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Characterization and mosquitocidal potency of a *Bacillus thuringiensis* strain of rice field soil of Burdwan, West Bengal, India

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ABSTRACT

Bacillus thuringiensis is the most popular mosquitocidal bacteria, strains of which are effective against almost all mosquito larvae. It has host specificity and thus, has no adverse effect on non-target species of the ecosystem. *Culex tritaeniorhynchus*, a vector of Japanese encephalitis (JE), breeds in vast area of rice fields in Burdwan district of West Bengal, India, which has already confronted JE epidemic.

Entomological investigation and ecological studies on this vector mosquito showed that JE epidemic may reoccur anytime in the area. A strain of Bt (BU55) was isolated from rice field soil, efficacy was tested against *Cx. tritaeniorhynchus* and mosquitocidal role was confirmed against *Cx. quinquefascistus* also. The LC_{50} of *Bacillus thuringiensis* BU55 against *Cx. tritaeniorhynchus* and *Cx. quinquefascistus* after 72 h was 8.59 ml (final dose 2.49 $x10^7$ CFU/ml) and 7.52 ml (final dose 2.20 x 10^7 CFU/ml), respectively. Insecticidal crystal protein profile of BU55 produced 136.89, 64.80, 43.45, 33.65 and 26.98 kDa bands. Among them 136.89, 64.29, 26.98 kDa proteins are comparable to actual toxins viz. Cry1Ac (138.3 kDa, Lepidoptera specific), Cry4D (68.0 kDa, Diptera specific) and Cyt (27.4 kDa, Diptera specific). The results clearly showed that the Bt strain is a potent dipteran larvicide and can be used against the JE vectors to control the disease.

1. Introduction

Bacillus thuringiensis (Bt) is the most popular mosquitocidal bacteria, which has high larvicidal activity and is used for decades against mosquito larvae as one of the most powerful biocide [1–6]. Bt is a Gram positive, rod shaped, motile bacterium that occurs commonly in soil, root surface, leaf litter, insect faeces or part of flora of many insect gut etc. [7–14]. Bt is fermentation friendly and therefore, commercially exploitable [15]. Broadly, the Bt strains are species or host specific or have narrow host range [1,2,4,5]. The Cry4A, Cry4B, Cry4D, Cry10A, Cry11A crystal proteins of *B. thuringiensis* have insect spectra limited to mosquitoes, as well as, blackflies [5,16]. Another Cry unrelated dipteran toxin, CytA (27 kDa) binds with the lipids of the membrane and acts synergistically with Cry toxin to enhance the toxicity [5,12,17]. Bt strains and toxins show outstanding diversity which may be due to its high degree of genetic plasticity [13–15,18]. Prolonged use throughout the world is now a days causing emergence of resistance against different Bt strains [19], though in a very slow rate. The resistance drift found in mosquitoes made scientists keen to find new strains of *B. thuringiensis* or other mosquitocidal bacteria. Thus, aim of the proposed study was to search new biocidal strains of Bt for biocontrol of *Cx. tritaeniorhynchus*, the predominant vector of JE in Burdwan, W.B., India.

2. Materials and methods

2.1. Isolation of the bacteria from soil

Nine (9) plots of rice fields were randomly chosen from the University Farm House; Tarabag (23°15′7″ N, 87°50′35″ E) for bacteria isolation. Sub-surface soil (up to 1 cm) were excavated and samples were collected in sterile plastic bags, brought to the laboratory for further processing for isolation of different strains of Bt [20].

Five (5) samples, 10 g each, were collected from each location from 5 spots of each plot. Collected soils were mixed thoroughly, air dried up to 20% moisture level, powdered, sieved (200 mesh), put in the polythene bags and stored in desiccators. Soil sample (1g) was suspended in 100 ml

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sterile (autoclaved at 121°C for 15 min as general practice unless otherwise mentioned) distilled water; serial logarithmic dilutions were prepared up to 10^{-6} level. Ten μl of 10^{-3} dilution suspension was added with 100 ml nutrient agar (NA) medium and pour plated. Plates were incubated for 72h at 30 ± 1°C in a BOD incubator. Crystal producing colonies were isolated confirming presence of crystal inclusions under 100X objective of a phase contrast microscope. Isolated bacteria were purified and confirmed for spore and crystal formation under the microscope, slants and stabs of pure cultures were prepared and incubated for 72h at 30 ± 1°C temperature. Each bacterial isolate was grown on a rotary shaker for 72h to attain about 0.20–0.24 O.D. at 620nm i.e. about 2.90 – 6.50 × 10^8 cfu/ml culture and concentrated up to 10^9 cfu/ml for bioassay.

2.2. Characterization of the bacteria

Morphological, cultural and staining characters viz. size, shape, colour, opacity of the colonies, margin and morphology and staining characters of the vegetative cells as well as physiological and biochemical characters such as NaCl tolerance, catalase, oxidase, nitrate reduction, citrate utilization, MRVP, indole production, urease, acid and gas production, extracellular enzymatic activity (starch, protein and lipid hydrolysis) of the isolates were recorded following standard methods of identification [21–23]. Antibiotics viz. gatifloxacin (5 μg/disc), levofloxacin (5 μg/disc), doxycycline (30 μg/disc), tetracycline (30 μg/disc), chloramphenicol (30 μg/disc), rifampicin (5 μg/disc), ofloxacin (5 μg/disc), kanamycin (30 μg/disc), nalidixic acid (30 μg/disc), gentamicin (10 μg/disc), ampicillin (30 μg/disc), and streptomycin (10 μg/disc) sensitivity of the organisms were also recorded. Diameter of the inhibition zone of sensitive bacteria was measured with an antibiotic zone scale and compared with a standard antibiotic sensitivity chart [22].

2.3. Scanning electron microscopic observation of the bacteria

The smears of bacteria were done on a cover glass and fixed over a flame for a few seconds, followed by glutaraldehyde (2.5%) treatment for 45 min, dehydrated the smears by passing through graded alcohol series (50, 70, 90 and 100% ethanol, 10 min each), followed by gold-coated the slides and observed under a Hitachi Model S-530 scanning electron microscope (SEM) [18].

2.4. Isolation, amplification of 16S rDNA and phylogeny analysis of the bacteria

The genomic DNA of the bacterium was isolated following Janssen [24]. Bacterial pellet (spun at 10000 rpm, 10 min, 4 ± 0.1 °C) obtained from 1.5 ml Luria Bertani (LB) broth (g/l: tryptose 10, yeast extract 5, NaCl 5, pH 6.8) culture was suspended in 576 μl TE buffer, 30 μl 10% SDS, 3 μl proteinase K (20 mg/ml in 0.5% SDS solution) were mixed, incubated for 1h at 37 ± 0.1 °C, 100 μl 5M NaCl was added mixed thoroughly, 80 μl CTAB/NaCl solution (10% CTAB in 0.7 M NaCl) was added, incubated for 10 min at 65 ± 0.1°C. Equal volume of chloroform/isoamyl alcohol mixture (1:1 vol mixture) was mixed, spun (4–5 min), aqueous viscous supernatant was collected, equal volume phenol-chloroform/isoamyl alcohol (1:1 vol mixture) was added spun for 5 min, supernatant was collected and 0.6 vol isopropyl alcohol was added, mixed gently to precipitate DNA, centrifuged, supernant was discarded, DNA pellet was washed with 70% ethanol to remove residual CTAB, re-centrifuged, pellet was collected and dried in a lyophilizer. Pellet was dissolved in sterile distilled water, and 0.1% SDS and 1–2 μg/ml RNase A were added, incubated at 37 ± 0.1°C for 1h and DNA was extracted with phenol–chloroform/isoamyl alcohol. About 15 kbp rDNA fragment was amplified by PCR using universal primers 27F (5′AGAGTTTGATCCCTGGCTCAG3′) and 1492r (5′AAGGAGGTG ATCCAGCCGCA3′) [24,25] following the PCR protocol of denaturation for 5 min at 95 °C once, followed by 30 cycles for 30 s at 95°C, 30 s at 55°C, 2 min at 72°C, final extension at 72°C for 10 min and 4°C for 10 min. The 16S rRNA gene was sequenced, BLAST (BLASTN) searched with NCBI database to investigate the most similar sequences, the sequences were aligned and analyzed by ClastalW programme [26]. Evolutionary distances were calculated according to Jukes and Cantor [27], topology was determined through the 'neighbour-joining' method [28] and phylogenetic tree was prepared following Tamura et al. [29].

2.5. Crystal protein production assessment

In addition to scanning electron microscopic visualization, assessment of crystal protein production was done by SDS-PAGE analysis. Seven (7) day old 100ml bacteria culture (at 30 ± 0.1°C) in nutrient broth (NB) was centrifuged (10000 rpm, 10 min, 4 ± 0.1°C), pellet was washed 3 times with 50 ml crystal wash (1 M NaCl having 0.1% SDS) solution and once with sterile distilled water. Then suspension was made in 3 ml sterile distilled water with the pellet and thoroughly mixed with same volume of alkaline solubilization buffer (50 mM anhydrous Na_2CO_3 containing 10 mM dithiotheitol (DTT), pH 10) and incubated for 12h at 37 ± 0.1°C. Neutralization of pH of the solubilized crystal was done with the 0.5 M HCl, treated with 1/10 vol aqueous trypsin (200 U/g potency) solution (1 mg/ml) and incubated at 37 ± 0.1°C for 3–4h, again equal amount of trypsin was added and incubated at 37 ± 0.1°C for 12h, centrifuged at 4 ± 0.1°C at 10000 rpm for 15 min and the supernatant was taken for further processing. Protein profile of trypsinized extract was determined by SDS–PAGE by the cellular protein analysis method [20].

2.6. Collection and maintenance of mosquito larvae

The immature mosquitoes were collected from Bt isolation plots of rice fields. Sampling was performed following 'stratified random sampling' [30,31] using plankton net (mesh size 200 μm) fitted with long wooden handled iron frame [32]. The collected larvae were poured out in the laboratory in enamel trays (46 × 32 × 6 cm) containing tap water and allowed to settle. Following settlement of mud, the larvae and pupae were segregated and placed in labelled glass vials. Segregated larvae were identified on the basis of generic characters [33]. *Cx. tritaeniorhynchus* larvae were segregated and used for bioassay experiments.

2.7. Bioassay of the bacteria

Bioassay was conducted following WHO [34] in disposable containers (500 ml, white) containing 100 ml of dechlorinated tap water with batches of 25 larvae (late 3rd instar), supplied with 4–5 grains of Tokyu® fish food, maintained at room temperature and a 12:12h LD photoperiod in the laboratory. Two types of positive control experimental sets were maintained along with the treatments, one with dechlorinated tap water mixed with nutrient broth (the ratio was applied at per with the doses, such as 5 ml, 10 ml, and so on) and stipulated food, whereas, the other control experiment had only dechlorinated tap water with stipulated food. Observations of the static bioassay tests were utilized for estimation of the LC_{50}. Crystal and spore forming bacteria were purified and slants and stabs of pure cultures were prepared and incubated for 72h at 30 ± 1°C temperature. Each bacterial isolate was grown on a rotary shaker for 72h to attain about 0.20–0.24 O.D. at 620 nm i.e. about 2.90–6.50 × 10^8 cfu/ml culture and concentrated up to 10^9 cfu/ml for bioassay. Five doses of BU55 strain i.e., 5 ml, 10 ml and 12 ml, 13 ml and 15 ml bacterial inoculum/100 ml water were then separately tested using the 2.90–6.50 × 10^8 cfu/ml bacterial suspension along with positive controls without bacteria for each mosquito species. At 24 h time intervals i.e. at 24, 48 and 72h, mortality was recorded from treatments of five different doses of the bacteria which were subsequently transformed to log values for probit analysis [35].

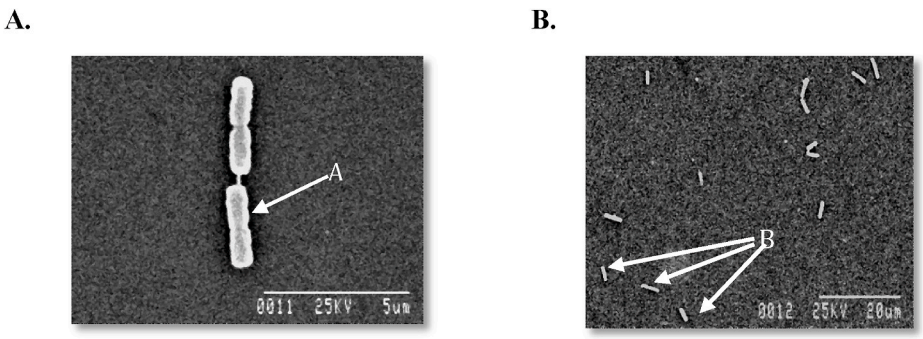

Fig. 1. SEM photograph of vegetative cell of BU55. A = 10000X, B = 1500X

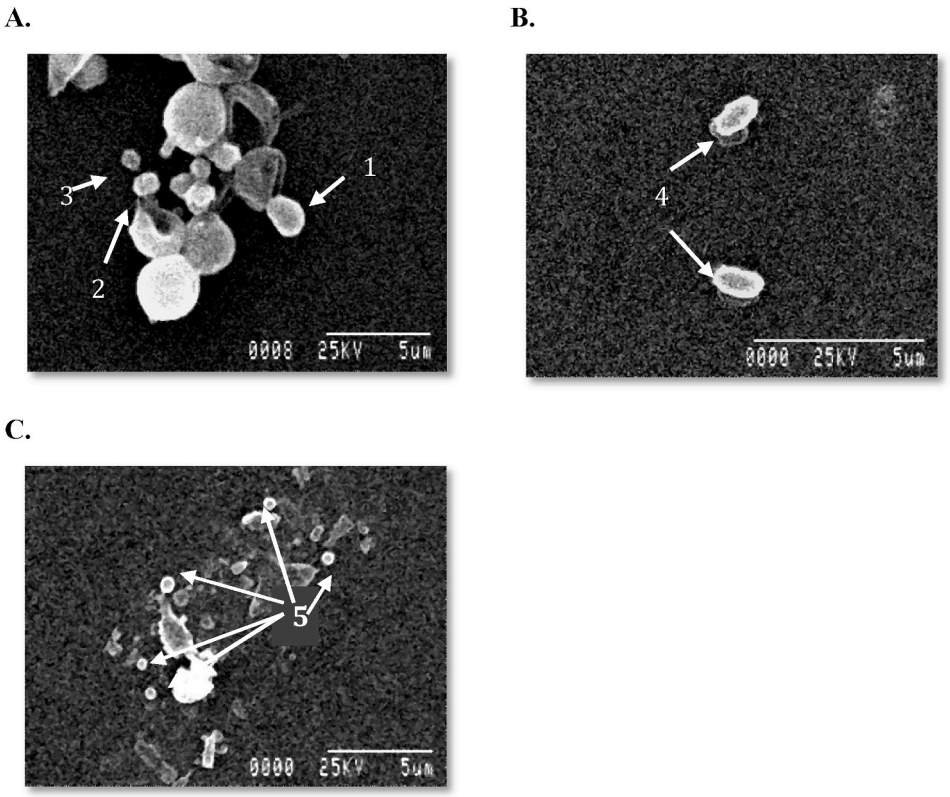

Fig. 2. SEM photographes of spores and crystals of BU55. A. 1 and 2 = spores with ruptured exosporium and 3 = spherical crystals (8000X), B. 4 = spores (10000X), C. 5 = spherical crystals (6000X).

3. Results and discussion

3.1. Morphological attributes

The crystal producing bacterial isolate BU55 produced elevated, gummy light brown colonies with entire margin measuring 4.152 ± 0.03 mm diameter. Vegetative cells were rod shaped with rounded ends, motile and Gram positive (Fig. 1). Scanning electron microscopic image showed elliptical spores [1.67 ± 0.09 × 0.98 ± 0.02 μm (length x breadth)].The organism produced spherical crystals (1.01 ± 0.01 μm in diameter) (Fig. 2). All the attributes are enlisted in Table 1.

3.2. Biochemical properties

BU55 tolerated and showed its growth upto 14% NaCl supplemented NA and at a temperature range of 4–50°C. The organism utilized fructose, sucrose and glucose as carbon sources but not mannose. It produced extracellular enzymes to digest cholesterol, Tween 80 and gelatin but not casein. It was catalase, methyl red, Voges-Proskauer, and nitrate reduction tests positive but negative for indole production, citrate utilization, urease and oxidase tests (Table 1). BU55 was resistant to penicilin G (10U), ampicilin (10 μg), nystatin (100U), nalidixc acid (30 μg) and doxycyclin hydrochloride (30 μg) (unit/disc). Sensitive to other enlisted antibiotics (Table 2). Resistance of the Bt to penicilin, ampicilin, nystatin, nalidixc acid and doxycyclin hydrochloride (Table 2) complied with other counterparts and approved that Bt are generally resistant to ampicillin and antifungal (nystatin) group of antibiotics [14,36,37]. Nevertheless, the phenotypic characters confirmed and identified BU55 as *B. thuringiensis* [38].

3.3. Phylogeny analysis

The phylogram of BU55 (KY978071) revealed that the BU55 (KY978071) branched with the cluster consisting of *B. thuringiensis*

Table 1
Cultural, morpho–physiological and biochemical characters of the isolate BU55.

Attributes	Observations
Colony character	Spherical, light brown, raised, entire, gummy,
Colony diameter	4.152 ± 0.03
Cell Shape	Rod
Cell length and bredth (μm)	3.5 ± 0.32, 2.32 ± 0.23
Motile	+
Filament	–
Rods/filaments curved	–
Cocci in tetrads or packets	–
Endospore	+
Spore character, length and bredth (μm)	Elliptical, 1.67 ± 0.09, 0.98 ± 0.02
Strict aerobe	–
Gram stain	+
Sporangium	NS
Crystal	+
Crystal character, diameter (μm)	Spherical, 1.01 ± 0.01
Facultative anaerobe or microaerobic	–
Strict anaerobe	–
Carbon sources used	Glucose, Sucrose, Fructose, Mannose
Extracellular enzymes produced	Protease (gelatin), lipase, amylase
Catalase	+
Oxidase	–
Marked acidity from glucose	+
Nitrate reduced to nitrite	+
Indole	–
MR	+
VP	+
Citrate	–
Urease	–
Remarks	
Genus	*Bacillus*
Species	*thuringiensis*

(+) = positive growth, (−) = negative growth, NS = non swollen.

Table 2
Antibiotic sensitivity of the isolate BU55.

Name of the antibiotic	Inhibition zone	Diameter (mm)
Vancomycin (30 mg)	S	17
Penicillin G (10 U)	R	**0**
Polymyxin B (300 U)	S	14
Norfloxacin (10 mg)	S	14
Bacitracin (10 U)	S	9
Ampicillin (10 mg)	R	0
Erythromycin (15 mg)	S	14
Gentamycin (10 μg)	S	14
Tetracycline (30 μg)	S	16
Amoxycillin (10 μg)	S	21
Nystatin (100 U)	R	0
Chlorotetracycline (30 μg)	S	14
Kanamycin (30 μg)	S	20
Chloramphenicol (30 μg)	S	14
Ciprofloxacin (5 μg)	S	28
Rifampicin (5 μg)	S	20
Streptomycin (10 μg)	S	20
Trimethoprin (30 mg)	S	13
Triple sulphas (300 mg)	S	13
Levofloxacin (5 μg)	S	20
Nalidixic Acid (30 μg)	R	10
Gatifloxacin (5 μg)	S	30
Doxycyclin hydrochloride (30 μg)	R	0
Ofloxacin (5 μg)	S	28

Data in the result column are the mean of inhibition zone diameter including the disc diameter (6 mm). R = resistant, S = sensitive.

(EF501373) with 30% bootstrap value (Fig. 3). The organism contained 53.10% GC and 46.90% AT in 16s rDNA sequence. Therefore, the phylogenetic identity confirmed the isolate BU55 (KY978071) as *Bacillus thuringiensis* [38,39].

3.4. Crystal protein profile

The insecticidal crystal inclusions of BU55 possessed different protein fractions i.e. 136.89, 64.80, 43.45, 33.65 and 26.98 kDa mol. wt. out of which 136.89, 64.29, 26.98 kDa fractions were comparable to actual toxins viz. Lepidoptera specific Cry1Ac (138.3 kDa), Diptera specific Cry4D (68.0 kDa) and Cyt (27.4 kDa) (Fig. 4, Table 3). Different types of toxins in the same crystal proved that the Bt BU55 would be a broad spectrum biocide [20,40,41] and would be effective against various pests of rice and other crops. Besides, production of different anti-pathogenic enzymes (Table 1) like amylase that hydrolyze α−1,3/1, 4 or β−1,3/β-1,6 glycosidic bonds of carbohydrates, protease that metabolize mannoproteins and glycoproteins, lipase that lyses lipoproteins, phospholipids etc. of cell wall of bacteria/fungi proved that BU55 would also give protection to plants from pathogens [39,41–43]. Thus, it is plausible that the Bt BU55 would be simultaneously effective against insect and plant pathogens.

3.5. Bioassay outcome

LC_{50} value of BU55 against *Cx. tritaeniorhynchus* at 72h was 8.59 ml (final dose 2.49×10^7 cfu/ml) with fiducial limit 7.77–9.82 ml (Table 4). Besides, the organism was virulent against *Cx. quinquefasciatus* and LC_{50} at 72h was 7.52 ml (final dose 2.20×10^7 cfu/ml) (Table 4). The pathogenicity results proved that the resident Bt was effective against *Cx. tritaeniorhynchus* that breeds in the rice field which suggests that the biocide would naturally infect the mosquito larvae and suppress the vector. Besides, the Bt was virulent to *Cx. quinquefasciatus* which fulfilled the proposition of broad host range of the organism and indicated wide vector control potency of the organism in general.

Nevertheless, the LC_{50} values were comparable to many other larvicidal bacteria such as *Aneurinibacillus aneurinilyticus* [44] as well as, other strains of Bt such as SB1 [18]. In both cases BU55 was proven more effective than those pathogens. BU55 showed LC_{50} final dose 2.20×10^7 cfu/ml against *Cx. quinquefasciatus*, whereas, SB1 showed 6.32×10^8 cfu/ml against *Cx. quinquefasciatus* [18] and *A. aneurinilyticus* showed LC_{50} value 22×10^9 cfu/ml against *Cx. quinquefasciatus* [44].

4. Conclusion

Present study depicted the effective larvicidal role of *Bacillus thuringiensis* strain BU55 against *Cx. tritaeniorhynchus* mosquito. Although the bacterial strains showed similarities to both *B. thuringiensis* and *Bacillus cereus* in respect to phenotypic and biochemical properties, but the presence of crystals and crystal proteins confirmed the strain as *B. thuringiensis*. Molecular phylogeny again confirmed the same. The synergistic action of Cry proteins and Cyt protein suppressed the population of mosquito larvae. The crystal proteins of *B. thuringiensis* BU55 were target specific and very much effective against the larvae of dipterans and lepidopterans also. So, this target specific, eco-friendly strain of *B. thuringiensis* may be explored in the management of *Cx. tritaeniorhynchus* larvae as well as in the control of Japanese Encephalitis in Burdwan and other parts of India.

Author contribution

SC and TKD contributed to Experiment designing, supervision of the research work, manuscript examining and rectifications. Lab work, data collection, data analysis, compilation, manuscript preparation, reviewing, editing was done by MR.

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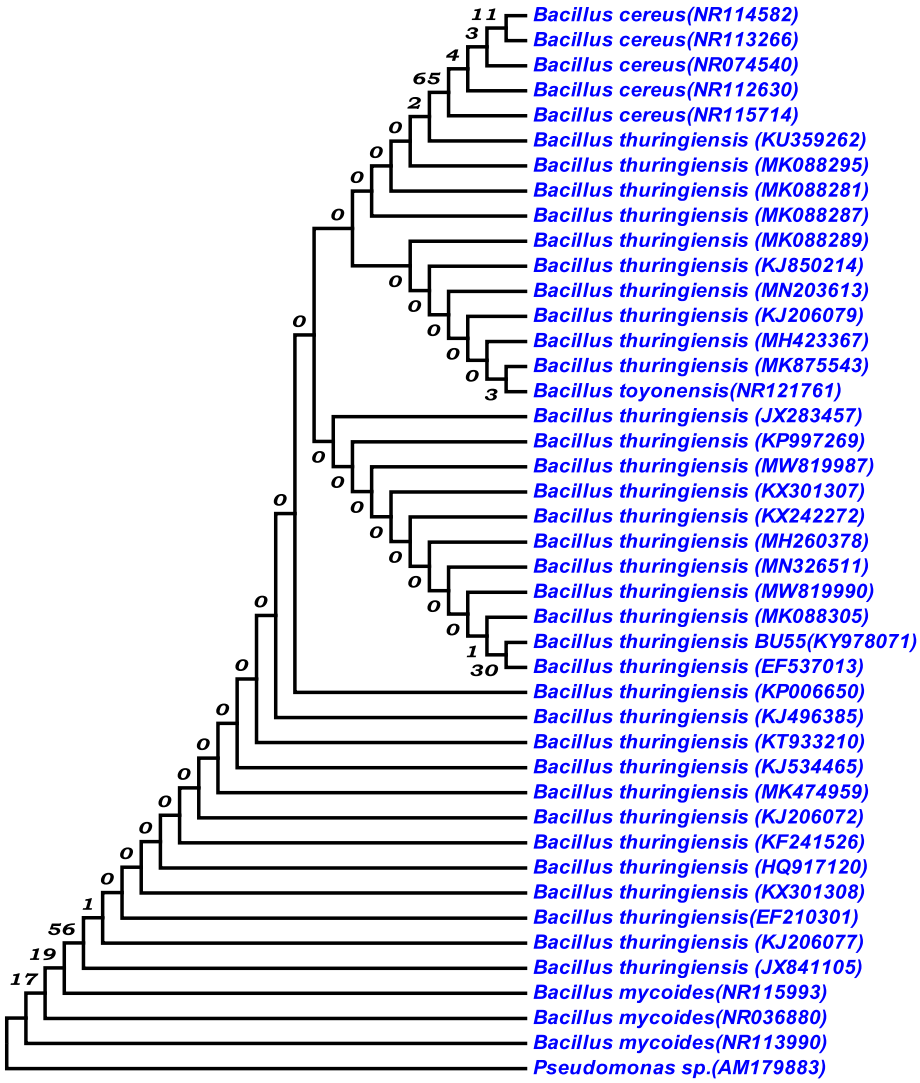

Fig. 3. Phylogram of BU55 (showing 30% bootstrap value).

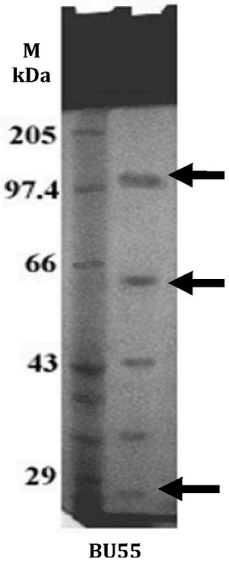

Fig. 4. Protein profile of BU55.

Table 3
Crystal protein profile of the isolate BU55.

No. of bands	Molecular weight (kDa)	Predicted toxin (kDa)	Actual toxin type (kDa)	Host specificity range
5	136.89, 64.80, 43.45, 33.65, 26.98	Cry1Ac −136.89 Cry4D −64.80 Cyt – 26.98	Cry1Ac −138.3 Cry4D – 68.0 Cyt – 27.4	Lepidoptera Diptera Diptera

Table 4
The LC_{50s} of the isolate BU55 against two mosquito species.

Culex tritaeniorhynchus (CT)			*Culex quinquefasciatus* (CQUI)		
Upper limit	LC_{50}	Lower limit	Upper limit	LC_{50}	Lower limit
9.82 ml (0.99)	8.59 ml (0.93)	7.77 ml (0.89)	8.32 ml (0.92)	7.52 ml (0.9)	6.61 ml (0.82)

The values given within parentheses are the log values.

Declaration of competing interest

The authors declare that the research was conducted in the absence of commercial or financial relationships that could be construed as a potential conflict of interest.

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