Characterization of locally isolated *Bacillus thuringiensis* for the Development of Eco-friendly Biopesticides in Bangladesh

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ABSTRACT

Bacillus thuringiensis (Bt) was investigated in three different habitats (vegetable and crops-cultivated soils, phylloplanes and insect guts) of Bangladesh. A total of 61 *Bacillus cereus*-like isolates were obtained by selective methods and 57 of those were identified as *Bacillus thuringiensis* isolates based on their hemolytic activity, presence of parasporal crystal proteins, plasmid profile and crystal protein profile. The prevalence of Bt was highest (60%) in soil samples followed by leaf and insects. Five different types of parasporal crystal proteins (spherical, bipyramidal, irregular pointed, cuboidal and irregular shaped) were observed among the isolates which indicates the diversity of the local Bt isolates examined. In addition, confirmation of the strain identification was done using 16S rDNA gene sequencing. Plasmid analysis recovered from the isolates yielded at least one 15kb DNA, which is well comparable to the reference strain, *B. thuriginesis kurstaki var.* HD-73. Further similarity between the test and standard strains was observed while analyzing crystal proteins on a SDS-polyacrylamide gel that produced major bands of Cry1, Cry2 and Cry9 type proteins. Bioassay performed with isolates Bt-01i, Bt-25f and reference strain, Bt *kurstaki var.* HD-73 against pulse beetles *Callosobrochus chinensis* resulted LC_{50} values of 0.30, 0.72 and 0.22 mg/ml crude proteins respectively which demonstrated their competency in Biopesticide production and application.

Key words: Bt, biopesticides, crystal proteins, 16S rDNA gene sequencing, SDS-PAGE.

INTRODUCTION

Bacillus thuringiensis (Bt) is a gram-positive, rod-shaped, motile, facultative anaerobic, spore-forming bacterium widely used as biocontrol agent against pests (Fernando *et. al.,* 2010). Bt produce parasporal crystalline inclusion bodies constituted by highly specific insecticidal toxins which are protein by nature. These toxins are mainly active against lepidopteran species and some also shows toxicity against dipteran and coleopteran species and other organisms (Vidyarthi *et al.,* 2002; Martin *et al.,* 2010).

So far more than 50,000 Bt strains have been isolated from different environments (Sadder *et al.,* 2006). It has been reported that Bt can be present in several different habitats such as soil, stored product dust, insect cadavers, grains, agricultural soils, olive tree related habitats, different plant and aquatic environments (Martin and Travers, 1989; Meadows *et al.,* 1992; Ben-Dov *et al.,* 1997; Theunis *et al.* 1998; Bravo *et al.,* 1998; Bel *et al.,* 1997; Mizuki *et al.,* 1999; Iriarte *et al.,* 2000, Maher *et al.,* 2004; Xavier *et al.,* 2007). In fact, each habitat may contain a novel Bt strains awaiting discovery which has a toxic effect on a target insect group. Therefore, Bt strains have been collected and characterized in order to evaluate their toxic potential against various insect orders (Chak *et al.,* 1994; Theunis *et al.,* 1998; Bravo *et al.,* 1998; Forsyth and Logan 2000; Uribe *et al.,* 2003; Özgür *et al.* 2005; Glen *et al.,* 2006). Powdered Bt biopesticide formulation and Bt transgenic plants are common measures in different parts of the world at present. Transgenic Bt cotton containing *cry1Ac* gene which offers resistance to major bollworms was first commercially released in the world in 1996 and during 2002 in India (Prasad *et al.,* 2009). Steven and Naranjo highlighted the impact of Bt on various organisms in an elaborate manner (Nethravathi *et al.,* 2010). Identification of *cry* gene content by PCR is the most effective techniques in screening large native collection when predicting insecticidal activities of individual strains (Ben-Dov *et al.,* 1997; Porcar *et al.,* 2003). Biological activity tests, plasmid contents, 16S rDNA analysis, PFGE analysis of chromosomal DNA, crystal morphology and protein profiling of Bt are also currently in use as complementary methods in the search for novel strains.

The use of Bt product is gradually being increased as the alternative to chemical pesticides, as organic pesticides cause too many ill effects to human beings when they consume insecticides treated products (Kandibane *et al.,* 2010). Therefore, the aim of this study is to initiate the establishment of the collection of indigenous Bt strains in Bangladesh, determining diversity among them and sorting out of the potential strains that are active against vegetable pests for their large scale production.

MATERIALS AND METHODS

Sample collection

A total of 99 samples were collected from 11 different places of Bangladesh targeting soil, leaf and insect as their habitat as shown in Table 1. Soil samples were collected from the places not exposed to sunlight or at 2 to 5 cm depth by scarping off surface material and were placed in plastic bags aseptically, transported to the laboratory and stored at 4°C until processed.

Bacterial strains:

B. thuringiensis subsp. *kurstaki* HD-73 (cry1Ac)**,** *B. thuringiensis* subsp. japonensis *Buibui* (cry8Ca), *B. thuringiensis* subsp. *sotto* (BGSC T84, cry1Aa) were received in slant culture, provided by Okayama University, Japan and used as reference strains.

Isolation and analysis of Bacillus thuringiensis

Isolation was carried out using two different methods with some minor modifications. To isolate *B. thuringiensis*, a 10% soil suspension in 0.9% NaCl was preheated at 80°C for 10 min and 100 µL of each samples was then transferred to 900µl Mannitol-egg yolk-polymyxin (MYP) broth for enrichment of environmental samples, a selective medium for the *Bacillus cereus* group (Oxoid, Ltd., Basingstoke, England), and incubated overnight at 30°C. Then the cultures were plated on MYP agar from broth and incubated at 30°C for 24 hrs. Those which formed irregular white colonies with a pink background, similar to reference isolates were primarily identified as *Bacillus thuringiensis*. Similar method was followed for leaf and insect samples. The acetate selection method described by Travers *et al.* (1987) was used to further screening whether or not germination of their spores was inhibited at 0.25M acetate. Isolates were then streaked on 5% Sheep blood agar plate and incubated overnight at 30ºC. Isolates possessing â-hemolytic activity were sub-cultured on Acras agar medium (Wei-Ming *et al.,* 1995) plates and incubated at 30ºC for 18-48 hrs at 30ºC allowing sporulation. They were then observed under Phase contrast microscope (Zeiss) at 1000 magnification for the presence of parasporal crystal protein. Observation for all isolates was recorded considering the presence of crystal proteins and crystal shapes.

16S rDNA gene sequencing

Colony PCR was performed with universal primers complementary to phylogenetically conserved portions of the 5' and 3' ends of the 16s rDNAs of *Bacillus thuringiensis*. Primers, 20F 5'-GAGTTTGATCCTGGCTCAG-3' (position 9-27), and 1500R 5'-GTTACCTTGTTACGACTT-3' (position 1509-1492) were used for 16S rDNA gene sequencing. The amplification and sequencing was carried out according to the method described by Ben-Dov *et al.* (1997). The 16S rDNA gene sequence of the purified PCR product was determined using 3 primers 20 F, 520 F and 920 F with an Applied Biosystems model 3130 DNA sequencer and the ABI PRISM cycle sequencing kit. The sequence obtained was compared with those acquired from GenBank using the BLAST program (Altschul *et al.*, 1990).

Plasmid profiling

Table 1. Sampling sites, origin of samples collected and number of samples yielding *Bacillius thuringiensis* (Bt).

Name of localities	Type of sample	Number of sample	Name of localities	Type of sample	Number of sample
Savar	Soil	11	Lakshmirchor, Jamalpur	Soil	11
	Leaves	03		Leaves	11
Keraniganj, Dhaka	Soil	04	Ati, Dhaka	Soil	04
Jessore	Soil	01		Leaves	04
Agargaon, Dhaka	Soil	02	Sonargaon	Soil	08
	Leaves	01		Leaves	08
Comilla	Soil	02		Insects	07
Nandipara, Dhaka	Soil	04	Norsinghdi	Soil	07
	Leaves	04	Chuadanga	Soil	07

Plasmid of the isolates was purified by alkaline lysis method (Sabine *et al.,* 2003) with some modifications. 2 ml of overnight culture grown in LB broth at 30°C was pelleted and resuspended in 100µL of TE buffer (40 mM Tris-HCl, 2 mM EDTA, pH 7.9). 200 µL of lysis solution [3% (w/v) SDS, 15% (w/v) sucrose, 50 mM Tris-hydroxide, pH 12.5] and was incubated for 30 min at 60°C. Then 5U of proteinase-K was added and mixed gently and incubated for another 90 min at 37°C. 1 mL of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the mixture and the tubes were inverted carefully several times. The samples were then centrifuged for 15 min at 13000 rpm and the supernatant fluid harboring plasmid DNA was separated carefully. Plasmids of the isolates were then electrophoresed through 0.5% agarose gel prepared in 1× TBE and visualized against UV in Gel Documentation machine (Bio-Rad).

Crystal protein weight determination

100 µL of sporulated culture grown in T_3- medium (per liter: 3 g tryptone, 2 g tryptose, 1.5 g yeast extract, 0.05 M phosphate buffer pH 6.8, and 0.005 g of $MnCl_2$) was pelleted and washed with 1.0M NaCl containing 5mM EDTA and then with 5mM EDTA alone (Ozturk F.*et al.* 2008). Washed crystals and spores was resuspended in 100 µL of SDS sample loading buffer (50 mM Tris-HCl, pH 7.5; 2% (w/v) SDS, 0.05% (w/v) bromophenol blue, 1mM EDTA, 10% (v/v) glycerol, 15 mM DTT) and boiled for 5 min at 100°C. Insoluble material was removed by centrifugation and supernatant containing ä–endotoxin was analyzed by SDS-PAGE loading 20ìl aliquots onto 7.5% acrylamide gels. Following electrophoresis, the gels were stained for 1 hr in 0.1% Coomassie Brilliant Blue G-250 and destained overnight in a solution containing 6.75% (v/v) glacial acetic acid and 9.45% (v/v) methanol. The molecular weights of proteins were determined by using protein molecular standards (Invitrogen, USA).

Bioassay

The adult pulse beetles (*Callosobrochus chinensis, Tribolium castaneum and Sitophilus oryzea*) were collected and kept at 28-32ºC and 70-80% RH for both multiplication and bioassay. Isolates with parasporal bodies were cultured in 100 ml of T_3- liquid medium and incubated for 7 days at 30°C with continuous shaking at 250 rpm. Liquid cultures were centrifuged at 5000 rpm for 15 min. Pellets (spores and parasporal protein crystals) were washed in 20 mL sterile distilled water and centrifuged at 5000 rpm for 5 minute. Washing procedure was repeated twice. The pellets were resuspended in 20 mL of sterile distilled water and kept at 4°C. Different concentrations of spore-crystal mixture of Bt was prepared by diluting with sterilized distilled water and the fresh pulse grain were soaked in it as food for the test

insects. The food was then dried and placed in a petri dish where 10 adult beetles were placed. The toxicity of each strain was assayed in triplicate for either the original toxin-spore suspension or the dilutions. The transformation was confirmed carefully to the test cases with soft brush to avoid physical trauma. Mortality was scored comparison with parallel control in which pulse grains were soaked in sterile distilled water instead of bacterial suspension and used to correct the test mortality by using Abbot's formula. The LC_{50} values were determined by probit analysis using SPSS for Windows. When the control mortality were more than 10%, the percentage mortalities was corrected following Abbott's (1925) formula. The dose response data were analyzed (LC_{50}, LC_{90}) and presented using SPSS-16, Biostat-2009, Microsoft Excel Program-2007. Computations performed by this program were based on Finney (1964).

RESULTS

***Bacillus thuringiensis* distribution**

A total of 99 samples were examined in this study (Table 1) and growth on MYP medium plate permitted 99 to cosidered as Bacilli. Acetate slection and hemolysis as Bt test, a means to distinguish between *B. anthracis, B.cereus* (Bc) and *B. thuringiensis,* identified 57 isolates as Bt and restof them as Bc. Randomly selected 15 isolates were allowed to sporulate and observed under Phase contrast microscope and presence of parasporal crystal proteins was confirmed. For these randomly selected 26 isolates, Bt index was calculated 0.6 ranging from 0.4 for leaf to 0.67 for soil.

Crystal composition of the isolates

Fifteen out of randomly selected 26 isolates from a total of 99, were observed under Phase contrast microscopy to be parasporal crystal protein producing (Fig.3). Five different types of crystal shapes were observed among the isolates such as spherical, cubical, bipyramidal, irregular pointed and irregular shaped. More than one type of crystal proteins were also found in some isolates (Table 2).

16S rDNA gene sequencing for identification

16S rDNA gene sequencing of isolates, numbered 01i, 25f and 48s yielded an 897 nucleotide-long amplicon with universal primer (5'-GAGTTTGATCCTGGCTCAG-3') which was compared with the sequences reported in Gene Bank of NCBI (National Centre for Biotechnology Information, http://www.ncbi.nih.gov/). From the BLAST search (Basic Local Alignment Search Tool) that provides a method for rapid searching of related sequences, the 16S rDNA sequences showed 99% sequence match with Bt strain BAC3042. The partial sequence of this strain was aligned with related

Figure 1. Presence of crystals under Phase-contrast microscope (A): 24 hrs (B): 48 hrs
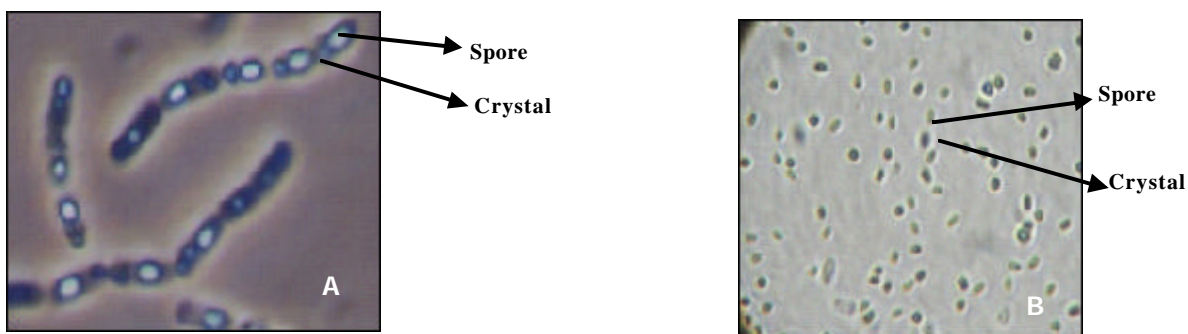
Table 2. Classification of Bt isolates based on crystal morphology and comparison with reference strains.

Morphology of crystals	Bt Isolates	Similarity with reference strains	% of samples
Bipyrmidal(BP)	01i, 26s, 45s	Sotto , HD-73	20%
BP, Spherical(SP)	06i, 27s, 48s,	Sotto , Buibui , HD-73	20%
SP	03i, 25f, 41s, 49s, 51s	Sotto , Buibui, HD-73	33%
SP, Irregular pointed(IP)	47s	Buibui, HD-73	7%
IP, Irregular shape(IS)	40f	Buibui	7%
BP , Cubic	44f	Sotto, HD-73	7%
BP , IP	47f	Sotto , Buibui, HD-73	7%

sequences (obtained from BLASTn) by CLUSTAL W 2.0.12 program CLUSTAL 2.0.12 multiple sequence alignment, resulted in the generation of highest score of 99% among the sequences.

Plasmid profiles of the isolates

The plasmid profiles of the Bt strains were compared to the reference strains which revealed a major plasmid band of 15 kb size was present in all isolates (Figure 4). In addition, plasmid bands varying in length between 10 and 22 kb were also observed in some local strains. For 49s strain, there were

4 plasmid bands of 8kb, 10kb- 15kb, and 22kb resulting in a unique array (lane 10) (Benintende *et al.,* 1999).

Characterization of crystal protein of the isolates

Crystal protein profile of thirteen native isolates and reference strains were analyzed by discontinuous SDS-PAGE (Laemmli, 1970) which revealed protein bands with molecular weight ranging from 19kDa to ~195kDa (Fig. 5). Analysis showed the presence of several major polypeptide bands within the isolates 25f, 1i, 27s, 48s, 47f, 45s, 49s, 51s and reference strains *Btk* HD-73 and Bt*sotto,* where three common prominent bands

Figure 2. Plasmid profiling of the Bt isolates; lane 1: *Bt*-40f; lane 2: *Bt*-6i; lane 3: *Bt*-47s; lane 4: *Bt*-25f: lane 5: *Bt*-48s; lane 6: *Bt*-1i; lane 7: *Bt*-27s; lane 8: Marker (PDK-9); lane 9: *Bt* japonensis *buibui*; lane 10: *Bt*-49s; lane 11: *Bt*-51s; lane 12: *Bt*- sotto; lane 13: *Btk* HD-73.
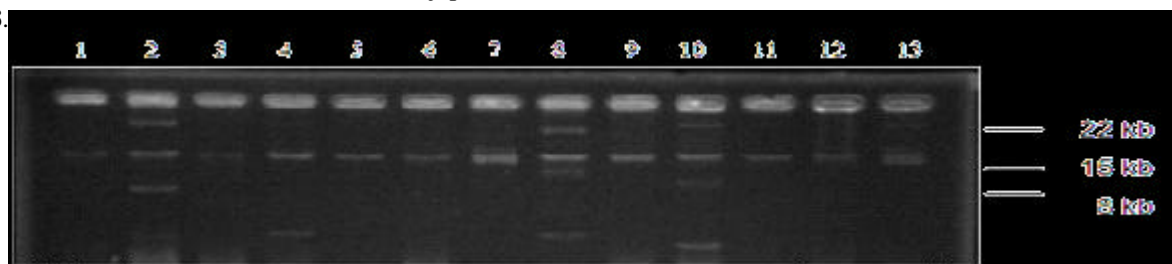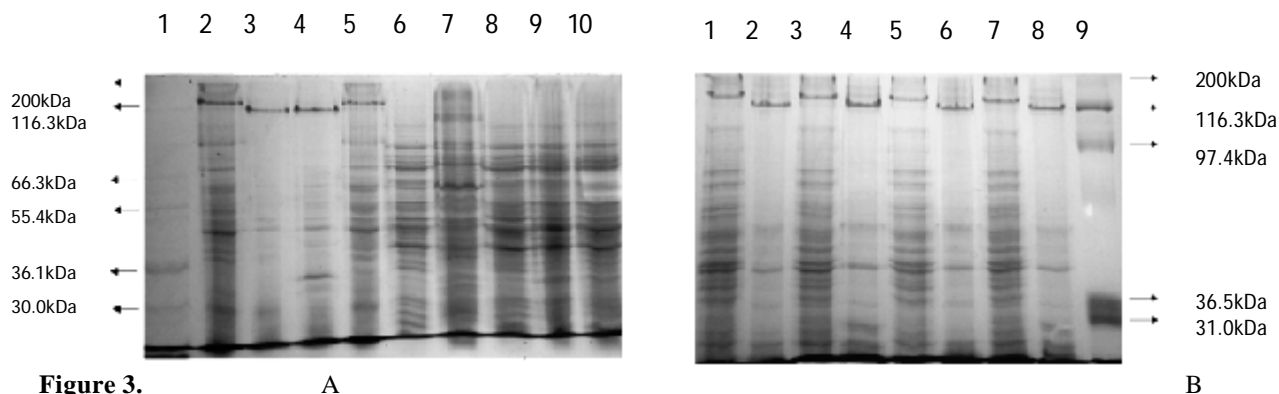
Figure 3.　　　　　　　　　A　　　　　　　　　　　　　　　　　　　B

Gel (A): 1: Marker; 2: HD-73; 3: Sotto; 4: 25f; 5: 1i; 6: 3i; 7: 47s; 8: 6i; 9:40f; 10: 44f
Gel (B): 1:47s; 2: 49s; 3: 27s; 4: Sotto; 5: 48s; 6: 51s; 7: 47f; 8: 45s; 9: Marker.

of ~65kDa, ~130kDa and ~135kDa suggest the presence of Cry1, Cry2 and Cry9 proteins (Crickmore *et al.*, 1998).

Bioassay

Static bioassay was conducted to determine the susceptibility level of the adult pulse beetles (*Callosobrochus chiniensis*, *Sitophilus oryzea* and *Tribolium castaneum*) against two Bt isolates (1i, 25f) and one reference strain *Btk* HD-73 where *C. chiniensis* was found to be more susceptible than *Sitophilus oryzea* and *T. castaneum*. Comparing LC_{50} and LC_{90} value at 72, 96 and 120 hrs it was found that strain *Btk* HD-73 was most active and 01i isolate's activity was impressive as its very close to the activity of reference strain. 25f isolate was also found to have good activity after 5 days exposure to the insects in comparison to the reference strain (Table 3). In *C. chiniensis* significant difference of efficacy among the strains was found only at 24 hrs (Test of Homogeneity, p=0.017). Significant variation (5%) among different doses was also found except 24 and 48 hours (p=0.729 and 0.242).

DISCUSSION

A total of 99 samples (61 from soil, 31 from phylloplanes and 7 from insect) were collected from local agricultural lands in Bangladesh mainly of different common vegetables. After antibiotic selection, no desired growth was observed in 34 (16 phylloplane, 4 insect and 14 soil) samples. Though polymyxin B sulfate is used to inhibit most of the Gram negative and Gram positive bacteria, *S. aureus, B. subtilis* and some other soil bacteria can grow along with *B. cereus and B. thuringiensis.* Crystal protein observation and analysis was performed under Phase contrast microscope to distinguish Bt and others. Bt was found in all selected habitats which suggests the ubiquity of Bt in Bangladesh. The abundance of *B. thuringiensis* was the highest in all soil samples with a Bt index maximum of 0.67.

Crystal morphology of Bt can provide valuable information on target insect spectra (Maeda *et al.* 2000). Spherical shaped crystal morphology was observed mostly among the isolates tested which indicate their toxicity to the dipterans. On the basis of the content of protein crystals the isolates were divided into 5 different groups (Table 2). 16S rDNA gene sequence analysis was performed to identify Bt isolates. Universal forward primers for 16S rDNA gene sequencing were used and three Bt isolates were identified as Bt. The genes coding for the insecticidal Cry proteins are normally associated with plasmid of large molecular mass (Gonzales and Carlton, 1980). Plasmid profiling was performed with nine

Table 3. Susceptibility of four Bt isolates (LC_{50} and LC_{90} mg/mL) against *Callosobrochus chiniensis*.

Time (hours)	1i		25f		HD-73	
	LC_{50}	LC_{90}	LC_{50}	LC_{90}	LC_{50}	LC_{90}
72 h	0.30	0.72	0.73	2.02	0.22	0.74
96 h	0.22	0.83	0.09	0.85	0.08	0.58
120 h	0.14	0.78	0.04	0.31	0.02	0.48
144 h	0.12	0.59	0.06	0.31	0.01	0.42

LC= lethal concentration (mg/ml)

Bt isolates and the result showed bands of different molecular weights ranging from 15 kb to 22 kb for the isolates and a major band of 15 kb was evident in all isolates. In crystal protein studies, the ranges of proteins and variations in their molecular weight suggest the diversity and probability of getting new Cry proteins from Bangladesh Bt isolates.

Bt isolates having Cry protein pattern similar to the reference strains were tested for their insecticidal activity against pulse beetles. In this study, a series of bioassays were performed by providing the adult insect with pulse grains soaked with the spores-crystals suspension of Bt isolates and reference. Spores and crystals were both included in suspensions because they produce higher level of mortality than either crystals or spores alone (Thomas *et al.,* 1983). *C. chiniensis* was found to be more susceptible to the spore-crystal suspension among the three tested. *Btk* HD-73 reference strain was most active against *C. chiniensis* at LC_{50} values 0.22 and 0.08 mg/mL of crude protein concentration after 72 and 96 hrs respectively. 01i was also found to be a good candidate in comparison to reference strain and LC_{50} value 0.30 and 0.22 mg/mL of crude protein concentration were recorded after 72 and 96 hrs, respectively. In*Callosobrochus chiniensis* significant difference of the effectiveness among the strains was found at 24 hours (Test of Homogeneity, p=0.017). Significant variation (pd"0.05) among different doses was also found except 24 and 48 hours (p=0.729 and 0.242). In case of *S. oryzea* and *T. castaneum* dose effectiveness was insignificant but strains were significantly different at (10%) except 24 and 120 hours for *Tribolium* sp. (p= 0.227 and 0.238).

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