# Effect of temperature, population density and shelf life of EPN *Heterorhabditis indica* (RCR) in sodium alginate gel formulation

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**ABSTRACT**

For long term storage of infective juveniles (IJs) of entomopathogenic nematode, *Heterorhabditis indica* were formulated in sodium alginate gel capsule and tested in vitro at four different temperatures (10, 15, 20 and 28 ± 1.72° C) and at 85% relative humidity. The experimental results showed that the survival of *Heterorhabditis indica* was higher at refrigerated condition (10ÚC) up to a density of 1,000 IJs per capsule and 90 days of storage followed by storage at 15° C. EPNs stored at room temperature (28.54 ± 1.72° C) in the capsules were all dead after two months of storage.

**Key words :** *Heterorhabditis indica*, long term storage, alginate capsule formulation, temperature

## INTRODUCTION

Entomopathogenic nematodes (EPN) of the genera Steinernema and Heterorhabditis are excellent candidates for biological control as they kill the insect pests by infecting them with symbiont entomopathogenic bacteria which they carry (Prabhu and John Sudheer, 2008; Teguh Santoso *et al.,* 2009; Sankar *et al.,* 2009; Javad Karimi *et al.,* 2010). One of the important criteria for a successful biocontrol agent is formulation of nematodes into a stable product, which has played a significant role in commercialization of these biological control agents in a given population density at optimum temperature, so that maximum IJs can survive for long periods before they are utilized for field application.

Entomopathogenic nematodes are successfully used against numerous soil inhabiting pests but their poor survival at room temperature storage is one of the main factors that prevents them from realizing their full potential as bio insecticides (Grewal, 2002). Infective juveniles of entomopathogenic nematodes do not feed and depend solely on stored reserves for their energy supply. Therefore, energy conservation is a vital factor in prolonging the survival of IJs, and extending their shelf lives of EPN-based bioinsecticides (Patel and Wright, 1997a, b; Patel *et al.,* 1997; Qiu *et al.,* 2000). Although the infective juveniles of entomopathogenic nematodes can be stored for several months in water in refrigerated bubbled tank, high cost and difficulties of maintaining quality prevent the practical use of this method. Several storage media have been developed to improve the survival of stored nematodes. Therefore, nematodes are usually formulated into solid or semiliquid substrates soon after they are produced. Kaya and Nelsen (1985) were the first to report the encapsulation of entomopathogenic nematodes with calcium alginate which is based on immobilizing the nematode, was a major breakthrough in formulation development. As a result, a number of agrochemicals companies introduced the formulation into various market segments including turf grass, greenhouse, berries, home and garden. This discovery subsequently led to the development of a commercial nematode product, which used thin sheets of calcium alginate spread over a plastic screen to trap nematodes (Georgis, 1990). Sodium alginate formulation of *Arthrobotrys robusta* for *Haemonchus placei* larvae management (Araujo *et al.,* 2000). Though several commercial formulations are available worldwide and easily acceptable in high value niche markets, no information is available for sodium alginate formulation of *Heterorhabditis indica.* Hence the present study was carried out to formulate EPN in alginate gels in order to evaluate the effect of temperature and density on shelf life of native strain *Heterorhaabditis indica* (RCR) for long term storage.

## MATERIALS AND METHODS

### EPN culture and encapsulation

Indigenous culture of *Heterorhabditis indica* were maintained in the lab on *Galleria mellonella* larvae as per Woodring and Kaya (1988). The infectives of *H. indica* were encapsulated in sodium alginate according to a method given by Kaya and Nelson (1985). A Solution of

the gel matrix was prepared by dissolving 2 g of sodium alginate in 100 ml of water with through blending for 10-15 min. To the gel matrix, different concentration of nematodes (*Viz.*, 100, 200, 400, 800, 1000, 2000, 4000, 8000, 10000 IJs /capsule) were added and mixed thoroughly. Drops of this solution through 1 ml hypodermic syringe was placed into a complexing solution (100mM solution of $CaCl_2$. $2H_2O$) with continuous stirring to get discrete capsules of calcium alginate of 0.4 cm diameter. Capsules were allowed to complex for 20-30 min and then were separated from the compelxing solution by sieving, rinsed in distilled water and stored in plastic Petri plates (90 dia x 10 ht mm) lined with wet blotting paper. The plastic petriplates were sealed with parafilm. Each petriplate was considered as a replication.

Observations on sodium alginate capsule formulation containing nematodes of various densities were stored at four different temperatures *viz.*, refrigerated condition (10ÚC), B.O.D temperatures of 15ÚC and 20ÚC and room temperature (28.54 ± 1.72 °C) (was recorded using a thermo - hygrometer). The actual number of nematodes was determined by dissolving five capsules in 9.5ml of 0.5M sodium citrate containing 0.1% Triton X-100.The capsules were stirred with magnetic spin bar until dissolution (about 30 minutes) , and the nematode in 1 ml of suspension were counted using a Hawksley counting slide. There was no growth of any contaminants around the capsule. Nematode mortality in the formulation was recorded till they suffered 50 per cent mortality. Maximum number of destructive samples was maintained and for every observation five samples were drawn from different densities of nematodes stored. The data obtained was tabulated and analyzed using two factorial completely randomized designs.

## RESULTS AND DISCUSSION

Since entomopathogenic nematodes entered commercial use for the biological control of insect pests, the problem of short shelf life has inhibited the expansion in the use of EPNs (Grewal, 1998, 2002). Temperature is the most important factor affecting nematode survival in formulations. In the present study as the density and period of storage increased there was decline in the survivability at all the storage temperatures. However, under refrigerated condition (10°C) the nematode stored at a density of up to 1,000 IJs per capsule recorded less than 30 per cent mortality compared to 2000, 4000, 8000 and 10000 IJs per alginate capsule which differed significantly ($P < 0.01$) with each other after 90 days of storage (Table 1). Chaerani *et al.* (2001) revealed that alginate formulation were able to maintain *H. indica* PLR2 viability up to 61.10 per cent after three months of storage at 10°C. *H. indica* survived for 18 weeks at 5°C and 12 weeks at 25°C. However, 100 per cent survival was recorded upto two weeks at 25°C and 4 weeks at 5°C (Umamaheshwari *et al.*, 2005)

At 15°C on 30$^{th}$ day of storage the densities of up to 4000 IJs per capsule recorded less than 20 per cent mortality. Similar findings were made by Gitanjalidevi (2007) who found that the viability of *H. indica* in alginate capsule was 95 per cent after being stored for 30 days at 15°C. On 90$^{th}$ day of storage highest mortality of 72.38 per cent was recorded in the density of 10,000 IJs per capsule, which was significantly ($P < 0.01$) superior over all other densities, whereas the lowest mortality of 24.67 per cent was observed in the density of 100 IJs per capsule (Table 2). Kim Yonggyun *et al.* (2003) found that more than 80 per cent of infective juveniles in the alginate capsules could survive at 15°C for 90 days. However, on 120 days of storage all the treatments recorded more than 50 per cent mortality. This indicated the direct relationship between the density, period of and the mortality.

At 20°C the infective juveniles of *H. indica* stored at a density of 100 IJs per capsule recorded significantly ($P < 0.01$) lowest mortality of 30.28 per cent compared to all other densities (Table 3).The results clearly indicate that the nematodes can be stored up to 60 days with less than 50 per cent mortality up to 4,000 IJs per capsule. The results are in confirmation with the findings of Hussaini *et al.* (2000) reported that the per cent survival of *H. indica* (PDBCEN 13.3) in alginate formulation was 43.3 per cent after 70 days of storage.

At room temperature (28.54 ± 1.72° C) in general, survival decreased significantly ($P < 0.01$) with increase in temperature and duration of storage (Table 4). The results clearly indicate that the nematodes can be stored up to 30 days at a density of 2,000 Ijs per capsule recording less than 50 per cent mortality. The sudden decline in survival rate may be due to excessive moisture loss in the capsule or increase in its metabolism or due to drying and hardening of the capsule which was found to be critical for the survival of the infective juveniles at room temperature.

Earlier *S. carpocapsae* products were the first to achieve a shelf life at room temperature, and this led to increased acceptability of nematodes in high value niche markets (Grewal, 1998). Georgis (1990) reported that that the formulated product could be stored up to 6 - 12 months at refrigerated condition and 1-3 months at room temperature. In the present study the survival of infective juveniles in sodium alginate capsule was better under refrigerated

**Table 1.** Per cent cumulative mortality of *H. indica* stored in alginate formulation at different densities under refrigerated condition (10°C)

| Density (IJs /capsule) | * % mortality at different days | | | | | | | Mean of A |
|---|---|---|---|---|---|---|---|---|
| | 7th | 15th | 30th | 60th | 90th | 120th | 150th | |
| 100 | 0.29 | 3.11 | 5.17 | 10.41 | 21.19 | 32.05 | 54.08 | 18.07 |
| | (3.09)$^{a}$ | (10.16)$^{b}$ | (13.14)$^{bc}$ | (18.82)$^{fg}$ | (27.41)$^{kl}$ | (34.48)$^{o}$ | (47.34)$^{s}$ | (22.06)$^{a}$ |
| 200 | 0.46 | 4.32 | 5.41 | 10.74 | 20.32 | 34.26 | 56.37 | 18.84 |
| | (3.90)$^{a}$ | (12.00)$^{b}$ | (13.45)$^{bc}$ | (19.13)$^{fgh}$ | (26.80)$^{k}$ | (35.83)$^{op}$ | (48.66)$^{stu}$ | (22.82)$^{a}$ |
| 400 | 0.76 | 3.40 | 6.53 | 11.82 | 22.65 | 32.17 | 58.73 | 19.43 |
| | (5.01)$^{a}$ | (10.63)$^{b}$ | (14.81)$^{de}$ | (20.11)$^{gh}$ | (28.42)$^{lm}$ | (34.56)$^{o}$ | (50.03)$^{uv}$ | (23.36)$^{a}$ |
| 800 | 0.92 | 4.29 | 7.32 | 12.63 | 23.15 | 34.27 | 57.03 | 19.94 |
| | (5.51)$^{a}$ | (11.95)$^{b}$ | (15.70)$^{de}$ | (20.81)$^{ghI}$ | (28.76)$^{lm}$ | (35.83)$^{op}$ | (49.04)$^{tu}$ | (23.94)$^{a}$ |
| 1000 | 1.35 | 4.66 | 6.16 | 14.29 | 25.02 | 35.37 | 60.27 | 21.01 |
| | (6.67)$^{a}$ | (12.46)$^{b}$ | (14.37)$^{de}$ | (22.21)$^{hij}$ | (30.02)$^{mn}$ | (36.49)$^{pq}$ | (50.93)$^{v}$ | (24.73)$^{ab}$ |
| 2000 | 1.50 | 5.63 | 8.28 | 17.49 | 30.54 | 42.30 | 63.34 | 23.28 |
| | (7.04)$^{a}$ | (13.73)$^{bc}$ | (16.73)$^{def}$ | (24.72)$^{j}$ | (32.92)$^{o}$ | (37.05)$^{r}$ | (52.74)$^{w}$ | (26.49)$^{b}$ |
| 4000 | 2.36 | 7.49 | 8.42 | 20.31 | 36.10 | 55.54 | 71.45 | 26.91 |
| | (8.84)$^{a}$ | (15.88)$^{de}$ | (16.86)$^{ef}$ | (26.79)$^{k}$ | (36.93)$^{q}$ | (48.18)$^{st}$ | (57.70)$^{X}$ | (28.57)$^{c}$ |
| 8000 | 5.09 | 10.82 | 15.36 | 28.48 | 40.21 | 64.23 | 84.33 | 34.26 |
| | (13.04)$^{b}$ | (19.20)$^{fgh}$ | (23.07)$^{j}$ | (32.25)$^{n}$ | (39.35)$^{r}$ | (52.89)$^{w}$ | (66.68)$^{y}$ | (34.53)$^{d}$ |
| 10000 | 9.75 | 14.43 | 20.82 | 31.76 | 52.83 | 72.85 | 91.60 | 42.01 |
| | (18.19)$^{e}$ | (22.32)$^{ij}$ | (27.15)$^{kl}$ | (34.30)$^{o}$ | (46.62)$^{s}$ | (58.60)$^{x}$ | (73.15)$^{z}$ | (40.04)$^{e}$ |
| Mean | 2.49 | 6.46 | 9.27 | 17.54 | 30.11 | 41.77 | 66.35 | |
| | (7.92)$^{a}$ | (14.25)$^{b}$ | (17.25)$^{c}$ | (24.34)$^{d}$ | (33.02)$^{e}$ | (39.84)$^{f}$ | (55.14)$^{g}$ | |

* Mean of five replications; Figures in parenthesis are 'arc sine' - transformed values

Means followed by the same letters in each column are non significant at P = 0.01 as per DMRT

**Table 2.** Per cent cumulative mortality of *H. indica* stored in alginate formulation at different densities at 15° C over a period of time

| Density (IJs /capsule) | * % mortality at different days | | | | | | Mean of A |
|---|---|---|---|---|---|---|---|
| | 7th | 15th | 30th | 60th | 90th | 120th | |
| 100 | 1.35 | 4.52 | 9.45 | 16.20 | 24.67 | 60.36 | 19.42 |
| | (6.68)$^{a}$ | (12.28)$^{cde}$ | (17.90)$^{ghi}$ | (23.73)$^{l}$ | (29.78)$^{no}$ | (50.98)$^{t}$ | (23.55)$^{a}$ |
| 200 | 2.19 | 5.38 | 9.09 | 18.30 | 24.81 | 61.13 | 20.15 |
| | (8.51)$^{ab}$ | (13.41)$^{de}$ | (17.55)$^{gh}$ | (25.33)$^{lm}$ | (29.87)$^{no}$ | (51.43)$^{tu}$ | (24.35)$^{a}$ |
| 400 | 2.28 | 4.18 | 10.63 | 17.31 | 26.03 | 63.00 | 20.57 |
| | (8.68)$^{ab}$ | (11.79)$^{de}$ | (19.03)$^{hij}$ | (24.59)$^{lm}$ | (30.67)$^{op}$ | (52.54)$^{v}$ | (24.55)$^{a}$ |
| 800 | 2.46 | 6.23 | 9.20 | 17.75 | 25.14 | 62.34 | 20.52 |
| | (9.03)$^{abc}$ | (14.45)$^{ef}$ | (17.66)$^{gh}$ | (24.92)$^{lm}$ | (30.09)$^{nop}$ | (52.14)$^{uv}$ | (24.71)$^{a}$ |
| 1000 | 3.52 | 6.34 | 11.40 | 18.69 | 26.32 | 63.50 | 21.62 |
| | (10.81)$^{bcd}$ | (14.58)$^{ef}$ | (19.73)$^{ij}$ | (24.11)$^{lm}$ | (30.87)$^{op}$ | (52.83)$^{v}$ | (25.48)$^{ab}$ |
| 2000 | 3.63 | 8.42 | 15.39 | 19.06 | 29.16 | 67.35 | 24.00 |
| | (10.98)$^{bcd}$ | (16.87)$^{g}$ | (23.10)$^{l}$ | (28.01)$^{m}$ | (31.41)$^{P}$ | (55.15)$^{w}$ | (27.58)$^{c}$ |
| 4000 | 4.54 | 7.61 | 18.24 | 26.72 | 40.14 | 72.63 | 27.59 |
| | (12.30)$^{cde}$ | (16.02)$^{fg}$ | (25.28)$^{m}$ | (31.12)$^{op}$ | (39.31)$^{r}$ | (58.45)$^{x}$ | (29.97)$^{d}$ |
| 8000 | 8.41 | 13.86 | 25.48 | 37.40 | 40.14 | 64.26 | 32.68 |
| | (16.86)$^{g}$ | (21.85)$^{k}$ | (26.19)$^{op}$ | (37.70)$^{q}$ | (39.31)$^{r}$ | (53.52)$^{v}$ | (33.31)$^{e}$ |
| 10000 | 11.04 | 18.25 | 34.42 | 54.67 | 72.38 | 88.08 | 46.47 |
| | (19.41)$^{hij}$ | (25.29)$^{lm}$ | (35.92)$^{q}$ | (47.68)$^{s}$ | (58.29)$^{x}$ | (69.81)$^{z}$ | (42.73)$^{f}$ |
| Mean | 4.38 | 8.31 | 15.92 | | 25.45 | 32.42 | 68.87 |
| | (11.47)$^{a}$ | (16.28)$^{b}$ | (22.48)$^{c}$ | | (29.68)$^{d}$ | (34.48)$^{e}$ | (56.43)$^{f}$ |

* Mean of five replications; Figures in parenthesis are 'arc sine' - transformed values

Means followed by the same letters in each column are non significant at P=0.01 as per DMRT

**Table 3.** Per cent cumulative mortality of *H. indica* stored in alginate formulation in different densities at 20° C over a period of 90 days.

| Density (IJs /capsule) | * % mortality at different days | | | | | Mean of A |
|---|---|---|---|---|---|---|
| | 7th | 15th | 30th | 60th | 90th | |
| 100 | 3.63 (10.98)$^{a}$ | 8.26 (16.71)$^{c}$ | 16.56 (23.23)$^{h}$ | 30.28 (33.39)$^{l}$ | 65.42 (53.98)$^{s}$ | 25.12 (27.83)$^{a}$ |
| 200 | 3.87 (11.35)$^{a}$ | 8.48 (16.93)$^{c}$ | 17.24 (24.53)$^{hi}$ | 32.51 (34.83)$^{m}$ | 67.24 (55.32)$^{t}$ | 25.86 (28.59)$^{a}$ |
| 400 | 4.29 (11.95)$^{a}$ | 10.37 (18.89)$^{d}$ | 16.72 (24.13)$^{h}$ | 32.60 (34.82)$^{m}$ | 68.70 (55.98)$^{t}$ | 26.53 (29.15)$^{ab}$ |
| 800 | 5.38 (13.41)$^{ab}$ | 9.83 (18.27)$^{d}$ | 16.34 (23.06)$^{h}$ | 31.74 (34.29)$^{m}$ | 70.69 (57.22)$^{u}$ | 26.50 (29.07)$^{b}$ |
| 1000 | 5.50 (13.56)$^{b}$ | 10.74 (19.13)$^{d}$ | 17.33 (24.60)$^{hi}$ | 34.76 (36.12)$^{n}$ | 72.47 (58.35)$^{v}$ | 28.16 (30.35)$^{c}$ |
| 2000 | 6.29 (14.53)$^{c}$ | 12.48 (20.69)$^{e}$ | 18.37 (25.38)$^{i}$ | 36.08 (36.92)$^{o}$ | 75.54 (60.36)$^{w}$ | 29.75 (31.57)$^{d}$ |
| 4000 | 8.57 (17.02)$^{d}$ | 16.39 (23.88)$^{gh}$ | 26.82 (31.19)$^{k}$ | 38.22 (38.18)$^{p}$ | 80.51 (63.80)$^{x}$ | 34.10 (34.81)$^{e}$ |
| 8000 | 10.48 (18.89)$^{f}$ | 21.65 (27.73)$^{j}$ | 32.15 (34.54)$^{m}$ | 53.27 (46.88)$^{q}$ | 84.62 (66.91)$^{y}$ | 40.43 (38.99)$^{f}$ |
| 10000 | 14.52 (22.40)$^{f}$ | 30.49 (33.52)$^{l}$ | 56.44 (48.70)$^{r}$ | 72.67 (58.48)$^{v}$ | 92.45 (74.05)$^{z}$ | 53.31 (47.43)$^{g}$ |
| Mean of B | 6.94 (14.89)$^{a}$ | 14.29 (21.75)$^{b}$ | 24.21 (28.81)$^{c}$ | 40.23 (39.32)$^{d}$ | 75.29 (60.67)$^{e}$ | |

* Mean of five replications; Figures in parenthesis are 'arc sine' - transformed values
Means followed by the same letters in each column are non significant at P = 0.01 as per DMRT

**Table 4.** Per cent cumulative mortality of *H. indica* stored in alginate formulation in different densities over a period of 60 days under room temperature (28.54 ± 1.72° C).

| Density (IJs /capsule) | * % mortality at different days | | | | Mean of A |
|---|---|---|---|---|---|
| | 7th | 15th | 30th | 60th | |
| 100 | 4.51 (12.26)$^{a}$ | 11.63 (19.94)$^{e}$ | 22.16 (28.08)$^{i}$ | 74.16 (59.45)$^{t}$ | 28.66 (30.29)$^{a}$ |
| 200 | 5.63 (13.73)$^{a}$ | 12.46 (20.67)$^{ef}$ | 23.39 (28.93)$^{j}$ | 75.11 (60.07)$^{t}$ | 29.14 (30.85)$^{a}$ |
| 400 | 6.84 (15.16)$^{b}$ | 12.24 (20.48)$^{e}$ | 25.63 (30.42)$^{lm}$ | 76.34 (60.89)$^{u}$ | 29.71 (31.37)$^{ab}$ |
| 800 | 7.65 (16.06)$^{b}$ | 14.28 (22.20)$^{g}$ | 24.47 (29.65)$^{k}$ | 76.53 (61.02)$^{u}$ | 30.73 (32.23)$^{b}$ |
| 1000 | 7.86 (16.28)$^{b}$ | 13.30 (21.39)$^{fg}$ | 26.32 (30.87)$^{m}$ | 80.57 (63.84)$^{v}$ | 32.01 (33.09)$^{c}$ |
| 2000 | 9.47 (17.92)$^{c}$ | 15.63 (23.29)$^{h}$ | 34.29 (35.84)$^{o}$ | 81.62 (64.62)$^{w}$ | 35.25 (35.41)$^{d}$ |
| 4000 | 10.54 (18.95)$^{d}$ | 24.62 (29.75)$^{kl}$ | 51.83 (46.05)$^{q}$ | 85.58 (67.69)$^{x}$ | 43.14 (40.61)$^{e}$ |
| 8000 | 15.48 (23.17)$^{h}$ | 32.66 (34.85)$^{n}$ | 64.21 (53.26)$^{r}$ | 89.43 (71.02)$^{y}$ | 50.44 (45.57)$^{f}$ |
| 10000 | 23.28 (28.85)$^{i}$ | 42.75 (40.83)$^{p}$ | 72.54 (58.40)$^{s}$ | 96.48 (79.19)$^{z}$ | 58.76 (51.81)$^{g}$ |
| Mean of B | 10.14 (18.04)$^{a}$ | 19.95 (25.93)$^{b}$ | 38.31 (37.94)$^{c}$ | 81.75 (65.31)$^{d}$ | |

* Mean of five replications; Figures in parenthesis are 'arc sine' - transformed values
Means followed by the same letters in each column are non significant at P = 0.01 as per DMRT

condition (10°C) compared to all other storage temperatures ($P < 0.01$). Trehalose accumulation at low temperatures appears to be common among entomopathogenic nematodes and may be a component of survival stratergy during environmental stress (Grewal, 2002).

Apart from temperature, anhydrobiosis is an important storage stability of entomopathogenic nematodes in formulations. Connick *et al*. (1993) dried granules based on wheat gluten matrix to low moisture content to prevent nematode migration and reduce the risk of contamination, but the granules rapidly lost their water during storage, which resulted in poor nematode survival. A water dispersible granule formulation consisting mixtures of various types of silica ,clays,cellulose,lignin and starches (Georgis *et al*., 1995; Silver *et al*., 1995) was found to induce partial anhydrobiosis after 4-7 days, and to reduce the nematodes oxygen consumption (Grewal, 2000a, b). Despite the induction of partial anhydrobiosis, nematodes require high moisture for survival in the formulations. Grewal (1998) reported that initial moisture content was positively correlated with survival of *S.carpocapsae* in water dispersible granules at 30°C. The shelf –life in most formulated products had been enhanced by reducing nematode activity and metabolism through physical trapping, metabolic inhibition, and cold storage or by induction of anhydrobiosis (Grewal, 2002).

Based on the studies, the best combinations of temperature, population density and duration of storage was 10ºC, up to 1000 IJs per capsule and 90 days of storage, respectively. However, greater understanding of nematode behaviour, physiology and biochemical process could lead to the further improvement of the formulations.

**ACKNOWLEDGEMENT**

The authors gratefully acknowledge the assistance of Ms. Sudha with statistical analysis.

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Received: September 2, 2010 Revised: October 15, 2010 Accepted: November 9, 2010