International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.2, No.3, pp 2048-2053, July-Sept 2010

Insecticidal Activity of *Bacillus thuringiensis* IBT- 15Strain against *Plutella xylostella*

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Abstract: *Bacillus thuringiensis* is a common soil bacterium was subsequently found that thousands of strains of *Bacillus thuringiensis exist.* Each strain produces its own insecticidal or toxin activity to control Plutella , which is a pest that causes severe damage to the crops. An attempt was made to characterize the indigenous isolate Bt IBT -15. The genotype of indigenous Bt isolate was evaluated through phase contrast microscopy, Biochemical characterization, plasmid profile, SDS and insecticidal activites. The plasmid and protein profile differs from the reference strain *Bacillus thuringiensis entomocoids.* Biochemical characterization revealed the isolate as Bt. The insecticidal toxicity of the isolate for *plutella xylostella* (Diamond black moth) is like that of the reference of strain. Furthur characterization are necessary to develop this strain as biopesticides.

Keywords: *Bacillus thuringiensis, plutella xylostella,* IBT -15 strain, Insecticidal Activity.

Introduction:

Bacillus thuringiensis (Bt) is a spore forming, gram-positive bacterium of ubiquitous occurrence. It produces proteinaceous crystal (cry) toxin. These proteinaceous inclusions of *Bacillus thuringiensis* are called as crystal protein or delta endotoxins. These delta endotoxins are activated by proteases in the alkaline condition of the midgut. These activated toxins binds with the receptor on the brush border membrane vesicle of the midgut epithelium and perforate the cell membrane, which leads to ionic imbalance and insect death. Since the early 1900's Bacillus group has received great attention for its use as the biopesticide against a variety of insect pest. Hence, preparation of *Bacillus thuringiensis* is being used as bioinsecticides for the control of certain insect species belonging to the orders of Lepidoptera, Ditera and Coleopatra[1] . It is also well documented that the encoded products of cry genes of certain Bacillus thuringiensis are toxic against other insect order such as Hymenoptera,Homoptera ,Orthptera and Mallophaga and against certain nematodes, mites and protozoa[2].

Classification of cry genes of *Bt* are grouped into 45 classes and it has taken various shapes[3] ; many possessing specific insecticidal activity against certain species of insects like cry1, cry9, cry3,cry7,cry8,cry14, cry4,cry10,cry11 and cry5,cry6,cry12-14 . All of these cry toxin genes are thought to have a common evolutionary origin because of the high amino acid homology [4] they share three dimentional structures of three insecticidal crystal proteins ,cry1A(a),cry3Acyt b have been elucidated by x-ray diffraction analysis[5].The activated cry 1A and cry3A are remarkably similar with respect to their structures and they share three structural domains [6]. Domain I is located at the N-terminal end of the activated protein and is made of bundle of eight hydrophobic and amphiphatic α-helices whereas domain II and III are are made of β – sheets. Domains I is thought to be responsible for pore

formation in the epithelial cell membrane.The main target pest of Bt insecticide include various lepidoptrous dipterous and individual coleopterous species. Some strains have also been found to kill off nematodes. Conventional Bt preparations such as those registered in germany but also world wide are mostly derived from the highly potent strain *Bacillus thuringiensis var*. kurstaki HD1, which was isolated in sixties. Bt genes can occur in different strains and in diverse combinations .almost all *Bt* strains are able to form more than one type crystalline inclusion body, and these inturn can be made up of several different δ-endotoxin molecule species . *Bt* intoxications b cry toxins in a susceptible host reveals various levels at which resistance might evolve in an insect population. The high degree of host specificity, as well as the complexity of *Bt* mode of action, results from the interaction of the toxin within the complex environment of the insect's midgut lumen and on the surface of the midgut epithelial cells . Researchers discovered relatively early that the midgut was the primary site of δ-endotoxin activity. A three dimentional model of the cry III A protein structure supports the hypothesis that the toxin causes pore or channels to form in the lipid bilayer.The immediate cessation of feeding observed in most insects after ingestion of *Bt* as well as the rapid regenerative capability of midgut epithelial cells , can allow damaged regions of the midgut to heal.At present, the primary mechanisms of resistance reported for *P.interpuntella*[7,8,9]and *P.xyllostella*[10] is a reduction in the binding of toxins to receptors on the midgut brush border membrane. Resistance mechanisms studies of btk- resistance field populations of *P. xyostella* demonstrated greatly reduced or lack of toxin binding to midgut receptors, suggesting a change or complete loss of the receptor[11,12,13] .Two separate studies of resistance in *H.virescens* found no relation between resistance to cry IA (b) or cry IA(c) and toxin – receptor binding[14,15.]

Materials and Methods:

Methodology:

Microscopic tests:

a. phase contrast Microscopy:

The strain *Bacillus thuringiensis* **I**BT -15 was grown on SCG agar medium at 37 degree celcius for 3 days. Bacterial cells were harvested and washed thrice in 0.5 M sodium chloride by centrifugation at 8000 g for 15 min. The pellet was suspended in sterile distilled water for further use. These cells were examined under phase contrast microscope the presence of cell wall structure, spores and crystals.

b. Gram staining:

Staining Procedure:

Thin bacterial smear was made on a clean glass slide, dried in air and heat fixed. The smear was covered with crystals violet and kept for one minute. The slide was washed, covered with gram's Iodine and let stand for one minute. The slide washed, decolourized with acetone, rocking the slide gently. The decolorization step was done very quickly. Immediately the slide was washed with water. Counterstained with safranine and let stand for 30 seconds. Washed with water, blot dried and examined under the microscope.

c. Spore staining:

It makes for very simple method distinguishing the endospore from the vegetative cell. Bacterial smear was made on a clean glass slide and allowed to dry in air and heat fixed. Flood smears with malachite green and placed on a warm hot plate for 2 to 3 minutes. The slide was replaced from hot plate, cooled and washed under running tap water. The slide was covered with safranine for 30 seconds. The slide was washed with tap Water, blot dried and examined under the microscope.

d. Crystal staining:

Samples were taken and inoculated into *Luria Bertani* media. Incubated at 37 degree celcius for hours. Smear was made on a glass slide and covered with crystal violet reagent. Washed with water, blot dried and examined under the phase contrast microscope.

e.Motility test:

Clean a cover glass and cavity slide grease free. A film of Vaseline was placed around the rim of glass cover. Old the cavity slide inverted . Turn the slide carefully upside down to make the drop hanging in the cavity. Using a high power under oil immersion observe the edge of the drop.

Biochemical test

a. Indole test:

This test is used to determine the ability of microorganisms to degrade the amino acid tryptophan. Using sterile techniques the organism was innoclated into tryptophan booth. This was incubated at 37 degree Celsius for 24-48 hours. After incubation 0.2 ml of kovac's reagent was added and shaken to observe the result.

b. Voges Proskauer test (V.P.test):

This is to determine the capacity of some microorganism to ferment carbohydrates with the production of non-acidic or neutral end products such as methyl carbinol as a product of glucose fermentation. MR/VP broth were prepared and sterilized, sample was inoculated into the MR/VP broth and incubated at 37 degree Celsius for 24 hours.

After incubation, 0.2 ml of VP reagent B was added into same culture tubes. Gently mixed well and allowed it to stand for 15 minutes.

c. Methyl red test:

This test is to determine the capacity of some organisms to ferment carbohydrates with the production of acidic end products depending upon the specific enzymatic pathway as a product of glucose fermentation. MR/VP broth were prepared and sterilized. Sample was inoculated into the MR/VP broth and incubated at 37 degree Celsius for 24 hours. After incubation, 5to 6 drops of methyl red solution was added into the same culture tubes.

d. Starch hydrolysis test:

Starch agar plates were prepared .Made a single line of streak on the starch agar plates. Plates were placed at 35 degree Celsius for 24 hours. After incubation, iodine solution was added into the growth plates to observe the result.

e. Citrate Utilisation test:

This test is used to differentiate among enteric organism on the basis of their ability to ferment citrate as sole carbon source. Using sterile technique, the organism was inoculated in simmons citrate agar slant. The tubes were incubated at 37 degree Celsius for 24 hours.

f. Catalase test :

This test is used to determine the ability of an organism to produce the enzyme catalase.

2-3 ml of H_2O_2 solution was poured into the test tube containing growth of microorganisms. Immediate liberation of air bubbles indicate that organism was was catalyse positive and no liberation of air bubbles indicates that the organisms was catalyse negative.

g. Gelatin test:

Gelatin is a incomplete protein, lacking the essential amino acid Tryptophan. This gelatin protein produced by hydrolysis of collagen is a connective tissue in humans and animals. Nutrient broth with gelatin tubes were prepared. The tubes were incubated at 37 degree Celsius for 24 to 48 hours. Following incubation, the tubes were placed in a refrigerator at 4 degree Celsius for 30 minutes.

h. Casein hydrolysis test :

Skim milk agar plates were prepared. A single line inoculation with test culture was made. The plate was incubated at 37 degree Celsius 24-48 hours. The formation of clear zone around the colonies indicates casein hydrolysis.

Plasmid Isolation:

Over night 3 ml culture was taken and pelleted. The alkaline lysis solution I,II,III were added one after the other and was centrifuged at 10,000 rpm for 15 min. The supernatantwas collected and transferred to a fresh tube. To this 600 ml of absolute ethanol or isopropanol was and centrifuged at 10,000 rpm for 15 min.

PCR Amplification:

Template DNA was extracted from 16 -18 hours culture in LB broth

Primers:

5'TGCGGCTGGATCCCCTCCTT-3
5'CCGGGTTTCCCCATTCGG3'

5'CAGATACCCTTGCTGGTGTAA3'
5'ATAGGCCCGTGCTCCACCAGG3'

These primers were designed previously. Reactions were carried out in 25:1 μM of template DNA was mixed with reaction buffer, 250 μM of each dNTP, 0.2-0.5 μM of each primer and 0.5 U of Taq DNA polymerase . Template DNA was denaturated and annealed to primers and extensions of PCR products were achieved at 72 degree Celsius for 2 minutes. Each experiment was accompanied by a negative (without DNA template) control. PCR amplicons of predicted size were easily identified by electrophorosis on 1.5% and 2% for cry II gene and intergene respectively.

SDS-PAGE Analysis:

SDS –PAGE was formed with 10% separating gel and 4% stacking gels. After electrophoresis, gels were stained with 0.05 % comassie brilliant blue R – 250 and destained.

Dot blot assay:

Antisera were raised in rabbits against solubilised inclusions protein of *Bt* reference strain. Solution of solubilised inclusion proteins was mixed with equal volume of Frend's complete adjuvant. Rabbits were immunized by four subcutaneous injections of the mixture weekly intervals. One week later, intravenous booster injections of adjuvant free proteins were given. Rabbits were bled one week after the last injection. Antiserum was heated at 56 degree Celsius for 30 minutes and stored at 20 degree Celsius until use. 10 ml of Sporulated cultures were spotted on the blotting membrane.

Insecticidal activity:

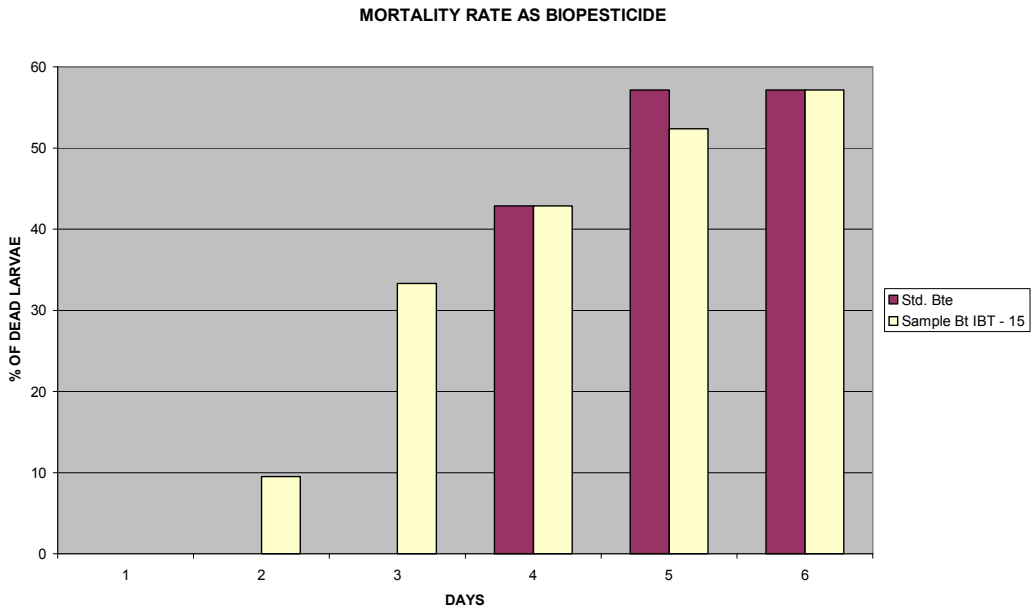
Plutella Xylostella were collected from cabbage field in coonoor. Insect larvae were acclimatized to the lab conditions. Isolated strain was allowed to sporulate 72 hours in SCG minimal media. The strain sample was applied on the leaf & the leaf was dried & given as feed for the pest.

TABLE - I: Microscopic and Biochemical tests

S.NO	TEST	RESULT
	MICROSCOPIC TESTS	
1	Gram staining	Gram positive
2	Spore staining	Presence of spores
3	Crystal staining	Bipyramidal crystals
4	Motility test	Positive
	Biochemical tests	
5.	Indole test	Positive
6.	V.P test	Positive
7.	M.R test	Negative
8.	Starch hydrolysis test	Positive
9.	Citrate utilization test	Positive
10.	Catalase test	Positive
11.	Gelatin test	Positive
12.	Caesin hydrolysis test	Positive

TABLE – II : Insecticidal activity

Days	% of Dead larvae	
	Std. *Bte*	**Sample *Bt* IBT – 15**
1	0	0
2	0	9.52
3	0	33.3
4	42.5	42.85
5	57.14	52.38
6	57.14	57.14



Results and Discussions:

Bacillus thuringiensis is well known for its entomo pathogenic activity against insect pests mainly of the order Lepidoptra, diptera and coleopteran. Its toxicity is associated with the production parasporal proteinaceous crystalline inclusions during sporulation. The proteinaceous crystals are in various shapes and depending on the sub species .Different crystals are toxic to different insect taxonomy . More recentely new isolates of *Bt* have been shown to toxic against Hymenoptra and orthoptera [16] .

Our study is consistent with these reports of new isolate having toxicity against *Plutella xylostella* , since resistance of plutella larvae to chemical insecticide is increasing ; several laboratories are searching for novel strains and toxins. One of the isolate IBT -15 appears as rod shape was particularly interesting because of its high toxicity against *Plutella xylostella* . The unknown isolate was gram stained and examined under phase –contrast microscopy . It is apparent that the isolate absorbed the crystal violet and did not decolourise indicating Gram-positive organism . This technique also indicated that the cell wall of the organism is composed of peptiglycon and had a distinct rod shape. The isolate IBT – 15 produces bipyramidal crystal , when observed under phase contrast microscopy. The spore forming bacteria IBT -15 bearing a parasporal inclusions body was screened to find their taxonomical position based on spore and cell morphology, biochemical and physiological characteristics according to the procedure described in bergrey's manual of systematic bacteriology.

The Bacillus thuringiensis serovar entomocidus used as the reference strain , both the strains showed positive result for motility, V -P test, indole test , starch hydrolysis test , casein test , catalyase test , citrate utilization and gelatin test and negative result for mannitol. Plasmid pattern of this strain was different *Bacillus thuringiensis entomocidus*. Toxin genes are often located on large self transmittance or mobilizable plasmid . DNA amplification was carried out with primers that are specific to internally transcribed spacer and cryII genes.

The results of the PCR indicated that this isolate is certainly a *Bt* Strain. SDS – PAGE showed that the crystals is composed of major protein of approximately 130 kDa and some minor protein in around 80 kDa .This indicated that this strain contain two or more genes encoding protein with different molecular weight . In this present study polyclonal antisera were raised against Bt IBT-15. Moreover immunoblot reaction of the isolate with the antisera was weak and it might be due to the presence of different type of crystals proteins and also indicates that it is a different strain from the reference strain .

Preliminary toxicity test was done using cells grown in SCG medium for 72 hours. Strain *Bte* was used as a positive control and larvae without inoculation of bacteria as negative control. The spore crystal of Bte and IBT – 15 were tested separately in *Plutella xylostella* .IBT -15 had a level of activity similar to that of Bte against *Plutella xylostella* . Preparation *of Bacillus Thuringiensis* usually as a mixture of cells , spores and parasporal crystals have been used as microbial insecticide for more than 30 years and no unexpected toxicity have been noted.

Conclusion:

The prospects offered new isolation of *Bt* strain with improved efficacy and specificity may be benefit in developing alternative control strategies against *Plutella xylostella*. This isolate might be a candidate for harbouring putative , novels crygene . The identification putative novel *Bt* strains could be the first step in the sequence of finding novel toxicity , since novel toxins may be toxic for new targets . The isolation and sequencing of novel cry genes should be encouraged,once the target insect is identified and more evidence on the novel toxin as biological control agents are available.

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