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# PROTEIN AND GENE PROFILING OF INDIGENOUS *Bacillus thuringiensis* ISOLATES

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ABSTRACT

*Bacillus thuringiensis* is a gram positive, spore forming bacterium. During sporulation, it produces intracellular crystal proteins (Cry proteins), which are toxic to insects. Because of its insecticidal activity, it has been used for nearly fifty years to control certain insect species among the orders *Lepidoptera, Coleopteran* and *Diptera*. However it is still necessary to search for more toxins to control other insect orders and to provide alternatives for coping with the problem of insect resistance. Each habitat may contain novel *B. thuringiensis* strains, which have some toxic effects on target spectra of insects. The aim of the study was to isolate indigenous *B. thuringiensis* strains, and analyze protein profile and of *cry* gene content using PCR. SDS-PAGE was optimized to detect protein profiles of crystals obtained from indigenous *B. thuringiensis* isolates. The protein profiles of *B. thuringiensis* isolates carrying many different *cry* genes produced proteins of different size *viz.,* 200,130, 97, 70, and 50 kDa. PCR technique was used to detect *cry* gene content in the indigenous *B. thuringiensis* isolate. Variation in the mass of crystal proteins showed the presence of diverse group of *cry* genes. Further cloning and characterization studies will be carried out.

Key words: *Bacillus thuringiensis,* cry proteins, SDS-PAGE, *cry* gene, PCR.

## 1. INTRODUCTION

The use of chemical substances to control pests was started in the mid-1800s. Early insecticides were some inorganic chemicals and organic arsenic compounds. Organochloride compounds, organophosphates, carbamatespyrithroids and formamides followed them [1] Certain properties made these chemicals useful, such as long residual action and toxicity to a wide spectrum of organisms [2]. The continuous use of chemical insecticides to control insect pests of agriculture, forestry and horticulture crop plants have caused many environmental problem including insect resistance, toxicity to humans and to beneficial insects[3]. Some insects have developed resistance to chemical insecticides. An alternative strategy used to control harmful insects is bio pesticides [4].

*Bacillus thuringiensis* is a gram positive, spore forming; ubiquitous, facultative, aerobic soil bacterium that is characterized by the production of insecticidal crystal proteins known as δ-endotoxins (Cry protein) that are specifically toxic for different insects. These proteins are encoded by *cry* genes. Nearly 300 *cry* genes have been identified and classified into 51 groups and subgroups on the basis of amino acid sequence similarity[5]. Significant sequence similarity exists among the *cry* genes of, *B. thuringiensis* however; individual gene products vary in their levels and spectra of toxicity to different insects[6] Continuous exposure to a similar kind of *B. thuringiensis* toxin can lead to the development of insect resistance[7]. Variation in single amino acid can significantly influence the level of toxicity in Cry proteins[8]. The toxicity of each *Bt* type is limited to one or two insect orders, and is nontoxic to vertebrates and many beneficial arthropods. The reason is that *B. thuringiensis* toxin works by binding to the appropriate receptor on the surface of midgut epithelial cells. Any organism that lacks the appropriate receptors in its gut cannot be affected by *B. thuringiensis* .The aim of our study is to investigate the protein profiling of the various isolated *B. thuringiensis* strains.

*Bacillus thuringiensis* is a major microorganism, which shows entamopathogenic activity[3,9]. *Bacillus thuringiensis* is the most valuable and environmental friendly bio pesticide that has been used as an alternative to conventional chemical pesticides. It occupies 90% of the word bio pesticide market[5, 10]. *B. thuringiensis* is remarkably non toxic to humans and to large extent on non target fauna. Hence it is easy to use making it popular alternative to chemical treatment, for crop protection[11, 12].

## 2. MATERIALS AND METHODS

### 2.1. COLLECTION OF SOIL SAMPLES

Soil samples were collected from five different places under different environmental

conditions. Samples were collected by scraping off the soil surface with sterile spatula and about 10g of soil was obtained at a depth of 2-5cm below the surface. All samples were kept aseptically in sterile plastic bags and stored at 4°C.

2.2. ISOLATION OF *Bacillus thuringiensis* STRAINS

One gram of soil sample was suspended in 10 ml of sterile distilled water ($10^{-1}$) in a boiling tube. The boiling tube was subjected for heat treatment at 65°C for 30 min and allowed to settle. Different dilutions were prepared. 1 ml aliquots of dilutions $10^{-1}$, $5^{-1}$ to $5^{-5}$ were taken in six different petriplates over which melted T3 agar medium (PH 6.5) was poured[13]. The plates were incubated at 30°C for 2-3 days. Finally the bacterial colonies were separated based on their colony morphology. *B. thuringiensis* like colonies which are usually described as cream-colored and have the experience of fried edge on plate were sub cultured in selective $T_3$ medium and incubated at 30$^0$C for 24 hrs. Then the culture was stored at 4$^0$C.

2.3. PREPARATION OF SPORE-CRYSTAL MIXTURE FROM *B.thuringiensis* STRAINS

Single colony of *B. thuringiensis* strains was inoculated into 5 ml of $T_3$ broth and incubated in a shaker and maintained in 30°C at 200 rpm. After overnight growth, 1 % inoculums was added to 250 ml flask containing 25 ml $T_3$ medium and incubated at 30°C in a rotatory shaker maintained at 200 rpm for nearly 48-60 hrs .The bacterial sporulation was monitored through Phase contrast microscope. When more than 90 % of cell lysed, the sporulated broth culture was transferred to 4°C, at least half-an-hour before harvesting. The $T_3$ broth containing spore-crystal mixture was centrifuged at 10000 rpm at 4°C. The pellet was resuspended in ice cold 25 ml Tris-EDTA buffer [Tris 10 mM, EDTA 1mM, pH 8.0 with 1mM phenyl methyl sulphonyl fluoride (PMSF)] and washed once with 25 ml ice-cold 0.5 M NaCl at 10000 rpm for 10 min followed by two washes with 25 ml Tris-EDTA buffer with 0.5 mM PMSF at the same speed and time. Finally, the spore-crystal pellet was suspended in 500 µl sterile distilled water containing 1 mM PMSF and stored in -20 °C [14].

2.4. SOLUBILIZATION OF CRY PROTEIN

Protein crystals were dissolved in 2ml 50 mM sodium carbonate (PH 9.6) containing 0.1% 2–mercaptoethanol by incubating the preparation for 2 hrs at 25°C and then centrifuged at 1000 rpm for 15 min[15]. The supernatant was collected and analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.5. ISOLATION OF GENOMIC DNA

A loopful over night grown culture was inoculated into 5ml of $T_3$ broth and kept at 30 ml broth culture was taken and centrifuged 8000 rpm for 10 minutes to collect the pellet and added 450 µl of the buffer. Next added 45 µl of 10% SDS, 5µl of 20 mg/ml of proteinase-k then this mixture was mixed gently and incubated for 1 hour at 37°C. After this incubation time added 500 µl of phenol chloroform and mixed well by inverting the tubes. The eppendrof tubes were centrifuged at 10000 rpm for 10 minutes. The aqueous layer was collected in separate eppendrof tube and equal volume of phenol chloroform was added. Then this mixture was mixed well and centrifuge at 10000 rpm for 10 minutes. After this centrifugation 50 µl of sodium acetate was added to the aqueous phase and mixed properly. 300 µl isopropanol was added and mixed gently to precipitate the DNA. Then this eppendrof tubes were centrifuged at 10000rpm for 10 minutes. The pellet was washed with 70% ethanol then centrifuged at maximum speed for 30 sec. Finally pellet was dissolved in 50 µl of TE buffer[15].

2.6. ANALYSIS OF DNA SAMPLE BY AGAROSE GEL ELECTROPHORESIS

PROCEDURE

0.8% agarose was prepared by adding 0.8g of agarose to 100 ml of 1x TAE buffer. The mixture was warmed in a flask at 100°C in water bath until the agarose was dissolved. The agarose solution was cooled until its temperature reached 50°C. 10µl of ethidium bromide was added to the agarose. The mixture was poured into the gel boat containing comb and was allowed to solidify. Boat was poured into the gel buffer reservoir in the electrophoretic apparatus to about 2mm above gel surface. DNA sample were mixed with loading dye at 5:1 ratio and loaded into respective gel wells. Electrophoresis was carried out at 50 V and the gel was examined on the transilluminator using UV light and documented using alpha imager[16].

2.7. AMPLIFICATION OF *cry2Aa* GENE

Amplification was carried out in a DNA thermal cycler (cyber cyber p series PCR peltier model p96+). Reactions were routinely carried out in 25 µl; 1 ml of template DNA was mixed with reaction buffer, 150 mM (each) deoxynucleoside triphosphate, 0.5 mM (each) primer (Un2D:GTTATTCTTAATGCAGATGAATGGG; 2AbR: TGGCGTTATTTGGGGGGAGAAA T) (2)., and 0.5 U of *Taq* DNA polymerase. Template DNA was denatured (4 min at 94°C) and annealed to primers (60°C), and extensions of PCR products were achieved at 72°C for 90 s.

Each experiment was associated with negative (without DNA template) and positive (with a standard template) controls. The reliability of the primers was verified a reference strains *B. thuringiensis* subsp. *kurstaki* HD-1 obtained from The Ohio State University, Columbus. The expected amplified PCR product was checked by 1.5 % agarose gel with a 100bp ladder. The gel was examined on the UV transilluminator and documented using Alpha imager.

## 3. RESULTS AND DISCUSSION

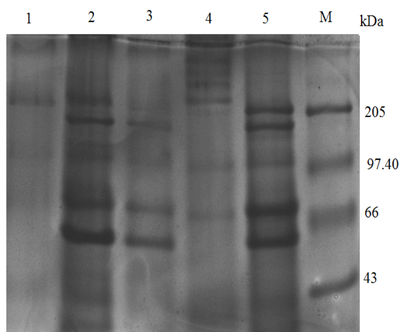
### 3.1.MORPHOLOGICAL IDENTIFICATION

Rod shaped colonies along with proteinaceous crystals were observed for 18 strains under 100X. *Bacillus thuringiensis* sub sp, *Kurstaki* HD1 and *Bacillus thuringiensis* subsp. israelensis 4Q2-81, obtained from The Ohio State University, Columbus, were used as reference strains. About five different structural morphologies and seven different types of crystalline inclusions have been already reported[17, 18].

### 3.2. SDS - POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

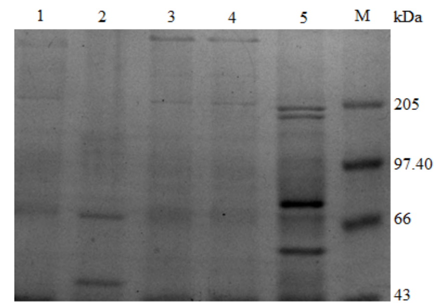
All the 18 isolates were studied for crystal protein profile(s) by SDS-PAGE[15]. The new isolates of *B. thuringiensis* showed four different types of crystal protein profile *viz.,* 200,130, 97, 70, and 50 kDa. Around 135 and 70 kDa proteins suggesting the presence of genes related to *cry1* or *cry4* and *cry2*. The four strains RA3, MCI2, PU1 and PU2 showed weak bands. The time for completion of sporulation, growth medium conditions, parasporal crystal formation was checked always before isolation. These results suggest that the variation in mass of crystal protein(s) purified from the isolates of *B. thuringiensis* may be due to various environmental conditions. (Fig: 1-4)

FIG 1: SDS-PAGE ANALYSIS OF SPORE CRYSTAL MIXTURE ISOLATED FROM *B. thuringiensis*



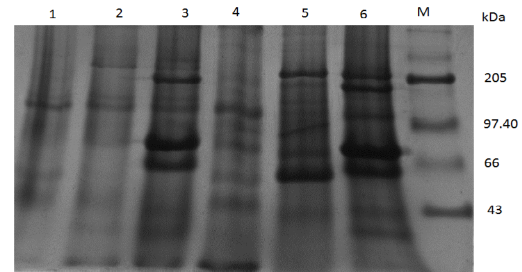

Lane 1: *Bacillus thuringiensis* strain PU3 showed weak bands
Lane 2: *Bacillus thuringiensis* strain KAR3 showed 130, 97, 70 & 50 kDa proteins
Lane 3: *Bacillus thuringiensis* strain SG2 showed 97, 70 & 50 kDa proteins
Lane 4: *Bacillus thuringiensis* subsp. *israelensis* 4Q2-81 showed weak bands (negative control)
Lane 5: *Bacillus thuringiensis* subsp, *Kurstaki* HD.1 (positive control)
Lane M: Protein molecular marker

FIG 2: SDS-PAGE ANALYSIS OF SPORE CRYSTAL MIXTURE ISOLATED FROM *B. thuringiensis*



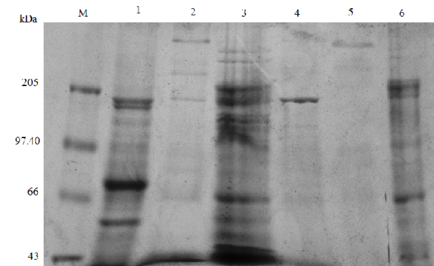

Lane M: Protein molecular marker
Lane 1-3: *Bacillus thuringiensis* strain MCI2 & PU2 showed weak bands.
Lane 2: *Bacillus thuringiensis* strain SG1 showed 70 & 50 kDa proteins.
Lane 4: *Bacillus thuringiensis* strain RA2 showed 70 kDa proteins
Lane 5: *Bacillus thuringiensis* subsp, *Kurstaki* HD1 (positive control).

FIG 3: SDS-PAGE ANALYSIS OF SPORE CRYSTAL MIXTURE ISOLATED FROM *B. thuringiensis*




Lane M: Protein molecular marker.
Lane 1: *Bacillus thuringiensis* strain MCI1 showed 130 & 50 kDa protein.
Lane 2 & 4: *Bacillus thuringiensis* strain RA1 & BBK1 showed 130 kDa protein.
Lane 3: *Bacillus thuringiensis* strain KAR2 showed 130 & 70 kDa proteins.
Lane 5: *Bacillus thuringiensis* strain KAR1 showed 50 kDa proteins.
Lane 6: *Bacillus thuringiensis* subsp, *Kurstaki* HD1 (positive control).

FIG 4: SDS-PAGE ANALYSIS OF SPORE CRYSTAL MIXTURE ISOLATED FROM *B. thuringiensis*




Lane M: Protein molecular marker
Lane 1: *Bacillus thuringiensis* subsp, Kurstaki HD1 (positive control)

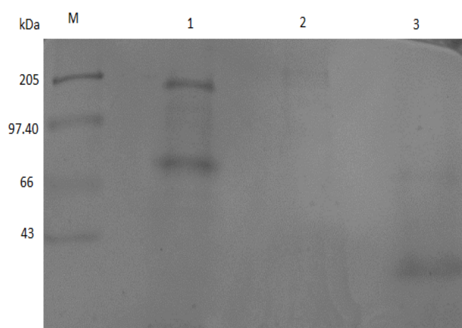
Lane 2-5*: Bacillus thuringiensis* strain PU1 & RA3 showed weak protein bands.
Lane 3*: Bacillus thuringiensis* strain BBK2 showed 70 kDa weak protein bands.
Lane 4*: Bacillus thuringiensis* strain MCI3 showed 200kDa protein bands.
Lane 6*: Bacillus thuringiensis* strain RA4 showed 97 & 70 kDa protein bands.

Similarly many different Cry proteins were studied and *cry* genes were examined. The results of SDS-PAGE displayed a common protein band at about 66, 43 and 35 kDa from different serovars as well as the new isolates in addition to distinct protein bands for each strain. Three native isolates produced major proteins of 130 and 65 kDa consistent with the *cry1* and *cry2* genes were confirmed[8].

### 3.3. SOLUBILISATION OF CRY PROTEIN

The Solubilised Cry protein of 4D1,RA4 and SG1 strains when analysed by SDS- PAGE showed two bands each viz. 4D1 (130kDa and 70kDa); RA4 (70kDa and 35kDa). However SG1 showed no bands after solubilisation. (Fig; 5)

FIG 5: SDS-PAGE ANALYSIS OF SOLUBLIZED CRYSTAL PROTEIN




Lane M: Protein molecular marker
Lane 1: *Bacillus thuringiensis* subsp, *Kurstaki* HD1 (positive control)
Lane 2: *Bacillus thuringiensis* strain SG1 no bands.
Lane 3: *Bacillus thuringiensis* strain RA4 showed 70 & 30 kDa size.

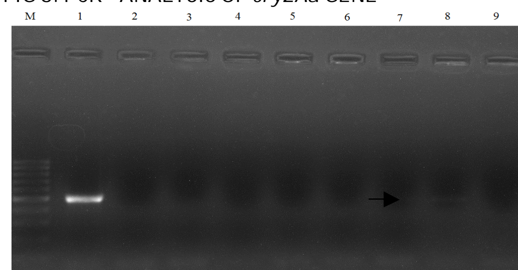
### 3.4. GENOMIC DNA ISOLATION FROM *B. thuringiensis* STRAINS

Total genomic DNA was isolated from 18 *Bacillus thuringiensis* strains along with reference strains 4D1 and 4Q7. It was analysed by agarose gel electrophoresis.

### 3.5. PCR AMPLIFICATION

The DNA of *Bacillus thuringiensis* strains was used as template for PCR amplification. PCR was performed with Un2 (d) and *cry2Aa*(r) primers. *Bacillus thuringiensis* sub sp, *Kurstaki* HD1 and *Bacillus thuringiensis* subsp. *israelensis* 4Q2-81, obtained from The Ohio State University, Columbus, were used as positive and negative control respectively. Reference strain 4D1 showed a prominent band of ~498bp. Out of 18 *B. thuringiensis* isolates, RA4 and SG1 only showed the positive results for PCR. (Fig: 6)

FIG 6: PCR - ANALYSIS OF *cry2Aa* GENE




Lane 1: 100kb marker
Lane 2, 8, & 9: Amplified product of the strains 4D1, SG1 & RA

## 4. CONCLUSION

*Bacillus thuringiensis* strains have been found worldwide from diverse habitats, including soil, insects, stored products, dust, deciduous and coniferous leaves, phyllospheres, and other miscellaneous habitats. In our report, soil has been used as a predominant source for isolation among the diverse habitats in different areas under different environmental conditions. Among 28 colonies, 18 strains were characterized using biochemical tests. In protein profiling study, all the 18 strains were tested; two strains showed the presence of 130 kDa and 6 strains showed 70 kDa proteins. The result suggested that the isolated strains may contain genes related to *cry1* or *cry4* and *cry2***.** The other 6 isolates showed the presence of 97 kDa, other five isolates showed around 50 kDa proteins. It implies the presence of *cry27* and *cry6* genes which are responsible for the toxicity in *Diptera* and *nematodes*[19]. The proteins of 70 and 97 kDa were predominantly present in most of the isolates from different environment. The insufficient protein concentration results in weak band formation and therefore, protein bands were not detectable with Coomassie brilliant blue staining. Out of 18 *B. thuringiensis* isolates, two strains; RA4 and SG1 only showed *cry2A* gene amplification. Furthermore, the bioactivity of crystal proteins purified from *B. thuringiensis* isolates will be examined on different insect groups. Plasmid profiling of *B. thuringiensis* isolates may help to characterize further because crystalline genes are mostly carried on the plasmid. Further, the genetic manipulation of new isolates of *B. thuringiensis* may be helpful in solving the problems such as insect resistance and narrow host range.

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