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Research Article

In vitro evaluation of microencapsulated *Bacillus thuringiensis* (Berliner) formulation against *Helicoverpa armigera* (Hubner)

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ABSTRACT: An experiment was conducted to evaluate the microencapsulated formulation of lyophilized spore crystal aggregate of native isolate BGC-1 and reference isolate HD-1 against second instar larvae of *Helicoverpa armigera*. The 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 para-aminobenzoic acid were evaluated by exposing microencapsulated *Bacillus thuringiensis* to UV A light at 365nm. Among four microencapsulated formulations, BGC-1 with melanin recorded significantly highest mortality of 95.00 per cent at 0h exposure, as time increased, the mortality decreased and HD-1 was on par with BGC-1.

KEYWORDS: *Bacillus thuringiensis,* bioassay, *Helicoverpa armigera,* microencapsulation, PCR, UV protectants

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INTRODUCTION

Bacillus thuringiensis is a gram-positive soil bacterium that is widely used as a biocontrol agent. Its toxicity is attributed to the production of delta endotoxins or Insecticidal Crystal Protein (ICP) by the sporulating cells. The inactive protoxins in the insect midgut are broken down by proteolysis to create active toxins. These toxins adhere to the cells in insects' midguts and cause cell lysis by opening ion pores, which stops the insects from eating and ultimately kills them (Gill *et al*., 1992). The Insecticidal Crystal Proteins (ICPs) have been categorised into four classes: Cry I and Cry II against lepidopteran insects, Cry III against coleopteran insects, and Cry IV against dipteran insects. This classification is based on their host range and sequence homology.

Some crystal proteins are also reported to be nematicidal. In addition to ICPs, some strains of *B. thuringiensis* have also been reported to produce specific insecticidal proteins called Vegetative Insecticidal Proteins (VIPs) and Cytotoxic proteins (Cyt) (Estruch *et al.,* 1996). Despite all of these benefits, several *B. thuringiensis* formulations are unable to spread effectively in crops because of a range of environmental stresses, such as temperature, precipitation, UV radiation and the physiology of foliage, of which radiation is the most significant (Myasnik *et al*., 2001). The inactivation of crystal proteins against insect pests is mainly caused by ultraviolet radiation from the UV-B (280-400 nm) and UV-C (100-280 nm) spectrum, according to Griego and Spence (1978). To increase the effectiveness and performance of biopesticides, several protective techniques (dust, granules, water-dispersible granules, wettable powders, emulsions, suspension concentrates, etc.) have been developed. While some of these techniques have been successfully tested, higher production costs and the possibility of environmental contamination have prevented them from being commercialised.

Among UV radiation protection approaches, microencapsulation was offered as a reliable, secure, and viable alternative that could be implemented on a large scale. (Poncelet *et al*., 1992). Microencapsulation is a novel technology that functions as a tool to regulate and protect the release of a substance. It has been effectively utilised to safeguard substances that are susceptible to fluctuations in temperature, photodegradation, oxidation, moisture, and other undesirable reactions, increasing the potential applications of micro-encapsulated products and offering an alternative for entomopathogen protection (Gonsalves *et al*.,

2009). For microencapsulation, gelatine, starches, cellulose, and other polymers like sodium alginate can all be utilised. Of these, immobilising biological materials with sodium alginate has been thoroughly investigated (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 (Sopeña *et al*., 2009). Large particle diameters, which are reliant on the extrusion element's diameter, are a drawback of this approach. Encapsulation in the form of microcapsules is being investigated extensively as a method to obtain smaller particles of regular size to improve the formulation's performance and efficiency as well as its aerial dispersion onto foliage and larval uptake. (Huang *et al*., 2007). The objective of this study was to investigate the microencapsulation process of a lyophilized Spore Crystal Aggregate (SCA) generated by native isolate BGC-1 and *B. thuringiensis* var. *kurstaki* HD-1 (*Bt*-HD1). The internal gelation/emulsification approach served as the foundation for the methodology (Poncelet *et al*., 1992) and the goal was to create tiny microcapsules (diameter ≤10 µm) that could protect spore viability and crystal activity from intense UV radiation. Bioassays using *H. armigera* were used to test the toxicity of the encapsulated bioinsecticide.

MATERIALS AND METHODS

Maintenance of *B. thuringiensis* culture

Both the reference strain HD-1 and the local strain *B. thuringiensis* were obtained from the Department of Agricultural Entomology, Bheemarayana Gudi. Once every three months, *Bt* strains were sub-cultured on Luria agar medium at 37°C for 48 hours and pure cultures were kept at 4°C for further studies.

Lyophilization of *B. thuringiensis* isolate pellet for bioassay

The bacterial cells were inoculated in Luria broth for five days at 37°C, the turbid solution was subjected to centrifugation at 10,000 rpm for 10 minutes, the supernatant was discarded and the pellets were exposed to lyophilization to make a 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 diluting up to 1×10^{-9}.

Preparation of microencapsulation formulation of *B. thuringiensis* isolates

To prepare *B. thuringiensis* microencapsulation, the emulsion gelling method as described by Rodrigues *et al*. (2006) was followed with minor modifications. 60 ml corn oil, 200 µl Span80 and 10 ml of three distinct polymers such as sodium alginate, starch, and gelatine at the concentrations of 2, 3, and 5 per cent w/w, as well as 100 mg SCA (10^8 spores/ml were blended. Agitation was maintained at 2000 rpm for ten minutes, after that, calcium chloride was added in two phases. In the phase first, 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. To precipitate the microcapsules, 150 ml of $CaCl_2$ (0.05M) was added in the second phase. Until the microcapsules formed, agitation was maintained at 2000 rpm for 45 minutes. After centrifugation at 10,000 rpm for 20 minutes, the microcapsules were retrieved and stored at 4°C after being rinsed with distilled water. SEM, or scanning electron microscopy, was used to assess the microcapsule morphology (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 *B. thuringiensis* isolates

Microencapsulated *B. thuringiensis* isolates (BGC-1 and HD-1) were tested at different concentrations against second-instar larvae of *H. armigera* viz., 0.5, 1, 1.5, 2 and 2.5 gm/l of water. The larvae fed with sterile distilled water served as control. Four replications were maintained for each concentration. Different concentrations of microencapsulated *B. thuringiensis* isolates (BGC-1 and HD-1) were tested against second instar larvae of *H. armigera* at different concentrations viz., 0.5, 1, 1.5, 2, and 2.5 gm/l of water. Control insects were fed with sterile distilled water. Four replications were maintained for each concentration. The diet was added in a thin layer to twelve multi-cavity trays with a surface area of 3.14 cm^2, and about 4 ml of diet per well. For every concentration, 146 µl of the bacterial suspension containing Tween-80 (0.02%) was applied to the diet surface in each well and it was left for an hour. Each well contained one pre-starved (4 hours) second instar larva. For every concentration, 40 larvae in total were used with 10 larvae per replication (4 replications) and control was also maintained. These trays were maintained in an insectary with 26 ± 1°C, 70 ± 5.0 per cent Relative Humidity (RH), and 16:8 hours of light to dark. At 24-, 48-, 72-, 96-, and 120-hours following

treatment, observations on mortality were made (Devi & Vineela, 2014). To calculate the median Lethal Concentration (LC_{50}), concentrations and mortality data were used.

The promising concentration of microencapsulated formulation was selected. The suspension was transferred to a 100ml capacity beaker. The beaker was exposed to UV-A light at 365nm (NARVA, LT 18W T8/073, made in Germany) for 0-, 6-, 12-, 24-, and 48-hours. There are three types of UV light viz., UV-A (320-400 nm), UV-B (290-320 nm) and UV-C (100-290 nm). Among these three types, UV-A is more commonly found in sunlight, which is the major responsible source of *B. thuringiensis* toxin degradation in nature. Hence, UV-A light (365nm) was chosen for conducting bioassay. The UV-A light exposed *B. thuringiensis* suspension was used to conduct bioassay against the second instar larvae of *H. armigera*.

After thoroughly mixing the bacterial suspension, one milligram of the sample was aseptically extracted and transferred into one ml of distilled water blank, and 0.1 ml of the sample was extracted and transferred into 0.9 ml of water blank. This allowed for the determination of the total number of viable cells in the bacterial suspension. Serial dilutions were made and 100µL of suspension was plated on LA medium plates. These plates were incubated at 30ºC for 24 hours in a B.O.D. incubator. Colonies appearing on the plates were counted and CFU per milliliter in the bacterial suspension was calculated. After making serial dilutions, 100µl of the suspension was plated onto LA medium plates. These plates were placed in a B.O.D. incubator and incubated for 24 hours at 30ºC. The number of colonies grown on the plates was recorded and Conoly forming units for each plate were computed.

RESULTS AND DISCUSSION

Morphology and particle size distribution of microcapsules of *B. thuringiensis* isolates

The surfaces of all the spore-crystal aggregates that were placed inside microcapsules were smooth and had a spherical shape. The *B. thuringiensis* microcapsules were verified by the shape of those particles observed by SEM analysis. 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 zeta sizer (Figure 1). a) The average particle diameter of BGC-1 Microencapsulated without UV A protectant was 3.2 µm or (3256 d.nm). b) The average particle diameter of HD-1 Microencapsulated without UV A protectant was 3.4 µm or (3407 d.nm). c) The average particle diameter of BGC-1 Microencapsulated with Melanin UV A protectant was 8.3 µm or (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 or (5486 d.nm). e) The average particle diameter of HD-1 Microencapsulated with Melanin UV A protectant was 7.3 µm or (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 or (4993 d.nm) (Figure 1).

Our results are in line with the reports of García *et al*., (2011), who conducted an experiment on the microencapsulation of spore crystal complex of *B. thuringiensis* var. *kurstak* HD-1 and reported that the diameter of microcapsules ranged from 3.1 ± 0.2 to 6.8 ± 0.4 µm. similarly (Khorramvatan *et al*., 2017) conducted

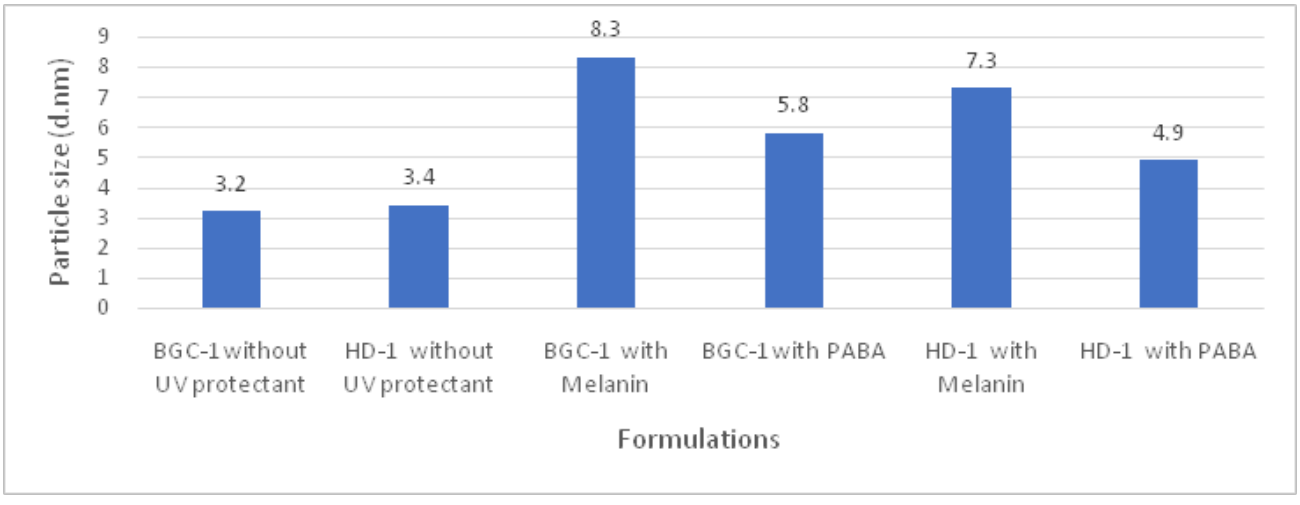

Figure 1. The particle size of microencapsulated formulations of *B. thuringiensis* isolates.

a study on optimizing microencapsulation formulation stability of *B. thuringiensis* subsp. *kurstaki* (*Bt*-KD2) against ultraviolet conditions and they revealed that the size of the microcapsules was in the range of 8 to 20 µm. Similarly, Gifani *et al*., (2015) evaluated the microencapsulated formulation of HaNPV against *H. armigera* to improve efficacy against ultraviolet radiation and the result revealed that the average size of microcapsules was 10±4.57µm.

Further microencapsulated formulations were evaluated against *H. armigera* at different temperatures and UV radiation at 365nm. The results obtained are discussed as follows.

Median lethal concentration (LC_{50}) of lyophilized *B. thuringiensis* isolates against *H. armigera*

LC_{50} for the native *B. thuringiensis* BGC-1 isolate was 0.66 g/l, whereas it was found to be the lowest with 0.50 g/l in the reference strain (*B. thuringiensis* var *Kurtaski* HD-1). The LC_{50} value of BGC-1 was very close to the LC_{50} value of the HD-1 strain. Similarly, LC_{95} of native isolate BGC-1 was found to be lowest with 9.29 g/l, followed by HD-1 **(**10.96 g/l) indicating that HD-1 LC_{95} value is slightly higher than BGC-1 (Table 1). The current results showed that increasing concentration of lyophilized powder exhibited increasing mortality of *H. armigera* was noticed and there was a direct relationship between mortality and concentration of lyophilized powder. Similar findings were observed by several researchers who noticed higher concentrations resulted in higher mortality (Sareen *et al.,* 1983). Both *B. thuringiensis* strains (reference HD-1 and BGC-1) did not exhibit 100 per cent mortality. Similar studies were also revealed by Lakshminarayana and Sujatha (2005), who noticed that none of the tested strains caused 100 per cent mortality at the highest concentration even up to 6 days after treatment. As the concentration of bacteria and the period of exposure varied, the degree of pathogenicity also varied (Savitri & Mohan, 2003). Devi and Vineela. (2015) studied the efficacy of suspension concentrate formulation (*B. thuringiensis* var. *kurstaki* + boric acid (75: 25)) against *H. armigera*. The formulation was highly effective with an LC_{50} value of 185 µL (53.36 mg). Kalantari *et al*., (2014) evaluated three native *B. thuringiensis* isolates against bollworms and the result revealed that the isolate KD-2 recorded significantly highest mortality with LC_{50} value of 1.2×10^7 spore/ml.

Microencapsulation formulation of BGC-1 with UV protectant melanin by exposing to UV A light (365 nm) at different time intervals UV LIGHT (365 nm)

Melanin-containing BGC-1 microencapsulated formulation was subjected to UV A light at 365 nm at various intervals between 0- and 48 hours, as well as a control treatment that did not receive any UV A light exposure. The UVA-treated microencapsulation formulation was exposed to second instar larvae of *H. armigera* and larval mortality was recorded at 24-, 48-, 72-, 96-, and 120-hours after treatment (Table 2). The result revealed that 0 to 48 hours of UV A light exposure had larval mortality ranging from 12.50 to 30.00 per cent after a 24 hours exposure. At 0 hours of UV A light exposure, the mortality was significantly highest at 30.00 per cent, followed by 20.00, 17.50, 15.00, and 12.50 per cent at 6-, 12-, 24-, and 48-hours of UV A light exposure. In the control treatment, there was no mortality was noticed. Following a 48 hours exposure period, the 0 h treatment had the highest larval mortality of 50.00 per cent, followed by the 6-, 12-, 24-, and 48-hours exposure times with 45.00, 37.50, 30.00, and 22.50 per cent, respectively. Seventy-two hours after exposure, the 0 h treatment recorded significantly highest mortality of 75.00 per cent. At 6-, 12-, 24-, and 48-hours following exposure, the per cent mortality for the remaining treatments ranged from 50.00 to 67.50 per cent. Larval mortality after 96 hours varied from 62.50 to 90.00 per cent. Significantly maximum mortality was recorded in 0 hours and 6 hours treatment with 90.00 per cent each and significantly lowest mortality was recorded with 62.50 per cent 48 hours after exposure. After 120 hours, the cumulative larval mortality ranged from 72.50 to 95.0 per cent. The 0 hours treatment recorded significantly highest mortality with 95.00 per cent, which was followed by 92.50, 80.00, 77.50, and 72.50 per cent in the 6-, 12-, 24-, and 48-hours after treatments. In the control treatment, there was no recorded mortality (Table 2). The result indicated that the microencapsulation of *B. thuringiensis* with Melanin protected the degradation of toxin up to 6 hours of exposure to UV A light at 365 nm.

Protein and CFU (colony forming units) estimation of BGC-1 with melanin as UV A protectant

BGC-1 microencapsulated formulation containing melanin as UV A protectant was exposed to UV A light (365 nm) at 0-, 6-, 12-, 24- and 48-hours intervals and samples were subjected to estimation of colony forming units (CFU/

Table 1. Concentration mortality response (LC_{50}) of microencapsulation formulation of *B. thuringiensis* against *H. armigera*

Sl. No.	Isolates	LC_{50} (g/l)	Fiducially limit		Regression equation	$\chi 2$ value	LC_{95} (g/l)	Fiducial limit	
			Lower	Upper				Lower	Upper
1	HD-1 (ref)	0.50	0.26	0.96	Y= 5.368+1.292x	5.22	10.96	2.45	49.09
2	BGC-1	0.66	0.42	1.02	Y= 5.259+1.481x	1.30	9.29	3.17	27.19

ml) and protein estimation by Lowry's method (Table 3). The number of colonies per milli litre ranged from 1.96×10^8 to 2.92×10^8 CFU/ml. The highest number of colonies (2.92×10^8) was recorded at 0 hours of UV A light exposure. The next highest CFU/ml 2.78×10^8 was recorded at 6 hours followed by cell counts of 2.48×10^8, 2.03×10^8 and 1.96×10^8 at 12-, 24-, and 48 hours after exposure, respectively (Table 3). Crude protein ranged from 128.26 to 198.85 µg/ml, significantly highest crude protein recorded at 198.85 µg/ml at 0h of UV A light exposure. The next highest was 184.78 µg/ml at 6 hours of exposure. The least recorded was 128.26 µg/ml at 48 hours of exposure (Table 3).

Microencapsulation formulation of BGC-1 with UV A protectant PABA against *H. armigera* at different time intervals

The BGC-1 microencapsulated formulation containing PABA was subjected to UV A light at 365 nm at different times of 0-, 6-, 1-2, 24-, and 48-hours. The control sample was not exposed to UV light, and the samples that were exposed to UV A light were fed to second-instar larvae of *H. armigera* and larval mortality was determined at 24-, 48-, 72-, 96-, and 120-hours after exposure. The larval mortality after 24 hours ranged from 5.00 to 17.50 per cent in the treatments of feeding and UV A light exposure of 365 nm from 0 to 48 hours (Table 2). The mortality rate was highest (17.50 per cent) at 0 hours of UV A light exposure, followed by 15.00, 12.50, 12.50, and 5.00 per cent at 6-, 12-, 24-, and 48-hours respectively of UV A light exposure. In the control treatment, there was no mortality observed. Larval mortality after 48 hours varied from 15.00 to 30.00 per cent. The 0 hours treatment recorded the highest mortality with 30.00 per cent, which was followed by the treatments 6-, 12-, 24-, and 48-hours with mortality of 27.50, 25.00, 22.50, and 15.00 per cent respectively. After 72 hours, the treatment 0 hours recorded significantly the highest mortality (52.50 per cent). The percentage mortality in the remaining treatments varied from 42.50 to 32.50 per cent in the 6 to 48 hours treatments. The 0 hours treatment showed the highest mortality of 80.00 per cent, which was followed by 70.00, 62.50, 55.00, and 47.50 per cent in the 6-, 12-, 24-, and 48-hours treatments, respectively. After 120 hours, the cumulative larval mortality ranged from 70.00 to 95.0 per cent. The 0 hours treatment exhibited the highest mortality of 95.00 per

Table 2. Bio-efficacy of microencapsulation formulation of native isolate *B. thuringiensis* (BGC-1) with UV protectants melanin and PABA against *H. armigera* to different times of exposure to UV A light (365nm)

Duration of sample exposed to UV light (365nm)	BGC-1 with UV-protectant melanin					BGC-1 with UV protectant PABA				
	Per cent mortality after									
	24 hours	48 hours	72 hours	96 hours	120 hours	24 hours	48 hours	72 hours	96 hours	120 hours
0 hours	30.00 (33.05)^a	50.00 (45.06)^a	75.00 (60.11)^a	90.00 (74.14)^a	95.00 (80.78)^a	17.50 (21.58)^b	30.00 (33.05)^a	52.50 (46.44)^a	80.00 (63.80)^a	95.00 (80.78)^a
6 hours	20.00 (26.19)^b	45.00 (42.11)^b	67.50 (56.03)^b	90.00 (74.14)^a	92.50 (78.75)^a	15.00 (22.50)^a	27.50 (31.54)^b	42.50 (40.61)^b	70.00 (56.94)^b	90.00 (74.14)^b
12 hours	17.50 (24.16)^c	37.50 (37.66)^c	60.00 (50.83)^c	72.50 (58.60)^b	80.00 (63.80)^b	12.50 (17.89)^d	25.00 (29.88)^c	40.00 (39.16)^{bc}	62.50 (52.33)^c	75.00 (60.63)^c
24 hours	15.00 (19.55)^d	30.00 (33.05)^d	52.50 (46.44)^d	65.00 (53.77)^c	77.50 (62.14)^{bc}	12.50 (20.46)^c	22.50 (28.22)^d	37.50 (37.66)^c	55.00 (47.88)^d	70.00 (56.94)^d
48 hours	12.50 (17.52)^c	22.50 (27.69)^c	50.00 (45.06)^d	62.50 (52.49)^c	72.50 (58.60)^c	5.00 (9.21)^e	15.00 (22.50)^e	32.50 (34.71)^d	47.50 (43.55)^e	70.00 (56.94)^d
Control	0.00 (0.00)^e	0.00 (0.00)^f	0.00 (0.00)^e	0.00 (0.00)^d	0.00 (0.00)^d	0.00 (0.00)^f	0.00 (0.00)^f	0.00 (0.00)^e	0.00 (0.00)^f	0.00 (0.00)^e
S.Em±	0.14	0.43	0.68	1.00	1.24	0.13	0.30	0.51	0.75	1.07
CD @ 1%	0.58	1.78	2.78	4.09	5.05	0.55	1.25	2.11	3.07	4.37

Note: Figures in the parentheses are "arcsine" transformed values. The values represented by the same alphabet are statistically on par with each other by DMRT.

Table 3. Effect of Ultraviolet light of 365nm on larvicidal activity, colony forming units and crude protein content of native. Isolate *B. thuringiensis* (BGC-1) microencapsulation formulation against *H. armigera* with melanin and PABA as UV A protectants

Duration of the sample exposed to UV A light at 365 nm	Melanin			PABA		
	Bioefficacy/ larvicidal activity at 120 h	Crude protein (µg/ml)	CFU/ml	Bioefficacy/ larvicidal activity at 120 h	Crude protein (µg/ml)	CFU/ml
0 hours	95.00 (80.78)^a	198.85^a	2.92×10^8	95.00 (80.78)^a	195.94^a	2.87×10^8
6 hours	92.50 (78.75)^a	184.78^b	2.78×10^8	90.00 (74.14)^b	183.33^b	2.69×10^8
12 hours	80.00 (63.80)^b	166.05^c	2.48×10^8	75.00 (60.63)^c	164.90^c	2.37×10^8
24 hours	77.50 (62.14)^{bc}	131.92^d	2.11×10^8	70.00 (56.94)^d	128.05^d	2.03×10^8
48 hours	72.50 (58.60)^c	128.26^d	1.96×10^8	70.00 (56.94)^d	127.56^d	1.87×10^8
S.Em ±	1.24	2.61	0.09	1.07	2.57	0.07
CD @ 1%	5.05	10.91	0.38	4.37	10.63	0.32

Note: Figures in the parentheses are "arcsine" transformed values. The values represented by the same alphabet are statistically on par with each other by DMRT.

cent, which was followed by the 6-, 12-, 24-, and 48-hours treatments, with mortality of 90.00, 75.00, 70.00, and 70.00 per cent, respectively. In the control treatment, no mortality was observed. The 0 hours treatment had the significantly highest mortality with 95.00 per cent (Table 2).

Protein estimation of BGC-1 microencapsulation formulation with PABA as UV A protectant

BGC-1 microencapsulated formulation containing PABA as UV A protectant was exposed to UV A light of 365 nm at 0-, 6-, 12-, 24-, and 48-hours and samples were subjected to protein analysis and CFU estimation (Table 3). The number of colonies per millilitre ranged from 1.87×10^8 to 2.87×10^8 CFU/ml, significantly highest number of colonies (2.87×10^8) were recorded at 0 hours UV A light exposure. The next highest was 2.69×10^8 CFU/ml which was recorded at 6 hours and the least number of colonies 1.87×10^8 was recorded at 48 hours after UV A exposure (Table 3). Significantly highest crude protein recorded was 195.94 µg/ml at 0h UV A light exposure. The next highest crude protein recorded was 183.33 µg/ml at 6 hours followed by 164.90 µg/ml, 128.05 µg/ml and 127.56 µg/ml at 12-, 24-, and 48-hours, respectively (Table 3).

Microencapsulation formulation of HD-1 with UV A protectant melanin against *H. armigera* at different time intervals

HD-1 microencapsulated formulation containing melanin UV A protectant was exposed to UV A light at 365 nm at different intervals 0-, 6-, 12-, 24-, and 48-hours and UV A treated samples were incorporated in *H. armigera* diet and were fed to second instar larva of *H. armigera* and larval mortality was recorded at 24-, 48-, 72-, 96-, and 120-hours (Table 4). Larval mortality after 24 hours, ranged from 20.00 to 10.00 per cent in the treatments of feeding in the UV A light of 365 nm exposed treatments of 0 to 48 hours. Significantly, the highest mortality of 20.00 per cent was observed at 0h UV A light exposure followed by 20.00, 15.00, 12.50 and 10.00 per cent at 6-, 12-, 24-, and 48-hours exposure. There was no mortality observed in the control treatment. Significantly the highest mortality of 45.00 per cent was recorded in the 0 h exposure, 48 hours after treatment. In the remaining treatments, larval mortality ranged from 22.50 to 32.50 per cent at 48 to 64 hour exposure. Larval mortality ranged from 67.50 to 45.00 per cent 72 hours after treatment. Among the different times of UV A exposure, the highest mortality of 67.50 per cent was recorded in the 0 hours treatment. The lowest mortality of 45.00 per cent was recorded in the 48 hours exposure. After 96 hours significantly highest mortality of 92.50 per cent was recorded in the 0-hour treatment followed by 82.50 per cent recorded in the 6-hours after exposure. The lowest of 70.00 per cent was noticed with 48 hours of UV A light exposure. Similarly, with 120 hours of UV A exposure larval mortality ranged from 92.50 to 80.00 per cent. Significantly highest mortality of 92.50 per cent was recorded in the 0 hours treatment followed by 90.00, 90.00, 85.00 and 80.00 per cent recorded in the 6-, 12-, 24-, and 48-hours treatments (Table 4).

Protein 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 UVA light of 365 nm at 0-, 6-, 12-, 24-, and 48 hours and samples were subjected to protein analysis and CFU estimation (Table 5). The number of colonies per milli litre ranged from 1.91×10^8 to 2.74×10^8 CFU/ml. Significantly highest number of colonies (2.74×10^8) was recorded at 0h UV A light exposure. The next highest was 2.59×10^8 CFU/ml which was recorded at 6 hours followed by 2.47×10^8, 2.32×10^8 and 1.91×10^8 CFU/ml was recorded at 12-, 24-, and 48 hours after exposure (Table 5). Crude protein was recorded ranging from 138.23 to 186.72 µg/ml. significantly highest crude protein recorded was 186.72 µg/ml at 0h UV A light exposure. Similarly, at 0-, 6-, 12-, 24-, and 48 hours crude protein recorded were 177.99 µg/ml, 163.93 µg/ml, 160.05 µg/ml and 138.23 µg/ml, respectively (Table 5).

Microencapsulation formulation of HD-1 with UV A protectant PABA (Para amino benzoic acid) against *H. armigera* at different time intervals

HD-1 Microencapsulated formulation with UV A protectant PABA was exposed to UV A at 365 nm at different intervals ranging from 0h to 48h and after UV A treatment samples were fed to the second instar larva of *H. armigera* and larval mortality was recorded at 24-, 48-, 72-, 96- and 120-hours (Table 4). The larval mortality after 24h ranged from 10.00 to 20.00 per cent in the treatments during 48 to 0 hours UV A light exposure. Significantly highest mortality of 20.00 per cent was observed at 0 and 6 hours UV A light exposure followed by 17.50, 15.00, and 10.00 per cent at 12-, 24-, and 48-hours UV A light exposure. After 48 hours larval mortality ranged from 20.00 to 30.00 per cent. The highest mortality of 30.00 per cent was recorded at 0 hours of UV A exposure and 20.00 per cent was the least mortality recorded at 48 hours of exposure. Similarly, 72 hours after treatment larval mortality ranged from 50.00 to 32.50 per cent. The highest mortality of 50.00 per cent was recorded at 0h and the remaining per cent mortality ranged from 47.50 to 32.50 in treatments with 6 to 48 hours of UV A exposure. Larval mortality ranged from 50.00 to 77.50 per cent after 96 hours of treatment. Significantly highest mortality of 77.50 per cent was recorded at 0 hours treatment and 50.00 per cent was the least mortality noticed at 48 hours of UV A exposure. After 120 hours larval mortality ranged from 75.00 to 90.00 per cent. There was no mortality was observed in the control

Table 4. Bio-efficacy of microencapsulation formulation of *B. thuringiensis* isolates (HD-1) with UV A protectant melanin and PABA against *H. armigera* to different times of exposure to UV A light at (365nm)

Duration of sample exposed to UV light (365nm)	HD-1 with UV-protectant melanin					HD-1 with UV protectant PABA				
	Per cent mortality after									
	24 hours	48 hours	72 hours	96 hours	120 hours	24 hours	48 hours	72 hours	96 hours	120 hours
0 hours	20.00 (26.19)^{a}	45.00 (42.05)^{a}	67.50 (55.50)^{a}	92.50 (78.50)^{a}	92.50 (78.75)^{a}	20.00 (26.19)^{a}	30.00 (33.05)^{a}	50.00 (44.93)^{a}	77.50 (62.14)^{a}	90.00 (74.14)^{a}
6 hours	20.00 (26.19)^{a}	32.50 (34.71)^{b}	57.50 (49.38)^{b}	82.50 (65.83)^{b}	90.00 (76.71)^{b}	20.00 (26.19)^{a}	30.00 (33.05)^{a}	47.50 (43.55)^{a}	62.50 (52.33)^{b}	90.00 (74.14)^{a}
12 hours	15.00 (19.55)^{b}	27.50 (31.39)^{c}	50.00 (45.00)^{c}	75.00 (60.63)^{c}	90.00 (76.71)^{b}	17.50 (21.58)^{c}	27.50 (31.54)^{b}	40.00 (39.16)^{b}	60.00 (50.83)^{b}	85.00 (70.44)^{b}
24 hours	12.50 (17.89)^{c}	25.00 (29.88)^{d}	47.50 (43.55)^{cd}	75.00 (63.58)^{b}	85.00 (70.44)^{c}	15.00 (22.50)^{b}	27.50 (31.39)^{b}	37.50 (37.66)^{b}	55.00 (47.94)^{c}	80.00 (66.75)^{c}
48 hours	10.00 (15.88)^{d}	22.50 (27.69)^{e}	45.00 (42.05)^{d}	70.00 (56.94)^{d}	80.00 (63.80)^{d}	10.00 (15.85)^{d}	20.00 (26.19)^{c}	32.50 (34.34)^{c}	50.00 (44.93)^{d}	75.00 (60.11)^{d}
Control	0.00 (0.00)^{e}	00 (0.00)^{f}	00 (0.00)^{e}	00 (0.00)^{e}	00 (0.00)^{e}	0.00 (0.00)^{e}	0.00 (0.00)^{d}	0.00 (0.00)^{d}	0.00 (0.00)^{e}	0.00 (0.00)^{e}
S.Em±	0.17	0.48	0.90	0.99	1.21	0.24	0.31	0.52	0.74	1.08
CD @ 1%	0.71	1.98	3.68	4.05	4.96	0.97	1.29	2.13	3.01	4.39

Note: Figures in the parentheses are "arcsine" transformed values. The values represented by the same alphabet are statistically on par with each other by DMRT.

Table 5. Effect of ultraviolet light at 365nm on larvicidal activity, colony forming units and crude protein content of *B. thuringiensis* isolate (HD-1) microencapsulated formulation against *H. armigera* with melanin and PABA as UV A protectant at different times of exposure.

Duration of the sample exposed to UV A light at 365 nm	Melanin			PABA		
	Bioefficacy/ larvicidal activity at 120 h	Crude protein (µg/ml)	CFU/ml	Bioefficacy/ larvicidal activity at 120 h	Crude protein (µg/ml)	CFU/ml
0 hours	92.50 (78.75)^{a}	186.72^{a}	2.74×10^{8}	90.00 (74.14)^{a}	183.33^{a}	2.69×10^{8}
6 hours	90.00 (74.14)^{b}	177.99^{a}	2.59×10^{8}	90.00 (74.14)^{a}	172.45^{b}	2.49×10^{8}
12 hours	90.00 (76.71)^{ab}	163.93^{b}	2.47×10^{8}	85.00 (70.44)^{b}	159.08^{c}	2.23×10^{8}
24 hours	85.00 (70.44)^{c}	160.05^{b}	2.32×10^{8}	80.00 (66.75)^{c}	121.74^{d}	1.84×10^{8}
48 hours	80.00 (63.80)^{d}	138.23^{c}	1.91×10^{8}	75.00 (60.11)^{d}	101.86^{e}	1.73×10^{8}
S.Em ±	1.21	2.47	0.06	1.08	2.56	0.06
CD @ 1%	4.96	10.39	0.28	4.39	10.55	0.25

Note: The values represented by the same alphabet are statistically on par with each other by DMRT.

treatment. Significantly highest mortality of 90.00 per cent was recorded in the 0 and 6 hours treatment (Table 4).

Protein estimation of HD-1 microencapsulation formulation with PABA as UV A protectant

HD-1 microencapsulated formulation containing PABA as UV A protectant was exposed to UV A light of 365 nm at different time intervals of 0-, 6-, 12-, 24-, and 48-hours and samples were subjected to estimation of colony forming units (CFU/ml) and protein quantification. (Table 5). The cumulative number of colonies per milli litre ranged from 1.73×10^{8} to 2.69×10^{8} CFU/ml, the significantly highest number of colonies (2.69×10^{8}) was recorded at 0 hours UV A light exposure and the least number of colonies noticed was 1.73×10^{8} CFU/ml at 48 hours after exposure (Table 5). The crude protein estimated ranged from 101.86 to 183.33 µg/ml. Significantly highest crude protein was recorded as 183.33 µg/ml at 0 hours UV A light exposure. The next highest crude protein recorded was 172.45 µg/ml at 6 hours and the least protein content of 101.86 µg/ml was noticed at 48 hours after exposure (Table 5).

Encapsulation is a safe and feasible technique, that protects biopesticides. It improves their stability against UV radiation by coating the *B. thuringiensis* spores and crystals with polymers. By adding UV A protectants (melanin/PABA) to the roencapsulation of *B. thuringiensis* formulation, we can enhance the protection of crystalline toxins from inactivation by UV irradiation. Hence, microencapsulation with UV A protectants was prepared for evaluation of bio-efficacy and standardization for the native isolate BGC-1 and reference isolate HD-1 against *H. armigera.*

In the present study, native isolate BGC-1 and the reference strain HD1 with UV protectants (melanin and PABA) were used for the preparation of microencapsulation formulations and exposed to UV A light at 365 nm for 0 to 48 hours intervals and the same were evaluated for their efficacy against *H. armigera*. Among four microencapsulated formulations, BGC-1 with melanin recorded significantly highest mortality at 0 hours exposure, as time increased, the mortality decreased and the lowest was observed at 48 hours after exposure, however, the reference strain HD-1 recorded

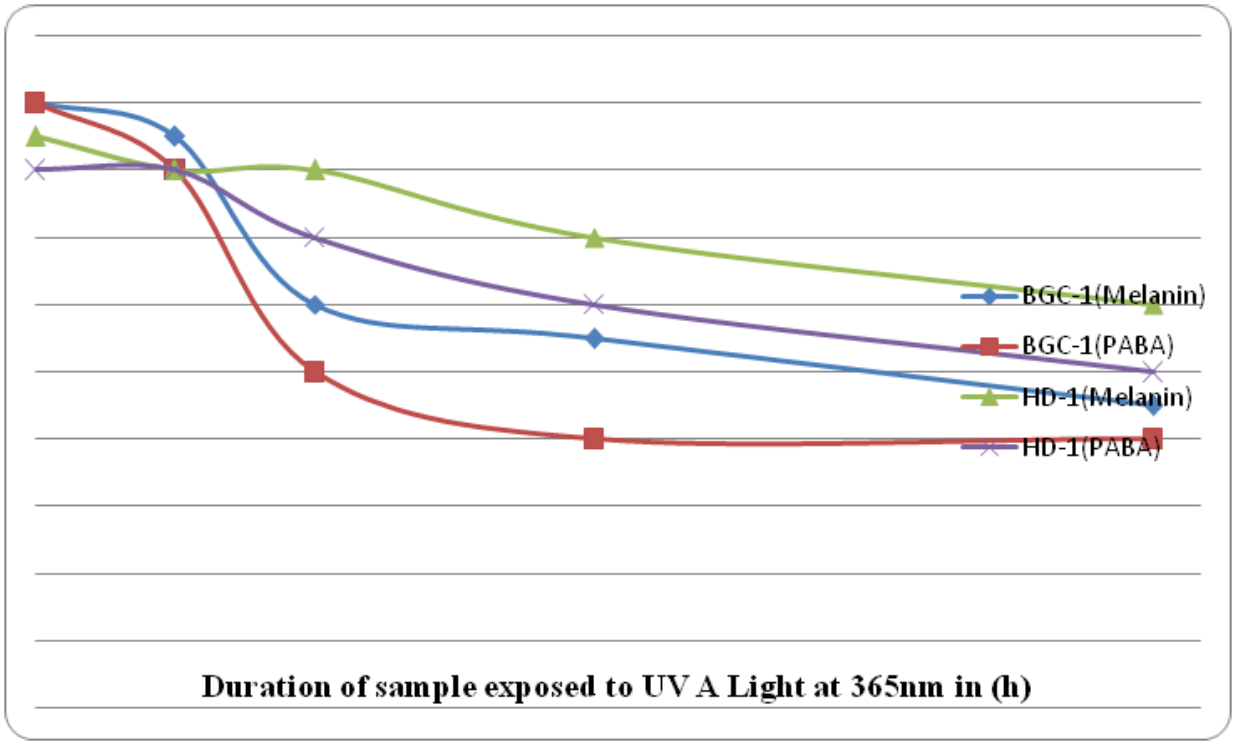

Figure 2. Comparison of bio-efficacy of microencapsulated formulation of *B. thuringiensis* isolates with UV A protectants exposed to UV A light at 365 nm for different durations against *H. armigera*.

mortality of 92.50 per cent, which was on par with BGC-1(Figure 2). Similar observations were recorded with *B. thuringiensis* with PABA. Significantly highest mortality of 95.00 per cent was recorded with BGC-1 (PABA) at 0 hours exposure followed by HD-1 PABA with 90.00 per cent. The study concludes that melanin performed better in protecting crystals and spores of *B. thuringiensis* compared to PABA this may be because melanin acts as a UV absorber. Melanin performed better in protecting the toxins and spores of *B. thuringiensis* (Figure 2).

The current results are in line with the reports of Ruan *et al.* (2004), who conducted an irradiation bioassay against *H. armigera* and exposed *B. thuringiensis* strains 94001 (with and without melanin) to UV radiation. They noticed that the *B. thuringiensis* strain 94001 without melanin had a significantly higher LC_{50} (81 µg/ml) than the strain with melanin (66 µg/ml). As a result, after irradiation, the potency of *B. thuringiensis* strains 94001 without melanin (19074 IU/mg) was lower than that of the same sample with melanin (22391 IU/mg). Ultimately, the long-term viability of *B. thuringiensis* preparations following UV radiation was found to be enhanced by melanin. This was proved experimentally by Liu *et al.* (1993) on the effectiveness of melanin in shielding *B. thuringiensis* subsp. *israelensis* mosquito larvicidal activity from UV radiation. Similarly Wan *et al.* (2007) documented the effectiveness of melanin in preventing the commercial bioinsecticide *B. thuringiensis* subsp. *israelensis* insecticidal activity from natural solar radiation as well as ultraviolet light.

The spore viability and larvicidal activity of *Bacillus sphaericus* ISPC-8 and 1593 spores were completely lost after 6 hours of exposure to UV-B radiation, and the formulations containing para-aminobenzoic acid and Congo red will protect the spore viability and larvicidal activity from UV B radiation for up to 168 hours (Hadapad *et al.*, 2009). Melanin was found to be an effective UV protectant against *B. thuringiensis* formulation spores and toxins, according to Sansinenea and Ortiz's (2014) report. Because melanin is an irregular, dark-coloured, hydrophobic, negatively charged biopolymer with a high molecular weight that is made up of polymerized phenolic and/or indolic compounds. Melanin's primary function is to provide resistance to ultraviolet light by absorbing a large portion of the electromagnetic spectrum and averting damage caused by light. Therefore, melanin is a natural pigment that is easily biodegradable compared to other chemicals (Zhang *et al.*, 2016). It has also been used commercially in photoprotective creams and eyeglasses, and it also shields several bacterial species from UV A radiation (Nosanchuk & Casadevall, 2003). Hence, melanin is a good UV A protectant for *B. thuringiensis* pesticides followed by Para Amino Benzoic Acid (PABA).

The microencapsulated formulation of *B. thuringiensis* strains BGC-1 and HD-1 with melanin and PABA were

evaluated for bioassay, simultaneously the exposed concentration of *B. thuringiensis* microencapsulation was also subjected to protein estimation by Lowry's method using the standard protocol total number of colonies forming units (CFU/ml) were also calculated. Among the different microencapsulated *B. thuringiensis* formulations, BGC-1 (melanin) recorded the highest protein content and CFU of 198.85µg/ml and 2.92×10^8 CFU/ml at 0h exposure respectively (Table 4). As the duration of exposure increases the protein content decreases from 198.85µg/ml to 128.26 µg/ml at 0 to 48 hours exposure. Similarly, the CFU counts were also decreased from 2.92×10^8 CFU/ml to 1.96×10^8 µg/ml at 0 to 4 hours of exposure. The protein concentration and CFU decreased as the exposure time increased. This may be due to the degradation of protein and inactivation of spores by UV radiation. However, melanin provided better protection against UV light compared to PABA, as evidenced by the results. Similar trend was observed in BGC-1(PABA), HD-1 (melanin) and HD-1 (PABA).

CONCLUSION

There was meagre research on the bio-efficacy of *B. thuringiensis* microencapsulation with UV protectants PABA and Melanin exposed to UV A light at 365nm. The present results revealed that protein content and colony count were significantly higher for BGC-1 with melanin formulation (159.94 µg/ml and 2.83×10^8 CFU/ml respectively) compared to BGC-1 with PABA (154.62 µg/ml and 2.78×10^8 CFU/ml, respectively). Therefore, Melanin could be used as a better UV protectant in the preparation of *B. thuringiensis* formulation to prevent the degradation of cry toxin from the UV A radiation in sunlight.

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