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Original Article

Larvicidal Activity of *Bacillus sphaericus* Strain SI-1 and Its Toxins against *Culex quinquefasciantus* Larvae

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A local isolate of *Bacillus sphaericus* strain SI-1 had been investigated for its larvicidal activity against *Culex quinquefasciantus* mosquito. Wet spores of 72-h grown culture of the strain were sufficiently potent as mosquitocidal agents 3rd instar larvae of *C. quinquefasciantus*. After lyophilization the toxic potency of the strain increased sharply (165 times). The toxic crystal proteins had been solubilized in a hydroxyl-chloride buffer (pH 12.0) by three successive extractions. The majority of the proteins were found to be solubilized in water under these conditions. Increase of pH in the media by addition of buffer and its effect on the mortality of *C. quinquefasciantus* larvae had been checked. The extraction buffer was found to be non-lethal although significant rise in pH up to 10.44 was observed. The lethal concentration fifty (LC_{50}) value of the crude protein was found to be 62.0 µg/ml.

Keywords: *Bacillus sphaericus* SI-1, Biopesticide, *Culex quinquefasciantus*, Larvicidal activity, Lyophilization

Introduction

Bacillus sphaericus and *B. thuringiensis* have been widely used as biopesticides. These bacterial strains produce a crystalline endotoxic protein during sporulation, which is toxic to Coleopteran and Dipteran organisms[1-5]. These toxins are very specific for target organisms but friendly to the environment being non-toxic to human being and other higher animals, plants and fishes[6]. A large number of *B. sphaericus* strains have been isolated until now. They have been found to be especially active against mosquito larvae[5]. But there are different species of mosquitoes in nature and bacterial strains active against multiple species of mosquitoes are very rare. The toxin produced by a particular strain is generally active against the larvae of one particular species of mosquito. Different workers have shown that isolates of these are different in their nature and extent of activity in different parts of the world.

Some bacterial strains, namely, *Bacillus thuringiensis krustaki* (BtK), *Bacillus thuringiensis israelensis* (Bti), *Bacillus thuringiensis galleria* and *Bacillus sphaericus* 1593 (Bs93) have been used as mosquitocidal agents. The isolates of *B. thuringiensis* and *B. sphaericus* obtained in our laboratory from local soils proved to be more effective in our screen tests. Only a few strains of *B. sphaericus* and *B. thuringiensis* have been found, which produce toxins active against *Culex* and *Aedes*. In general, the toxin from *B. sphaericus* is active against *Culex, Aedes* and *Monsonia,* whereas that from *B. thuringiensis* is occasionally active against *Anophelis*[7]. Locally isolated *B. sphaericus* strain SI-1 appeared to be very effective against the *Culex* species. This has been isolated and characterized partially at biochemical and morphological level in this laboratory. Its mosquitocidal activity, toxicity on some other animals and sporulation time has been determined[8]. It has been reported that 16-h grown vegetative cells of *B. sphaericus* strain SI-1 at a concentration of 4.8 x 10^9 cfu/ml have no toxicity against *C. quinquefasciantus* larvae, but the spores of this strain showed significant mosquitocidal activity. This suggests that the toxin be synthesized during sporulation. This confirms the findings of other workers using different *B. sphaericus* strains[9-12] have further specified the association between sporulation and toxicity using specific mutants and showed that the toxin is produced at the later stages of sporulation.

The aim of the present study was to determine the effectiveness of the spore and the crude toxin of *Bacillus sphaericus* SI-1 in killing the larvae of *Culex quinquefasciantus.*

Materials and Methods

Bacterial strains

Bacillus sphaericus strain SI-1 and the corresponding spores were collected from the Molecular Biology Laboratory of the Department of Biochemistry, University of Dhaka[8] and were stored at -20°C in a conical flask covered with parafilm and aluminium foil.

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Bioassay of wet spores using mosquito larvae

Either the late third instar or the early fourth instar larvae of *Culex quinquefasciantus* was used in the routine examination of mosquito larvicidal toxicity of the spores throughout the study. At the beginning of the experiment, the larvae were checked for any infection. Any larvae showing any sort of abnormalities (*e.g.*, a fuzzy appearance) were discarded. The larvae were washed repeatedly with sterile tap water to remove any attached impurities. Highly concentrated suspension of spores of 72-h grown culture was used for the tests. One gram of these spores was resuspended in 6 ml sterile tap water. A series of five sterile glass jars, each containing 5 ml sterile tap water taken. To each glass jar ten mosquito larvae were added. Then, 25, 50, 100 and 200 µl of spore suspensions were added to glass jar 2, 3, 4, and 5 respectively. Glass jar 1 was kept as control that contains sterile tap water instead of spore suspension. The status of the larvae (dead or alive) was monitored for 32 h.

Lyophilization of spores obtained from Bacillus sphaericus strain SI-1

About 30-40 g of spores (on wet weight basis) was collected in Sorvall tubes. It was transferred into a conical flask and liquid nitrogen was added to freeze the contents. The lyophilization was then carried out in a Lyovoc GT-2 lyophilizer till the spores were obtained in power form. The lyophilized spores were then preserved at 4°C for further use.

Bioassay of lyophilized spores

Twenty milligrams of lyophilized spores were resuspended in 100 ml sterile tap water. A series of 5 sterile glass jars, each containing 5 ml sterile tap water was taken. Ten mosquito larvae were added to each glass jar. Then, 25, 50, 100 and 200 µl of spore suspensions were added to glass jar 2, 3, 4, and 5 respectively. The first glass jar was kept as control. Survival observation of the larvae was monitored every 6 h.

Extraction of crude toxic protein

Four Sorvall tubes were sterilized and 500 mg of lyophilized spores were taken in each tube. Ten millilitres of hydroxyl-chloride buffer (pH 12.0) was added and vortexed for 10 min. It was kept at 4°C overnight and then centrifuged at 10,000 rpm at 4°C for 45 min. Supernatant from each tube were pooled together in a conical flask. Two more successive extractions were carried out using the residues from each tube and then the supernatants were added to the pooled suspension. The protein content of the pooled supernatant was estimated by Lowry method[13].

Mosquito larvicidal activity of crude toxin protein

A series of six small glass jars containing 5 ml, 4.990 ml, 4.950 ml, 4.850 ml and 4.900 ml of sterile tap water was taken. Hydroxyl-chloride buffer (100 µl) was introduced into glass jar 6. To glass jars 2, 3, 4 and 5 the amount of buffer extracted crude protein of 5, 10 , 50, 100 and 150 µl were added respectively. Glass jar 1 and 6 served as controls. To each of the six glass jars 10 mosquito larvae were added. The mortality of the larvae was monitored every 6 h.

Results and Discussion

Sporulation of *Bacillus sphaericus* strain SI-1 was completed when grown on G-media for 72 h[8]. Samples from this sporulated species were used in this study. Determination of the larvicidial activity of the wet spores was done against 3rd instar larvae of *Culex quinquefasciantus.* The strain under study was found to be fairly toxic as wet spores, killing 100% of the larvae in 24 h at spore concentration 6.4 mg/ml (Table 1).

After lyophilization of wet spores, the dried spores in powder form were obtained. Table 2 shows that 5 µg/ml of lyophilized spores kill 10 larvae in 18 h, while 0.828 mg/ml of the wet spore and 32 h of exposure time is needed to get the same time effect (Table 1). The lyophilized spores are thus 165 times more potent than the wet spores.

In the wet spores the toxic proteins exist in their natural form. The function of the globular protein is very sensitively related to its 3-dimensional structure. Evan a slight change in the 3-dimensional structure of a protein may adversely affect its functional efficacy and effectiveness[14]. The aqueous environment within the spores could be thought of as a stabilizing factor for the toxin proteins. In the lyophilized process, the water is extracted completely and the product is obtained in a powder form. This could have led to

Table 1. *Mosquito larvicidal activity of wet spores of Bacillus sphaericus strain SI-1 against Culex quinquefasciantus*

No. of glass jar	Sterile tap water (ml)	Spore suspension added (µl)[b]	Amount of spores present (mg)	Spore concentration (mg/ml)	% of dead larvae after		
					18 h	24 h	32 h
1[a]	5.0	0	-	-	0	0	0
2	5.0	25	4.16	0.828	20	50	100
3	5.0	50	8.33	1.649	40	60	100
4	5.0	100	16.66	3.266	50	80	100
5	5.0	200	33.33	6.409	70	100	100

No. of mosquito larvae added = 10. [a]Control. [b]From the stock suspension of 1 g spore suspended in 6 ml water.

Table 2. *Mosquito larvicidal activity of lyophilized spores of Bacillus sphaericus strain SI-1 against Culex quinquefasciantus*

No. of glass jar	Sterile tap water (ml)	Spore suspension added (µl)	Amount of spores present (µg)	Spore concentration (µg/ml)	% of dead larvae after		
					18 h	24 h	32 h
1[a]	5.0	0	-	-	0	0	0
2	4.975	25	5.0	1.0	0	40	80
3	4.875	125	25.0	5.0	10	60	100
4	4.750	250	50.0	10.0	10	70	100
5	4.500	500	100.0	20.0	10	80	100

No. of mosquito larvae added = 10. [a]Control.

a shrinking of the spores leading to a change in the conformation of the toxin proteins. In that case the activity of the protein should be decreased. However, in the present study the results showed a tremendous (165 times) increase in activity precluding such a possibility. The increase in this case can be explained by an increase in the effective concentration of the spores due to removal of water through lyophilization.

The fact that the activity increase sharply when the spores are in dry powder form is encouraging, because commercial utilization of such spores requires that they be available in a form which is easy to store, transport and spray in the field. The storage stability of the spores of *B. sphaericus* strain SI-1 has been determined. The lyophilized spores in form of water suspension were found to retain its activity at room temperature without any loss for more than six months. The powered form is therefore expected to remain stable at least for more than one year. This makes this strain more effective for commercial exploration for the control of mosquitoes.

Hydroxyl-chloride buffer at pH 12.0 can solubilize crystalline toxin from *B. sphaericus* strain SI-1. The toxin along with other bound and soluble proteins were extracted with this buffer at this pH. Two grams of spores yielded 372 mg of protein after three successive extractions. Thus, about 5.5% of the total spore mass was extracted in this process. After the 4th extraction it was observed that only a negligible amount of protein was extracted and that its activity was very low[15]. Thus only triple extraction of the spores with the buffer would be used for the extraction of the toxic proteins although Baumann *et al.*[16] reported solubilization of crystal from *B. sphaericus* strain 2362 by centrifugation at 40,000x *g* using NaOH-Tris-HCl buffer for 30 min.

The results presented in Table 3 show that only 20% of the larvae died within 18 h when the protein concentration in the medium was 31.0 µg/ml. The percentage of deaths increased to 80% as the protein concentration was raised to 93.0 µg/ml. On the other hand, an increase of 6 h of exposure time (24 h) caused 70% deaths without any rise in protein concentration (31.0 µg/ml). After another 6 h, *i.e.*, after a total of 32 h of exposure time 100% of the larvae died. In glass jar 6, 100 µl buffer was added instead of protein solution. No larvae died even after 32 hours showing that the effect (*i.e.*, the death of larvae) was actually due to the protein and not due to the buffer solution. Table 3 also shows that up to 62.0 µg/ml protein concentration the time of exposure was the main factor for killing the mosquito larvae, whereas at 93 µg/ml the protein concentration itself was sufficient for 80% efficiency at 18 h.

In this study, hydroxyl-chloride buffer (pH 12.0) was used. Sodium hydroxide was one of the components of this buffer that was found to be lethal for *Culex quinquefasciantus* larvae. There was a possibility that the free OH^- ions might interfere with the growth and maintenance of the organism. The activity of the toxin was measured in terms of number of dead larvae under the given conditions of toxin concentration and time of contact with the

Table 3. *Mosquito larvicidal activity of crude protein of the spores of Bacillus sphaericus strain SI-I against Culex quinquefasciantus*

No. of glass jar	Sterile tap water (ml)	Spore suspension added (µl)	Final protein concentration in media (µg/ml)	Buffer (µg)	% of dead larvae after		
					18 h	24 h	32 h
1[a]	5.0	0	-	-	0	0	0
2	4.990	10.0	6.2	-	0	0	20
3	4.950	50.0	31.0	-	20	70	100
4	4.900	100	62.0	-	30	80	100
5	4.850	150	93.0	-	00	90	100
6[a]	4.900	-	-	100	0	0	0

No. of mosquito larvae added = 10. [a]Glass jar 1 and 6 were controls.

larvae. Sodium hydroxide was present in the medium as a component of the hydroxyl-chloride buffer. Therefore, there is a possibility that this might have influenced the results. In order to test this possibility, an experiment was designed in which the larvae were put under these conditions with increased pH of up to 10.44. Any effect of the buffer components under these conditions should be manifested as a death of larvae. But no larvae died even after 32 h, which was the longest exposure time employed for the assays (Table 4). Therefore, it can be concluded that all the deaths in the assay were due to the toxin added and not due to the NaOH or any other components in the assay medium employing the hydroxyl-chloride buffer.

Table 4. *Change of pH due to addition of buffer and its effect on the mortality of mosquito larvae*

No. of glass jar	Sterile tap water (ml)	Buffer added (µl)	Measured pH	No. of larvae taken	% of dead larvae after 32 h
1[a]	5.0	0	8.32[b]	10	0
2	5.0	50	8.98	10	0
3	5.0	100	9.30	10	0
4	5.0	200	9.65	10	0
5	5.0	300	9.90	10	0
6	5.0	1,000	10.44	10	0

No. of mosquito larvae added = 10. [a]Control. [b]pH of normal tap water was 6.6 ± 0.02; after autoclave the pH rose to 8.3 ± 0.02.

Table 5 shows the LC_{50} of the buffer extracted crude protein. The protein concentration required to kill 50% of the larvae in 20 h was defined as LC_{50}. From this table the LC_{50} value of the crude protein was found to be 62.0 mg/ml.

Table 5. *Determination of the lethal concentration fifty (LC_{50}) value of crude protein of the spores of Bacillus sphaericus strain SI-I against Culex quinquefasciantus*

No. of glass jar	Sterile tap water (ml)	Crude protein solution (µl)	Protein concentration in media (µg/ml)	Mortality after 20 h
1[a]	5.0	0	-	0
2	4.950	50	31.0	30
3	4.925	75	46.5	40
4	4.900	100	62.0	50
5	4.875	125	77.5	70

No. of mosquito larvae added = 10. [a]Control.

In conclusion, it can be said that *Bacillus sphaericus* strain SI-1 is a very promising strain as far as the control of mosquitoes in the country is concerned. It is very effective against *Culex quinquefasciantus* that is the most abundant species in Bangladesh, especially in the city of Dhaka. It can be stored in dry power form over a very long period and is highly effective as such so that it does not need any extra processing to make it active before use. It was isolated locally and is hence cheap, easy to produce and easy to handle. Thus this strain of *B. sphaericus* provides a very attractive biological control agent against mosquito.

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