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Evaluation of *Bacillus thuringiensis* isolates against *Diaphania indica* (Saund.) (Lepidoptera: Crambidae)

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Abstract

A study was conducted to develop a commercial formulation of *Bacillus thuringiensis* and evaluate its bio-efficacy for the management of pumpkin caterpillar, *Diaphania indica* (Saund.). Twenty native isolates of *B. thuringiensis* obtained from the Western Ghats of Kerala and maintained in the repository of the Department of Agricultural Microbiology, College of Horticulture, Thrissur, Kerala were characterized by morphological, biochemical and molecular tests. PCR assay for insecticidal genes revealed the presence of *cry*1 gene in 35 per cent of the isolates. Blastn analysis of *cry*1 amplicons revealed homology with *cry*1A, *cry*1Ac and *cry*1Aa genes, ranging from 93 to 96 per cent identity. The native isolate KAU-2189 showed highest per cent mortality against *D. indica* at all the spore concentrations tested. Coconut water was found to be the best low cost substrate for the mass production of *B. thuringiensis* and the formulation based on coconut water broth was effective in controlling *D. indica* in pot culture experiment, on little gourd (*Coccinia indica*). The shelf life of the formulations was three months.

Key words: *Bacillus thuringiensis*, Coconut water, *Cry* gene, *Diaphania indica*, Soy flour broth, T3 broth.

Introduction

Chemical pesticides play a key role in crop protection. However, due to the adverse effect of these chemical molecules on the environment as well as the possibility of insects developing resistance, eco-friendly alternatives are gaining importance. Microorganisms pathogenic to insects are varied and diverse and can replace these chemical pesticides.

B. thuringiensis (Bt) are Gram positive, spore forming entomopathogenic bacteria belonging to the *Bacillus cereus* group of bacilli producing insecticidal crystal proteins during sporulation, which make them distinguishable in *B. cereus* group (Rasko et al., 2005). The crystal protein acts as stomach poison in insects.

More than 500 isolates of *B. thuringiensis* obtained from the Western Ghats of Kerala are being maintained in the repository of the Department of Agricultural Microbiology, College of Horticulture, Thrissur as per project funded by the Department of Biotechnology (Government of India). The insecticidal activity of these isolates was earlier assessed on the basis of PCR screening and bioassay on pumpkin caterpillar. A few isolates resulted in 100 per cent mortality in larvae (Girija and Mathew, 2009). Though Kerala Agricultural University had developed several microbial inoculants, there is no formulation containing *B. thuringiensis*. In this context, the present study was undertaken during 2014-2016 at the Department of Agricultural Microbiology, College of Horticulture, Thrissur with the objective to develop a commercial formulation of *B. thuringiensis* and

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to evaluate its bio-efficacy for the management of *D. indica* (Saund.).

Materials and Methods

Twenty native *B. thuringiensis* isolates (KAU-4, KAU-11, KAU-14, KAU-37, KAU-41, KAU-76, KAU-152, KAU-158, KAU-187, KAU-214, KAU-234, KAU-283, KAU-295, KAU-321, KAU-324, KAU-474, KAU-2182, KAU-2189, KAU-2184 and KAU-2203) were purified on Luria-Bertani (LB) agar. *B. thuringiensis* var. *Kurstaki* HD-1 was used as a reference strain in all the experiments. All the isolates were subjected to Gram staining, endospore staining (Schaeffer-Fulton method) and Coomassie brilliant blue staining (Sharif and Alaeddinoglu, 1988). The number of crystal proteins present in each isolate was recorded. Colony morphology of the isolates was observed on LB agar. The biochemical characteristics were studied by using starch hydrolysis, Voges-Proskauer test, esculin hydrolysis and sucrose fermentation test (Cappuccino and Sherman, 1992). The isolates were screened for the presence of *cry* gene content by using lepidopteran specific primers such as *cry*1 and *cry*2 (Ben-Dov et al., 1997) and *cry*9 (Juarez-Parez et al., 1997). Colony PCR was carried out using the reaction volume of 25 µL consisting of master mix (12.5 µL), template (2.0µL), forward and reverse primers (0.5µL each) and dH_2O (9.5µL). The steps followed in PCR were initial denaturation (94°C for 2 min), denaturation (94°C for 1 min) followed by annealing (61°C for 1 min for *cry*1, 59°C for 1 min *cry*2 and 53°C for 1 min for *cry*3) and primer extension at 72°C for 1 min. The above steps were repeated for 30 cycles. Final extension at 72°C was given for 10 min. The PCR products were checked by agarose gel electrophoresis (Sambrook et al., 1989). The Base Local Alignment Search Tool for Nucleotides (Blastn) programme (http://blast.ncbi.nlm.nih.gov/Blast) was used to find out the homology of the nucleotide sequences with the accessions available in database.

Based on the abundance of crystal proteins and *cry* gene content, three native isolates were selected for laboratory bioassay against *D. indica* using diet contamination method (Schesser et al., 1997), along with *B. thuringiensis* var. *kurstaki* HD-1 as reference. Semi-synthetic diet was prepared as per Mathew et al. (2010). Three different concentrations *viz*., 10^7, 10^8 and 10^9 spores per mL were tested. Crude extract of spores and cry toxin (1 mL) were mixed with 9 mL artificial diet and fed to the larvae after solidification. Three replications were used with ten larvae in each replication. The per cent larval mortality was recorded every 12 h for one week.

Best isolate that recorded maximum mortality in the bioassay experiment was selected for low cost liquid formulation. Soy flour (5 g in 200 mL distilled water) and coconut water (100 per cent) broth were used as low cost media for multiplying *B. thuringiensis*, with T3 broth as reference. Sporulation were assessed at 0 h, 72 h and 96 h after inoculation using haemocytometer.

The media with highest sporulation were selected as media for preparing the formulation. Additives including tween 80 (0.28%), microcrystalline cellulose (0.3%), poly ethylene glycol (0.58%), antifoam silicon emulsion (0.05%) and tea extract (0.13%) were also added to the liquid formulation, following Sushitha (2014), with slight modification in the concentration. The shelf life of the formulations was studied upto six months at monthly intervals by checking the spore count by haemocytometer.

A pot culture experiment was designed in completely randomized design (CRD) to evaluate the bio-efficacy of the low cost liquid formulations on *D. indica*, with little gourd (*Coccinia indica*) var. *Sulabha* as the test crop. The different treatments used in the experiment were formulation of selected *B. thuringiensis* isolate in low cost medium, formulation of BtK HD-1 (reference isolate) in T3 broth, formulation of selected *B.*

thuringiensis isolate in low cost medium, formulation of BtK HD-1 (reference isolate) in T3 broth and commercial liquid formulation of *B. thuringiensis* (BtK from Abtec, Kottayam, Kerala).

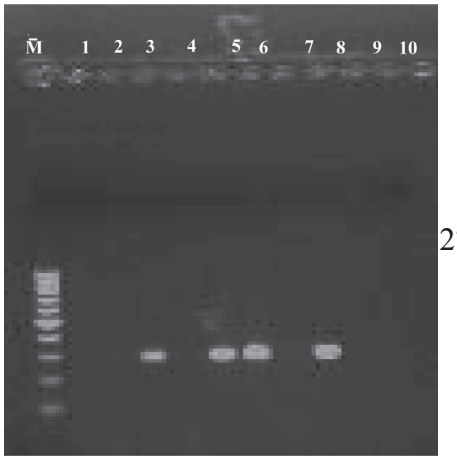
Each treatment was replicated four times, each replication having five plants. Ten larvae (3rd instar) of *Diaphania indica* per plant were released one month after planting (MAP) and 3MAP. *B. thuringiensis* formulations were sprayed at a concentration of four per cent, one day after the release of larvae. The observations on larval mortality were recorded upto 10 days after spraying. Per cent leaf damage was also calculated after both the sprayings by using the formula:

$$\text{Per cent leaf damage (\%)} = \frac{\text{Number of leaves damaged}}{\text{Total number of leaves in the plant}} \times 100$$

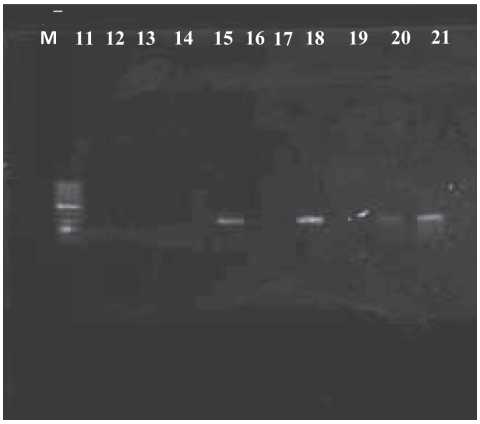
Results and Discussion

Gram staining revealed purple coloured rod shaped cells arranged in chains whereas, pink coloured vegetative cells and green endospores were observed on endospore staining. Coomassie brilliant blue staining revealed the presence of three types of crystal protein *i.e.*, irregular (60%), bipyramidal (40%) and circular (35%). Thirty per cent of the isolates produced a complex of crystal proteins (either spherical + irregular or bipyramidal + irregular). Large number of crystal proteins (50-80 crystals per microscopic field) were present in the isolates KAU-11, KAU-41, KAU-76, KAU-474, KAU-2182, KAU-2189 and KAU-2203. The colonies appeared creamy white in colour having circular form, entire to undulate margin and flat elevation. Biochemical characterization of the isolates showed positive reaction to starch hydrolysis, sucrose fermentation and esculinase hydrolysis, whereas were negative to Voges-Proskaeur test. Similarly, Das et al. (2015) reported *B. thuringiensis* strains which were Gram positive and endospore formers with white to off white coloured colonies having regular margin and slightly raised elevation. They also observed the presence of spherical and rhomboidal shaped crystal proteins. Biochemical characterization revealed positive reaction to starch hydrolysis and sucrose fermentation, and negative reaction to Voges-Proskaeur test in some of the strains studied.

PCR assay revealed that seven isolates (KAU-11, KAU-234, KAU-321, KAU-474, KAU-2182,



M: MOLECULAR MARKER (100bp ladder): 1: KAU-4; 2: KAU-14; 3: KAU-11; 4: KAU-37; 5: KAU-234; 6: KAU-321; 7: KAU-152; 8: KAU-474; 9: KAU-187; 10: KAU-214



M: MOLECULAR MARKER (100bp ladder): 11: KAU-158; 12: KAU-283; 13: KAU-295; 14: KAU-41; 15: KAU-2182; 16: KAU-76; 17: KAU-2184; 18: KAU-2189; 19: KAU-324; 20: KAU-2203; 21: HD-1

Plate 1. Cry gene profile of *B. thuringiensis* isolates

Table 1. Sequence analysis of *cry* gene amplicons

Sl. No.	Isolate	Accession No.	Gene showing maximum homology	Identity(%)
1	KAU-11	HQ439790.2	PS9- D12 *cry*1A like protein	96
2	KAU-234	GQ202005.1	AR- 6 *cry*1Ac gene	95
3	KAU-321	KC158223.1	Lip plasmid insecticidal crystal protein gene (*cry*1Aa gene)	96
4	KAU-474	HQ439790.2	PS9- D12 *cry*1A like protein	96
6	KAU-2182	KC16668.1	ARP 102 pesticidal protein gene	93
6	KAU-2189	KC16668.1	ARP 102 pesticidal protein gene	93
7	KAU-2203	KC16668.1	ARP 102 pesticidal crystal protein	95

KAU-2189 and KAU-2203) yielded a single band of 277 bp size, indicating the presence of *cry*1 gene (Plate 1). None of the isolates yielded amplicons corresponding to *cry*2 and *cry*9 genes, indicating the absence of these genes in the test isolates. Sequence analysis of the *cry*1 amplicons showed homology towards *cry*1A, *cry*1Ac and *cry*1Aa genes (Table 1). Hence, the presence of *cry*1 was confirmed in the seven isolates. Earlier workers have also used PCR for the detection of *cry* gene content (Khojand et al., 2013; Shishir et al., 2014).

Based on the presence of *cry* gene and abundance of crystal proteins, three isolates (KAU-2189, KAU-11 and KAU-474) were selected for bioassay experiment. The results showed differential toxicity levels between 10 and 100 per cent when tested at three different concentrations (10^7, 10^8 and 10^9 spores/mL) (Figs. 1 and 2). Among the native isolates tested, KAU-2189 produced highest per cent mortality against second and third instar larvae of *D. indica* at all the concentrations used. However, second instar larvae were more susceptible and killed faster than the third instar larvae. No mortality was observed in control up to one week after which they emerged as moths. These results were in accordance with the findings of Lalitha and Muralikrishna (2012) who reported mortality per cent ranging from 10.00 to 93.33 per cent against first and third instar larvae of *Spodoptera litura* among the 114 native *B. thuringiensis* isolates tested. They also observed that highest mortality percentage was recorded in early instars of larvae.

In the present investigation, the per cent mortality generally increased and the time required to attain 100 per cent mortality was reduced with increase in concentration of crude protein of all the isolates. This result is in agreement with the findings of Lalitha et al. (2012) who described the increase in per cent mortality with the increase in spore concentration. Jacob (2008) also described the increase in per cent mortality with the increase in time.

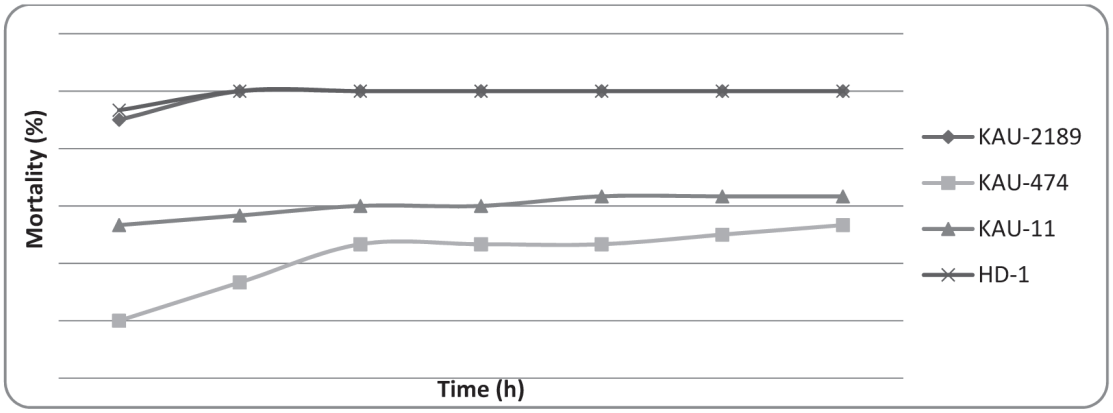
Table 2. Effect of different media on sporulation of *Bacillus thuringiensis* isolates

Sl. No.	Isolate	Medium	Spore count	
			72 h($\times 10^7$ spores /mL)*	96 h($\times 10^7$ spores /mL)*
1	KAU-2189	T3 broth	12.06 (7.08)[a]	12.93 (8.11)[a]
		Soy flour broth	3.14 (6.50)[b]	3.45 (7.54)[b]
		Coconut water broth	11.80 (7.07)[a]	12.23 (8.08)[a]
		CD(0.05)	0.05	0.05
2	HD-1	T3 broth	12.70 (7.10)[a]	12.90 (8.11)[a]
		Soy flour broth	2.60 (6.41)[b]	2.71 (7.43)[b]
		Coconut water broth	12.30 (7.09)[a]	12.53 (8.10)[a]
		CD(0.05)	0.04	0.05

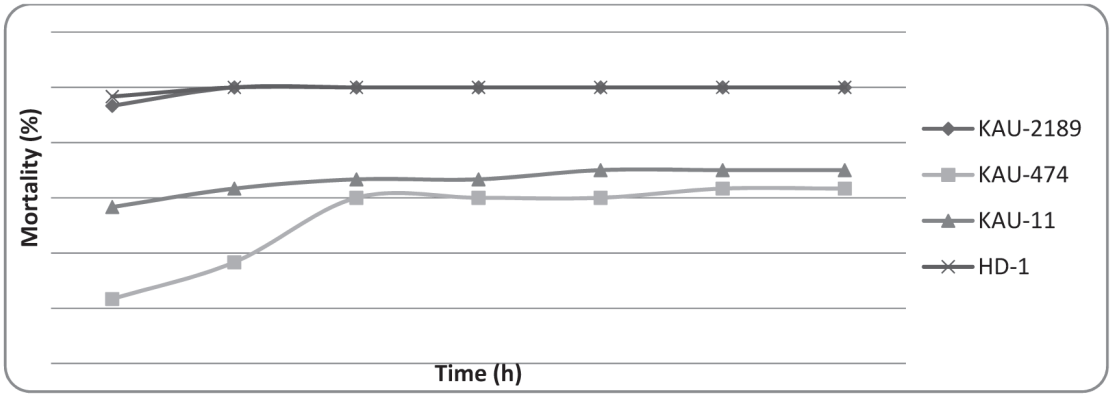
*Mean of three replications

The values given in parentheses are log transformed values

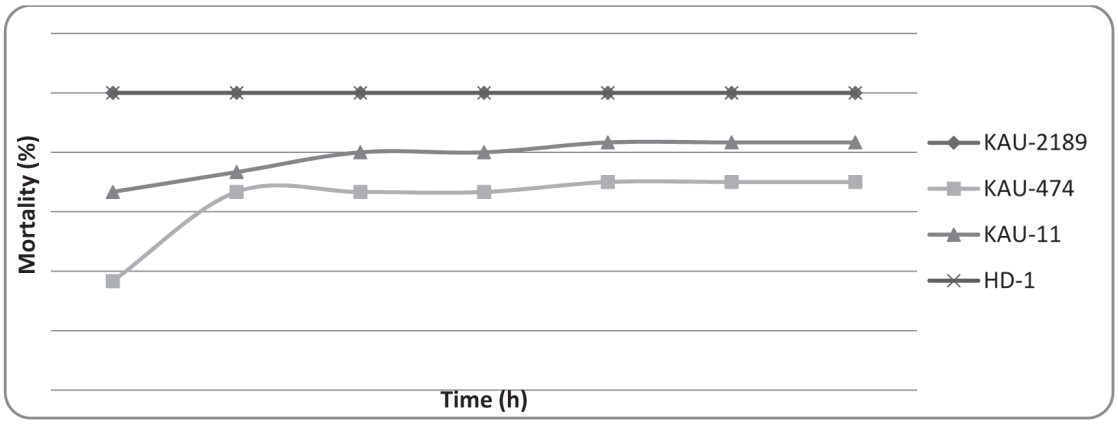
Figures with same superscripts do not differ significantly. Subgrouping is based on CD values



A) At 10^7 spores/mL

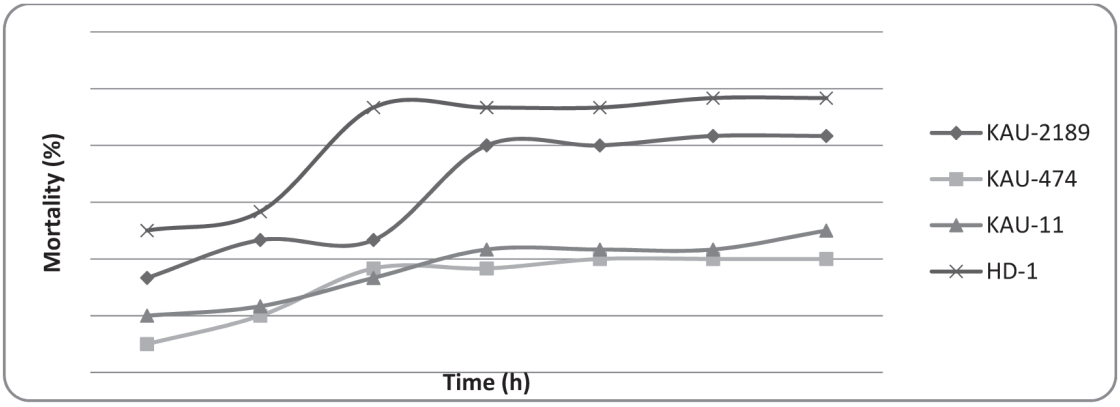


B) At 10^8 spores/mL

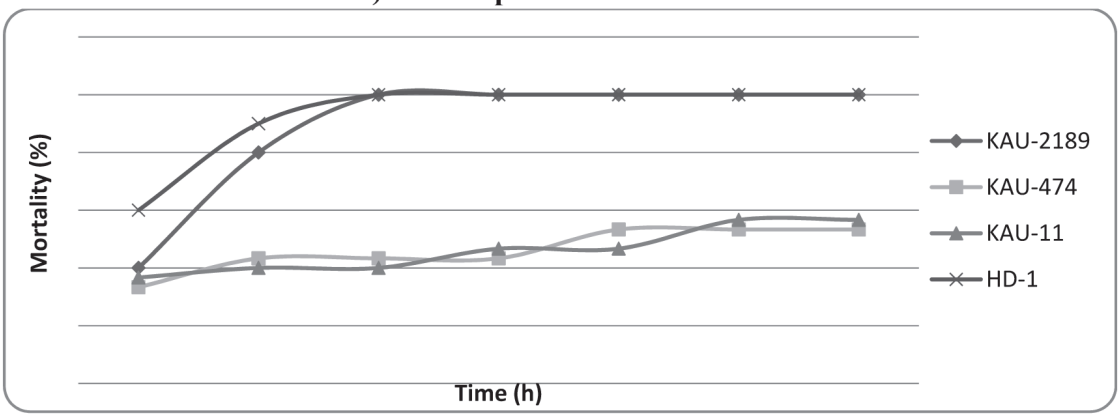


C) At 10^9 spores/mL

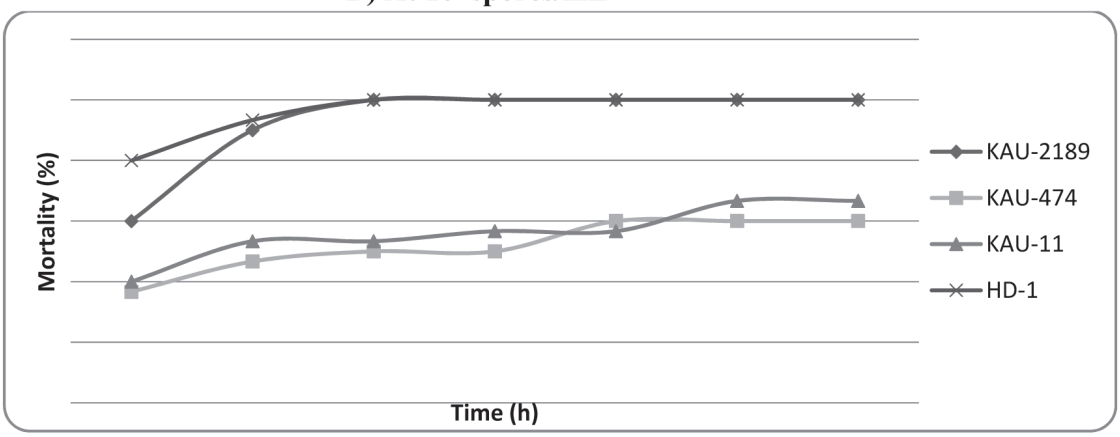
Figure 1. Mortality of *Diaphania indica* (2nd instar larvae) at different concentrations of selected *Bacillus thuringiensis* isolates at different time intervals



A) At 10^7 spores/mL



B) At 10^8 spores/mL



C) At 10^9 spores/mL

Figure 2. Mortality of *Diaphania indica* (3rd instar larvae) at different concentrations of selected *Bacillus thuringiensis* isolates at different time intervals

Table 3. Effect on spore count of native *Bacillus thuringiensis* formulations on storage

Sl. No.	Formulations	At the time of inoculation	Spore count ($\times 10^8$ spores/mL)*					
			1st month	2nd month	3rd month	4th month	5th month	6th month
1	HD-1 in T3 broth	26.96(9.43)	26.63(9.43)	26.00(9.42)	25.63(9.41)	2.65(9.42)	1.30 (8.11)[a]	0.12(7.08)[a]
2	HD-1 in coconut water broth	26.40(9.42)	26.20(9.42)	26.16(9.42)	25.46(9.41)	2.50(9.40)	1.13 (8.05)[b]	0.10(7.03)[b]
3	KAU- 2189 in T3 broth	26.50(9.42)	26.43(9.42)	26.23(9.42)	25.23(9.40)	2.56(9.40)	1.24 (8.09)[a]	0.11 (7.07)[a]
4	KAU- 2189 in coconut water broth	26.30(942)	26.13(9.41)	26.03(9.42)	25.03(9.40)	2.42(9.38)	1.03 (8.01)[c]	0.09(7.00)[c]
	CD(0.05)	NS	NS	NS	NS	NS	0.03	0.03

*Mean of three replications
The values given in parentheses are log transformed values
Figures with same superscripts do not differ significantly. Subgrouping is based on CD values

Among the different media evaluated, both the isolates KAU-2189 and HD-1 in coconut water broth yielded more spores than the standard medium (T3 broth) (Table 2). The results of the present study were in accordance with the results obtained by Prabakaran et al. (2008) who reported that coconut water based culture medium yielded cell mass of 3.1 g/L and spore count of 3.4×10^{11} spores/mL with a 30 h old culture of *B. thuringiensis* var. *israelensis* which was similar to that obtained with conventional medium (Nutrient Yeast Salt Medium).

In the present study, spore count of the liquid formulations decreased from fourth month, revealing its shelf life to be three months (Table 3). Dhingra (2012) also reported a decline in the viable cell number of *B. thuringiensis* cells from 3.0×10^{12}cfu/mL to 1.4×10^{10}cfu/mL after 90 days in glycerol based formulation of *B. thuringiensis* isolate Bt_{III}.

Pot culture experiment revealed that during both the sprayings, second highest mortality was recorded for the native isolate KAU-2189 in coconut water and this was statistically on par with HD-1 in coconut water (Table 4). The lowest per cent mortality was observed for the untreated control followed by the commercial formulation, BtK (Abtec). Zafar et al. (2000) reported that formulation of indigenous strain of *B. thuringiensis* (CAMB) was effective in controlling *Helicoverpa armigera* on tomato plants. Tamez-Guerra et al. (1998) also reported that a cost effective nixtamalized corn flour based granular formulation of *B. thuringiensis* at two per cent produced higher pest reduction than Dipel 2X (commercial formulation) against three lepidopteran crop pests, *Heliothis virescens* (F.), *Spodoptera exigua* (Hubner), and *Trichoplusia ni* (Hubner).

Table 4. Effect of different formulations of *Bacillus thuringiensis* on *Diaphania indica*

Sl. No.	Formulations	Per cent mortality * (on 3rd instar larvae)	
		1st spraying (1 MAP)	2nd spraying (3 MAP)
1	BtK (ABTEC)	42.50[c]	43.00[c]
2	HD-1 in T3 broth	49.00[b]	49.50[b]
3	HD-1 in coconut water broth	56.00[a]	55.00[a]
4	KAU-2189 in T3 broth	47.00[b]	47.00[b]
5	KAU-2189 in coconut water broth	54.00[a]	54.00[a]

*Mean of four replications MAP: Months after planting
Figures with same superscripts do not differ significantly. Subgrouping is based on CD values

In the present investigation, per cent leaf damage observed in coconut water formulations of KAU-2189 and HD-1 was minimum and comparable with each other (Table 5). The highest per cent leaf damage was observed for the untreated control followed by commercial formulation, BtK (Abtec). A study conducted by Kandibane et al. (2010) revealed that treatment containing *B. thuringiensis*

Table 5. Per cent leaf damage caused by *Diaphania indica*

Sl. No.	Formulations	Per cent leaf damage *	
		1st spraying (1 MAP)	2nd spraying (3 MAP)
1	BtK (ABTEC)	63.10[b]	48.63[b]
2	HD-1 in T3 broth	61.64[c]	46.95[bc]
3	HD-1 in coconut water broth	59.99[d]	45.93[c]
4	KAU- 2189 in T3 broth	61.88[bc]	47.20[bc]
5	KAU-2189 in coconut water broth	60.26[d]	46.30[c]
6	Control	69.09[a]	57.32[a]
	CD(0.05)	1.26	1.96

*Mean of four replications MAP: Months after planting
Figures with same superscripts do not differ significantly. Subgrouping is based on CD values

formulation @ 2.5 kg/ha had the lowest leaf folder damage of 8.21 per cent and was comparable with monocrotophos 36 WSC @ 500 g ai/ha at 7 days after spraying (DAS). The lowest leaf folder damage of 13.65 per cent was recorded in *B. thuringiensis* formulation @ 2.5 kg /ha at 10 DAS. The study revealed that the native isolates have the potential to be developed into a biopesticide. Coconut water could be used as an ingredient for low cost liquid formulation. Further evaluation under field conditions is required to confirm the efficiency of KAU-2189 as a biopesticide.

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