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ORIGINAL CONTRIBUTION

New *Bacillus thuringiensis* strain isolated from the gut of Malabari goat is effective against *Tetranychus macfarlanei*

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Keywords
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Abstract

This study illustrates a novel strain (designated as BPU5) of *Bacillus thuringiensis* (*Bt*) isolated from the rumen of Malabari goat, capable of producing polymorphic δ-endotoxin crystals concomitantly with sporulation in Luria–Bertani medium (LB), and the δ-endotoxin was efficient to combat *Tetranychus macfarlanei,* a devastating mite. Polymorphic δ-endotoxin crystals produced were assessed by scanning electron microscopy and monitored its production concomitantly with sporulation in LB with or without sugar supplements. Toxicity of the δ-endotoxin was assessed on *T. macfarlanei* using leaf disc bioassay method. Mortality rate was determined by comparing the survival of mites on the diet (prepared in 10% sucrose and powdered rice husk) containing different concentrations (1–10 mg/ml) of 72-h-old crude pellet (dried mixture of δ-endotoxin (17 mg/g pellet), endospores and a few vegetative cells) or control diet with autoclaved pellet. The maximum production (1.39 mg/ml) of δ-endotoxin was observed at 72 h in LB. Among the sugars (glucose, sucrose, maltose or lactose) tested as additional carbon source, glucose (8 g/l) enhanced (1.82 mg/ml) the production of δ-endotoxin by 30%. The lethal concentration (LC_{50}) required to kill 50% mites was estimated as 8.024 mg/ml. The δ-endotoxin produced by *B. thuringiensis* BPU5 is shown to efficiently combat *T. macfarlanei,* a devastating mite infesting agricultural fields.

Introduction

Bacillus thuringiensis (*Bt*) is a soil-borne, entomopathogenic bacterium showing cosmopolitan distribution. *Bt* strains have been isolated from naturally occurring samples such as insect cadavers (Chilcott and Wigley 1993), insect larvae (Brownbridge and Margalith 1986) and stored grains (Delucca et al. 1982), coupled with these, other habitats of *Bt* include the following: leaves (Ohba 1996), faeces of wild shika deer (Ohba and Lee 2003), guano of penguin (Forsyth and Logan 2000), aquatic and terrestrial environments (Martínez and Caballero 2002), etc. This ubiquitous nature of *Bt* makes its exploitation in the area of pest management. But the prevalence of bacterium in the digestive system of animals is lower than those reported in soil, litters of leaf, sediments, etc. *Bt* may enter into the gut of animals along with the food they consume. Species of *Bacillus* (not *B. thuringiensis*) were isolated from ruminants such as sheep, goat and cow (Oyeleke and Okusanmi 2008). Rumen harbours a complex consortium of different microbial taxa, living in symbiotic relationship with the host. In fact, this study is a report describing the inhabitance of a strain of *B. thuringiensis* in the rumen of a goat, the Malabari goat, along with its utility to control mite.

The characteristic insecticidal activity of *Bt* is solely due to the crystalline proteins (δ-endotoxin), produced concomitantly with endospore – both are toxic to the larvae of various insects belonging to

lepidoptera, coleopteran and diptera; of them, lepidopterans have exceptionally been well studied (Crickmore et al. 1998; Schnepf et al. 1998). The δ-endotoxins produced by *Bacillus* sp. are often insect species specific and they do not contaminate the environment, as these toxins have lesser residual efficacy and are generally safe for non-target organisms (Jisha et al. 2013).

Spider mites (*Tetranychus macfarlanei* Baker and Pritchard) often spin characteristic protective silky webs and attack a number of crops, which include vegetables, cotton, tea, fruit, ornamentals and more than 300 other plant species. Higher population of spider mite cause extensive leaf damage, resulting in reduced crop yield and quality (Jeppson et al. 1985). Spider mites are very tiny; the adult females are only 0.3–0.5 mm long, and the males are even smaller. They are often red, brown, green or yellow in colour, and they can be seen clearly with the aid of a lens. Their immature stages resemble the adults except in size and legs (they bear three pairs of legs at the larval stage and four pairs of legs at pre-mature and adult stages). During summer, the spider mites lay eggs on the abaxial side of the leaves and multiply rapidly during the hot and dry weather; however, their population declines at the onset of rains. The rapid rate of multiplication of mites under ideal conditions demands the application of control measures, rather than the post-affected treatments.

The medium for the large scale production of *Bt* biopesticide is generally composed of complex carbon and nitrogen sources such as commercial media, corn steep liquor (Saalma et al. 1983), gruel-based media (Zouari and Jaoua 1999), fish meal or their combinations (Zouari et al. 2002) and potato flour (Smitha et al. 2013). As a preliminary step in the formulation of media for the industrial production of *Bt*-based biopesticides, it is also significant to evaluate the effect of supplements, especially carbon sources in the medium on the growth and the production of δ-endotoxin. It is possible to exploit the toxicity potentials of *Bt* for efficiently combating the devastating spider mites. Unlike in larvae, the bioefficacy studies of *Bt* on mites were carried out with nymphal and adult stages. Relative toxicity of δ-endotoxin *Bt* var. *tenebrionis* was evaluated on adult *T. urticae* Koch (Chapman and Hoy 2009). Similarly, effect of thuringiensin on mortality, fecundity and feeding habit of *T. urticae* was evaluated by Royalty et al. (1990). Toxicity to immatures and the sublethal effects suggested that thuringiensin may control field populations of *T. urticae,* despite low initial mortality (Royalty et al. 1990). Spray-dried powder of *Bt* toxin was used at a concentration of 5 mg/ml to combat *T. urticae* (Payne et al. 1993). Although a few toxicity studies with *Bt* or its formulations have been performed on *T. urticae*, no such studies are seen conducted on other mites or *T. macfarlanei,* the target of this study.

In the light of aforesaid background, this study is focused on the isolation and characterization of a novel *Bt* strain with the following specific objectives: to isolate and characterize a novel *B. thuringiensis* strain (BPU5) from the rumen of Malabari goat; to optimize the production of δ-endotoxin in the Luria–Bertani medium; to purify the δ-endotoxin produced by it; to demonstrate the δ-endotoxin produced and the bioefficacy of *Bt* strain BPU5 against *T. macfarlanei*, the spider mites seen in the locality of this study.

Materials and Methods

Isolation and characterization

Rumen content of both male and female Malabari goats (naturally grazing) was collected aseptically from the local slaughter house, as we described previously (Priji et al. 2013). Briefly, the rumen content was extracted with sterile distilled water and serially diluted, and 100 μl of each dilution was spread on nutrient agar (Peptone-1 g/l, NaCl-1 g/l, beef extract-0.5 g/l, pH-7) plates and incubated at 37°C. The isolated colonies were examined morphologically, and the purity of the isolate was confirmed by repeated subcultures. The pure cultures were maintained on nutrient agar medium at 37°C.

Morphological characterization

The morphological characterization was made by observing colour, texture, etc. of the colony, including the staining techniques using Gram's iodine, malachite green-safranine and coomassie brilliant blue (CBB).

Biochemical characterization

Fermentation reactions were carried out in carbohydrate fermentation broth containing glucose, sucrose or lactose as carbon source. Tests on indole production, methyl red and Voges-Proskauer; citrate and acetate utilization, motility, nitrate reduction, hydrolyses of starch and casein were also carried out.

Molecular characterization

The isolate was subjected to molecular characterization by 16S rRNA PCR amplification. Forward and reverse DNA sequencing reactions of PCR amplicon were carried out with 8F and 1492R primers using

BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer (Xcelris Labs Ltd., Ahmedabad, India) under following conditions: 5 min of denaturation at 94°C followed by 25 cycles of amplification with a 1-min denaturation at 94°C, 45 s of annealing at 45°C and 2 min of extension at 72°C. For sequence, the length and proportion of GC contents were estimated, sequence alignment was done and trimming was done using BioEdit tool (Hall 1999). Homology searches were performed with GenBank BLAST algorithm (Altschul et al. 1997). Out of a total of 1288 bases, a final alignment of 1223 bases of the rDNA sequence was used in the analyses, using ClustalW2 programme (Larkin et al. 2007).

Phylogenetic analysis was performed using maximum likelihood, neighbour joining and UPGMA method with MEGA5 (Tamura et al. 2007), parameters were fixed for a bootstrap analysis with 10 000 replicates. The majority rule consensus of the bootstrap replicates was calculated in Consense and seqboot in the PHYLIP package (Felsenstein 1993). *Paenibacillus vortex* strain V453 with GenBank accession number HQ005270.1 was designated as outgroup for the sequence, as it comes outside of ingroup, preferably sister group of ingroup.

Preparation of medium and seed culture

For preparing stock culture, the isolate was grown on nutrient agar plates and stored at 4°C. Luria–Bertani (LB) broth (composition: 1 g/l tryptone, 1 g/l NaCl, 0.5 g/l yeast extract; pH 7.0) was used throughout the study for the production of δ-endotoxin. The 12-h-old culture (4.8×10^6 cfu/ml) in LB was used as a standard inoculum for all studies described herein.

Pattern of endospore formation under different incubation periods

Endospore production pattern of the isolate in LB was monitored at an interval of 12 h. Endospores were visualized by coomassie brilliant blue staining (Jisha et al. 2014) and observed with the aid of phase contrast microscope (Zeiss, China).

Production and purification of δ-endotoxin

The δ-endotoxin was purified by the biphasic separation method (Pendleton and Morrison 1966) with minor modifications. This purified δ-endotoxin was quantified as we described previously (Smitha et al. 2013).

Effect of sugars on the production of δ-endotoxin

To investigate the effect of individual sugars on the production of δ-endotoxin, different sugars (glucose, sucrose, maltose or lactose) at a concentration of 8.0 g/l were individually added to the LB medium as additional carbon source. Cultures were harvested at 72 h for the quantification of δ-endotoxin. To detect the optimum concentration of glucose, LB was supplemented with glucose at 4, 6, 8 and 10 g/l. Cellular morphology of the isolate in the LB medium containing a sugar was also scored.

Scanning electron microscopy (SEM)

Purified crystals were mounted on a metal support using carbon tape and dried by keeping it in a desiccator for 3 h; which subsequently sputtered with gold for 60 s at 40 mA (SEM, Hitachi SU 6600, Japan).

Mites and collection

Spider mites (*Tetranychus macfarlanei* Baker and Pritchard) were collected from highly infested leaves of *Vigna unguiculata* (L.)Walp (cowpea) from cultivated fields. Mites were identified by Prof. N. Ramani, Department of Zoology, University of Calicut, Kerala.

Crude δ-endotoxin preparation

The 72-h culture was harvested and centrifuged at 9440 ***g***, 4°C to collect the pellet. The pellet thus obtained (consisted of spore-crystal mixture, a few vegetative cells and cell debris) was dried (40°C 12 h, in an oven) and used as crude *Bt* toxin for bioassay.

Rearing of mites

Mites were cultured in Petri dishes containing moist cotton, above which fresh and pest-free healthy leaves of *V. unguiculata* (cowpea) were placed carefully. These culture dishes were incubated in a big chamber (hood), where temperature was maintained at 35°C using 100 V tungsten lamps. The mites were subcultured (transferred using a fine brush) on fresh cowpea leaves twice a week, and this standardized culture was used for toxicity assay. Various growth stages of the mite obtained from this culture set-up were used to illustrate its life cycle using images taken with the aid of a stereomicroscope (Leica M80, Germany).

Bioassay (acaricidal assay)

Different quantities of 72-h-old culture pellet (1–10 mg/ml) in 10% sucrose were prepared for the bioassay. Finely powdered and pre-sterilized rice husk was used as the inert carrier for holding the δ-endotoxin. To make the slurry (artificial diet) for feeding the mites, 5 ml of the above preparation in sucrose was mixed with 1 g of powdered rice husk. A specially designed bioassay set-up with artificial diet method was chosen for this experiment. The set-up was prepared as follows: A circular glass ring was placed on a circular glass disc having surface area 3.46 cm^2 and the joint was sealed with parafilm to prevent the leakage of diet and the escaping of mites. Specific quantity of the diet (with or without δ-endotoxin) was placed as a spread in each glass ring, subsequently a blotting paper was placed above the feed as a cover. The blotting paper was moistened occasionally with 10% sucrose solution to maintain the slurry nature of the diet constantly. Using a fine brush, 15 healthy adult mites (random mix of males and females) were carefully released into the glass ring (on the tissue paper in the feeding set-up). The whole set-up (fig S1) was then covered with a glass disc (on the top), and the entire set-up placed over a raised glass support. A negative control was also prepared, in which the autoclaved (15 psi, 121°C for 15 min) spore–crystal complex (pellet) was mixed with artificial diet as above, that is, the normal toxin was replaced by autoclaved toxin. Eco Trol™ was used as a positive control in this study. The mites were allowed to feed for 24 h, which were then transferred to Petri dishes containing fresh cowpea leaves, for monitoring their survival. The number of surviving mites and their mortality was observed every 24 h up to 7 days through stereomicroscope; each experiment was repeated thrice, and mortality rate on day 4 was scored for probit analysis.

Statistical analyses

The softwares like SPSS version 20, Microsoft Excel 2007, Photoshop CS2 were used for the data analysis and presentation. Data on bioassay were scored on different dates (replicates) under the same assay conditions were pooled and used to construct the probit model (Finney 1971). Subjecting fourth day mortality, lethal concentration (LC_{50} value) required to kill 50% of spider mites was estimated. The time of death in the different groups was summarized as survival functions using Kaplan–Meier curves. The estimates were compared among the groups with a log-rank test.

Results

Characterization of isolate

Morphological characterization

Colonies were found to be white to off-white in colour with smooth texture and flat to slightly elevated margins on nutrient agar medium at 24 h of incubation. Spores and crystals were evidenced by staining techniques (fig. 1a–c). The size of the vegetative cells and spores was ranged between 2.64 to 2.93 μm and 1.61 to 1.86 μm, respectively.

Biochemical characterization

Fermentation reactions showed that *Bt* strain BPU5 utilized glucose and sucrose, but not lactose and maltose. The isolate oxidized glucose in methyl red, indole, Vogues-Proskauer tests; citrate, acetate utilization and motility tests were found negative. Nitrate reduction, starch hydrolysis and casein hydrolysis showed positive responses.

Molecular characterization

The isolate was identified as *B. thuringiensis*, which was given a strain name BPU5 (GenBank accession number KF 550913; Microbial Type Culture Collection (MTCC) number 5922). The isolate was further confirmed by nucleotide homology and phylogenetic analysis. The sequence was 1288 nucleotide in length. The GC content (%) was 51.55. The frequencies of nucleotide (%) were A (22.44), T (29.35), C (22.20) and G (26.01). For final alignment correction, 1223 bases of rDNA were used. Based on maximum likelihood method, the consensus phylogenetic tree (fig. 1d) was prepared with the twelve species showing more than 98% of similarity. The *Bt* strain BPU5 was isolated from the rest of the *Bacillus* species, but the genus identity was confirmed as it was grouped within the *Bacillus* cluster and showed close relationship supported by strong bootstrap values. However, the out-group *Paenibacillus vortex* strain V453 was grouped separately from the rest of the *Bacillus* species within this cluster.

Endospore production patterns of *Bt* strain BPU5 vs. incubation period

At 12-h incubation of *Bt* strain BPU5 in LB, all cells were in the vegetative phase, arranged compactly as pseudofilamentous structures (fig. 2a). At 24 h, vegetative cells were started to transform into sporangia, as indicated by hyaline dots (fig. 2b), which gradually enlarged to form sporangium in 32 h of

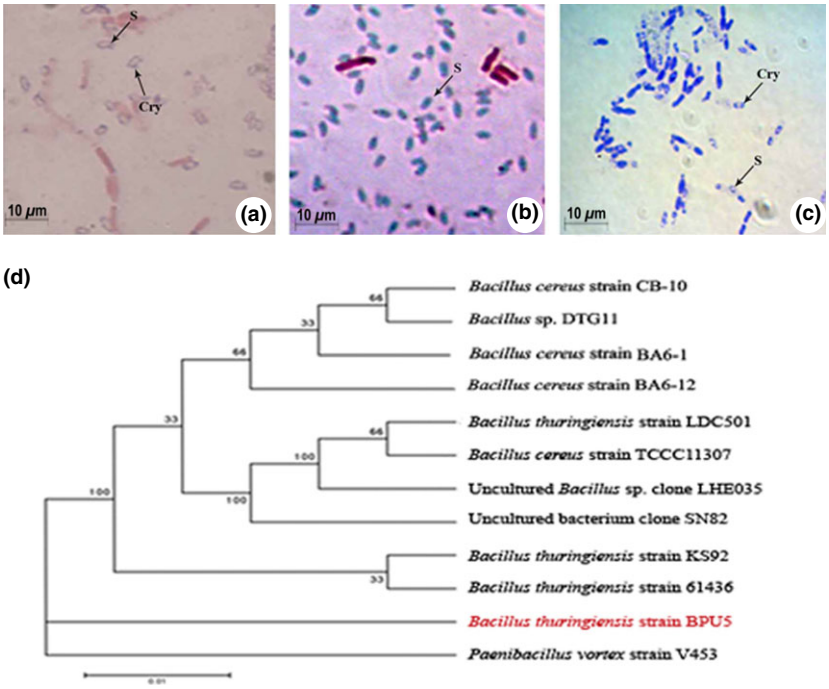

Fig. 1 Morphological characterization of *Bt* strain BPU5. (a). Gram's iodine; (b). malachite Green safranin; (c). coomassie brilliant blue; S indicates spore and *Cry* indicates the parasporal crystals. (d). evolutionary tree of *Bt* strain BPU5

incubation (fig. 2c). At 32 h, a few mature sporangia enclosing endospore were seen. Sporangia underwent lysis and started releasing endospores at about 48 h, coupled with some intact sporangia. At 48 h, the δ-endotoxin crystals were seen darkly stained by CBB, juxtaposed to hyaline endospores (fig. 2d). At 72 h of incubation, almost all cells were transformed into spores, which were released in the medium subsequently. At this stage, the maximum spore count with δ-endotoxin was observed (fig. 2f). All sporangia got ruptured, and endospores were released completely in 84 or 96 h of incubation (fig. 2g and h). The CBB staining enabled to differentiate the endospores from parasporal crystals by phase contrast microscopy.

Quantification of δ-endotoxin

The purified δ-endotoxin was quantified, that is at 48, 72 and 96 h (at 24-h interval). The maximum production of δ-endotoxin was observed at 72 h (1.39 mg/ml). At 48 and 96 h, the quantity of δ-endotoxin produced were 1.18 mg/ml and 0.367 mg/ml, respectively. Thus, for all further studies, cultures were harvested at the 72 h, the time of the maximum production of δ-endotoxin (fig. 3a).

Effect of sugars on the production of δ-endotoxin

Significant increase in the production of δ-endotoxin was also observed when sugars (glucose, sucrose,

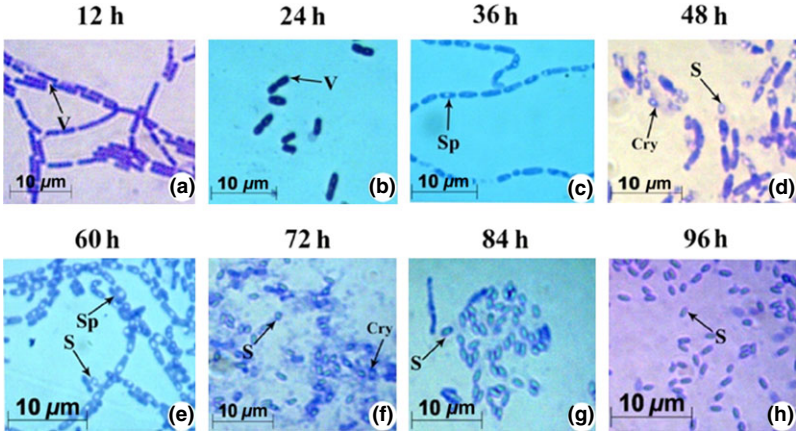

Fig. 2 Pattern of endospore production at different incubation periods and its visualization by coomassie brilliant blue staining: vegetative cells (V), sporangium (Sp), δ-endotoxin crystal (*Cry*) and endospore (S) are marked.

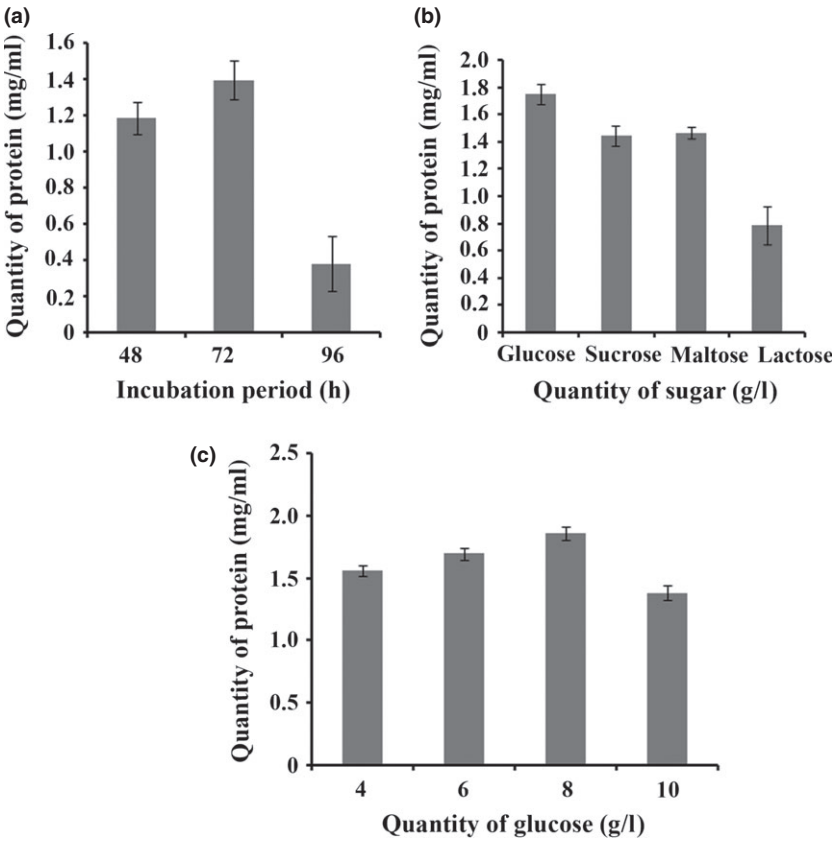

Fig. 3 Quantification of δ-endotoxin produced by *Bt* strain BPU5: (a). Quantification of δ- endotoxin at different incubation periods; (b). effects of various sugars (8 g/l) on the production of δ-endotoxin by *Bacillus thuringiensis* strain BPU5; and (c). effect of different concentrations of glucose on the production of δ-endotoxin.

maltose or lactose) supplemented in the LB medium. Among these, the presence of 8 g/l glucose as supplement in LB medium was found to be more effective for the production of δ-endotoxin (1.79 mg/ml), that is 30% increase (fig. 3b). Upon addition of maltose in LB medium, δ-endotoxin production was increased from 1.39 mg/ml to 1.45 mg/ml (4.5% increase). However, addition of sucrose showed only marginal enhancing effect (1.44 mg/ml), that is by 3.5%.

Upon increase in concentration of glucose, yields of δ-endotoxin gradually increased, but further increase in the concentration beyond 8 g/l showed retardation (1.29 mg/ml at 10 g/l). The maximum production of δ-endotoxin (1.82 mg/ml) was observed with 8 g/l glucose, followed by 6 g/l (1.75 mg/l) (fig. 3c). Along with enhanced production of δ-endotoxin, glucose also influenced the cellular morphology, that is, the length of vegetative cells in glucose supplemented LB was more (~3.64 μm) than those in unsupplemented LB medium (~2.94 μm).

Scanning electron micrographs

The δ-endotoxin crystals showed variations in size, shape and morphology. The crystals were small with bipyramidal, rectangular, rhomboidal to polygonal shapes, as displayed in fig. 4.

Bioassay

Cowpea is a locally available leguminous crop, appeared to be infested by the spider mites, and it causes heavy loss in the yield; hence, this mite was chosen for the bioassay in this study. The preliminary step of this study was the identification of all stages of development in the life cycle of mites.

The effect of homogenous preparation of parasporal crystal of *Bt* (1.7% δ-endotoxin) on mites is given in fig. 5. The mites fed with 1 to 4 mg/ml crude toxin did not show any symptoms of mortality after 24 h of feeding (fig. S2). They were quite active and egg laying. Subsequent developmental stages such as larvae, protonymphs, deutonymphs and intermediate quiescent stages were observed; this pattern was similar to that of control mites, whose diet contained autoclaved toxin; after 7 days of observation, they reproduced at exponential rates, passed through all 5 developmental stages and number of mites increased exponentially. The population size of mites was enlarged from the initial number 15, which included all stages of the development.

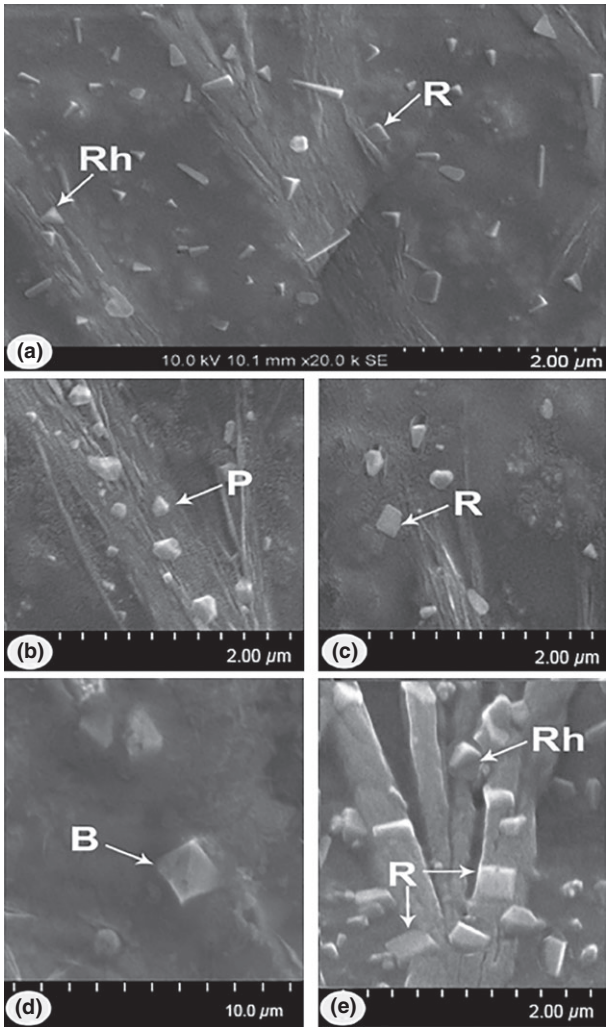

Fig. 4 (a–e) Scanning electron micrographs of δ-endotoxin crystals produced by *Bt* strain BPU5 in LB. Shapes of crystals are denoted by letters: rectangular (R); rhomboidal (Rh); polygonal (P) and bipyramidal (B).

From toxin concentrations 5 mg/ml onwards, the mites started showing symptoms of mortality such as slow movement, paralysis and crumbling of legs, and when the concentration of parasporal crystals were increased (6–10 mg/ml), the mortality rate increased exponentially. At 5 mg/ml concentration of the crude toxin, 50% mortality rate was observed on day 5; upon feeding 7–10 mg/ml toxin, 50% of mites died within day 3 after exposure.

Pattern of the death of mites corresponding to the increasing level of toxin is depicted. Mortality percentage of spider mites increased with increasing concentration of *Bt* toxin in the diet. Even after feeding, some mites laid eggs on the next day, which were degenerated in subsequent days. It clearly indicates the effect of crude toxin even on eggs, as they were inhibited from further development. The mortality rate of mites on the day 4 was taken for probit analysis. Number of dead mites on the day 4 of exposure to toxin was tabulated (Table 1). The observed and expected responses of the mites after exposure to toxin (generated by the SPSS) were closely related, which confirms the accuracy of the data observed. The mean of 50% lethal concentration value for crude toxin against spider mite obtained was log 0.9044, that is 8.024 mg/ml (its antilog) (fig. S3). The LC_{50} value (for adult mites) obtained by probit analysis was 8.32 mg/ml (LC_{50} value of the positive control, Eco Trol^{TM} was 0.02 mg/ml), that is this much concentration was sufficient to kill 50% of the adult mites under the assay conditions. Kaplan–Meier curve showing the probability of survival of *T. macfarlanei* after treatment with different concentrations of δ-endotoxin was evaluated (fig. 6). Upon increasing concentration of toxin, probability of survival was reduced gradually. The mites survived beyond the treatment period were considered to be 'censored'. By log-rank test, the level of significance was observed as $P < 0.05$. The survival distributions of mites at different concentrations of δ-endotoxin were not equal in populations showing the statistical significance of the experiment.

Discussion

This study focused on the purification of δ-endotoxin (almost 100% purity) produced by a novel *Bt* strain, isolated from the goat rumen using biphasic purification strategy, as demonstrated by us recently (Jisha et al. 2014). Among the various sugars supplemented to LB, glucose (8 g/l) was found effective for enhancing the production of δ-endotoxin by 30% (1.79 mg/ml). Using artificial diet bioassay method, the efficacy of the crude pellet was demonstrated against the spider mite, *T. macfarlanei*.

Purification of δ-endotoxin crystal protein is a major aspect, while considering its production from *Bt* strains. Various methods are commonly employed for the purification of δ-endotoxin with the maximum purity. From literature, it seems that various investigators (Valicente et al. 2010; Poopathi and Archana 2011) reported the yield of *Bt* toxin as a crude mixture of δ-endotoxin and spores (very often the total pellet), in a range of 1–5 g/l medium in liquid cultures; wherein commercial, synthetic or semisynthetic media were used. However, higher quantities of δ-endotoxin yield were reported from solid media, for instance, our group showed sixfold and 37-folds enhancement in the yield of δ-endotoxin with *Bt*

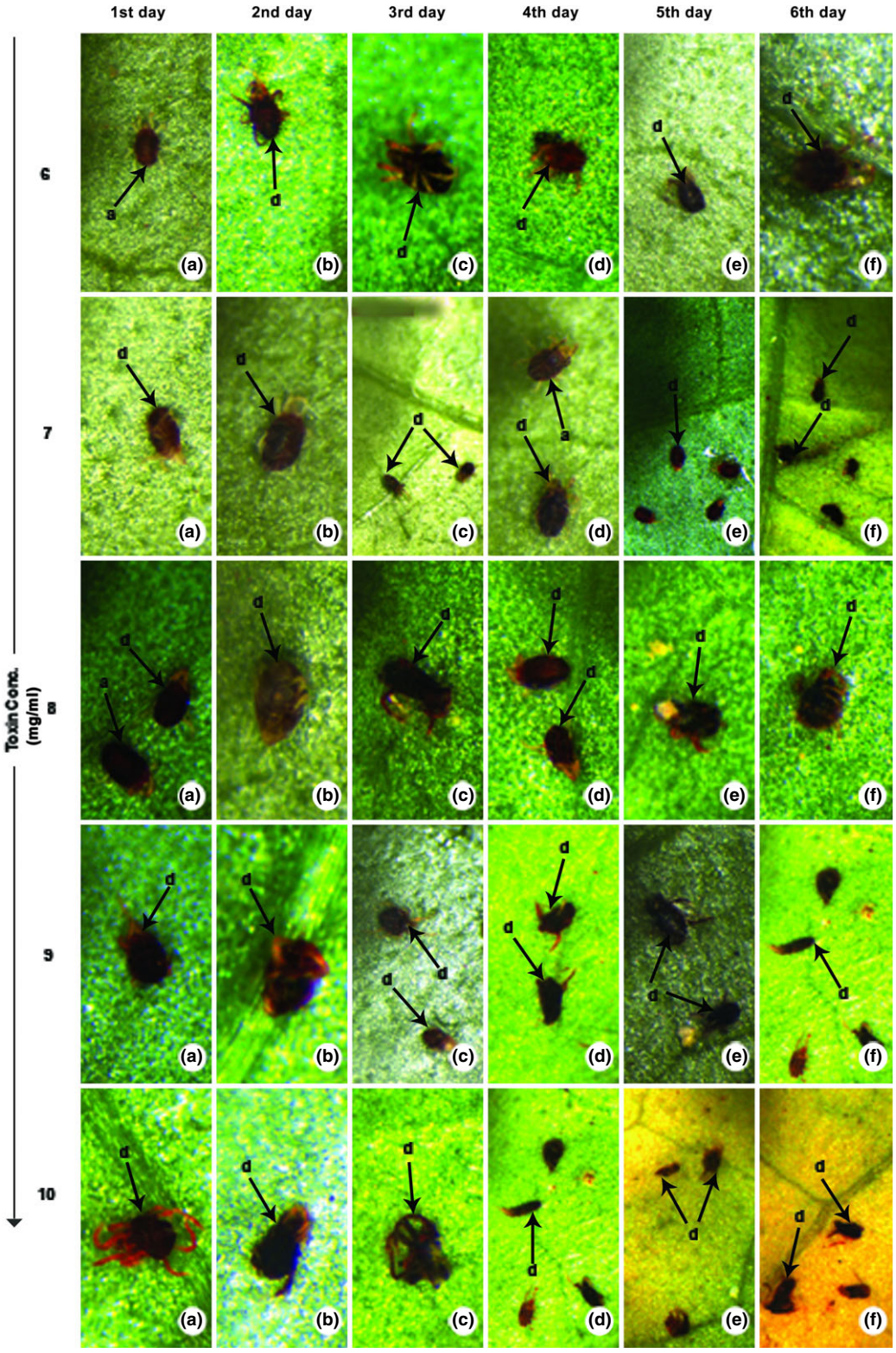

Fig. 5 Effect of different concentrations (6–10 mg/ml) of δ-endoxin (crude toxin pellet). larva (*l*), protonymph (*pn*), deutonymph (*dn*), quiscent stage (*q*), dead mite with crumbled limbs (*d*), alive mite (*a*); female (*f*) and male (*m*) mites (images by Leica M80, stereomicroscope).

Table 1 Observed and expected responses of mites on 4th day after exposure to toxin

Toxin concentration (mg/ml)[1]	No. of subjects[2]	Observed responses[3]	95% confidence limits		Expected responses
			Lower bound	Upper bound	
5	15	6	2.70	7.31	5.88
6	15	6	5.32	9.94	7.41
7	15	10	7.25	20.91	8.72
8	15	10	8.63	60.26	9.80
9	15	12	10.74	337.05	10.70
10	15	10	11.03	421.30	11.43

[1]*Bt* toxin of 72-h-old culture used for bioassay.
[2]Total number of mites subjected for assay.
[3]Number of mites died in the experiment.

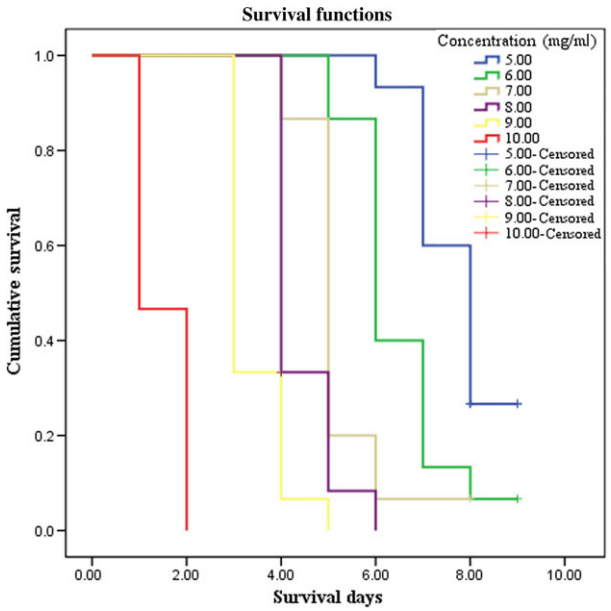

Fig. 6 Kaplan–Meier plot showing survival probability of *Tetranychus macfarlanei* after treatment with different concentrations of δ-endotoxin.

subsp. *kurstaki* on 10% potato flour (Smitha et al. 2013) and 30% soya bean flour (Jisha et al. 2014) supplemented LB medium (solid-state fermentation), respectively, against unsupplemented LB liquid medium. Polymorphic δ-endotoxin crystals were produced by the *Bt* strain reported in this study; similarly bipyramidal, cuboidal and spherical crystals or the combinations of these structures were produced by various strains of *Bt* (Attathom et al. 1995). Interestingly, spindle-shaped crystals (as reported herein) were also produced by a non-toxic strain of *Bt* subsp. *yunnanensis* (Murty et al. 1994).

This study also demonstrated that the sugars used as additional carbon source enhanced the production of δ-endotoxin, as reported for the strains of *Bt* (Zouari et al. 2002). However, higher concentration of glucose inhibited the formation of crystals, which may be due to catabolite repression, compensated by the limitation of protein synthesis in the presence of higher concentration of readily accessible carbon sources. In addition to glucose, optimum aeration also has a decisive role in enhancing the yield of δ-endotoxin (Ghribi et al. 2007). From various reports, the optimum glucose concentration for the enhancement of δ-endotoxin production was ranged from 6 to 8 g/l (Yousten and Rogoff 1969; Scherrer et al. 1973), which was in close agreement with the present data, that is 8 g/l. From this, it is very clear that glucose at appropriate concentration to be incorporated in the medium to accelerate the growth of *Bt* for maximizing the production of δ-endotoxin.

While evaluating the acaricidal potential of the *Bt* toxin, life cycle of the mite is an important parameter to be considered. Duration of time required for the development of egg to adult in acarids varies greatly depending on temperature (Ullah et al. 2012). As demonstrated, the life cycle of *T. macfarlanei* was completed within 10 days at 35°C (laboratory condition), the average temperature of the place where this study was conducted. Moreover, the present bioassay provided a reasonable evaluation on the effects of the crude *Bt* toxin on the spider mites. Almost all *Bt* biopesticide formulations are composed of spore–crystal complex (crude culture pellet), obtained after the centrifugation of the culture medium (Tang et al. 1996). Commercial formulation of *Bt* biopesticides is a physical mixture of living entities with inert ingredients, which may be toxic than the purified crystals or spores alone. However, the difference in toxicity may also involve other factors, for example, activated and purified crystals may be more readily degraded by enzymes in the midgut of the pests, and thus, less

toxic. Similarly, the use of purified and activated crystals entails a greater risk of lesser activity and its decline during storage, than the use of a crude spore and δ-endotoxin mixture. Upon ingestion of δ-endotoxin, the alkaline condition in the insect gut facilitates the solubilization of the crystal and subsequent cleavage; thus, the inactive δ-endotoxin crystal form (protoxin) is converted into an active and toxic form (Jisha et al. 2014).

We adopted the leaf disc method for rearing the mites, wherein artificial diet feeding assay system was followed (Payne et al. 1993). A few studies have exhibited the toxicity of *Bt* on mites. Normally, specific larval stages are used for bioassay procedures. However, apart from larval stages, adult and nymphal stages of mites were usually chosen for experiments with bioefficacy studies of *Bt* on acarids (Chapman and Hoy 2009). Bioassay of *Bt* var. *tenebrionis* was evaluated at laboratory conditions with adult mites infesting stored grains, that is *Acarus siro, Dermatophagoides farina, Lepidoglyphus destructor* and *Tyrophagus putrescentiae*. The fitted dose of *Bt* for 50% lethality of these mites was 25–38 mg crude toxin per g diet (we showed LC_{50} for *T. macfarlanei* as 8.024 mg). However, no remarkable difference on the lethality among these species was observed (Erban et al. 2009). In fact, only one study is seen in literature, wherein a *Tetranychus* sp. (*T. urticae*) was used for the toxicity studies of *Bt* or its formulation. They fed the mites with 5 mg spray-dried powder of *Bt* broth (a mixture of spores, crystals, cellular debris) in 1 ml sucrose (10%) containing preservatives and biosurfactant (Payne et al. 1993).

The dose–mortality relationship of an insect to a toxin is typically expressed as an LC_{50} value, which is the toxin concentration required to kill 50% of the population in a specified period. The lower the LC_{50} value, the greater the toxicity is. Therefore, LC_{50} value can be characterized by the slope of the log-probit curve. The slope of the dose–mortality curve is a measure of variability in response to treatment within the insect population tested. As the value of the slope increases, mortality associated with changes in concentration of toxin also increases. Conversely, as the value of slope decreases, less change in mortality is seen per unit change in concentration of the toxic substance (Jyothi and Gary 1999).

The effect of *Bt* for a certain period against any insect is judged by a single criterion, that is death (Dulmage 1981). The potentiality of *Bt* strain indicates the degree of susceptibility against certain insects. In addition to the lethality, the parasporal crystal also affects the development of egg after exposure to the toxin. Fully developed mites after feeding with the toxin laid eggs, but no further development and emergence of corresponding nymphal stages (Dulmage 1981). Such failure in the emergence pattern may be a crucial factor concerned with the integration of *Bt* as a biocontrol agent with chemical pesticides.

Briefly, this study evaluated the potential of *Bt* strain BPU5 for the production of δ-endotoxin crystal proteins in conventional LB in the presence of glucose as additional carbon source. The bioassay demonstrated herein shows it as a good candidate for controlling the devastating spider mite infesting the agricultural fields, offering its utility as an effective biological control agent against other pests too. Also suggests that, *Bt* strain BPU5 is an additional candidate along with already available *Bt* strains to be explored further as an alternative to chemical pesticides.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Feeding set-up.

Figure S2. Concentration dependent toxicity of the raw toxin from *Bt* strain BPU5 on *Tetranychus macfarlanei*: effect of different concentrations (1–5 mg/ml) of δ-endoxin (raw toxin pellet).

Figure S3. Mortality of *Tetranychus*: (a). mortality of mites *vs*. different toxin concentrations; and (b). probit analysis showing LC_{50} value of δ-endotoxin.