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Bio-efficacy of Indigenous *Bacillus thuringiensis* JSd1 against Melon Fly, *Zeugodacus cucurbitae* (Coq.) (Diptera: Tephritidae: Dacinae)

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Abstract
A sustainable development of *Bacillus thuringiensis* based Biopesticide against *Zeugodacus cucurbitae*, a detrimental vegetable pest susceptible to *Cry* proteins, more lab-scale and field bioassay is required to replace hazardous chemical pesticides. In this concern, 44 indigenous *Bt* strains harboring different *cry* genes were assayed *in vivo* against the 3rd instar larvae of *Z. cucurbitae* following enhanced expression of *Cry* proteins in T_3 broth. Of the tested isolates, 9 causing more than 70% larval mortality were *Bt* JSd1 (95%), SaS6 (87%), JDc1 (86%), TaSa4 (82%), NaSc3 (80%), RaSa2 (79%), FhSb3 (77%), Ksa2 (75%) and MuSc2 (72%) respectively. Based on the Lethal concentration (LC_{50}, LC_{99}) (Logarithmic value of spore count) and Lethal time (LT_{50}, LT_{99}) as estimated by Probit analysis, the efficacy of the potential strains were compared. LC_{50} was determined lowest for JSd1 (0.429) and highest for MuSc2 (0.752) ml/gm whereas the LC_{99} values as determined lowest for JDc1 (3.792) and highest for MuSc2 (6.546) ml/gm. The LT_{50}, the time required for half of the larvae to be killed, was estimated lowest for *Bt* JSd1 (54.16 hr) whereas for the LT_{99}, lowest 239.47 hr was required for *Bt* RaSa2 to cause 99% mortality. *Btk* HD-73 was used as reference strain in this study. The dose and time response based bioassay thus identify the effective *Bt* strains *viz*. *Bt* JSd1 in controlling *Z. cucurbitae* larvae with lowest of dose and quickest of time in the laboratory which upon field trial will be suitable for large-scale production and sustainable delivery to the farmers.

Keywords: bio-efficacy, mortality, tephritidae, *cry* protein, *Bacillus thuringiensis*, *Zeugodacus cucurbitae*

Introduction
Bangladesh is an agricultural country, having a subtropical climate produces various vegetables and crops. Fruit flies are one of the most crucial agricultural pest species distributed world-wide, infesting a vast range of fresh fruits and vegetables [1] throughout the tropical and sub-tropical countries. Tephritidae, the most destructive insect pests on horticultural crops throughout the tropical and subtropical regions [2-5]. Flies lay eggs under the skin of fresh fruits and fleshy vegetables. Their larvae feed in the decaying fruit flesh, infested hosts quickly become rotten and inedible or drop to the ground prematurely, thus causing 10-30% losses of annual agricultural productions in the country and causes severe economic losses of cucurbitaceous vegetables and fruits varies from 30 to 100% depending on the fruit species and season [6, 7]. Due to the infestation of these Tephritids species, many countries have imposed quarantine restriction on the imports of products from countries infested with particular fruit fly species, and/or require that fruits and vegetables undergo quarantine treatment before their importation is allowed [8] Due to the suppression of fruit flies have been a prime goal to pest control programs. A number of new country records, including a major pest species, *Bactrocera carambolae* and a new species, *Zeugodacus madhupuri* increasing the number of species of Tephritidae from 27 to 37 (29 Dacini and 8 from other tribes) in Bangladesh [9-13]. Dacini is a very diverse group of fruit flies, with 939 described species, including 83 pests of cultivated fruit and cucurbits [14]. Of these, 118 are known to occur on the Indian subcontinent [15-19]. 210 species of *Bactrocera* were reared on more than 811 host species among them 73 species were found economically significant which were categorized into 4 groups on the basis of their severity, invasiveness, host range and frequency of infestation [20]. Among these species the melon fly, proposed as *Zeugodacus cucurbitae* (Coquillett) (Diptera: Tephritidae) [21], was formerly known as *Bactrocera cucurbitae* (Coquillett) is one of the major insect pest species which is our concern in this study that distribute in various parts of the world and cause remarkable damage to vegetables and fruits.

B. thuringiensis, a gram-positive spore-forming soil bacterium produces proteinaceous insecticidal crystals or δ-endotoxins during sporulation, which can specifically kill the insects belonging to the Lepidoptera, Diptera, Coleoptera, Diptera, Hymenoptera, Hemiptera, Homoptera, Orthoptera and Mollaphaga as well as some invertebrates at their larval stage [22-24]. It is widely distributed and approximately 70% of all soil samples from all countries were found with this bacterial species and are especially plentiful in Asian samples. This is a entomo-pathogenic bacterium which has been preferred to chemical pesticides for environment-friendly pest management [25] as they are free of recalcitrant residues due to bioaccumulation and biomagnification, might become carcinogenic, mutagenic, teratogenic or allergenic [26]. Thus, *Bt* biopesticides were proved to be valuable alternatives to the synthetic chemical pesticides in agriculture, forestry and mosquito control for the last many decades [27-30]. *Bt* biopesticide is a key component in Integrated Pest Management (IPM) strategies targeting to ensure sufficient number of biological enemies

of pests considering insect resistance to chemicals [31]. In USA, the *Bt* biopesticide preparations were the most widely used microbial insecticides since 1960 and it can be found in various habitats like soil, insect cadavers, stored product dust, grains, olive tree related-habitats, agricultural soils, plant and aquatic environments [25,31-35]. Since Bangladesh is an agriculture-based country and *Bt* biopesticide is still absent in its IPM, potential *Bt* strains were isolated and characterized as a national study and their novel toxicity was also established against the melon fruit fly, *Bactrocera cucurbitae* [36-39]. Recently, indiscriminate use of chemical insecticides containing long residual action and high toxicity against a large number of insects causes environmental pollution, breaking of food-chain, ecosystem failure, resistance increasing in agricultural pest and human diseases. So, therefore the public demand for environment-friendly pest management and organic farming using *Bt* biopesticide could be more applicable for insect pest management in Bangladesh which would be more sustainable and cost-effective. The present study was therefore, conducted to evaluate the entomopathogenic activity, identify and characterize the efficacy of potential indigenous *Bt* strains against melon fly, *Z. cucurbitae* as well as develop field level application of *Bt* biopesticide to control Tephritid fruit flies eco-friendly.

Materials and Methods

Bacillus thuringiensis (*Bt*) strains and culture conditions

44 *Bt* isolates, *B. thuringiensis, kurstaki (Btk) & indiana* strains, i.e. AgS2, ChSd2, CiSa5, DSe1, DSf4, DSh4, FhSb2, FhSb3, JDb1, JDc1, JSd1, KSa2, KSb1, KSe2, MeSa1, MeSb1, MuSa1, MuSc2, MuSc4, MuSe4, NaSc3,NaSd2, NoS2, NoS3, NsSe1, NsSe2, RaSa1, RaSa2, RaSb1, RaSc1, RaSd1, RhSa2, RhSb2, SaS4, SaS6, SaS7(19s), Soi1(li), SpSc1, SSb2, SSe2, TaSa4, TaSb3, TaSc1, USc3 were used in this study. These strains were collected from Lab 215, Department of Microbiology, University of Dhaka and the reference *Bt kurstaki* HD-73, *Bt sotto* T84A1 and *Bt japonensis* Buibui strains collected from *Bt* stock collection of Okayama University, Japan. LB agar was used for culture maintenance, subculture and spore count of the *Bt* strains. Incubation temperature was maintained at 30ºC for all types of cultures and the liquid cultures were incubated in an orbital shaker at 180 rpm.

Preparation of spore crystal mixture

The Spore-crystal mixture for bioassay was prepared from the tested *cry1* gene positive isolates by inoculating them in 100ml of T_3-liquid medium [40] and incubating them for 3-4 days at 30°C with continuous shaking at 250 rpm. Before that bacterial cultures were prepared in LB broth. Cultures were centrifuged at 5000 rpm for 15 min to separate the culture from the medium. Pellets (spores and crystal protein mixture) were washed with 50 ml of cold sterile distilled water and centrifuged at 5000 rpm for 5 min. The pellets were re-suspended in 50 ml of sterile distilled water and incubated for another 2 days at 30°C with continuous shaking at 250 rpm and used for bioassay. Protein concentration from the supernatant was estimated by the Bradford method [41]. A cost effective medium was developed by statistical method for *Bt* biopesticide production using potential strain for bioassay [42, 43].

Inoculum preparation

Bt strains were streaked on LB-agar plate from the slant and were incubated overnight at 30°C. An isolated colony was picked from the LB-agar plate aseptically with a loop and was inoculated into a 100 ml Erlenmeyer flask containing 20 ml of LB broth and incubated overnight at 30°C and 180 rpm. The cell density of the culture medium was measured after overnight incubation at OD_{600nm} using uninoculated LB broth as blank. The overnight culture was then used as inoculum for all relevant sporulation experiments which was performed in T3 medium (or modified youstin agar medium) and each time, inoculum was added into the medium in such a manner that the culture medium starts with an OD_{600nm}= 0.1 if not otherwise stated.

Preparation of the Doses Suspensions

The spore-crystal suspension of *Bt* strains, with average percentage of mortality more than 50%, will be serially diluted in sterile distilled water up to 2^{-1}, 2^{-2} and 2^{-3} folds and mixed with the diet. Number of dead larvae in different concentrations of different treatments (a spore-crystal mixture of *Bt* strains) will be recorded for further analysis to determine LC_{50} and LC_{99} values and the test will be performed in triplicate.

Egg Collection from fruit flies

To collect a huge number of eggs some mature ripens and fresh pieces of fresh greenish sweet gourd were cut into several pieces and placed inside the *Z. cucurbitae* adult rearing cages for oviposition. After half an hour all the pieces of sweet gourd were collected and put into plastic bowls spread with dry sawdust bellow for further development of *Z. cucurbitae* on sweet gourd. Fresh sweet gourds were supplied as food time to time for further larval development up to early 3rd instars.

Insect Rearing

Larvae are maintained on laboratory developed larval diets. Adult flies stocked in a stainless steel framed cage (120×120×90) cm^3 and (183 X 76 X 38) cm^3 covered with a stainless steel net. Generally, 4000-6000 and 6000-9000 adult flies were maintained in a stock cage respectively. Newly hatched larvae fed on the preferred host for further development. Emerged adults were usually supplied with two different laboratory developed artificial diets i.e., liquid (baking yeast: sugar: water = 1:3:4) and dry (yeast extract: casein: sugar=1:1:2). Water is kept in a conical flask soaked with cotton into the cage for humidity. Temperature and relative humidity (RH) of the rearing room maintained at 28±2°C and 70-80% respectively. Insect rearing was maintained at Insect Biotechnology Division, Institute of Food and Radiation Biology, Atomic Energy Research Establishment, Savar, Dhaka.

Bioassay

The toxicity of the *Bt* strains was analysed *in vivo* against the 3rd instar larvae of melon fruit fly, *Z. cucurbitae* by bioassay. Spores crystal mixtures prepared with *cry1A* gene positive isolates [40] of which 1.0 ml spore-crystal mixture mix with10gm of boiled and mashed sweet gourd paste on which 20 early 3rd instar larvae were placed in each petri dish and were kept and fed at 28±2°C and relative humidity (RH) at 70±10%, with a photoperiod of 16:8 (L: D). Then mortality was recorded for the *Bt* strains along with a

parallel control diet supplemented with sterile distilled water as well as it is used to correct the test mortality using Abbot's formula [44] up to 7 days. *Btk* HD-73 was used as reference strain. Bioassays performed in triplicate in all the cases.

Data collection and Statistical analysis:

Bioassay consisted of two steps including screening for potential strains in the initial step and determination of lethal concentration values in the final step for the strains causing more than 50% mortality. In the initial step, the undiluted spore-crystal suspension was mixed with the larval diet standardizing the volume (keeping approximately equal spore) for each strain and numbers of dead larvae were recorded. Mortality data were corrected using Abbot's formula [44]. It was performed in triplicate and the average percentage of mortality was determined for each strain. *Bt* strains causing more than 40% mortality were tested again in the same manner and strains exerting death to more than 50% of the larvae were selected for the next step. In the final step, the spore-crystal suspension of *Bt* strains, with an average percentage of mortality more than 50%, was serially diluted in sterile distilled water up to 2^{-1}, 2^{-2} and 2^{-3} folds and mixed with the diet. The number of dead larvae in different concentrations of different treatments (a spore-crystal mixture of *Bt* strains) were recorded for further analysis to determine LC_{50} and LC_{99} values and It was also performed in triplicate. As the concentration of spores in the suspension was the basis for estimation of Lethal Concentration values, the logarithmic scale was used for simplification. From the data of dead larvae for different treatments, concentrations causing the death of 50% and 99% of larvae were determined by Probit Analysis-Finney Method [Lognormal Distribution] [45, 46] for the treatments using Statplus 2009 software for Windows. All bioassays were repeated thrice, and means were analyzed using one-way analysis of variance and compared as least significant differences (LSD). The acceptance level of statistical significance was α=0.05.

Results

Among the 44 indigenous *Bt* strains tested against *Z. cucurbitae*, nine isolates were found to cause the highest mortality against the 3rd instar larvae. Certain strains were very close in case of toxicity and maximum mortalities were recorded for indigenous *Bt* JSd1 (95%), SaS6 (87%), JDc1 (86%), TaSa4 (82%), NaSc3 (80%), RaSa2 (79%), FhSb3 (77%), Ksa2 (75%) and MuSc2 (72%) respectively while the reference strain, *Btk* HD-73 exhibited 95% mortality. Some larvae were found to die in control i.e. without any treatment which was used to correct the test mortalities. Total tested indigenous *Bt* strains against *Z. cucurbitae* larvae as well as nine of them FhSb3, JDc1, JSd1, KSa2, MuSc2, NaSc3, RaSa2, SaS6 and TaSa4 causing more than 70% mortality with the reference strain, *Btk* HD-73 were shown in Fig. 1, 2 & 3.

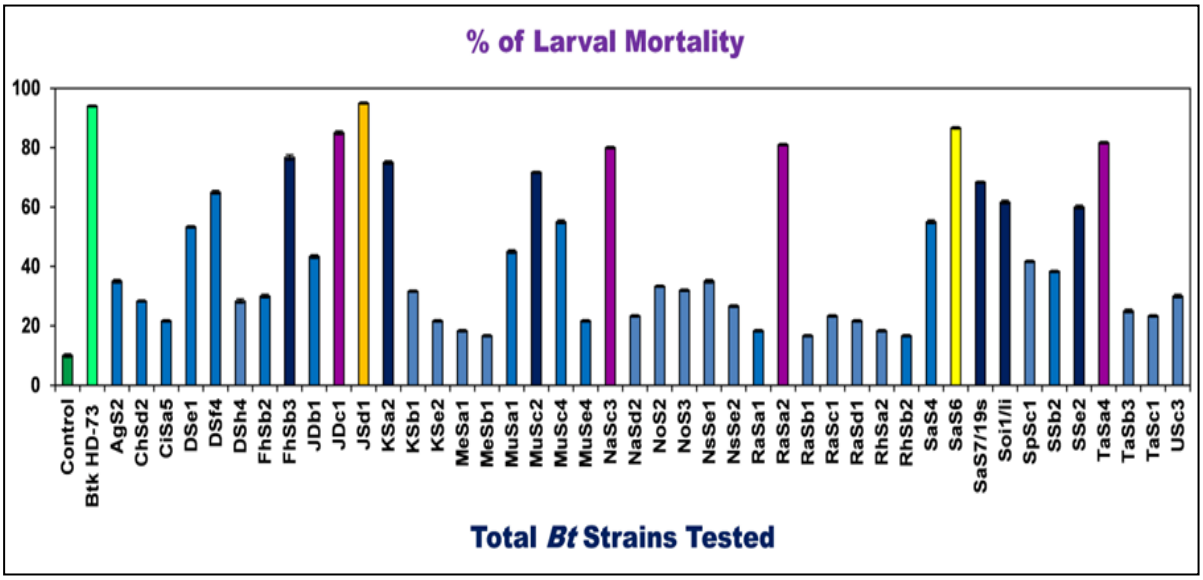

Fig. 1: Mortality caused by the spore-crystal protein mixture of different indigenous *Bt* strains tested against *Z. cucurbitae* (3rd instar larvae).

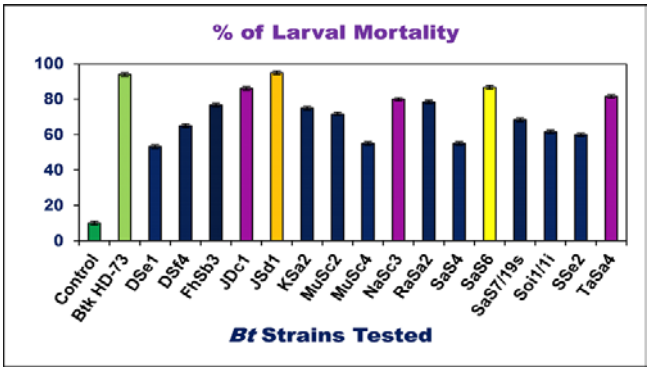

Fig 2: Mortality (>50) caused by the spore-crystal protein mixture of potential *Bt* strains against *Z. cucurbitae* (3rd instar larvae).

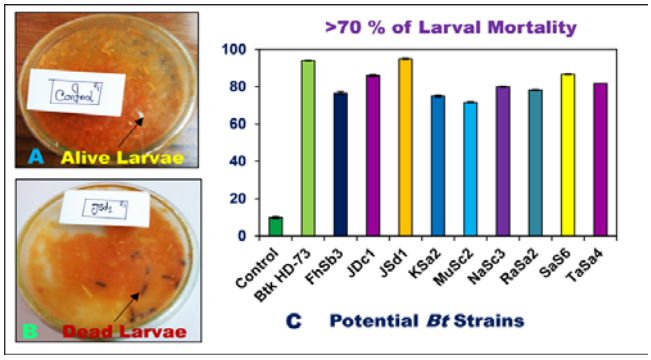

Fig 3: Bioassay performed with spore-crystal protein mixture of potential *Bt* strains against *Z. cucurbitae* (3rd instar larvae) caused >70% mortality.

Determinations of Lethal Concentrations (LC)

The bioassay was repeated thrice with the *Bt* strains causing more than 70% mortality for the determination of LC_{50} and LC_{99} values. In this connection, the original spore-crystal mixture was diluted for each strain up to 2^{-1}, 2^{-2} and 2^{-3} dilutions. The spore concentration for each strain was calculated in the logarithmic scale and the mortality for each concentration was recorded (**Table 1**).

Table 1: LC_{50}, LC_{99} values, 95% confidence limits and χ^2 values for potential indigenous *Bt* strains against *Z. cucurbitae* (3rd instar larvae)

Bt Strains (Potential)	LC_{50} values	LC_{99} values	Regression equations	95% confidence limits		χ^2 values (df)
				lower	upper	
FhSb3	0.655	4.963	Y=2.5479+3.0040 X	0.555	0.771	0.5606 (2)
JDc1	0.564	3.792	Y=2.6557+3.1192 X	0.485	0.656	2.7459 (2)
JSd1	0.429	4.278	Y=3.2285+2.7970 X	0.307	0.600	9.4796 (2)
KSa2	0.689	5.458	Y=2.4486+3.0419 X	0.583	0.814	1.0185 (2)
MuSc2	0.752	6.546	Y=2.3149+3.0627 X	0.632	0.896	2.1265 (2)
NaSc3	0.621	4.727	Y=2.5989+3.0265 X	0.529	0.728	3.3091 (2)
RaSa2	0.658	5.075	Y=2.5529+2.9885 X	0.558	0.777	2.9309 (2)
SaS6	0.531	4.958	Y=2.9898+2.7719 X	0.451	0.625	4.5069 (2)
TaSa4	0.522	5.447	Y=2.9204+2.7435 X	0.484	0.676	3.9764 (2)
Btk HD-73	0.489	4.478	Y=3.3285+2.8970 X	0.378	0.630	9.5796 (2)

The LC_{50} and LC_{99} values varied from 0.429 to 0.752 ml/gm and from 3.792 to 6.546 ml/gm respectively. The lowest LC_{50} value was observed for the indigenous *Bt* strain JSd1 (LC_{50}- 0.429) indicated the highest potency in causing the death of 50% of the larvae. On the other hand, MuSc2 exhibited maximum spore requirements (LC_{50}-0.752). The LC_{99} values i.e. causing 99% larval mortality, JDc1 (LC_{99}-3.792) was found to be potentials and MuSc2 (LC_{99}- 6.546) was the highest. The regression equation, 95% confidence limits (lower & upper value) and χ^2 values were subjected to LC_{50} values whereas the value for reference strain *Btk* HD-73 was (LC_{50}-0.489) and (LC_{99}-4.478) as shown in **Table 1**.

Determination of Lethal Time (LT)

The LT_{50} and LT_{99} values for the potential *Bt* strains were varied from 54.16 hours to 148.96 hours and from 239.47 hours to 861.34 hours respectively. The lowest LT_{50} value was observed for the indigenous *Bt* strain JSd1 (LT_{50}- 54.16 hr) indicated the highest potency in causing the death of 50% of the larvae within four days. On the contrary, MuSc2 exhibited maximum time requirements (LT_{50}- 148.96 hr). For LT_{99} values i.e. causing 99% larval mortality, Rasa2 (LC_{99}-239.47 hr) was found to be potentials. KSa2 and MuSc2 (LT_{99}- 861.34 hr) was the higher value while the LT_{50}- 56.19 hr and LT_{99}- 148.96 hr were recorded for the reference strain *Btk* HD-73 (Table 2).

Table 2: LT_{50} and LT_{99} values estimated for the Potential indigenous *Bt* strains against *Z. cucurbitae* (3rd instar larvae)

Bt Strains (Potential)	LT_{50} (hrs)	LT_{99} (hrs)
FhSb3	136.80	329.38
JDc1	136.06	344.86
JSd1	54.16	329.38
KSa2	136.06	861.34
MuSc2	148.96	861.34
NaSc3	136.80	466.45
RaSa2	148.36	239.47
SaS6	93.09	344.76
TaSa4	136.80	466.45
Btk HD-73	56.19	335.41

Phenotypic Characterization of Potential Isolates

Among the total tested *Bt* strains against *Z. cucurbitae*, nine isolates were found to cause the highest mortality against the 3rd instar larvae. So, therefore, Out of 44 tested indigenous *Bt* strains only nine potential strains were used in this experiment, were consider to observed the phenotypic characteristics with the phase contrast microscope upon sporulation. Number of spores/ml suspension as well as the concentration of protein mg/ml of the effective strains were also determined and recorded as shown in **Table 3**.

Table 3: Phenotypic Characteristics of the potential indigenous *Bt* strains tested against *Z. cucurbitae* (3rd instar larvae)

Bt Strains (Potential)	Size	Shape	Color	Margin	Consistency	Elevation
FhSb3	Medium to large	Round	Off white	Wooly	Opaque	Raised
JDc1	Medium to large	Round	Off white	Wooly	Opaque	Raised
JSd1	Medium to large	Round	Off white	Wooly	Opaque	Raised
KSa2	Medium to large	Round	Off white	Wooly	Opaque	Raised
MuSc2	Medium to large	Round	Off white	Wooly	Opaque	Raised
Nasc3	Medium	Round	Off white	Entire	Opaque	Raised
RaSa2	Large	Round	Off white	Entire	Opaque	Raised
SaS6	Large	Round	Off white	Entire	Opaque	Raised
TaSa4	Medium	Round	Off white	entire	opaque	flat

Spore Counting and Protein Concentration Estimation

Of these 44 tested indigenous *Bt* strains only nine effective strains were used in this experiment, were considered to determine the number of spores/ml suspension as well as the concentration of protein mg/ml and were recorded as shown in Table 4.

Table 4: Spores count and estimation of Protein concentration of the potential indigenous *Bt* strains tested against *Z. cucurbitae* (3rd instar larvae)

***Bt* Stains (Potential)**	**Spore count/ml**	**Protein concentration (mg/ml)**
FhSb3	2×10^5	0.244
JDc1	4×10^9	0.265
JSd1	8×10^9	0.257
KSa2	3×10^9	0.562
MuSc2	113×10^9	0.421
NaSc3	32×10^6	1.226
RaSa2	3×10^6	0.342
SaS6	35×10^7	0.033
TaSa4	5.9×10^8	0.017

Discussion

This fly is known to infest over one hundred varieties of vegetables and fruits [47]. The most preferred plant species of *Z. cucurbitae* is the cucurbitaceous plant. The cucurbitaceous vegetables are the largest groups in the vegetable kingdom with their wide adaptation from arid to the humid tropic environments and *Z. cucurbitae* cause more than 60% yield loss of cucurbits in the many parts of the world as a devastating pest [48]**.** The usage of chemical insecticides in forest and agricultural sector to control pests gave negative effects that lead to bad soil management, deforestation and pollution. Biopesticide derived from *Bacillus thuringiensis* can solve this problem [49]**.** This research was, therefore, carried out to determine the efficacy of certain indigenous *Bt* strains, that can be potential against the melon fly, *Z. cucurbitae*, an important vegetable damaging pest in Bangladesh. Larval stage of this fly is considered as the most damaging stage. So this experiment was concerned with the use of effective *Bt* strains against the larval stage of *Z. cucurbitae* which will in turn be helpful eradicate this melon fly. Another study was to manage this fly by using the botanicals against the pupal stage [50]. There was a report also to control *B. cucurbitae* by using the Soybean Trypsin Inhibitor (SBTI) against the 2nd instar larvae of this fly [51]. Bio-insecticidal activity of Ethiopian native *Bt* isolates against *Galleria mellonella* L. and tomato whitefly, *Bemisia tabaci* (Genn.) were published very recently [52].

B. thuringiensis, the entomo-pathogenic bacterium, has gained considerable attention since 1960 and is widely preferred over chemical insecticides for eco-friendly pest management as it is environmentally benign [25]. Development of novel potential *Bt* strains is required to solve the problem of resistance that has been reported in many pests against different *Bt* biopesticide formulations and transgenic *Bt* crops [38]. Hence, the screening for potential *Bt* strains that are highly toxic against different insect orders including resistant species is a rudimentary and continuous process all over the world and many such novel *Bt* strains have been recovered from numerous sources such as soil, grain dust, diseased insect larvae, animal feed mills and aquatic environments [25]. *Bt* is widely distributed and approximately 70% of soil samples from all continents have been found to produce this bacteria, and are especially plentiful in Asian samples. Because of this, the particular interest of this research was to isolate and identify potential *Bt* strains with novel toxicity against the melon fly, *Z. cucurbitae*, an important vegetable-damaging pest in Bangladesh. *Bt* strains used in this study were observed with the phase contrast microscope upon sporulation. Crystal proteins and the spores were distinguished from each other as the lights of different phases were passed through the specimens that revealed glowing spores and dark crystal proteins. *Bt* has also been reported to produce parasporin, another type of parasporal crystal protein which has anti-cancer cell activity. It was shown that parasporal proteins from non-hemolytic *Bt* strains are mainly noninsecticidal but may have anti-cancer cell activity [53]. That is why, hemolytic strains were mainly used in *cry1* and *cry1A* gene detection. All of the biotypes describing different subspecies were found to consist of both hemolytic and non-hemolytic *Bt* strains [38]. It was reported from the previous studies that the *cry1* gene was present mainly in the biotypes of *kurstaki, thuringiensis, sotto, dendrolimus, morrisoni, galleriae* and *darmstadiensis* [54]. *Bt* toxin was reported to cause mortality (> 65-80%) to the olive fruit fly, *Bactrocera oleae* [36]. However, there has been no report of *Bt* toxicity against the melon fly, *Z. cucurbitae*, nor is it listed in the toxin specificity data summary. Though Dipteran insect orders have been found to be susceptible to *Bt* subsp. *israelensis* and mostly to *Cry4*, *Cry10* and *Cry11* proteins, *Cry1Ab* and *Cry1Ac* proteins were also found to exert toxicity against them (The Canadian Forest Service: http://cfs.nrcan.gc.ca/projects/119/6). In these concerns, the toxicity of *Bt* strains harbouring *cry1A*-type gene was tested against the early 3rd instar larvae of *Z. cucurbitae* in this study. The larvae were fed on sweet gourd paste in which a *Bt* spore-*Cry* protein suspension was mixed. The larvae were observed up to 7 days as the unaffected larvae grew up into pupae and finally matured into fly. On the other hand, the effect of *Cry* toxins over the larvae was evidenced as their movement and feeding gradually stopped, and finally they turned black and died (Fig.3B). The result of the bioassay performed in this experiment revealed that the indigenous *Bt* JSd1 and reference *Btk* HD-73 were highly toxic to the melon fly larvae. The experiment was repeated thrice, and each time it was done in triplicate to evaluate the results statistically. *Bt* strains causing more than 50% mortality were studied to determine the lethal concentrations. Thus, nine potential indigenous *Bt* strains and one reference strain, *Btk* HD-73, were used. The logarithmic value of the concentration of spores was the basis for LC_{50} and LC_{99} determination instead of proteins because actual amount of the active proteins should be confirmed for the strains expressing more than one protein. The LC_{50} value of *Bt* JSd1 and the LC_{99} value of *Bt* JDc1 were found to be highly comparable to those of *Btk* HD-73 (Table 1). 16S rRNA gene sequence analysis has been used as a molecular identification tool for *Bt* and the claims of its ability to discriminate *Bt* in different H-serotypes also was reported [55-60].

The insecticidal potential of a *Bt* strain can be more appropriately ascertained by detection of *cry* genes present followed by analysis of crystal proteins produced by that strain [61]. The synergistic effects of *Cry* proteins encoded by *cry1*, *cry2*, *cry3* and *cry9* are the causes of novel toxicity of the indigenous *Bt* strains against *B. cucurbitae* and that the $_{Cry}$1Ac protein is the toxic agent in *Btk* HD-73, already has been reported [39]. Therefore, it can be concluded that the traditional control measures using chemical insecticides have many disadvantages, such as pesticide residues, expensive and the inability of the insecticides to penetrate infested fruits to kill larvae. Moreover, the use of

environmentally friendly *Bt* Biopesticide is the present public demand for insecticide free fresh fruit and vegetables has been encouraged for pest control program.

Conclusion

The findings from this experiment will be a valuable resource for further inspiration and research for the isolation of efficient indigenous *Bt* strains i.e., *Bt* JSd1 as pest controlling agents and replacement of hazardous chemical pesticides by environment-friendly *Bt* Biopesticide which is today's burning public demands. So, the advancement work in larger volume is required which would be most promising to lead to open a new door in the future biological control strategies to solve the Melon flies problem eco-friendly which in turn will prevent bioaccumulation and biomagnification of toxic substances from the food chain as well as enhance the food safety and security in Bangladesh.

Conflict of Interest

The authors declare that they have no conflict of interests.

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