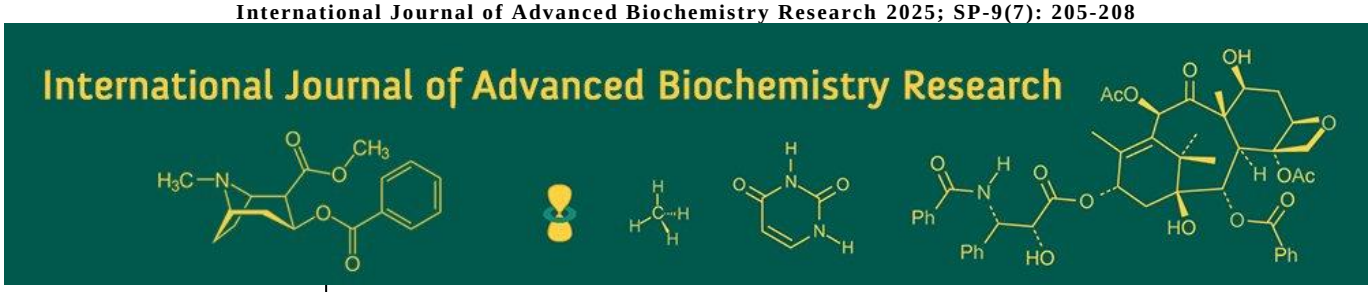

ISSN Print: 2617-4693
ISSN Online: 2617-4707
IJABR 2025; SP-9(7): 205-208
www.biochemjournal.com
Received: 02-05-2025
Accepted: 06-06-2025

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Shelf life assessment of EPN *Heterorhabditis indica* infective juveniles in different formulations under laoratory conditions

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DOI: https://www.doi.org/10.33545/26174693.2025.v9.i7Sc.4784

Abstract
Entomopathogenic nematodes (EPNs), particularly *Heterorhabditis indica*, are gaining prominence as eco-friendly biological control agents against insect pests. However, the effective use of EPNs in field applications depends largely on their viability and survival during storage. This study was conducted to assess the shelf life of *Heterorhabditis indica* infective juveniles (IJs) suspended in various formulations under laboratory conditions over a 60-day period. The treatments included Calcium alginate (T_1), *Aloe vera* (T_2), Hydrogen peroxide (T_3), Polyvinyl propyl (T_4), Glycerol (T_5), Triton X-100 (T_6), and a water-based control (T_7). Survival of IJs was recorded at four intervals: 15, 30, 45, and 60 days after treatment (DAT).
At 15 DAT, the highest IJ survival was observed in the *Aloe vera* (T_2) (44.67 IJs) and Glycerol (T_5) (45.33 IJs) formulations, followed closely by Hydrogen peroxide (T_3) and the control (T_7), indicating early-stage viability benefits. As the storage period progressed, a gradual decline in IJ survival was noted across all treatments. At 30 DAT, Glycerol (T_5) maintained the highest viability (42.33 IJs), followed by *Aloe vera* (41.00 IJs). This trend continued through 45 DAT, where Glycerol again demonstrated superior performance (38.67 IJs), suggesting its stabilizing effect on nematode longevity. At 60 DAT, Glycerol-treated suspensions retained the highest survival (35.33 IJs), significantly outperforming the control (24.00 IJs) and other formulations. In contrast, Polyvinyl propyl (T_4) and Triton X-100 (T_6) consistently showed the lowest survival rates across all time intervals.
The results indicate that Glycerol and *Aloe vera* are promising substrates for enhancing IJ survival during storage, with potential to improve field efficacy and ease of commercial use.

Keywords: Entomopathogenic nematodes, *Heterorhabditis indica*, shelf life, suspension formulation, infective juveniles, *Aloe vera*, Glycerol, laboratory storage, biological control

Introduction
Entomopathogenic nematodes (EPNs), particularly those belonging to the genera *Heterorhabditis* and *Steinernema*, are widely recognized as eco-friendly biocontrol agents for insect pests. Their use has been integrated into pest management strategies worldwide (Kaya and Gaugler, 1993; Kavya *et al.*, 2006) [4, 11]. However, the commercial success of EPN-based biopesticides is constrained by the short shelf life of infective juveniles (IJs), especially in aqueous suspensions (Gokulakrishnan *et al.*, 2021) [10].
Among the EPNs, *Heterorhabditis indica* is widely recognized for its efficacy in tropical and subtropical climates. This nematode forms a mutualistic association with the bacterium *Photorhabdus luminescens*, which is released into the insect hemocoel after infection, leading to rapid insect mortality (Shapiro-Ilan *et al.*, 2020) [14]. The infective juvenile (IJ) stage is the only free-living and infective stage, making the viability and pathogenicity of IJs crucial for successful field application.
Despite the promising biocontrol potential, one of the critical constraints limiting the wider adoption of EPNs is their short shelf life and sensitivity to environmental conditions such as temperature, oxygen, and desiccation (Kaya *et al.*, 2020) [14]. In the context of commercial formulation and distribution, it is essential to ensure that EPNs retain viability and virulence over extended periods under storage.

The formulation of EPNs in aqueous suspensions offers a practical and economical approach for their application and distribution. However, the effectiveness of this method depends heavily on the stability of the suspension and the maintenance of nematode viability and pathogenicity during storage. Loss of infectivity over time not only compromises pest control efficacy but also affects the economic feasibility of mass production.

To enhance EPN stability, various formulation approaches have been explored, including natural polymers, cryoprotectants, and encapsulation techniques (Garcia *et al.*, 2008) [9]. Substances such as glycerol, *Aloe vera*, and hydrogen peroxide have shown promise in increasing IJ longevity by minimizing desiccation and microbial contamination (Thube *et al.*, 2023) [13]. This study aimed to assess the viability of *Heterorhabditis indica* IJs stored in six different carrier formulations over a 60-day period under laboratory conditions.

Materials and Methods

Nematode Culture

Heterorhabditis indica IJs were mass-produced using *Galleria mellonella* larvae and harvested using the White trap method. Freshly collected IJs were standardized to 64 IJs per 100 µl suspension.

Treatments and Experimental Design

Sr. No.	Particulars	Details
1.	EPN Species	*Heterorhabditis indica*
2.	Design	CRD
3.	Replication	3
4.	Treatments	7

Storage of *Heterorhabditis indica* Suspension

Suspensions of *Heterorhabditis indica* infective juveniles (IJs) were prepared and stored in sterilized plastic bottles using different formulations—

Seven treatments were prepared:

T_1: Calcium alginate (5%)
T_2: *Aloe vera* (5%)
T_3: Hydrogen peroxide (5%)
T_4: Polyvinyl propyl (5%)
T_5: Glycerol (5%)
T_6: Triton X-100 (5%)
T_7: Sterile water (control)

Each bottle contained a known concentration of IJs and was stored at room temperature (30 ± 2 °C). The viability of IJs were assessed at regular intervals.

Each treatment was replicated thrice, and stored at room temperature (30±2 °C). Viability counts were taken at 15, 30, 45, and 60 days after treatment (DAT) using a stereomicroscope.

Method of Viability Test

The viability of *Heterorhabditis indica* infective juveniles (IJs) was assessed at regular intervals to evaluate the shelf life of the suspension under laboratory conditions. A 100 µL of the EPN suspension was taken using a micropipette and placed on a glass cavity slide for observation. Counts were taken at 15, 30, 45, and 60 days after treatment (DAT). Viability was determined by counting live (actively moving) IJs under a stereomicroscope. Three replications were maintained for each observation, and data were expressed as number of IJs survive in formulations.

Results

The survival of *Heterorhabditis indica* infective juveniles (IJs) under different formulations was evaluated over a 60-day period under laboratory conditions. Significant differences ($p \leq 0.05$) were observed among treatments at all observation intervals (15, 30, 45, and 60 days after treatment, DAT).

At 15 DAT, *Aloe vera* (T_2) showed the highest IJ survival (44.67 IJs), followed closely by Glycerol (T_5) with 45.33 IJs and Hydrogen Peroxide (T_3) with 43.67 IJs. The lowest survival was recorded in Triton X-100 (T_6) and Polyvinyl Propyl (T_4), with numbers of 24.33 IJs and 26.00 IJs per 100 µl, respectively.

At 30 DAT, *Aloe vera* (41.00 IJs) and Glycerol (42.33 IJs) again exhibited superior survival, while the Control (T_7) showed moderate survival (38.00 IJs). The lowest survival was once again observed in Triton X-100 (23.67 IJs).

At 45 DAT, the highest IJ survival was maintained in *Aloe vera* (36.67 IJs), followed by Glycerol (35.67 IJs) and Hydrogen Peroxide (34.00 IJs). The Control treatment recorded a significant drop (27.33 IJs per 100 µl), and Polyvinyl Propyl and Triton X-100 continued to show the lowest survival values.

By 60 DAT, a further decline was noted across all treatments. However, Glycerol (33.33 IJs), *Aloe vera* (33.33 IJs), and Hydrogen Peroxide (30.67 IJs) still supported significantly higher IJ survival compared to the Control (24.00 IJs). The lowest survival was again noted in Triton X-100 (16.33 IJs) and Polyvinyl Propyl (17.33 IJs per 100 µl).

Overall, *Aloe vera* and Glycerol-based formulations exhibited the most consistent and prolonged IJ viability, whereas Polyvinyl Propyl and Triton X-100 were the least effective.

Table 1: Survival of *Heterorhabditis indica* IJs In Numbers at Different Intervals

Sr. No	Treatment Details	Survival of IJs In Numbers at Different Intervals			
		15 DAT	30 DAT	45 DAT	60 DAT
T_1	Calcium Alginate	36.33 (37.07)	31.33 (34.04)	28.67 (32.36)	25.33 (30.19)
T_2	*Aloe vera*	44.67 (41.94)	41.00 (39.81)	36.67 (37.27)	33.33 (35.26)
T_3	Hydrogen Peroxide	43.67 (41.36)	39.33 (38.84)	34.00 (35.66)	30.67 (33.62)
T_4	Polyvinyl Propyl	26.00 (30.65)	23.33 (28.88)	20.67 (27.03)	17.33 (24.60)
T_5	Glycerol	45.33 (42.32)	42.33 (40.59)	38.67 (38.45)	35.33 (36.47)
T_6	Triton X-100	24.33 (29.56)	21.67 (27.72)	18.67 (25.59)	16.33 (23.84)
T_7	Control	43.33 (41.17)	38.00 (38.05)	27.33 (31.52)	24.00 (29.32)
	F Test	sig**	sig**	sig**	sig**
	SE (m)	0.07	0.07	0.08	0.10
	CD @ 0.05%	0.30	0.33	0.34	0.40
	CV	2.01	2.35	2.64	3.27

(Figures in parentheses are arcsine transformed values, **F test highly significant at 5% level of significance)

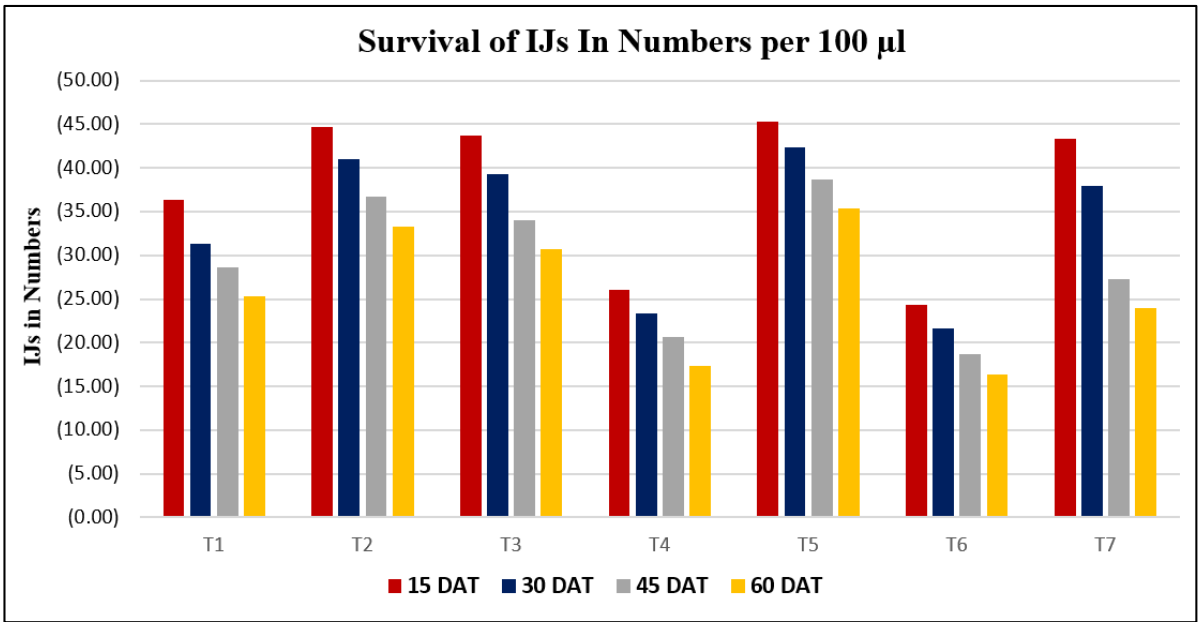

Fig 1: Survival trend of IJs of *H. indica* in numbers per 100 µl

Discussion

The present study revealed significant differences in the survival of *Heterorhabditis indica* IJs among various formulations over time, emphasizing the importance of suitable carriers and stabilizers in maintaining nematode viability. The results clearly demonstrate that formulations containing *Aloe vera* (T_2) and *Glycerol* (T_5) provided superior protection to IJs, significantly extending their shelf life under laboratory conditions.

These findings are in agreement with those of Painkra *et al.* (2016) [7], who reported that the inclusion of stabilizing matrices such as alginate and natural polymers helped prolong the shelf life and infectivity of EPNs. Similarly, Shapiro-Ilan *et al.* (2020) [14] emphasized that the use of organic additives can improve EPN survival by maintaining adequate moisture and reducing desiccation stress.

The beneficial effects of *Aloe vera* can be attributed to its gel-like nature and natural antimicrobial properties, which may create a favorable microenvironment for nematode longevity. This observation aligns with the findings of Kaya and Gaugler (1993) [4], who stressed that the survival of EPNs is highly dependent on the moisture content and protection from harmful environmental fluctuations. Furthermore, Premchand *et al.* (2020) [8] also documented improved survival of EPNs when stored in *Aloe vera*-based formulations due to the compound's hygroscopic nature and bio-compatibility.

Glycerol (T_5) was also effective in maintaining IJ viability, corroborating the work of Mahmoud (2018) [6], who demonstrated that glycerol-based formulations preserved EPN viability for longer durations, possibly by reducing osmotic stress and acting as a cryoprotectant. The hygroscopic and humectant nature of glycerol likely helps prevent desiccation, as also supported by Koppenhofer *et al.* (2000) [9], who reported enhanced shelf life in glycerol and clay-based formulations of *H. bacteriophora*.

Hydrogen peroxide (T_3) showed moderate effectiveness, likely due to its antimicrobial properties, which help reduce microbial competition in storage media. This agrees with the observations of Grewal (2000) [3], who noted that oxidative agents could help suppress harmful microorganisms, indirectly improving EPN stability.

In contrast, treatments with Polyvinyl Propyl (T_4) and Triton X-100 (T_6) consistently showed lower survival rates, suggesting their incompatibility with nematode biology. These findings support the conclusions of Georgis *et al.* (2006) [2], who emphasized that formulation components must be biologically compatible with nematodes to avoid toxicity or physical damage to their cuticle.

The control (T_7), which lacked any additives, consistently performed poorly across all time intervals. This underlines the necessity of incorporating appropriate carriers for the successful commercial storage and field application of EPNs, a conclusion echoed by Glazer and Lewis (2000) [1], who stated that without formulation support, IJ survival and infectivity drop significantly during storage.

In summary, the present findings strongly support the use of *Aloe vera* and Glycerol as effective formulation additives for prolonging the shelf life of *H. indica* suspensions. These results are consistent with a range of earlier studies and contribute valuable insights toward developing more stable and commercially viable EPN formulations.

Conclusion

The experiment demonstrated that the formulation significantly influences the shelf life and viability of *Heterorhabditis indica* IJs under laboratory conditions. Among all tested carriers, Glycerol (T_5) proved to be the most effective in preserving IJ survival over a 60-day period. *Aloe vera* (T_2) and Hydrogen Peroxide (T_3) were also effective alternatives, whereas Polyvinyl Propyl (T_4) and Triton X-100 (T_6) were the least effective. These findings suggest that using biocompatible carriers like Glycerol can greatly enhance the storage stability of entomopathogenic nematodes, offering practical advantages for their application in biological pest control.

Acknowledgements

This study is part of thesis research of first author submitted to Entomology Section, College of Agriculture, Nagpur Dr. PDKV Akola. Authors are thankful to the Associate Dean. College of Agriculture, Nagpur for providing necessary laboratory facilities. Authors are also grateful to the

student's advisory committee members for their critical comments and valuable suggestions during the study.

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