Original Research Article

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**cry** Gene Profile of Native Entomopathogenic *Bacillus thuringiensis* from Soil

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**ABSTRACT**

*Bacillus thuringiensis* produces different insecticidal crystal proteins which are encoded by **cry** genes. Isolation was carried out from 17 diverse locations’ soil; nine bacterial isolates were separated and selected on the basis of presence of parasporal body. Isolate ABt 10 was screened for different **cry** genes with universal and gene specific primers. Results indicated the presence of **cry1Ac** and **cry1Ab**, **cry4**, and **cry1D** genes in native isolate ABT 10. All these clearly indicated the specificity of these isolates for Lepidopteran insect pests. The search and selection of native isolates will be useful in the generation of a transgenic plant with an indigenous **cry** gene that offers resistance to the serious local pests.

**Keywords**

*Bacillus thuringiensis*, **cry** gene, *H. armigera* and *S. litura*.

**Article Info**

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**Introduction**

*B. thuringiensis* comprises of gram-positive, big rod shaped, spore-forming bacterium. It contains novel properties regarding toxin genes and their expressions. They have unusual property of producing a parasporal protein crystal (δ-endotoxin or Cry protein) which is toxic for many insect pests (Sarker and Mahbub, 2012).

Number of strains have been isolated and used to control pests of agricultural importance because of the insecticidal activity. Efforts have been made in many countries to isolate new strains with increased potency against target pest insects having a wider host range. These toxins have not only shown activity against Lepidoptera, Diptera, Hymenoptera, Isoptera, Orthoptera and Coleoptera but also against nematodes, mites, lice, aphids and ants (Rosas-Garcia, 2009).

The commercial *B. thuringiensis* products are powder based mixture of dried spores and toxin crystals which applicable to leaves or other plant parts where the insect larvae feed. The toxin genes have also been genetically engineered into several crop plants (Brookes and Barfoot, 2013; James, 2013). Scientists have identified at least 29 different crystals and δ-endotoxin effective against specific insects. *B. thuringiensis* can produce one or more type of Cry proteins (Sanahuja *et al*., 2011).
Materials and Methods

Sample collection

Representative soil samples (approximately 100 g) were collected from 17 different locations of Anand, Gujarat in sterile HDPE bags, brought to the laboratory and stored at 4°C till further processing.

Isolation of B. thuringiensis from soil samples and preliminary toxicity test

Native B. thuringiensis were isolated following the modified method described by Patel et al., (2013). The cultures were then transferred on Nutrient agar slants and stored at 4°C for further studies.

Test insects belonging to Lepidopteran group H. armigera and S. litura were selected and efficacy of isolate was tested following food contamination technique (Navon et al., 1990). Haemolymph of the dead larvae was observed under 400X in Phase-Contrast Microscope for the presence of parasporal body of B. thuringiensis by preparing wet mounts.

Identification and characterization of native B. thuringiensis isolates

All the isolates were identified based on their morphological, cultural and biochemical characteristics using 9th edition of Bergey’s Manual of Determinative Bacteriology and standard literature (Halt et al., 1994).

Screening of cry genes by PCR analysis

Screening of cry gene of one native B. thuringiensis isolate was performed by PCR analysis. PCR was performed as per method given by Carozzi et al. (1991). Appendix shows sequences of the general and specific primers used to identify specific cry genes. The oligonucleotides were synthesized at MWG Eurofins Genomics India Pvt. Ltd. PCR products were subjected to submarine gel electrophoresis with marker DNA of known molecular weight, in 2% agarose gel at voltage of 6V/cm using 1X TBE buffer and Ethidium bromide (0.5µg/ml) staining. Gels were viewed under UV light and photographed using Gel Documentation system.

Results and Discussion

This study was undertaken in order to carry out isolation of potential native B. thuringiensis from soil; its cry gene characterization.

Isolation of native B. thuringiensis from soil

Total 109 colonies on MYP agar plate were obtained from 17 soil samples and only 9 colonies designated as ABt 2, 10, 17, 21, 33, 49, 54, 61 and 63 were confirmed as B. thuringiensis (Table 1).

Nine isolates showed the presence of bipyramidal parasporal crystal inclusion body and successfully proved Koch’s postulates in the laboratory against H. armigera and S. litura as target insects. Isolate ABt 10 gave maximum mortality 80% and above (Table 2).

Characterization and identification of native B. thuringiensis isolates

All the nine isolates were found gram positive, straight, thick, sporulating rods, occurring in long chains. On Nutrient agar the colonies were round, medium sized, elevated with irregular margins and the color was creamish white, which later on showed dark center. All the test isolates were confirmed B. thuringiensis by biochemical characters (Table 3).
### Table 1 Isolation of *B. thuringiensis*

| Sr. No | Villages | Total soil samples collected | Total colonies on MYP agar plate | Bt like colonies | Confirmed Bt |
|--------|----------|------------------------------|----------------------------------|------------------|--------------|
| 1      | Anand    | 1                            | 09                               | 06               | 01           |
| 2      | Bakrol   | 1                            | 05                               | 03               | 00           |
| 3      | Bedva    | 1                            | 08                               | 05               | 00           |
| 4      | Chikhodara | 1                         | 03                               | 01               | 00           |
| 5      | Kambaholaj | 1                         | 07                               | 03               | 01           |
| 6      | Navli    | 1                            | 12                               | 09               | 02           |
| 7      | Anklav   | 1                            | 03                               | 02               | 00           |
| 8      | Bhetasi  | 1                            | 06                               | 04               | 01           |
| 9      | Kahanwadi | 1                         | 04                               | 01               | 00           |
| 10     | Umet     | 1                            | 07                               | 05               | 00           |
| 11     | Kantharia | 1                         | 10                               | 06               | 00           |
| 12     | Napa     | 1                            | 08                               | 05               | 00           |
| 13     | Dali     | 1                            | 04                               | 03               | 01           |
| 14     | Palol    | 1                            | 02                               | 01               | 01           |
| 15     | Kanawada | 1                            | 05                               | 03               | 01           |
| 16     | Rhinza   | 1                            | 07                               | 04               | 00           |
| 17     | Tarapur  | 1                            | 09                               | 06               | 01           |
| **Total** | **17** | **109**                     | **67**                           | **09**           |             |

### Table 2 Bioactive compounds Efficacy of isolates against *H. armigera* and *S. litura*

| Sr. No. | Isolate No. | Mortality |         |         |
|---------|-------------|-----------|---------|---------|
|         |             |           | *H. armigera* | *S. litura* |
| 1       | ABt 2       | ++        | ++      | ++      |
| 2       | ABt 10      | ++++      | ++++    | ++++    |
| 3       | ABt 17      | ++        | ++      | ++      |
| 4       | ABt 21      | +         | +       | +       |
| 5       | ABt 33      | +++       | +++     | +++     |
| 6       | ABt 49      | +         | +       | +       |
| 7       | ABt 54      | ++        | ++      | ++      |
| 8       | ABt 61      | ++        | ++      | ++      |
| 9       | ABt 63      | +         | +       | +       |

**Note:** + = Poor effect, ++ = Moderate effect, +++ = Good effect, ++++ = Excellent effect.
Table 3 Biochemical characterization of native isolates

| Biochemical test | ABt 2 | ABt 10 | ABt 17 | ABt 21 | ABt 33 | ABt 49 | ABt 54 | ABt 61 | ABt 63 |
|------------------|-------|--------|--------|--------|--------|--------|--------|--------|--------|
| Malonate         | -     | -      | -      | -      | -      | -      | -      | -      | -      |
| Voges Proskaur's | -     | -      | -      | -      | -      | -      | -      | -      | -      |
| Citrate          | +     | +      | +      | +      | +      | +      | +      | +      | +      |
| ONPG             | -     | -      | -      | -      | -      | -      | -      | -      | -      |
| Nitrate reduction| +     | +      | +      | +      | +      | +      | +      | +      | +      |
| Catalase         | +     | +      | +      | +      | +      | +      | +      | +      | +      |
| Arginine         | +     | +      | +      | +      | +      | +      | +      | +      | +      |
| Sucrose          | +     | +      | +      | +      | +      | +      | +      | +      | +      |
| Mannitol         | -     | -      | -      | -      | -      | -      | -      | -      | -      |
| Glucose          | +     | +      | +      | +      | +      | +      | +      | +      | +      |
| Arabinose        | -     | -      | -      | -      | -      | -      | -      | -      | -      |
| Trehalose        | +     | +      | +      | +      | +      | +      | +      | +      | +      |

Note: + positive reaction; - negative reaction

Fig. 1 PCR Amplification of native isolate ABt 10 produced by cry gene primers

| Lane No. | Isolate No. | cry Primer |
|----------|-------------|------------|
| M        | 100 bp Ladder |            |
| 1        | HD 73 (Std.)  | cry 1 Ac   |
| 2        | ABt 10       | cry 1 Ac   |
| 3        | ABt 10       | cry 1 Ab   |
| 4        | ABt 10       | cry 1 D    |
| 5        | ABt 10       | cry 4      |
| M        | 100 bp Ladder |            |
Appendix
General Primers for cry and cyt gene detection

| No. | Gene     | Sequence (5'→3')                  | Direct | Mer | Reverse | Mer |
|-----|----------|-----------------------------------|--------|-----|---------|-----|
|     | cry1     | TGT AGA AGA GGA AGT CTA TCC A     | 22     |     | TAT CGG TTT CTG GGA AGT A | 19  |
|     | cry 3,7,8| TTA ACC GTT TTC GCA GAG A         | 19     |     | TCC GCA CTT CTA TGT GTC CAA G | 22  |
|     | cry4     | CAA GCC GCA AAT CTT GTG GA        | 20     |     | ATG GCT TGT TTC GCT ACA TC | 20  |
|     | cry 5,12,14,21 | TTA CGT AAA TTG GTC AAT CAA GCA AA | 23     |     | AAG ACC AAA TTC AAT ACC AGG GTT | 24  |
|     | cry 9    | CGG TGT TAC TAT TAG CGA GGG CGG  | 24     |     | GTT GGA GCC GCT TCA CAG CAA TCC | 24  |
|     | cry11    | TTA GAA GAT ACG CCA GAT CAA GC   | 23     |     | CAT TTG TAC TTG AAG TTG TAA TCC C | 25  |
|     | cry13    | CTT TGA TTA TTG AGG TTT AGT TCA A | 25     |     | TTG TAG TAC AGG CTT GTG ATT C | 22  |
|     | cyt1     | AAC CCC TCA ATC AAC AGC CAA G    | 22     |     | GGT ACA CAA TAC AT ACG CCA CC | 23  |

Specific Primers for cry 1 group of genes

| No. | Gene     | Sequence (5'→3')                  | Direct | Mer | Reverse | Mer |
|-----|----------|-----------------------------------|--------|-----|---------|-----|
|     | cry1A    | CCG GTG CTG GAT TTG TGT TA        | 20     |     | AAT CCC GTA TTG TAC CAG CG | 20  |
|     | cry1B    | CTT CAT CAC GAT GGA GTA A         | 19     |     | CAT AAT TTG GTC GTT CTT TT | 20  |
|     | cry1C    | AAA GAT CTG GAA CAC CTT T         | 19     |     | CAA ACT CTA AAT CCT TTC AC | 20  |
|     | cry1D    | CTG CAG CAA GCT ATC CAA           | 18     |     | ATT TGA ATT GTC AAG GCC TG | 20  |
|     | cry1Aa   | TGC ATA GAG GCT TTA AT            | 17     |     | CAG GAT TCC ATT CAA GG     | 17  |
|     | cry1Ab   | TCG GAA AAT GTG CCC AT            | 17     |     | AAT TGC TTT CAT AGG CT     | 17  |
|     | cry1Ac   | GGG ACT GCA GGA GTG AT            | 17     |     | CAG GAT TCC ATT CAA GG     | 17  |
|     | cry1Ad   | CAG CCG ATT TAC CTT CTA           | 18     |     | TTG GAG CTC TCA AGG TGT AA | 20  |

Screening of cry genes by PCR analysis

The development of molecular tools has paved the way for rapid and specific identification of specific gene or markers (Carozzi et al., 1991), thus PCR was run to predict insecticidal activities to identify cry gene, determine their distribution and detect new cry genes in B. thuringiensis strains. Native isolate ABt 10 was analyzed for
presence of different cry genes using general primers in the present study. B. thuringiensis spp. kurstaki HD-73 (standard) gave amplified product of approximately 655 bp size using specific primer of cry IAc. Similarly, isolate ABt 10 was positive for cry IAc gene (Bourque et al., 1993; Morris et al., 1998 and Purani, 2005). Primers for cry 1Ab, cry 1D and cry 4 specific gene produced amplicon of ~ 858 bp, ~ 290 bp and 797 bp, respectively. Isolate ABt 10 gave same desired product. So, it was proved that isolate ABt 10 possessed cry 1Ab, cry 1D and cry 4 gene, which shows Lepidopteran specific insecticidal activity. Similar findings were also reported by Bourque et al., (1993), Ceron et al., (1994) and Ben-Dov et al., (1999) (Figure 1).

PCR analysis was also carried out for general primers of cry 3, 5, 7, 8, 11, 12, 13, 14, 21 and cyt 1. But none of the isolate found positive for presence of the above genes; so it was concluded that these genes are absent in native population.

In this study, we have focused only on preliminary cry gene characterization through PCR. Isolate ABt 10, which shown best performance during primary toxicity test in laboratory condition. This isolate can be tested in field for its potency and can be developed commercially as a formulation. The comparison of cry gene with standard strain and of our new isolate may lead to a better candidate for cloning and developing transgenic crop. The research work presented here opens the door for future research on developing a novel B. thuringiensis of Middle Gujarat for exploitation by farmers for pest control.

In conclusion, based on the results obtained from present investigations, it is concluded that modified method applied for isolation of B. thuringiensis from soil revealed good results and can be very useful in future. PCR is found suitable technique for rapid and specific detection of cry genes from B. thuringiensis. Even though, in the present study few selected primers for cry genes have been used, screening of more number of primers are essential for identification of different genes from this and other collections.

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