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, *B. kurstaki var. HD-73* against pulse beetles *Callosobrochus chinensis* resulted LC50 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; Özkür 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.
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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 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 30°C. Isolates possessing â-hemolytic activity were sub-cultured on Acras agar medium (Wei-Ming et al., 1995) plates and incubated at 30°C for 18-48 hrs at 30°C 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 Bacillus thuringiensis (Bt).**

<table>
<thead>
<tr>
<th>Name of localities</th>
<th>Type of sample</th>
<th>Number of sample</th>
<th>Name of localities</th>
<th>Type of sample</th>
<th>Number of sample</th>
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<tbody>
<tr>
<td>Savar</td>
<td>Soil</td>
<td>11</td>
<td>Lakshmichor,</td>
<td>Soil</td>
<td>11</td>
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<tr>
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<td>Leaves</td>
<td>03</td>
<td>Jamalpur</td>
<td>Leaves</td>
<td>11</td>
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<tr>
<td>Jessore</td>
<td>Soil</td>
<td>04</td>
<td>Ati, Dhaka</td>
<td>Soil</td>
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<tr>
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<td>Soil</td>
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<td>Sonargaon</td>
<td>Soil</td>
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<tr>
<td>Comilla</td>
<td>Leaves</td>
<td>01</td>
<td>Sonargaon</td>
<td>Leaves</td>
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<td>Soil</td>
<td>04</td>
<td>Norsinghti</td>
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<td></td>
<td>Leaves</td>
<td>04</td>
<td>Chuadanga</td>
<td>Soil</td>
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</table>
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 protease-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 Tz- 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 MnCl2) was pelleted and washed with 1.0M NaCl containing 5mM EDTA and then with 5mM 
EDTA alone (Ozturk 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 ß-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) 
acrylamide 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 oryzae) were collected and 
kept at 28-32°C and 70-80% RH for both multiplication 
and bioassay. Isolates with parasporal bodies were cultured 
in 100 ml of T medium (per liter: 3 g tryptone, 2 g tryptose, 1.5 g 
yeast extract, 0.05 M phosphate buffer pH 7.5; 2% (w/v) 
acetate, 0.005 g of MnCl2) was 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 
distilled water and centrifuged at 5000 rpm for 5 minute.

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 selection 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 microscope 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 ampiclon 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
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 satu, where three common prominent bands

![Figure 1. Presence of crystals under Phase-contrast microscope (A): 24 hrs (B): 48 hrs](image)

![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.](image)
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 oryzae* 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* *oryzae* 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-crystal 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 LC50 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 LC50 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 of the strains was found at 24 hours (Test of Homogeneity, p=0.017). Significant variation (pd"0.05) among different doses of the strains was found at 24 hours (Test of Homogeneity, p=0.017). Significant variation (pd"0.05) among different doses of the strains was found at 24 hours (Test of Homogeneity, p=0.017). Significant variation (pd"0.05) among different doses of the strains was found at 24 hours (Test of Homogeneity, p=0.017). Significant variation (pd"0.05) among different doses of the strains was found at 24 hours (Test of Homogeneity, p=0.017).

ACKNOWLEDGEMENT

This work was supported by a Grant-in-Aid from the USDA as a project entitled ‘Production of *Bacillus thuringiensis* biopesticides by biotechnological approach for the control of vegetable pests in Bangladesh.’

REFERENCES


Cambridge, 318 P.


Bacillus thuringiensis - An eco-friendly biopesticide


Porcar, M., Juarez-Perez, V. 2003. PCR-based identification of Bacillus thuringiensis pesticidal crystal genes. FEMS Microbiol Reviews, 26: 419-432.


Uribe, D., Martinez, W. and Ceron, J. 2003. Cry genes in negative strains of Bacillus thuringiensis obtained from different ecosystems from Colombia. Journal of Invertebrate Pathology, 82: 119-127.

