African Journal of Microbiology Research Vol. 6(18), pp. 3980-3983, 16 May, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.898
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Biotoxicity assay of *Bacillus thuringiensis* (spores) against *Tribolium castaneum*

Kausar Malik*, Farhana Munir, Khansa Fakhar-un-Nisa, Shagufta Naz, Saima Sharif, Farzana Rashid and Khadija Awan

Lahore College for Women University Lahore, Pakistan.

Accepted 19 December, 2011

Bacillus thuringiensis **spore forming, gram positive aerobic bacteria whose spores consist of the crystal proteins, are found to have the insecticidal activity to the specific insect orders.** ***B. thuringiensis*** **are considered to be cosmopolitan in nature and a great difference occurs between the two strains from different habitat. The present study was conducted on the collection of samples and isolation of the** ***B. thuringiensis*** **from different samples. These isolates were identified on the basis of microscopic and staining techniques. Culturing of these isolates was made on the specific media such as T3. Culturing of these bacteria was done to get enough amount of the** ***B. thuringiensis*** **spore cell. Rearing of the** ***Tribolium castaneum*** **a flour beetle was done by understanding the physiology of the beetles. Then biotoxic effect of these bacteria was determined by the process of Biotoxicity assay of the** ***B. thuringiensis*** **spores against** ***T. castaneum*** **in glass vials in triplicates. The seven day repeated observation of these isolates was presented in the tabular form and LC_{50} was calculated by the Probit analysis programme. LC_{50} vary from isolate to isolate for the insect under consideration, giving different mortality rates.**

Key words: *Bacillus thuringiensis, Tribolium casteneum,* Biotoxicity, assays, spore**.**

INTRODUCTION

Bacillus thuringiensis was first discovered in 1901 by the Japanese biologist whose name was Shigetane Ishiwatari (Aizawa, 2001). In 1911, Ernst Birliner discovered the *B. thuringiensis* who isolated it from a patient of schlaffsucht (Barfoot, 2006).

B. thuringiensis has the ability to form δ-endotoxins or commonly called Cry proteins which are considered as effective insecticides against different types of insects and mosquito larvae. This bacterium is of great importance, because it can biodegrade or detoxify the harmful substances therefore, it is of great value to know the cooperative behavior of *B. thuringiensis* strains under different environmental conditions and the presence of *B. thuringiensis* in different localities (Silveria et al., 2005; Cappello et al., 2006; Ohba et al., 2009).

B. thuringiensis strains are ubiquitous in nature although, they are widely distributed in soil, they are also present in the atmosphere (Merrill et al., 2006; Lues et al., 2007), aquatic environment (Ichimatsu et al., 2000), foodstuff of livestock (Anadon et al., 2006) and farming crop.

Important pests of the agriculture are considered to be the Coleopterans. Members of several families such as *Chrysomelidae, Cruculionidae, Tenebrionadae,* and *Scarebeidae* have already been found effective to the toxin crystals of *Bacillus thuringiensis*. Mutagenesis in the DNA sequences of the plasmid of the *B. thuringiensis* have resulted in the improvement of pesticidal activity or in the alternation of pest specificity when it is given to the plant for insect control (Abad et al., 2003). Crystal proteins of the *B. thuringiensis* have been found very toxic to a red flour beetle (*Tribolium*) (Malik and Riazzuddin, 1998). *Tribolium castaneum* a Coleopteran is greatly influenced by the environmental cues, if the conditions of the environment are adverse and other elements such as parasites or predators are present, they will stimulate the maturity earlier (Day and Rowe, 2002).

*Corresponding author. E-mail: kausarbasit7576@yahoo.com.

A parasite can either benefit from the changes in the pattern of life style of this beetle (Hurd et al., 2001) and they can also be injured or harmed as well (Agnew et al., 2000; Gandon et al., 2002).

Bioassay and the immunoassays are the most common two types of the assays. Lethal doses (LD_{50}) or lethal concentrations (LC_{50}, LC_{90}) are the general terminologies which are used to determine the insecticidal activity of a particular strain now a day.

By the procedure of bioassay, toxicity of particular isolate of *B. thuringiensis* against the *T. castaneum* can be determined by observing the mortality rate and also by LC_{50}.

MATERIALS AND METHODS

Sample collection of *B. thuringiensis*

Samples of organically rich soil, animal dung, bird droppings and grain dust were collected from different areas of the Pakistan. About 200 samples were collected in complete sterile glass jars which were properly labeled. Soil samples were taken after digging the ground and from one inch underneath the ground level.

Isolation and biochemical characterization of the *B. thuringiensis*

Samples were collected from different habitats of the Punjab were further processed for the isolation of the *B. thuringiensis* according to the technology proposed by Martin and Travers (1989). 0.5 g of each sample was suspended in 10 ml of LB media containing 0.2 M sodium acetate. The composition of the LB medium is Tryptone 10 g/L, yeast extract 5 g/L and NaCl 5 g/L. Then, the samples with LB medium and sodium acetate were shaken well and incubated at 30℃ for about 4 h. Filter paper having the pore size of 0.25 nm was used in order to filter the incubated samples and the filtrate was heated at 80℃ for 15 min in order to isolate the sp ore forms.

1:2 dilutions were made of the above treated samples. After it was spread on the LB agar plates and the samples were kept in the incubator for overnight at 30℃. Those colonies were picked which have *B. thuringiensis* like morphological characteristics such as entire margin, off white color, dry and rich growth of colony and LB agar plates were streaked with these colonies and these plates were incubated for 24 h at a temperature of 30℃.

For microscopic examination of the *B. thuringiensis* strains, these were grown on the media plates at 30℃ for 18 h for v egetative culture and for 3 days in order to get the spore culture. In order to determine the position of endospore and extra cellular protein particles culture were examined under the light microscope. Different staining techniques such as Gram staining and endospore staining with Malachite green and acid fuschin were applied on the bacteria. Biochemical characterization of the Gram-positive rods and spores formers was done.

Preparation of the *B. thuringiensis* spores

Spores of bacterial isolates were prepared according to the method prescribed by Makino et al. (1994), for which single isolated *B. thuringiensis* colony was inoculated in LB broth and this was kept in incubator at 37℃. The inoculum from the above cultu re which was grown for 24 h and streaked on the sporulation medium T3. The chemical composition of the T3 is Tryptone 3 g/L, Yeast extract 1.5 g/L, Tryptose 2 g/L, $MnCl_2$ 0.005 g/L, NaH_2PO_4 6.9 g/L, $NaHPO_4$ 8.9 g/L and agar 15 g/L. After streaking the plates, these were incubated at 30℃ for about 72 h. After incubation p eriod of 72 h growth was seen on the plates and the growth was collected from the plates.

Rearing of the *Tribolium castaneum*

Tribolium can be reared in the medium containing semolina and 10% yeast extract. 50 adult of *Tribolium* were placed in each jar containing ¼ of flour of jar (the jar was closed with muslin cloth). Jars should be placed in insectory set at 30±1¢ and provide it with continuous humidity. Then the larvae on exposing to *B. thuringiensis* show the affect that can be noticed. From this stock of insect of different life stage were used for the purpose of bioassay.

Susceptibility of *T. castaneum* against *B. thuringiensis*

All 200 samples were checked by bioassay technique against *T. castaneum.* Different strains of *B. thuringiensis* had toxic effect at different concentrations on the stored grain pests. These four isolates of *B. thuringiensis* named accordingly as FM4, FM5, FM6 and FM7, were found toxic for the *Tribolium castaneum.* Virulence of each strain varies from each other according to the composition of the crystal proteins or parasporal bodies in the spores of *B. thuringiensis.*

Initial step of the bioassay was to check the mortality with equal amount of artificial diet and equal amount of *B. thuringiensis* culture. In order to check out the mortality ratio, a control group diet was prepared by mixing 0.1 g (10%) of the yeast extract and 0.9 g (90%) of semolina and ten 2nd instar larvae were released to feed on this artificial diet in glass vials (1inch wide and 2 inch in length).

First bioassay was made taking 1 g of diet (0.1 g yeast extract and 0.9 g of semolina) and 1.25 g of the culture of every strain of *B. thuringiensis* from FM4 to FM7 in glass vials. The diet and culture of strain were thoroughly mixed; air dried and converted it to powdered form. Then put ten 2nd instar larva in each glass vial including control. Three set of control group and three 3 sets or triplicates of each isolate were designed. The mortality was observed for about 7 days continuously. This whole procedure was repeated 5 times in order to get best average values. Same procedure was repeated for the different concentrations of cultures from 1 to 0.25 g in descending orders in 1 g of diet.

RESULTS

Biotoxicity assay

All the isolates were checked for their insecticidal activity. Only four of these were found to be toxic according to the bioassay toxicity analysis for the coleopteran, showing that it may contain the Cry3 A protein due to the fact that it is toxic to the flour beetle. Insecticidal activity of these isolates showed that it may contain Cry3 A protein and this protein is found to be toxic for coleopteran. Other isolates can be toxic for other group of insects containing their respective crystal toxic proteins. Isolates which give the mortality assigned the names as FM4, FM5, FM6 and FM7 as they vary in their toxicity due to the change in composition and amount of the crystal proteins in their structure.

Table 1. LC_{50} of different isolates on the 3rd day of observations.

S/n	Isolates	Source	LC_{50} g/g	LC_{50} 95% confidence limits	
				Lowest	Highest
1	FM4	Soil	1.403	1.146	2.177
2	FM5	Cow dung	0	0	0
3	FM6	Wheat dust	0	0	0
4	FM7	Soil	0	0	0

Table 2. LC_{50} of different isolates on the 4th day of observations.

S/n	Isolates	Source	LC_{50} g/g	LC_{50} 95% confidence limits	
				Lowest	Highest
1	FM4	Soil	0.989	0.820	1.287
2	FM5	Cow dung	1.481	1.154	2.924
3	FM6	Wheat dust	1.274	1.067	1.795
4	FM7	Soil	0	0	0

Table 3. LC_{50} of different isolates on the 5th day of observations.

S/n	Isolates	Source	LC_{50} g/g	LC_{50} 95% confidence limits	
				Lowest	Highest
1	FM4	Soil	0.903	0.705	1.229
2	FM5	Cow dung	1.022	0.858	1.319
3	FM6	Wheat dust	1.075	0.9081	1.394
4	FM7	Soil	1.413	1.180	2.044

Table 4. LC_{50} of different isolates on the 6th day of observations.

S/n	Isolates	Source	LC_{50} g/g	LC_{50} 95% confidence limits	
				Lowest	Highest
1	FM4	Soil	0.579	0.430	0.694
2	FM5	Cow dung	0.739	0.619	0.858
3	FM6	Wheat dust	0.749	0.601	0.897
4	FM7	Soil	1.112	0.982	1.327

Table 5. LC_{50} of different isolates on the 7th day of observations.

S/n	Isolates	Source	LC_{50} g/g	LC_{50} 95% confidence limits	
				Lowest	Highest
1	FM4	Soil	0.522	0.372	0.632
2	FM5	Cow dung	0.674	0.548	0.787
3	FM6	Wheat dust	0.53	0.416	0.622
4	FM7	Soil	1.036	0.903	1.245

Results of the periodic determination of the LC50 are given in the tabular forms that are calculated by the Probit analysis programme (Tables 1 to 5).

DISCUSSION

In the present study, the main emphasize was on the use

of the pellet of the *B. thuringiensis* spores in order to check its insecticidal activity on the red flour beetle *T. castaneum*. Previous work by Malik and Riazuddin (1998), elaborates that the use of the crystal proteins isolated from the spores of *B. thuringiensis* show a considerable rate of mortality when applied against the *Tribolium castaneum*. Present study relates with the previous work. The previous work was conducted on the use of isolated insecticidal protein to check the mortality. In both cases, mortality of the insects was obtained.

The present work was done by using the pellets of the spores of *B. thuringiensis* instead of using the isolated crystal proteins, as the isolation of these proteins is very tedious and time consuming, and the mortality was obtained with the use of pellet only. This study provides a more convenient method of use of *B. thuringiensis* as a biopesticide. The spore forms in the pellet contain variety of crystal proteins. One of these proteins, is the Cry 3 A which is really very toxic for the Coleopterans, members of this order are stored grain pests and are economically of great threat to the stored grains. Other proteins in the *B. thuringiensis* formulation can also be toxic to the some other stored grain pests and can be helpful in getting rid of an infestation.

Pellet used in this study is not only effective for the Coleopteran; it can also prove toxic to the other insect orders such as Lepidoptera, Isopteran, Dipterans etc. and can be used in order to get rid of an infection or infestation caused by the members of crystal proteins these Orders as pellet consist of a lot of toxic proteins.

B. thuringiensis-based biopesticide production depends on high quality and high formulation processes. The formulation used in this study was safe, easy to use, have long shelf time and very effective product. The active ingredient in the formulation was the spore crystal complex, which is more effective to use and cheaper to obtain than the crystal alone, which are frequently used in experimental tests.

Conclusion

In this study, only the spore forms of *B. thuringiensis* when mixed in the diet of *Tribolium castaneum* showed its toxic affect instead of crystal proteins isolation which is relatively a complex process. The study provides a convenient method which is time saving and economical. This study recommends that, *B. thuringiensis* at spore stage provide good mortality percentage. Probit analysis programme was applied to calculate the LC_{50} values of *B. thuringiensis* against *Tribolium castaneum*.

RECOMMENDATIONS

1. Isolation of insecticidal crystal proteins (ICPs) from these studies can be used in molecular biological studies in future research work.

2. One more future perspective is the development of genetically modified (GM) crops based on these *B. thuringiensis* isolates to make insect resistance crops.

REFERENCES

Abad A, Duck NB, Feng X, Flannagan RD, Khan TW, Sims LE (2003). Sporulation activity of the *Bacillus thuringiensis*. Appl, Microbiol., 9(3): 544-567.

Agnew P, Haussy C, Michalakis Y (2000). Effects of density and larval competition on selected life history traits of *Culex pipiens* quinquefasciatus (Diptera: Culicidae). J. Med. Entomol., 37: 732–735.

Anadon A, Larranaga MR, Aranzazu MM (2006). Probiotics for animal nutrition in the European Union. Regulation and safety assessment. Regul. Toxicol. Pharmacol., 45: 91-95.

Barfoot P (2006). GM crops: the first ten years – global socio- economic and environmental impact. Final PG Economics., 11-23.

Cappello M, Bungiro RD, Harrison LM, Bischof LT, Griffitts JS, Barrows BD, Aroian RV (2006). A purified *Bacillus thuringiensis* crystal protein with therapeutic activity against the hookworm parasite *Ancylostoma ceylanicus.* Proc. Natl. Acad. Sci. USA., 103*:* 15154-15159.

Day T, Rowe L (2002). Developmental thresholds and the evolution of reaction norms for age and size at life-history transitions. Am. Nat., 159: 338–350.

Gandon S, Agnew P, Michalakis Y (2002). Coevolution between parasite virulence and host life-history traits. Am. Nat., 160: 374–388.

Hurd H, Warr E, Polwart A (2001). A parasite that increases host life span. Proc. R. Soc. London Ser., 268: 1749–1753.

Ichimatsu T, Mizuki E, Nishimura K, Akao T, Saitoh H, Higuchi K, Ohba M (2000). Occurrence of *Bacillus thuringiensis* in fresh waters of Japan. Curr. Microbiol., 40: 217-220.

Lues JF, Theron MM, Venter P, Rasephei MH (2007). Microbial composition in bio aerosols of a high-through put chicken-slaughtering facility. Poul. Sci., 86: 142-149.

Makino S, Ito N, Inque T, Miyata S, Moriyama R (1994). A spore-lytic enzyme released from *Bacillus cereus* spore during germination. Microbiology, 140: 1403-1410.

Malik K, Riazuddin S (1998). Insecticidal activity of some *Bacillus thuringiensis* strains against *Tribolium castaneum* (Coleopteran; Tenebrionidae). Punjab. Univ. J .Zool., 15: 31-34.

Martin PAW, Travers RS (1989). Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl. Environ. Microbiol., 55(10): 2437–2442.

Merrill L, Dunbar J, Richardson J, Kuske CR (2006). Composition of *Bacillus* species in aerosols from 11 U.S. cities*.* Forensic Sci*.,* 51: 559-565.

Ohba M, Mizuki E, Uemori A (2009). Parasporine, a New Anticancer Protein Group from *Bacillus thuringiensis*. Anticancer. Res*.,* 29: 427-433.

Silveria MM, Molina MAB (2005). Indirect estimation of *Bacillus thuringiensis* var *israelensis* biomass concentration using oxygen balance data. Braz. J. Chem. Eng*.,* 22: 495-500.