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Research Article

Characterization and evaluation of two indigenous *Bacillus thuringiensis* isolates against *Helicoverpa armigera* Hubner

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ABSTRACT: Two isolates of *Bacillus thuringiensis* isolated from dead lepidopteran larvae from a tea garden in Jorhat, Assam and one isolated from soil sample from Rajasthan, obtained in a nationwide screening program showed bipyramidal crystal morphology. These two isolates named as NBAII-BTAS and NBAII-BTG4 were characterized by their high level of toxicity against diamond back moth (*Plutella xylostella*). The PCR amplification of these two isolates revealed the expected size of the PCR product for *cry*1Aa, *cry*1Ab, *cry*1Ac, *cry*1E, *cry*1G, *cry*1I, and *cry*2 of 390 bps, 1111bps, 238 bps, 540 bps, 300 bps, 468 bps, and 1170 bps respectively. Purified *cry* proteins from each of these two cultures were subjected to SDS-PAGE analysis, where, two distinct bands of 130-140 Kda and 65 Kda corresponding to *cry*1 and *cry*2 proteins were observed. Toxicity studies was carried out using trypsin activated purified proteins against *Helicoverpa armigera*, where NBAII-BTG4 derived crystal proteins displayed more toxicity (0.93µg\ml) than NBAII-BTAS.

KEY WORDS: cry genes, PCR, specific cry primers, bipyramidal, toxicity

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INTRODUCTION

One of the most promising alternatives to the use of chemical pesticides is the applications of *Bacillus thuringiensis* (Beron and Salerno, 2006). Recent discoveries of new varieties of *B. thuringiensis* suggest that naturally occurring microorganism found in the soil may provide a treasure chest of microbes with untapped, unknown potential for agriculture. In 1987, two scientists at the US Department of Agriculture (USDA) announced the discovery of 72 new varieties of *B. thuringiensis*. Since, only about 24 varieties of *B. thuringiensis* were previously known, the identification of new *B. thuringiensis* germplasm could radically change (Schnepf *et al*., 1998). More than 300 ICPs have been cloned, sequenced and classified into 53 groups based on amino acid homology. Establishment of a *B. thuingiensis* collection is to have a method which allows for rapid and accurate characterization of its crystal inclusions so that its specificity and toxicity is determined. Analysis of these genes by bioassay has proved to be an exhaustive, time-consuming process, since, it is necessary to screen all target insect isolates. Different methods have been developed in an effort to reduce the number of bioassays, such as (i) Southern blot analysis in search of known homologous genes (Kronstad and Whiteley, 1986), (ii) analysis of reactivity to different monoclonal antibodies (Hofte *et al*., 1988) or (iii) electrophoretic analysis of PCR products using specific primers (Carozzi *et al.,* 1991). From the above-mentioned methods, PCR analysis is considered to be the best choice, because, it allows rapid determination of the presence or absence of a sequence and it is highly sensitive, relatively fast, and can easily be used on a routine basis (Ceron *et al.*, 1994; 1995).

PCR-based methodologies with universal primers and sets of primers directed against specific regions of type-specific *cry* genes have been proposed, and these approaches allow the detection of *cry* genes and prediction of their insecticidal activities (Ceron *et a*l., 1995; Ben Dov *et al.*, 1999; Beron *et al.*, 2005). Recently, Aly (2007) and Thammasittirong and Attathom (2008) designed primers to detect specific *cry* genes based on the nucleotide sequences available at NCBI and proposed that *cry* specific genes could be identified using PCR techniques. *B. thuringiensis* strains isolated from avocado orchards exhibit a low toxic activity towards *Argyrotaenia* sp. larvae, in spite of their specific *cry* gene content (Rosas-Garci´a *et al.*, 2007).

During screening for native *B. thuringiensis* isolates, two bipyramidal forming isolates were encountered from soil and insect cadaver respectively. We undertook a study to characterize these two isolates for their *cry* gene profile using PCR based methodologies and also evaluate their toxicity against *Helicoverpa armigera*. We used the standard HD-1 strain for comparison.

MATERIALS AND METHODS

Bacterial strains

Two indigenous *B. thuringiensis* isolates NBAIIBT-G4 (from rhizosphere soil of groundnut, Sri Ganganagar, Rajasthan) and NBAIIBT-AS (purified from dead lepidopteran larvae, Jorhat, Asssam) were used in the study. The isolation was carried out according to the protocol of Obeidat *et al*. (2004). Microscopic analysis revealed that they produced bipyramidal crystals, spore forming and Gram positive as revealed in Gram staining and Amido black staining. They were previously found to be toxic to diamondback moth (*Plutella xylostella)*. They were identified and characterized by PCR reactions with universal and specific primers.

DNA extraction

The cultures of two *B. thuringiensis* isolates, namely NBAII-BTAS and NBAII-BTG4, were grown overnight in LB broth in rotary shaker at 250 rpm. 3ml of culture was pelleted and DNA was extracted by HiPura reagents (HiMedia) with mini-prep column formation as per Sambrook (2001). Integrity of DNA was tested by running in 1.25% agarose gel for 1hr at 100V.

PCR amplification and primers designed of *cry* genes

Polymerase chain reaction was carried out in a Thermo cycler (Quantarus) for 30 reaction cycles each. PCR reactions were carried out in 25 ìl containing 50 ng of DNA mixed with 1X Taq reaction buffer, dNTP mix- 50 mM, Primer- 0.4 µM (Both forward & reverse), Taq DNA Polymerase (Genei)- 1.5 U, $Mgcl_2$- 14 ì M. Template DNA was denatured for one minute at 94ºC, one minute of annealing at 54-59ºC and elongation at 72ºC. An extra denaturation and elongation step was provided at 94ºC for 2.5 minutes and at 72ºC for 5 minutes respectively (Mahadi *et al.,* 1998). PCR products were separated by running on 1.25% agarose gel with 4ìl/100ml of ethidium bromide for 1hr at 100V. The fragments were visualized under gel documentation unit (DNR MiniLumi). Sizes of PCR product was determined by the ladder of 100 bps and of 1 kb as required in each according to the sizes. The specific primers are designed by Aly (2007), and the universal primers by Bravo *et al*. (1998) and Thammasittirong and Attathom (2008) (Table 1).

Purification of *cry* proteins and SDS-PAGE analysis

Whole cell proteins were isolated from sporulating bacterial culture by centrifugation and dissolution in specific buffers. Respective cultures were allowed to grow on T3 agar medium containing (per litre) tryptose 3g, peptone 2g, yeast extract 15g, magnesium chloride 0.005g, agar powder 15g, sodium phosphate 0.05M and pH 6.8 at 30°C for 5 days until sporulation. The lawn culture was scraped using sterile cotton swabs and dissolved in equal amount of sterile water, washed twice with distilled water (8000 rpm for 8 minutes), dissolved in 500µl of lysis buffer containing 100mM Tris HCl (pH 7.0), 20mM EDTA, 5mg/ml lysozyme, 2% SDS and it was centrifuged at 8000 rpm for 7 minutes. Pellet was resuspended in 200µl resuspension buffer (0.1% SDS + 10mM EDTA), further diluted with treatment buffer (2.0%SDS +5% â mercaptoetranol+130mM Tris HCl, pH 10.0) and incubated at 90ºC for 7 minutes (Morris *et al*., 1998). Protein concentration was measured as per Lowry *et al.* (1951) and used for SDS PAGE. Approximately, 150ì g of proteins were loaded in each well of 12% stacking gel and run at 50V for 4 hours in denaturing conditions. The gel was stained for 1 hour in staining solution and destained overnight. Gel was visualized in a gel documentation system (DNR Mini-Lumi, Israel).

Activation of *cry* proteins (protoxins)

Protoxins were converted to active toxins by trypsin digestion. Since, trypsin works at neutral pH, the pH 10.0 of the solubilized protoxin was adjusted to pH 7.0 using 1N HCl. 1µg of trypsin (stock: 1mg/ml in distilled deionized water) for each 20 µg of protoxin was mixed well and incubated at 37ºC for 3 hours.

Biotoxicity analysis of *cry* protein

NBAII-BTAS and NBAII-BTG4 were screened against *H. armigera* using the semi-synthetic diet bioassay method. The concentration of the activated *cry* proteins was adjusted to 2mg/ml and then dilutions were made up to 10^{-6}. Around 100µl were applied to each vial containing about 5ml diet and air dried. Two second instar larvae of *H. armigera* were introduced into each vial and 10 replications were maintained for each dilution. Larval death was recorded up to 92 hours and LC_{50} values were calculated by subjecting the data to probit analysis using SPSS software version 10.

Table 1. Primers used in the study for identification of *cry* genes

Primers	Genes recognized	Product Size (bp)	Sequence	Tm (ºC)	References
Universal *cry* 1 primers	*cry* 1A, *cry* 1B, *cry* 1C etc.	558	5'CTGGATTTACAGGTGGGGATAT '3 5'TGAGTCGCTTCGCATATTTTGACT '3	52	Bravos *et al*, 1998
Universal *cry* 2 primers	*cry* 2Aa, *cry* 2Ab, *cry* 2Ac, *cry* 2Ad	1170	5'TACCTTTATTTGCACAGGCA '3 5'CTACCGTTTATAGTAACTCG '3	54	Thammasittirong and Attathom (2008)
Universal *cry* 3 primers	*cry* 9Aa, *cry* 9Ba, *cry* 9Ca, *cry* 9Da etc	440	5'CACATGAGTTTTCTTCCTAT '3 5'AGATACGATGCTTGTTGTAA '3	54	Thammasittirong and Attathom (2008)
cry 1Aa specific primers	*cry* 1Aa	398	5'ATTATCATATTGATCAAGTTC '3 5'CATAAGGAACCCGTACCTGG '3	52	Aly *et al.*, 2007
cry 1Ab specific primers	*cry* 1Ab	1111	5'GGACCAGGATTTACAGGAGG '3 5'GTTCTCCTACTAATGGTTTCC '3	52	Aly *et al.*, 2007
cry 1Ab specific primers	*cry* 1Ac	238	5'CTCAATGGGACGCATTTCTT '3 5'CGGTTGTAAGGGCACTGTTC '3	52	Aly *et al.*, 2007
Universal *cry* 1C primers	*cry* 1Ca, *cry* 1Cb	432	5'TAATCCACAGTTACAGTC '3 5'TATTATCCTCAGGCGGTAA '3	57	Thammasittirong and Attathom (2008)
Universal *cry* 1D primers	*cry* 1da, *cry* 1Db	641	5'AAGGGAAGGAAATACAGAGC '3 5'CGAACGAACGAGATGTTAG '3	54	Thammasittirong and Attathom (2008)
Universal *cry* 1E primers	*cry* 1Ea, *cry* 1Eb	540	5'CAGCTATTCCTCTTTTTTCAGT '3 5'ATGAGAAGTTACACGATGCC '3	55	Thammasittirong and Attathom (2008)
Universal *cry* 1F primers	*cry* 1Fa, *cry* 1Fb	1080	5'TACTGGCAGATTACCGTTAG '3 5'AAATGTTCGGGTGTGGTTCG '3	55	Thammasittirong and Attathom (2008)
Universal *cry* 1G primers	*cry* 1Ga, *cry* 1Gb, *cry* 1Gc	300	5'AATCTTCATTCAGGTGCCAC '3 5'GAAAAGGTAAATGGAGTAGTAA '3	58	Thammasittirong and Attathom (2008)
Universal *cry* 1H primers	*cry* 1Ha, *cry* 1Hb	1500	5'GGGGAGTTATTGGTCCTGAT '3 5'GTTATTGGTGTGAAAAGAGTTG '3	55	Thammasittirong and Attathom (2008)
Universal *cry* 1I primers	*cry* 1Ia, *cry* 1Ib, *cry* 1Ic, *cry* 1Id, *cry* 1Ie	468	5'TGAATATGTGGGGAGGACA '3 5'CTAATGGTATTTGTGTAATGCT '3	55	Thammasittirong and Attathom (2008)
Universal *cry* 9 primers	*cry* 9Aa, *cry* 9Ba, *cry* 9Ca etc	440	5'CACATGAGTTTTCTTCCTAT '3 5'AGATACGATGCTTGTTGTAA '3	54	Thammasittirong and Attathom (2008)

RESULTS AND DISCUSSION

Isolation

The two *B. thuringiensis* isolates were purified from soil and insect cadaver during routine screening. The isolate NBAII-BTG4 was isolated from rhizosphere soil (Rajasthan) during screening of 158 soil samples whereas the NBAII-BTAS was isolated from an infected lepidopteran larvae from Assam. Microscopic analysis revealed that the indigenous *B. thuringiensis* isolates NBAIIBT-G4 and NBAIIBT-AS were spore forming, Gram positive and produced bipyramidal crystals.

B. thuringiensis was isolated from many environmental sites, e.g. soil, insect habitats, insect larvae, stored product and leaf surface (Martin and Travers, 1989; Ohba and Aizawa, 1986 and Smith and Couche, 1991). Bipyramidal shaped crystals show a greater degree of toxicity than all other types and the majority of lepidopteran active *B. thuringiensis* produce such bipyramidal crystal inclusions (Chilcott and Wigley, 1994).

PCR studies

To date, specific DNA sequences of a known *cry* gene sequence used either as probes or PCR primers to

identify the potential *cry* gene sequences have been used as a common strategy for cloning *cry* genes (Shang Kuo *et al.*, 2000). Carozzi *et al*., (1991) initially performed PCR analysis to identify different delta-endotoxins and reported the sequences of 12 PCR primers that distinguished three major classes of ICP genes (*cry*I, *cry*III, and *cry*IV). In the present study, analysis of *cry* genes present in the two indigenous *Bt* isolates namely NBAII-BTAS and NBAII-BTG4 was characterized based on primers indicated in Table 1. BT-HD-1 was used as positive standard for the cry genes- *cry* 1, *cry* 2, *cry* 1Aa, *cry* 1Ab and *cry* 1Ac. For the other *cry* genes namely cry1I, cry1G and cry1E, we only did a preliminary study using universal primers and standard validation was done only based on the size of the PCR product as mentioned in the quoted papers. The results of the PCR studies revealed that both the isolates expressed the *cry*1 gene (558bp) but NBAII-BTG4 also expressed the *cry*2 gene (1.17kb) which is supposed to have dual toxicity against Lepidopterans and Dipterans (Fig. 1). The *cry* gene profile of the two *Bt* isolates were also analyzed using *cry* specific primers and the PCR analysis revealed that the two isolates harbored *cry*1A, *cry*1Ab (1111bp) and *cry*1Ac (238bp) genes, but, the NBAII-BTG4 also expressed the *cry*1Aa (398bp) gene (Fig. 2 to 4).

Analysis was also carried out using the universal *cry* primers for detection of other *cry* genes. Results established that both were positive for *cry*1I which is having supposed to have dual toxicity against Lepidopterans and Coleopterans (Fig. 5). The NBAII-BTAS isolate, however, also showed positive for the genes *cry*1E (Fig. 6) and *cry* 1G (Fig. 7). The comprehensive *cry* gene profile of the two isolates is indicated in Table 2.

PCR-based techniques have been proposed to identify different *cry* genes in *B. thuringiensis* strains (Porcar and Juarez-Perez, 2003). The insecticidal pathogenicity of *B. thuringiensis* can be predicted from the presence of *cry* genes in the bacterial genome (Carozzi *et al.,* 1991 and Ben Dov *et al*., 1999). The profile of *B. thuringiensis* *cry* genes can be estimated by the polymerase chain reaction (PCR) method with specific primers (Konecka *et al.,* 2007).

We also undertook partial sequencing of the specific primer products for *cry*1Aa, *cry*1Ab and *cry*1Ac. Blast analysis revealed that the *cry*1Aa gene of NBAII-BTG4 shares 99 per cent homology with the reference sequence of *B. thuringiensis* strain BLB1 *cry*1Aa gene (GenBank Acc. No. GU322940.1). Similarly, for *cry*1Ab and *cry*1Ac specific genes partial PCR products share a homology of

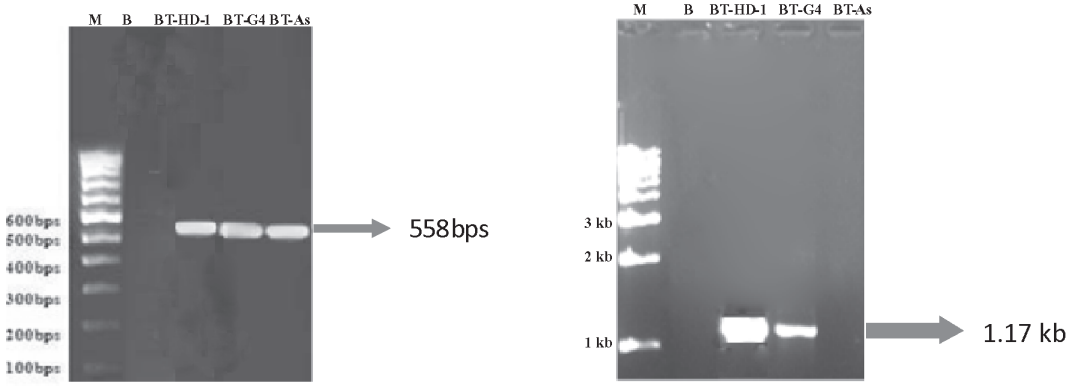

Fig. 1: Detection of *cry* 1 and *cry* 2 genes

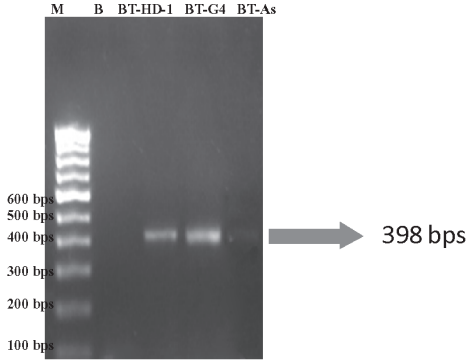

Fig. 2: Specific *cry 1Aa* gene PCR product

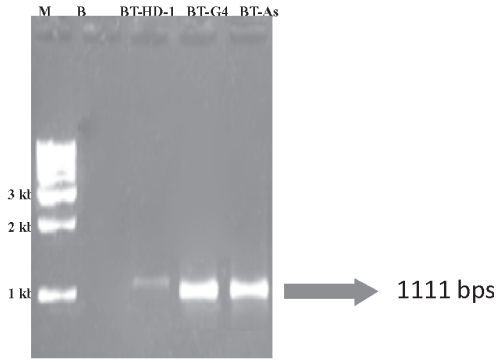

Fig. 3: Specific *cry 1Ab* gene PCR product

at least 99% for the reference strain GU322940.1 and EU250285.1 respectively.

The GenBank accession numbers assigned for *cry*1Aa from NBAII-BTG4 is JN120765 and *cry*1Ac from NBAII-BTAS is JN120764. Similarly, *cry*1Ab partial cds obtained from NBAII-BTG4 and NBAII-BTAS were submitted and the respective accession numbers are JN120763 and JF501457.

SDS-PAGE analysis

The crystalline delta-endotoxins are predominantly synthesized as long inactive protoxins that are activated by proteolysis in the insect gut. These toxins include *cry1*, *cry4A*, *cry4B* having molecular weights ranging from 130-140 kDa and are processed to active 65-70 kDa toxins while, *cry2A*, *cry3A*, *cry10A* and *cry11A* are naturally truncated toxins with molecular weights ranging from 65-80 kDa. However, as observed in *cry2A* and *cry3A*, proteolytic cleavage at the N and C termini can also process these naturally truncated toxins to active 60-65 kDa toxins (Gill *et al*., 1992; Hofte and Whiteley, 1989). In our studies SDS-PAGE analysis of whole cell protein was carried out and the results revealed that the strains synthesize a protein or a group of proteins with molecular weights between 130 and 140 kDa (consistent with the presence of a *cry*1 gene), and a further protein of 65 kDa (consistent with the presence of a *cry*2 gene) (Fig. 9).

Toxicity studies

The trypsin activated toxins of the indigenous *Bt* namely NBAII-BTG4, NBAII-BTAS was evaluated against second instar larvae of *H. armigera* and it was observed that the standard *Bacillus thuringiensis kurstaki* HD-1 and NBAIIBTG4 were on par showing a LC_{50} value of 0.92 and 0.93 µg/ml respectively (Fig. 9). The least toxic was NBAII-BTAS which showed a LC_{50} value of 4.6 µg/ml. The lowest LC_{50} values (highly toxic) were recorded in the *Btk* isolates (BtNg13, BtAm2 and BtPl4) from Tamil Nadu (0.03–1.82 mg ml^{-1}) when tested against *H. armigera* (Anitha *et al.*, 2011). Toxicity and larval growth inhibition of 11 insecticidal proteins of *B.thuringiensis* against neonate larvae of *H. armigera*, revealed that the most active toxins were *cry* 1Ac and *cry*2Aa1 with LC_{50} values of 3.5 and 6.3 ìg/ml, respectively (Avilla *et al*., 2005). The *cry* I proteins belonging to different subgroups could exert different insecticidal properties against different lepidopteran pests (Gill *et al.*, 1992). In our studies, the isolate NBAII-BTG4 carried diverse types of *cry* genes and was more toxic. Carrozi *et al.* (1991) has stated that one can predict insecticidal activity of *B. thuringiensis* strains by polymerase chain reaction product profiles.

The present study established that *cry* gene profiling of new *B. thuringiensis* isolates can be readily done using PCR based techniques. Based on the *cry* gene profile one can determine their toxicity status.

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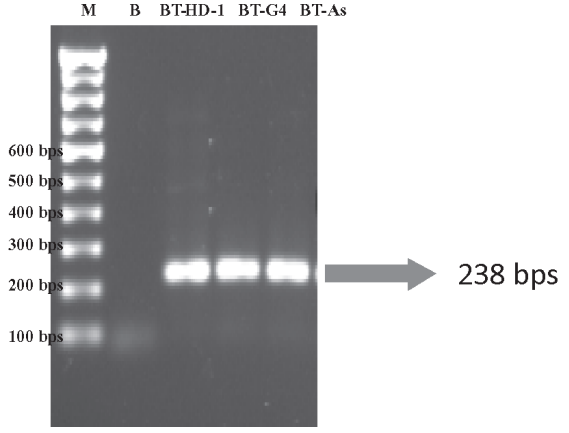

Fig. 4: Specific *cry 1Ac* gene PCR product

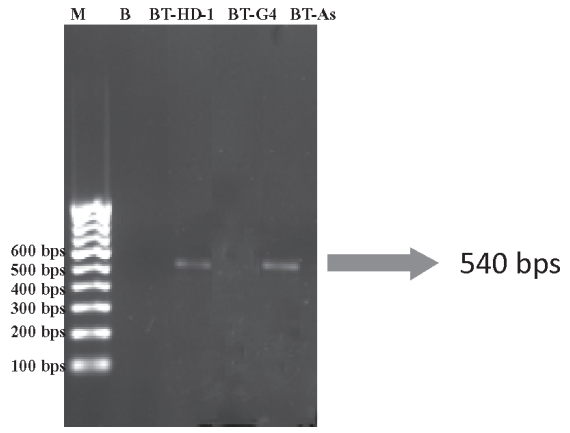

Fig. 5: *cry 1E* specific primer PCR product

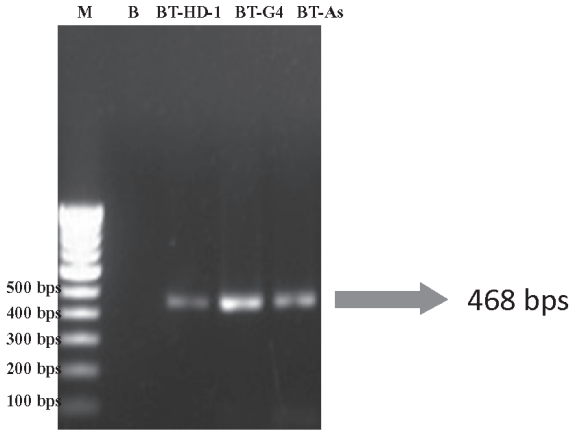

Fig. 6: *cry 1I* specific primer PCR product

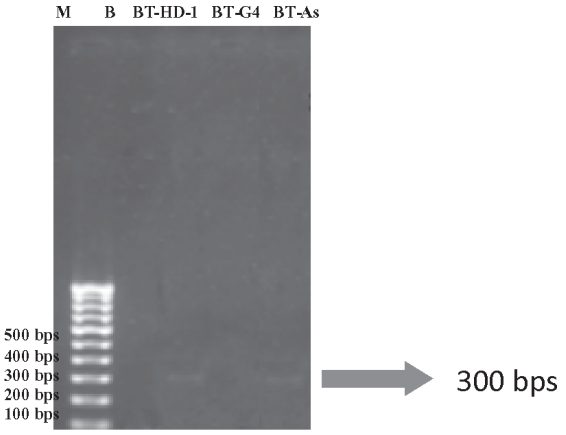

Fig. 7: *cry 1G* specific primer PCR product

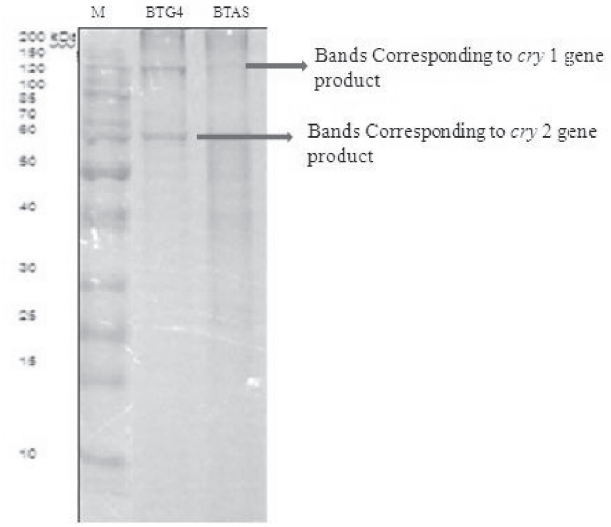

Fig. 8. SDS-PAGE analysis of the purified cry proteins

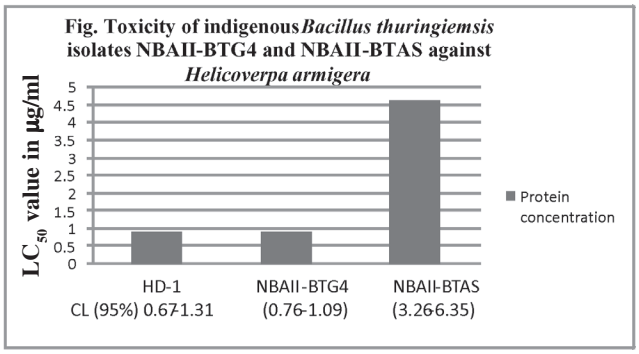

Fig. 9: Evaluation of trypsin activated toxins of indigenous *Bacillus thuringiensis* isolates NABII-BTG4 and NBAII-BTAS against *Helicoverpa armigera*

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Table 2. *cry* gene profile of the indigenous *Bacillus thuringiensis* isolates NBAIIBT-AS and NBAIIBT-G4

Genes	NBAII-BTAS	NBAII-BTG4	Active against
Universal *cry*1 primers	✓	✓	Lepidopteran
Universal *cry*2 primers		✓	Lepidopteran & Dipteran
Universal *cry*1A primers	✓	✓	Lepidopteran
*cry*1Aa specific primers		✓	Lepidopteran
*cry*1Ab specific primers	✓	✓	Lepidopteran
*cry*1Ac specific primers	✓	✓	Lepidopteran
Universal *cry*1B primers			Lepidopteran & Coleopteran
Universal *cry*1C primers			Lepidopteran
Universal *cry*1E primers	✓		Lepidopteran
Universal *cry*1F primers			Lepidopteran
Universal *cry*1G primers	✓		Lepidopteran
Universal *cry*1H primers			Lepidopteran
Universal *cry*1I primers	✓	✓	Lepidopteran & Coleopteran
Universal *cry*3 primers			Coleopteran
Universal *cry*9 primers			Lepidopteran

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