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An alginate-based encapsulation enhances shelf life and bioactivity of the entomopathogenic fungus, *Metarhizium anisopliae*

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Abstract

Background This study evaluated the shelf life and effectiveness of *Metarhizium anisopliae* encapsulated in Na (sodium)-alginate matrix. Air-flow-dried and freeze-dried beads containing conidia of *M. anisopliae* produced in submersion in the liquid broth were fabricated and characterized. Shelf life and efficacy of fabricated beads against three species of the white grubs, namely *Holotrichia serrata*, *Adoretus* sp., and *Lepidiota mansueta,* were evaluated under greenhouse conditions.

Results The mean size of the wet beads ranged between 1.4 and 3.2 mm. The mean size of beads that were dried through freeze drying ranged between 0.55 and 2.3 mm. However, the mean size of beads dried through air flow ranged between 0.55 and 2.35 mm. Viability of *M. anisopliae* was more in freeze-dried beads stored at 8 °C compared to air-flow-dried beads with 20.0×10^7 and 16.00×10^7 CFU/g, respectively, after two weeks of drying. Further, freeze-dried beads supported *M. anisopliae* conidial viability till 10 months with 2.0×10^7 CFU/g. The LC_{50} values of the freeze-dried beads with *M. anisopliae* after 21 days of treatment were 0.35 (2.1×10^8 CFU/g), 0.31 (2.1×10^8 CFU/g), and 0.76 mg (2.1×10^8 CFU/g) against *H. serrata, Adoretus* sp., and *L. mansueta,* respectively.

Conclusion The results of this study support the use of Na-alginate beads followed by freeze drying to maintain the viability of *M. anisopliae*. The effectiveness of freeze-dried beads with *M. anisopliae* against white grubs indicates that these beads can be an alternative option of synthetic insecticides against soil dwelling insect pests. In addition, this study offers a steppingstone for further advancement of sodium alginate encapsulated formulations for soil insect control.

Keywords Encapsulated formulation, Freeze-dried, Air-flow-dried, *Holotrichia serrata*, *Adoretus* sp., *Lepidiota mansueta*

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Background

The use of beneficial microorganisms in the field comes with a number of issues related to unpredictable environmental circumstances (Lorenz et al. 2020). Encapsulation serves as a tool for substance protection and release modulation, and it has been successfully used to protect chemicals sensitive to heat, light, oxidation, moisture, and other unfavorable reactions, increasing the potential for use of encapsulated products (Faria et al. 2009) and offering a good alternative for the protection of fungal biocontrol agents, including entomopathogens, in recent years (Lei et al. 2022). Research into innovative formulation techniques, particularly bioencapsulation, has significantly grown in recent years because of the increasing demand for microbial biocontrol agents (Shah et al. 2022a). Among the carriers used for bioencapsulation, alginate, a biopolymer derived from macroalgae, has been widely used (Harmann et al. 2021). In addition to facilitating application and distribution in soil, sodium (Na) alginate also protects the conidia from external factors that disrupt them (Shah et al. 2022b). Out of the entomopathogens are explored, *Metarhizium anisopliae* (Metch.) Sorokin is widely used or in practice for pest management and reported to be pathogenic to more than 200 insect pest species (Pandey et al. 2021). Bioinsecticide based on *M. anisopliae* is globally available either in the form of a wettable powder or as a liquid formulation (Pandey et al. 2021). The inconsistent performance of many entomopathogens as microbial biocontrol agents is largely due to the shorter shelf life, effect of biotic stresses, and organic carbon content of soil (Rodrigues et al. 2017). Therefore, it has opened the area to search for novel bioformulations (Ahmad et al. 2020).

A white grub or root grub is a larva of a scarab beetle commonly referred to as cock chafers, leaf chafers, chafer beetles, or May beetles. Grubs are subterranean and feed on living roots. In temperate and tropical climates, they damage food crops by feeding on the roots of both cultivated and uncultivated plants (Ravinder et al. 2018). The 3rd instar grubs are most destructive stage of the insect. The yield loss due to white grubs was reported to be as high as 100% in food crops growing in India (Thamarai Selvi et al. 2010). White grubs *Holotrichia* spp. (Coleoptera: Scarabaeidae) are noxious subterranean pests damaging the root systems of several crops and can cause yield losses of 12 to 60% (Sankaranarayanan et al. 2019) and 10 to 60% plant mortality (Ramanujam et al. 2021). The other species of white grubs such as *Adoretus* sp., *Adoretus brachypygus* Burro., and *A. duvavceli* Bl (Coleoptera: Scarabaeidae) cause damage by eating semi-ripened fruits like apple, peach, palm and apricot in India (Batra et al. 1973) and Philippines (Calcetas et al. 2021). White grub, *Lepidiota mansueta* (Burmeister) (Coleoptera: Scarabaeidae) has been emerged as a significant severe pest of many field crops in Assam, India, and reported to appear as a severe pest of potato (42 to 48%), sugarcane (15 to 20%), green gram (30 to 35%), and colocasia (35 to 40%) in Majuli district of Assam, India (Bhattacharyya et al. 2015).

Application of insecticides and adults' collection are the major strategies that have been adopted for the management of white grub species (Ramanujam et al. 2021). The widespread concern about ground water contamination and the use of synthetic insecticides worldwide in agriculture raised the public awareness against the uncontrolled use of insecticides. Hence, to counter conventional pest control practices, different integrated and biological pest management strategies have been developed (Wagiyana et al. 2021). Eco-friendly pest management programs are the possible way to manage white grubs in a sustainable way. Although biocontrol measures have been deployed against the insect pests, their viability in soil and shelf life of the formulations are the major concerns for effective control of pests. For commercial use, inoculum of beneficial microbes should be supported by appropriate formulation, which prevents a rapid decline of introduced microorganisms and extend their shelf life (de Oliveira Lopes et al. 2020). There are several types of formulations that contain entomopathogenic fungi, such as wettable powders, granules, water dispersible granules, baits, sprinkle powders, powders for contact, oil dispersions, suspension concentrates, miscible suspension concentrates in oil, and ultra-low volume suspensions (Gharsallaoui et al. 2007). In the last few decades, increasing attention has been paid to hydrocolloid beads from a range of materials and in various sizes appropriate for the immobilization of beneficial microorganisms, including entomopathogens (Humbert et al. 2017). Living biological control microbes can be safely entrapped in nontoxic alginate matrix by a process known as ionotrophic gelation (de Oliveira Lopes et al. 2020). However, use of appropriate ingredients for extended shelf life remains the concern for the encapsulated formulations for entomopathogenic fungi. Further, although several encapsulated products based on entomopathogenic fungi have been evaluated against insect pests (Felizatti et al. 2021), investigation on efficacy of encapsulated *M. anisopliae* against the white grub species is very scanty. Therefore, the objectives of the present study were: (i) to develop an alginate-based formulation of *M. anisopliae,* (ii) to analyze its shelf life and viability, and (iii) to examine its efficacy against three species of white grubs that infest various crops in northeast India.

Methods

Reagents and chemicals

Potato dextrose agar (PDA), yeast extract, peptone, glucose, and antibiotics used for the isolation of *M. anisopliae* were obtained from HiMedia, Mumbai, India. Solvents and other encapsulation materials used for fabrication of the entomopathogen were procured from Sisco Research Laboratories Pvt. Ltd, Mumbai, India.

Fungal strain and growth conditions

In the present study, *Metarhizium anisopliae* (OL375172) strain was isolated from an infected white grub collected from Umiam, Meghalaya (27.7 N, 91.9 E, 916 m), India, in 2019 (Fig. 1A). The culture was maintained on PDA slants until sporulation for 7 days at 28 °C and 70 to 80% relative humidity (RH). For production of conidial suspension, a 6-mm mycelial disk of *M. anisopliae* taken from a 6-day-old culture on agar plate was inoculated in YPG (yeast peptone glucose) broth medium (yeast extract 0.3%, glucose 1.0%, and mycological peptone 0.5%) in triplicate and incubated at 28 °C in a BOD (Biological Oxygen Demand) incubator shaker (ThermoFisher, Mumbai, India) at 180 rpm for 72 h. The grown submerged conidia were harvested by centrifugation for 10 min at 5000 rpm and used for further needs. At the time of encapsulation, the viability of conidia was determined following the method of Oliveira et al. (2015). The conidial suspension was aseptically prepared in distilled water, and the conidial density of suspension was adjusted to 10^9 conidia/ml using a hemocytometer.

Encapsulation of *M. anisopliae* in Na-alginate beads

Preparation of alginate–conidia admixture

The admixture comprised presterilized sodium alginate as a gelling material dissolved in sterile Millipore water wherein kaolin as an inert material, glycerol as a protectant, and conidial suspension of *M. anisopliae* were added. During the course of the preparation of admixture, inside laminar airflow, sodium alginate (5% w/v) was added to the sterile Millipore water in a sterilized beaker and continuously stirred for 5 to 10 min and aseptically kept for 30 min in laboratory at 28 ± 2 °C. Then 1% (w/v) kaolin, 10% (m/v) submerged conidial suspension (10^9 conidia/ml) of *M. anisopliae*, and 5% (w/v) glycerol were added with the Na-alginate admixture, and the whole content was stirred continuously under aseptic conditions for 10 to 15 min for homogenization.

Encapsulated bead formation

The alginate-conidia admixture was extruded by using medical grade syringe of 10 ml/15 ml having needle orifice of 0.55 mm or by using a micropipette tip of 200 µl. Under aseptic conditions inside laminar air flow, the admixture was extruded dropwise over the cooled calcium chloride solution (0.5 M) kept in a beaker which was stirred continuously for uninterrupted bead formation. The admixture drops instantaneously formed beads on reaching the calcium chloride solution. These beads entrapped or encapsulated the active conidia of *M. anisopliae* that were added in the admixture. The formed beads were allowed to settle in the calcium chloride solution for 30 min as bead hardening time. The beads formed were washed in sterile Millipore water with three changes to remove the calcium chloride residue and

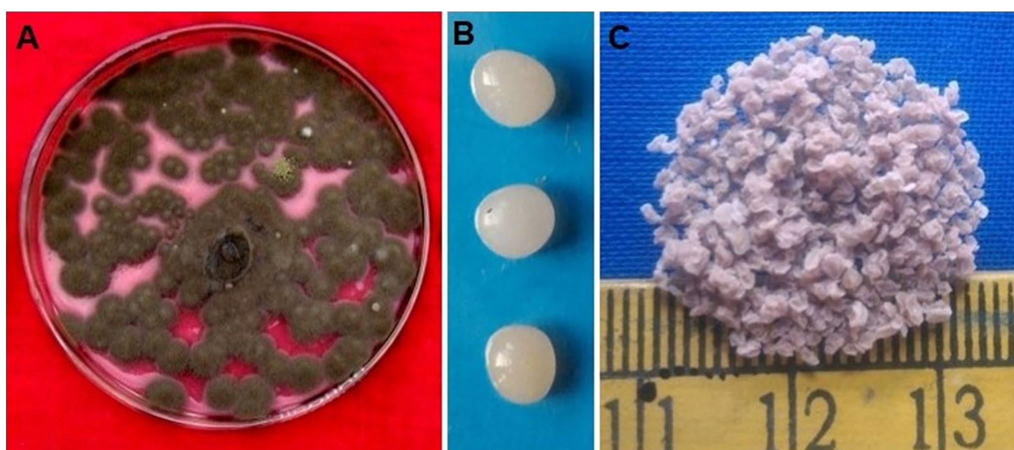

Fig. 1 An active culture of *Metarhizium anisopliae* (**A**—GenBank Accession Number OL375172) and encapsulated freeze-treated dried beads with *M. anisopliae* of different sizes (**B**, **C**)

allowed to dry. In normal air-drying method, the beads were dried inside laminar air flow cabinet for 32 to 42 h at 28 ± 2 °C.

Freeze-treated drying of fabricated alginate beads with M. anisopliae

An attempt was made to develop an easy, short and proper drying method of the encapsulated beads to replace the air-flow drying which usually requires long drying periods with no control over moisture content. After three times washing with water and draining, the wet encapsulated beads were taken in a glass container with a lid and kept in a refrigerator at −4 °C. The containers were kept in such condition for three different durations of 6, 8, and 12 h. After this cold treatment, the beads were spread over the Petri plates (15 cm diameter) inside laminar air flow for 2 h. and the percolated water was drained out. Then the water strained/removed beads were dried in a ventilated cabinet dryer (ICT, Kolkata) till 10 h at 29 ± 2 °C. The beads were also kept at a refrigerated temperature, i.e., 4 °C in freeze for comparison of drying hours.

Characterization of the Na-alginate beads with *M. anisopliae*

Determination of swelling ratio

The formulated encapsulated beads were tested for its swelling capability when soaked in water. Fifty beads were weighted first and placed in sterile Petri plates. The beads were soaked in sterile water in Petri plates and sealed with parafilm. The Petri plates along with soaked beads were kept under room temperature for 3, 6, and 24 h. The water was drained out from the Petri plates, and the beads were placed over a pre-weighed glass slide. Extra water was absorbed with blotting paper and the slide was kept for 20 min to wipe out the outer surface water. Then the beads were weighted with a balance (Mettler Toledo, PL303), and the amount of water imbibed by the beads was calculated. The entire test was replicated for five times and repeated. The percent swelling ratio was computed compared to dried beads.

Determination of size

The average size of encapsulated intact and freeze-dried beads was determined following the methodology of de Oliveira Lopes et al. (2020). Twenty-five beads were randomly selected, placed on paper, and photographed with a digital DSLR camera (Canon EOS 70 D) with a 100 mm macro-lens. A length scale was added while taking the image.

Evaluation of mycelial growth and sporulation capability

Mycelial growth and sporulation capability of *M. anisopliae* encapsulated formulation was measured by the method of Moslim et al. (2009) with slight modification. ELISA plate was first surface sterilized with 100% ethyl alcohol and then sterilized under UV radiation. Beads were graded into three groups, i.e., small size (0.5 to 1.2 mm), medium size (1.2 to 2.0 mm), and large size (2.0 to 2.5 mm) based on the size of individual bead. Individual encapsulated bead was then placed in the well of previously surface sterilized ELISA plate. The beads were then wetted with 40 µl of sterile water and wrapped with a polythene bag and incubated in a BOD incubator for 7 days at 28 °C. Five ELISA plates were taken as five replications. After incubation, the number of beads observed with growth of whitish hyphae and the number of beads producing conidia were counted. Encapsulated beads with high number of *M. anisopliae* conidia (> 50%) were considered as qualitative beads.

Stability and shelf life

The stability or viability of *M. anisopliae* in the encapsulated beads was determined at 0, 4, 24, 168 h for 1 week, and 336 h for 2 weeks after beads formation that were maintained at room temperature (24 ± 2 °C). Petri plates containing Whatman no. 1 filter paper as per the size of the Petri plates (90 mm) were sterilized properly. The filter paper was then soaked with 1.0 ml sterile water, and 10 to 20 encapsulated beads were placed over the soaked filter paper. The plates were then incubated at 28 ± 2 °C in a BOD incubator for 5 to 7 days. In order to determine the viable conidia count, incubated three beads (approximately 10 mg in weight) were suspended in 10 ml sterile water and vortexed (Kaith vortex shaker, KE-1). Serial dilution of the bead suspension was made, and 0.1 ml aliquot from 10^{-7} dilution of bead suspension was spread onto the PDA medium and incubated at 28 ± 2 °C in a BOD incubator. The number of fungal colonies appeared on the agar plate after 3 to 4 days of incubation was recorded as CFU/g of beads and expressed as log CFU/g encapsulated beads wherever necessary. The plate count was conducted in five replicates and repeated. The encapsulated beads were further checked for its viability with *M. anisopliae* till 10 months of storage. After every 30 days to till 10 months, 100 beads were drawn from each of the storage duration and viability was recorded as CFU/g of beads.

Determination of moisture content

The moisture content of the intact and Na-alginate beads was assessed in accordance with Instituto Adolfo Lutz (2008). An amount of 3 g of encapsulated beads was dried in an electric hot air oven (Thermo Fishers, Mumbai, India), for 3 h at 105 °C. The samples were weighed after chilling in a glass desiccator at room temperature. This process was repeated until a constant weight was

reached. The assays were carried out five times and repeated.

Determination of the yield

In the beginning of experiments, ten centrifuge tubes of 50 ml volume were previously weighed for each treatment group, such as intact beads, with or without *M. anisopliae.* As described above, 20 ml of beads were transferred to tubes for freeze drying. The difference between intact beads and freeze-dried beads was determined by weighing the tubes again after freeze drying.

Pathogenicity effect of fabricated freeze-dried alginate beads with M. anisopliae against white grubs

The developed freeze-dried alginate beads with *M. anisopliae* were evaluated against three species of white grubs, namely *Holotrichia serrata* (Fabricius), *Adoretus* sp., and *Lepidiota mansueta* under greenhouse conditions with 28/18 °C (day/night) temperature with a 70–75% of RH and 14-h photoperiod. These species of white grubs were selected because they were infesting majority of tuber crops in northeast India. The 1st and 2nd grub instars were collected from the infested areas of Jorhat and Majuli districts of Assam state, India. Collected grubs were maintained in earthen pots and fed on cut potatoes for 2 to 5 days. The efficacy of the encapsulated beads with *M. anisopliae* was evaluated in vitro by using plastic bowl (1 l) for *H. serrata*, small earthen pots (1 l) for *Adoretus* spp., and plastic glass (1 l) for *L. mansueta*. The containers were first surface sterilized with ethyl alcohol and filled with 500 g of sterilized soil. For *L. mansueta*, 500 g of native soil of infested area of Majuli was filled to the individual plastic glasses. Encapsulated beads were added to each bowl/pot/glass as per the treatment and mixed with the soil. The treatments included T_1; Control, T_2; 250 mg (2.1×10^8 CFU/g) alginate-based beads with *M. anisopliae*, T_3; 500 mg (2.1×10^8 CFU/g), alginate-based beads with *M. anisopliae*, T_4; 1000 mg (2.1×10^8 CFU/g), alginate-based beads with *M. anisopliae*, T_5; Chloropyriphos 20 EC @ (at the rate of 0.02%), T_6; 3% conidial suspension of *M. anisopliae* @ 10^9 spores/ml. Third instar white grubs were released to the bowl or pot @ 5 grubs/bowl or pot. In case of *L. mansueta*, 3rd instar grub of *L. mansueta* was released individually to each glass containing native soil. Here, five glasses containing single *L. mansueta* grub, each were made one replication. Sliced potato @ 2–3 small pieces were provided as food materials for the grubs in the pot/bowl/glass. The bowls/pots/glasses were covered with a thin layer of muslin cloth, separately. Sterile Millipore water was sprinkled in pot/bow/glass whenever necessary to maintain soil moisture (20–30%). Soil without any encapsulated formulation was served as a control. All the treatments were replicated for five times and repeated under the same biological conditions.

Data analysis

Based on the results of repeated experiments, non-significant variances were observed between them ($P>0.05$), and errors within and between groups were homogeneous; therefore, data from each repeated experiment were combined for statistical analysis. Normality distribution of all variables was verified using the Shapiro–Wilk test. The corrected mortality was evaluated as per the Abbott's formula (Abbott 1925). The paralyzed and dead grubs were picked and washed with sterile water and kept in moist chamber for confirmation of fungal infection. Data were subjected to Probit analysis as per Finney (1964) for determination of LC_{50} value. ANOVA analysis of the data was carried out using R-software (R core 2020).

Results

Bead structure

The size of the developed formulation was measured with the mean sizes of the wet beads ranged between 1.4 and 3.2 mm, whereas the size of the dried beads through airflow drying ranged between 0.55 and 2.35 mm. Besides, the mean sizes of the freeze-dried beads ranged between 0.55 and 2.30 mm and were non-significantly ($P<0.001$) different from the beads developed through air-flow-drying method (Fig. 1B, C).

Freeze-treated drying of encapsulated alginate beads with *M. anisopliae*

The wet encapsulated beads were subjected to cold temperature for definite period before drying under ventilated dryer. The results revealed that for the wet formulation (bead) when kept at refrigerated temperature, i.e., 4 °C for 12 h in freeze, it required 8 h to dry the encapsulated beads with moisture content of 6 to 7%. On the other hand, for the wet formulation when kept in the freeze at freezing temperature, i.e., −4 °C for 8 h, it reduced the drying time to 5 h with the encapsulated bead moisture content of 6%. The same result was also observed when the wet formulation was kept at −4 °C for 12 h with drying time of 5 h with bead moisture content of 6%. However, considering the time factor of cold treatment duration, the freeze treatment of wet encapsulated formulation at −4 °C for 8 h found to be the best treated condition to dry the formulation within 5 h.

Swelling ratio of alginate beads with *M. anisopliae*

The capability of encapsulated beads in terms of swelling ratio is presented in Table 1. The average swelling ratio of air-flow-dried beads was increased from 37.2 to 67.4% from 3 to 24 h of soaking. In case of freeze-treated

Table 1 Swelling ratio of encapsulated Na-alginate beads with *Metarhizium anisopliae* at 3, 6 and 24 h of soaking

Method of drying	Swelling ratio (%)		
	3 h. of soaking	6 h. of soaking	24 h. of soaking
Air-flow drying	37.2a	52.0b	67.4c
Freeze-treated drying	43.0a	63.0b	79.2c

Data with the same letter within column are non-significantly different at $P<0.05$

beads, the average swelling ratio was increased from 43.0 to 79.2% upon soaking in water from 3 to 24 h. Freeze-treated dry beads were swelled significantly ($P<0.001$) more compared to air-flow-dry beads from 3 to 24 h. The interaction of method of drying and time also had a significant effect ($F=53.28$; $P<0.0001$).

Growth and sporulation of *M. anisopliae* encapsulated in alginate beads

The revival of the entrapped *M. anisopliae* in encapsulated beads was checked in terms of mycelial growth and sporulation. The mycelial growth and sporulation of the encapsulated beads were observed by placing the encapsulated beads individually on ELISA plate. The result of the bead quality based on mycelial growth and sporulation of the encapsulated *M. anisopliae* beads is presented in Table 2. The mycelial growths were 96.87, 94.53, and 89.84% and sporulations were 93.74, 90.62, and 89.06% for small, medium, and large air-flow-dried beads without statistical difference ($F=31.57$, $P<0.0001$), respectively. On the other hand, in case of freeze-treated dry beads, mycelial growths were 100.00, 98.43, and 96.89% and sporulations were 98.96, 96.09, and 92.23% for small, medium, and large beads, respectively, with non-significant difference ($F=28.43$, $P<0.0001$).

Viability/stability of encapsulated alginate beads with *M. anisopliae*

The data presented in Table 3 show that the viability of the *M. anisopliae* was marginally reduced from just after drying (0 h) to 336 h of storage with 17.8×10^7 CFU/g to 16.0×10^7 CFU/g colonies for air-flow-dried beads and 21.6×10^7 CFU/g to 20.0×10^7 CFU/g colonies for freeze-treated beads. Although data were non-significant ($F=12.71$, $P<0.0001$), *M. anisopliae* were more viable in freeze-treated dry beads with 20.0×10^7 CFU/g colonies than air-dried beads, which had 16.0×10^7 CFU/g colonies after 336 h of storage.

Shelf life of encapsulated alginate beads with *M. anisopliae*

The storage life of the encapsulated formulation stored at room temperature (24 ± 2 °C) was determined in terms of number of colonies of *M. anisopliae* produced by the encapsulated formulation after different months of storage (Fig. 2). The results presented in Table 4 show that storage life of encapsulated formulation gradually declined from the date of storage to 10 months of storage. The encapsulated beads that were dried by freeze treatment dry showed the viability of *M. anisopliae* with population 18.2×10^7 CFU/g after one month of storage at room temperature which was gradually

Table 2 Mycelial growth and sporulation *of Metarhizium anisopliae* in encapsulated beads

Bead size	Mycelial growth (%)		Sporulation (%)	
	Air flow dry	Freeze-treated dry	Air flow dry	Freeze-treated dry
Small size (0.5 to 1.2 mm)	96.8a	100.0a	93.7a	98.9a
Medium size (1.2 to 2.0 mm)	94.5a	98.4a	90.6a	96.09a
Large size (2.0 to 2.5 mm)	89.8a	96.8a	89.06a	92.2a

Data with the same letter within column are non-significantly different at $P<0.05$

Table 3 Viability of *Metarhizium anisopliae* in encapsulated beads at different time interval

Drying method	Initial spore load in the admixture ($\times10^8$ spore/ml)	Population of *M. anisopliae* ($\times10^7$ CFU/g) at different time interval ± Standard error				
		0 h	4 h	24 h	168 h	336 h
Air flow dry	1.00	17.8 ± 2.2a	17.2 ± 1.9a	17.0 ± 0.9a	16.0 ± 1.2a	16.0 ± 0.5a
Freeze-treated dry	1.00	21.6 ± 1.5a	21.2 ± 1.3a	21.2 ± 1.2a	20.8 ± 0.8a	20.0 ± 1.2a

Data with the same letter within column are not significantly different at $P<0.05$

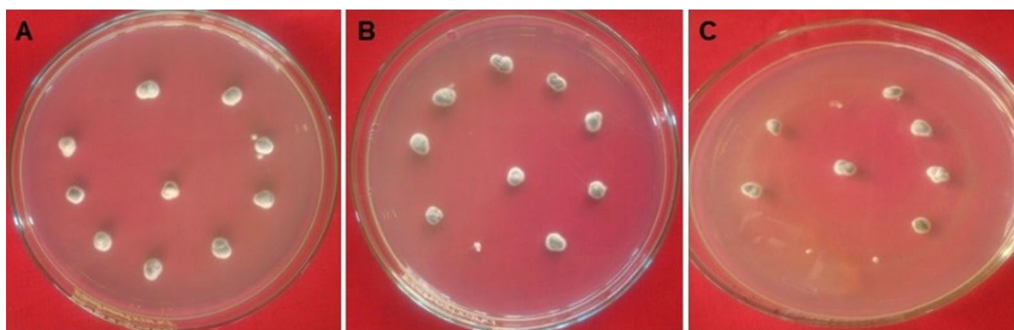

Fig. 2 Growth of *Metarhizium anisopliae* encapsulated in freeze-treated dried Na-alginate beads after different duration of storage **A** 1 month of storage, **B** 7 months of storage, **C** 10 months of storage

Table 4 Viability of *Metarhizium anisopliae* in encapsulated beads along 10 months of storage

Month of storage	*M. anisopliae* population (×10^7 CFU/g) in encapsulated beads ± Standard error	
	Freeze-treated dry	Air flow dry
0	21.2 ± 0.5a	17.0 ± 1.2a
1	18.2 ± 0.8a	14.8 ± 1.4b
2	14.0 ± 1.3b	11.0 ± 0.5b
3	11.8 ± 1.2b	9.0 ± 0.9bc
4	9.6 ± 1.3c	6.6 ± 1.2bc
5	7.0 ± 0.5c	4.4 ± 1.1c
6	6.4 ± 0.9c	4.0 ± 0.5c
7	6.0 ± 1.1c	2.2 ± 0.9d
8	5.2 ± 1.5d	1.0 ± 0.2 cd
9	4.0 ± 1.0d	0.6 ± 0.1 cd
10	2.0 ± 0.5e	0.2 ± 0.1 cd

Data with the same letter within column are non-significantly different at $P < 0.05$

declined to 6.4×10^7 CFU/g after 6 months and then to 2.0×10^7 CFU/g after 10 months of storage. The beads that were dried by air-flow drying showed the viability of *M. anisopliae* with population 14.8×10^7 CFU/g after one month of storage and which has become 4.0×10^7 CFU/g after 6 months of storage. The viability of the *M. anisopliae* encapsulated beads dried through air-flow drying drastically reduced to 0.2×10^7 CFU/g than the freeze-treated dry beads. From the above results, it was observed that freeze-treated drying method can support more viability of *M. anisopliae* with longer shelf life. The storage time also had a significant effect in both air-flow drying (F = 47.37, P < 0.0001) and freeze-dried (F = 53.58, P < 0.0001) methods.

Pathogenicity of encapsulated freeze-dried alginate beads with *M. anisopliae* against white grubs

The mortality of grubs of *H. serrata* was increased as the days of treatment increased (Table 5). The highest mortality of 80.0% was recorded when the encapsulated formulation was used at 500 (2.1×10^8 CFU/g) and 1000 mg (2.1×10^8 CFU/g) concentrations. The mortality due to the developed beads at 250 mg (2.1×10^8 CFU/g) concentration and due to the conidial suspension of *M. anisopliae* was 48.0 and 44.0%, respectively, after 21 days of treatment. Chlorpyriphos showed 100% mortality after 14 days of treatment. The mortality caused

Table 5 Efficacy of freeze-treated dry Na-alginate beads with *Metarhizium anisopliae* against white grubs

Treatments	Corrected mortality (%)											
	Holotrichia serrata				*Adoretus* sp.				*Lepidiota mansueta*			
	3 DAT	7 DAT	14 DAT	21 DAT	3 DAT	7 DAT	14 DAT	21 DAT	3 DAT	7 DAT	14 DAT	21 DAT
Control	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
250 mg * beads	0.0a	8.0b	28.0b	48.0b	4.0b	20.0b	40.0b	52.0b	0.0a	4.0b	12.0b	28.0b
500 mg * beads	4.0b	24.0c	52.0c	80.0c	8.0b	32.0c	72.0c	80.0c	4.0b	8.0c	32.0c	40.0c
1000 mg * beads	4.0b	44.0d	64.0c	80.0c	12.0c	52.0d	72.0c	88.0c	8.0c	24.0d	40.0c	60.0d
Chlorpyriphos 20 EC	16.0c	64.0e	100.0d	100.0d	20.0d	68.0e	100.0d	100.0d	12.0d	32.0d	56.0d	84.0e
M. anisopliae SS*	0.0a	8.0b	28.0b	44.0b	0.0a	24.0b	32.0c	48.0b	0.0a	4.0b	8.00b	28.0b

DAT days after treatment, Data with the same letter within column are non-significantly different at $P < 0.05$., *SS* spore suspension, *2.1×10^8 CFU/g

Table 6 LC_{50} value of freeze-treated dry Na-alginate beads with *Metarhizium anisopliae* against white grubs

White grub species	LC_{50} values in mg (2.1×10^8 CFU/g)			
	3 DAT	7 DAT	14 DAT	21 DAT
Holotrichia serrata	2.66	1.02	0.66	0.35
Adoretus sp.	2.43	0.89	0.47	0.31
Lepidiota mansueta	1.94	1.42	1.05	0.76

LC lethal concentration, *DAT* day after treatment

Fig. 3 White grub (*Adoretus* sp.) infected with *Metarhizium anisopliae* encapsulated in freeze-treated dried Na-alginate beads in laboratory test. The arrow represents growth of *M. anisopliae* over grub

due to 500 and 1000 mg concentrations of the encapsulated formulation was statistically ($P<0.0001$) *at par* as seen in the experiment. The LC_{50} value as shown in Table 6, was decreased from 2.66 mg (2.1×10^8 CFU/g) to 0.35 mg (2.1×10^8 CFU/g) after 21 DAT, as the mortality increased.

As presented in Table 5 and Fig. 3, the mortality of treated grubs of *Adoretus* sp. was increased from 3 to 21 DAT with the highest mortality of 88.0% at 1000 mg (2.1×10^8 CFU/g) concentration of encapsulated formulation. The corrected mortality was 52.0 and 80.0% when treated with 250 (2.1×10^8 CFU/g) and 500 mg (2.1×10^8 CFU/g) concentrations of encapsulated formulation, respectively, after 21 days of treatment. Chemical treatment showed complete mortality after 14 days of treatment, whereas spore suspension showed 48.0% mortality after 21 days of treatment. The LC_{50} value as presented in Table 6 was decreased from 2.43 mg (2.1×10^8 CFU/g) to 0.31 mg (2.1×10^8 CFU/g) 21 DAT as the mortality increased.

The result of the efficacy of encapsulated formulation against 3rd instar white grub, *L. mansueta* is presented in Table 5. The results showed that the mortality rates of the white grubs were 28.0, 40.0, and 60.0% when encapsulated formulation used at 250 (2.1×10^8 CFU/g), 500 (2.1×10^8 CFU/g), and 1000 mg (2.1×10^8 CFU/g) concentrations after 21 DAT, respectively. The mortality caused due to the application of encapsulated formulation was comparatively low toward this white grub species compared to other two species of white grubs (*H. serrata* and *Adoretus* sp.) evaluated under the study. Even the chemical application could cause 84.0% mortality of grubs after 21 DAT. The spore suspension showed only 28.0% mortality of grubs at 21 DAT. The LC_{50} value was decreased from 1.94 mg (2.1×10^8 CFU/g) to 0.76 mg (2.1×10^8 CFU/g) after 21 DAT as the mortality increased (Table 6). At 21 DAT, there was non- significant difference between the 500 (2.1×10^8 CFU/g) and 1000 (2.1×10^8 CFU/g) mg beads treatments for *H. serrata* ($F=13.6$, $P<0.0001$) and *Adoretus* sp. ($F=17.5$, $P<0.0001$); however, there was a significant difference between these two treatments for *L. mansueta* ($F=47.9$; $P<0.0001$). Additionally, at 21 DAT, these both treatments differentiated significantly than insecticide and the *M. anisopliae* spore suspension tested in the present study.

Discussion

Earlier investigations report that encapsulation can protect sensitive beneficial microorganisms from adverse environmental conditions including pH, temperature, water stress, and harmful organisms (Shah et al. 2022b). Additionally, it is easier to handle, provides increased safety for farmers through low dusting, and is compatible with other management practices as well as agricultural operators (Rodrigues et al. 2017). Conidia are the most preferred form of beneficial fungi including entomopathogens used in the preparation of biopesticides. Therefore, the possibility of encapsulation of submerged conidia instead of using mycelium was explored to enhance shelf life and application. Our goal was to develop a bioencapsulation and drying method that would increase the shelf life and effectiveness of conidia/spores of *M. anisopliae*.

In the present study, mean sizes of beads developed through air-flow-drying and freeze-drying methods were within the range reported by earlier investigators (Liu and Liu 2009). In contrast, the mean sizes of Ca-alginate beads developed with active spores of *Trichoderma asperellum* before and after freeze drying were 2.5 and 1.1 mm (de Oliveira Lopes et al. 2020), which were lesser than the sizes reported in this investigation. This might be due to use of different chemical materials, methodology used and incubation conditions of fabricated beads. The freeze-treated and dried encapsulated beads were becoming wrinkled with rough in surface and their structure was porous making them slightly brittle to touch. The cold treatment was accorded to desiccate the encapsulated beads in order to sublimate the cold treated water from the alginate matrix during post drying at normal

temperature. Again, the method greatly reduced the drying time of air-flow-drying method of the encapsulated formulation by 12 to 30 h without affecting the viability of *M. anisopliae*.

As far as swelling ratio is concerned, increased swelling ratio of lyophilized beads than air-dried beads were reported by Li et al. (2016), while working on sorption capability of alginate beads. Their average swelling ratios were around 50 to 65% with alginate–CNC beads by air-drying method after 48 h of soaking in distilled water. Freeze-dried alginate–xylan beads reached 190%, while alginate–CNC showed an average swelling ratio of 135%. Similar results were reported in present investigation; freeze-treated dry beads were swelled significantly more compared to air-flow-dry beads. The air-dried beads were more compact in structure compared to freeze-treated beads upon cross linking with calcium chloride. The probable pore spaces enabled the freeze-treated dry beads to take up more water and to revive more rapidly. The present method did not require sophisticated and high-cost machineries and can be done by simple equipment available in common laboratories and, thus, found very effective for production of macro encapsulated formulation in shorter period of time.

Similar to our study, Krell et al. (2018) also fabricated mycelium of *Metarhizium brunneum* strain BIPESCO5 in calcium alginate/starch beads and confirmed that fungal mycelium/spore encapsulation enhanced drying survival by 31.5%. For alginate beads, increasing the initial cell concentration in the formulation could prevent abrupt loss of viability during storage because freeze drying is always associated with long-term storage of cell suspensions of a particular microorganism. After freeze drying for maintenance, Jin and Custis (2011) found that a survival rate of 0.1% of the original cell population is acceptable for survivors. Saha and Pan (1995) reported that the population of *Gliocladium virens* in the sodium alginate biomass–bentonite-based prills varied from 4.0 to 5.0×10^8 CFU/ml to 0.6 to 1.0×10^8 CFU/ml in initial period and 48 h of storage, respectively and again declined afterward. Likewise, Fravel et al. (1985) also reported the gradual decline of *G. virens and Trichoderma viride* from initial population of 6.7×10^5 to 6.8×10^6 /pellet to 12.0 to 0×10^3 /pellet, respectively, till 12 weeks. In the present study, it was observed that the encapsulated formulation could support the viability of *M. anisopliae* with constant high population during initial period till 336 h of bead storage. These variations in viability might be due to variation in formulation methods and species of beneficial microbes used during fabrication.

In a study of starch-based encapsulation, Badgujar et al. (2009) reported that maize starch-encapsulated *M. anisopliae* conidia showed more viability for up to 180 days than free conidia, although the viability decreased from 20 to 180 days. In another study, Adzmi et al. (2012) reported that encapsulated *T. harzianum* was viable for 3 months only under room temperature with population 7.716 log CFU at 1 month storage and 5.643 log CFU at 3 months of storage with initial spore concentration of 1.97×10^{10} spores/ml. In their experiment, Locatelli et al. (2018) revealed 14 months viability of *Trichoderma* sp. with 2.4×10^6 CFU/g of encapsulated alginate–polyphosphate–pectin formulation. Pereira and Roberts (1991) while working on alginate and corn starch formulation and after 13 weeks of storage there were tenfold reduction of spore production of alginate formulation of *M. anisopliae* at room temperature compared to storage at 4 °C. However, most of the above experiments were conducted utilizing different modes of drying. Przyklenk et al. (2017) found that autoclaved baker's yeast and a combination with starch enhanced the conidiation to 1.0×10^8 conidia/bead of *M. brunneum* encapsulated in corn starch and viable up to the six months. Conversely, in this study, the encapsulated beads showed viability up to 10 months. The differences in viability might be due to different species of *Metarhizium* used in encapsulation and chemical materials.

The main bottleneck in the production of any microbial biopesticide formulation for agricultural improvements is the shelf life, which required to be increased as the number of inoculums eventually decreases during the storage. The results showed that the encapsulated formulation utilized only three simple carrier components: sodium alginate as a structural carrier, kaoline as an inert material to give physical strength, and glycerol as a protective material to the formulated beads. The highest mycelial growth and sporulation in the present experiment indicated the good quality of the encapsulated formulation. The formulation could also support the viability of *M. anisopliae* till 10 months of storage with 10^7 CFU/g of spore density which is above the required population being fixed for the fungal biocontrol agent before application to the target pests (John et al. 2014).

In the present study, encapsulated freeze-dried alginate beads with *M. anisopliae* were evaluated against three species of white grubs for its efficacy. Badgujar et al. (2009), while working on the maize starch-based formulation, reported that Maize starch formulation (SM-II) showed 100% mortality of *H. consanguinea* at lower concentration (2.5×10^7 CFU/g soil). No investigations on efficacy of encapsulated alginate beads with *M. anisopliae* against *H. serrata* have been reported so far. The present study revealed that encapsulated formulation at 500 mg (2.1×10^8 CFU/g) could cause grub mortality of 80.0% with LC_{50} value of 0.35 mg (2.1×10^8 CFU/g) after

21 days of treatment. Further, there is no earlier report found on the efficacy of encapsulated alginate beads with *M. anisopliae* against *Adoretus* sp. However, Rodrigues et al. (2017), while working on the bioinsecticide action of encapsulated conidial formulation of *B. bassiana* and *M. anisopliae,* reported that dry encapsulated conidia of *B. bassiana* caused 79.6% mortality and *M. anisopliae* caused only 10.0% against *Diatraea saccharalis* caterpillar of sugarcane. This is the first report of efficacy of encapsulated alginate beads with *M. anisopliae* against *Adoreus* sp. Present study revealed that encapsulated formulation at 500 (2.1×10^8 CFU/g) and 1000 mg (2.1×10^8 CFU/g) concentrations could cause 80.0 and 88.0% mortality of *Adoretus* sp., respectively, after 21 DAT in in vitro test. The ability of the grubs to withstand adverse conditions including stress soil environment, probably made the *L. mansueta* grubs less susceptible to the formulations. So far, no work has been reported on the effect of encapsulated alginate beads with *M. anisopliae* against *L. mansuata.* The present study revealed that the developed encapsulated *M. anisopliae* formulation was comparatively less affective against *L. mansueta* even at a higher concentration. However, more investigations on the field efficacy can provide further acceptable results.

Conclusions

The present study developed a freeze-dried formulation of *M. anisopliae* based on Na-alginate. In freeze-dried beads, *M. anisopliae* showed better viability and potential efficacy against white grub species, namely: *H. serrata*, *Adoretus* sp., and *L. mansueta*. In conclusion, the results represent the first evidence for encapsulating *M. anisopliae* with alginate beads, their survival rates after freeze drying at different storage intervals, and their potential pathogenic activity against white grubs. Further research is necessary to improve alginate beads formulation aspects for *M. anisopliae* submerged propagules, to provide increased protection against soil dwelling insect pests, and to enhance plant growth. In order to improve this encapsulation system for *M. anisopliae* stored at non-refrigerated temperatures, future studies regarding initial cell loading in the formulation, the addition of cell protectants, cost-effective drying methods, and controlled atmospheric packaging are warranted.

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Author contributions
BDS and KCP conducted experiment after significant inputs from PD, PD performed review and edit, and AKP performed original draft preparation, data analysis, and review and edit.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no conflict of interest.

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