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Cry Protein Profiling of *Bacillus thuringiensis* Isolated from Different Agro-Climate Soils of Punjab

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ABSTRACT

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Insect pests caused the yield loss in agricultural crops and forestry plants world widely. Commercial biopesticides based on *Bacillus thuringiensis* (Bt) have been used for pest management. The most important factor that makes Bt as commercial biopesticides is its remarkable ability to produce large quantities of larvicidal proteins (known as Cry protein or delta-endotoxin) that form crystalline inclusion bodies during sporulation. However, use of Bt based biopesticides is limited due to high production cost of Bt based biopesticides. Therefore, using alternative raw materials with the low cost is very important. Differences amongst the strains of *B. thuringiensis* have been reported among 21 strains of Bt, reported three protein banding patterns ranging from low molecular mass (35-66 kDa), medium molecular mass (66-80 kDa) and high molecular mass only (80-116 kDa).

Introduction

Microbial insecticides are being developed as biological control agents during the last three decades. The widely known and used bacteria as insect pathogens belong to *Bacillus* genus and these are Bt, *B. lentimorbus*, and *B. sphaericus*. The most common biopesticides applied in many agro ecosystems are the commercial formulations of Bt (Entwistle *et al.*, 1993). Cry1, Cry2, and Cry9 proteins show strongest toxicity to Lepidoptera (Crickmore 2000). Proteins belonging to the class Cry4 and Cry11 are specifically toxic to Diptera. Cry3, Cry7, Cry8, Cry14, Cry18,

Cry34, and Cry35 (de Maagd *et al.*, 2001) proteins show insecticidal activity against Coleoptera. Some Cry proteins on the other hand display toxicity to more than one insect order. Each insecticidal crystal protein is the product of a single gene. The genes synthesizing these endotoxins are often located on large, transmissible plasmids.

Cry proteins are the predominant type. Two types of δ -endotoxin are produced by Bt strains. They are named Cry and Cyt proteins. Cry and Cyt proteins differ structurally. The most important feature of these proteins is their pathogenicity to insects and each crystal

protein has its distinct host range. The crystal proteins are encoded by cry genes. The accumulation of Cry protein in a mother cell can make up 20-30% of the dry weight of the sporulated cells (Agaisse and Lereclus, 1995). Each crystal protein has its own insecticidal spectrum. Therefore, Cry proteins have been classified on the basis of their host specificity and their amino acid compositions (Jensen *et al.*, 2003).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis is generally used to compare protein profiles of *B. thuringiensis* isolates. For example, Bel *et al.*, (1997) used SDS-PAGE analysis in order to determine the differences among the *B. thuringiensis* isolates collected from different olive tree environments. Hofte and Whiteley (1989) reported that the size of Cry 1 and Cry 2 proteins of *B. thuringiensis* subsp. *kurstaki* were different, Cry 1 type of protein was between 130-150 kDa; Cry 2 type protein was at 70 kDa; Cry 3 type protein was at 75 kDa and Cry 9 type protein was at 130-140 kDa.

Over synthetic pesticides, the advantages of this organism include lack of polluting residues, high specificity to target insects, safety to non-target organisms such as mammals, birds, amphibians and reptiles as well as its relatively low costs of development and registration (Flexner and Belnavis 1999). In spite of these economical benefits, the use of Bt-based biopesticides is much less than synthetic chemicals.

Materials and Methods

Collection of soil sample and isolation of *Bacillus thuringiensis*

Isolation of Bt strains was carried out from soil samples collected from different locations of the Punjab state following the method of Travers *et al.*, (1987). About 5 gm of soil

sample was added to 100 ml of Luria broth buffered with 0.25 M sodium acetate. The mixture was shaken for 4 h at 250 rpm at 30° C. A sample was then taken and heat treated at 80° C for 3 min. About 0.2 ml suspension was plated on Luria agar and incubated over night at 30 ± 2° C. Random sample of colonies were then picked on T₃ medium and allowed to sporulate at 30 ± 2° C. Cultures were checked for the presence of crystals (Ohba and Aizawa, 1986) using a Phase Contrast Microscope (Nikon, Trinocular).

Biochemical characterization of bacteria

The isolates of *Bt* from soil samples were categorized on the basis of these biochemical tests viz. carbohydrate utilization, Voges Proskauer test, Starch hydrolysis, casein hydrolysis, methyl-red test, catalase positive test, citrate and nitrate reduction test.

Confirmation of crystal protein presence

Cultures of approximately five days were analyzed by light microscopy (Nikon E-200 Eclipse) by staining with Coomassie blue (0,25% (w/v) in 60% ethanol (v/v) and 7% of acetic acid (v/v), for the confirmation of crystal structure. The smear was prepared on glass slide and heat fixed. Later it was stained and washed in running tap water. The stained cultures were observed through light microscope for the presence of spore formation.

Polyacrylamide gel electrophoresis

Crystal-spore preparations were analyzed for the presence of the δ -endotoxins in 10% SDS-PAGE gels. A single colony was inoculated into Luria broth and allowed to grow at 30 ± 2° C, 200 rpm for 48-72 h. Once the cells sporulated, 1.5 ml of roth was harvested by centrifugation and washed once with distilled water. The pellet was suspended in T.E. buffer

(pH 8.0). About 45µl sample was mixed with SSB (1% w/v SDS, 0.1 % v/v β-mercaptoethanol, 10 %v/v glycerol and bromophenol blue). They were heated in a boiling water bath for 5 mins before electrophoresis. About 50 µl of the sample was loaded into each well with a stringe and gel was run overnight at 40v (Vertical slab gel electrophoresis, Atta, Japan).

The molecular weight of the Cry proteins was determined by comparing with protein markers of known molecular weight (BR Biochem 161-0304).

Results and Discussion

A total of 68 soil samples were collected from 19 different regions of Punjab. Samples were collected by scraping off the soil surface with sterile spatula and about 10g of soil were obtained at a depth of 2-5cm. All samples were kept aseptically in sterile plastic bags and stored at 4°C. One hundred and forty *Bacillus thuringiensis* were iaolated from these soil samples according to sodium acetate selection method.

Bt colonies which are usually as cream-colored and have the appearance of fried egg on plate were sub cultured on Bt specific T3 medium (per liter, Tryptone-3g, Tryptose-2g, Yeast extract-1.5g, Sodium monophosphate-6.9g, Disodium hydrogen phosphate-8.9g, MnCl₂-0.005g, and pH 6.8) and incubated at 30°C for 24hrs. Then the culture was stored at 4°C for further work.

Various biochemical tests exhibited abundant growth of the isolates on nutrient agar (pH 7.5), producing acid from glucose but lactose, arabinose, rhamnose, mannitol, sorbitol negative. Starch and casein hydrolysis and gelatin liquification was also observed in all the cultures. Catalase production was also observed. The cultures also utilized citrate and

reduced nitrate to nitrite. Based on their biochemical characterization, they were identified to be strains of *Bacillus thuringiensis* (Table 1). Some of these tests have been used in the studies of Cinar *et al.*, 2005 to characterize their native isolate as *Bt*.

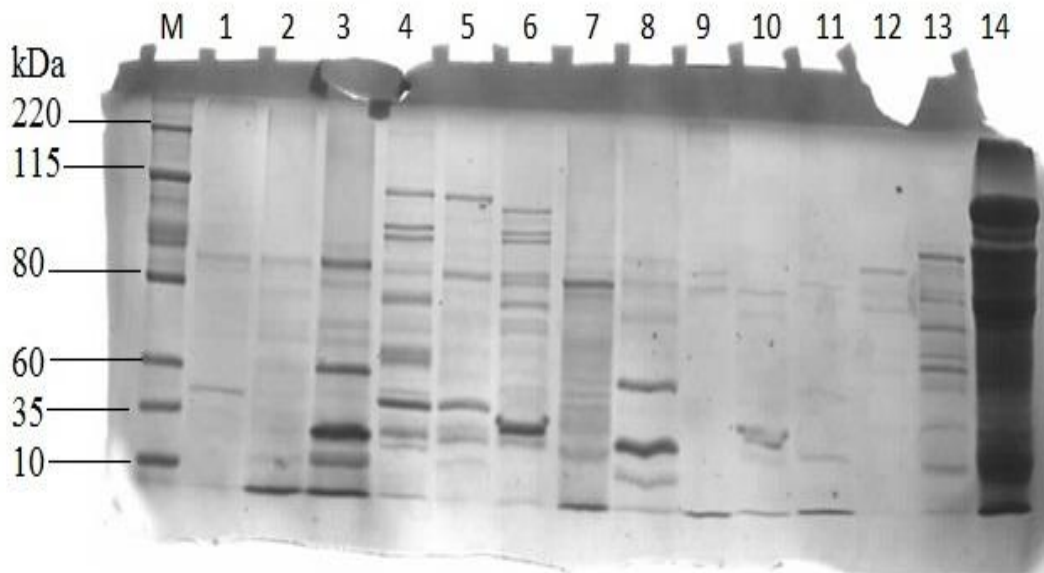
Biotypes of *B. thuringiensis* had the presence of shining crystals in the endospores, which were seen under light microscope after staining with Coomassie blue, made sure Bt strains harboring crystalline inclusions that might have potential for use as bioinsecticidal agent.

Ten per cent sodium dodecyl sulphate, Polyacrylamide gel electrophoresis was carried out with freshly sporulating cultures to determine the number and molecular mass of the 21 positive crystal polypeptides using 4AA1 as reference strain. In the present study, significant differences amongst the 21 strains of *B. thuringiensis* have been reported. *B. thuringiensis* showed existence of 115 kDa, 110 kDa, 100 kDa, 90 kDa, 85 kDa, 80 kDa, 75 kDa, 70 kDa, 68 kDa, 60 kDa, 40 kDa, 35 kDa, 25 kDa, 20kDa and 10 kDa. The reference strain *B. thuringiensis* subsp. *morissoni* LM 79 showed protein band of 25-220 kDa. These results (Plate 13) revealed that Bt isolates generated proteins with molecular weight of 10-115 kDa which were almost similar to those of the reference strains. Bt 34 and Bt 35 produced dense band of a 115 kDa protein (consistent with the presence of a cry3 gene product); most of the isolates synthesized a protein or a group of proteins with molecular weights between 25 and 90 kDa. The presence of proteins or group of proteins with molecular weights 25 - 75 kDa and 75- 115 kDa suggested that the isolated strains contain genes related to cry3 which are known to be active against coleoptera. However, a few isolates showed varying protein pattern. Some weak bands were also observed during toxin protein analysis (Fig. 1).

Table.1 Cultural and biochemical characteristics of *Bacillus thuringiensis* isolates

| Test Character | <i>Bacillus</i> isolated from Luria Agar medium |
|---------------------|---|
| Gram's reaction | +ve |
| Shape | Round, white and smooth |
| Pigment | +ve |
| Pigment colour | Creamish |
| Starch hydrolysis | +ve |
| Casein hydrolysis | +ve |
| Catalase production | +ve |
| Methyl red test | -ve |
| Citrate utilization | -ve |
| Nitrate reduction | +ve |

Fig.1 SDS-PAGE, protein profiling of native *B. thuringiensis* with reference strain. Lane 1: Protein MW Marker, 2: Bt15, 3: Bt16, 4: Bt20, 5: Bt35, 6: Bt34, 7: Bt36, 8: Bt60, 9: Bt39, 10: Bt38, 11: Bt54, 12: 47, 13: Bt50, 14: Standard strain



Insufficient protein concentration could be a reason for that, and therefore protein bands were not detectable with Coomassie brilliant blue staining. Ellis *et al.*, 2002, suggested, the crystal morphology of the bacterium referring to protein Cry35Ba, 44kDa could be associated to toxicity against coleopteran. In another study of Silva N *et al.*, 2010 reported,

a protein identified by SDS-PAGE analysis showed 120 kDa protein band which indicated a new Cry3 protein.

Comparison of the protein pattern has been reported to be useful in evaluation of the relationship between *Bt* isolates (Swiecicka and De Vos, 2003).

Ellis *et al.*, 2002, suggested that this crystal morphology of the bacterium referring to protein Cry35Ba, 44kDa could be associated to toxicity against coleopteran. In another study of Silva N *et al.*, 2010, identified protein by SDS-PAGE analysis and reported a 120 kDa protein band for one isolate which indicated a new Cry protein. Moreover, their study highlights the relations between the genetic profiles for gene *cry35Ba*, the 44 kDa proteins and the spherical morphology are present in all isolates effective against *S. oryzae*. Several *B. thuringiensis* subspecies produce a series of other toxins besides the Cry proteins (Caballero and Ferré 2001). However this was not the aim of the present work that was carried out using spore/crystal suspension in the bioassays, with the possibility of one or more Cry proteins being expressed at one time as described by Fatoretto *et al.*, (2007).

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