Evaluation of UV protectants for wettable powder formulation of native *Bacillus thuringiensis* (Berliner) isolate against *Helicoverpa armigera* (Hubner) in the Laboratory

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**ABSTRACT:** Radiation severely inactivates the potency of *Bacillus thuringiensis* spores and crystals present in sprayed formulations leading to decreased efficacy in field. Incorporation of UV protectants to biopesticides is one of the methods to protect against radiation damage. Keeping this as objective, a native isolate BGC-1 was selected for preparation and evaluation of wettable powder formulation against second instar larvae of *Helicoverpa armigera*. Median lethal concentration of the BGC-1 was 1.05 g/l and assigned biopotency value was 15428.57 ITU/g. UV protectants viz., melanin, para-amino benzoic acid, polyvinyl alcohol and Congo red were evaluated by exposing formulated solution to sunlight at different intervals of time. Among four UV protectants, melanin showed an excellent UV protecting ability with the mortality of 86.67 per cent and, 116.49 µg/ml of crude protein at 5 h sunlight exposure with temperature of 43.6°C and light intensity of 4.93×10⁵ lux followed by 80.00 per cent mortality, 1.74×10⁸ CFU/ml and 109.40 µg/ml of crude protein in para-amino benzoic acid UV protectant. Next best UV protectant was congo red with the mortality of 73.33 per cent and, 90.76 µg/ml of crude protein and 1.26×10⁸ CFU/ml. It is concluded that melanin was found to be an effective UV protectant for *B. thuringiensis* WP formulations against *H. armigera*.

**INTRODUCTION**

*Bacillus thuringiensis* Berliner (Bt) is being used as a biological control agent (Magda and Bendary, 2006). Most entomopathogens are not stable against natural environmental stresses such as Ultra Violet (UV) radiation, rain and temperature. Radiation is the main limitation that severely inactivates the potency of *B. thuringiensis* crystals against different insect pests. Formulation of biopesticides by adding various UV protectants, adjuvants or phago stimulants, is one of the methods to overcome these limitations (Amoura *et al.*, 2009), however, in adjuvant-based formulations, the contact between the protectants and active ingredient is not always adequate (Brar *et al.*, 2006).

*Helicoverpa armigera* commonly known as gram pod borer, American bollworm of cotton, tomato fruit borer, maize cob borer and sunflower head borer is a polyphagous pest. This pest causes extensive losses in cotton, pulses, oilseeds and certain vegetable crops in India. In India, total losses in both pulses and cotton exceed $530 million annually and the extent of losses in chickpea and pigeonpea worldwide has been estimated over $927 million annually (Ragesh *et al*., 2015). Due to excessive and injudicious use of chemical pesticides this pest has started developing resistance to most chemical pesticides. Hence, *B. thuringiensis* is an effective insecticide, relatively harmless to natural enemies, safe to the higher animals and environmentally acceptable.

Limited study on the effect of *B. thuringiensis* formulations on the *H. armigera* is a serious drawback in the field of *H. armigera* management. There is an urgent need for screening of newer adjuvants that may be incorporated in formulations to improve the efficacy of *B. thuringiensis*. Hence, the present study was taken to develop effective *B. thuringiensis* formulation against *H. armigera*. The main objective was to compare the protective effect of the UV protectants on the stability of formulations exposed to UV radiation. Spore viability and the crude protein content of WP formulations also evaluated.

**MATERIALS AND METHODS**

**Site of experiment**

All experiments in this research work were carried out in the Department of Agricultural Entomology, A.C, Bheemarayanagudi and NFSM lab, UAS Raichur during the year of 2016–17.
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Maintenance of Bacillus thuringiensis culture

The native Bacillus thuringiensis strain BGC-1 along with the reference HD1 strain were taken from Department of Agricultural Entomology, AC, B’gud. B. thuringiensis strains were sub cultured on Luria agar medium at 30°C for 48 h and stored at 4°C for the further studies.

Lyophilization of bacterial pellets

The bacterial cells were suspended in five liter Luria broth for five days at 30°C. The turbid solution was centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and pellets were subjected to lyophilization to technical powder as shown in flowchart. The lyophilized powder was stored at 4°C. Lyophilized B. thuringiensis technical powder was used to prepare six different concentrations of (1 × 10⁻⁴, 1 × 10⁻⁵, 1 × 10⁻⁶, 1 × 10⁻⁷, 1 × 10⁻⁸ and 1 × 10⁻⁹) for conducting bioassay against second instar larvae of Helicoverpa armigera.

Mass rearing of Helicoverpa armigera

Mass rearing of cotton bollworm, H. armigera was reared in the laboratory on the chickpea based artificial diet till pupation (Kranthi, 2005). Newly formed pupae were collected on daily basis and they were sexed into male and female pupae based on their genital structure and maintained. After the emergence, adults were introduced into ovipositional chamber. An each alternative day, fresh honey solution was prepared and soaked in a cotton wad and hanged in ovipositional chamber. Later the ovipositional chamber was covered with sterile black muslin cloth and secured with rubber band. Similarly, fresh black muslin cloth was provided on each alternate day for oviposition. Later, egg mass along with muslin cloth was transferred to a rearing box with moist sponge pad to facilitate emergence of neonate larvae. After emergence, the neonate larvae were released on birdbox containing artificial diet for two days and then transferred to multi cavity tray containing artificial diet. Second instar larvae were used for further laboratory bioassay studies.

Flow chart of lyophilization of Bacillus thuringiensis

B. thuringiensis pellet were transferred to the petriplate with sterilized the spatula and spread it uniformly

Cover with aluminum foil and holes were made on foil

Kept in deep freezer at - 20°C for 2-3 hr

Again Kept in deep freezer at - 80°C for 2-3 hr

liquid nitrogen of 10-20 ml was added

After 10 min, petriplate containing B. thuringiensis pellet flakes were grinded with help of mortar and pestle

Bioassay of promising native lyophilized Bacillus thuringiensis technical powder

The diet was poured as a thin layer into 12 celled multi cavity trays, approximately 4 ml per well with a surface area of 3.14 cm². The bacterial suspension containing Tween-80 (0.02%) at 146 µl was overlaid on the diet surface in each well for all concentrations and kept for one hour. One pre-starved (4 hours) second instar larvae were released in each well. A total of 40 larvae were used for each concentration @ 10 larvae/replication (4 replication including control). These trays were kept in an insectary at 25±1°C, 70±5.0 per cent Relative Humidity (RH) and with light: dark as 16 : 8 hours. The observations on mortality were recorded at 24, 48, 72, 96 and 120 hrs after treatment (Vimaladevi and Vineela, 2014). In addition, an untreated check was also maintained in order to get corrected mortality. The per cent mortality was calculated as per Abbott (1925) using the standard formula. Concentrations and mortality data were used for determination of median lethal concentration (LC₅₀).

Development of wettable powder formulation without UV protectant

The Wettable Powder (WP) formulation was prepared in a aseptic condition. A concentrated 2 gm WP formulation was prepared by mixing 0.4 gm lyophilized powder with the other ingredients (Gouder, 2011). Initially 0.4 gm lyophilized powder and 0.26 gm boric acid both are mixed thoroughly with the help of mortar and pestle. 10 mg of sucrose, 60µl of tween-80 and 40µl of triton X-100 and finally 15 mg of silica gel were added mixed thoroughly with the help of mortar and pestle and the prepared formulation was stored at 4°C used for bioassay.

Evaluation of wettable powder formulation against Helicoverpa armigera

The WP formulation of B. thuringiensis (BGC-1 and HD-1) were tested against H. armigera with different dosages viz., 0.5 gm/l, 1 gm/l, 1.5 gm/l, 2 gm/l and 2.5 gm/l of distilled water. The methodology for bioassay studies is same as mentioned above. The observations on larval mortality were recorded at an interval of 24 h for five days. Concentrations and mortality data were used for determination of median lethal concentration (LC₅₀). The insecticidal potency (ITU) of the sample was calculated by using the standard formula (Dulmage et al., 1971).

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\text{ITU of sample} = \frac{\text{LC}_{50}\text{ of standard} \times \text{Reference standard ITU}}{\text{LC}_{50}\text{ of sample}}
\]

Screening of different UV protectants for Bacillus thuringiensis formulations

Three different WP formulations were prepared with containing different UV protectants viz., Melaninand Congo red along with control (without UV protectant). Effective dose of the
formulation was selected for further testing at different intervals of sunlight. This is to find out the efficacy of UV protectants to protect the toxin when exposed to sunlight. Fifty ml effective dosage of WP formulation with different UV protectants was prepared in 100 ml plastic cups, exposed to sunlight at different intervals of time (0h, 1h, 2h, 3h, 4h and 5h). Solar light intensity was measured by solar pyranometer on planer surface and it is designed to measure the solar radiation in W/m² then, it was converted to lux.

The exposed solution was fed to second instar larvae of H. armigera as mentioned above. The observations on larval mortality were recorded at an interval of 24 h for five days. Colony forming units and soluble protein of the solution were calculated.

**Quantification of colony forming units (CFU/ml)**

The total number of viable cells in the bacterial suspension was determined. The contents of the suspension was mixed thoroughly, one milligram of the sample was aseptically extracted and transferred into 1 ml of distilled water blank and 0.1 ml of the sample was as extracted and transferred into 0.9 ml of water blank. Serial dilutions were made and 100µL of suspension was plated on LA medium plates. These plates were incubated at 30ºC for 24 h in a BOD incubator. Colonies appearing on the plates were counted and CFU per milliliter in the bacterial suspension was calculated.

**Quantification of protein by Lowry’s method**

The aromatic amino acids present in a protein like tyrosine, tryptophan react with phosphomolybdo-phosphor tungstate (FCR) reagent to produce a blue coloured complex at 660 nm. Soluble proteins were extracted from dissolving 0.2 gm of formulation using distilled water. One ml sample was taken in test tube and five ml of alkaline copper reagent was added and kept for 10 minutes. Then 0.5 ml of 1 N FCR was added and kept in dark place for 30 minutes. Per cent absorbance was read at 660 nm. Soluble proteins were calculated using Bovine Serum Albumin (BSA) standards (20–100 µg) (Lowry et al., 1951).

**Statistical analysis**

Analysis of the bioassay results was carried out for the dose mortality response (LC₅₀) using the method proposed by Finney (1952) with the help of MLP package. The data generated from the laboratory experiments were subjected to statistical analysis by Completely Randomized Design (CRD) described by Yates (1937).

**RESULTS AND DISCUSSION**

**Standardization of dosages of native Bacillus thuringiensis isolates**

Based on LC₅₀ values established against Helicoverpa armigera by employing standard bioassay method the Bacillus thuringiensis isolates which caused higher mortality were selected for further studies. The concentration mortality response data showed a progressive increase in the dose required to cause 50 per cent mortality as reported by earlier workers in bioassay studies with entomopathogens (Zaz, 1989).

Among the three isolates (BGC-1, GBP-2 and BGM-2) native B. thuringiensis isolate BGC-1 was more virulent possessing lowest LC₅₀ value of 9.14 ng/ml with fiducial limit ranging from 3.50 to 23.85 value and was comparable to the reference strain HD1 with LC₅₀ value of 6.08 ng/ml with fiducial limit ranging from 2.28 to 16.20 value followed by the isolates GBP-2 (19.21 ng/ml with fiducial limit ranging from 6.80 to 54.31) and the isolate BGC-2 which showed highest LC₅₀ value of 36.23 ng/ml with fiducial limit ranging from 13.90 to 94.45 (Table 1). As the technical powder concentration increased the mortality also increased in all the isolates. The presents finding are in conformity with the results of Malik et al. (2013) who have reported the LC₅₀ value of 9 ng/mg of artificial diet was exhibited by local Bt isolates HW 4.4 and INS 2.25 against second instar larvae of H. armigera and Lakshminarayana and Sujatha (2003) reported that Bt toxin was superior even at lower concentration (125 ng/cm²) at 48h.

Contrastingly Sharma et al. (2014) reported that Bt technical powder caused high mortality of H. armigera with LC₅₀ value of 12µg/ml. Similarly, the LC₅₀ of pure crystals, pure spores and the spore-crystal complex were 22.1µg/ml, 23.2µg/ml and 20.2µg/ml, respectively against second instar larvae of Spodoptera exigua (Yang et al., 2007). Praca et al., 2013 reported that the LC₅₀ of three B. thuringiensis strains S1905, S2122 and S2124 that were toxic to Plutella xylostella varied between 2.336 to 4.842 µg/ml. Degree of pathogenicity was varied with concentration of bacteria as well as the period of exposure (Savitri and Muralimohan, 2003). The differences in the efficacy of different isolates of B. thuringiensis has been suggested to be due to the difference in the carbohydrate affinity of the domain II which results in variable binding specificity with the receptors at the brush border membrane of the insect larvae, causing difference in toxicity of the cry protein (Burton et al., 1999). Present findings showed that higher mortality was registered with increasing concentration of lyophilized powder and there was a direct relationship between mortality and concentration of lyophilized powder and increase in feeding period. According to Ashfaq et al. (2001) the length of the larval developmental period increased linearly with an increase in feeding time.

**Evaluation of wettable powder formulation against Helicoverpa armigera**

Bacillus thuringiensis has been extensively used for four decades in biopesticide formulations due to its safe environmental and human health records and solid formulation was more effective than liquid formulation (Lalitha et al., 2012), they are being sold as either wettable powder or granules or suspension of spores (Bernhard and Utz, 1995) and commercially available primarily as WP formulations with effective dosage ranging
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In the present study, isolate BGC-1 and the reference strain HD1 were used for the preparation of Wettable Powder (WP) formulations and the same were tested for their efficacy against H. armigera. In both WP formulations, the larval mortality in the experiment was low or nil upto 24 h but increased with time. The HD1 WP formulation elicited an LC50 value of 0.9 g/l and assigned a biopotency of 18,000 ITU/g against second instar larvae of H. armigera. The BGC-1 WP formulation exhibited an LC50 value of 1.5 g/l and assigned a biopotency of 15428.57 ITU/g (Table 2).

The LC50 value of HD-263 was 0.53µg/g and the assigned 42,264 IU/mg of bioprotection was reported by Navon et al., 1990. Bioprotectivity of 53000 IU/mg in Delfin, 17600 IU/mg in Dipel and 15000 IU/mg in Centari were found against P. xylostella (Justin et al., 2001). The LC50 of Bacopsorine was 0.97-1.35 g/L and Dipel was 1.441.65 g/L reported by Sharma and Reddy (1993). Similarly, a report by Kashyap and Amla, 2007 found that LC50 value for H. armigera was 0.04 µg of HD73, 0.031 µg of HD1, 0.011 µg of Dipel and 0.008 µg of HD1 Dipel.

In the present study, UV protectants like melanin and congo red were screened for B. thuringiensis WP formulations by exposing the solution to sunlight from 0 to 5 h. In the formulation without UV protectants, the per cent mortality recorded was 60.00 and number of colonies counted are 1.10×108 in (BGC-1) and 1.10×108 in (HD1) and crude protein content was 69.55 µg/ml (BGC-1) and 74.76 µg/ml (HD1) (Table 3). The mortality of 86.67 per cent in both formulation, 1.89×108 (BGC-1) and 2.01×108 (HD1) number of colonies and 116.49 µg/ml (BGC-1) and 113.97 µg/ml (HD1) of crude protein was recorded in melanin UV protectant (Table 4).

In congo red, 73.33 per cent mortality was recorded in both formulation with 1.26×10 8 (BGC-1) and 1.28×10 8 (HD1) number of colonies and 90.76 µg/ml (BGC-1) and 94.88 µg/ml (HD1) of crude protein (Table 5).

The exposure of B. sphaericus ISPC-8 and 1593 spores to UV -B radiation for 6 h resulted in complete loss of spore viability and 50 per cent reduction in larvicidal activity (Hadpad et al., 2008) and the formulations containing para-amino benzoic acid and congo red will protect the spore viability and larvicidal activity up to 168 h from UV B radiation (Hadpad et al., 2009). Similarly, at 0.015 per cent melanin acts as UV protectant (Zhang et al., 2016).

### Table 1. Concentration mortality response (LC50) of Helicoverpa armigera to lyophilized Bacillus thuringiensis powder

| Sl. No. | Isolates   | LC50 (ng/ml) | Fiducial limit | Regression equation | χ² value |
|---------|------------|--------------|----------------|---------------------|----------|
| 1       | HD-1 (ref) | 6.08         | 2.28           | 16.20               | Y = 4.60611 + 0.50347x | 9.87    |
| 2       | BGC-1      | 9.14         | 3.50           | 23.85               | Y = 4.50634 + 0.51363x | 5.18    |
| 3       | GBP-2      | 19.21        | 6.80           | 54.31               | Y = 4.38282 + 0.48077x | 3.26    |
| 4       | BGM-2      | 36.23        | 13.90          | 94.45               | Y = 4.12841 + 0.55900x | 2.56    |

### Table 2. Concentration mortality response (LC50) of Helicoverpa armigera to WP formulation of Bacillus thuringiensis

| Sl. No. | Isolates | LC50 (g/l) | Fiducial limit | Regression equation | χ² value | Biopotancy (ITU/mg) |
|---------|----------|------------|----------------|---------------------|----------|---------------------|
| 1       | HD-1 (ref) | 0.90       | 0.75           | 1.10                | Y = 5.11411 + 2.77073x | 8.38    | 18,000.00           |
| 2       | BGC-1    | 1.05       | 0.87           | 1.28                | Y = 4.94197 + 2.41427x | 9.33    | 15428.57            |

from 1 to 2 kg/ha for management of lepidopteron pests (Vimala Devi and Vineela, 2014). The potency of bioassay for selecting B. thuringiensis preparation against agricultural insect pests was developed by Navon et al. (1990).

The present findings are in comparison with Hadpad et al. (2009) and Zhang et al. (2016). The exposure of B. sphaericus ISPC-8 and 1593 spores to UV-B radiation for 6 h resulted in complete loss of spore viability and 50 per cent reduction in larvicidal activity (Hadpad et al., 2008) and the formulations containing para-amino benzoic acid and congo red will protect the spore viability and larvicidal activity up to 168 h from UV B radiation (Hadpad et al., 2009). Similarly, at 0.015 per cent melanin acts as UV protectant (Zhang et al., 2016).
Table 3. Effect of sunlight and temperature on larvicidal activity, Colony count and Crude protein of native *Bacillus thuringiensis* strain BGC-1 and reference strain HD1 formulation against *Helicoverpa armigera* (without UV protectant)

| Time exposure (h) | Native strain *B. thuringiensis* BGC-1 formulation | Reference strain HD1 formulation |
|------------------|---------------------------------------------------|----------------------------------|
|                  | Temperature (°C) | Light Intensity (lux) | Per cent mortality at 120 h | CFU/ml | Crude protein (µg/ml) | Temperature (°C) | Light Intensity (lux) | Per cent mortality at 120 h | CFU/ml | Crude protein (µg/ml) |
| 0                | 28.5             | 2.02×10³             | 96.67 (79.48)a | 2.42×10⁴ | 164.91 | 29.0               | 2.04×10³             | 96.67 (79.48)a | 2.89×10⁴ | 145.06 |
| 1                | 36.1             | 2.13×10³             | 86.67 (68.58)b | 2.03×10⁴ | 122.10 | 34.2               | 2.06×10³             | 86.67 (68.58)b | 2.64×10⁴ | 126.49 |
| 2                | 38.2             | 3.02×10³             | 73.33 (58.91)c | 1.80×10⁴ | 111.15 | 37.4               | 3.00×10³             | 83.33 (65.91)bc | 2.37×10⁴ | 118.78 |
| 3                | 39.1             | 3.70×10³             | 73.33 (58.91)c | 1.43×10⁴ | 106.89 | 38.9               | 3.50×10³             | 73.33 (58.91)cd | 2.26×10⁴ | 107.84 |
| 4                | 41.4             | 4.69×10³             | 60.00 (50.76)d | 1.23×10⁴ | 81.77  | 42.0               | 4.52×10³             | 60.00 (50.76)d | 1.19×10⁴ | 89.55  |
| 5                | 43.6             | 4.93×10³             | 60.00 (50.76)d | 1.08×10⁴ | 69.55  | 43.5               | 4.86×10³             | 60.00 (50.76)d | 1.10×10⁴ | 74.76  |
| S. Em ±          | 1.75             | 0.04                 | 2.23             | CD @ 1% | 7.57   | 0.20               | 9.42             | CD @ 1% | 7.82   | 0.23   |

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

Table 4. Effect of sunlight and temperature on larvicidal activity, Colony count and Crude protein of native *Bacillus thuringiensis* strain BGC-1 and reference strain HD1 formulation against *Helicoverpa armigera* with Melanin as UV protectant

| Time exposure (h) | Native strain *Bacillus thuringiensis* BGC-1 formulation | Reference strain HD1 formulation |
|------------------|---------------------------------------------------|----------------------------------|
|                  | Temperature (°C) | Light Intensity (lux) | Per cent mortality at 120 h | CFU/ml | Crude protein (µg/ml) | Temperature (°C) | Light Intensity (lux) | Per cent mortality at 120 h | CFU/ml | Crude protein (µg/ml) |
| 0                | 28.5             | 2.02×10³             | 96.67 (79.48)a | 2.83×10⁴ | 159.94 | 29.0               | 2.04×10³             | 93.33 (75.03)a | 2.92×10⁴ | 143.40 |
| 1                | 36.1             | 2.13×10³             | 90.00 (71.57)a | 2.68×10⁴ | 133.11 | 34.2               | 2.06×10³             | 90.00 (71.57)a | 2.88×10⁴ | 138.35 |
| 2                | 38.2             | 3.02×10³             | 90.00 (71.57)a | 2.42×10⁴ | 124.45 | 37.4               | 3.00×10³             | 90.00 (71.57)a | 2.63×10⁴ | 126.49 |
| 3                | 39.1             | 3.70×10³             | 86.67 (68.58)b | 2.23×10⁴ | 120.77 | 38.9               | 3.50×10³             | 86.67 (68.58)b | 2.32×10⁴ | 115.28 |
| 4                | 41.4             | 4.69×10³             | 86.67 (68.58)b | 2.19×10⁴ | 118.78 | 42.0               | 4.52×10³             | 86.67 (68.58)b | 2.13×10⁴ | 114.58 |
| 5                | 43.6             | 4.93×10³             | 86.67 (6.58)b  | 1.89×10⁴ | 116.49 | 43.5               | 4.86×10³             | 86.67 (68.58)b | 2.01×10⁴ | 113.97 |
| S. Em ±          | 2.22             | 0.07                 | 2.49             | 1.99     | 0.08   | 2.42             |
| CD @ 1%          | 9.61             | 0.30                 | 10.50            | 8.62     | 0.35   | 10.46            |

Note: Figures in the parentheses are “arcsine” transformed values. The values represented by same alphabet are statistically on par with each other by DMRT.
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One of the main disadvantages is that the insecticidal activity of B. thuringiensis formulation is unstable and rapidly loses its activity under field conditions due to UV radiation (Sansinenea and Ortiz, 2014). The major role of the melanin is that it confers resistance to UV light, absorbing a broad range of the electromagnetic spectrum and preventing photo induced damage. Therefore, melanin has been commercially used in photoprotective creams and eye glasses and at the same time protects several bacterial species from UV radiation (Nosanchuk and Casadevall, 2003).

To compare cost benefit ratio among different uv protectants tested, melanin was found to be more expensive than other uv protectants like para amino benzoic acid, congo red and polyvinyl alcohol. But melanin is a natural pigment, is easily biodegradable in the nature, and absorbs uv radiation consequently photo protection of Bt. Congo red is an azo dye, it is toxic and a suspected carcinogen and mutagen. Hence there is need to reduce the production cost of melanin. Therefore, melanin could be used as new UV protectants for developing Bt formulation to protect Bt toxins under field conditions.

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Table 5. Effect of sunlight and temperature on larvicidal activity, colony count and crude protein of native Bacillus thuringiensis strain BGC-1 and reference strain HD1 formulation against Helicoverpa armigera with Congo red as UV protectant

| Time exposure (h) | Native strain Bacillus thuringiensis BGC-1 formulation | Reference strain HD1 formulation |
|-------------------|-------------------------------------------------------|----------------------------------|
|                   | Temperature (°C) | Light Intensity (lux) | Per cent mortality at 120 h | CFU/ml | Crude protein (µg/ml) | Temperature (°C) | Light Intensity (lux) | Per cent mortality at 120 h | CFU/ml | Crude protein (µg/ml) |
| 0                 | 29.0            | 2.04×10⁴            | 93.33 (75.03)a                | 2.80×10⁵ | 159.57               | 26.6              | 1.54×10⁵            | 96.67 (79.48)a                | 2.87×10⁴ | 135.78               |
| 1                 | 34.2            | 2.06×10⁴            | 86.67 (68.58)ab              | 2.68×10⁵ | 120.43               | 28.9              | 1.96×10⁵            | 83.33 (65.91)b              | 2.63×10⁶ | 124.42               |
| 2                 | 37.4            | 3.00×10⁴            | 80.00 (63.43)bc              | 2.45×10⁵ | 115.28               | 31.6              | 2.04×10⁵            | 83.33 (65.91)b              | 2.46×10⁴ | 115.75               |
| 3                 | 38.9            | 3.50×10⁴            | 76.67 (61.11)c               | 2.09×10⁵ | 107.84               | 35.8              | 2.05×10⁵            | 80.00 (63.43)b              | 2.01×10⁴ | 108.83               |
| 4                 | 42.0            | 4.52×10⁴            | 76.67 (61.11)c               | 1.85×10⁵ | 105.08               | 36.5              | 2.15×10⁵            | 76.67 (61.11)b              | 1.63×10⁶ | 102.33               |
| 5                 | 43.5            | 4.86×10⁴            | 73.33 (58.91)c               | 1.26×10⁵ | 90.76                | 41.6              | 4.52×10⁵            | 73.33 (58.91)b               | 1.28×10⁶ | 94.88                |

| S. Em ±          | 2.22            | 0.07                | 1.71                         | 0.06 | 2.48                | 0.08              | 2.42 |
| CD @ 1%          | 9.61            | 0.30                | 7.19                         | 0.26 | 10.72               | 0.35              | 10.46 |

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