Pakistan J. Zool., vol. 43(4), pp. 701-713, 2011.

Cloning and Expression of *cry11B* Gene From a Local Isolate of *Bacillus thuringiensis* and its Mosquitocidal Activity

Farah Rauf Shakoori,[1] Muhammad Tariq Zahid,[1] Dil Ara Abbas Bukhari[2] and A.R. Shakoori[2]*
[1] *Department of Zoology, Government College University, Lahore*
[2]*School of Biological Sciences, University of the Punjab, Lahore, Pakistan.*

Abstract.- The present paper describes *cry11B* gene from a local isolate of *Bacillus thuringiensis (B.t.). Escherichia coli* DH5α was transformed with recombinant DNA comprising pTZ57 and *B. t. cry11B* gene (1.9kb) amplified from a local *B.t.* isolate SBS *B.t.* 48 for cloning. The cloned 1.9kb gene was sequenced and then ligated in the expression vector pT7-7 for transformation of *E. coli* $BL21C^{+}$. For expression of the recombinant organism containing *cry11B* gene, the conditions were optimized with respect to IPTG concentration, time of induction and incubation temperature. The optimized expression was recorded at 1.0mM IPTG incubated at 37°C for 3.5 h. The toxicity of *B.t.* spores, recombinant organisms and crude recombinant Cry11B proteins was determined against the third instar larvae of mosquito, *Anopheles stephensi*. The LC_{50} of SBS *B.t.* 48 spores, recombinant organisms and recombinant Cry11B protein was, respectively, 700 µg /ml, 525 µg/ml and 390 ng/ml as against 850, 550 and 470 of HD500 standard *B.t.* strain. The toxicity of crude Cry11B recombinant protein was 1346 fold higher than that of the recombinant organism.

Key words: *Bacillus thuringiensis*, *cry11B* gene, expression optimization of expression, IPTG.

INTRODUCTION

Biological control through entomo-pathogenic microorganisms are now becoming important in crop, forest protection and in insect vector control (Carlton, 1988; Spear, 1987; Ertola, 1988). The genus *Bacillus* comprises gram-positive rods, grow aerobically and forms heat-resistant spores. The vegetative cells are large and straight and often grow in filamentous chains (Green, 1989). Different species of *Bacillus* have been studied and among them special attention is given to *Bacillus thuringiensis* (*B.t.*) and its various strains regarding toxicity. *B.t.* is a gram-positive, rod-shape, aerobic, and spore-forming bacterium that produces crystal protein during sporulation. *B.t.* is one of the important organism and widely used as bioinsecticides (Chak and young, 1990; Morris and Trollier, 1990).

The sporulating cells of *B.t.* release crystal proteins into the surrounding medium and after exposure of alkaline environment the crystal protein is activated. The C-terminal end of the protein recognizes the receptor site in the gut membrane and then its N-terminal end undergoes conformational changes and loop like structure is formed which ultimately insert in the membrane and makes pore(s). Knowles and Ellar (1987) observed that after insertion into the membrane and pore formation, an influx of the water with the ions occurs that causes cells to swell and finally leads to their lysis. The lysis of the cells due to the formation of nonspecific pores causes the paralysis of the gut and the larvae stop feeding. This brings about larval death (Schwartz *et al*., 1993; Lorence *et al*., 1995; Pietrantonio and Gill, 1996).

During the past years mosquito control programs worldwide have been evaluating by using *B.t.* subsp. *israelensis (Bti), B.t. darmstadensis, B.t. kyushuensis, B.t. morrisoni , B.t. fukuokaensis, B.t. medellin , B.t. canadensis, B.t. shandongiensis, B.t. amagiensis, B.t. jegathesan* and *B.t. higo* are reported to be toxic to mosquitoes namely *Culex quinquefasciatus, C. tritaeniorhynchus, C. sitiens, Anopheles stephensi* and *Aedes aegypti*

B.t. subsp. *israelensis* (*Bti*) is one of the leading candidates to replace the wide spectrum environmentally unsafe chemical insecticides (Porter *et al.*, 1993; Goldberg and Margalit, 1977; Padua *et al.*, 1994; Orduz *et al.*, 1992, 1994; Seleena *et al.*, 1995; Ragni *et al.*, 1996). The parasporal

* Corresponding author: arshak@brain.net.pk
0030-9923/2011/0004-0701 $ 8.00/0

crystalline inclusions of *Bti* comprises four major polypeptides with molecular masses of 135, 125, 68 and 28 kDa, which are referred to as CryIVB, CryIVA, CryIVD, and CytA, respectively, according to the classification designed by Höfte and Whiteley (1989) and assigned to Cry4A, Cry4B, Cry11A, Cry11B and Cyt1A according to the new proposed classification. The genes encoding the four crystal polypeptides of *Bti* have been cloned and the analysis of cloned gene products has showed the interaction of the different crystal proteins and their resulting mosquito larval toxicity (Angsuthanasombat *et al*., 1992; Delécluse *et al.,* 1993; Poncet *et al.,* 1993; Wu *et al.,* 1994).

In the present study the *cry11B* gene (1.9kb) from well characterized local *B.t.* isolate SBS *B.t.* 48 and positive control HD500 was amplified. The amplified gene was cloned and sequenced. The amplified *cry11B* gene was cloned in pT7-7 and expressed in *E. coli* BL21C^{+}. The biotoxicity assays showed significantly high mosquitocidal activity of recombinant Cry11B protein.

MATERIALS AND METHODS

Bacterial strains and plasmids

Local *B.t.* isolate SBS *B.t.* 48 which was previously characterized by biochemical tests (Baig *et al.,* 2010) was used to amplify the *cry11B* gene. The reference strain HD500 kindly provided by Daniel R. Zeigler, Director, Bacillus Genetic Stock Centre, Columbus, Ohio, USA was used as positive control of *cry11B* gene. Both these strains were maintained on LB agar medium. *E. coli* DH5α for plasmid propagation and BL21C^{+} was used for expression. pTZ57R/T (Fermentas) was used for initial cloning and sequencing of gene. pT7-7 was used for expression of *cry11* gene. Selection of positive transformants of *cry11B* gene was made on LB agar medium containing X-gal (270 µg/mL), IPTG (120 µg/mL) and ampicillin (100µg/mL).

Microscopy of B.t. *isolate*

The isolate was Gram stained and stained for endospore according to details in Bukhari and Shakoori (2010).

Protein profiling of SBS B.t. *48*

Proteins of *B.t.* were isolated according to Alberolla *et al*. (1999). *B.t.* strains were streaked on LB agar plates and grown at 30°C for 48 h until sporulation occurred and inclusions were visible under the microscope. Two to three colonies (2-3mm diameter) of each strain were removed from the plate with loop and transferred to 1ml of ice-cold 0.5M NaCl. The tubes were whirl mixed or sonicated briefly in sonic bath to create homogenous suspension of material, then spun at 13,000rpm (6500 x g) for 5 min. The supernatant was discarded, and pellets were suspended in 140 µl of lysis buffer (1% SDS, 0.01% mercaptethanol) by whirl mixing or sonication and boiled for 10 min. During boiling process the tubes were again sonicated briefly for dissolving the crystals. The tubes were spun for 10 min at 13,000 (6500 x g). Supernatant was saved and frozen at-20°C for SDS-PAGE.

Cloning of cry11B *gene*

The *cry11B* gene (1.9 kb) was amplified from SBS *B.t.* 48 and positive control HD500.

Total DNA was isolated by the method of Kronstad *et al*. (1983). Single isolated 24 hours old colony of *B.t.* from LB agar was inoculated in 250ml of LB broth and incubated at 37°C for 24 h. Centrifugation was done at 5000 rpm (3000 x g) for 15 min at 4°C. The supernatant was discarded and 10 ml of wash buffer (NaCl, 100mM Tris –HCl, 10mM EDTA, 10mM) was added. The mixture was vortexed to suspend the pellet and then centrifuged at 5000rpm (3000 x g) for 10 minutes. Five ml of solution II (NaCl 150mM, EDTA 100mM, lysozyme 2.5mg) was added to the pellet and incubated at 37°C in shaking incubator for 40 minutes. Later, 6.25ml of solution III (Tris–HCl, 100mM NaCl, 100mM, SDS, 2%) was added and incubated at 60°C for 1.5 hours in water bath. This reaction mixture was centrifuged at 5000rpm (3000 x g) at 4°C. Pellet was discarded, whereas 10ml of phenol:chloroform (25:24) was added to supernatant, gently inverted two to three times, and then centrifuged at 5000rpm (3000 x g) for 10 min at 4°C. The phenol chloroform treatment was done twice. At the end, 30-40ml of chilled absolute ethanol was added to the supernatant to precipitate the DNA. The tubes were centrifuged again at 5000rpm (3000xg) at 4°C and the DNA pellet was

dissolved in autoclaved distilled water and stored at -20°C.

PCR reaction cycle

Following primers were used for the amplification of *cry11B* gene (1.9kb) from SBS *Bt* 48 and reference strain HD500.

F 5 ATGCAATAACAACTTTAATACCACAG 3`
R 5` CTATGGTTTTAATGGAATCTGAGTCG 3

The PCR reaction mixture (50µl) comprised 5 µl of 10X Taq buffer with KCl (1mM Tris.HCl pH 8.8 and 500mM KCl), 200ng of DNA, 250 µM of dNTPs, 2.5mM of $MgCl_2$, 150pmoles of primer concentration. The amplification was done in a thermal cycler (Applied Biosystem 2720) with initial denaturation at 94°C for 5min followed by 35 cy, each of denaturation at 94°C for 2 min, annealing at 45°C for 2 min, and extension at 72°C for 2min, with final extension at 72°C for 5min.

PCR products of *cry11B* gene (1.9 kb) was cloned in pTZ57R/T cloning vector and then sequenced. For cloning, the PCR products were visualized in 1% agarose gel and then isolated from the low melting point agarose gels following method described by Sambrook *et al.* (1989). *cry11B* gene was ligated in T/A cloning vector pTZ57R/T, already digested with *Eco*R1 and *Hind*III by using Fermentas DNA ligation kit (#K1214). For ligation pTZ57R/T and DNA were used in 3:1 ratio at 20°C for 18 h. The competent cells of *E. coli* DH5α were transformed with recombinant DNA containing *cry11B* gene (Sambrook *et al.*, 1989). The transformed cells (200 µl) were spread on dried LB agar plates containing X-gal (270 µg/ml), IPTG (120 µg/ml) and ampicillin (100 µg/ml). The plates were then incubated at 37°C for 24 hours to screen blue and white colonies. Cloning was confirmed by two methods *viz*., colony PCR and restriction analysis of recombinant plasmid DNA containing *cry11B* gene.

Expression of cry11B *gene*

The amplified *cry11B* gene previously cloned in pTZ57R (T/A cloning vector) was expressed in *E. coli* $BL21C^+$. For the expression *cry11B* gene was first cloned in expression vector. For this purpose the amplified and purified *cry11B* (1.9kb) gene was ligated using T4 ligase in the expression vector, pT7-7 cut with *Eco*R1 and *Hind*III. *E. coli* $BL21C^+$ competent cells were made and pT7-7 containing *cry11B* gene was transformed. The positive transformants were screened on LB agar ampicillin (100µg/ml) plate. The cloning of *cry11B* gene in expression vector was confirmed by colony PCR and digestion of recombinant plasmids containing *cry11B* with *Eco*R1 and *Hind*III. The expressed recombinant Cry11B protein was isolated from 1.5ml culture broth previously inoculated with positive transformants and negative control (pT7-7 with out insert) and spun at 13,000rpm (6500 x g) for 5 min. Pellets were washed once with autoclaved distilled water, and then sonicated in 140µl of lysis buffer (1% SDS, 0.01% mercaptoethanol) before being boiled for 10 min. During boiling the tubes were again sonicated briefly for solubilization of crystals. All tubes were again spun for 10 min at 13,000rpm (6700 x g) and supernatants were removed very carefully in new tubes with out disturbing the pellet. The expressed protein was analyzed by SDS-PAGE, for which 15µl sample was run on 12% SDS-PAGE (5% stacking and 12% resolving) along with the control sample.

Optimization of the expression of cry11B *gene*

Different conditions such as concentration of IPTG, incubation time, and incubation temperature were optimized for the good expression of recombinant Cry11B protein. For optimum IPTG concentration three different concentrations of IPTG ranging 0.25, 0.5 and 1.0 mM were used during incubation at 37°C for 3 h. Likewise four different incubation temperatures (*viz*. 28°C, 30°C, 35°C, and 37°C) were tried at IPTG concentration of 0.5mM for 3 h for determination of optimum temperature. The incubation time was also optimized at IPTG concentration of 0.5mM and incubation temperature at 37°C. Three different incubation periods *viz*. 2, 3.5 and 5 h were tried to determine the optimum incubation time for maximum expression. $BL21C^+$ transformed with pT7-7with out insert was used as controls.

Biotoxicity assays with different preparations of SBS B.t. 48

Three different preparations *viz.*, bacterial

spores, recombinant organisms and crude preparation of recombinant Cry 11B protein of SBS *B.t.* 48 and HD500 were used for determination of biotoxicity against 3rd instar larvae of mosquito, *An. stephensi*.

Bacterial spores

Spores of bacterial isolates were prepared according to Makino *et al*. (1994), for which single isolated colony of *B.t.* was inoculated in LB broth and incubated at 37°C. After 24 hours, the above inoculum was streaked on sporulated medium T3 (tryptone 3g/L, yeast extract 1.5g/L, tryptose 2g/L, $MnCl_2$ 0.005g/L, NaH_2PO_4 6.9g/LNa_2HPO_4 8.9g/L and agar15g/L) and incubated at 30°C for 72 h. After 3 days, the spores were harvested from the medium and washed five times with autoclaved distilled water**.** Then the pellet was incubated at 37°C for 40 min in 10 ml of 2M KCl:10mM sodium phosphate (1:1) pH 7.5. The spores were washed twice with the autoclaved distilled water, and then incubated at 37°C for 30 min in 10ml of 5 mM of urea : 5mM CHES (1:1) pH 9.3. This was followed by the addition of 25 mM of 2-mercaptethanol. The spores were then washed five times with autoclaved distilled water and stored at 4°C.

The bioinsecticidal activities of sporulated form of SBS *B.t.* 48 determined in nine replicates against third instar larvae of *An. stephensi* obtained from Malaria Research Center, Birdwood Road, Lahore. Different concentrations of *B.t.* spores *viz.*, 0, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 µg/ml were added to eleven wide mouthed cups, each containing 100 ml of autoclaved distilled water with 100 third instar larvae and incubated at 25°C. After 24 hours the mortality of larvae was recorded in each cup. The larvae which were knocked down at the bottom of cup and failed to swim to the surface of water, were considered dead. Assessment of the toxicity was determined through Log-Probit analysis (Finney, 1952).

Recombinant E. coli *transformed with* cry11B *gene*

E. coli BL21C+ transformed with *cry11B* gene was first grown in 250ml LB broth supplemented with ampicillin (100µg/ml) and IPTG (1.0mM) and incubated at 37°C for 3.5 hours in shaking incubator. Cells were harvested by centrifugation at 10,000rpm (3500 x g) and washed twice with autoclaved distilled water. Pellet was then weighed and desired concentrations *viz.* 50, 100, 150, 200, 250µg/ml ….and up to 500µg/ml were added in sterile cups containing 20ml autoclaved distilled water. Twenty mosquito larvae (*An. stephensi*) were kept in this water at 25°C for 24 h to check the toxicity of the organism, expressing Cry11B protein. The number of dead larvae counted and % mortality calculated.

Recombinant Cry11B *protein*

The above transformed organism was first grown in 250ml LB broth supplemented with ampicillin (100 µg/ml) and IPTG (1.0mM) and incubated for 3.5 h at 37°C in shaking incubator. The cell pellet was washed thoroughly with buffer (10 mM Tris-HCl pH 7.2, 10 mM NaCl and 1% Tween-20), disrupted by sonication at 4°C, and cell-free extract prepared by centrifugation. Different concentrations (*viz.*, 100, 200, 300, ……1000ng/ml) of total protein of recombinant organism were added in sterile cups containing 20ml autoclaved distilled water and 20 third instar larvae of mosquito, *An. stephensi* at 25°C for 24 h. The number of dead larvae was counted and % mortality calculated.

RESULTS

Characteristics of SBS B.t. *48*

These were Gram positive rods which were purple in colour. The cells sporulated after about 18h (Fig.1A). The vegetative cells containing spores were visible (Fig.1B), and sporulation seemed to be complete after 48 h (Fig.1C), when most of the *B.t.* cells contained spores and crystals. Sporangia were released after 72h (Fig. 1D). HD500 also showed the same stages of sporulation at 18, 24 and 48 hours (Figs. 1E, F and G). During endospore staining with Malachite green of SBS *B.t.*48 endospores green in colour released in medium after 48 and 72h (Fig. 1I and J) while some vegetative cells containing spores are visible inside the cell (Fig.1H). The endospores staining of positive control HD500 showed the same pattern of sporulation. The vegetative cells have spore inside the cell (Fig. 1K) and almost 75% culture got sporulated after 48 h (Fig. 1L). Malachite green

stained the spore green and acid fuchsin gave deep pink colour to protein crystal and vegetative cells.

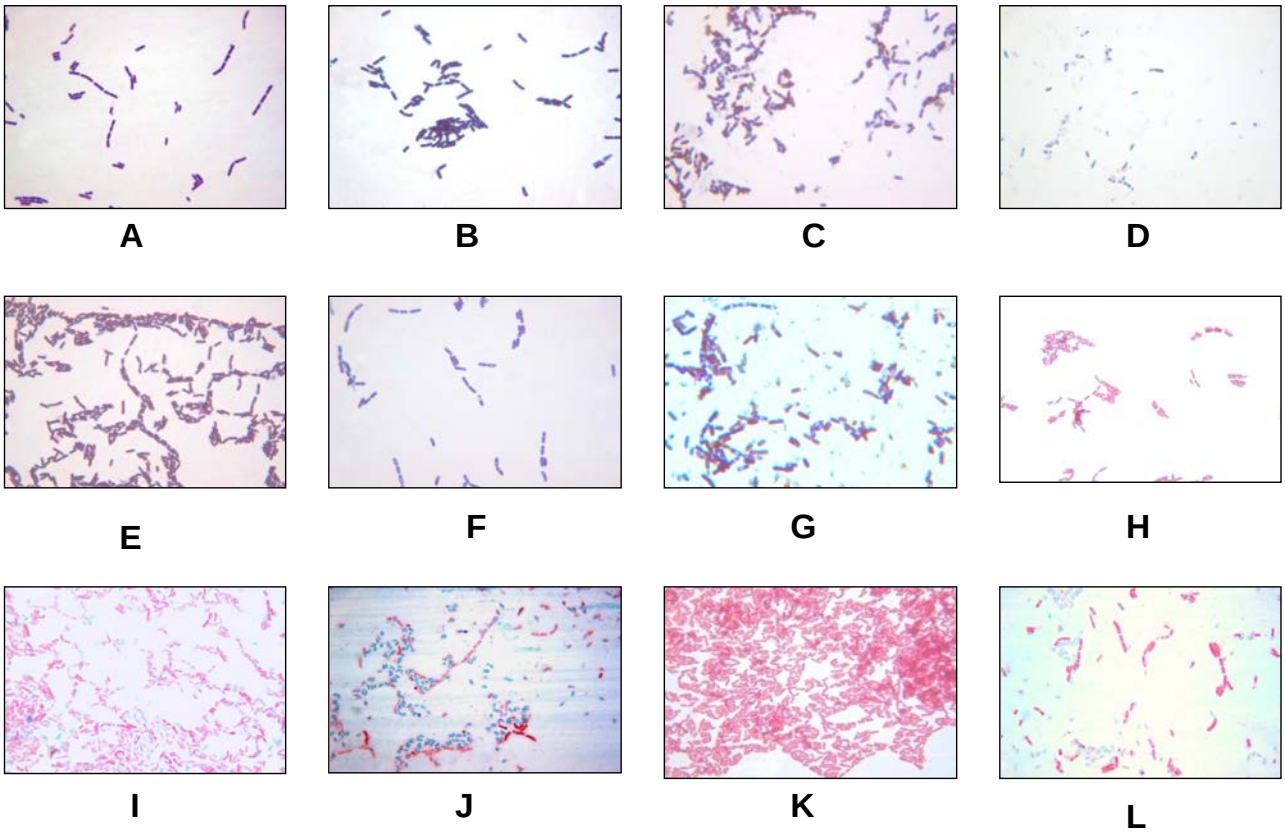

Fig. 1. Gram staining and endospore staining of SBS *B.t.*-48 and HD 500 at different hours of growth in LB medium at 37°C.

The endospore was paracentral or sub terminal and all had extra cellular protein crystals.

Protein profile of SBS B.t. *48*

Figure 2 shows the SDS-PAGE protein profile of SBS *B.t.* 48. The most important bands were proteins of 130kDa, 70kDa, 68kDa, and 44kDa. The protein bands with low molecular weights of 29kDa and 14kDa, were also present in the total protein profile of *B.t.* isolate SBS *B.t.* 48 and positive control HD 500.

cry11B *gene*

The 1.9 kb PCR product of *cry11B* gene (Fig. 3) was ligated in pTZ57R (T/A cloning vector) and *E. coli* DH5α cells were transformed with this recombinant DNA. The clones were confirmed by digestion with *Eco*RI and *Hin*dIII (Fig. 4). *cry11B* gene of SBS-*B.t.* 48 was sequenced. Its total length was 1.9 Kb and encoded 646 amino acids with molecular weight of 70494.9 (approximately

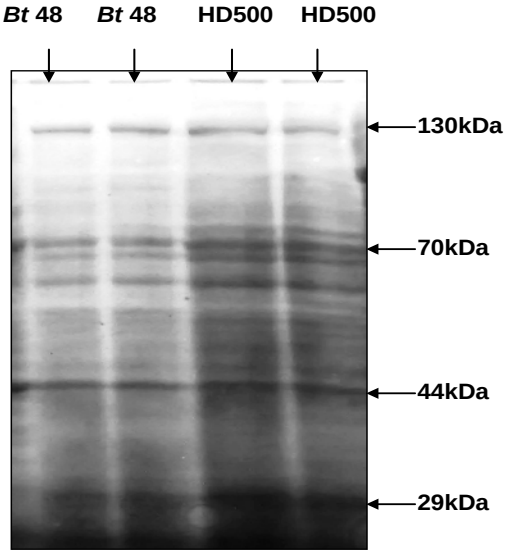

Fig. 2. Polyacrylamide gel electrophoretic pattern of total proteins of sporulated *B.t.* isolate SBS *B.t.* 48 and HD500.

70 kDa). The *cry11B* gene sequence was deposited in the GenBank DNA Database with accession number HQ 845255. The sequence was aligned with already known sequences using BLAST (Basic Local Alignment Search Tool), which showed 99% homology with X86902.1 *Bacillus thuringiensis* subsp. *jegarthesan*.

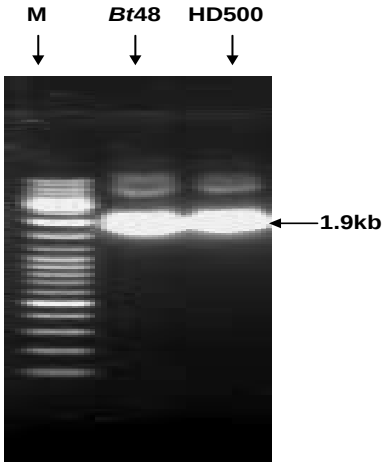

Fig. 3. PCR product of *cry11B* gene (1.9Kb) from *B.t.* isolates. M, DNA Marker (1Kb).

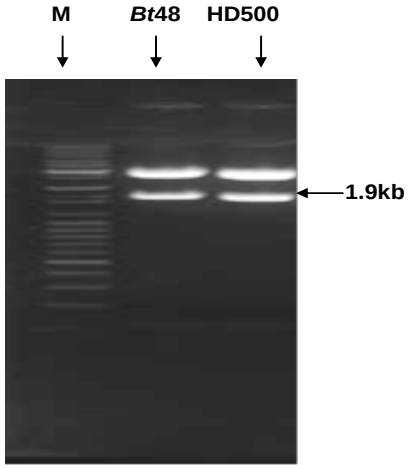

Fig. 4. Restriction digestion, with *Eco*RI and *Hin*dIII, of recombinant plasmid containing 1.9 kb toxic region *cry11B* gene of *B.t.* isolate SBS *B.t.*48 and HD500,

Expression of cry11B *gene in* E. coli *BL21C*$^+$

For the expression of *cry11B* gene, *E. coli* strain BL21C$^+$ was transformed with recombinant DNA comprising pT7-7 expression vector ligated with *cry11B* gene encoding 70kDa protein through T7 promoter.

Optimum expression was at 1.0mM IPTG at 37^{o}C for 3.5 h. pT7-7 vector without *cry11B* gene and transformed in *E. coli* was used as control. The total cell protein was isolated and run on 12% SDS–PAGE. The expressed 70kDa Cry11B protein bands were present in the total protein profile of all the organisms (BL21C$^+$ transformed with pT7-7 containing *cry11B* gene) and absent in the protein profile of control containing only transformed expression vector (pT7-7) with out *cry11B* gene (Fig. 5).

Biotoxicity of B.t. *formulations*

B.t. *spores*

Table I shows the effect of spores of SBS *B.t.*48 against 3rd instar larvae of *An. stephensi*. The isolate showed 50% mortality at 700µg spores/ml, as against 850 µg of spores/ml of HD-500 for the same mortality (Fig. 6).

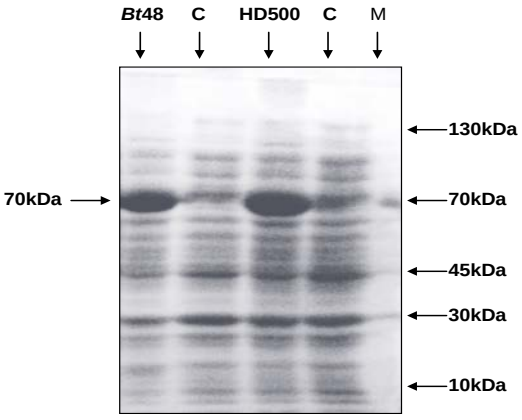

Fig. 5. Optimized protein expression profile of induced *E. coli* samples with 1.0mM of IPTG in comparison to uninduced *E. coli* samples (lane 1 and 3) and control sample (lane2 and 4) did not have any insert of *cry11B* gene but have vector grown at 37°C for 3.5 hours. The induced 70 kDa Cry11 band is very prominent in induced samples in comparison to all other samples.

Recombinant organism

Bio-toxicity assay of recombinant organisms was done to check the toxicity of individual *cry11B* gene against mosquito larvae of *An. stephensi*. Different concentrations of transformed organism were added in 20ml water containing 20 mosquito larvae of 3rd instar *An. stephensi*. Toxicity was calculated as percentage mortality of triplicate readings. It was found that LC_{50} (525 µg/ml) of SBS *B.t.* 48 is bit less then LC_{50} of HD500 (550µg/ml) (Fig. 6). *cry11B* gene from SBS *B.t.* 48 is comparatively more toxic as compare to *cry11B* gene of positive control HD500 (Table I).

Table I.- Toxicity (% mortality) of sporulated form of *B.t.* 48, recombinant *E. coli* BL21C$^+$ transformed with *cry11B* and crude recombinant protein Cry11B against 3rd instar larvae of *An. stephensi* exposed for 24 h.

No	Dose (µg/ml)	Spores		Recombinant organism*		Dose (ng/ml)	Crude recombinant protein Cry11B	
		SBS *B.t.* 48	HD 500	SBS *B.t.* 48	HD 500		SBS *B.t.* 48	HD 500
1	0	1.67±1.11	0	1	0	0	0	0
2	100	15.0±2.88	16.67±3.33	11.67±2.33	11.87±1.93	100	17.51±2.13	15.01±1.06
3	200	16.67±4.01	18.33±1.67	25.0±1.89	18.33±2.30	200	24.0±1.09	22.89±1.21
4	300	23.33±3.33	15.0±2.89	33.33±2.05	25.0±2.45	300	33.65±3.43	29.55±1.90
5	400	30.0±2.87	20.0±5.77	41.67±2.67	36.67±1.37	400	46.87±2.39	35.87±2.54
6	500	28.33±1.67	26.67±1.67	48.33±1.57	46.67±2.76	500	51.45±2.67	44.43±1.95
7	600	36.67±5.01	26.67±4.41	60.0±2.55	55.0±1.48	600	60.77±1.98	57.09±2.86
8	700	50.0±4.77	33.33±1.67	66.67±2.09	63.33±2.93	700	72.77±3.71	67.35±1.64
9	800	52.0±3.08	40.0±5.0	74.09±1.73	77.0±5.0	800	83.09±2.93	79.0±2.88
10	900	55.0±3.77	55.0±5.77	87.55±2.44	85.65±7.1	900	92.0±2.04	90.55±1.75
11	1000	58.33±1.41	60.33±6.01	99.0±3.3	100	1000	100	100

* *E. coli* BL21C$^+$ transformed with *cry11B.*

Recombinant Cry11B protein

Biotoxicity assay of crude recombinant proteins Cry11B protein was done against 3rd instar larvae of mosquito *An. stephensi*. The toxicity of crude recombinant protein had increased several folds when compared with that of organism transformed with specific genes. Biotoxicity assays with expressed Cry11B protein from SBS *B.t.* 48 showed LC_{50} at 390ng/ml while with recombinant organism the LC_{50} value was 525µg/ml so the increase in toxicity is 1346.15 folds. The LC_{50} value of recombinant organism containing *cry11B* gene from positive control HD500 was 550µg/ml and with crude recombinant Cry11B protein was 470ng/ml so the increase in toxicity is 1170.2 folds (Table I).

Three dimensional structure of Cry11B protein

The activated region of the delta-endotoxin Cry11B is composed of three structural domains range 50 to 633 (Fig 7) but among them only the N-terminal helical domain is involved in membrane insertion. It is only conserved region which involved in pore formation. The second and third domains are not conserved and involved in receptor binding (Fig.8). Domain I of Cry 11B toxin is composed of 15-256 residues consists of 9ά -helices and two small ß-strands. All the helices in the Cry11B were slightly shorter and less amphiphilic than other Cry proteins. The ά4 and ά5 helices of Cry 11B toxin insert into the membrane in an antiparallel manner as a helical hairpin. Domain II of Cry11B toxin comprised by residues 257-478, one helix (ά8, Ala279-Ala285) and 11 ß-strands (ß2 Ser292-Asn305, ß3 Pro319-Ser332, ß4 Ile341-Lys343, ß5 Thr365- Iso369, ß6 Val374-Phe381, ß7 Trp389-Leu396, ß8 Asn401-Arg407, ß9 Ile418-420, ß10 Pro437-Thr450, ß11 Tyr458-Val468 and ß12 Phe470-Lys476). The ß2 and ß3 and the N-terminal part is mostly hydrophobic, while the C-terminal half (Thr312-Thr318) is polar. The charged residues present at the tip of the loop are important of insect specificity. The domain III is highly conserved and consists of ß-strands *viz.*,ß13a (Tyr485-Asn490), ß3b (Ile495-Ala497), ß14 (Ala501-Val503), ß15 (Pro513-Ala516), ß16 (Ser520-Gly529), ß17

(Lys533-Asn543), ß18 (Thr546-Arg553), ß19 (Lys555-Ala562), ß20 (Gly579-Glu583), ß21 (Ile592-Leu601), ß22 (Thr608-Val619).The domain III only determine the specificity of toxin.

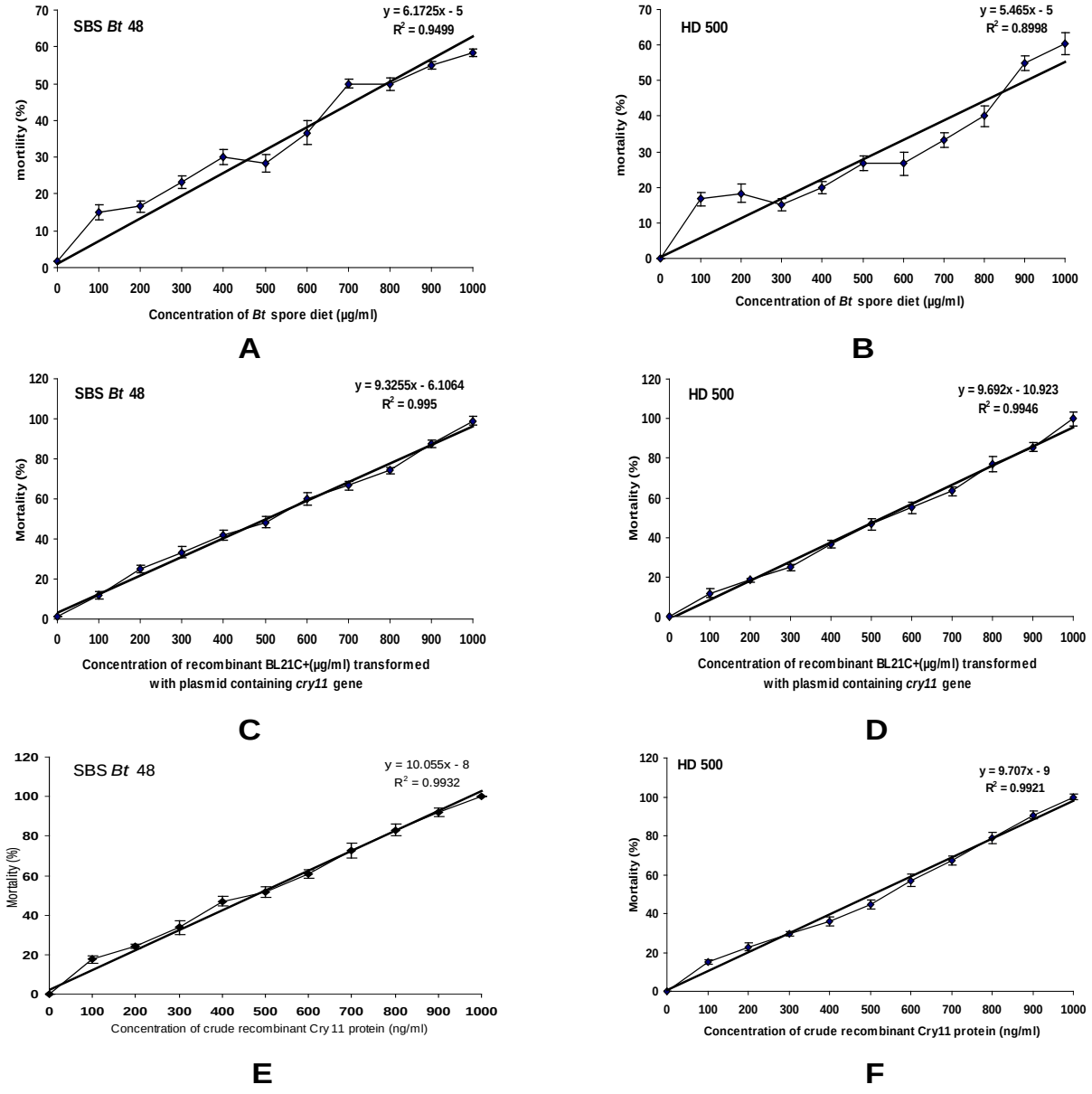

Fig. 6. Toxicity (%) mortality of *B.t.* spore (A, B), recombinant organisms containing *cry11* gene (C, D) and crude recombinant Cry11 (E, F) protein.

DISCUSSION

We have reported expression of 1.9kb *cry11B* gene and the mosquitocidal activities of various formulations of SBS *B.t.* 48.

Many reports on the frequency of occurrence of *B.t.* from natural environments indicate a high possibility of being able to isolate a novel strain with improved mosuitocidal activity (Iriate *et al.*, 2000). The diversity of *B.t.* in natural population is

explained on the presence of a vast majority of *cry* genes on transmissible plasmids effective against their specific pest. Improvement in *B.t.* based insecticides is expected from the discovery of natural isolates with novel, and more competent combinations of ICPs or formation new combinations using molecular techniques like DNA cloning, PCR amplification, Hybridization analysis etc (Akiba *et al.*, 2006).

The *cry11B* genes encode 67-94 kDa proteins highly active against different species of mosquito larvae, which are vectors of tropical diseases such as yellow fever, malaria and dengue (Sauka *et al.*, 2010).

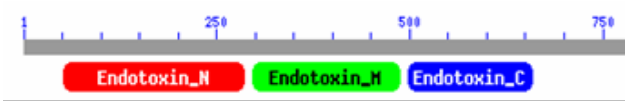

Fig. 7. Linear form of three conserved domains of Cry11B of SBS *B.t.* 48. N-terminal domain is involved in membrane insertion, M and C domains are involved in receptor binding.

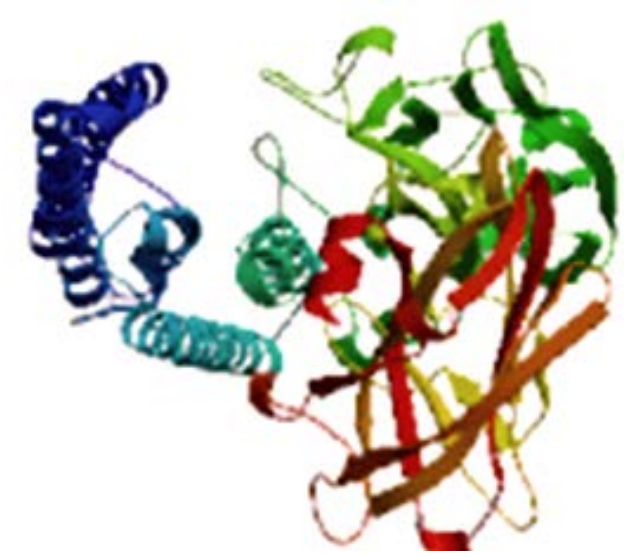

Fig. 8. Overall view of the Cry11B toxin from SBS *B.t.* 48, which is composed of three domains. Pore-forming domain I is blue, putative receptor binding domain II is red, and C-terminal domain III is green.

The first report became available about the cloning of *B.t.* subsp. *kurstaki* crystal protein expressed in *E. coli* (Schnepf and Whiteley, 1981.).

E. coli is an effective expression system for *cry* genes, because the parameters of the biosynthetic processes are better understood in this bacterium. There are a lot of reports in which the cloning and expression of novel *B.t.* toxin genes have been performed mainly to attempt their characterization (Ben-Dov *et al.*, 1995; Brizzard *et al.*, 1991; Ge *et al.*, 1990; McPherson *et al.*, 1988; Sanchis *et al.*, 1989; Shimizu *et al.*, 1988). The use of *E. coli* as an expression host allows the selective production of Cry proteins with particular biocidal specificity for their individual study. In addition, it can also be used as an effective vehicle for industrial production of crystal proteins, functionally improved by genetic and protein engineering (Aronson *et al.*, 1995; Bosch *et al.*, 1994; Caramori *et al.*, 1991; De Maagd *et al.*, 1996). Ge *et al.* (1991) have optimized the culture conditions for suitable hyper-expression in *E. coli* of the gene *cry1Ac73* encoding the Cry1Ac insecticidal crystal protein. P*tac* promoter was found to have the best performance for the production of protoxins to high levels on pBR322 derivatives (pKK223–3) in *E. coli* host JM103. The expression in *E. coli* of the truncated *cry1A* gene versions encoding the ICP toxic fragments has also been achieved (Vazquez *et al.*, 1995).

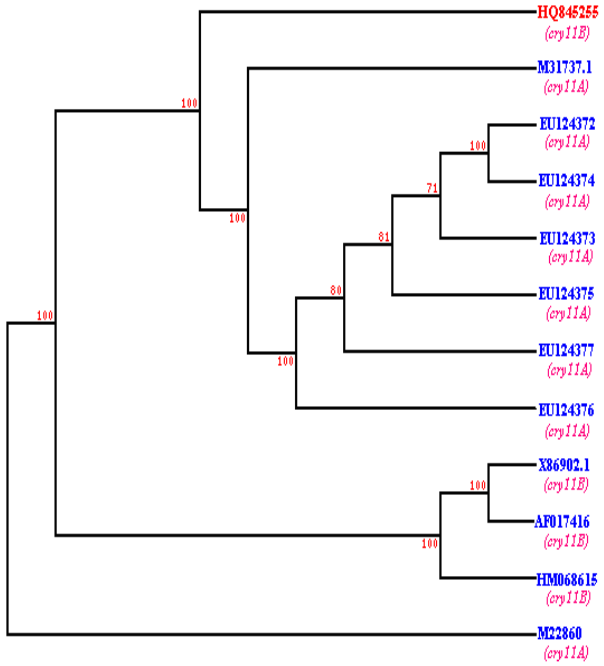

Fig. 9. Dendrogram showing the relatedness of *cry11B* gene of SBS *Bt* 48 with accession no HQ845255 with already reported genes in the GENbank data base. The values shown at the base of divergent lines are bootstrap values.

In present work, we amplified and cloned the *cry11B* gene. Bioassays and molecular detection by PCR represent an alternative method to western blot and ELISA technique (Tilquin *et al*., 2008). After sequencing this gene was expressed in *E. coli* strain $BL21C^{+}$ to study the expression pattern. pTZ57R vector was used to clone the 1.9kb *cry11B* gene and pT7-7 vector was used as expression vector to transfer the cloned *cry11B* gene into the *E. coli* host organism after restriction digestion by using *Eco*R1 and *Hin*dIII restriction enzymes. Various promoters are used to increase the expression level of the inserted gene like heterologous promoter was used for amylase gene of *B. subtilis, cry3Aa* and *cry3Bb* (Agaisse and Lereclus**,** 1995). In our case, we used T7 promoter in pT7-7 for controlling the expression of *cry11B* gene inserted next to it. The T7 promoter is recognized by the viral T7 RNA polymerase encoded by genomic DNA of the *E. coli* and is under the strict control of strong *lacuv* promoter. Now, in *E. coli* the sporulation dependent *cry11* gene becomes independent of asporulation phase and expressed as normal cellular gene in *E. coli.*

The condition of good expression of *cry11B* gene was optimized into the *E. coli* host with respect to the incubation time, incubation temperature and IPTG concentration. After 3.5 hours of incubation time, at 37^{o}C and by using 1.0mM IPTG concentration maximum expression was obtained in induced as compared to uninduced samples. The presence of proteins in uninduced samples could be because of some leakage of inducible T7 RNA polymerase gene promoter. This kind of constitutive expression has also been reported by Ge *et al.* (1990). As it was reported that T7 promoter is recognized by viral RNA polymerase encoded by host genomic DNA. In host cell, chromosomal DNA encodes viral RNA polymerase is itself under strict regulation of *lacuv* promoter. IPTG is an artificial inducer of this promoter being having structural homology to the natural inducer lactose. This inducer activates *lacuv* promoter which in turn transcribes more RNA polymerase gene and later on more viral RNA polymerases were translated from these transcripts by using host cell machinery. Thus large number of RNA polymerases now bind to T7 promoters to transcribe the downstream located gene accordingly and by this switching and indirect activation by IPTG, expression of the cloned *cry11*B gene was enhanced exponentially within very short time period of 3.5 hours. Thus by using *E. coli* as host cell, we can get the expression of sporulation dependent genes even earlier than normal cellular gene expression by induction mechanism.

The phylogenetic relationship of *cry11B* gene with other *cry11* genes reported earlier on from this laboratory (Bukhari and Shakoori, 2008) and other laboratories is shown in Figure 9. *cry11* reported from our laboratory are distinctly different from the ones reported elsewhere. The cluster of *cry11* genes reported from our laboratory seem to have evolved differently from the other reported *cry11* genes.

The activated Cry11 toxin consists of three structural domains Domain I consists of 9ά-helices and two small β-strands (Gutiérrez *et al.*, 2001).The most exposed helices are ά1 ά2a, ά2b, ά3, and ά6; ά4 and ά5 insert into the membrane in an antiparallel manner as an helical hairpin with their polar sides exposed to the solvent (Gutiérrez *et al.*, 2001). Domain II consists of 257-478 residues and is formed of three "Greek key" β-sheets arranged in a β prism topology. The N terminal of this protein is mostly hydrophobic, while the C-terminal half is polar. This protein interacts with the receptor through both hydrophobic and electrostatic interactions (Gutiérrez *et al.*, 2001). The Domain III is characterized by conservation of residues and determines the specificity of toxin. Homology based three dimensional structure of Cry11 protein form local *B.t.* isolate was made by using deduced amino acid sequences. The activated region of the delta-endotoxin Cry11B from SBS *Bt* 48 is composed of three structural domains range 50 to 633. Domain I of Cry11B toxin is composed of 15-256 residues consists of 9ά -helices and two small ß-strands. All the helices in the Cry11B were slightly shorter and less amphiphilic than other Cry proteins. The N-terminal helical domain is involved in membrane insertion. It is only conserved region which is involved in pore formation. The second and the

third domains are not conserved and are involved in receptor binding.

Biotoxicity assay with *E. coli* BL21C$^+$ transformed with plasmid containing *cry11*B gene against third instar larvae of mosquito, *An. stephensi* showed that *cry11B* from SBS *B.t.* 48 was more toxic (LC_{50}=525 µg/ml) as compared to *cry11* from HD500 (LC_{50}=550 µg/ml). Biotoxicity assay with crude recombinant Cry11B protein of SBS *B.t.* 48 showed LC_{50} of 390ng/ml while LC_{50} value of Cry11B protein from HD500 was 470ng/ml.

The increased level of toxicity from recombinant organism to crude recombinant protein was 1346.15 folds in the case of SBS *B.t.* 48, while the increase in toxicity was 1170.2 fold in HD500. Bukhari and Shakoori (2008) reported the high level of mosquito-larvicidal activity of crude recombinant Cry11 protein as compared to recombinant organisms containing *cry11B* gene.

To conclude the present study was molecular characterization of *cry11B* gene from local *B.t.* isolate and the determination of toxicity of Cry 11B protein as bioinsecticide against mosquito larvae.

ACKNOWLEDGMENTS

We are grateful to Prof Dr. Daniel R. Zeigler, Director, Bacillus Genetic Stock Centre, Columbus, Ohio, USA for providing us positive strain HD500 of *Bacillus thuringiensis* and to Ms. Ghazala, Director, Malaria Centre Birdwood Road, Lahore, for providing us mosquito larvae for bioassays.

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(Received 7 July 2010, revised 31 December 2010)