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Characterization and Screening of *Bacillus thuringiensis* Isolated from Nashik and Ahmednagar Region of Maharashtra

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***Abstract* – *Bacillus thuringiensis* is a Gram positive, facultative anaerobe, Soil dwelling, spore forming bacteria that produces toxic against different insect species. This feature makes it the widely used biological control agent in agriculture. The aim of this study was to isolate and cloned *B. thuringiensis cry* genes and check the toxicity level against Helicoverpa *armigera*. Soil samples were collected Nashik and Ahmednagar region of Maharashtra. The fried egg type colonies which were obtained from this soil sample were identified as *Bacillus thuringiensis*. The crystal morphology of isolates was observed under phase contrast microscope during spore formation and rectangular type crystal morphology was present in both the cultures. The bioassay was done to check mortality percentage in *Helicoverpa armigera*. For genotypic characterization, the *cry gene* content of the isolates was screened by polymerase chain reaction (PCR) analysis using universal primers as per Ben-Dov, *et al.*, 1997. Both isolates were screened for *cry* genes and it was found that both isolates showing the band size more than the expected amplicon size, thus it was hypothesized that particular gene been amplified in different manner. So it is concluded that it may be a *cry* gene or Vegetative insecticidal Proteins (Vips) genes.**

***Keywords* – *Bacillus thuringiensis*, Colony Morphology, Crystal Morphology, DNA Isolation, SDS-PAGE, Protein Harvesting.**

Highlight* – In the Present study the Bt isolates of Nashik and Ahmednagar was screened for *cry* genes by PCR amplification which were examined for their insecticidal activity against *Helicoverpa armigera.

I. INTRODUCTION

Bacillus thuringiensis (*Bt*) is a typical aerobic, rod-shaped bacterium that occurs naturally in soil, dead insects, water, and grain dust (Lambert and Peferoen, 1992)[1]. *Bt* is a Gram positive spore forming bacteria grouped into the *Bacillus cereus* group of *Bacilli* which produces proteinaceous insecticidal crystals during sporulation which is the distinctive feature between it and other members of the *Bacillus cereus* group (Read *et al.*, 2003; Rasko *et al*., 2005)[2]. This endospore forming microorganism *Bacillus thuringiensis* produces the best known insect toxin which has great potential for the control of various *Lepidopteron, Dipteron* and *Coleopteron* insects (Aronson *et al*., 1986, Hofte and Whiteley, 1989)[3]. The parasporal inclusions are formed by different insecticidal crystal proteins (ICP). When orally ingested by insects, this crystal protein is solubilized in the midgut, forming proteins called delta-endotoxins. A partial correlation between crystal morphology, ICP composition, and bioactivity against target insects has been established.

The toxicity of these Crystals to the insects is determined by the presence of the specific receptors in the midgut epithelium (Bravo *et al.*, 2007)[4].Because delta-endotoxins are generally safe to vertebrates and beneficial arthropods and are often highly toxic to insect pests at relatively low doses. Genes encoding these proteins (*Cry* genes) were among the first to be used in genetic engineering of plants for enhanced insect resistance (Roh *et al*., 2007)[5]. Recent findings have demonstrated the toxicity of *Bacillus thuringiensis* to a number of other classes of pests such as nematodes, aphids and sheep fleas (Feitelson *et al*., 1992)[6]. There are now at least 29 distinct Crystal protein genes and still *Bacillus thuringiensis* pesticidal activity spectrum is not at all exhausted since much more interesting specificity is being identified in nature.

II. MATERIAL AND METHODS

2.1 Collection of soil sample

Soil Sample collected from two regions of Maharashtra i.e. Nashik And Ahmednagar.

2.2 Isolation of Bt

Different dilutions of soil sample prepared and 1ml aliquots of dilutions were taken in different petri-plates over which melted T3 agar medium was poured and mixed clockwise and anticlockwise direction (pour plate technique). The plates were incubated at 30°C for 48hr. Colony showing *Bacillus thuringiensis* like colonies which are yellowish-white, spread out and seems to fried egg on plate (Travers *et al*., 1987) were labeled and subcultured. Subculturing from one individual colony was repeated until pure culture obtained.

2.3 Crystal morphology

A loopful culture from 48hr inoculate is smeared on the slide & heat fixed. After heat fixing, drops of the Coomassie Brilliant Blue stain were added and kept as such for 1 min. Then the smear is washed gently in running tap water. After the blot drying with blotting paper, the stained cultures were observed through bright field microscopy for presence of crystalline inclusion. The isolate showing presence of crystalline inclusion was selected as *Bt.*

2.4 Protein harvesting

The *Bt* culture was inoculated in 5ml T3 broth tube and incubated at 30°C for overnight 12-14hrs. The 1% of

mother culture (250µl) was re-inoculated into 25ml T3 broth and incubated at 30ºC for 48hrs. Monitored the spore suspension of *Bt* through a phase contrast microscope. When >90 % lysis occurred, the conical flask was placed at 4ºC for 30 min before harvesting. The 48h grown culture (>90 % lysed culture) was poured in 30 or 50ml centrifuge tube. The sample was centrifuged for 10 minutes 10,000 rpm, at 4ºC, the supernatant was discarded. Pellet was washed with 1X TE containing 500µl of 100mM Phenymethylsulfonyl fluoride (PMSF). (100mM). Then it was centrifuged 10 minute 10,000 rpm, at 4ºC. Again the supernatant was discarded and pellet was washed with 25ml of 0.5M NaCl. The sample was centrifuged for 10 minutes 10,000 rpm, at 4ºC, the supernatant was discarded. Again the pellet was washed with 1X TE containing 250µl of PMSF (100mM). Again the sample was centrifuged for 10 minutes 10,000 rpm, at 4ºC, the supernatant was discarded and pellet was washed with 1X TE containing 250µl of PMSF. Again the centrifugation was given for 10 minutes at 10,000 rpm at 4ºC and the supernatant discarded. Empty wash was given for 5 minutes at 10,000 rpm at 4ºC. The final pellets were dissolved in distilled water containing 100mM PMSF and homogenize the pellet.

2.5 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out which is used for the separation of proteins .In SDS PAGE two gels are used one of them is the separating gel of 8% and stacking gel 5% as described by (Davis, 1964).

2.6 Bioassay against Helicoverpa armigera

2.6.1 Preparation of diet

Artificial diet was prepared as given by Armes *et al.*, (1992) for Bioassay studies.

2.6.2 Isolation of genomic DNA from Bt isolate

Genomic DNA was extracted by combining two methods (Ausubel *et al.* 1994; Cardinal *et al.* 1997)[9]. DNA Isolated from cells grown in 10 ml of nutrient broth (NB) at 37ºC overnight and quantify at 0.8% agarose by agarose gel electrophoresis. After complete electrophoresis the gel was observed into the Gel Documentation under UV light.

2.6.3 Oligonucleotide primers for polymerase chain reactions (PCR)

In this study, universal primers reported by Ben-Dov *et al*. (1997, 1999)[7], for *cry1* and *cry2* genes were used these primers synthesized by Eurofins Genomics India Pvt Ltd.

2.6.4 Screening of Cry gene by polymerase chain reactions (PCR)

All PCR reactions were carried out in 25µl reaction volumes. Amplifications were carried out in a DNA thermal cycler. Thirty cycles were carried out for the amplification of *cry* gene fragments. After amplifications, 5µl of each PCR product was electrophoresed on 1 % agarose-ethidium bromide gel. Gels were visualized in a gel documentation system (UviTec).

2.6.5 Ligation and Transformation in E.Coli Host

T/A vector and insert DNA was used in ratio of 1:3 for ligation reaction. Transformation of *E. coli* with ligated mixture by heat shock method and the selection of transformed colonies of *E. coli* by Alpha Complementation with 0.1M IPTG and 40µl of X-Gal.

III. RESULT AND DISCUSSION

3.1 Colony and Crystal Morphology

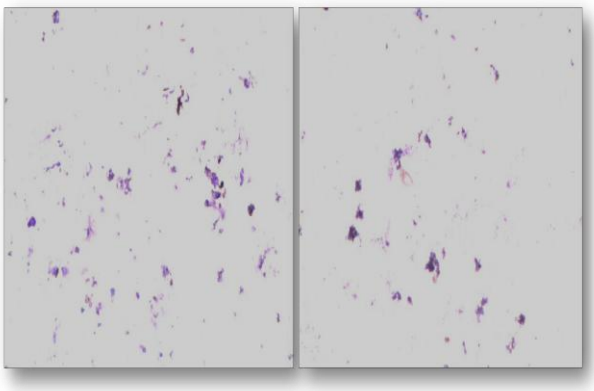
The samples of two regions *i.e.*, Nashik and Ahmednagar were examined in this study. The colony morphology was checked as per Travers *et al*., 1987, *Bacillus thuringiensis* like colonies which are yellowish-white, spread out and seems to fried egg on plate were observed.

Bt Isolates from Nashik region *Bt* Isolates from Ahmednagar region

Fig.1. Colony morphology of Bt isolates

Both of the samples were examined with the phase contrast microscope (PCM) for spore formation and crystal morphology. *Bacillus thuringiensis* strains produce parasporal crystal inclusions with different morphologies, sizes and numbers. Based on literature, some distinct morphologies were apparent; bipyramidal crystals, related to Cry 1 proteins: cuboidal inclusions, related to Cry 2 proteins: flat and square crystals, related to Cry 3 proteins; amorphous and composite crystals, related to Cry 4 proteins (Federici *et al*., 1990)[8].

In both samples rectangular, cuboidal and bipyramidal type of crystals were observed.



Bt isolate of Nashik region *Bt* isolate of Ahmednagar region

Fig.2. Crystal morphology of *Bt* isolates

3.2 SDS-PAGE

SDS-PAGE (8% separating and 5% stacking) was done to separate the protein from nucleic acids and for determination of molecular weight of the specific protein (Laemmli, 1970). In this study, the protein i.e. crystal protein was separated through SDS-PAGE. Molecular weight of crystal protein in Nashik region sample is near about 135-140kDa and in Ahmednagar region sample is 140kDa and ~35kDa. (Figure 3)

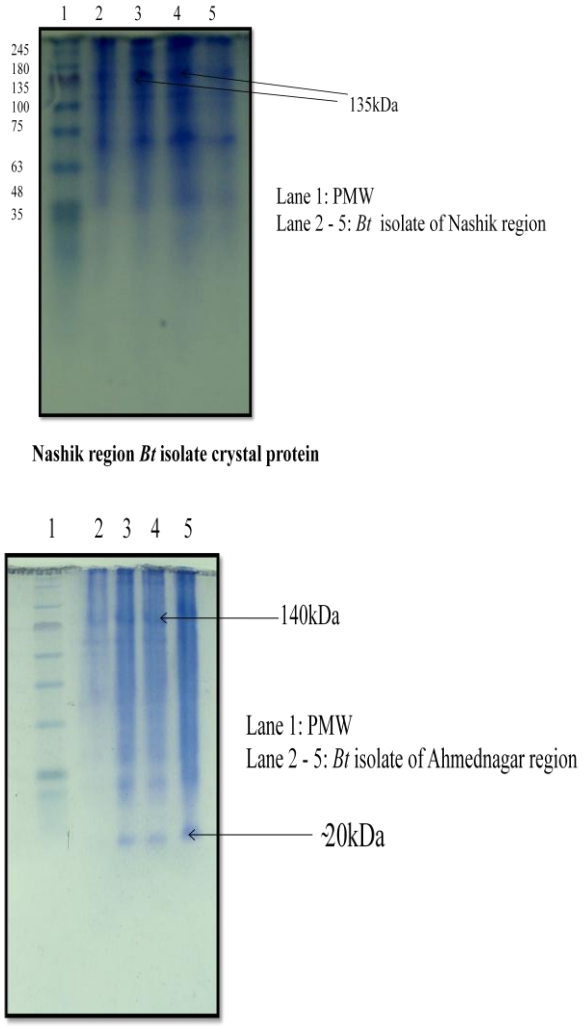


Fig.3. SDS-PAGE of *Bt* isolates

3.3. Bioassay against Helicoverpa armigera

Artificial diet was prepared for *Helicoverpa armigera*. In diet crystal protein was added in minute quantity and checked for seven days and the mortality of that *Helicoverpa armigera* was checked in percentage, in both the regions 65% mortality was observed.

$$\text{Mortality Percentage} = \frac{\text{No. of died}}{\text{No. of inoculated}} \times 100$$

3.4 DNA isolation

DNA was isolated from both Nashik and Ahmednagar region as both shown 65% mortality in *Helicoverpa armigera*. The method combining two methods (Ausubel et al. 1994; Cardinal et al. 1997) was used for DNA isolation. The DNA isolated was electrophoresed for the determination of molecular weight of that DNA. (Figure 4)

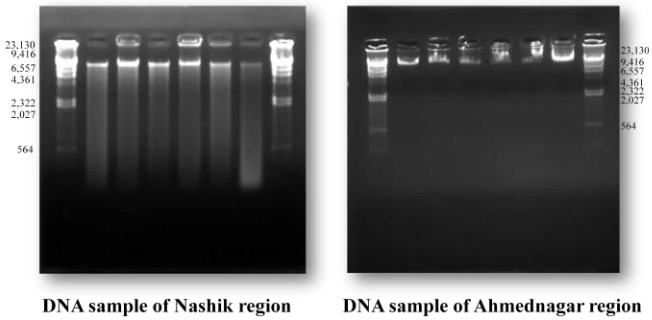


Fig.4. DNA of *Bt* isolates

3.5 Screening of Cry genes using PCR

Toxic crystal proteins are encoded by *cry* genes and one *Bacillus thuringiensis* strain can contain one or more *cry* genes. The *cry* gene contents of both crystal producing isolates were determined by PCR analysis of *cry1* and *cry2* genes Porcar and Jaurez-Perez, 2003[9]. Genomic DNA extracted from each *Bt* isolate was used as template in PCR reactions and target gene fragments were amplified by using the universal primers specific to each group of *cry* gene. The DNA from Nashik region was given amplification in both of the primers for *Cry1* and *Cry2*. (Figure 5)

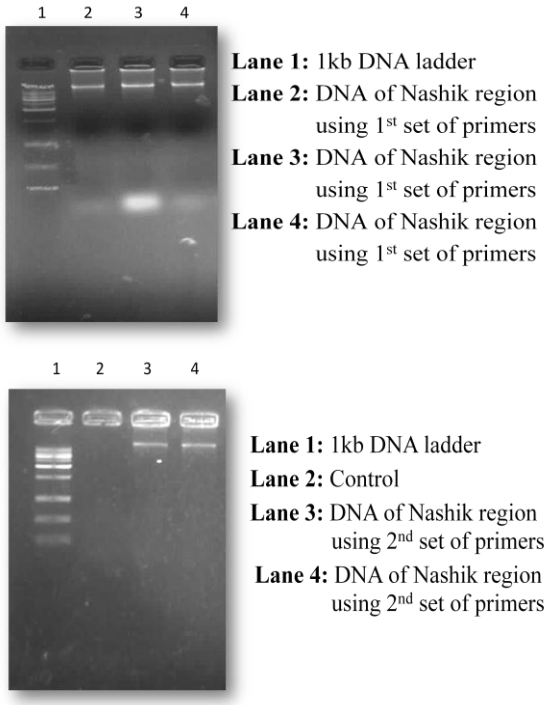


Fig.5. Screening using universal primers for *Cry* genes

V. CONCLUSION

From the present study it was concluded that the Bt isolate of Nasik and Ahmednagar region contains the Crystal protein,further the Bt isolates were screened for the presence of *cry 1* genes by PCR amplification which were examined for their insecticidal activity against *Helicoverpa armigera.*

REFERENCES

[1] Lambert B., Peferoen, M. (1992).Insecticidal promise of *Bacillus thuringiensis*, Facts and mysteries about a succesful biopesticide, *Bioscience*, 42, 112-122.
[2] Rasko D. A., Altherr M. R., Han C. S., Ravel J. (2005), Genomics of the *Bacillus cereus* group of organisms, *FEMS Microbiol. Rev 29* (2):303–329.
[3] Aronson A., (2002). Sporulation and δ-endotoxin synthesis by *Bacillus thuringiensis, Cell. Mol. Life Sci.*, 59 417-425.
[4] Höfte H. and Whiteley H. R., (1989). Insecticidal crystal proteins of *Bacillus thuringiensis, Microbiol, Rev*., 53, 242-255.
[5] Bravo A., Gill S. S., Soberon M.,(2007). Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon*. Mar. 15, 49(4): 423-435.
[5] Roh J. Y., Jae Y. C., Ming S. L., Byung R. J., Yeon H. E. (2007), *Bacillus thuringiensis* as a specific, safe, and effective tool for insect pest control, *Microbiol Biotechnol* 17: 547-559.
[6] Feitelson J. S., Payne J., Kim L., (1992). *Bacillus thuringiensis*: insects and beyond. *Bio/Technology*, 10, 271-275.
[7] Ben-Dov E., Zaritsky A., Dahan E., Barak Z., Sinai R., Manasherob R., Khamraev A., Troitskaya E., Dubitsky A., Berezina N., Margalith Y. (1997). Extended screening by PCR for seven cry-group genes from field-collected strains of *Bacillus thuringiensis*. *Appl. Environ Microb.* 63: 4883-4890.
[8] Federici B. A., Park H. W., Sakano Y. (2006), Insecticidal protein crystals of *Bacillus thuringiensis*, Inclusions in Prokaryotes-Microbiology Monographs, 1, pp 195-236.
[9] Porcar and Jaurez-Perez,(2003).PCR-based identification of *Bacillus thuringiensis* pesticidal crystal genes, *FEMS Microbiology Reviews* 26, 419-432.

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