Plutella xylostella* to different concentrations of *Bacillus thuringiensis* Yevur isolate

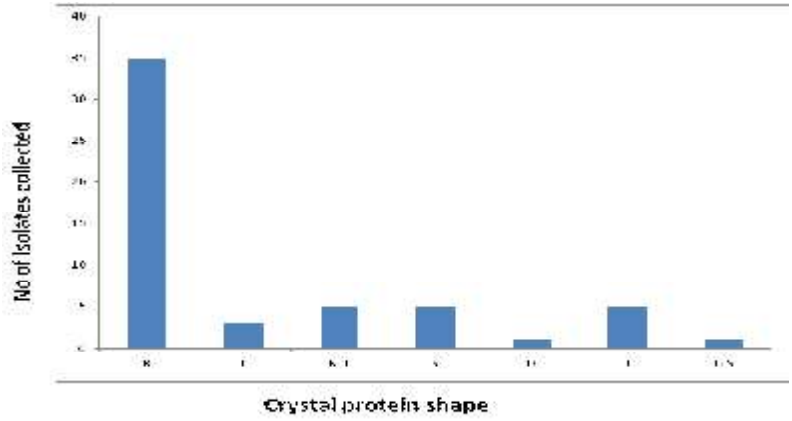
Conc	Regression equation	LT_{50}	LT_{50} at 95 % CI	Chi-square
1×10^{-1}	y=-0.36808+2.96x	1.33	1.1-1.53	1.33
1×10^{-2}	y=-0.5951+2.19x	1.87	1.55-2.19	0.059
1×10^{-3}	y=-0.903+1.66x	3.51	2.82-5.16	0.279
1×10^{-4}	y=-1.14+1.37x	6.74	4.44-23.05	0.335
1×10^{-5}	y=-1.23+1.10x	13.34	6.29-736.700	0.014

Table 5. Time mortality response of *Plutella xylostella* to different concentrations of *Bacillus thuringiensis* Karkalli-1 isolate

Conc	Regression equation	LT_{50}	LT_{50} at 95 % CI	Chi-square
1×10^{-1}	y=-0.4975+3.30x	1.41	1.21-1.60	2.33
1×10^{-2}	y=-0.5820+2.21x	1.83	1.51-2.41	0.357
1×10^{-3}	y=-0.841+1.62x	3.31	2.67-4.79	0.422
1×10^{-4}	y=-1.18+1.53x	5.91	4.16-14.44	0.216
1×10^{-5}	y=-1.4649+.233x	6.28	4.77-12.91	0.02

Table 6. Time mortality response of *Plutella xylostella* to different concentrations of *Bacillus thuringiensis* Gogi isolate

Conc	Regression equation	LT_{50}	LT_{50} at 95 % CI	Chi-square
1×10^{-1}	Y=-0.3554+2.475x	1.39	1.11-1.63	0.309
1×10^{-2}	Y=-0.5673+2.078x	1.87	1.53-2.201	0.059
1×10^{-3}	Y=-0.9963+1.754x	3.70	2.98-5.43	0.422
1×10^{-4}	Y=-1.304+1.732x	5.66	4.15-11.48	0.602
1×10^{-5}	Y=-1.362+1.264x	11.98	6.20-184.98	0.21



B: Bipyramidal, T: Triangular, B+T : Bipyramidal + Triangular, S : Spherical, O : Ovid, I : Irregular, T+S: Triangular+ Spherical

Fig. 1. Shape of parsporal bodies

DISCUSSION

The greatest successes in microbial pesticides have come from the uses of *Bacillus thuringiensis* (*B. t.*). Commercial preparations of *B. thuringiensis* have been shown to be the most successful biological control products worldwide (Carlton, 1988).

The isolation of *B. thuringiensis* was carried out according to the method described by Travers *et al* (1989) with modification. The modification was based on the results of Andrezejczak and Lonc (2008) who used NaCl in the protocol for isolation and enhanced temperature duration from 3 min to 20 min, moreover used L-syrine amino acid for selective isolation, which allows to grow only *B. thuringiensis*. We have devised a method to isolate *B. thuringiensis* was different from the earlier workers in that temperature for heating was increased from 3 to 20 min at 80 ^{o}C and additionally, Nacl was used to improve the isolation efficacy. Finally the solution was plated on to the M9 media supplemented with L-syrine aminoacid at 0.20mM. Similarly Singer and Rogoff (1986) provided evidence that L-syrine amino acid inhibit *B. thuringiensis* growth. Contrastingly Lachowicz et al (1996) reported that *B. thuringiensis* strains were completely resistance to the inhibitory action of L-serine.

B. thuringiensis isolates were collected from soil samples of rice fields in UKP area. As many as 72 soils were collected among them 56 soils produced *B. thuringiensis* isolates. The isolates collected showed varied morphology. This was confirmed by subjecting *B. thuringiensis* isolates to micrometer by calculating cell size and spore size. The cell sizes varies from 1.45x0.83 µm to 3.49x0.63 µm where as endospore size varies from 0.83-0.66 µm to 1.66-0.66 µm. This in line with reports of Das et al (2008) who isolated *B. thuringiensis* from saline soils of three rice fields and two fallow lands of coastal Orissa, India. Spore-forming bacteria were relatively more abundant with 1.00- 21.2 x10^5 CFU/gram soil. Similarly Chatterjee *et al* (2007) and Thaphan *et al* 2008.

The shape of the parasporal bodies of the collected bacteria also varied viz bipyramidal, irregular, triangular, spherical and ovoid. Among them 64 % of collected bacterial isolates produced bipyramidal shape crystal protein and other isolates produced ovoid to irregular shape. The results are in line with the reports of Chatterjee *et al (*2007) who classified *B. thuringiensis* isolates, out of 11 isolates, 3 were polymorphic, 7 were spherical and 1 was bipyramidal type of crystal. Similarly Das *et al* (2008) isolated and classified.

The isolates of Thanegundi, Karkalli, Gogi and Yevur were used for conducting bioassay to establish LC_{50} and LT 50. Among the isolates collected Thanegundi was most virulent with lowest LC_{50} value of 1.9x10^{-4}. This was followed by Karkalli , Yevur and Gogi isolates. Simultaneously the LT_{50} values were also calculated. As the concentration of bacteria increase from 1x10^{-1} to 1x10^{-5}, LT_{50} values were also increased, this reveals that time taken to kill 50 per cent population increases as concentration decreases. The LT_{50} for Thanedundi isolate was lowest and this was followed by Karkalii, Yevur and Gogi isolates. The virulence of Thanegindi isolate may be because of production of more quantity of toxic protein and specific protein. The results are in line with reports of Thaphan *et al* (2008) who tested *B. thuringiensis* isolates against *Plutella xylostella* larvae and found that JCPT74 isolate was the most effective against *P. xylostella* with LC_{50} of 1.13x10^2 spore/ml. Similarly, results are also in accordance with Chanpaisaeng *et al*. (2001).

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