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Investigation of *Bacillus thuringiensis* var. *israelensis* (*Bti*) endotoxin production and analysis of efficiency of *Bti* against mosquito larvae

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ABSTRACT

Mosquitoes are dangerous to mankind and are threatening human life worldwide. Insect specific toxins, which are commonly produced by *Bti*, are becoming an important component of biological strategies to control mosquito's population that causes communicable or life threatening diseases. These insect specific toxins are commonly known as biolarvicides. Biolarvicides of strain *Bti* are highly effective against mosquito's larvae at very low doses and show no harmful effects to other non-target organisms. Therefore, *Bti* has been extensively used in mosquito control programs. No field resistance has been observed in mosquitoes populations treated with *Bti*. This suggests that *Bti* will be an effective biocontrol agent for years. In the present study, we evaluated the efficacy of *Bti* against mosquito larvae (*Culex quinquefasciatus*) commonly found in central India, Bhopal, Madhya Pradesh. The formulation was effective in killing mosquito larvae and its international toxic unit was found to be 5200 ITU/mg.

Key words: *Bacillus thuringiensis var. israelensis*, biolarvicide, larvae, *Culex quinquefasciatus*, mosquito control

Introduction

Mosquito borne diseases form a major part of communicable diseases (filariasis, malaria, Japanese encephalitis and dengue) in India and in other Asian countries. Several strategies have been implemented to control the various types of diseases transmitted by mosquitoes. Chemical insecticides have been used for past several decades to control population of mosquito larvae and to reduce diseases transmitted by mosquitoes. The use of chemical insecticides has been reduced because of multiple problematic factors such as physiological resistance, environmental pollution, food chain contamination and harmful effects on other beneficial insects. Therefore, the use of biological control agents has been increased in recent years. The discovery of *Bti*, which is very toxic to dipteran larvae, has opened up the possibility to use *Bti* in mosquito control programme (Goldberg & Margalit, 1977; Kalfon et al., 1983). This strain of bacteria has many advantages over conventional chemical insecticides in mosquito eradication operation. It is safe against non-target organisms including humans. It is highly specific and also eco-friendly. *Bti* is found to be most active microbial control agent effective against mosquito larvae. It forms intracellular crystal inclusions, which contain multiple protein components having molecular weights 27, 67, 125 and 134 kDa. These proteins are observed to be toxic to mosquito larvae when cloned individually. However, a combination of these proteins is more effective and possesses higher toxicity than any of the individual components (Ramírez & Ramírez, 2012). On ingestion the spore/crystal complex is solubilized and proteolytically cleaved in the alkaline pH of the mosquito's midgut. The active form of toxins (crystals) released in the midgut bind to specific receptors present in the midgut (brush border membrane) and leads to death of mosquitoes (Bravo et al., 2007).

In the present investigation, we produced *Bti* and evaluated the efficacy of this insecticide against mosquito larvae commonly found in central India (Bhopal, Madhya Pradesh). The trials were conducted during the period from

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October 2013 to April 2014. This is the first study in which *Bti* has been used for mosquito larvae control in central India, Bhopal, Madhya Pradesh.

Materials and Methods

Mosquitoes

Mosquitoes' larvae used in the present study were collected from local drains at Govindpura Industrial area in Bhopal.

Bacterial strain and preservation

Bti used in the present study was obtained from the culture collection of Vector Control Research Centre (Indian Council of Medical Research), Pondicherry, India. The strain was maintained in the Nutrient agar media and agar slants (10.0 g glucose, 5.0 g sodium chloride, 5.0 g peptone, 3.0 g beef extract, 0.5 g yeast extract, 0.203 g $MgCl_2$, 0.01 g $MnCl_2$, and 0.102 g $CaCl_2$ (Sigma) per litre of distilled H_2O, pH 7.0).

Bacterial culture medium

Medium for bacterial strain consisted of 2% soybean flour. The ingredient of medium was dissolved in distilled water and filtered through a plastic strainer (mesh size 100) to remove all the insoluble solid particles present in the medium. 21.3 g $MgCl_2$, 10.3 g $CaCl_2$, and 1.0 g $MnCl_2$ per 100 ml of distilled water were added into the medium. The medium was stirred untill it got homogeneous. The pH of the medium was adjusted to 7.2 prior to inoculation. The culture medium was suspended in Erlenmeyer flasks (250 ml capacity). The culture medium was autoclaved at 120°C / 20 lb / in^2 for 20 min.

Bacterial growth condition

A loopful culture of *Bti* was transferred into the prepared soya flour medium and was kept in shaker (150 rpm at 30°C for 48 hours) under constant agitation. Culture sample from the respective medium was drawn (0.5 ml) every 12 h, untill the end of the bacterial growth (48 h).

Endospore staining

Small amount of bacteria was smeared onto a clean slide with the help of inoculation loop and diluted with a drop of distilled water. The slide was dried in flame. The dried slide was flooded with Malachite green dye (prepared by dissolving 5 g of Malachite green dye in distilled water and volume was made up to 100 ml) and then immediately steamed over a water bath for 5 minutes. On cooling, the slide was rinsed with distilled water. The slide was counterstained with Gram's Safranin solution (Sigma-Aldrich) for 2 minutes and finally rinsed with distilled water. Once the slide was dried, the specimen was observed under microscope using oil immersion.

Harvesting, drying and powder formation

The culture (100 ml) was centrifuged at 10 000 g for 30 minutes at 4°C. The supernatant of centrifuged solution was discarded and the cell pellet was stored. The pellet was spread on a plastic paper in form of a thin layer and was allowed to dry in dark room at normal room temperature. The dried form of *Bti* was crushed with mortar and pestle. The powder obtained was stored in refrigerator (4°C).

Bacterial protein extraction

10 mL of bacterial culture (48 h) was taken in falcon tube (15 mL) and was centrifuged at 4000 rpm for 10-12 minutes. The supernatant was discarded and the pellet was dissolved in 500 µL of 1 M NaCl solution. The pellet containing 500 µL of 1 M NaCl solution was transferred into an eppendorf tube and was centrifuged at 7000 rpm for 7 minutes. The supernatant was discarded, 250 µL of TE buffer was added in the pellet and the mixture was centrifuged at 7000 rpm for 7 minutes. The pellet obtained was dissolved in 250 µL of distilled H_2O and was centrifuged at 7000 rpm for 7 minutes. The supernatant was discarded and the pellet was resuspended in 150 µL of 10 mg/mL lysozyme solution in TE buffer and the mixture was incubated at 37°C for 30 minutes. Then, 25 µL of 10% SDS (sodium dodecyl sulphate) solution was added into the suspension and was vortexed for 30 seconds. The suspension was centrifuged at 6000 rpm for 10 minutes. Finally, 100 µL (0.2% SDS solution) was added to the pellet. It was stored at -20°C.

Bacterial protein estimation

A small volume (10 ml) of bacterial sample was centrifuged at 10 000 g for 15 minutes at 4°C. The supernatant was discarded and the pellet was dissolved in solubilisation buffer (10 mM dithiothreitol and 50 mM $NaHCO_3$ adjusted to pH 10). The sample containing solubilisation buffer was incubated for 1-2 h, at 30°C. The solution was centrifuged at 10 000 g for 15 minutes at 4°C. The pure solubilised protein was quantified for protein concentration taking bovine serum albumin (Sigma) as a standard protein(Lowry et al., 1951).

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SDS-PAGE Analysis

For denaturation, 60 µL of gel loading buffer (autoclaved glycerol: 5 ml, 10% SDS: 0.5 ml, β-mercaptoethanol: 0.5 ml, stacking buffer: 2.5 ml, sterile distilled water: 11.5 ml, Bromophenol blue: 10 mg) was added to 30 µL of suspension stored at -20°C during protein extraction. This suspension was incubated in a water bath maintained at 90°C for 7 minutes and then placed on ice until it cools. This procedure was repeated thrice. It was separated on SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) unit (Genei, India). The gel was stained with Coomassie Brilliant Blue dye (prepared by dissolving 0.8 g Coomassie Brilliant Blue dye dissolved in 200 ml methanol and 40 ml glacial acetic acid and volume was made up to 400 mL with distilled water) and destained with destaining solution (methanol / glacial acetic acid / distilled water : 200 mL / 100 mL / 700 mL). Finally, the bands of protein were visualized.

Biolarvicide test

Biolarvicide test, for calculating toxicity of *Bti*, were conducted with the larvae of *Culex quinquefasciatus* collected from the local drains at industrial area in Bhopal.

Bti solution was formulated according to the standard procedure (De Barjac & Larget-Thiéry, 1984; Dulmage et al., 1990). Accordingly, 50 mg of powder was dissolved in 10 ml of distilled water and homogenized on a vortexer for 10 minutes using glass beads. It was diluted with distilled water to form working solution and again serial dilutions were prepared (0.001 to 0.05 mg/mL). A Pasteur pipette having narrow tip was used to transfer 25 individual larvae in disposable wax coated paper cups, containing 150 mL of distilled water with appropriate dosage of *Bti*. A control experiment was always run at each step of the procedure. The experiments were performed in duplicates. The biolarvicide test was conducted at room temperature (25-30°C). The mortality of larvae was observed after 24 hours.

The biopotency of product was assigned by comparison with lyophilized standard powder of *Bti* obtained from Vector Control Research Centre, Pondicherry. This standard lyophilized powder has toxicity of 13000 ITU/mg. The potency of shake flask product against this standard powder was calculated according to the Abbott formula:

Potency (A-ITU/mg) = Potency (Std) x LC_{50} (Std)/LC_{50} (A)

where (A) is the product and (Std) is the standard.

Results

The biomass of *Bti* produced using soya bean flour and Luria broth media is given below (Table 1). Medium containing soya flour mixed with mineral salts (SF+mineral salts) produced highest biomass (3.26±0.14 g/L) compared to reference medium (LB). The experiment was repeated several times and same results were obtained on repetition. Therefore, advance studies on bacterial growth and biolarvicide assays (on laboratory scale) were performed only with soya medium containing mineral salts.

Table 1: *Biomass production of Bti from soya flour based culture media*

No.	Culture media	Biomass (dry weight g/L)
1.	SF+ mineral salts	3.26±0.14*
2.	LB	1.86±0.19

* Average performance of six individual observations, SF= soya flour, LB= Luria broth

Bacterial growth analysis was observed from the culture media (SF+mineral salts and LB), that the exponential phase of *Bti* begun from the sixth hour onwards. This phase extended up to 48 hours after that *Bti* entered into stationary phase of growth. Bacterial sporulation started after completion of 48 hours of growth and it completed in 50 hours. Within 56 hours the spores were observed to be released from the bacterial cells. A sporulation of 90% was observed (Figure 1). The bacteria were able to completely digest all the culture media by 60 hours. Microscopic observation of *Bti* revealed that the rate of sporulation from soya flour was as good as that of the LB medium.

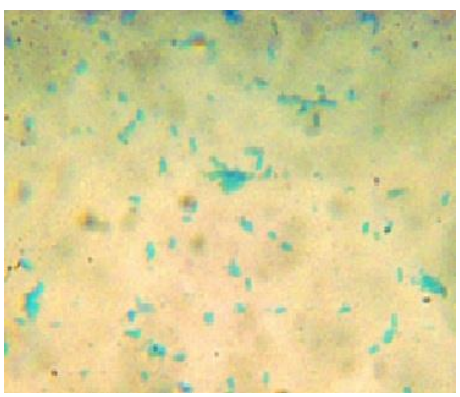

Figure 1: *Spores released from the Bti cells after 56 hours.*

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During sporulation, *Bti* produces protein parasporal inclusions bodies that are mainly responsible for its cytotoxic effect against mosquito's larvae. In Figure 1, the small green colour crystal represents the spores which have been released by the bacterial cells after 56 hours.

Protein content (approximately 10 mg/mL) was observed in the standard *Bti* powder and around 0.08 mg/mL protein was produced in *Bti* grown using soya flour medium.

SDS-PAGE protein profile of *Bti* from the two culture media were observed by SDS-PAGE having 10% of gel concentration. The major mosquitocidal polypeptides (27, 67, 125 and 134 kDa) synthesized from the parasporal crystal proteins of *Bti* were observed as bands in SDS-PAGE (Figure 2).

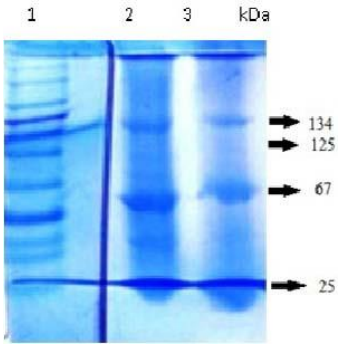

Figure 2: *Expression of Bti toxins produced using culture media (SF+mineral salts).*

Figure 2 depicts crystal protein present in the *Bti* culture. 1st lane of gel represents marker, four dark blue bands in 2nd lane represents crystal protein present in standard *Bti* powder and four dark blue bands in 3rd lane represents crystal protein present in sample *Bti* powder. These crystal proteins are responsible for killing the mosquito's larvae.

The effect of *Bti* on larvae of *Culex quinquefasciatus*is is depicted below (Table 2 and Table 3).

The biolarvicide has generated significant mortality in mosquito larvae during the experiment. The reduction in larval population was observed in each experiment, except the control experiment (Table 4). The efficiency of the standard and tested samples is shown in Table 2 and Table 3. From Table 2 could be seen that 50% of mosquito larvae got killed at 0.008 µg/ml of standard *Bti* powder. This concentration represented the LC_{50} value of the standard *Bti* powder against the 3rd instar larvae of *Culex quinquefasciatus*. In the same manner, LC_{50} value of the culture medium protein (sample *Bti* powder) was deduced from Table 3. It was observed that 50% of 3rd instar larvae of *Culex quinquefasciatus* got killed at 0.02 µg/ml of culture medium *Bti* protein. The total population of mosquito larvae in control experiment (Table 4) remained same throughout the observation. The population of live larvae was 25 during the time interval between 0-48 h and 0% mortality was observed.

The International Toxic Unit was calculated by using standard mathematical formula (Abbott formula). The LC_{50} value of both, the standard *Bti* protein and culture medium *Bti* protein, was put in the Abbott formula. The ITU of the culture medium *Bti* protein was found to be 5200 ITU/mg. Accordingly, it was concluded that culture medium *Bti* protein was effective in killing mosquito's larvae.

Discussion

Mosquito borne diseases pose major health issues around the world. Therefore, mosquito control program is an essential part to control spreading of disease and it relies on the use of *Bti* (biopesticide). Biopesticides has revolutionized mosquito eradication programs. High cost of conventional media components makes it necessary to use cheap and commonly existing material to produce biopesticides on a large scale through simple technique.

Table 2: *Larvicidal activity of different concentrations of standard Bti powder on larvae of Culex quinquefasciatus*

No.	Particulate	Concentration (µg/ml)	Total larvae	Dead larvae	Live larvae
1	S 1	0.04	25	25	0
2	S 2	0.03	25	24	1
3	S 3	0.02	25	21	4
4	S 4	0.01	25	18	7
5	S 5	0.008	25	15	10
6	S 6	0.005	25	8	17

S = Standard *Bti* powder

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Table 3: *Larvicidal activity of different concentrations of sample Bti powder on larvae of Culex quinquefasciatus*

No.	Particulate	Concentration (µg/ml)	Total larvae	Dead larvae	Live larvae
1.	L 1	0.04	25	21	4
2.	L 2	0.03	25	19	6
3.	L 3	0.02	25	15	10
4.	L 4	0.01	25	9	16
5.	L 5	0.008	25	8	17
6.	L 6	0.005	25	4	21

L=Culture medium protein (sample *Bti* powder)

Table 4: *Data showing the result of control experiment (disposable wax coated paper cups without Bti).*

Total larvae	25	25	25	25	25	25	25	25	25
Time elapsed (h)	0	6	12	18	24	30	36	42	48
Live larvae	25	25	25	24	25	25	25	24	25
Percent mortality	0	0	0	0	0	0	0	0	0

Till date, efficiency of *Bti* synthesized using different conventional media (NYSM and Luria Bertani) has been tested for controlling mosquito larvae population. Additionally, cost effective and cheap media has been utilized to produce biopesticides at large scale. The media formulated using seeds of legumes (cow pea of black and white varieties, groundnut cake, soya bean and Bambara beans), were found to be ideal for production of *Bti* (Obeta & Okafor, 1984). Coconuts, potatoes, cornsteep liquor, fishmeal and soyabean have also been reported for the production of biopesticides (Saalma, 1983, Poopathi et al., 2002; Poopathi et al., 2003; Poopathi & Anur, 2004). In our studies, mosquitocidal toxins produced using soya flour with mineral salts indicated that the production of endotoxins were significantly comparable with that produced using conventional media. *Bti* toxins showed considerable mosquitocidal activity against mosquito larvae. The toxicity of toxins of *Bti* is due to the binding of active crystal toxins to specific receptors present in the midgut. It generates spores in the cell membrane of mosquito larvae, disturbs the osmotic balance and cell gets lysed. Mosquito larvae stop feeding and die within few hours of ingestion of *Bti* toxins (Chilcott & Ellar, 1988).

This study has revealed several factors regarding mosquitocidal activity of *Bti*. Firstly, cheap medium having high content of carbon and nitrogen source is essential for large scale production of *Bti*. Secondly, bacterial growth and sporulation conditions should be observed carefully. Thirdly, minimum concentration of *Bti* should be effective in killing mosquito larvae. Further investigation can be done to improve biomass yield of *Bti.*

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