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Isolation and Identification of *Bacillus thuringiensis* and Corroborate Its Insecticidal Property

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Abstract

Novel Cry genes are potential candidates for resistance management strategies. Strains of *Bacillus thuringiensis* were isolated from various soil and water samples. Strains which were gram positive, endospore forming were selected for further work. Cry2 gene novel potential candidates were selected for resistance management strategies. Cry2 gene protein was used against cotton pathogen *Helicoverpa armigera*.

Keywords: *Bacillus thuringiensis*; PCR; Cry2 gene

Introduction

Bio preparation based on spore crystals mixture of *Bacillus thuringiensis* seems to be good alternative for chemical pesticides. They are environment friendly, do not have any negative influence on no target animals, including vertebrates and are effectives in reducing the number of insect pests. *Bacillus thuringiensis* is a gram-positive bacterium, which produces insecticidal crystal sporulation. It produces proteinaceous inclusion of *Bacillus thuringiensis* are called as crystals. These crystal proteins are activated by proteases in the alkaline condition of the midgut of insects. These activated toxin binds with the receptor on the brush border membrane vesicle of the midgut epithelium and perforate the cell, which leads to ionic imbalance and insect death. Cry2 gene is isolated from *Bacillus thuringiensis* and used against pathogen from the cotton (*Helicoverpa armigera*). *Helicoverpa armigera* is a larva which feeds on a wide range of the plants including many important cultivated crops. It is major pest in cotton and one of the most polyphagous and cosmopolitan pest species. The greatest damage is caused to cotton, tomatoes, maize, peas and tobacco [1-3].

Materials and Methods

Isolation and identification of *Bacillus thuringiensis*

The soil and water samples were collected from different fields where crops were being cultivated. The soil and water were inoculated on SMM (Spizien's Minimal Medium) for isolation of *Bacillus thuringiensis*. The isolated organisms were identified by gram staining, spore staining, crystal staining and biochemical tests.

Isolation of plasmid DNA

24-hour bacterial culture was taken in fresh Eppendorf tube and centrifuged at 8000 rpm for 10 minutes. Supernatant was discarded, and 0.2 ml of alkaline lyses buffer was added, vortexed and centrifuged at 8000 rpm for 5 minutes. Supernatant was discarded. Alkaline lyses buffer 2 was added and gently vortexed and left for 5 minutes at room temperature. Alkaline lyses buffer 3 was added. Kept for 10 minutes on ice. Ethanol was added to the supernatant and centrifuged at 8000 rpm for 2 minutes. Supernatant was discarded, and pellet was air dried. 1 × TE buffer was added and loaded in 1% agarose gel [4-5].

Protein extraction

Two loops full of bacterial culture was added to ice cold sodium hydroxide; centrifuged at 13000 rpm for 5 minutes. The pellet was resuspended into SDS buffer and ß-Mercaptoethanol and kept at room temperature for 10 minutes. Centrifuged at 13000 rpm for 10 minutes. TCA was added for 15 minutes. Equal amount of acetone was added. Centrifuge at 12000 rpm for 5 minutes. Pellet was dissolved in PBS buffer.

SDS PAGE

The protein was loaded in a 10% polyacrylamide gel at 150 Volts. After gel was fixed, acetic acid and ethanol 100%, with Coomassie brilliant blue was used for staining.

PCR and isolation of Cry2 gene

Primer for Cry2 gene was procured which is

Forward 5'GTTATTCTTAATGCAGATGAAT3'

Reverse 5'CGGATAAAATAATCTGGGAAATA3'

Condition of hot start PCR

Initial denaturation – 95°C/15 minutes

Cycle denaturation – 95°C/30 minutes

Annealing – 60°C/40 seconds

Cycle extension – 72°C/45 seconds

Final extension – 72°C/10 minutes

Hold – 4°C/infinity

No. of cycles – 30

Results and Discussion

Isolation and identification of *Bacillus thuringiensis*

Morphology of *Bacillus thuringiensis*–Gram staining: Gram positive purple rod-shaped bacteria was seen under microscope. It showed parasporal crystals as spore under microscope in Gram staining.

Spore staining: The spores are stained by the malachite green and safranin which differentiated the vegetative cells, which were stained in pink color and the spores were stained in green color.

Crystal staining: When the crystal staining was performed, the crystal violet stained the parasporal proteinaceous which reported as crystal protein. These crystals are activated by the protease which made the alkaline condition in the midgut of the insect and leads to the death.

Citrate utilization test: The Bacteria can convert citrate to pyruvate after the pyruvate enter to the metabolic cycle of the organism to produce the energy. In metabolism the citrate was converted to the ammonia. The change in color showed the positive result.

Motility test: Bacteria present in the young culture was showing motility. Motility resultant that the Bacteria is motile (Figure 1).

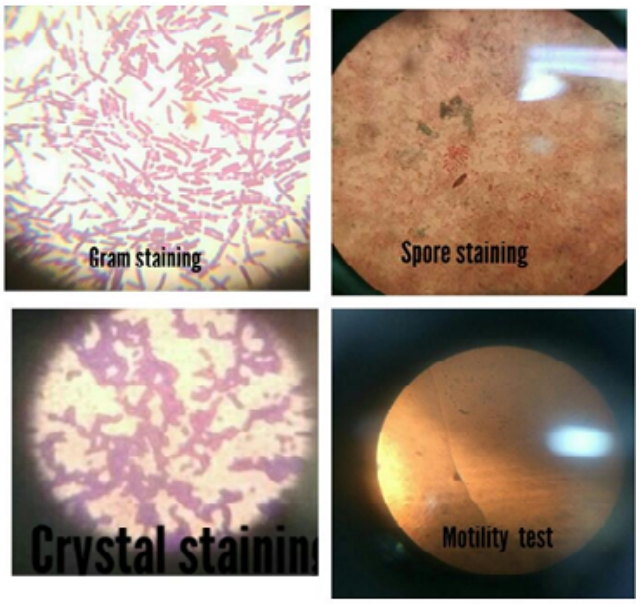

Figure 1: Staining of bacteria.

S.NO.	Tests	Results
1	Indole	Positive
2	VP	Positive
3	MR	Negative
4	Starch hydrolysis	Positive
5	Citrate utilization	Positive
6	Catalase	Positive
7	Gelatin	Positive
8	Casein hydrolysis	Positive
9	Oxidase	Positive

Table 1: Biochemical tests.

Indole test: This test is used to determine the ability of the Bacteria to degrade the amino acid tryptophan to indole. The enzyme tryptophanase which converted the amino acids tryptophan to pyruvate and indole.

VP test: A natural formed from pyruvic acid to the glucose fermentation. VP reagents were added, and cherry red color ring was observed in the tube.

Methyl red test: This test was performed to see the ability of the microorganism presented in the medium to maintain the acid and product glucose fermentation.

Starch hydrolysis test: After incubation of 48 hours when iodine solution was added to the plate it was showing the zone of clearness.

S.NO.	Antibiotics	Results
1	Penicillin	Resistance
2	Methicillin	Resistance
3	Tetracycline	Sensitive (22mm)
4	Carbenicillin	Resistance

Table 2: Antibiotics tests.

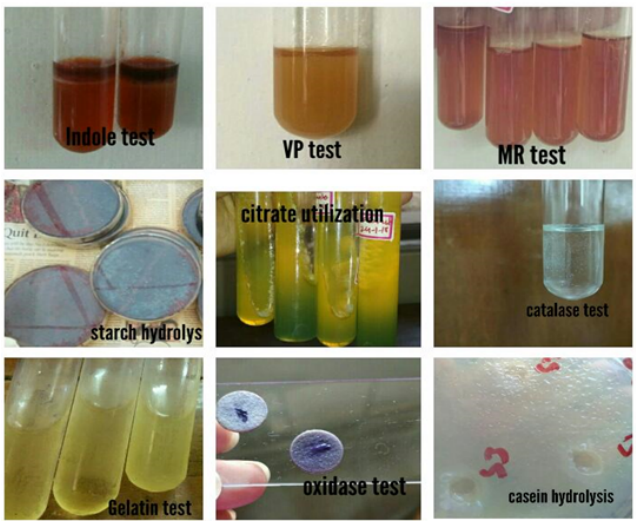

Figure 2: Bacteria identifying tests.

Catalase test: During aerobic respiration the microorganism break down the hydrogen peroxide into oxygen and water resultant bubbles are appeared.

Gelatin test: To see the ability to produce gelatinase that liquify gelatin. Hydrolysis of the gelatin indicated the presence of gelatinase.

Casein hydrolysis test: The organism produced the proteolytic enzyme due to that the zone of clearness was appeared. (Table 1 and Figure 2).

Antibiotic Susceptibility: The antibiotic suitability showed the resistance to the penicillin, methicillin, carbenicillin, Bacteria was sensitive to the tetracycline (Table 2).

Isolation of plasmid DNA: The orange color bands shows the presence of plasmid under the UV trans illuminator in the sample which had Bacterial culture.

Protein Extraction and estimation By Lowry's Method: The blue color showed presence of the protein in the sample have Bacillus thuringiensis.

SDS-page: The blue bands indicated the protein ladder and the fed blue color indicated the presence of the protein in the sample.

PCR and Cry2 gene isolation: The PCR products were checked for amplification with 2% agarose gel. *Bacillus thuringiensis* sample for the Cry2 gene isolated which have the size 676bp was observed [6-9].

Conclusion

Insecticidal activity of *Bacillus thuringiensis* on the cotton pathogen

The insect was killed by the *Bacillus thuringiensis* isolated Cry2 gene protein, when insect was in water it lived for more than 2 hours however with the Cry2 gene protein within 5 seconds. *Bacillus thuringiensis* is a gram-positive bacterium. The morphology of the *Bacillus thuringiensis* was confirmed by the microscopic test and the genius was found by the biochemical test. *Bacillus thuringiensis* have different types of crystal protein which kills many insects. Each protein has different insecticidal activity. Cry2 gene was confirmed using the specific primer which code for the Cry2gene. When the PCR amplification was done the size of the protein of Cry2 gene was same as the primer by this it was confirmed that the protein that present in the organism is Cry2 gene. The protein was confirmed by the Lowry's method and SDS-PAGE. The blue color appeared in the tube which showed that protein is present in the sample. The insecticidal activity was performed against the cotton pathogen *Helicoverpa armigera*. When the protein was dissolved in phosphate buffer and poured on the insect it died in 5seconds but the insect in the water was living more than 2 hours. The isolated Cry2 gene was killing the *Helicoverpa armigera* (Figure 3) [10].

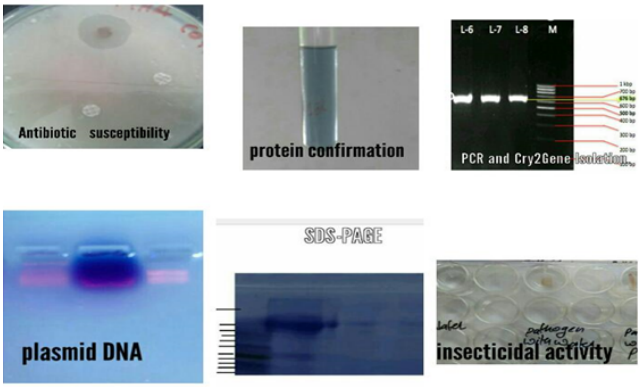

Figure 3: Antibiotic and insecticidal activity of *Bacillus thuringiensis*.

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