

Isolation, Molecular Characterization and Toxicity of *cryIC* Gene Harboring *Bacillus thuringiensis* From Different Habitats and Localities of Pakistan

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Abstract.- *Bacillus thuringiensis* (*Bt*) is Gram positive bacterium which forms endospores and produces parasporal crystals, which are toxic to specific lepidopterous and dipterous insects. In this study, 70 spore former rods were selected from 470 bacterial colonies, obtained from 72 soil and sand samples from different habitats and parts of Pakistan. Out of these 70 strains, 48 gave positive tests specific for *Bt*. Only three strains were positive for *cryIC* gene. Three isolates (MS-SBS-*Bt*1-3) were found positive for *cryICa*, and two (MS-SBS-*Bt*4-5) were found positive for *cryICb* genes. All *cryIC* genes were 99% homologous when aligned on alignment tool BLAST. The optimum growth conditions and antibiotic sensitivity of the isolates were determined. Bioassays showed that MS-SBS*Bt*1 was more toxic to *Spodoptera litura*, compared to control HD137, more toxic to *Musca domestica* compared to control HD29, and less toxic to *Heliothis virescens* compared to HD29.

Keywords: *Bacillus thuringiensis*, *cryIC* gene, bioinsecticides, *cryICa*, *cryICb*.

INTRODUCTION

Bacillus thuringiensis (*Bt*) is a Gram positive bacterium which forms endospores and produces parasporal crystals. These parasporal crystals known as Cry proteins are specific proteins which are toxic to different target insects, nematodes and some other organisms (DeMaagd *et al.*, 2001; Siegel, 2001; Frederiksen *et al.*, 2006) but are harmless to non-target insects (Torres and Ruberson, 2008; Grisolia *et al.*, 2009). Cry toxins kill larvae of the orders Lepidoptera, Coleoptera, Diptera, Hymenoptera, Homoptera, Mallophaga and organisms belonging to the phyla Nematoda, Platyhelminthes and Sarcomastigophora (Bravo *et al.*, 2007; Gatehouse, 2008). So they are very useful natural killers of target insects and are environment friendly. For these specific reasons *Bt* isolated from different sources are widely used in biopesticide industry (Carozzi *et al.*, 1991; Burges and Hurst, 1977).

The insecticidal Cry proteins from *Bt* are

encoded by *cry* genes, which include more than 230 different host ranges. They have been classified into 28 families according to the degree of amino acid identity shared by their corresponding proteins (Crickmore *et al.*, 2008). Regardless of the fact that many families of Cry proteins have been identified, only some families are toxic towards a specified insect order. For Lepidoptera, only Cry1, Cry2, and Cry9 families are toxic (Bravo, 1997). Some *Bt* strains bear a single *cry* gene *e.g.*, strain HD73 has this gene located on a 77 kb plasmid (Gonzalez *et al.*, 1981), whereas others have complex gene combinations (Bravo and Soberon, 2008).

Some insects like *Plutella xylostella* has been reported to have developed resistance against *Bt* harboring Cry1Aa, Cry1Ab and Cry1Ac proteins, but have very low level of resistance to Cry1B, Cry1C and Cry1D proteins (Tabashnik *et al.*, 1997). *Bt* formulations containing *cryIC* toxin gene are more effective pesticides (Liu *et al.*, 1996).

The Cry1C cluster of *Bt* proteins has 10 extremely homologous proteins of the Cry1Ca subset. Those insects which show complete or partial resistance to all available *Bt* crops in the market can be controlled by using *cry1Ca* toxin (Avisar *et al.*, 2009). Xue *et al.* (2005) showed that Cry1Aa and Cry1C have synergistic effect against *Spodoptera exigua* and *Helicoverpa armigera*. Cry1Ca is active against a large variety of pests

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such as *Spodoptera* spp., *Mamestra configurata*, *Heliothis virescens*, *Pectinophora gossypiella*, *P. xylostella* and *Phthorimaea operculella* (Avisar *et al.*, 2009).

The objective of present study was to isolate, screen and characterize the *Bt* from local environment and to determine the toxicity of *cryICa* harboring *Bt* isolate against *S. litura* in the green house.

MATERIALS AND METHODS

Isolation of local Bt strains

Bt were isolated from different habitats and characterized according to procedures described in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994).

Soil and sand samples were collected from various localities of Karachi, Multan, Lahore, Jhang and Sargodha. Samples were taken from one inch below the surface area. The soil containing reasonably high organic content was used as the bacterial source. The soil and sand samples were brought to the laboratory and stored in separately labeled zipped bags.

Screening of soil samples for Bt. strains

Bt. strains were isolated according to the method described by Travers *et al.* (1987). For each sample, 1.0 g soil was added in 20 ml of LB medium (Tryptone 10g/L, yeast extract 5g/L, NaCl 5g/L) and mixed well and sodium acetate was also added to the mixture above up to final concentration of 0.3 M and incubated in shaking incubator at 37°C and 250 rpm for 5 h. The sample was heated at 80°C for 20 min. Serial dilutions of treated sample were prepared and 250µl of dilute sample was spread on nutrient rich LB agar plates (Tryptone 3g/L, Yeast extract 1.5g/L, Tryptose 2g/L, MnCl₂ 0.005g/L, NaH₂ PO₄ 6.9g/L, Na₂HPO₄ 8.9g/L and Agar 15g/L) and incubated for 24 hrs at 37°C. Colonies with *Bt.* like morphology (entire margin, off white color, dry and rich growth of colony) were picked up at random and purified by restreaking on LB agar plates.

Characterization of isolated Bacillus species

Bacillus species were identified by following the diagnostic plan specified in Bergey's Manual of

Determinative Bacteriology (Holt *et al.*, 1994). The isolated *Bacillus* spp were characterized for Gram and endospore staining, and tested for Voges-Proskauer (V-P), catalase activity, motility, indole, citrate utilization, tyrosine decomposition, nitrate reduction, phenylalanine deamination, acid production from glucose, casein and starch hydrolysis, and growth at Sabouraud dextrose agar, 7% NaCl, and in 0.001% lysozyme. Procedures for these biochemical tests were taken from Collee and Miles (1989), Benson (1994) and Himeno *et al.* (1985).

PCR based detection of cryIC gene

The initial screening of local *Bt.* isolates for *cryIC* gene was done with Universal Primers cj 10 and cj 11 to amplify 130bp fragment (Table I).

For isolation of crude DNA for PCR a loop full of overnight grown *Bt* culture on LB agar plate was mixed with 200 µl distilled water in 0.5ml eppendorf tube, vortexed and then tube was placed in boiling water bath for 10 minutes to lyse the cells. Eppendorf was then placed at -70°C for 10 min. Heating and freezing was repeated twice for complete lysis of cells. Lysed cells were centrifuged at 13,000 x g for 10 minutes. PCR was performed by using 15 µl of supernatant as crude DNA as described by Carozzi *et al.* (1991).

PCR was done by a modified procedure described by Saiki *et al.* (1988) in which 50µl of PCR mixture contained 2.0mM MgCl₂, 10mM Tris-HCl pH 8.0, 200µM of deoxyribonucleoside triphosphate (dNTPs), 100 pmoles of each forward and reverse primer, 2.5 units of Taq DNA polymerase and 100-150 ng of crude DNA. Total volume of reaction mixture was made 50µl by adding sterile water and dispensed in 200µl tube.

DNA was amplified by using thermocycler (Applied biosystem 2720), set at 94° for 6 mins for initial denaturation and then 40 cycles each of 45 sec denaturation at 96°C, 30 sec annealing at 52°C and 30 sec extension at 72°C. Final extension was at 72°C for 10 mins.

The amplified product was cloned and recombinant *cryIC* plasmid was purified using QIAprep spin miniprep kit (QIAGEN, Hilden, Germany). Miniprep was done by following the instructions provided with the kit.

Table I.- Primers used in this study

Genes	Primers	Primer sequence	Product size
<i>cry1Ca</i>	F CJ10 R CJ11	5` AAAGATCTGGAACACCTTT 3` 5` CAAACTCTAAATCCTTTCAC 3`	130 bp
<i>cry1Ca</i>	F R	5` ATTGGGGGAGGACATCGAGTAATATCTAGCCTTA 3` 5` ACTCCTGTGGATGCCGCTCCTGTTAATACTAT 3`	670 bp
<i>cry1Cb</i>	F R	5` GGACTTTATCAAATCCTACTTTTACACCT 3` 5` TCCTCAAATGTAGAAGTAACTGGGAGAA 3`	730 bp

Amplification of shorter fragments of cry1Ca and cry1Cb

Specific universal primers (Table I) were used for amplification of short fragments of *cry1Ca* and *cry1Cb*. The composition of reaction mixture was the same as above. The reaction cycle comprised an initial denaturation only once at 94° for 5 min and then 40 cycles, each of 45 seconds denaturation at 96°C, 1 min annealing at 57°C for *cry1Ca* and 52°C for *cry1Cb* and 1 min extension at 72°C. Final extension was done at 72°C for 10 min.

Nucleotide sequencing

Nucleotide sequencing was as recommended by the supplier (Beckman Coulter, USA) with the kit (Genome Lab™ DTCS-Quik Start Kit, cat#608120). In one sequencing reaction 180-220 ng of template DNA, 3.3 pmol of M13 forward and reverse primers, 8.0 µl of DTCS quick start master mix. Distilled water was dispensed in the tube to make final volume of above mixture up to 20 µl. The sequencing reaction was performed in a thermal cycler at 96°C for 20 sec (denaturation), 50°C for 20 sec (annealing) and 68°C for 1 min for 30 cycles (extension).

Two µl of 100 mM Na₂-EDTA (pH 8.0), 2 µl of 3 M sodium acetate (pH 5.2) and 1 µl of 20 mg/ml of glycogen (supplied with the kit) was mixed to make stop solution. Five µl of the above solution was dispensed in the sequencing reaction and mixed well. 60 µl of cold 95% ethanol was mixed with the above mentioned solution, centrifuged at 13000x g 4°C for 20 min. The supernatant was discarded and the pellet was washed 2 times with chilled 70% ethanol. Pellet was air dried, dissolved in 40 µl of the sample loading

solution (provided in the kit), shifted to the well of the sample plate (P/N 609801) and one drop of mineral oil (provided in the kit) was put on the mixture to avoid any evaporation during reaction. The sample plate was put into the instrument (Beckman-Coulter CEQ 8000) and the program was started to get the sequence on computer.

Determination of optimum growth conditions of Bt isolates

cry1C harboring isolates proving most toxic to *S. litura* were chosen for optimization of growth conditions.

Optimum temperature

Optimum temperature was determined by inoculating 10 ml sterilized LB broth in seven sets, each of three test tubes, with 200µl of overnight grown cells of bacteria and kept at different temperatures *i.e.*, 22, 26, 32, 35, 37, 40 and 42°C for 12 h. Absorbance at 600nm after 12 hours was measured with spectrophotometer. A graph was plotted between O.D and temperature to determine optimum temperature.

Optimum pH

Autoclaved LB broth (5ml) in 8 sets, each of three test tubes with different pH *viz.*, 4, 5, 6, 7, 8, 9, 10 and 11 was inoculated with 100µl of overnight grown bacterial culture and were kept at 37°C for 12 hours in shaking incubator. Absorbance was measured at 600nm by spectrophotometer to determine the bacterial growth after 12 hours. A graph was plotted between O.D and pH to determine optimum pH.

Optimum inoculum size

Autoclaved LB broth (5ml) in 10 sets, each of three test tubes, inoculated with 100µl of overnight grown bacteria at a concentration 0.1%, 0.2%, 0.3%, 0.4%, and 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 1.2%, 1.5% and 2.0% and were kept at 37°C for 12 hours in shaking incubator. Absorbance was measured at 600nm by spectrophotometer to determine the bacterial growth. A graph was plotted between O.D and % inoculum to determine optimum inoculum size.

Preparation of growth curve

For preparation of growth curve 80 ml autoclaved LB broth dispensed in each of the three 250 ml flasks, were inoculated with 1% (0.3ml) inoculum of overnight grown bacterial culture of test organism at 37°C in shaking incubator. After every hour one ml sample was taken out from each of three flasks, and O.D was taken at 660nm. Graph was plotted between time and O.D to determine the growth curve.

Determination of antibiotic sensitivity and resistance

The antibiotic sensitivity/resistance of *Bt* isolates was determined by preparing LB agar plates. Test organism spread on the plates. Antibiotic discs of kanamycin (30µg), streptomycin (30µg), chloramphenicol (30µg), ampicillin (30µg), amoxicillin (30µg), bacitracin (30µg), oxytetracycline (30 µg) tetracycline (30µg) and erythromycin (30 µg) were placed on the surface of solid culture and then incubated at 37°C for 20 to 22 h. The diameter of zone of inhibition around the antibiotics discs was measured to determine antibiotic sensitivity/resistance.

Bioassays of local Bt. isolates

Toxicity of local *Bt* isolates harboring *cry1C* was determined against different insect pests.

Determination of spore concentration

Amount of spores was determined by a procedure described by Clara *et al.* (2005). Ten milligram of spores were taken in three 1.5 ml tubes for each sample, set aside at 65°C for 30 h, shifted

to desiccators, dried and weighed. The dried spores were resuspended in 1 ml of sterile water and serial dilutions were made till the concentration of 100ng/ml. Suspension was heated at 90°C for 10 minutes in water bath to kill vegetative cells, 100µl was spread on T3 agar plates and incubated at 37°C. After 72 h, the colonies were counted to determine the number of spores.

Bioassays with Helicoverpa armigera

Artificial diet for *H. armigera* was prepared according to Shao *et al.* (2001). This diet had following ingredients: 22.7% chick pea powder, 2.5% (w/v) baking yeast powder, 0.1% formaldehyde, 0.4% (w/v) choline chloride, 4% (w/v) sucrose, 0.5% (v/v) wheat germ oil, 1% (w/v) ascorbic acid, 0.03% (w/v) thiamine hydrochloride, 0.03% (w/v) folic acid, 0.03% (w/v) sorbic acid, 0.02% (w/v) cholestrine, 0.4% (w/v) methyl 4 hydroxy benzoate and 4% agar. 3 ml of diet was poured in high quality plastic transparent bottles. One *Bt* colony was inoculated in 5ml LB broth and placed in shaking incubator set at 37°C and 100rpm for 16 hours. This 5ml inoculum was dispensed in 1L of T3 sporulation medium (tryptone 3g/L, yeast extract 1.5g/L, tryptose 2g/L, MnCl₂ 0.005g/L, NaH₂ PO₄ 6.9g and Na₂HPO₄ 8.9g/L) in 4L flask and incubated at 28°C for 96 hours. The spores were harvested by centrifugation at 6632×g and washed four times with cold 0.5M NaCl and four times with autoclaved distilled water. Weight (wet and dry) of pellet was determined and stored at -20°C.

Bioassays were performed with ten different concentrations of spores and crystals mixture such as 100 µg/ml, 200 µg/ml, 300 µg/ml 1000 µg/ml, were spread on surface of the diet. Ten first instar larvae *H. armigera* were used in one glass vial with perforated lid and each dose was tested in triplicate. These experiments were performed twice to confirm the results. The experiment was performed in the Bioassay lab set at 27±2°C, 55±5% relative humidity and 12L:12D photoperiod. Larval mortality was determined after 96 hrs. Toxin free diet was used as control to correct the readings with Abbot formula (Abbott, 1925). Linear line equation of regression curve was used to determine the LC50 of local *Bt* isolates.

Bioassays with Army worms (Spodoptera litura)

Bioassay setup was the same as described in the above section.

Bioassays were performed on cotton and tomato plants with *S. litura* in green house. Sugarcane molasses was found to be a cheap source of nutrients for growth of *Bt*. The formulation, which gave the best results, had water 40ml, sugar 4g, antifoam solution 400 μ l, wheat flour 5g, milk powder 5g, and gum arabica 1g as ingredients.

Bioassays with Musca domestica

Two types of artificial diets were prepared, one for maintaining culture and the other for performing bioassays.

Diet for egg laying was prepared according to Shakoori *et al.* (1998). One volume of ground sugar and two volumes of skimmed milk were mixed, and then autoclaved water was added to make its paste. The paste was autoclaved and then added in autoclaved glass jars. Sterile moist tissue papers were placed in jars. Flies (70-80) were transferred to the jars. Mouths of the jars were closed with cheese cloth. Temperature was maintained at 25 \pm 2. Flies laid eggs on tissue papers and cheese cloth. Eggs were collected for bioassays.

Diet for bioassays was prepared according to Shakoori *et al.* (1998). Diet was made by mixing maize flour 25g, meat extract 1.25g, yeast extract 1.25g, molasses 1/2 table spoon, agar 1g and distilled water 200ml and put this all mixture on a gas stove for 20 mins. Five drops of propionic acid was added to above cooked mixture. Fifty ml diet was poured in each jar and autoclaved it for further use.

Bioassay diet was mixed with 50 μ g/ml, 100 μ g/ml, 200 μ g/ml, 300 μ g/ml 1500 μ g/ml of *Bt* spore/crystal suspension. Moist tissue paper was placed on one side of jar containing diet. About sixty eggs were transferred to each jar on moist tissue paper. Mouths of jars were closed with cheese cloth and incubated at 25 \pm 2 $^{\circ}$ C. Results were recorded after 12 days on bases of number of flies emerged from eggs. Reduction in the number of flies compared to the control flask was taken as mortality.

RESULTS*Characteristics of Bt isolates*

Seventy two soil samples were taken from different places of Pakistan. Four hundred and seventy colonies were picked from all samples after treating them with selective growth procedure. Seventy four colonies were found Gram positive and spore former rods. The Gram positive rods were selected for biochemical characterization. All *Bt* isolates were motile could grow on 7% NaCl, Sabouraud Dextrose Agar and lysozyme (0.001%), could hydrolyze starch and casein, deaminate phenylealanine, decompose tyrosine, utilize citrate but do not grow at 65 $^{\circ}$ C. They were also positive for Voges Proskauer and negative for indole test. All isolates follow the general criteria of biochemical tests (positive or negative) for *Bt* except isolate A18 (negative for acid production), isolate A23 (negative for casein hydrolysis) and isolate A46 (positive for indole test).

cry1Ca and cry1Cb harboring Bt isolates

Three *Bt* isolates (MS-SBS*Bt*1-3) gave positive results for *cry1Ca* gene (Fig. 1). Figure 2 shows PCR amplification of domain 3 of *cry1Ca* gene of *Bt* isolates MS-SBS-*Bt*1-3 (Fig. 2A) and *cry1Cb* of *Bt* isolates MS-SBS-*Bt*4-5 (Fig. 2B).

Growth conditions of cry1Ca gene harboring MS-SBS-Bt.1

The growth conditions were optimized for local *Bt*. isolate, MS-SBS-*Bt* 1, positive for *cry1C* gene. Absorbance of the test culture was taken as indicator of bacterial growth. Figure 3 shows the temperature of 37 $^{\circ}$ C, inoculum size of 1% and pH 7 as optimum growth conditions for *Bt*. isolate MS-SBS-*Bt*.1.

Growth curve of Bt isolate

Growth curve was determined for local *Bt*. isolate. Lag phase vary from 1-2 hrs, log phase vary 7-9 h followed by the 7-8 h stationary phase and after that decline phase (Fig.4).

Antibiotic resistance/sensitivity profile of cry1C positive local Bt Isolates

Three *cry1C* positive *Bt* isolates (MS-SBS-

*Bt*1-3) and HD137 were resistant to amoxicillin, bacitracin, ampicillin and tetracyclin and sensitive to polymixin and streptomycin.

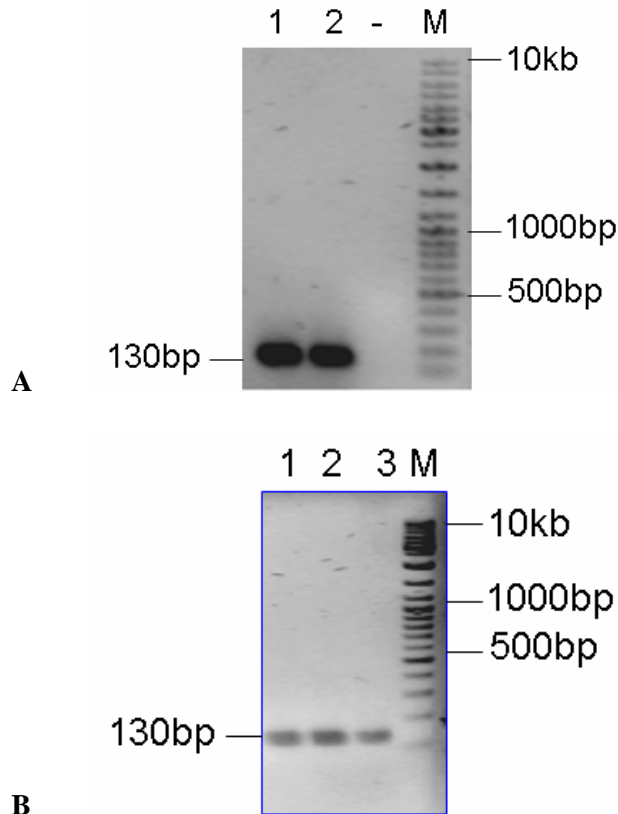


Fig. 1. PCR amplified product of *cry1Ca* of local *Bt* isolates. A, Lane#1, MS-SBS *Bt*1; Lane#2, HD137 (Positive control); B, Lane#1, MS-SBS *Bt*2; Lane#2, MS-SBS *Bt*3; Lane#3, HD137 (Positive control). M, DNA marker (Fermentas cat# SM0403).

MS-SBS-*Bt*.1 and 2 were partially to completely sensitive to neomycin and chloramphenicol and completely to partially resistant to kanamycin. Two *Bt* isolates (MS-SBS-*Bt*4 and 5) were resistant to kanamycin, ampicillin, tetracyclin and oxytetracyclin and sensitive to polymixin, streptomycin, bacitracin and neomycin (Table II).

Bioassays

Toxicity of MS-SBS-*Bt*1 was checked against *M. Domestica*, *H. virescens* and *S. litura* and the results compared with positive controls (HD 137

and HD 29). (Table III, Fig.5). LC_{50} was calculated using regression curve. The LC_{50} of MS-SBS-*Bt*. 1 was 248 $\mu\text{g/ml}$, 131.4 $\mu\text{g/ml}$ and 430.022 $\mu\text{g/ml}$ against *S. litura*, *M. domestica* and *H. virescens*, respectively.

Regression curves were prepared by plotting the log dose against the percent mortality (5) and

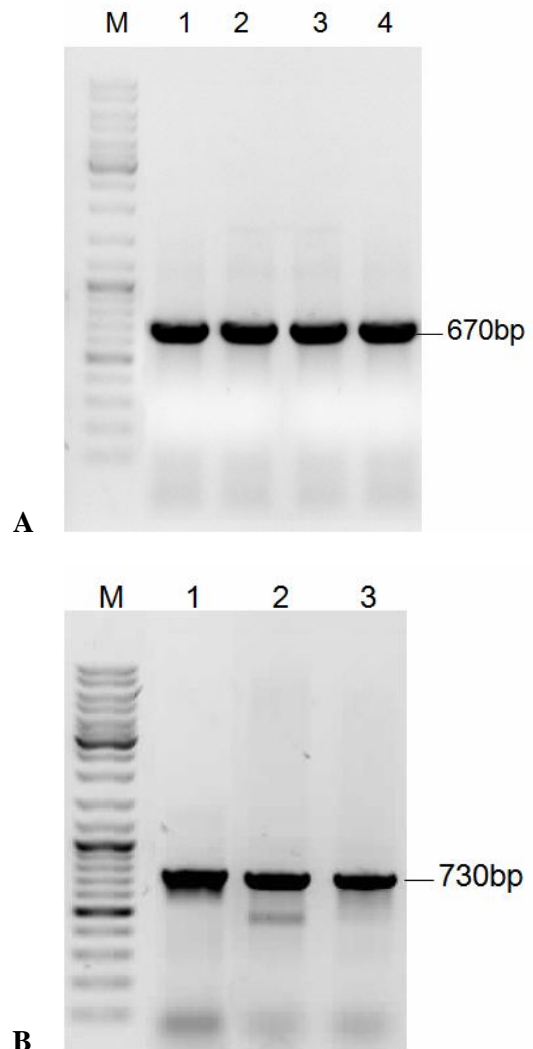


Fig. 2. PCR amplification of domain 3 of *cry1Ca* (A) and *cry1Cb* (B) genes of *Bt* isolates. A: Lane#1, MS-SBS-*Bt*. 1; Lane#2, MS-SBS-*Bt*. 2; Lane#3, MS-SBS-*Bt*. 3; Lane#4, HD137 (Positive control). B: Lane#1, MS-SBS-*Bt*. 4; Lane#2, MS-SBS-*Bt*. 5; Lane#3, HD29 (Positive control). M, DNA marker (Fermentas cat# SM0403).

Table II.- Antibiotic resistance/sensitivity of *cryIC* positive local *Bt* isolates.

Strain name	Baci	Amp	Am	Kan	Ery	Strep	Chl	Tetcyc	P.mix	Neo	Oxytet
MS-SBS- <i>Bt</i> -1	R	R	R	I	R	S	S	R	S	S	R
MS-SBS- <i>Bt</i> -2	R	R	R	R	S	S	S	R	S	S	R
MS-SBS- <i>Bt</i> -3	R	R	R	S	I	S	I	R	S	I	S
MS-SBS- <i>Bt</i> -4	S	R	S	R	S	S	S	R	S	S	R
MS-SBS- <i>Bt</i> -5	S	I	R	R	I	S	I	I	S	S	I
HD137	R	R	R	I	R	S	I	I	S	R	R

Abbreviations used; Amp, Ampicillin; Am, Amoxicillin; Baci, Bacitracin; Chl, Chloramphenicol; Ery, Erythromycin; I, intermediate; Kan, Kanamycin; Neo, Neomycin; Nal, Nalidixic acid, Oxytet, Oxytetracycline; P.mix, Polymixin; R, resistant; S, sensitive; Strep, Streptomycin; Tet Cyc, Tetracycline; and Van, Vancomycin.

Table III.- Effect of different concentrations of spore/crystal mixture of *Bt* MS-SBS-*Bt*-1; positive for *cryICa* against armyworms (*Spodoptera litura*), house fly (*Musca domestica*) and *Heliothes virescens* after 3 days of incubation. Toxicity is shown as % mortality.

Strain name	Different concentrations of spore/crystal mixture ($\mu\text{g/ml}$)										LC ₅₀	
	100	200	300	400	500	600	700	800	900	1000		
<i>Spodoptera litura</i>												
MS-SBS- <i>Bt</i> -1	35%	60%	80%	95%	100%	100%	100%	100%	100%	100%	100%	248
HD.137	9%	20%	29%	38%	45%	53%	61%	70%	81%	90%	566%	
<i>Musca domestica</i>												
MS-SBS- <i>Bt</i> -1	46%	58%	70%	85%	98%	100%	100%	100%	100%	100%	100%	131
HD29	25%	39%	52%	63%	74%	86%	94%	100%	100%	100%	100%	242
<i>Heliothes virescens</i>												
MS-SBS- <i>Bt</i> -1	7%	21%	34%	43%	54%	62%	73%	85%	94%	98%	430	
HD29	89%	100%	100%	100%	100%	100%	100%	100%	100%	100%	<100	

then LC₅₀ value was calculated by equation $y = ax + b$. LC₅₀ values show that local isolate MS-SBS- *Bt*.1 was more toxic to *S. litura* than HD137, more toxic to *M. domestica* than HD29 and less toxic to *H. virescens* than HD29.

DISCUSSION

The Cry proteins have been used as biopesticide for more than 50 years, and their safety has been demonstrated (Schnepf *et al.*, 1998; Ferre *et al.*, 2008). In this study overall 72 samples of garden soil, dry and moist soil cultivated with different crops, uncultivated soil and beach sand were used to isolate *Bt*. From these samples, 470 colonies showed *Bacillus* like morphology, of which 74 colonies were spore formers and Gram-positive.

After biochemical characterization 48 colonies were selected for screening of *cryIC* genes. From amongst 48 *Bt* isolates 37%, 23%, 15%, 15% and 10% were isolated from dry soil, moist soil, cattle waste, dry wheat straw and coastal area soil, respectively. Dry and moist soil was rich source of *Bt*. Most of the isolates were obtained from the samples from cultivated areas. It is difficult to correlate the occurrence of *Bt* with specific habitat.

The isolated bacterial strains showed optimum growth temperature at 37°C pH. 7.0. The results correlated with the previous studies with the isolation and growth of *Bacillus* strains (Xavier *et al.*, 2007).

The isolated *Bacillus* strains were further confirmed by the amplification of 130bp DNA fragment for *cryIC* with specific universal primers.

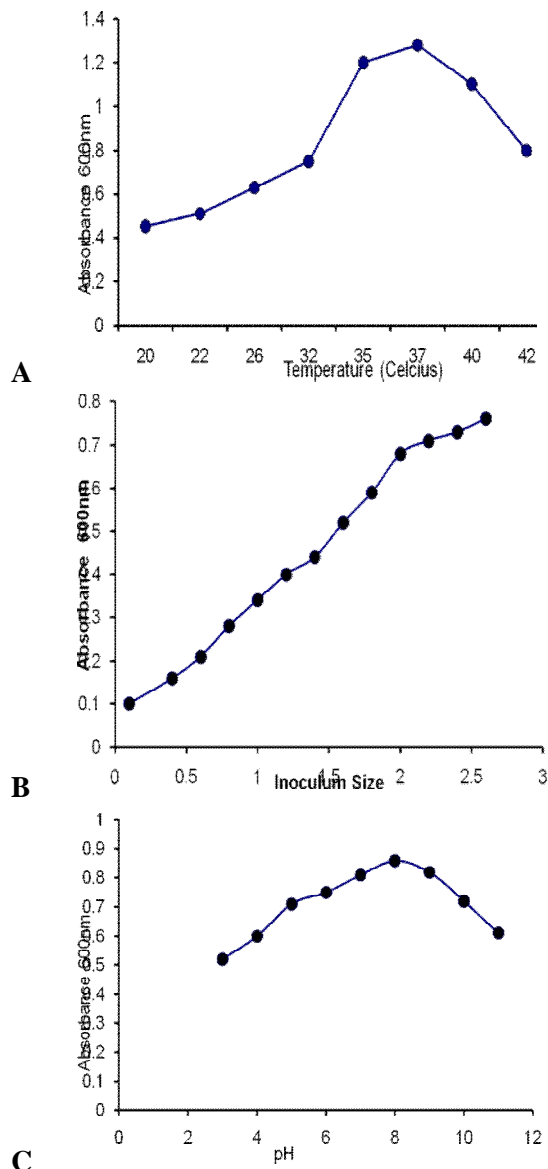


Fig. 3. Growth of *cry1Ca* positive *Bt* isolate (MS-SBS-*Bt.1*) at different temperatures (A) inoculum size (B) and pH (C). For determination of optimum temperature, 8 flasks containing LB medium were inoculated with overnight culture and incubated at different temperatures for 12 h. Optical density was measured at 600 nm after 12h (A). Similarly for determination of optimum inoculum size, different amounts of inocula were used to inoculate broth culture (B) and for determination of optimum pH, cultures were grown at different pH (C).

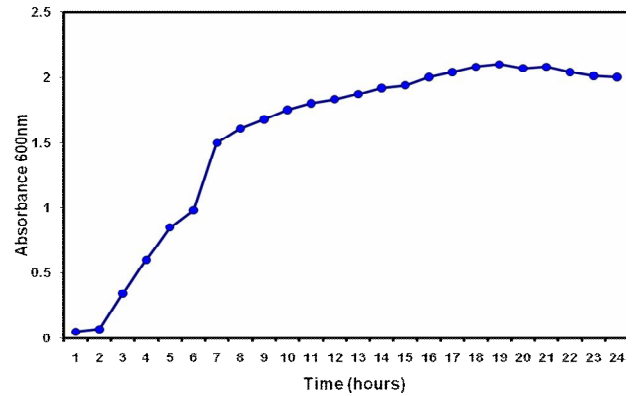


Fig. 4. Growth of *cry1Ca* positive *Bt* isolate (MS-SBS-*Bt.1*) at optimum inoculum size (1%), pH (7) and temperature (37°C).

The DNA fragment was T/A ligated into pTZ57R/T plasmid which was cloned in DH5 α strain of *E. coli*. The DNA fragment was analyzed for nucleotide sequences for the further confirmation of *Bacillus* strains. The sequence of the clones was 99% similar to *cry1Ca1* gene (accession# X07518.1), when aligned on Basic Local Alignment Tool (BLAST).

It shows that occurrence of *cry1C* gene is very low compared to *Cry2* and *Cry4* genes in Pakistan (Saleem, 2007; Bukhari, 2007).

The isolated *Bacillus* strains were further confirmed by the amplification of 130bp DNA fragment for *cry1Ca* with specific universal primers. The isolated *Bacillus* strains were also confirmed for the presence of *cry1C* genes by PCR amplification of the genes encoding portion of *cry1Ca* (670bp) and *cry1Cb* (730bp) with gene specific primers using the genomic bacterial DNA as template. The gene sequence for *cry1Ca* (670bp) was 99% similar to *cry1Ca1* gene (accession# X07518.1) and gene sequence for *cry1Cb* (730bp) was also 99% homologous to *cry1Cb1* gene (accession# M97880.1), when aligned on Basic Local Alignment Tool (BLAST).

Bioassays with spore/crystal mixture against *S. litura* in Lab were carried out by using cotton and tomato plants, grown in plastic pots in green house as the host plants for the insects. The results indicated that the formulations prepared and applied in the present study have high potency for the control of insects. The results are comparable with

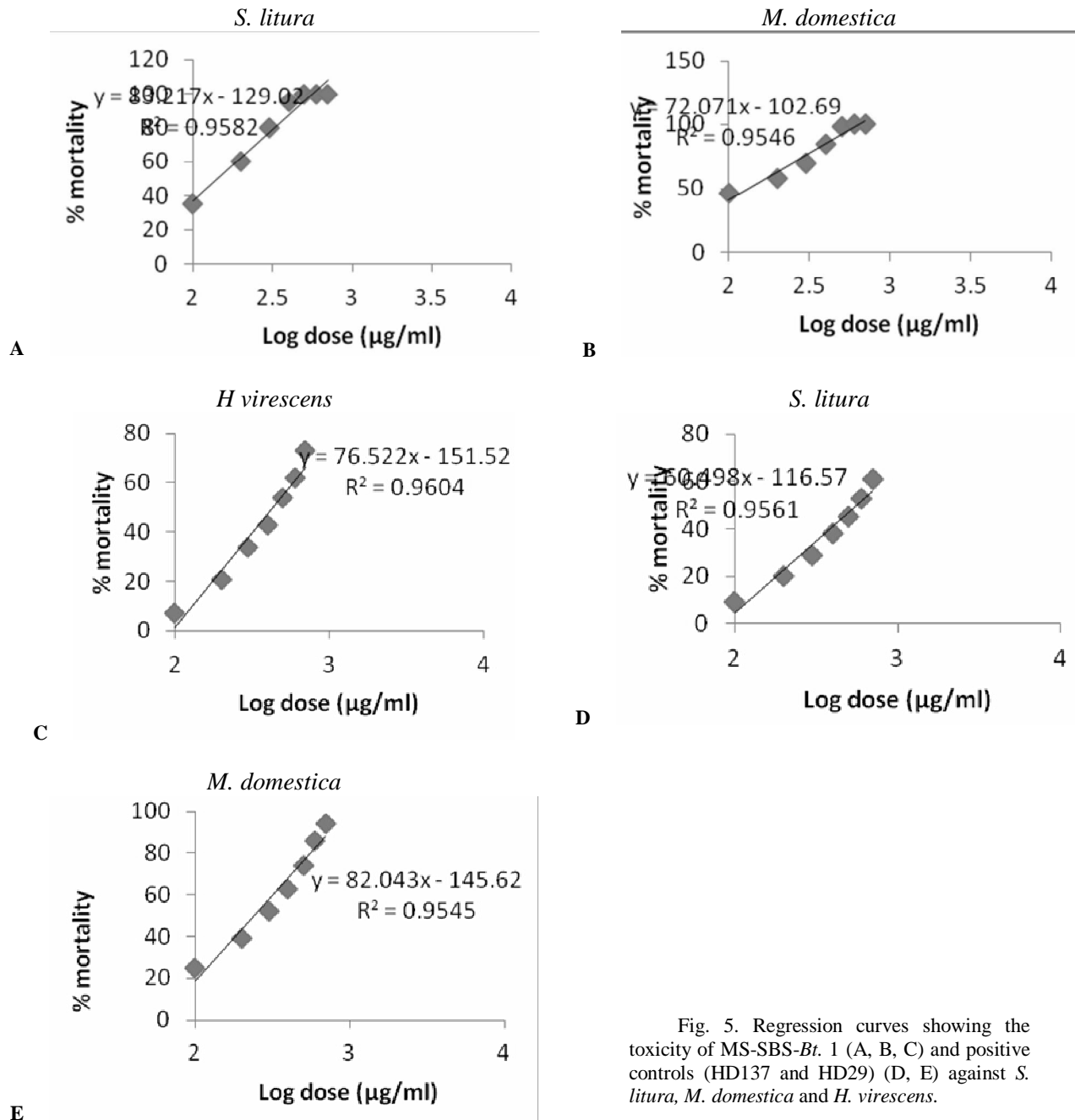


Fig. 5. Regression curves showing the toxicity of MS-SBS-*Bt*. 1 (A, B, C) and positive controls (HD137 and HD29) (D, E) against *S. litura*, *M. domestica* and *H. virescens*.

the previous studies with such formulations which contain different additives like dispersants, chemo-attractants, phagmostimulants, UV blockers and toxicity enhancers (Satinder *et al.*, 2006; Rowe and Margaritis, 2004; Keller *et al.*, 2001; Darnon *et al.*, 2003; Zhou *et al.*, 2004; Bravo *et al.*, 2007). For

cost affectivity of formulation, culture media must be of very low cost. Among different culture media one is wastewater (WW)/wastewater sludge/ (WWS) and other is sugarcane molasses. These media are exploited by many scientists and according to their opinion these are cost effective

(Tirado-Montiel *et al.*, 2001, 2003; Lachhab *et al.*, 2001; Tyagi *et al.*, 2001; Vidyarthi *et al.*, 2002; Yezza *et al.*, 2004; Sachdeva *et al.*, 2000).

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