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Research Article

In vitro evaluation of microencapsulated *Bacillus thuringiensis* exposed to different temperatures against *Helicoverpa armigera* (Hubner)

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ABSTRACT: The experiment was conducted to prepare and evaluate microencapsulation of lyophilized Spore Crystal Aggregate (SCA) of native *Bacillus thuringiensis* isolate BGC-1 and standard isolate HD-1 against second instar larvae of *Helicoverpa armigera* at the Department of Agricultural Entomology, Bheemarayanagudi. The zetasizer analyzer results revealed that the microcapsule diameter ranged from 3.2 to 8.3 µm. Median lethal concentrations of the BGC-1 and *Bt*-HD1 were 0.66 g/l and 0.50 g/l, respectively. UV protectants *viz*., melanin and (PABA) para-amino benzoic acid were evaluated by exposing microencapsulated *Bacillus thuringiensis* to temperature regimes of 25°C, 30°C, 35°C, 40°C and 45°C in the B.O.D at different intervals of time. Among four microencapsulated formulations, BGC-1 with melanin recorded the highest mortality of 95 % at zero h exposure. As time increased, the mortality decreased and HD-1 was on par with BGC-1. HD-1 melanin showed significantly higher mortality of 62.50 to 92.50 % followed by BGC-1 (melanin) which ranged from 70 to 90 %. Even though formulations were exposed to different temperatures, because of encapsulation, potential to cause insect mortality was retained.

KEYWORDS: *Bacillus thuringiensis, Helicoverpa armigera,* melanin, microencapsulation, para-aminobenzoic acid, UV protectants

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INTRODUCTION

Ecologically sound pest management practices involve the use of entomopathogens to control insect pests in crops. Microbiological agents such as fungal pathogens and insect viruses are being used, still, *Bacillus thuringiensis* (Berliner) seems to have the most potential in this regard. About 90% of microbial insecticides on the market today are commercially produced from *B. thuringiensis*, and transgenic plants carrying the insecticidal proteins of the plant have already been made available for use in agriculture (Samsonova *et al*., 1997). Gram-positive soil bacterium *B. thuringiensis* is a well-known biocontrol agent. Its toxicity results from the production of delta endotoxins or Insecticidal Crystal Protein (ICP) in the sporulating cells. In the insect midgut, the inactive protoxins undergo proteolytic digestion to produce active toxins. These toxins attach to the midgut cells of insects and induce cell lysis via the creation of ion pores in the midgut, which results in the stoppage of eating and eventual death (Gill *et al*., 1992).

The physiology of foliage, temperature changes, rain and UV radiation are some of the environmental stressors that prevent many *B. thuringiensis* formulations from dispersing efficiently in crops, despite their benefits. Of these, radiation is thought to be the most important (Myasnik *et al*., 2001). In 1978, Griego and Spence claimed that the inactivation of crystal proteins against insect pests was primarily caused by UV-B (280-400 nm) and UV-C (100-280 nm) ultraviolet radiation (Griego *et al.*, 1978). To increase the effectiveness and performance of biopesticides, several protective methods (dust, granules, water-dispersible granules, wettable powders, emulsions, suspension concentrates, etc.,) have been developed. However, even though some of these methods have been successfully tested, they are not commercialized because of the higher production costs and potential for environmental contamination. Microencapsulation was promoted as a reliable, safe and viable solution for UV radiation protection methods that could be used on an industrial scale (Poncelet *et al*., 1992). Microencapsulation

is an innovative method that works as a tool for material protection and release modulation. It has been effectively utilized to safeguard materials that are susceptible to temperature changes, photodegradation, oxidation, moisture, and other unwanted reactions, increasing the potential applications of micro-encapsulated products and offering a viable substitute for entomopathogen protection (Gonsalves *et al.,* 2009). It is possible to achieve microencapsulation using gelatin, starches, cellulose, and additional polymers like sodium alginate. Sodium alginate is one of these that have been studied extensively for the immobilisation of biological materials (Brar *et al.*, 2006).

The majority of encapsulation techniques in biological control that have been documented are based on the extrusion of biological material suspensions (Sopena *et al.,* 2009). The disadvantage of this method is the large particle diameter, which is determined by the diameter of the extrusion element. Nowadays, encapsulation in the form of microcapsules is being investigated in depth as a way to produce smaller particles of uniform size to improve not only the formulation's performance and efficiency but also its aerial dispersion onto foliage and the uptake by larvae. (Huang *et al*., 2007).

This work aimed to examine the microencapsulation procedure of a lyophilized spore crystal aggregate (SCA) produced by *B. thuringiensis* var. *kurstaki* HD-1 (*Bt*-HD1) and native isolate BGC-1. The approach, which was centred on creating tiny microcapsules (≤10 µm diameter) that could shield spore viability and crystal activity from temperature changes, was based on the emulsification/ internal gelation process (Poncelet *et al*., 1992). *H. armigera* was used in bioassays to test the bioinsecticide's toxicity. Considerable work has been carried out in the Department of Entomology at UAS, Raichur on the isolation, identification and characterization of native *B. thuringiensis* isolates from different cropping system and their bio-efficacy against Diamondback moth and *H. armigera* under the laboratory condition (Praveen 2014; Saroja *et al.,* 2018). With the above background, the present work on the development of microencapsulation formulation of *B. thuringiensis* toxins to withstand high temperature and their efficacy against *H. armigera* in the laboratory was studied.

MATERIALS AND METHODS

Maintenance of *B. thuringiensis* culture

The reference HD-1 strains and the native *B. thuringiensis* strain were obtained from the Department of Agricultural Entomology, Bheemarayanagudi. Every three months, *Bt* strains were subcultured on Luria agar medium at 30°C for 24 hours, and pure cultures were kept at 4°C for further study.

Lyophilization of *B. thuringiensis* isolate pellet for bioassay

Bacterial cells were suspended in a five-litre Luria broth for five days at 30°C. The turbid solution was centrifuged for ten minutes at 10,000 rpm. After discarding the supernatant, the pellets went through lyophilization to produce technical powder. The process of lyophilization was carried out at GKVK college, Biotechnology Department laboratory (Lyophilizer model: vir Tis wizard 2.0). The lyophilized powder was stored at 4ºC and used for conducting bioassay. *Bt* suspension was prepared by dissolving one milligram of lyophilized powder in one millilitre of sterile distilled water and serially diluted up to 1×10^{-9}.

Preparation of microencapsulation formulation of *B. thuringiensis* isolates

The emulsion gelling method described by Rodrigues *et al.* (2006) was used, with some modifications, to prepare microencapsulated *B. thuringiensis*.

In 60 ml corn oil and 200 µl Span80, a mixture of 10 ml of three different polymers, sodium alginate, starch, and gelatin at concentrations of 2, 3, and 5% w/w, and 100 mg SCA (108spores ml-1) was blended. For ten minutes, the agitation was maintained at 2000 rpm. After that, calcium chloride was added in two stages.

1. At first, 70 ml from the mixture of 37.5 ml $CaCl_2$ (0.3 and 0.1M) with 37.5 ml ethanol Initially, the agitating solution was mixed with 70 ml of 37.5 ml $CaCl_2$ (0.3 and 0.1M), 37.5 ml ethanol, and 1.0 ml acetic acid.

2. To precipitate the microcapsules, 150 ml of $CaCl_2$ (0.05M) was added in the second step. Until the microcapsules formed, the agitation was maintained at 2000 rpm for 45 minutes.

3. After centrifugation at 10,000 rpm for 20 minutes, the microcapsules were separated and stored at 4°C after being rinsed with distilled water and ether.

4. The microcapsule morphology was evaluated by (SEM) scanning electron microscopy (Carle zeiss EVO-18). Zeatasizer (ZETA sizer, nano383 issue 5.0, Malvern, England) was used in the dynamic light scattering apparatus to measure the average particle diameter (µm) of microcapsules of *B. thuringiensis.*

Bioassay of microencapsulated formulations of *Bacillus thuringiensis* isolates

Tests were conducted using microencapsulated *B. thuringiensis* isolates (BGC-1 and HD-1) at varying concentrations particularly 0.5, 1, 1.5, 2, and 2.5 gm/l of water.

against second-instar larvae of *H. armigera.* The control larvae were fed sterile distilled water. For each concentration, four replications were kept. The diet was poured in a thin layer into 12 celled multi cavity trays with a surface area of 3.14 cm^2 and a volume of approximately 4 ml per well. For all concentrations, a bacterial suspension containing Tween-80 (0.02 %) was overlaid at 146 l on the diet surface in each well and left for one hour. In each well, one pre-starved (4 h) second instar larva was released. For each concentration, 40 larvae were used at a rate of 10 larvae per replication (4 replications including the control). These trays were kept in an insectary at 26 1°C, 70% Relative Humidity (RH), and a light: dark ratio of 16:8 hours. Mortality was observed at 24, 48, 72, 96, and 120 hours after treatment (Devi & Vineela, 2014). The median lethal concentration (LC50) was calculated using concentration and mortality data.

A microencapsulated *B. thuringiensis* suspension was prepared and transferred to a beaker with a capacity of 100ml. For 48 hours, the beakers were placed in a B.O.D incubator at temperatures of 25°C, 30°C, 35°C, 40°C, and 45°C. A temperature-exposed *B. thuringiensis* suspension was used in a bioassay against *H. armigera* second instar larvae. The observations on larval mortality were made at 24 hours intervals up to 5 days, and the percentage mortality was calculated. The total number of viable cells in the bacterial suspension was determined by thoroughly mixing the suspension, aseptically taking 1 mg of the sample and transferring it into 1 ml of distilled water blank, and taking 0.1 ml of the sample and transferring it into 0.9 ml of water blank. After serial dilutions, 100L of suspension was plated on LA medium plates. These plates were placed in a B.O.D. incubator and incubated for 24 hours at 30ºC. The number of colonies on the plates was counted, and the CFU per ml in the bacterial suspension was calculated.

RESULTS AND DISCUSSION

Morphology and particle size distribution of microcapsules of *Bacillus thuringiensis* isolates

All of the spore-crystal aggregates loaded in microcapsules had a spherical shape and smooth surface. The SEM analysis of the particles confirmed the form of the *B. thuringiensis* microcapsules. Similarly, the particle size of the microcapsules was observed by Zetasizer and the size of the microcapsules was in the range of 3.2 to 8.3 µm (Figure 1).

The microcapsules were characterized for average particle diameter from the intensity distribution analysis by using a zetasizer (Table 1).

a. The average particle diameter of BGC-1 Microencapsulated without UV A protectant was 3.2 µm (3256 d.nm).

b. The average particle diameter of HD-1 Microencapsulated without UV A protectant was 3.4 µm (3407 d.nm).

c. The average particle diameter of BGC-1 Microencapsulated with Melanin UV A protectant was 8.3 µm (8324 d.nm).

d. The average particle diameter of BGC-1 Microencapsulated with PABA (Para amino benzoic acid) UV A protectant was 5.4 µm (5486 d.nm).

e. The average particle diameter of HD-1 Microencapsulated with Melanin UV A protectant was 7.3 µm (7349 d.nm).

f. The average particle diameter of HD-1 Microencapsulated with PABA (Para amino benzoic acid) UV A protectant was 4.9 µm (4993 d.nm)) (Table 1).

Our findings are consistent with the experiment carried out by Garica *et al*. (2011) on the microencapsulation process of a spore crystal aggregate generated by *B. thuringiensis* var. *kurstaki* HD-1, wherein they reported that the microcapsules' diameter ranged from 3.1 ± 0.2 to 6.8 ± 0.4 µm. Similar to this, Khorramvatan *et al.* (2017) found that the size of the microcapsules ranged from 8 to 20 µm in their investigation on enhancing microencapsulation formulation stability of *B. thuringiensis* subsp. *kurstaki* (*Bt-KD2*) against ultraviolet conditions. Similarly, Gifania *et al.* (2015) studied the microencapsulated variant of the *H. armigera* nuclear polyhedrosis virus (Ha NPV), which was created to increase the virus's stability when exposed to ultraviolet (UV) light. They found that the microcapsules' average size was 10 ± 4.57 µm. Further microencapsulated formulations were tested at varying temperatures and UV radiation levels at 365 nm against *H. armigera.*

Standardisation of dosages of *B. thuringiensis* isolates against *H. armigera*

BGC-1

Following a 24 hour treatment period, the larval death rate varied between 0.00 and 25.00 %. The concentration of 2.5 g/l had the highest mortality rate of 25.00 %, which was followed by concentrations of 2.00 g/l and 1.5 g/l with 23.00 and 18.00 % mortality, respectively. At 0.5 g/l, the lowest death rate of 10.00 % was noted. In the control treatment, there was no mortality noted (Table 2). Following 48, 72,

Table 1. The particle size of microencapsulation formulations of *B. thuringiensis* isolates

Sl. No.	Sample	Particle size (d.nm)
1.	BGC-1 Microencapsulation without UV protectant	3256 (3.2 µm)
2.	HD-1 Microencapsulation without UV protectant	3407 (3.4 µm)
3.	BGC-1 Microencapsulation with Melanin	8324 (8.3 µm)
4.	BGC-1 Microencapsulation with PABA	5846 (5.8 µm)
5.	HD-1 Microencapsulation with Melanin	7349 (7.3 µm)
6.	HD-1 Microencapsulation with PABA	4993 (4.9 µm)

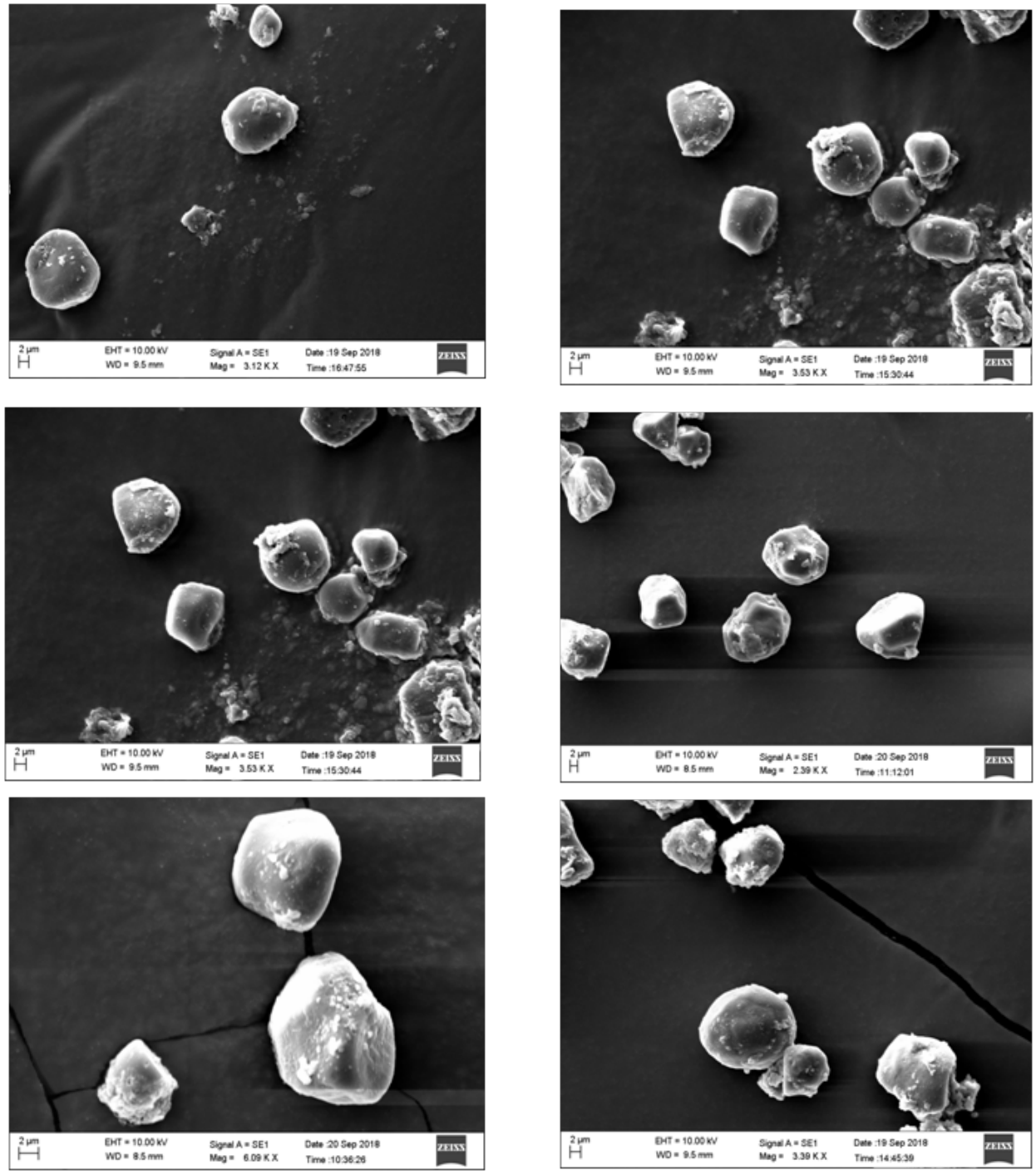

Figure 1. SEM images: **(a).** BGC-1 Microencapsulation without UV protectant, **(b).** HD-1 microencapsulation without UV protectant, **(c).** BGC-1 microencapsulation with melanin, **(d).** BGC-1 Microencapsulation with PABA, **(e).** HD-1 Microencapsulation with Melanin and **(f).** HD-1 Microencapsulation with PABA.

Table 2. Bioefficacy of microencapsulation formulation of native isolate (BGC-1) against *H. armigera* at different dosages

Concentration g/l	Per cent mortality after				
	24 h	**48 h**	**72 h**	**96 h**	**120 h**
0.5	10.00 (15.85)^{e}	17.50 (24.53)^{d}	22.50 (28.22)^{d}	37.50 (37.72)^{e}	45.00 (42.11)^{e}
1.0	15.00 (22.50)^{d}	27.50 (31.54)^{c}	37.50 (37.66)^{c}	47.50 (43.55)^{d}	60.00 (50.83)^{d}
1.5	18.00 (24.53)^{c}	32.50 (34.55)^{b}	45.00 (42.11)^{b}	50.00 (45.00)^{c}	65.00 (53.77)^{c}
2.0	23.00 (28.22)^{b}	40.00 (39.10)^{a}	52.50 (46.50)^{a}	62.50 (52.49)^{b}	72.50 (58.97)^{b}
2.5	25.00 (29.88)^{a}	40.00 (39.16)^{a}	55.00 (47.94)^{a}	67.50 (55.44)^{a}	85.00 (67.50)^{a}
Control	0.00 (0.00)^{f}	0.00 (0.00)^{e}	0.00 (0.00)^{e}	0.00 (0.00)^{f}	0.00 (0.00)^{f}
S.Em ±	**0.12**	**0.23**	**0.55**	**0.95**	**1.13**
CD at 1%	**0.49**	**0.95**	**2.24**	**3.86**	**4.26**

Note: The values in parenthesis are "arcsine" transformed values. The same alphabet values are statistically comparable to one another according to DMRT.

and 96 hours of treatment, a similar pattern of mortality was seen; following 120 hours of exposure, the mortality rate ranged from 0.00 to 85.0%. The concentration of 2.5 g/l had the highest death rate of 85.00 %, which was followed by concentrations of 2.00 g/l at 72.50 % and 0.5 g/l at 45.00 %. No mortality was noticed in the untreated control Table 2). According to the results of the bioassay tests, larval mortality rises with increasing concentration and exposure duration.

HD-1

In general, the mortality increased with the incubation period and maximum mortality was recorded after 120 h of feeding with a larval mortality range from 60 to 87.50 % against second-instar larvae of *H. armigera* (Table 3). The larval mortality after 24 hours of exposure ranged from 0.00 to 25.00. The highest mortality of 25.00 % was recorded in the concentration of 2.5 g/l followed by 17.50 % in the concentrations of 2.00 g/l and 1.5 g/l each. There was no mortality was observed in the control treatment. A similar trend of mortality was noticed after 48, 72 and 96 hours of treatment (Table 3). After 120 hours of exposure, larval mortality ranged from 0.00 to 87.50 %. The highest mortality of 87.50 % was recorded in the concentration of 2.5 g/l followed by 82.50 % in the concentrations of 2.00 g/l. The next best concentration was 1.5 g/l which recorded 67.50 %. There was no mortality in control. The bioassay studies revealed that larval mortality increases with an increase in concentration and exposure time.

The findings are similar to that of Khorramvatan *et al*. (2017) who reported that microencapsulated formulation of *B. thuringiensis* subsp. *Kurosaki* showed a mortality rate of 85.00 % against *Ephestia kuchniella* larvae. Even though both the isolates showed greater than 85 % mortality at 120 hours of exposure, maximum mortality was observed in the reference strain HD1, which was on par with the native *B. thuringiensis* isolate BGC-1. Larval death was minimal during the first 24 hours after feeding. Due to the stomach poisoning properties of *B. thuringiensis*, endotoxins after consumption attach selectively to receptor sites on the cell walls of the mid-gut, rupturing the membranes. At higher doses, this results in a lack of control in the ion exchange between the gut lumen and epithelial cells, which rapidly leads to death. Lower doses, on the other hand, may cause the gut lumen's pH to drop before spores can germinate, multiply quickly during vegetative stages and penetrate the hemocoel, causing severe septicemia and finally killing the insect (Glare & Callaghan, 2000). This process might take longer than twenty-four hours. According to Knowles (1994), *B. thuringiensis* larval mortality is often sluggish, taking two to three days for the larvae to cause death. Our results are confirmatory with Guerra *et al*. (2000) reported the insecticidal activity of microencapsulated formulation of *B. thuringiensis* killed 78.5 % *Ostrinia nubilalis* test larvae. Similarly, Bashir *et al*. (2016) observed the highest larval mortality 97.9 % for microencapsulated *B. thuringiensis kurstaki*. Garica *et al*. (2011) experimented with the microencapsulation process of a spore crystal aggregate produced by *B. thuringiensis* var. *kurstak* HD-1 and reported 46 % larval mortality of the *Spodoptera frugiperda* population. According to Haggag and Yousef (2010), the native *B. thuringiensis* strains generated mortality rates ranging from 25.00 to 100 %. According to Lalitha *et al.* (2012), the reference strain HD1 showed the highest mortality rate, which was comparable to that of the native B. thuringiensis strain 281 which was isolated from the Northern Telangana Zone. The percentage of mortality varied from 16.67 to 94.44 after 98 hours. Similarly, Gujar *et al.* (2000; 2004) found similar outcomes, showing that after 96 hours of treatment, HD-1 at 100 ppm and 500 ppm caused 62.3 and 91.7% death, respectively. Thirty *B. thuringiensis* strains were isolated from soil samples from a sericulture environment by Xavier *et al.* (2007). The strains were tested in preliminary larvicidal bioassays against *H. armigera* second instar larvae at 300µg/mL and revealed mortality rates ranging from 40 to 100%.

Table 3. Bioefficacy of microencapsulation formulation of *B. thuringiensis* isolates (HD-1) against *H. armigera* at different dosages

Concentration g/l	Per cent mortality after				
	24 h	**48 h**	**72 h**	**96 h**	**120 h**
0.5	10.00 (15.85)^{d}	32.50 (34.71)^{d}	50.00 (45.00)^{d}	52.50 (46.44)^{e}	60.00 (50.83)^{d}
1.0	15.00 (22.50)^{c}	37.50 (37.72)^{c}	52.50 (46.50)^{c}	57.50 (49.38)^{d}	60.00 (50.83)^{d}
1.5	17.50 (24.53)^{b}	40.00 (39.16)^{b}	52.50 (46.50)^{c}	62.50 (52.33)^{c}	67.50 (55.28)^{c}
2.0	17.50 (24.53)^{b}	40.00 (39.16)^{b}	57.50 (49.38)^{b}	70.00 (56.94)^{b}	82.50 (65.46)^{b}
2.5	25.00 (29.88)^{a}	45.00 (42.11)^{a}	60.00 (50.83)^{a}	75.00 (60.48)^{a}	87.50 (72.10)^{a}
Control	0.00 (0.00)^{e}	0.00 (0.00)^{e}	0.00 (0.00)^{e}	0.00 (0.00)^{f}	0.00 (0.00)^{e}
S.Em ±	**0.10**	**0.20**	**0.45**	**0.84**	**1.04**
CD at 1%	**0.42**	**0.84**	**1.85**	**3.43**	**4.23**

Note: The values in parenthesis are "arcsine" transformed values. The same alphabet values are statistically comparable to one another according to DMRT.

Table 4. Bioefficacy of microencapsulation formulation of native isolate *Bacillus thuringiensis* (BGC-1) with UV A protectant melanin against *H. armigera* at different temperatures

Sample exposed to different temperature	Per cent mortality				
	24 h	**48 h**	**72 h**	**96 h**	**120 h**
25^{o}C	27.50 (31.39)^{a}	40.00 (39.16)^{a}	55.00 (47.94)^{a}	72.50 (58.60)^{a}	90.00 (74.14)^{a}
30^{o}C	25.00 (29.88)^{b}	35.00 (36.15)^{b}	47.50 (43.55)^{b}	65.00 (53.99)^{b}	82.50 (68.41)^{b}
35^{o}C	22.50 (27.69)^{c}	35.00 (36.00)^{b}	45.00 (42.05)^{b}	57.50 (49.38)^{c}	75.00 (60.48)^{c}
40^{o}C	20.00 (26.19)^{d}	27.50 (31.39)^{c}	40.00 (39.10)^{c}	52.50 (46.50)^{d}	75.00 (60.48)^{c}
45^{o}C	15.00 (19.55)^{e}	25.00 (29.88)^{d}	37.50 (37.66)^{c}	52.50 (46.44)^{d}	70.00 (57.16)^{d}
Control	0.00 (0.00)^{f}	0.00 (0.00)^{e}	0.00 (0.00)^{d}	0.00 (0.00)^{e}	0.00 (0.00)^{e}
S.Em ±	**0.16**	**0.38**	**0.74**	**0.95**	**1.08**
CD at 1%	**0.67**	**1.44**	**3.03**	**3.87**	**4.41**

Note: The values in parenthesis are "arcsine" transformed values. The same alphabet values are statistically comparable to one another according to DMRT.

Evaluation of microencapsulation formulation of BGC-1 with UV A protectant melanin exposed at different temperatures against *H. armigera*

BGC-1 Microencapsulated formulation containing melanin UV A protectant was exposed to temperatures 25^{o}C, 30^{o}C, 35^{o}C, 40^{o}C and 45^{o}C in the B.O.D incubator for 48 hours. After 48 hours of exposure samples were incorporated into the *H. armigera* diet and fed to second instar larva of *H. armigera* and larval mortality was recorded at 24, 48, 72, 96 and 120 hours after treatment (Table 4). Larval mortality after 24 hours ranged from 15.00 to 27.50 %. Significantly highest mortality of 27.50 % was observed at 25^{o}C followed by 25.00, 22.50, 20.00 and 15.00 % at 30^{o}C, 35^{o}C, 40^{o}C and 45^{o}C exposure. Significantly highest mortality of 40.00 % was observed at 25 after 48 hours of exposure. Next, the highest mortality were 35.00 and 35.00, % at 30^{o}C and 35^{o}C treatment, respectively. The lowest per cent mortality of 25.00 was recorded at 45^{o}C exposure. After 72 hours larval mortality ranged from 37.50 to 55.00 %. Significantly highest mortality of 55.00 % was observed at 25^{o}C and the remaining per cent mortality ranged from 37.50 to 47.50 at 30^{o}C to 45^{o}C exposure. At 96 hours of feeding the cumulative mortality ranged from 52.50 to 72.50 %. The larval mortality after 120 hours ranged from 70.00 to 90.00 %. Significantly highest mortality of 90.00 % was observed at 25^{o}C followed by 82.50, 75.00, 75.00 and 70.00 % at 30^{o}C, 35^{o}C, 40^{o}C and 45^{o}C exposure, respectively. There was no mortality in the control treatment (Table 4).

Protein and CFU estimation of BGC-1 microencapsulated formulation with melanin as UV A protectant

BGC-1 microencapsulated formulation containing melanin as UV A protectant was exposed to temperature regimes of 25^{o}C, 30^{o}C, 35^{o}C, 40^{o}C and 45^{o}C in the B.O.D incubator for 48 hours. After 48 hours samples were subjected to quantification of protein and estimation of colony-forming units (Table 5).

Colony forming units (CFU/ml)

The significantly highest number of colonies 2.37×10^{8} CFU/ml was recorded at 25^{o}C exposure. The next highest 2.09×10^{8} CFU/ml was recorded at 30^{o}C and the least

Table 5. Effect of different temperatures on larvicidal activity, colony forming units and crude protein content of native isolate *B. thuringiensi* (BGC-1) microencapsulated formulation against *H. armigera* with melanin as UV A protectant

Sample exposed to different temperature	Bioefficacy/larvicidal activity at 120 h	Crude protein (µg/ml)	CFU/ml
25°C	90.00 (74.14)[a]	185.27	2.37×10^8
30°C	82.50 (68.41)[b]	174.60	2.09×10^8
35°C	75.00 (60.48)[c]	167.33	1.83×10^8
40°C	75.00 (60.48)[c]	160.05	1.69×10^8
45°C	70.00 (57.16)[d]	132.41	1.36×10^8
S.Em ±	**1.08**	**2.31**	**0.05**
CD at 1%	**4.41**	**9.83**	**0.24**

Note: The values in parenthesis are "arcsine" transformed values. The same alphabet values are statistically comparable to one another according to DMRT.

Table 6. Bioefficacy of microencapsulation formulation of native isolate *Bacillus thuringiensis* (BGC-1) with UV A protectant PABA against *H. armigera* at different temperatures

Sample exposed to different temperature	Per cent mortality				
	24 h	48 h	72 h	96 h	120 h
25°C	15.00 (19.55)[b]	30.00 (28.44)[c]	50.00 (45.00)[a]	60.00 (50.83)[a]	80.00 (63.80)[a]
30°C	17.50 (24.16)[a]	27.50 (31.93)[ab]	37.50 (37.66)[b]	52.50 (46.44)[b]	70.00 (56.94)[b]
35°C	12.50 (17.89)[c]	27.50 (31.93)[a]	32.50 (34.55)[c]	45.00 (42.11)[c]	67.50 (55.50)[b]
40°C	10.00 (15.85)[d]	25.00 (29.88)[abc]	30.00 (33.05)[c]	40.00 (39.10)[d]	52.50 (46.44)[c]
45°C	10.00 (15.85)[d]	25.00 (29.88)[bc]	30.00 (33.05)[c]	35.00 (36.22)[e]	52.50 (46.44)[c]
Control	0.00 (0.00)[e]	0.00 (0.00)[d]	0.00 (0.00)[d]	0.00 (0.00)[f]	0.00 (0.00)[d]
S.Em ±	**0.26**	**0.50**	**0.66**	**0.92**	**1.20**
CD at 1%	**1.07**	**2.06**	**2.70**	**3.74**	**4.90**

Note: The values in parenthesis are "arcsine" transformed values. The same alphabet values are statistically comparable to one another according to DMRT.

number of colonies 1.36×10^8 CFU/ml was recorded at 45°C temperature exposure (Table 5).

Quantification of crude protein

The crude protein estimated ranged from 132.41 to 185.27 µg/ml. A significantly higher crude protein recorded was 185.27 µg/ml at 25°C exposure. The next highest crude protein content recorded was 174.60 µg/ml at 30°C and the lowest concentration of 132.41 µg/ml was noticed at 45°C (Table 5).

Evaluation of microencapsulation formulation of BGC-1 with UV A protectant PABA (Para amino benzoic acid) exposed at different temperatures against *H. armigera*

BGC-1 Microencapsulated formulation containing PABA UV A protectant was exposed to different temperatures of 25°C, 30°C, 35°C, 40°C and 45°C in the B.O.D incubator for 48h. After 48h samples were fed to the second instar larva of *H. armigera* and larval mortality was recorded at 24, 48, 72, 96 and 120 hours of treatment (Table 6). The larval mortality after 24 h ranged from 10.00 to 17.50 %. Significantly highest mortality of 17.50 % was observed at 30°C followed by 15.00, 12.50, 10.00 and 10.00 % at 25°C, 35°C, 40°C and 45°C exposure, respectively. At 48, 72 and 96 hours of feeding, the cumulative larval mortality ranged from 25.00 to 30.00 %, 30.00 to 50.00 % and 35.00 to 60.00 %, respectively. The larval mortality after 120h ranged from 80.00 to 52.50 %. Significantly, the highest mortality of 80.00 % was observed at 25°C followed by 70.00, 67.50, 52.50 and 52.50 % at 30°C, 35°C, 40°C and 45°C exposure.

Protein and CFU estimation of BGC-1 microencapsulated formulation with PABA as UV A protectant

BGC-1 microencapsulated formulation containing PABA as UV protectant was exposed to different temperature regimes of 25°C, 30°C, 35°C, 40°C and 45°C in the B.O.D incubator for 48 h after which the samples were subjected to protein analysis and CFU estimation (Table 7).

Colony forming units (CFU/ml)

The number of colonies per ml ranged from 1.08×10^8 to 2.07×10^8 CFU/ml. The highest number of colonies 2.07×10^8 CFU/ml was recorded at 25°C temperature. The next highest CFU/ml of 1.80×10^8 was recorded at 30°C followed by CFU counts of 1.38×10^8, 1.21×10^8 and 1.08×10^8 which were recorded at 35°C, 40°C and 45°C exposures (Table 7).

Table 7. Effect of different temperatures on larvicidal activity, colony forming units and crude protein content of native isolate *B. thuringiensis (*BGC-1) microencapsulated formulation against *H. armigera* with PABA as UV A protectant

Sample exposed to different temperature	Bioefficacy/larvicidal activity at 120 h	Crude protein (µg/ml)	CFU/ml
25°C	80.00 (63.80)[a]	173.63	2.07×10^8
30°C	70.00 (56.94)[b]	165.87	1.80×10^8
35°C	67.50 (55.50)[b]	143.56	1.38×10^8
40°C	52.50 (46.44)[c]	90.22	1.21×10^8
45°C	52.50 (46.44)[c]	89.25	1.08×10^8
S.Em ±	**1.20**	**2.57**	**0.07**
CD at 1%	**4.90**	**10.74**	**0.34**

Note: The values in parenthesis are "arcsine" transformed values. The same alphabet values are statistically comparable to one another according to DMRT.

Table 8. Bioefficacy of microencapsulated formulation of *B. thuringiensis* isolate (HD-1) with UV protectant melanin against *H. armigera* upon exposure to different temperatures

Sample exposed to different temperature	Per cent mortality				
	24 h	48 h	72 h	96 h	120 h
25°C	32.50 (34.55)[a]	52.50 (46.44)[a]	62.50 (52.33)[a]	75.00 (60.11)[a]	92.50 (78.75)[a]
30°C	30.00 (33.05)[b]	45.00 (42.11)[b]	52.50 (46.44)[b]	62.50 (52.33)[b]	77.50 (62.14)[b]
35°C	25.00 (29.88)[c]	40.00 (39.16)[c]	50.00 (45.00)[b]	60.00 (50.83)[b]	65.00 (53.77)[c]
40°C	25.00 (29.88)[c]	37.50 (37.66)[d]	50.00 (45.06)[b]	52.50 (46.72)[c]	65.00 (53.99)[c]
45°C	20.00 (19.55)[d]	37.50 (37.66)[d]	42.50 (40.61)[c]	55.00 (47.94)[c]	62.50 (52.49)[c]
Control	0.00 (0.00)[e]	0.00 (0.00)[e]	0.00 (0.00)[d]	0.00 (0.00)[d]	0.00 (0.00)[d]
S.Em ±	**0.21**	**0.34**	**0.68**	**0.81**	**1.04**
CD at 1%	**0.89**	**1.39**	**2.77**	**3.30**	**4.25**

Note: The values in parenthesis are "arcsine" transformed values. The same alphabet values are statistically comparable to one another according to DMRT.

Quantification of crude protein

The crude protein ranged from 89.25 to 173.63 µg/ml. Significantly highest crude protein recorded was 173.63 µg/ml at 25°C exposure and the remaining protein concentration ranged from 165.87 µg/ml to 89.25 µg/ml at 30°C to 45°C temperature exposure (Table 7).

Evaluation of microencapsulation formulation of HD-1 with UV A protectant melanin exposed at different temperatures against *H. armigera*

BGC-1 Microencapsulated formulation containing melanin UV A protectant was exposed to different temperatures of 25°C, 30°C, 35°C, 40°C and 45°C in the B.O.D incubator for 48 hours. After 48 hours samples were incorporated into *H. armigera* diet and fed to the second instar larva of *H. armigera* and larval mortality was recorded at 24, 48, 72, 96 and 120 hours (Table 8).

The highest mortality of 32.50 % was recorded at 25°C temperature in treatment of 24h exposure, followed by 30.00, 25.00, 25.00 and 20.00 % at 30°C, 35°C, 40°C and 45°C exposures, respectively. After 48 hours larval mortality was significantly highest with 52.50 %, at 25°C. Least mortality of 37.50 % was observed at 40°C and 45°C. Larval mortality ranged from 42.50 to 62.50 % 72 hours after exposure to 25°C. Significantly highest mortality of 62.50 % was observed at 25°C followed by 52.50, 50.00, 50.00 and 42.50 % at 30°C, 35°C, 40°C and 45°C exposures, respectively. Similarly, after 96 hours exposure to 25°C, larval mortality was significantly highest at 75.00 %. The remaining treatments recorded mortality ranging from 52.50 to 62.50 % at 40°C to 30°C exposure. At 120 hours after exposure, larval mortality ranged from 62.50 to 92.50 %. Significantly highest mortality of 92.50 % was observed at 25°C followed by 77.50, 65.00, 65.00 and 62.50 % at 30°C, 35°C, 40°C and 45°C exposures respectively (Table 8).

Protein and CFU estimation of HD-1 microencapsulated formulation with melanin as UV A protectant

HD-1 microencapsulated formulation containing melanin as UV A protectant was exposed to different temperatures of 25°C, 30°C, 35°C, 40°C and 45°C in the B.O.D incubator for 48 hours. After 48 hours samples were subjected to CFU estimation and protein estimation (Table 9).

Table 9. Effect of different temperatures on larvicidal activity, colony forming units and crude protein content of *B. thuringiensis* isolates (HD-1) microencapsulated formulation against *H. armigera* with melanin as UV A protectant

Sample exposed to different temperature	Bioefficacy/larvicidal activity at 120 h	Crude protein (µg/ml)	CFU/ml
25°C	92.50 (78.75)[a]	194.48	2.41×10^8
30°C	77.50 (62.14)[b]	173.93	1.94×10^8
35°C	65.00 (53.77)[c]	166.66	1.86×10^8
45°C	65.00 (53.99)[c]	158.65	1.37×10^8
45°C	62.50 (52.49)[c]	127.89	1.26×10^8
S.Em ±	**1.04**	**2.58**	**0.04**
CD at 1%	**4.25**	**10.72**	**0.22**

Note: The values in parenthesis are "arcsine" transformed values. The same alphabet values are statistically comparable to one another according to DMRT.

Table 10. Bioefficacy of microencapsulation formulation of *Bacillus thuringiensis* isolates (HD-1) with UV protectant PABA against *H. armigera* to different temperature exposures.

Sample exposed to different temperature	Per cent mortality after				
	24 h	48 h	72 h	96 h	120 h
25°C	15.00 (22.50)[a]	32.50 (34.71)[a]	50.00 (45.00)[a]	62.50 (52.33)[a]	80.00 (58.60)[a]
30°C	12.50 (20.46)[b]	27.50 (31.54)[b]	42.50 (40.67)[b]	62.50 (52.33)[a]	72.50 (57.16)[b]
35°C	10.00 (15.85)[c]	22.50 (28.22)[c]	40.00 (39.16)[b]	52.50 (46.37)[b]	70.00 (53.77)[c]
40°C	7.50 (13.82)[d]	22.50 (28.22)[c]	32.50 (34.71)[c]	47.50 (43.55)[c]	65.00 (50.83)[d]
45°C	5.00 (9.21)[e]	22.50 (28.22)[c]	32.50 (34.71)[c]	45.00 (42.11)[c]	60.00 (47.83)[e]
Control	0.00 (0.00)[f]	0.00 (0.00)[d]	0.00 (0.00)[d]	0.00 (0.00)[d]	0.00 (0.00)[f]
S.Em ±	**0.13**	**0.29**	**0.62**	**0.79**	**1.02**
CD at 1%	**0.54**	**1.19**	**2.56**	**3.23**	**4.19**

Note: The values in parenthesis are "arcsine" transformed values. The same alphabet values are statistically comparable to one another according to DMRT.

Colony forming units (CFU/ml)

The cumulative number of colonies per milli litre ranged from 1.26×10^8 to 2.41×10^8 CFU/ml. The significantly highest number of 2.41×10^8 CFU/ml was recorded at 25°C and the lowest of 1.26×10^8 CFU/ml was recorded at 45°C (Table 9).

Quantification of crude protein

The crude protein estimated ranged from 127.89 to 194.48 µg/ml. Significantly highest crude protein was recorded as 194.48 µg/ml at 25°C. Similarly, at 30°C, 35°C, 40°C and 45°C temperature, crude protein content was estimated as 173.93, 166.66, 158.65 and 127.89 µg/ml respectively (Table 9).

Evaluation of microencapsulation formulation of HD-1 with UV A protectant para amino benzoic acid (PABA) exposed to different temperatures

HD-1 microencapsulated formulation containing PABA UV A protectant was exposed to various temperatures of 25°C, 30°C, 35°C, 40°C and 45°C in the B.O.D incubator for 48 hours. After 48h samples were incorporated into the diet of the second instar larva of *H. armigera* and larval mortality was recorded at 24, 48, 72, 96, and 120 hours (Table 10).

The larval mortality after 24 hours ranged from 5.00 to 15.00 %. Significantly highest mortality of 15.00 % was observed at 25°C temperature followed by 12.50, 10.00, 7.50 and 5.00 % at 30°C, 35°C, 40°C and 45°C exposure. Larval mortality after 48h ranged from 22.50 to 32.50 %. Significantly highest mortality of 32.50 % was observed at 25°C. The remaining per cent mortality ranged from 27.50 to 22.50 % at 30°C to 45°C exposure. At 72 hours and 96 hours of feeding, the cumulative larval mortality ranged from 32.50 to 50.00 % and 45.00 to 62.50 %, respectively. Similarly, larval mortality after 120 hours ranged from 60.00 to 80.00 %. Significantly highest mortality of 80.00 % was observed at 25°C followed by 72.50, 70.00, 65.00 and 60.00 % at 30°C, 35°C, 40°C and 45°C exposures, respectively (Table 10).

Protein and CFU estimation of HD -1 microencapsulated formulation with PABA as UV A protectant

HD-1 microencapsulated formulation containing PABA as UV protectant was exposed to different temperatures of

Table 11. Effect of different temperatures on bioefficacy, colony forming units and crude protein content of *Bacillus thuringiensis* isolates (HD-1) microencapsulated formulation against *H. armigera* with PABA as UV A protectant

Sample exposed to different temperature	Bioefficacy/larvicidal activity at 120 h	Crude protein (µg/ml)	CFU/ml
25°C	80.00 (48.86)^{c}	181.87	2.11×10^{8}
30°C	72.50 (58.60)^{a}	168.15	1.79×10^{8}
35°C	70.00 (57.16)^{a}	156.84	1.54×10^{8}
40°C	65.00 (53.77)^{b}	151.60	1.33×10^{8}
45°C	60.00 (50.83)^{bc}	114.11	1.17×10^{8}
S.Em ±	**1.02**	**2.64**	**0.03**
CD at 1%	**4.19**	**10.83**	**0.19**

Note: The values in parenthesis are "arcsine" transformed values. The same alphabet values are statistically comparable to one another according to DMRT.

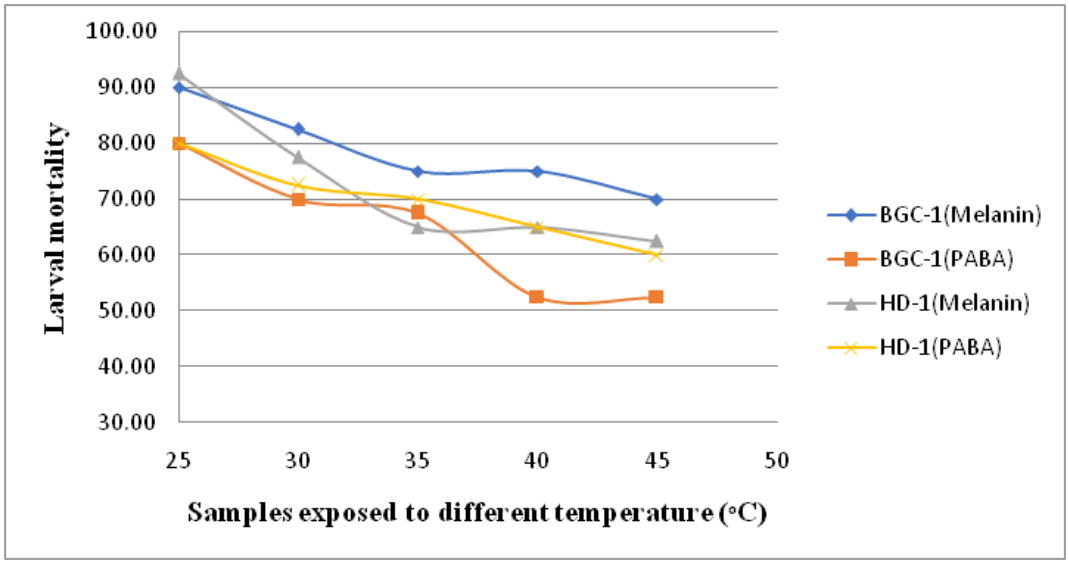

Figure 2. Comparison of bioefficacy of microencapsulated formulation of *B. thuringiensis* isolates with UV A protectants exposed to different temperatures against *H. armigera*.

25°C, 30°C, 35°C, 40°C and 45°C in the B.O.D incubator for 48 hours. After 48 hours samples were subjected to protein and CFU estimation (Table 11).

Colony forming units (CFU/ml)

The number of colonies per milli litre ranged from 1.17×10^{8} to 2.11×10^{8} CFU/ml. Significantly highest estimation of 2.11×10^{8} CFU/ml was recorded at 25°C. The next highest was 1.79×10^{8} CFU/ml which was recorded at 30°C and 45°C number of colonies recorded was 1.17×10^{8}/ml respectively (Table 11).

Quantification of crude protein

Significantly highest crude protein recorded was 181.87 µg/ml at 25°C and the remaining protein concentration ranged from 168.15 to 114.11 µg/ml at 30°C to 4°C (Table 11).

In the present study both reference strain HD-1 and local isolate BGC-1 were microencapsulated along with UV protectants (melanin and PABA) and exposed to different temperatures ranging from 25°C to 45°C in the B.O.D incubator and the same were tested for their efficacy against *H. armigera.* Among the different microencapsulated formulations, BGC-1 (melanin) recorded the highest mortality of 70.00 to 90.00 % at 25°C to 45°C exposure. This was followed by HD-1 (melanin) with 62.50 to 92.50 % mortality. However, BGC-1 (PABA) and HD-1 (PABA) recorded mortality ranging from 52.50 to 80.00 and 60.00 to 80.00 %, respectively (Figure 2).

Microencapsulation not only protects UV radiation but also protects formulation from temperature. The increased thermal stability of microencapsulated *B. thuringiensis* may be due to sodium alginate encapsulation since sodium alginate is a natural polymer and can build cell walls of microcapsules. A cross-linked matrix is created when calcium ions ($CaCl_2$) and sodium alginate are mixed. The calcium ions take the place of the sodium ions in the polymer.

Bacterial exopolysaccharides, or Extracellular Polymeric Substances (EPSs), are naturally occurring high molecular weight polymers that bacteria secrete into their surroundings to form bonds with the encapsulation matrix core surface. These bonds provide the bacterium with protection against desiccation and heat inactivation, as well as thermo stability.

In favour of our findings *B. thuringiensis* Berl. *var. israelensis* (B.t.i.), a mosquito and black fly pathogen that contains spores and a toxin crystal, was successfully encapsulated in an insolubilized carboxymethyl cellulose (CMC)-aluminium matrix to create a controlled-release bead formulation (Cokmus and Elcin, 1995). The larvicidal activity of the encapsulated B.t.i. was shown to be more robust to high temperatures (50°C) than that of the unformulated B.t.i. The formulation may work effectively in the field, based on the high reaction of Culex larvae to the encapsulated B.t.i. According to bioassays, unformulated B.t.i. can cause toxin inactivation after 60 days at 50°C, resulting in a 30% reduction in larval mortality. The encapsulated form maintained 100% lethality against the target larvae at the same temperature.

Elcin (1995) evaluated calcium alginate encapsulated *Bacillus sphaericus* 2362 against mosquitoes at the highest temperature of 50ºC and the study revealed that spore viability was decreased drastically to the tune of 10^4 folds in non-encapsulated *B. sphaericus* 2362 suspension, whereas, encapsulated *B. sphaericus* 2362 with calcium alginate at 1% improved the spore viability (from 6×10^8 to 6×10^6 spores/ml). Maximum spore viability of 6×10^8 was retained at 1.5% and 2 % in calcium alginate-encapsulated *B. sphaericus* 2362.

CONCLUSION

In the present study both reference strain HD-1 and local isolate BGC-1 were microencapsulated along with UV protectants (melanin and PABA) and exposed to different temperatures ranging from 25ºC to 45ºC in the B.O.D incubator and the same were tested for their efficacy against *H. armigera.* Among the different microencapsulated formulations, BGC-1 (melanin) recorded the highest mortality of 70.00 to 90.00 % at 25ºC to 45ºC exposure, followed by HD-1 (melanin) with 62.50 to 92.50 % mortality. Significantly highest number of colonies per ml was 2.11×10^8 CFU/ml at 25ºC, and the lowest was 1.17×10^8/ml CFU/ml at 45ºC. This indicated that microencapsulation not only protects UV radiation but also protects formulation from temperature.

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