Pak. J. Biochem. Mol. Biol., 2022, 55(1):1-11

Research paper

Isolation of *Bacillus thuringiensis* for the Production of Insecticidal Endotoxin

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ABSTRACT

Pakistan’s economy greatly relies on the agriculture sector as it contributes 24 percent to the GDP and absorbs 50 percent labor force. Hence, this sector needs to take steps to achieve a high yield and good quality crops. As the world has adopted the advancement in the agriculture sector by producing GMO plants having desirable traits, usage of synthetic pesticides, insecticides, and other adopting measures to produce a high yield of crops. The present study aimed to isolate an indigenous thermophilic bacterium namely, *Bacillus thuringinesis* from soil samples and to screen and optimize its insecticidal activity to evaluate its effective application as a bioinsecticide. We collected 5 soil samples from different vicinities of Karachi and Mardan. Standard microbiological techniques were adopted, and bacterial isolates were selected based on morphological characteristics. A total of 53 colonies were isolated and microscopic examination showed 37 isolates as spore former. These 37 isolates were selected for bioassay against *Tribolium casteneum*. During bioassay, 4 isolates (SMA 56, SMB 77, SMB 80, and SMB 82) showed a more than 50% mortality rate.

KEYWORDS: *Bacillus thuringinesis*, Insecticidal endotoxin.

INTRODUCTION

Pakistan's agriculture is one of the important sectors as it plays a crucial role in the economy of the country and contributes 24 percent to GDP and absorbs 50 percent of the labor force [1], while worldwide it accounts for 4 percent of GDP. For most developing countries like Pakistan, this sector is an important source of foreign exchange earnings while at the same time it helps and promotes the growth of other sectors as well.

Agricultural industry faces severe damage from insects and pests. It is estimated that worldwide 20 to 40 percent of crop yield is lost due to pests and insects [2]. In 2016, a study was conducted on 1300 invasive pests and it was estimated that their potential damage to the global agriculture industry is over US$540 billion if precautionary steps not be taken [3]. Globally pest is controlled by several methods but the most conventional and widely used are synthetic pesticides. It is estimated that the overall expenditure of pest control agents is US$40 billion per total consumption of pesticides is 2.5 million tons per year in which 45% is used in Europe, 25% is used in the USA, 4% is used by India and rest of 26% is used globally year [4] .

Synthetic pesticides have an adverse effect on the ecosystem. Exposure to pesticides causes acute (short term) or chronic (long term) effects and allergic effects on animals and human health, especially in the reproductive, endocrine, and central nervous systems [5]. Most of the synthetic pesticides are carcinogenic. According to US EPA, there are over 160 different pesticides used worldwide which has a carcinogenic effect. While on the other side there are over more than 500 species of pests and insects which developed resistance against synthetic pest control agents. This increasing resistance also increases the use of synthetic pesticides by combining different pesticides or moving toward more toxic agents.

To overcome and address this huge drawback of synthetic pesticides make the scientific community think about more ecologically safer, price-efficient ways to reduce the adverse impacts of synthetic pesticides. To overcome this situation, organic farming approaches are being in practiced across the world. Since the early 20th-century scientist has discovered micro-organism which has insecticidal activity and identified it as *Bacillus thuringiensis*. Subsequently a lot of research work has been carried out to study, explore and manipulate the potential of *Bacillus thuringiensis* in order to develop various techniques for reducing the practice of synthetic pesticides to overcome adverse impacts of xenobiotics on environment [6].

Bacillus thuringiensis is a well-known spore-forming bacterium that produces crystals protein called cry proteins which effect the guts of insects and produces toxic and lethal effect to many species of insects. *Bacillus thuringiensis* strains can be found almost anywhere in the world because it is natural habitat in soil [7]. There are thousands of different *Bacillus thuringiensis* strains found worldwide producing over 200 different types of cry proteins that are active and produces toxic effect against an extensive range of insects and some other invertebrates [8] (Table 1). Surveys have indicated that *Bacillus thuringiensis* is distributed in the soil sparsely but frequently worldwide. *Bacillus thuringiensis* has been spread in the environment and can be found in all types of terrain, including beaches, deserts, and tundra habitats. For this unique property against insects the *Bacillus thuringiensis* is a matter of interest in the production of Bio-pesticide and producing such organisms which are resistant to insects.

The advancement in molecular biology creates a gateway to produce GMO plants in which the plant has been manipulated at the genomic level to produce desired nutrients and also help plants to produce resistance against plant viruses and help them to prevent insects due to genetic manipulation [9]. Bt crops are being produced which are resistant to insects and produce their own insecticidal protein in their own tissue which helps in reducing the use of synthetic pesticides. Over this extensive manipulation of plants at the genomic level make a lot of concern on one side this manipulation has advantages like acquiring such trait which is lack in the native state on the other side this manipulation produces allergic reaction, cancer, antibiotic resistance over the population and it may also possess the risk of outcrossing [9].

In this study, we aim to produce Biopesticide by using recombinant technology and manipulation of the cry gene of *Bacillus thuringiensis* at the genomic level. As the genetically modified plant has a lot of Cons over the pros so this study may help us to overcome these disadvantages.

To achieve our aim in this study we have processed 5 different samples including soil and partially digested leaf sample. Adopting standard microbiological techniques, we have isolated 53 different isolated Bacillus like colonies which were screened by using gram staining and all isolates were gram positive after that endospore staining was done to screen spore former and 37 isolates were found spore former. Which were further screened by conducting Bioassay against *Tribolium casteneum.* After bioassay four SMA 56, SMB 77, SMB 80 and SMB 82 isolates shows mortality rate more than 50%.

Table 1: Bt gene and its crystal proteins

Gene	Crystal Shape	Protein Size (Kda)	Activity
Cry I [Sub group A(a) A(b), A(c),B,C,D,E,F,G]	Bipyramidal	130-138	lepidoptera larvae
Cry II [subgroups A, B, C]	Cuboidal	69-71	lepidoptera and diptera
Cry III [subgroups A, B, C]	flat/irregular	73-74	Coleopetra
Cry IV [subgroups A, B, C, D]	Bipyramidal	73-134	Diptera
Cry V-IX	Not Defined/ Irregular	35-129	Various

MATERIAL AND METHODS

Collection of various ecological samples

Different ecological samples were collected for isolation of desired specie. Sample include soil, water and leaf. Soil and water samples were collected from shaded places while leaf sample must be partially digested and contaminated with soil. Soil sample collected from the depth of approximately 5cm to 7cm and tag with important information. The sample size was 5-10gm and collected in plastic container and stored at 4 °C.

Isolation of *Bacillus thuringiensis*

World health organization (WHO) recommended procedure for isolation of *Bacillus thuringiensis* were adopted for isolation, and isolates are identified by its morphological characteristic and further classified by microscopic examination. 1g of soil mixed and homogenized in 10ml of Normal saline (0.9% of NaCl) solution. the sample is serially diluted in sterile saline solution. 1 ml of an aliquot is collected after serial dilution, subjected to heat shock at 80°C for 12 minutes [10], and then chilled in ice for 5 minutes in a microcentrifuge tube. This step killed all the vegetative cells and only spores are left. After subjected to heat shock 100ul of solution is poured and transferred to petri dishes containing Luria betani agar (growth medium). After transferring and pouring the solution was spread evenly on plates by using Driglaski spatula [11]. Petri plates were inverted and incubate at 30°C for 48 hours in laboratory incubator.

The colonies that show common morphological characteristics as Bacillus species were selected for further screening by growing colonies in Luria Bertani broth (pH 6.8) containing penicillin as antibiotic at 30°C at 180rpm in shaking incubator for 48 hrs. The colonies were observed by fixing grown isolates on glass slides and visualize under phase contrast microscopes for identification of parasporal crystals. The isolates were preserved in glycerol solution at -20°C.

Preparation of bacterial culture for use in the bioassays:

The Isolates that containing spores or crystal were prepared for bioactivity against insects. Each isolated strain was grown in Luria Bertani broth at optimum temperature 30°C at 180 rpm for 72 hours (3days). The culture was grown until the 90% sporulation achieved. After the period of sporulation, the viable cell count is performed on petri plates, and the remaining suspension containing spores was used for conducting bioassay against indicator organism. The sporulation was observed using a phase contrast microscope or by endospore staining.

Bioassays using *Tribolium casteneum* larvae as the indicator organism

For insecticidal activity bioassay was performed. The indicator organism *Tribolium casteneum* third-instar larvae was obtained. The larvae were grown and nourished under controlled environment and conditions. The controlled environment has mean temperature 28 ± 2 °C, and relative humidity around 85% [11]. The larvae reared in controlled environment subjected to carry out selective and quantitative bioassay against each isolate. The screening bioassays were conducted with all the isolates to confirm insecticidal activity of the isolates. For evaluation of insecticidal activity of each isolate, four replicates have been formed each of the two replicates containing petri-dishes with 1 gram wheat flour and 10 third-instar larvae grown and nourished in controlled environment. Both replicates were contaminated with 1ml of total bacillus culture, while 2 replicated were form as negative control which has no inoculated bacterial culture. This replicate act as a negative control for entire bioassay experiment. For positive control one replicate has been added containing inorganic pest control agent. The mortality rate was documented at an interval of 24 and 48 hours. Once the mortality rate of each isolate was determined the isolates were selected for quantitative bioassay. Selection criteria for quantitative bioassay was 50% mortality rate of insects.

RESULTS AND DISCUSSION

Isolation and screening of *B. thuringiensis*

Bacillus thuringiensis commonly known as "Bt" is an indigenous soil dwelling, gram-positive, spore forming micro-organism that has activity against variety of insects by producing toxicity upon ingestion of microbe or its spores. This toxic effect against variety of insects makes this microbe very famous among scientific community. There are many GMO are produced by manipulating the cry gene of Bt and transform it into plant. By transforming cry gene plant produces toxic effect against insects. While, it is matter of discussion that such type of GMO's is safe for human consumption or not to answer that burning question scientific community are constantly doing research to solve that matter. Scientist has produced Bt cotton, Bt Corn, and Bt Brinjals. In future this indigenous microbe is consider as alternate of inorganic pest control agents.

Bacillus thuringiensis has been isolated from different types of ecological samples and varieties of substrates. In this study, four different ecological soil samples collected from different localities and one partially digested leaf were also collected and then processed. Soil samples was collected from different sites includes fields and marshy and shady places. The sample were collected from vicinities of Karachi, and mardan.

Adopting standard microbiological techniques, we have isolated 53 different isolated Bacillus like colonies. The colonies were selected by considering the morphological characteristics of *Bacillus thuringiensis.*

Table 2: Reference for selecting colony based on morphological characteristics.

Species	*B. thuringiensis*
Size	Medium
Shape	Round
Color	White
Margin	Regular
Texture	Moist
Elevation	Raised

Total of 53 isolates were form mix culture screened by gram staining and all isolates were found Gram positive. All isolates were screened by for ability to form dormant spore and after endospore staining 37 were found spore formers. Isolates that are spore former are SM13, SM14, SM15, SD21, SD22, SD27, LS31, LS32, LS33, LS34, LS35, LS37, LS38, LS39, SMA52, SMA53, SMA55, SMA56, SMA59, SMA60, SMA61, SMA63, SMA64, SMA65, SMA67, SMA68, SMB69, SMB70, SMB73, SMB74, SMB75, SMB77, SMB78, SMB79, SMB80, SMB81, and SMB82. The morphological characteristic of all isolates varies which were discussed in (Table 2).

In this study the index for obtaining Bacillus spore former is around 68% while, in similar studies in China and Brazil likewise index for obtaining spore former was 70%-75%. In this study index for obtaining *Bacillus* *thuringiensis* was 8.9% while, similar study conducted in Brazil and China has Index 11.8% and 13.5% respectively. As variation in Bt index has been different region of world. There must be multiple factors involve like climate condition, soil property, complex environmental condition. These factors may affect the microbial population in eco-system [12].

Table 3: Morphological characteristics of selected colonies.

Isolate	Size	Shape	Color	Margin	Texture	Elevation	Spores
SM11	Small	Round	Opaque	Regular	Mucoid	Convex	No
SM12	Medium	Irregular	White	Wavy	Dry	Flat	No
SM13	Medium	Round	Opaque	Regular	Dry	Flat	Yes
SM14	Small	Punctiform	Off-white	Regular	Moist	Raised	Yes
SM15	Medium	Round	Opaque	Regular	Dry	Flat	Yes
SD21	Small	Irregular	Off-white	Wavy	Dry	Raised	Yes
SD22	Small	Punctiform	Off-white	Regular	Moist	Flat	Yes
SD23	Medium	Irregular	Opaque	Filiform	Dry	Umbonate	No
SD24	Medium	Round	Translucent	Irregular	Viscid	Raised	No
SD25	Medium	Irregular	Opaque	Irregular	Dry	Concave	No
SD26	Medium	Irregular	Opaque	Wavy	Dry	Flat	No
SD27	Medium	Round	Translucent	Regular	Viscid	Raised	Yes
LS31	Small	Filamentous	White	Wavy	Dry	Raised	Yes
LS32	Large	Irregular	Translucent	Regular	Moist	Flat	Yes
LS33	Large	Round	Opaque	Regular	Moist	Flat	Yes
LS34	Small	Punctiform	Opaque	Regular	Moist	Raised	Yes
LS35	Medium	Round	Opaque	Regular	Moist	Flat	Yes
LS36	Medium	Filamentous	Opaque	wavy	Moist	Raised	No
LS37	Medium	Rhizoid	Opaque	wavy	Dry	Raised	Yes
LS38	Large	Filamentous	Opaque	wavy	Moist	Craterform	Yes
LS39	Medium	Irregular	Opaque	wavy	Moist	Raised	Yes
SMA51	Large	Round	Opaque	Regular	Mucoid	Convex	No
SMA52	Large	Irregular	White	Wavy	Dry	Flat	Yes

SMA53	Large	Round	Opaque	Regular	Dry	Flat	Yes
SMA54	Small	Punctiform	Off-white	Regular	Moist	Raised	No
SMA55	Medium	Round	Opaque	Regular	Dry	Flat	Yes
SMA56	Medium	Irregular	Off-white	Wavy	Dry	Raised	Yes
SMA57	Medium	Punctiform	Off-white	Regular	Moist	Flat	No
SMA58	Large	Irregular	Opaque	Filiform	Dry	Umbonate	No
SMA59	Large	Round	Translucent	Irregular	Viscid	Raised	Yes
SMA60	Large	Irregular	Opaque	Irregular	Dry	Concave	Yes
SMA61	Small	Round	Opaque	Regular	Mucoid	Convex	Yes
SMA62	Large	Irregular	White	Wavy	Dry	Flat	No
SMA63	Large	Round	Opaque	Regular	Dry	Flat	Yes
SMA64	Small	Round	Opaque	Regular	Mucoid	Convex	Yes
SMA65	Medium	Irregular	White	Wavy	Dry	Flat	Yes
SMA66	Medium	Round	Opaque	Regular	Dry	Flat	No
SMA67	Large	Punctiform	Off-white	Regular	Moist	Raised	Yes
SMA68	Large	Round	Opaque	Regular	Dry	Flat	Yes
SMB69	Small	Irregular	Off-white	Wavy	Dry	Raised	Yes
SMB70	Medium	Round	Opaque	Regular	Mucoid	Convex	Yes
SMB71	Medium	Irregular	White	Wavy	Dry	Flat	No
SMB72	Medium	Round	Opaque	Regular	Dry	Flat	No
SMB73	Large	Punctiform	Off-white	Regular	Moist	Raised	Yes
SMB74	Large	Round	Opaque	Regular	Dry	Flat	Yes
SMB75	Large	Irregular	Off-white	Wavy	Dry	Raised	Yes
SMB76	Small	Punctiform	Off-white	Regular	Moist	Flat	No
SMB77	Medium	Irregular	Opaque	Filiform	Dry	Umbonate	Yes
SMB78	Large	Round	Translucent	Irregular	Viscid	Raised	Yes
SMB79	Large	Irregular	Opaque	Irregular	Dry	Concave	Yes
SMB80	Large	Round	Opaque	Regular	Mucoid	Convex	Yes
SMB81	Small	Irregular	White	Wavy	Dry	Flat	Yes
SMB82	Medium	Round	Opaque	Regular	Dry	Flat	Yes

Bt is a spore former so gram staining is not sufficient to differentiate between Bt and Bacillus cereus. Similar study conducted in India used selective media MYP agar to select Bt isolate. On MYP agar Bacillus cereus give pink colony while, Bt give yellowish colony. In this study Endospore staining was conducted to identify Bt on the basis of its ability to form para-sporal inclusion. Endospore staining is not enough to differentiate between Bt and Bacillus cereus both specie is spore former.

Bioassay for insecticidal activity:

Tribolium castaneum (Commonly known as red flour beetle) belongs to order Coleoptera and widely used as model organism for scientific studies. *T. castaneum* is mainly consider as stored grain pest. Infestation of T.castaneum can occur throughout the year as it need warm environment. Life cycle of *T. castaneum* consist of 40-80 days and adult can live up-to 3 years.

For exploring the activity of Bt against *T. castaneum* multiple studies are conducted. In one study it is reported that Bt has ability to grow in cadaver of *T. castaneum* and reported 460 folds increase of Bt spores. Which concluded that Bt has high toxicity for *T. castaneum* and Bt has ability to grow in cadaver of insect [13]. By keeping in view of previous studies in this study we used *T. castaneum* for bioassay for elucidating larvicidal action of Bt.

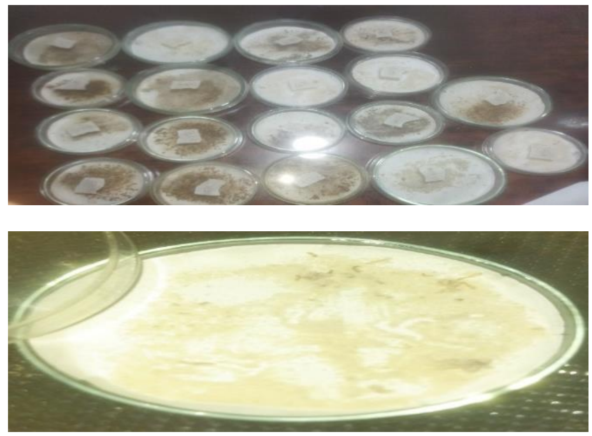

Figure 1: Bioassay for insecticidal activity

These 37 isolated strains were tested against *T. castaneum* 3rd instar larvae for activity. The activity was checked and recorded after intervals of 24 hrs, 48hrs, and 72 hrs (Figure 1 and Table 4). For Bioassay 1 ml broth culture media was centrifuged at 10,000 rpm for 3 mins and the supernatant was discarded and bacterial palette was mixed in 1 gram of wheat flour. Bioassay were conducted in duplicate such as for each isolate 2 replicates was constituted and each replicate were infested with 10 laboratory reared *T. castaneum* larvae.

Table 4: Bioassay observations

Isolates	Replicate 1			Replicate 2		
	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs
SM13	-	-	-	-	-	-
SM14	-	-	-	-	-	-
SM15	-	-	-	-	-	-
SD21	-	-	-	-	-	-
SD22	-	-	-	-	-	-
SD27	-	-	-	-	-	-
LS31	-	-	-	-	-	-
LS32	-	-	-	-	-	-
LS33	-	-	-	-	-	-
LS34	-	-	-	-	-	-
LS35	-	-	-	-	-	-
LS37	-	-	-	-	-	-
LS38	-	-	-	-	-	-
LS39	-	-	-	-	-	-
SMA52	-	-	-	-	-	-
SMA53	-	-	-	-	-	-
SMA54	-	-	-	-	-	-
SMA55	-	-	-	-	-	-
SMA56	+	+	+	+	+	+
SMA59	-	-	-	-	-	-
SMA60	-	-	-	-	-	-
SMA61	-	-	-	-	-	-
SMA63	-	-	-	-	-	-
SMA64	-	-	-	-	-	-
SMA65	-	-	-	-	-	-
SMA67	-	-	-	-	-	-
SMA68	-	-	-	-	-	-
SMB69	-	-	-	-	-	-
SMB70	-	-	-	-	-	-
SMB73	-	-	-	-	-	-
SMB74	-	-	-	-	-	-
SMB75	-	-	-	-	-	-
SMB77	+	+	+	+	+	+
SMB78	-	-	-	-	-	-
SMB79	-	-	-	-	-	-
SMB80	+	+	+	-	+	+
SMB81	-	-	-	-	-	-
SMB82	+	+	+	+	+	+

In this study the index for larvicidal activity of 37 samples was around 9% which is same as study conducted in Brazil. In contrast in one study the larvicidal activity is reported less than 1% for 300 isolates. In this study for larvicidal activity, we used *Tribolium castaneum* larvae while another study conducted in Brazil uses *A. aegypti* larvae which is a vector for dengue virus.

Bioassay was conducted in duplicate to assess any false positive or false negative results. During Bioassay 4 isolate show larvicidal activity. SMA56 and SMB80 show maximum activity and show larvicidal action in first 24hrs in both replicates. While, SMB 77, and SMB 82 shows larvicidal action within 24hr in replicate 1 and in replicate 2 SMB77 the activity was observed after 48hrs and SMB82 shows activity after 72hrs. In similar studies combined action of Bt, boric acid and cypermethrin and neem powder was assessed and show higher percentage of mortality[14] While, another similar study report that some classes of Cry endotoxin are active against *Tribolium casteneum* which includes Cry3Aa, Cry37Aa, Cry22Aa, Cry51Aa, and Cry3Ba [15].

CONCLUSION

In this study 5 ecological samples were screened for Bacillus like specie and 53 different isolates were isolated. These 53 isolates further screened for spore former by endospore staining. After Endo spore staining 37 isolates were found as spore former. These 37 isolates were selected for bioassay and bioassay was against *Tribolium casteneum* larvae. Larvicidal activity has been reported in SMA 56, SMB 77, SMB80 and SMB 82. Reported samples will be sequenced for specie level identification. During this study on Bt, we characterized 4 indigenous isolates by conducting bioassay on *Tribolium castaneum*. In future, strains will be used to transform cry gene into a vector system and express its insecticidal endotoxin for larvicidal activity. This expression in vector system will enable us to attempt and explore new ways to protect plants or crops from pest damages. Reduce damages to plants and crops will also boost-up economy and crop yield can be increased.

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