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Diversity and Toxicity of *Bacillus thuringiensis* from Shifting Cultivation (Jhum) Habitat

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Bacillus thuringiensis (*Bt*) strains were isolated from jhum-agriculture, jhum-forest, aquatic and fallow soil samples from Mizoram by acetate selection method. Isolates were characterized for biochemical typing, cry gene and protein profiling, growth curve study and toxicity against *Culex tritaeniorhynchus*. *Bt* frequency was high in jhum-agriculture land (69.56%) whereas low in jhum-forest soils (31.57%). *Bt* was found to be abundant in jhum shifting cultivation soil with an index ranging between 0.010 and 0.015. Majority of the isolates from jhum soils produced oval and spherical crystals and showed eleven types of crystal proteins groups. PCR analysis revealed predominance of dipteran-active cry genes (*cry4* and *cry9*). The variations in crystal morphology, cry genes and Cry protein(s) from the isolates of *Bt* revealed molecular diversity. Higher mortality, lower lethal dose, and lesser time to kill were observed in *Bt* isolates from jhum soils than aquatic and fallow habitats. Based on the toxicity test, SC1 and HP7 isolates containing *cry 4* and *cry 9* genes showed higher activity. Growth curve analysis showed significant variations among *Bt* isolates to reach the sporulating stage. Higher growth index and lower mean generation time were observed in SC1 and HP7 *Bt* isolates. *Bt* strains express different endotoxin genes and crystal proteins and their harvesting time also varied from strain to strain. Significant variation was found in *Bt* isolates from jhum habitats in relation to the cry gene composition, protein profiling and toxicity. Results from this study suggest that novel *Bt* entomopathogens may complement for regulating mosquito vectors.

Key words : cry gene / Cry protein / Native *Bacillus thuringiensis* isolates / Shifting cultivation / *Bacillus thuringiensis* toxicity.

INTRODUCTION

Shifting cultivation or slash and burn agriculture (locally called as Jhum) is the main form of agriculture in the hills of North-east India and is strongly based on traditional knowledge. The jhum cycle (the intervening period between two successive slashes, 10-15 year cycle) got shortened in order to meet the food demand which resulted in decrease in crop yield. This necessitated converting more virgin forest areas to wastelands as a result of repeated jhum having very short cycles (2-3 years) (Tripathi and Barik, 2003).

Bacillus thuringiensis (*Bt*) is useful as microbial control

of pest insects and the entomopathogenic activity of *Bt* is mainly due to insecticidal crystal proteins (ICPs) during sporulation (Feitelson et al., 1992). Each type of crystal protein has a specific host range and based upon differences in sequence, ICPs (over 300 cry genes) have been classified into 40 families, 55 groups and different subgroups (Crickmore et al., 2012; Schnepf et al., 1998). Although there is some correlation between subspecies and pathogenicity, at the moment it is known that strains of different subspecies may be toxic to insect of various orders, and even that strains from the same subspecies show differences in toxicity. The toxicity spectrum of *Bt* subspecies is determined by the different ICPs (cry genes) carried by their strains, and by the encoded proteins (Cry proteins) (Hofte and Whiteley, 1989); thus assessment of Cry proteins is a good basis

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to study insecticidal activity of *Bt* and is an important component of studying *Bt* resources. *Bt* shows genetic diversity with different toxic potential (Crickmore et al., 2012; Bernhard et al., 1997; Quesada-Moraga et al., 2004) and each habitat may contain a novel *Bt* strain which is toxic on a target insect group. Therefore, large screening programs, leading to important collections of isolates, have been conducted worldwide from different environments and characterized to evaluate their toxic potential against various insect orders (Bernhard et al., 1997; Quesada-Moraga et al., 2004; Ramalakshmi and Udayasuriyan, 2010; Uribe et al., 2003). *Bt* strains are characterized in a number of ways such as biochemical typing (Martin et al., 1985), flagellar serotyping (Chilcott and Wigley, 1993), profiling plasmid arrays or proteins, use of monoclonal antibodies and hybridization or PCR amplification, based on sequences of known *cry* genes (Porcar and Juarez-Perez, 2003) or by molecular fingerprints (Zara et al., 2006).

Mizoram ($21^{\circ} 57' - 24^{\circ} 30'$ North and $92^{\circ} 15' - 93^{\circ} 26'$ East) is a part of the 34 mega-biodiversity hotspots of the world (Indo- Burma global biodiversity hotspot). The altitude, temperature and annual rainfall range from 500 to 2157 m, 7° to 34°C and 2,000-4,000 mm, respectively (Myers et al., 2000) which gives unique geographical features and abundant biological resources. It is necessary to search for natural strains and toxins in jhum shifting cultivation regions of Mizoram (Northeast India), since a significant number of pests are not controlled with the available pest management programs. Hence, the present study was carried out to explore diversity of *Bt* present in jhum shifting cultivation soil samples of eight districts in Mizoram state, India, with the objectives: i) the isolation and distribution of *Bt* from soils of different habitats; ii) determination of crystal protein composition by microscopy studies and biochemical typing for rapid screening; iii) insecticidal *cry* gene determination by PCR; and iv) SDS-PAGE method in spore-crystals mixture to determine the distribution, diversity and toxicity; v) toxicity analysis against *Culex tritaeniorhynchus* and vi) growth rate and generation time studies to standardize the conditions for *Bt* production.

In the present study the following questions were addressed: 1) Is *Bt* present in the soils of shifting cultivation habitat? 2) What type of crystals were produced by *Bt* isolates? 3) Which *cry* gene was predominant? 4) How many types of crystal proteins present in the *Bt* isolates? 5) What is the degree of toxicity of the *Bt* isolates against mosquito vector, *C. tritaeniorhynchus*? 6) Is there any variation in growth curve pattern among *Bt* isolates?

MATERIALS AND METHODS

Soil samples

A total of 55 soil samples from four different habitats [jhum forest, jhum agriculture land, aquatic, and fallow] covering eight districts (Champhai, Lunglei, Mamit, Kolasib, Aizawl, Serchhip, Saiha, Lawngtlai-geographic coordinates $21^{\circ} 57' - 24^{\circ} 30'$ North and $92^{\circ} 15' - 93^{\circ} 26'$ East) in Mizoram, India (Fig.1) were used for isolation of *Bt*. No commercial *Bt* based product had been used in any of the sampled areas. All the soil samples (200 g) were collected aseptically from top to a depth of 10 cm after scrapping off the surface material with a sterile spatula. Samples were stored in labeled sterile plastic bags at room temperature until processed.

FIG. 1. Details of soil sample collection from thirty nine spots covering eight districts in Mizoram state, India for the isolation of *Bt*.

1, Champhai; 2, Bungtlang; 3, Khawzawl; 4, Rih Dil; 5, Kolasib; 6, Thingdawl; 7, Bairabi; 8, Dawrpui; 9, Tanhril; 10, Chanmari West; 11, Chawlhmun; 12, Zotlang; 13, Chawnpui; 14, Kannan; 15, Vaivakawn; 16, Zarkawt; 17, Ramrikawn; 18, Seling; 19, Sailam; 20, MZU Campus; 21, Sairang; 22, Sihhmui; 23, Tuivamit; 24, Serkawn; 25, Lunglei; 26, Zobawk; 27, Chhingchhip; 28, Chhiahtlang; 29, Thenzawl; 30, Serchhip; 31, Khengkawn; 32, Saiha; 33, Lawngtlai; 34, Chhintuipui; 35, Lengte; 36, Lengpui; 37, West Phaileng; 38, Rawpuichhip; 39, Chhippui.

Isolation and maintenance of *Bt* isolates from soil samples

Acetate selection method (Travers et al., 1987) was used to isolate the *Bt* strains. One gram of soil sample was suspended in 10 mL of Luria Bertani (LB) broth (Yeast extract 5 g/L, trypton 10 g/L, sodium chloride 5g/L, pH 7.0-7.2) buffered with 0.25 M sodium acetate and incubated for 4 h. After incubation, the sample was subjected to heat treatment at 80°C for 3 min. Serial dilutions (up to 10^{-4}) were made and 20 μ L of each serial dilute was spread on LB agar to observe *Bt* colonies. The colonies resembling *Bt* (cream-colored and have the appearance of a fried egg on a plate) were selected and gram stained, sub-cultured as ribbon streak (four colonies per plate) on T₃ agar medium (tryptone-3 g, tryptose-2 g, yeast extract-1.5 g, sodium phosphate-0.05 M, manganese chloride-0.005 g, agar-15 g, distilled water-1000 mL, pH-6.8). After 72 h of incubation crystal staining was done (Chilcott and Wigley, 1988).

The isolates showing the presence of crystalline inclusions were selected as *Bt* and streaked on T₃ agar medium for single colony purification. Broth culture was made from the isolated single colonies of crystal positive *Bt* isolates. Glycerol stocks were prepared using 24 h old broth culture and stored at -70°C for further studies. Standard strains of *Bt* subsp *kurstaki* (*Btk*-4D1), *Bt aizawai* (*Btai*-4J3), *Bt israelensis* (*Bti*-4Q1) and *Bt alesti* (*Btal*-4C1) were supplied by Bacillus Genetic Stock Center (The Ohio State University, Columbus, Ohio) and used as reference.

Morphological, physiological and biochemical characterization of *Bt* and *Bt* index

Morphological characters of the *Bt* colonies were studied following the standard microbiological methods (Pelczar et al., 1957). The isolates were streaked on nutrient agar plates, incubated for 72 h at $30 \pm 0.1^\circ\text{C}$. The shape, size, colour, margin and opacity were recorded from isolated colonies. Morphology of the 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 (spore and crystal staining, anaerobic growth, indole production, Vogues Proskauer, raffinose, catalase, D-mannitol, nitrate, esculin, tryptophan, xylose, arginine dihydrolase, starch, gelatin and casein hydrolysis) were studied. Biochemical and physiological tests were observed in 24 h cultures. Endospore and crystal protein were observed after 72 h under a phase-contrast microscope. The *Bt* index was calculated for each sample as the number of isolates of *Bt*/number of isolates of sporulated bacilli.

Biochemical typing

Four highly relevant biochemical tests including esculin utilization, acid formation from salicin and sucrose, and lecithinase production (Table 2) were used to subdivide the crystal-forming bacteria into eighteen biochemical types including six undescribed combinations (Martin and Travers, 1989).

Isolation of spore-crystal mixture from *Bt* isolates

The spore-crystal mixture was isolated from the *Bt* strains along with four standard strains. For each *Bt* strain, a single colony was inoculated into 5 mL T₃ broth and incubated in a rotatory shaker, maintained at 30°C at 200 rpm for nearly 96 h, and the bacterial sporulation was monitored through a phase contrast microscope. After 96 h, the *Bt* cells were found to be lysed and crystal toxins were released into the medium. The sporulated broth containing spore-crystal mixture was centrifuged for 10 min at 10,000 *g* at 4°C. The pellet was washed once with 5 mL of ice-cold Tris-EDTA buffer [Tris 10 mM, EDTA 1 mM, pH 8.0 with 1 mM Phenyl methyl sulphonyl fluoride-PMSF], once with 5 mL of ice-cold 0.5 M NaCl followed by two more washes with 5 mL of Tris-EDTA buffer with 0.5 mM of the protease inhibitor PMSF by centrifuging at the same speed and time. Finally, the spore-crystal pellet was suspended in 100 μ L of sterile distilled water containing 1 mM PMSF and stored in -20°C (Laemmli, 1970).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein content of spore-crystal mixture (5 μ L) of *Bt* isolates were analyzed by SDS-PAGE (Laemmli, 1970) using 10% acrylamide separating gels. Samples (15 mg) of washed spore-crystal mixtures were placed in 2 $\times$ concentrated sample buffer and heated at 80°C for 10 min and loaded onto the gel immediately before electrophoresis. Gels were stained in a solution containing 50% (v/v) ethanol, 10% (v/v) acetic acid and 0.1% (w/v) Coomassie brilliant blue R250 for 40 min, and then destained in a solution containing 6.75% (v/v) glacial acetic acid and 9.45% (v/v) ethanol.

Dendrogram and cluster analysis

Protein markers were scored in a binary form as presence or absence of protein bands (respectively, 1 and 0) for each sample. Cry protein profile data was used to construct a dendrogram following the method of Neighbour joining (NJ). Nei's genetic distances were calculated between each pair of the 27 *Bt* isolates using the Binary data. The genetic distance matrix was used to generate a phylogenetic dendrogram using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Consistency of tree was checked by a bootstrap value

of 1000 at 95% confidence intervals using NTSYSpc 2.1 software. The genetic similarity matrix of twenty seven *Bt* isolates was estimated using Jaccard's coefficient and was run on SAHN using the NJ clustering algorithm to generate dendrogram. All computations were performed using the NTSYSpc 2.1 (Rohlf, 1998).

Larvicidal bioassay

Mosquito maintenance

Culex tritaeniorhynchus larvae were collected from a fish pond in Lengpui, Aizawl, Mizoram and reared at the Department of Biotechnology, Mizoram University, Aizawl, Mizoram (Martin and Travers, 1989). The third instar larvae used in these assays belonged to the second generation and were maintained at $27 \pm 2^\circ\text{C}$ with $65 \pm 5\%$ relative humidity (RH) and 12 h light (12:12 L : D).

Selective assay

Bioassay was carried out in a biological oxygen demand chamber, under the same conditions described above. One hundred and seven *Bt* isolates were collected from different soil ecosystem of Mizoram. They were grown in 50 mL of nutrient broth yeast extract and salt medium (NYSM- a medium composed of nutrient broth, yeast extract, MnCl_2 , MgCl_2 and CaCl_2) medium, incubated at 37°C for 72 h at 200 rpm. The suspension was centrifuged at 5,000 rpm and washed 3 times with sterilized water. The concentration of bacterial spores was determined in a Neubauer (double improved) chamber using phase contrast microscopy at $400\times$. The number of cells was counted in each of the 4 corner squares of the counting chamber. The normal error in Neubauer chamber counting was less than 10%. Viable cells were counted from 1 mL of each of the samples from NYSM medium and serially diluted up to 1/1000 with milliQ water. The diluted sample (100 μL) was spread onto the surface of LB-agar poured into petri dishes and incubated at 37°C for 24 h. One mL of the broth containing 8×10^5 cells/mL were added to 250 mL deionized water previously put in a 500 mL glass beaker (10×5 cm), where larvae of third instar of *C. tritaeniorhynchus* were individualized (50 insects per isolate). In controls, the broth was replaced by 100 μL of sterile deionized water (WHO, 1996). The mortality was evaluated up to seven days after treatment. The data were corrected (Abbot, 1925) and submitted to Tukey's multiple range test ($P = 0.05$) (Snedcor and Cochran, 1989).

Toxicity assay-Concentration response bioassay (LC_{50})

Twenty five *Bt* isolates were chosen for LC_{50} bioassay based on the mortality obtained in the selective assay.

A concentration-response bioassay was carried out for assessing mortality of *C. tritaeniorhynchus* third instar larvae upon *Bt* exposure (along with reference strains- *Btal* 4C1, *Btk* 4D1, *Bti* 4Q1 and *Btai* 4J3) using three replicates of 50 third instar larvae for each *Bt* isolate concentration (50 insects $\times$ 9 concentrations including control $\times$ 3 replicates = 1350 insects per *Bt* isolate). Eight *Bt* concentrations were tested (8×10^4 , 1×10^4 , 8×10^3 , 1×10^3 , 8×10^2 and 1×10^2 , 8×10^1 and 1×10^1 cells/mL), in addition to a control with water only to correct the data for natural mortality. To achieve the initial concentration of 8×10^4 and 1×10^4 cells/mL, the above mentioned method in selective assay for cell counting was used. All other cell concentrations were obtained through dilutions of this suspension. The exact number of spores was determined in a Neubauer chamber. The amount of suspension and the method for growth of microorganism were the same as in selective assays. These concentrations were tested to allow establishing a concentration-response (causal) relationship as evidence of direct insecticidal activity, if existent. The mortality was monitored for seven consecutive days. Lethal concentration (LC_{50}) was calculated by probit analysis (Finney, 1971).

Toxicity assay-Time-mortality bioassay (LT_{50})

The acute lethal toxicity of twenty five *Bt* isolates, *Btal* 4C1, *Btk* 4D1, *Bti* 4Q1 and *Btai* 4J3 to the third instar larvae of *C. tritaeniorhynchus* was assessed in relation to time. A concentration of 8×10^4 *Bt* cells/mL along with untreated control with water were used to estimate lethal time (LT_{50}). Three replications were carried out (50 insects $\times$ 2 concentrations including control $\times$ 3 replicates = 300 insects per *Bt* isolate). Lethal time (LT_{50}) was calculated by probit analysis (Finney, 1971).

cry gene detection by PCR technique

Total DNA was extracted from the *Bt* strains and was used as a template for PCR (Table 2). The cultures were incubated overnight in LB agar at 37°C . After 16-20 h one loop full of culture was transferred to 300 μL of milliQ water, vortexed and kept in -80°C for 15 min. The frozen cells was immediately transferred to boiling water and kept for 10 min (Ben-Dov et al., 1997). The resulting cell lysate was spun at 6000 rpm for 3-4 s. The supernatant was used as the DNA template. Identification of lepidopteran, dipteran and coleopteran *cry* genes (*cry1*, 2,3,4 and 9 with expected product size of 276, 700, 600, 430, 350 bp, respectively) was done using the following PCR reaction mixture: *Bt* DNA (250 ng), Buffer ($1 \times$ Tris - 20 mM with 1.5 mM MgCl_2), Taq DNA polymerase (1 U), deoxynucleoside triphosphate (0.2 mM each), and forward and reverse primer (0.5 mM of specific primer for *cry* genes) (Biosciences, India) (Ben-

Dov et al., 1997). Amplification was done in an Eppendorf thermal cycler under the following conditions: 3 min of denaturation at 94°C followed by 30 cycles of amplification with a 1 min denaturation at 94°C, 45 s of annealing at 54-62°C, and 30 s of extension at 72°C. A final extension step of 5 min at 72°C was added after completion of the 30 cycles. PCR products were analyzed by 1.5% agarose gel electrophoresis in Tris-borate-EDTA (TBE) buffer (0.6 g of agarose was dissolved in 40 mL of 1 × TBE buffer and melted in micro wave oven for 1-2 min) and stained with 10 mg/mL ethidium bromide (Sambrook et al., 1989). DNA samples were run at 50 volts and PCR products were visualized under UV transilluminator and the sizes of the fragments were estimated based on a DNA ladder of 100 base pairs. The *Bt* isolates were compared with standard strains. The *cry* primers used were obtained from Ben-Dov et al. (1997). Nucleotide and deduced amino acid sequences were analyzed with the Blast tools (www.ncbi.nlm.nih.gov/BLAST). BioEdit (version 7.0.4.1) was used for sequence editing and analysis.

Growth curve studies

Growth curve study was performed, to value the mass scale production of effective *Bt* isolates having 70-100% mortality, in ten representative *Bt* isolates along with three reference strains (Table 5). LB broth (10 mL) was inoculated with 3 µL of 24 h old *Bt* culture and incubated at 37°C for overnight. After 12 h, LB broth containing *Bt* was subjected to turbidimetric observations at regular intervals (2 h) and OD was measured at 600 nm. OD readings were taken for all the *Bt* cultures for 48 h. The data was plotted on a graph with OD vs time, and the vegetative growth phase which is equivalent to the exponential phase was determined (Pelczar et al., 1957). Both time (h) and absorbance were plotted on the graph and absorbance on a logarithmic scale. For the growth rate formula two points on the straight line drawn were chosen through the exponential phase and made note of the time interval between them (t). Two points were chosen for which the logs are obtained (Higher CFU/mL = X_t = at final hours of exponential hours; Lower CFU/mL = X_0 = at initial hours of exponential phase; Time interval (in h) between the 2 points = t). Growth rate constant (μ), mean generation time (g) and growth rate index (C) were calculated (Pelczar et al., 1957) as follows: 1) calculation of growth rate constant (μ). It is the number of generations (doublings) per hour was found as Growth rate constant (μ) = $[\log_{10}N_t - \log_{10}N_0] \times 2.303 / [t_t - t_0]$ where, N_0 - Initial OD value of exponential phase; N_t - Final OD value of exponential phase; t_t - final time and t_0 - initial time, 2) calculation of mean generation time (g): It is the time it takes for the population to double was calculated by

using the following formula: Mean generation time (g) = $[\log_{10}N_t - \log_{10}N_0] / \log_{10}2$, where, N_0 - initial OD value of exponential phase; N_t - final OD value of exponential phase, 3) calculation of growth rate index (C): Growth rate index, C, is a measure of the number of generations (the number of doublings) that occur per unit of time in an exponentially growing culture. $C = \ln 2 / g$, where $\ln 2$ is the natural log of 2 (0.693) and g is the time in hours taken from the population to double during the exponential phase of growth.

RESULTS AND DISCUSSION

Isolation of *Bt* from soil samples

Bt isolates from four different habitat soils (jhum-forest, jhum-agriculture, aquatic and fallow) were isolated by acetate selection process from 39 locations in eight districts of Mizoram (Fig.1). Acetate inhibited the germination of *Bt* spores allowing other spores to germinate, while the growing cells and other non-spore-forming bacteria were eliminated by heat treatment. *Bt*-like colonies, which are usually described as cream-colored and have the appearance of a fried egg on a plate, were labeled and sub-cultured (Fig.2A). Out of 55 soil samples, *Bt* isolates were obtained from 29 soil samples (52.72% frequency) as shown in Table 1. The highest frequency of *Bt* was recorded in aquatic soil samples (71.42%), while 33.33% in fallow land samples. Between jhum cultivation habitats, jhum-agriculture soil registered

FIG. 2. Morphological characterization of *Bt* isolates. (a) Fried egg colony morphology (b) *Bt* crystals and (c) *Bt* spores.

TABLE 1. Distribution of (*Bt*) isolates from soil sample collected from different habitats.

S. No.	Habitat	Location	Number of samples			Number of colonies		
			Examined	<i>Bt</i>	%	Total	With <i>Bt</i>	<i>Bt</i> index [@]
1	Jhum-Forest (under jhum cycle and abandoned)	MZU Campus, Kolasib, Chanmari West, Chawlhmun, Khengkhang, Tanhril, Zotlang, Chawnpui, Kannan, Serkawn, Lunglei, Zobawk, Lawngtlai, Chhingchhip, Chhiahtlang, Bungtlang, Khawzawl, Lengte, Chhimtuipui	19	6	31.57	1731	24	0.014
2	Jhum-Agriculture land	Lunglei, Kolasib, Dawrpui, Thenzawl, Lengpui, Champhai, Sailam, Serchhip, Tuivamit, MZU Campus, Saiha, Hmunpui, Bairabi, Seling, West Phaileng, Rawpuichhip, Chhippui, Sairang, Sihmui	23	16	69.56	5099	58	0.012
3	Aquatic	Chhimtuipui, Lengpui, Ramrikawn, Kolasib, Thenzawl, Rih Dil	7	5	71.42	1273	19	0.015
4	Fallow	MZU Campus, Lunglei, Vaivakawn, Zarkawt, Ramrikawn	6	2	33.33	573	6	0.010
Total			55	29	52.72	8676	107	0.012

@The *Bt* index was calculated for each sample as the number of isolates of *Bt*/number of isolates of sporulated bacilli.

69.56% while 31.57% in jhum-forest soils. The overall *Bt* index of the soil samples was 0.012 (Table 1).

Soil samples from jhum, aquatic and fallow habitats were used as a source material for isolation of native *Bt* strains which led to the discovery of isolates with higher toxicity (Ramalakshmi and Udayasuriyan, 2010; Martin and Travers, 1989; Liang et al., 2011; Pinto et al., 2012; Xue et al., 2008). Results showed that about 52.38% of the 42 samples were positive for *Bt* and yielded 82 isolates in jhum-forest and jhum-agriculture soils. Earlier studies reported varied frequency for isolation of *Bt* from soil samples ranging from 3 to 85% (Ramalakshmi and Udayasuriyan, 2010; Martin and Travers, 1989; Wang et al., 2003). Shifting cultivation is responsible for loss of soil nutrients, lowers soil acidity, organic matter and total nitrogen, but enhances phosphorous and cations (Tawnenga et al., 1997). But, in the present study, moderate frequency (52.38%) of *Bt* was observed in jhum soils, allowing optimum survival and growth of *Bt*. This is the first report of characterization of *Bt* in jhum cultivation soil. Soil is a very important source of *Bt* providing a large genetic resource for its use in the development of bioinsecticide to control insect pests (Quesada-Moraga et al., 2004; Liang et al., 2011). The *Bt* index from Mizoram ranged from 0.010 to 0.015 in the soil samples studied. The *Bt* index ranged from 0 to 0.2 in United States (DeLucca et al., 1981), 0.2 to 0.5 in New Zealand (Chilcott and Wigley, 1993), 0.75 in Bangladesh (Hossain et al., 1997), 0.009 to 0.380 in Thailand (Martin and Travers, 1989) and 0.15 to 0.18 in Western Ghats, India (Mahadevaswamy et al., 2013;

Ramalakshmi and Udayasuriyan, 2010).

Biochemical typing of *Bt* isolates

Using the biochemical typing method, all the *Bt* strains isolated were divided into eleven biochemical types (Table 2). In some cases, undescribed combinations of biochemical types (six numbers) were yielded which were referred to by numbers. Among the biochemical types, *israelensis* subspecies was observed at higher frequency (30.84%) and undescribed biochemical type combinations ranged from 3.73-14.0% (Table 2). Biochemical characterization and typing were used as a method for rapid screening of large numbers of samples and identification of *Bt* for further molecular characterization (Martin and Travers, 1989).

Biochemical characterization and morphology of crystalline inclusions in *Bt* isolates from jhum cultivation

All the one hundred and seven isolates were rod shaped, gram positive, having both spores and crystals, positive to catalase, urease, VP, and negative to indole, arginine dihydrolase and tryptophan. All the *Bt* isolates reduced nitrate to nitrite, hydrolysed starch to glucose, utilized glucose for growth, tolerated 5% NaCl and did not ferment D- mannitol. They were able to produce amylase and protease due to which starch hydrolysis occurred forming a clear zone around the colonies (Data not shown). All the isolates were crystal forming rods. The shape of the crystalline inclusions varied among the *Bt* isolates. Based on crystal morphology, the 107 isolates

TABLE 2. Biochemical typing of (*Bt*) strains from jhum cultivation^a.

Biochemical type (described subspecies)	Biochemical and physiological test result ^b				Number of <i>Bt</i> isolates and (% frequency)
	Hydrolysis of		Utilization of		
	Esculin	Lecithin	Sucrose	Salicin	
<i>thuringiensis</i>	+	+	+	+	5 (4.67)
<i>aotto</i>	+	+	+	—	0 (0)
<i>alesti</i>	—	+	+	+	0 (0)
<i>kurstaki</i>	+	+	—	+	4 (3.73)
<i>aizawai</i>	+	+	—	+	4 (3.73)
<i>indiana</i>	+	—	+	+	0 (0)
<i>dendrolimus</i>	+	+	—	—	1 (0.93)
<i>galleriae</i>	+	—	—	+	3 (2.80)
<i>morrisoni</i>	+	—	+	—	1 (0.93)
<i>darmstadiensis</i>	+	—	—	—	0 (0)
<i>israelensis</i>	—	+	—	—	33 (30.84)
<i>ostrinae</i>	—	—	—	+	0 (0)
1*	—	+	+	—	10 (9.34)
2*	—	—	—	—	10 (9.34)
3*	—	—	+	—	15 (14)
4*	—	+	+	—	4 (3.73)
5*	—	+	—	+	12 (11.21)
6*	—	—	+	+	9 (8.41)

^a Martin and Travers, 1989 (modified).^b (+) Positive reaction; (—) Negative reaction.

* undescribed combinations of biochemical types.

of *Bt* fall into two major groups: spherical (49.53%) and oval (34.57%) (Fig.2B, 2C and Table 3). Crystal morphology of *Bt* can provide valuable information on target insect spectra (Maeda et al., 2000). *Bt* strains produce ICPs with different morphologies as bipyramidal crystals, related to Cry 1 proteins; cuboidal inclusions, related to Cry 2 proteins; flat and square crystals, related to Cry 3 proteins; and amorphous and composite crystals, related to Cry 4 and Cry 9 proteins (Ohba and Aizawai, 1986; Hernstand et al., 1986). In the present study, 107 bacterial colonies showed the presence of crystalline inclusions and were characterized into two major groups viz., spherical and oval. These findings differ from the earlier reports, wherein strains with bipyramidal (46%) and cuboidal (26.9%) crystals were predominant (Bernhard et al., 1997; Ramalakshmi and Udayasuriyan, 2010; Martin and Travers, 1989). The morphology of crystalline inclusions of *Bt* suggested predominance of spherical and oval in the *Bt* isolates from jhum soils. The diversity observed in crystal morphology for a given isolate and between isolates could be related to the presence of novel endotoxins (Mahadevaswamy et al., 2013).

Toxicity, cry gene and protein profiling of *Bt* from jhum soils

Bt isolates obtained from the jhum soils were studied for crystal protein profile(s) by SDS-PAGE (Tables 3, 4 and Fig.3). The reference strains, 4C1, 4D1, 4Q1 and 4J3 showed two crystal proteins particularly 135 and 65 kDa. The new isolates of *Bt* from jhum soil showed eleven different types of crystal protein profiles. Sixty isolates exhibited three major polypeptide bands with molecular weights in the range of 65 kDa (18.69%), 135 kDa (16.82%) and both 65 and 135 (20.56%) kDa as in the case of reference strains. Whereas, 19 isolates belonging to groups I – III (43 kDa, 30 kDa and both 43 and 30 kDa) were observed at a lower frequency (between 3.73 and 8.41%). Four *Bt* isolates did not show any distinct band of crystal protein(s). Based on the protein profile analysis, a dendrogram was obtained by UPGMA clustering method. Two major clusters were observed showing high similarity among the *Bt* isolates (Fig.4). Both the clusters represent different isolates with varying toxicity. The profiles of all PCR products were compared with those of standard strains. An isolate was considered to contain a determined gene only when

TABLE 3. Grouping of (*Bt*) strains based on Cry protein, cry gene and types of crystalline inclusions in jhum shifting cultivation habitats.

Cry Protein profiling*			Crystal morphology (%) *
Group number	Molecular weight of crystal protein(s) (kDa)	<i>Bt</i> strain (%)	
I	43	8.41	Spherical (49.53)
II	30	3.73	
III	43,30	5.60	
IV	65	18.69	
V	135	16.82	
VI	65,135	20.56	Oval (34.57)
VII	17, 60	8.41	
VIII	19	1.86	
IX	27,95	4.67	Bipyramidal (9.34)
X	14,25	4.67	
XI	22,106	2.80	
	No discrete band	3.73	Cuboidal (6.54)
cry gene profiling*			
Cry gene	No. of <i>Bt</i> isolates	Frequency (%)	
Cry 1, Cry 2, Cry 4	07	6.54	
Cry 1, Cry 4, Cry 9	06	5.60	
Cry 2, Cry 3, Cry 9	11	10.28	
Cry 2, Cry 9	11	10.28	
Cry 3, Cry 9	09	8.41	
Cry 4, Cry 9	15	14.01	
Cry 4	21	19.62	
Cry 9	27	25.23	

*Percent values calculated from a total of 82 *Bt* strains.

the amplification product was of the expected size (*cry1*, 2, 3, 4 and 9 with expected product size of 276, 700, 600, 430, and 350 bp, respectively). Analysis of the *Bt* isolates indicated that most contained *cry9* (25.23%), *cry4* (19.62%), as well as *cry4* and 9 (14.01%) genes. Twenty-two of the 107 isolates (10.28%) have a high tendency to occur in combinations of *cry 2*, 9 and *cry 2*, 3, 9 genes. The study of *cry* gene combinations in *cry* gene-containing isolates showed that 55.14% (59 isolates) harbored more than one type of *cry* gene (Table 3). The partial nucleotide sequences of the active *cry* gene obtained from jhum soils of Mizoram, Northeast India were submitted to NCBI GenBank database (Accession No. *cry 2A*-JN215465 and *cry 1*-JN596969). Pre-selective assays against *C. tritaeniorhynchus* showed that twenty five *Bt* isolates were pathogenic (60-100% mortality) which were used to determine the concentration response and time mortality response bioassays.

The isolates HP7 and SC1 showed 100% of mortality followed by CHP5, CAMP1 and RK1 with 95%, SE1 with 90%, SL1 with 85%, CHP1, KK1, LP1, RP1 and TZ1 with 80%, CHTP1, LL1, CH1, and RD2 with 75%, LP4, LT1, SK1, SL2, SR1 and WP1 with 70% and LP2, LP3 and SH1 with 60% of mortality at the fifth day after the treatment. The reference strains, *Btal* 4C1 and *Bti* 4Q1, showed 90% mortality, while 55% and 60% mortality was observed after *Btk* 4D1 and *Btai* 4J3 treatments (Table 4). The virulence assays showed that SC1 and HP7 were the most active isolates with an LC_{50} of 2.46×10^1 and 3.90×10^1 cells/mL, respectively. The median lethal time (LT_{50}) for *Bt* varied according to the concentrations (cells/mL) and was inversely proportional to the concentration. The LT_{50} for the 25 *Bt* isolates ranged between 2.36 and 8.75 d (Table 4). The pathogenicity of *Bt* isolates against *C. tritaeniorhynchus* was confirmed by presence of *Bt* cells in larval

TABLE 4. Cry gene and protein profiling in (*Bt*) isolates and their LC₅₀ and LT₅₀ against third instar of *Culex tritaeniorhynchus*.

Habitat	<i>Bt</i> source	Code	Cry gene & (crystal Morphology) [#]	Protein (kDa)	M* (%)	LC ₅₀ ** (cells/mL)	LT ₅₀ *** (days)
Reference-Soil	Bacillus Genetic Stock Centre, Ohio, USA	<i>Bt alesti</i> 4C1	Cry 1 (B)	25, 27, 34, 43, 65, 135	90	2.70 x 10 ²	4.11
		<i>Bt kurstaki</i> 4D1	Cry 1, 2 (B, C)	25, 29, 35, 65, 135	55	4.12 x 10 ²	9.98
		<i>Bt israelensis</i> 4Q1	Cry 4, 11 (O)	27, 35, 65, 135	90	1.08 x 10 ²	4.03
		<i>Bt aizawai</i> 4J3	Cry 1, 2, 7, 8, 9 (B, C, O)	14, 19, 45, 65, 135	60	3.67 x 10 ⁴	7.71
Jhum-Agriculture land	Chhippui	CHP 1	Cry 2, 9 (C, O)	25, 27, 43, 65	80	3.12 x 10 ³	6.42
		CHP 5	Cry 4, 9 (O)	30, 43, 65	95	2.70 x 10 ²	5.71
	Hmunpui	HP 7	Cry 4, 9 (O)	23, 27, 35, 65, 135	100	3.90 x 10 ¹	3.69
		LP-1	Cry 2, 9 (O)	14, 17, 19, 27, 43, 65	80	7.19 x 10 ³	6.83
	Lengpui	LP-2	Cry 9 (O)	19, 27, 43	60	5.03 x 10 ⁴	7.71
		LP-3	Cry 9 (O)	19, 27, 43	60	5.06 x 10 ⁴	8.00
		LP-4	Cry 9 (O)	14, 27, 30, 65	70	3.08 x 10 ⁴	7.56
	Rawpuichhip	RP 1	Cry 4, 9 (O)	14, 17, 19, 30, 43, 65, 106	80	1.02 x 10 ³	6.72
	Seling	SE 1	Cry 4, 9 (O)	18, 20, 29, 43, 65	90	1.57 x 10 ²	4.75
	Sihhmui	SH 1	Cry 4 (O)	17, 19, 29, 43, 65	60	5.94 x 10 ⁴	8.75
	Sairang	SR 1	Cry 9 (O)	17, 27, 43, 65	70	4.35 x 10 ⁴	8.53
	West Phaileng	WP 1	Cry 4 (O)	17, 27, 43, 65	70	2.69 x 10 ⁴	8.19
	Serchhip	SC 1	Cry 4, 9 (O)	17, 20, 27, 43, 65, 135	100	2.46 x 10 ¹	2.36
	Sailam	SL-1	Cry 9 (O)	18, 24, 29, 43, 65	85	2.09 x 10 ²	4.75
		SL-2	Cry 4 (O)	29, 43	70	1.94 x 10 ⁴	8.71
Jhum-Forest	MZU Campus	CAMP 1	Cry 4 (O)	23, 27, 35, 65	95	2.03 x 10 ²	4.51
	Khengkhangng	KK 1	Cry 4 (O)	22, 27, 35, 43, 65	80	4.20 x 10 ³	6.70
	Lengte	LT 1	Cry 9 (O)	14, 17, 19, 30, 43, 65, 106	70	4.20 x 10 ⁴	7.89
	Serkawr	SK 1	Cry 1, 2, 4 (B, C, O)	29, 43, 65	70	3.27 x 10 ⁴	8.74
Fallow	Lunglei	LL 1	Cry 1, 4, 9 (B, O)	25, 36, 40, 46, 65	75	1.06 x 10 ⁴	7.92
Aquatic	Champhai	CH 1	Cry 3, 9 (O)	14, 17, 19, 30, 43, 65, 106	75	5.72 x 10 ⁴	8.18
	RihDil	RD 2	Cry 4, 9 (O)	19, 29, 43, 65	75	5.13 x 10 ⁴	8.75
	Ramrikawn	RK 1	Cry 2, 3, 9 (C, O)	19, 27, 43, 65, 106	95	2.46 x 10 ²	4.64
	Thenzawl	TZ 1	Cry 4 (O)	17, 27, 43, 65	80	3.12 x 10 ³	6.78
	Chhimtuipui	CHTP 1	Cry 2, 3, 9 (C, O)	25, 27, 30, 65	75	5.82 x 10 ⁴	8.30

*Mortality at the 5th day after the *Bt* application (8 × 10⁵ cells/mL)**at the 5th day after the *Bt* application (1,350 insects per *Bt* isolate)****Bt* application (8 × 10⁴ *Bt* cells/mL)[#]B- Bipyramidal; C – Cuboidal; O – Ovoidal and spherical.

midgut under microscope observation on the fifth day after treatment.

Significant difference in toxicity (mortality, LC₅₀ and LT₅₀) was observed between 25 isolates in relation to habitats (jhum, aquatic, fallow). ANOVA analysis showed higher mortality (F value – 88.526, df 14, P < 0.0001), lower lethal dose (F value – 5.39 x 10¹⁰, df 14, P < 0.0001), and lesser time to kill (F value – 691.87, df 14, P < 0.0001) were observed in jhum soils than

aquatic and fallow habitats. All the *Bt* isolates possess *cry4* and *cry9* genes, and shared 43 and 65 kDa proteins. Significant differences in terms of toxicity and cry gene and protein profiling were observed in the *Bt* isolates from within the jhum soils (e.g. Lengpui, Chhippui and Sailam) (Table 4). Grouping of *Bt* isolates according to crystal protein(s) profile studied by SDS-PAGE will give a prelude for the presence of diversity in cry genes. The lepidopteran-active *cry 1* (130-140 kDa), lepidopteran and dipteran-active *cry 2* (65-75 and 135

FIG. 3. SDS-PAGE analysis of spore-crystal mixture of *Bt* (jhum/shifting cultivation) (a) lanes 1-9 (b) lanes 10-18 and (c) lanes 19-27 showing 30, 43, 135 and 65, 135, 95, and 65 kDa protein(s). M – Marker; Lane 1, *Bt alesti* 4C1; 2, Chhippui CHP1; 3, Chhippui CHP5; 4, Chhimtuipui CHTP1; 5, MZU campus CAMP1; 6, Hmunpui HP7; 7, *Bt israelensis* 4Q1 Israel; 8, Khengkawng KK1; 9, Lunglei LL; 10, Lengpui LP1; 11, Lengpui LP2; 12, Lengpui LP3; 13, Lengpui LP4; 14, Lengte, LT1; 15, Champhai CH1; 16, RihDil RD2; 17, Ramrikawn RK1; 18, Rawpuichhip RP1; 19, Serchhip SC1; 20, Seling SE1; 21, Sihhmui SH1; 22, Serkawr SK 1; 23, Sailam SL1; 24, Sailam SL2; 25, Sairang SR1; 26, Thenzawl TZ1; 27, West Phaileng WP1.

FIG. 4. Dendrogram based on protein profiling. C1, *Bt alesti* 4C1; C2, Chhippui CHP1; C3, Chhippui CHP5; C4, Chhimtuipui CHTP1; C5, MZU campus CAMP1; C6, Hmunpui HP7; C7, *Bt israelensis* 4Q1 Israel; C8, Khengkawng KK1; C9, Lunglei LL; C10, Lengpui LP1; C11, Lengpui LP2; C12, Lengpui LP3; C13, Lengpui LP4; C14, Lengte LT1; C15, Champhai CH1; C16, RihDil RD2; C17, Ramrikawn RK1; C18, Rawpuichhip RP1; C19, Serchhip SC1; C20, Seling SE1; C21, Sihhmui SH1; C22, Serkawr SK1; C23, Sailam SL1; C24, Sailam SL2; C25, Sairang SR1; C26, Thenzawl TZ1; C27, West Phaileng WP1.

kDa), coleopteran active – *cry 3* (66-73 and 70-75 kDa), dipteran-active *cry 4* (125-145, 68, 72, 78, 128, 130-140 and 135 kDa), *cry 10* (80 kDa) and *cry 11* (67-94 and 80 kDa), *cry 5-9* gene (129, 73, 75, 35

and 38 kDa) show toxicity to different insect orders (Crickmore et al., 2012; Alves and Lemos, 2000; Mahadeva Swamy et al., 2013). Therefore, analysis of crystal proteins(s) profile could be useful to predict the

TABLE 5. Mean generation time, growth rate constant and growth index of (*Bt*) isolates* from different habitats in comparison with the reference strains.

Habitat	Name of the <i>Bt</i> isolate**	<i>Bt</i> cells/mL (during harvest)	Mean generation time	Growth rate constant in 39 h	Growth rate index	Correlation coefficient	
						r	r ²
Reference-soil	<i>Bt kurstaki</i> 4D1	5.78 × 10 ⁶	1.48 ± 0.30	0.02 ± 0.63	0.46 ± 0.72	0.9955	0.9909
	<i>Bt israelensis</i> 4Q1	1.85 × 10 ⁶					
Aquatic	Thenzawl TZ1	1.66 × 10 ⁵	2.03 ± 0.65	0.03 ± 0.60	0.34 ± 0.19	0.9752	0.9511
Reference-soil	<i>Bt alesti</i> 4C1	2.09 × 10 ⁶					
Agriculture	Sailam SL1	2.66 × 10 ⁶	3.32 ± 0.22	2.18 ± 0.75	0.20 ± 0.85	0.9920	0.9841
	Chhippui CHP1	2.02 × 10 ⁵					
Forest	Khengkhawng KK1	1.17 × 10 ⁵	2.65 ± 0.74	0.04 ± 0.72	0.26 ± 0.07	0.9830	0.9663
	Serkawr SK1	3.51 × 10 ⁵					
Aquatic	Chhimtui CHTP1	2.36 × 10 ⁵	0.90 ± 0.53	0.01 ± 0.60	0.76 ± 0.54	0.9909	0.9819
Agriculture	Sailam SL2	6.96 × 10 ⁵					
Forest	Lengte LT1	3.65 × 10 ⁵	0.90 ± 0.53	0.01 ± 0.60	0.76 ± 0.54	0.9909	0.9819
	Serchhip SC1	7.53 × 10 ⁶					
Agriculture	Hmunpui HP7	1.62 × 10 ⁶					

*Mean of five observations.

**Inoculum dose - 1 × 10³ cells/mL for all the *Bt* isolates.

presence of *cry* gene. In the present study, 60 of the 107 isolates are having 135 and/or 65 kDa proteins suggesting the presence of genes related to *cry* 4 and *cry* 9 families. Other isolates showed that the presence of 43 (group I) or 30 (group II), or 43 and 30 (group III), or 17 and 60 (group VII) or 19 (group VIII), or 27 and 95 (group IX), or 14 and 25 (group X), or 22 and 106 (group XI) kDa proteins indicating the presence of other novel *cry* genes also. Among the eleven groups identified, groups IV-VI are more predominant (56.07%) and group VII-XI are novel *Bt* isolates (22.42%). These results led us to suggest the presence of diversity in *Bt* isolates of Mizoram (Table 3 and Fig.4). *Cry* proteins that could be of particular interest for this study are: *cry* 4 and *cry* 9 since they have been reported as effective against dipterans especially mosquitoes (Ben-Dov et al., 1997; Pinto and Fiuza, 2003) and most abundant in jhum soils. The characterization for most of the *Bt* collections were based on bioassays against different insect larvae without identification of the *cry* genes present in the *Bt* strains (Schnepf et al., 1998). In this study, 25 isolates were selected for toxicity, *cry* gene and protein profiling. All the *Bt* isolates showed 60-100% mortality, low LC₅₀ values and possess *cry* 4 and/or 9 genes. New variants of the already known *cry* gene subgroups could encode crystal proteins with significant difference in the level and spectrum of toxicity due to variation in their sequences (Xue et al., 2008; Zhu et al., 2009). Naturally occurring entomopathogens in agroecosys-

tems have been shown to provide significant regulation of pest populations (Pinto and Fiuza, 2003). The *cry* gene and protein profiling and toxicity (mortality, LC₅₀ and LT₅₀) of *Bt* isolates were significantly varied within habitats (Liang et al., 2011). The differences in toxicity of *Bt* isolates to *C. tritaeniorhynchus* may be related to crystal protein production and their toxic potential. *Bt* isolates SC1 and HP7 have *cry* 4 and *cry* 9 genes related to high toxicity (low LC₅₀, lesser LT₅₀, shorter time for crystal production and harvesting and 100% mortality), while CHTP1, LL1, CH1, RD2, LP4, LT1, SK1, SL2, SR1, WP1, LP2, LP3 and SH1 isolates have one to three *cry* gene combinations (*cry* 4/*cry* 9/*cry* 3, 9/*cry* 4, 9/*cry* 1, 2, 4/*cry* 1, 4, 9 and *cry* 2, 3, 9) and showing high LC₅₀, longer LT₅₀, longer harvesting time and 60-75% mortality. Distribution of *cry* gene and proteins and their toxicity to *C. tritaeniorhynchus* varied between *Bt* isolates which may be influenced by habitat, interaction between *cry* genes, expression of *cry* gene, crystal morphology and production and bacterial growth conditions. Further studies on proteomic and gene expression are needed to verify the aforementioned fact.

Growth curve studies and determination of harvesting time

The bacterial growth curve with identifiable lag, exponential and stationary phases were obtained as OD was measured at regular intervals. The maximum OD (referred as log phase) was seen between 24-42 h for *Bt* stan-

dard strains and between 18-24 h for *Bt* native isolates. The *Bt* cultures reached the stationary phase between 24 and 48 h. But after 48 h *Bt* growth was inhibited, due to the depletion of media components and may be due to release of secondary metabolites (Table 5). The growth of the bacterium was monitored for 48 h, as *Bt* reached its sporulating stage within that time. Further, positive correlation was observed during log phase in all the chosen *Bt* cultures ($r = 0.9752-0.9990$ and $r^2 = 0.9511-0.9909$). In *Btk* and *Btal*, the log phase was observed between 24 and 42 h with OD values of 1.139-1.251 and 0.766-1.251, respectively. The log phase was attained within 18 - 21 h in SC1 (OD value – 0.66-0.67) and 21-24 h in KK1 (OD value – 0.66-0.79) and SL2 (OD value – 0.742-0.78), respectively. Higher growth index (0.76), lower mean generation time (0.9) and growth rate constant (0.01) were registered in SC1 and HP7, whereas, lower growth index (0.2), higher mean generation time (3.32) and growth rate constant (2.18) were observed in KK1, SK1 and CHTP1 isolates. SC1 and HP7 *Bt* isolates took lesser time to reach stationary phase (sporulating stage), whereas, longer time was observed in KK1, SK1, CHTP1, SC2 and LT1 isolates (Table 5). The production of *Bt* in commercial scale is economically important and in order to develop its production it is necessary to determine the time to harvest. *Bt* strains express different endotoxin genes and crystal proteins and their harvesting time also varied from strain to strain (Alves and Lemos, 2000). OD is an indication of cell numbers in liquid media, enables the time necessary to reach stationary phase and crystal formation to be determined. The growth curve analysis was performed to determine the time required by the bacterium to reach its vegetative or exponential phase in a batch culture with shaking. In growth curve studies, culturing conditions such as incubation time (24-48 h) and growth medium (LB agar) significantly influenced final yield. Further, log phase varied between *Bt* cultures which is the major variable that determines the population size, production of Cry toxin and their toxicity (Crickmore et al., 2012). From the growth curve it is evident that the bacterium remains in its vegetative or exponential phase from 21-42 h. The result of this experiment was very much in accordance to the growth pattern demonstrated by Sattar et al. (2008) for a different *Bt* strains. Thus the protein was harvested from the culture supernatant after 24 h of culture, expecting maximum yield of vegetative proteins secreted by the *Bt* strain. The time taken to harvest the fermentation varied among the *Bt* cultures used. In this study, we found that soils of jhum cultivation are abundant with novel *Bt* isolates and to produce *Bt* toxins mostly specific to dipteran insects (*cry* 4 and *cry* 9 genes) acted to increase mortality and affected the

development of *C. tritaeniorhynchus*. Nonetheless, the effects observed here illustrate the potential compatibility of naturally occurring *Bt* isolates for regulating pest populations. In jhum soils, spherical and oval types of crystals are predominant and eleven different types of molecular weight Cry proteins were observed. Significant variation was observed in jhum soil habitats in relation to cry gene and protein profiling, toxicity (mortality, LC_{50} and LT_{50}) and growth curve pattern of *Bt* isolates than aquatic and fallow habitats.

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