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CHARACTERIZATION OF *BACILLUS THURINGIENSIS* FROM COTTON FIELDS AND ITS EFFECTIVENESS AGAINST *SPODOPTERA LITURA*

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ABSTRACT

Cotton is a cash crop of many countries as it serves as a source of fiber, edible oil, and seedcake. It is grown in a diverse range of environment. *Bacillus thuringiensis* (Bt) is a gram positive bacterium found in different habitats but mostly found in soil. This bacterium produces endotoxin which is harmful for various insects. Numerous crystal-forming strains of Bt exist in nature, but not all of them are efficient against insect pests. Bt cotton is prevalent internationally because of less insect attack. The key objective of this research was to analyze different *Bacillus thuringiensis* isolates residing in soil of different cotton fields of Multan and Bahawalpur districts and to identify them on the basis of morphology. To attain this, different soil samples were collected from Multan (Gup wala, Mosey Wala, Taloki Wala, Hafiz Wala, Choudary Wala, Basti Mangla Mari, Sharkha Wala, Lawain Wala, Ghulamo Wala) and Bahawalpur district (Basti Deewan Wali, Check No 8, Basti Tariqabad, Basti Khaji Wala, Basti Khandin, Basti Rammo Wali, Sultanpur, Khurampur, Jahanpur). About 178 colonies of different bacteria from eighteen soil samples appeared on Nutrient Agar plates. The screening of colonies based on morphology yielded 18 colonies of Bt. We performed endo-toxin production experiment and confirmed the presence of parasporal crystals in identified *B. thuringiensis* colonies. Colony forming units of soil samples collected from Multan and Bahawalpur represented statistically significant results. Based on morphology, eight colonies of *B. thuringiensis* were confirmed out of eighteen colonies. Results of the current study revealed that out of 18 isolates, 8 bacterial isolates that were grown on the media had an increased elevation, a round, white, slimy morphology, and smooth edges. Toxicity test for resistance to cotton leaf worm second instar larvae revealed that two strains (BtS2, BtS7) of *B. thuringiensis* were toxic and showed 39.25% mortality. This study will help to identify the local Bt isolates that can be used for the indigenous insect pest control.

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INTRODUCTION

Bacillus thuringiensis (Bt) is a naturally existing bacterium which has been demonstrated to be one of the most effective biocontrol agents. This bacterium

possesses Bt genes that is able to synthesize the insecticidal proteins both during the vegetative phase and throughout the sporulation stage. Many crystal-forming Bt strains exist in nature, but not all of them are

efficient against insect pests (Roh et al., 2007). The identification as well as classification of native Bt soil strains to find novel toxins that are efficient against agricultural pests has been the subject of numerous investigations.

The three domains of the Cry proteins have sequence similarity in their tertiary structures of the active toxin, despite of the differences within the cry genes. Lepidoptera, Coleoptera, and Diptera pests can be safely controlled using the proteins synthesized from these cry genes. Owing to proteolytic degradation during extraction, uneven spraying within the crop canopy, and destruction by ultraviolet sun rays, the effectiveness of these bio-chemicals is limited. As a result, the idea of genetically modifying crop plants to introduce codon-optimized variants of the Bt gene to create plants with built-in resistance to specific pests was born. As a result, many Bt transgenic crops that express the cry gene have been created and made available for sale, notably Bt cotton, Bt corn, and Bt soybean (Kumar et al., 1996). These Bt crops are well-liked all over the world, which led to fewer insecticide applications in the field and improved financial gains for the farming community. A great way to boost crop as well as vegetable plant productivity while achieving sustainable development is to make them more resistant to harmful organism infections.

Toxins with high degrees of toxicity are sought after, as are new toxins to prevent pests from developing resistance as a result of the usage of current Bt toxins. These goals are the main reasons for the interest in discovering novel Cry proteins. As parasporal bodies, the crystals are put together by the cry genes. Additionally, it is fascinating to note that a single Bt strain may include many crystal protein genes (Noguera and Ibarra, 2010). Toxins produced by a number of microorganisms can be employed to reduce the toxicity of a variety of plant infections. Crystal inclusions are produced during sporulation by the soil bacterium Bt. The inclusions are hazardous proteins that have been demonstrated to have harmful effects against several insect, nematode, and protozoa groups and are encoded by Cry genes (Abo-Bakr et al., 2020). The most effective method for constructing crop resistance to chewing or lepidopteran insects has been achieved by introducing insecticidal crystal proteins (ICPs) obtained from the soil bacterium, *B. thuringiensis* into crop varieties (Azam et al., 2021). Transgenic crops that produce Bt crystal toxins for insect pest control have

transformed global agriculture since their commercial release in 1996 (Choudhary et al., 2021).

Numerous pest species have developed resistance to these toxins as a result of insufficient control of insect pests in the field and ongoing planting, which may compromise the overall efficiency of this technique. To increase the insecticidal spectrum of pest management programs and prevent the development of resistance, innovative insecticidal proteins with increased toxicity are required (Beegle and Yamamoto, 1992; Güneş et al., 2016). Investigating new Bt species will offer an alternative strategy for dealing with the populations of pests that are resilient to the already used Bt bio pesticides (Hassan et al., 2021). Based on the information that is presently accessible, research regarding the usage of Bt to combat insects has revealed that Pakistan does not have a commercially accessible bio-pesticide based on native Bt. On the other hand, bio-pesticides are successfully used at higher levels in a variety of nations including the USA, Australia, India, as well as China (Pinnock et al., 1991). In the current study, we aimed to survey, isolate, and identify *B. thuringiensis* isolates based on morphology from different cotton fields located in Multan and Bahawalpur districts. The efficacy of isolated Bt isolates against cotton leaf worm was also tested.

MATERIALS AND METHODS

Collection and preparation of soil samples

Different areas of Multan district notably Gup Wala, Mosey Wala, Taloki Wala, Hafiz Wala, Choudary Wala, Basti Mangla Mari, Sharkha Wala, Lawain Wala, Ghulamo Wala and Bahawalpur district like Basti Deewan Wali, Chak No 8, Basti Tariqabad, Basti Khaji Wala, Basti Khandin, Basti Rammo Wali, Sultanpur, Khurampur, Jahanpur were surveyed and total eighteen soil samples were collected from the cotton fields. Approximately 200 g of soil samples were collected from surface and subsurface soil at the depth of 5-6 cm by removing upper layer of soil from different locations. The collected soil samples were brought to diagnostic lab of MNS University of Agriculture, Multan and placed at room temperature under the cover and was subjected to isolation of *B. thuringiensis* (Bt) (Bibi, 2010).

Isolation of *B. thuringiensis* from soil

Nutrient Agar was used as a medium for the isolation of bacterial isolates. One liter NA media was prepared for the isolation of bacteria from the soil. This media

constituted distilled water (1000 mL), Beef extract (3 g), Peptone (5 g), Agar (15 g), and NaCl (5 g) (Khodair et al., 2008). The isolation of *B. thuringiensis* from the soil was done by the procedure illustrated by Obeidat et al. (2004). Each sample was treated for 30 min at 80°C with one gram of soil suspended in 9 mL of sterile water. By adding

1 mL of every suspension to 10 mL of NA broth buffered with 0.25M CH₃COONa, Suspensions were incubated for 24 h at 30°C and then heated at 80°C for 15 min. The suspensions were saturated, placed on NA media and also incubated for 24 h at 30°C. The colonies attained were examined under the microscope (Figure 1).



Figure 1: Pure culture of *Bacillus thuringiensis*.

Screening of *B. thuringiensis*

For few days, soil samples were kept at room temperature in order to balance the moisture contents. In a test tube with NA broth (20 mL), 1 g soil samples were suspended. After the heat treatment at 55°C for 10 min, the samples were inspected for the presence of non-spore formers in the soil. The samples were diluted 10⁻⁴ times before being placed on the medium. For at least three days, the plates were incubated at 30°C. Microscope was used to identify the colonies morphology comparable to *B. thuringiensis* (Chak et al., 1994). Plates with the bacterial growth were then incubated for 48 h at a temperature of 30°C to ensure proper growth. These plates were then placed at 4°C for future use (Bibi, 2010).

Seed culture preparation

For the preparation of seed culture, 50 mL of nutrient broth and 0.03 percent (w/v) yeast extracts in 250-mL Erlenmeyer flasks were inoculated with a loopful of the tested culture. The flasks were shaken at 150 rpm with an orbital shaker and incubated at 30 ± 1°C for 24 h.

Shake flask experiments

In selected isolates, NA broth media was used to produce both endotoxins and high level of parasporal bodies. About 1% active second stage seed culture was introduced into 250 mL flasks containing 100 mL of

sterile media and the propagation was carried out. Using a shaking incubator at 150 rpm, the flasks were incubated for three days at 30±1°C. Centrifugation at 10,000 rpm for 15 min was done to separate the *B. thuringiensis* biomass after 72 h of growth, and the sediment was cleaned twice with distilled water before being dried at 70°C for constant mass. The culture was heated at 80°C for 15 min, serially diluted, and then poured on nutrient agar. *B. thuringiensis* colonies were enumerated and expressed as CFU/mL after plates were incubated at 30°C for an overnight period. The CFU was calculated by following formula that was given below;

$$\text{Colony forming Units} \left(\frac{\text{cfu}}{\text{mL}} \right) = \frac{\text{No. of colonies} \times \text{dilution factor}}{\text{Volume of culture plate}}$$

Toxicity test

The toxicity of *B. thuringiensis* was tested using pellets, culture supernatant, and the whole culture on 2nd instar cotton leaf worm larvae (Khodair et al., 2008). The entomo-toxicity of the samples was determined by comparing the mortality rates of cotton leaf worm (*Spodoptera litura*). A total of 15 min at 5000 rpm centrifugation was performed on the samples (10 mL). To separate the spores and protein crystals, the pellets were centrifuged for five minutes at 5000 rpm in sterile distilled water before centrifugation. After that, the washing cycle was repeated three times. They were then resuspended in sterilized distilled water and kept

at a temperature of 4°C until needed (Carozzi et al., 1991). Toxicities of *B. thuringiensis* isolates against 2nd stage cotton leaf worm larvae were assessed using pellets, culture supernatant, and whole culture. In order to prepare the larvae's diet, one gram of cotton leaf sections was soaked in a 10 mL bacterial suspension for five minutes. After that, the food was dried and put into disposable cups with 10 larvae each. The toxicity of every strain was scrutinized three times. Disposable cups were incubated at 25°C for 24 h and 48 h (Abbott, 1925). A parallel control group used cotton leaf pieces soaked in sterile distilled water instead of bacterial suspension was used to compare mortality results. When a blunt needle was inserted into a larva and it did not move, it was supposed that it had died (Bourgouin et al., 1990).

Morphological characterization

The experimental parameters were colony shape, colony color, colony edge, colony surface, and colony evaluation. By utilizing the accompanying catalyst test, gram response test, and microscope observation, *B. thuringiensis* bacteria were classified morphologically. Descriptive data were employed to describe the findings of the biochemical tests.

Gram staining

Gram staining technique was performed for the identification of *B. thuringiensis*. Gram staining was carried out by placing 1 scratch of bacterial isolate on top of a microscope slide with the help of sterilized needle and made smear on slide with distilled water. A drop of crystal violet was placed on slide for 30 s followed by washing with sterilized water to remove unbounded crystal violet. Two drops of gram iodine were stained on slide for 30 s and washed with distilled water followed by 95% ethanol. The slide was then treated with two drops of safranin for 30 s before being washed with distilled water. Using blotter paper, the slide was dried and examined under the microscope (Jarial et al., 2011).

KOH Test

The KOH test was performed to classify between gram positive and gram negative bacteria. KOH test was done using a bacterial colony which was placed on glass preparations and was smeared with 3% KOH solution. If the bacterium was not sticky (slimy) then it was considered as gram positive (Afriani et al., 2018).

Catalase Test

Catalase test was performed by taking and placing the

end of a needle culture of Bt bacteria on a prepared glass that had been smeared with a hydrogen peroxide solution (H₂O₂). The primary goal of this test was to evaluate the catalytic activity of the bacteria under investigation (Afriani et al., 2018).

Starch hydrolysis test

The medium of starch agar was prepared, then autoclaved and poured into petri plates. The plates were put in incubator for two days at 37°C prior to incubation. Plates were gently saturated with a diluted iodine solution. The presence of clear zone along the streaks and the rest of the plates as a blue color showed the hydrolysis of starch (Faiz, 2018).

RESULTS

Confirmation of *B. thuringiensis*

The streak-plate method was used to subculture suspected colonies on solid NA media in sterilized Petri plates and incubated at 30°C for 72 h and analyzed under the microscope after 24, 48, and 72 h. *B. thuringiensis* was identified by the presence of spores and crystals in the mother cells, but also based on unswollen sporangia. On the basis of morphology, 8 bacterial colonies were confirmed as *B. thuringiensis* (Table 1). The remaining isolates had no effect on the larvae's toxicity. Morphologically, these isolates resembled Bt and these bacterial isolates were also subjected to endotoxin production. *B. thuringiensis* strains that were grown on the media had an increased elevation, a round, white, slimy morphology, and smooth edges as explained by Astuti et al. (2018).


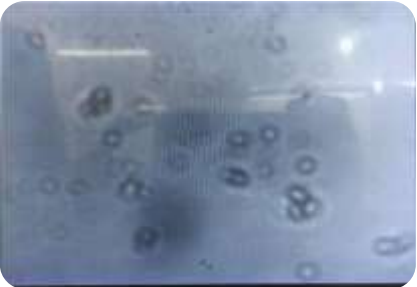

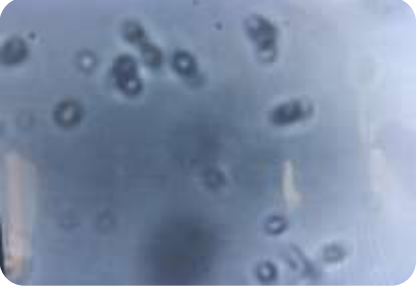

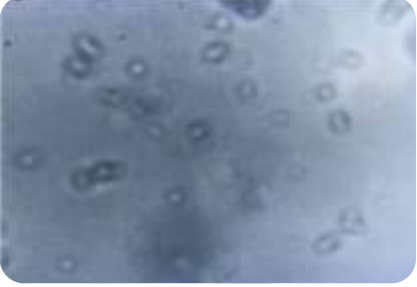

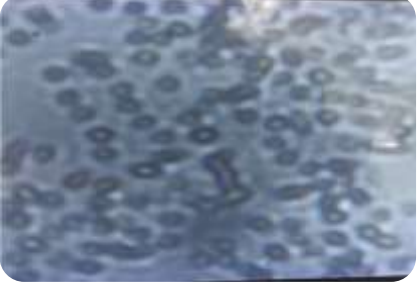


Endotoxin production and toxicity test of *B. thuringiensis*

As a result of the endotoxin production experiment, *B. thuringiensis* colonies with parasporal crystals were formed (Figure 2). These colonies were calculated by colony forming units. The subsequent identified strains were then subjected to toxicity test to check the resistance of cotton leaf worm second instar larvae. After toxicity test, two strains i.e. BtS2, and BtS7 of *B. thuringiensis* were toxic (39.25% mortality) against cotton leaf worm.

Results demonstrated that colony forming units obtained from soil samples of Bahawalpur and Multan were highly significant (Table 2). BtS1 has highest (5.4×10^{-5}) cfu/ mL followed by BtS6 (4.30×10^{-5}) and BtS5 (3.83×10^{-5}) whereas BtS4 showed 1.33×10^{-5} (least) cfu/mL from Bahawalpur samples. Moreover,

Multan samples also depicted varied results where BtS7 had highest cfu/mL i.e. 6.23×10^{-5} followed by BtS3 with 5.67×10^{-5} whereas BtS2 had least i.e. 2.17×10^{-5} cfu/mL.

Table 1: *Bacillus thuringiensis* isolated from soil samples of Multan and Bahawalpur.

Sr. No	Sampling area	Isolate of BT	Spore Shape
1	Mosey Wala, Shujabad, Multan		
2	Choudary Wala, Jalapur Pirwala Multan		
3	Sharkha Wala, Multan		
4	Ghulamo Wala, Multan		
5	Chak No. 8, Bahawalpur		

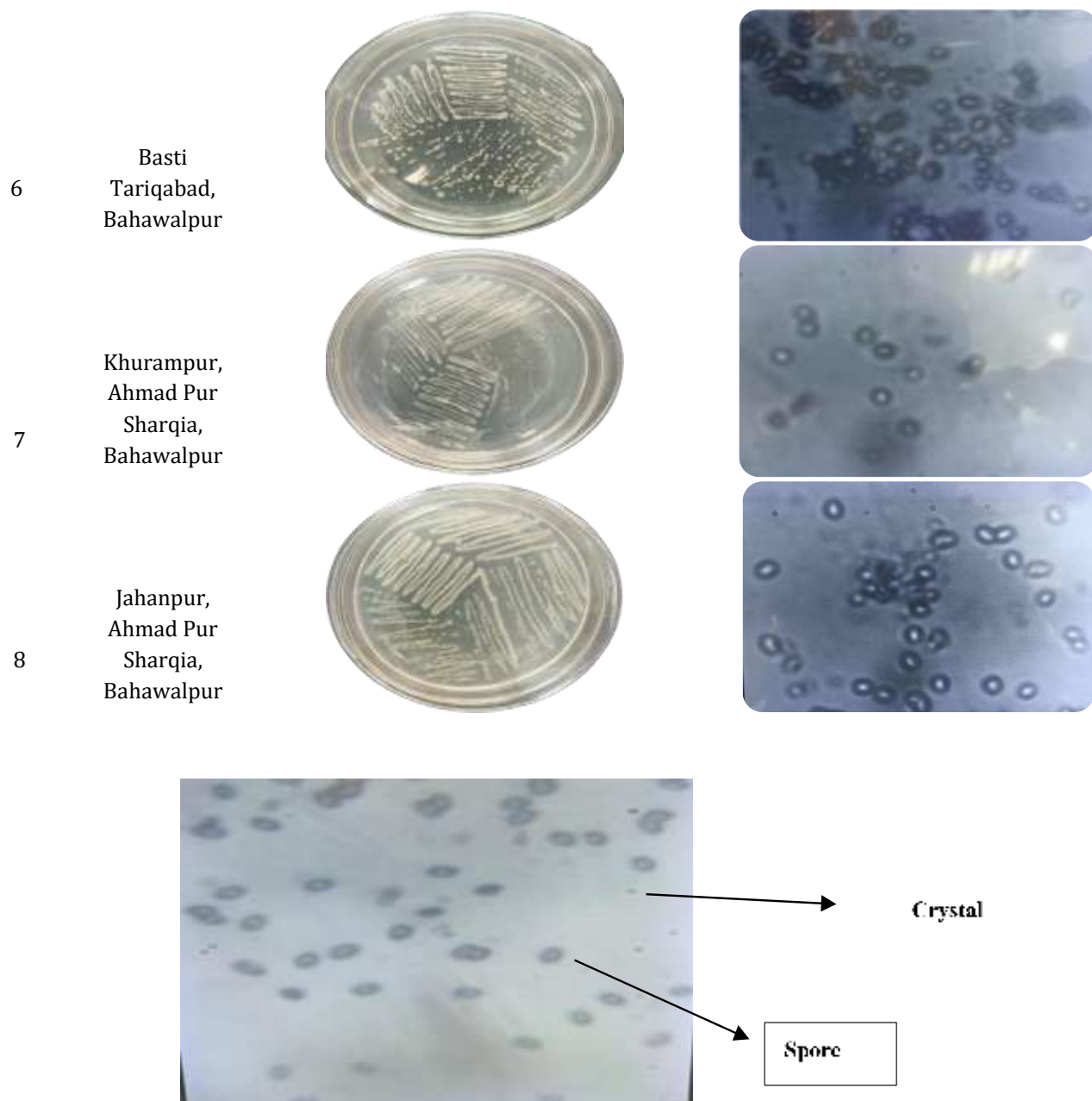


Figure 2: Indigenous *B. thuringiensis* isolate after 48-hours on a 100X magnification.

Table 2: Comparison of cfu/ml of various Bt isolates obtained from Bahawalpur and Multan.

Isolates	Bahawalpur	Multan
BtS1	5.40×10^{-5} a	2.87×10^{-5} a
BtS2	3.00×10^{-5} cd	2.17×10^{-5} f
BtS3	3.03×10^{-5} c	5.67×10^{-5} ab
BtS4	1.33×10^{-5} f	4.07×10^{-5} cd
BtS5	3.83×10^{-5} b	2.80×10^{-5} ef
BtS6	4.30×10^{-5} b	4.67×10^{-5} bc
BtS7	1.63×10^{-5} ef	6.23×10^{-5} a
BtS8	2.23×10^{-5} de	2.30×10^{-5} ef
BtS9	2.17×10^{-5} e	3.30×10^{-5} de

Biochemical tests

Gram staining confirmed gram-positive purple rod-shaped bacteria. The catalase analysis confirmed that the isolated samples were gram positive. After 48 h of incubation, the zone of clearness was apparent when the iodine solution was poured to the plate confirming that the sample is gram positive. This clearly demonstrated that the starch hydrolysis test was effective. When KOH solution was added to the slide on bacteria after 30 sec, the bacteria was not sticky (slimy) indicating that it was gram positive (Figure 3).

DISCUSSION

Bt toxins are very specific, accounting for more than half of the market (Lacey et al., 2015). One of the most effective pest management techniques in the world involves screening soil for new and potent Bt strains (Apaydin et al., 2005; Ammouneh et al., 2011; Hamedo, 2016). In the present study, About 18 soil samples were

obtained and screened. Out of 18 samples, eight bacterial colonies tested were positive for *B. thuringiensis*. To terminate the vegetative cells of other spore precursors and exterminate non-spore producing bacteria, a simple heat treatment of soil suspension at 80°C for 10 min was used as the isolation technique. Similar findings were evident by Morris et al. (1998). In another study, various researchers collected 56 soil samples from several Egyptian governorates, and *B. thuringiensis* was identified. Out of 56 soil samples, only 16 samples showed the positive results (Abo-Bakr et al., 2020). Previously, a total of 2671 colonies from 93 Egyptian soil samples were investigated, where 40 samples were identified as positive (Salama et al., 2012). In another study, 59 soil samples were obtained from 13 different locations in Egypt by various researchers (Ahmed et al., 2015). These studies are in line with our results as out of 18 bacterial colonies, 8 were identified as *B. thuringiensis*.

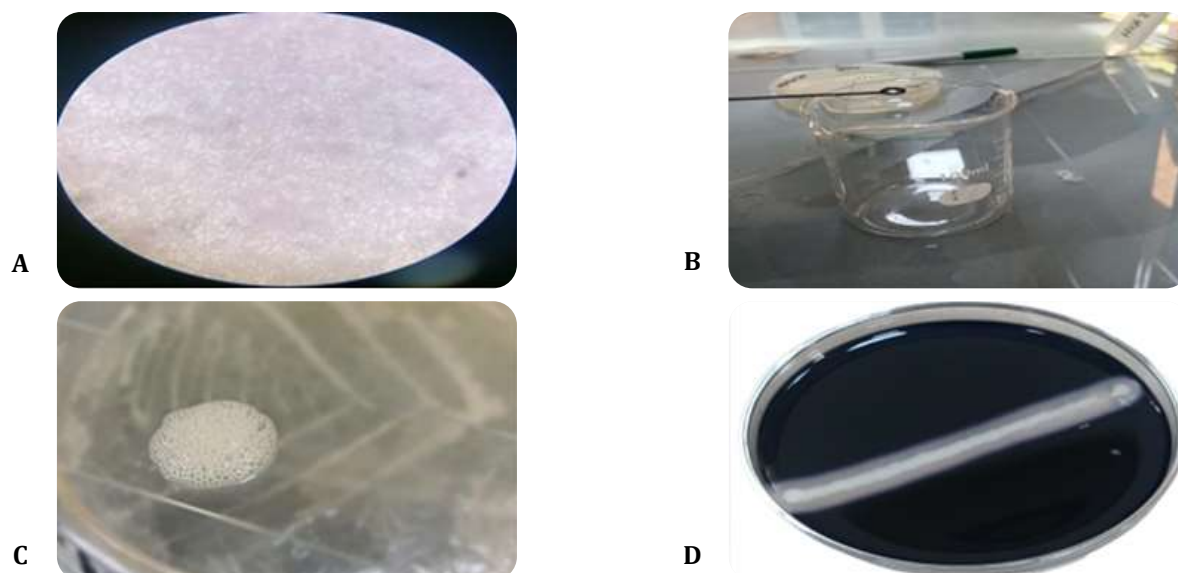


Figure 3: (A)Gram-staining (B)KOH test (C)Catalyze test (D)Starch hydrolysis test

Table 3: Overall results of biochemical test.

Sr. No	Test	Result
1	KOH	Positive
2	Catalase	Positive
3	Gram Staining	Positive
4	Starch Hydrolysis	Positive

Previous studies have shown that when *B. thuringiensis* is exposed to ultraviolet light, it becomes inactive, but

spores and crystals deactivate at different rates (Jarrett, 1980). Present study confirmed these findings as the soil

samples collected at a depth of 5-6 cm yielded positive results. This could be due to the fact that the samples recovered from the depths contained less ultraviolet light. Positive results were obtained from the soil samples collected at a depth of 5-6 cm. This could be because the samples retrieved from the depths had less effect of ultra-violet light.

In present study, smooth or lobate semi-transparent edges, creamy, white or off white, flat or thick, oval, round, or irregular in shape, and creamy, white or off white were seen in the majority of isolated colony morphology of *B. thuringiensis* strains. These results were in line with the results of various researchers (Iriarte et al., 2000; Chatterjee et al., 2007; Salaki, 2010). In the present study, crystal-forming colonies were observed using a microscope. A similar study was also reported earlier (Ohba and Aizawa, 1986). The appearance of the isolated bacteria, as well as the results of the biochemical assays confirmed that the 8 identified bacterial strains were Bt. Similar findings were obtained in a previous study (Cinar et al., 2008). The most significant toxins produced by *B. thuringiensis* are characterized as parasporal crystal proteins (ICP or delta-endotoxins), and they play an important role in the bacteria's distinctive insecticidal activity (Entwistle et al., 1993). During current studies, a specific crystal was identified in every isolated *B. thuringiensis*. The findings were consistent with those reported by Ohba and Aizawa (1986). During current study, the detection of parasporal crystal proteins (PCP) in the purified spore suspensions of the isolated bacteria confirmed that they are Bt. This finding was consistent with previous research on Bt protein crystals (Yilmaz et al., 2017). In another study, 1000 samples from multiple locations in Pakistan were collected in order to identify new *B. thuringiensis* strains resilient to Lepidoptera. There were 400 crystal formers identified as *B. thuringiensis* in all of the samples (Shaikh, 1978). This study was in line with the current studies.

In the present research, two strains of *B. thuringiensis* (BtS2, BtS7) were confirmed lethal (39.25% mortality) against cotton leaf worm after toxicological analysis. These findings corroborated with the findings of other researchers who reported that two strains were toxic to cotton bollworm second instar larvae (Obeidat et al., 2004). Present studies showed the severe pathogenicity levels of Bt parasporal crystal proteins against larvae of *Spodoptera litura*. Similar findings were also reported by

other scientists (Ammounh et al., 2011; Palma, 2015). During present study, biochemical assays such as gram staining, catalase test, KOH test, as well as starch hydrolysis test were carried out to ensure that the isolated bacteria was *B. thuringiensis*. Some of these tests were also conducted by other researchers for the identification of *B. thuringiensis* (Chatterjee et al., 2007). *B. thuringiensis*, which accounts for up to 90% of bio-insecticide preparations and over 2% of the market for insecticides overall, is the most effective agent used to control pests. Compositions based on *B. thuringiensis* are currently becoming more prevalent, and this market is anticipated to keep expanding. Biological control is required to prevent all of the detrimental consequences of chemical pesticides. There are nations like Pakistan which are endeavoring to manufacture *B. thuringiensis*-based products on their own (Karim et al., 2000). It is also a step towards the development of our own *B. thuringiensis*-based bio pesticides. This study will be helpful to formulate bio-insecticides with local Bt isolates that could be used for the indigenous insect pest control.

AUTHORS' CONTRIBUTIONS

MAM, MMA, and MA, conceived the idea, designed experiments, collected soil samples, contributed reagents/materials and supervised the research, MAK collected and processed samples and performed experiments, MI, NA, and AH, performed toxicity test and analyzed the data, MAK, MAM, MMA, MA, and AR wrote the manuscript. All the authors read and approved the final draft.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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