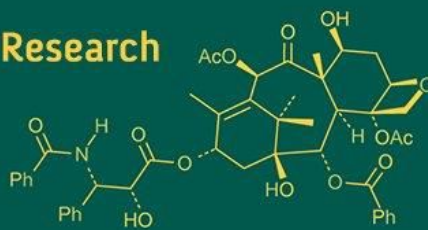
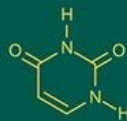
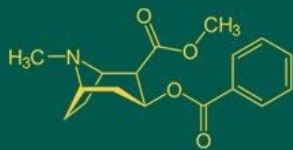


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Stability and shelf life of *Metarhizium anisopliae* in capsule formulation: Impact of storage conditions

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Abstract

Metarhizium anisopliae is crucial for sustainable pest management as a natural biocontrol agent, effectively targeting harmful insects while being safe for humans, animals and plants. The carrier-based capsule formulations were stored at room temperature for 180 days to assess their shelf life. The carriers used in the experiment were talc, kaolin, charcoal, sorghum grain, maize grain, lignite and fly ash. The month-wise colony-forming unit (CFU) counts of *Metarhizium anisopliae* in various carrier-based formulations stored at room temperature for six months revealed a gradual decline in spore viability over time. Initial cfu counts (0 days) for all formulations ranged from 17.55×10^6 to 19.40×10^6 cfu/g, indicating good initial viability of the spores. The sorghum grain capsules retained the highest CFU count at 1.33×10^6 CFU/g, while the fly ash capsules showed the greatest decline, dropping to 0.27×10^6 CFU/g.

Keywords: *Metarhizium anisopliae*, shelf life, formulations

Introduction

Metarhizium anisopliae entomopathogenic fungi belong to Kingdom: Fungi, Subdivision: Ascomycota, Class: Sordariomycetes, Order: Hypocreales, Family: Clavicipitaceae, Genus: *Metarhizium* and Species: *anisopliae*. The first known species of the genus *Metarhizium* Sorokin was isolated from infected insects in Ukraine in the late 1870s. It was originally named *Entomophthora anisopliae* based on the generic name of its scarab host, *Anisoplia austriaca* (Metschnikoff, 1879). The new generic name, *Metarhizium* was proposed for this fungus by Sorokin (1883). *Metarhizium* is a cosmopolitan fungus and have been isolated from infected insects and soil of all continents except Antarctica. There are more than 750 species of entomopathogenic fungi that can be used for the biological control of pests. Among the most important genera are *Metarhizium*, *Beauveria*, *Aschersonia*, *Entomophthora*, *Zoophthora*, *Hirsutella*, *Fusarium* and *Verticillium* (Acuna *et al.*, 2015) [1] and *Metarhizium anisopliae* is one of the most used commercially biopesticide.

The global biopesticide market size was valued at USD 7.54 billion in 2023 and is projected to grow from USD 8.73 billion in 2024 to USD 28.61 billion by 2032, exhibiting a CAGR of 15.99% during the forecast period. North America dominated the biopesticide market with a market share of 36.6% in 2023. Moreover, the biopesticide market size in the U.S. is projected to grow significantly, reaching an estimated value of USD 7.34 billion by 2032, driven by rising demand for organic food and increased use of sustainable agro-chemicals to grow crops.

The India biopesticide market was valued at USD 64.73 million in 2021 and is expected to grow to USD 130.37 million by 2029, with an annual growth rate of 9.38%. The market experienced a drop of 5.10% in 2020 due to the COVID-19 pandemic, but demand is now recovering (Source: <https://www.fortunebusinessinsights.com>).

In the year 2022-23, India's total consumption of chemical pesticides was 52,466 metric tons. The states of Uttar Pradesh, Maharashtra, Punjab, Telangana and Haryana are the top consumers of chemical pesticides in India, driven by their large-scale agricultural production, particularly in crops like cotton, rice and wheat, which require intensive pest control to ensure high yields.

During 2022-23, the demand for biopesticide in India was 11,211 metric tons (MT), while the consumption was 7,248 MT, highlighting a gap between the demand for and actual usage

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of biopesticide, suggesting potential for increased adoption in the future. (DPPQS 2022-23).

Day by day the application of chemical pesticides is rapidly increasing and it is very big problem for our environment and for our health. The excessive use of chemical products in agricultural crops is a problem, since some insects have developed resistance to them (Lezama *et al.*, 2012) ^[2]. Therefore, it has become necessary to look for alternative means. Biological control is one of good option for control pests; it will minimize the use of chemical pesticides (Mirhaghparsat *et al.*, 2013) ^[3]. Biological control provides additional tools for the management of pests that break the resistance cycles of insects.

The fungus infects and kills insects by penetrating their cuticle and proliferating within their bodies, ultimately leading to death. Unlike chemical pesticides, *M. anisopliae* is environmentally friendly, non-toxic to humans and animals and has a lower likelihood of resistance development in target pest populations. The secondary metabolites of *Metarhizium anisopliae* play a crucial role in its use as a biocontrol agent against pests, providing an eco-friendly alternative to chemical pesticides. These metabolites also promote plant growth, enhance soil fertility, and have potential applications in pharmaceuticals due to their antimicrobial and anticancer properties. Therefore, it represents a promising solution to the growing challenges of pest resistance and environmental degradation associated with chemical pesticide use.

Materials and Methods

Mass multiplication of *M. anisopliae*

1. Grain media

The virulent Ma-20 fungal isolate was used for mass multiplication. Sorghum and maize grains were soaked in water overnight to allow them to absorb sufficient moisture, promoting optimal fungal growth. After soaking, the grains were dried on paper to remove excess moisture, ensuring they were suitable for sterilization. The dried grains were then autoclaved to eliminate any potential contaminants, creating a sterile environment for the Ma-20 isolate. A 5 mm disc from a 7-day-old culture of the Ma-20 isolate was inoculated onto the sterilized grains, introducing the fungal culture. The inoculated grains were incubated for 12 to 15 days, during which the fungus spread, covering the grains with dark green mycelium and producing spores. After the incubation period, the grains, now fully colonized with mycelium and spores, were harvested. These grains were finely powdered and the resulting powder, rich in fungal biomass, was used in capsule formulations.

2. Sabouraud dextrose broth

The mass multiplication of *Metarhizium anisopliae* in sabouraud dextrose broth (SDB) begins with the preparation of 250 ml of SDB in a 500 ml conical flask, which is sterilized by autoclaving at 121 °C for 15 minutes. A 5 mm disc from a 7-day-old culture of *M. anisopliae* is aseptically inoculated into the sterilized broth. To prevent bacterial contamination, streptomycin sulfate (200 mg/L) was added. The flask was then plugged with sterilized cotton to ensure aeration while preventing external contamination and incubated at 28±2 °C for 7 days. After incubation, a thick mycelial mat was observed, indicating successful fungal growth.

The mycelial biomass was harvested by carefully decanting or filtering the liquid, followed by washing with sterile distilled water to remove any residual medium. The biomass was then dried under sterile conditions, such as in a laminar airflow or an oven set at 40 °C. The dried mycelial mat further processed into a powder or combined with carriers to prepare bio formulations suitable for use as a biocontrol agent. This process provides an efficient method for large-scale production of *M. anisopliae* for agricultural applications.

3. Capsule preparation

A well-designed formulation is essential for maintaining the viability of conidia and for the successful commercialization of biopesticides. Hard gelatin capsules, measuring 20 mm in length, 8 mm in diameter and with a radius of 4 mm, were used for capsule formulation. The volume of each cylindrical capsule was 1.0048 cm³. The capsules consisted of two parts: the body and a shorter cap, both supplied by the Department of Plant Pathology, Dr. P.D.K.V., Akola. The cap fits securely over the open end of the capsule body. These hard gelatin capsules were made from a mixture of gelatin, sugar and water. The body of the capsule was green and it had a cylindrical shape.

The Ma-20 isolate was grown in sabouraud dextrose broth and the resulting mycelial mat was dried and used to fill the capsules. A seven-day-old culture, showing a thick mycelial mat and abundant sporulation, was ground in a grinder. These mycelial mats, along with spores, were then mixed with autoclaved carriers, such as talc, charcoal, kaolin, fly ash, lignite, sorghum and maize grains. The substrate was further mixed with Carboxymethyl cellulose at 0.5 g and dextrose at 1 g per 100 g of the mixture. This mixture was then filled into each empty capsule and stored at room temperature. The capsules were kept under aseptic conditions for 48 hours to dry.

Three replications were maintained for each carrier to determine the initial number of colony forming units (CFUs) of *M. anisopliae* in the formulation. A sample of one gram of the product was drawn from each capsule just before packing. The sealed capsules were stored at room temperature for six months and the population was estimated at monthly intervals using the serial dilution technique. One gram samples from each carrier at respective intervals (30, 60, 90, 120, 150 and 180 days) were taken and serially diluted up to 10⁶ using sterilized distilled water. An aliquot of 0.1 ml was transferred onto each SDAY plate and the colony-forming units (CFUs) were counted after 5-6 days of incubation.

Results and Discussions

The carrier-based capsule formulations were stored at room temperature for 180 days to assess their shelf life. The carriers used in the experiment were talc, kaolin, charcoal, sorghum grain, maize grain, lignite and fly ash. The month-wise colony-forming unit (CFU) counts of *Metarhizium anisopliae* in various carrier-based formulations stored at room temperature for six months revealed a gradual decline in spore viability over time. Initial cfu counts (0 days) for all formulations ranged from 17.55 × 10⁶ to 19.40 × 10⁶ cfu/g, indicating good initial viability of the spores.

Among the carriers, sorghum grain capsules exhibited the highest spore viability throughout the storage period. The initial cfu count of 19.09±0.83 × 10⁶ cfu/g remained

relatively high, with only a gradual decline over six months, reaching 1.33×10^6 cfu/g by day 180. Similarly, Maize grain capsules also showed strong performance, starting with 18.90×10^6 cfu/g and maintaining good spore viability until the end of the study, with 1.24×10^6 cfu/g after six months.

In comparison with talc powder capsules showed good but slightly lower spore viability, with an initial cfu count of 17.60×10^6 cfu/g, which declined steadily to 1.05×10^6 cfu/g by 180 days. Charcoal and kaolin capsules also exhibited moderate stability, with final cfu counts of 1.17×10^6 cfu/g and 1.24×10^6 cfu/g, respectively, at the end of six months, showing their effectiveness in preserving spore viability, but not as effectively as sorghum and maize.

On the other hand, lignite capsules showed a significant loss of CFUs over time. The initial cfu count of 18.63×10^6 cfu/g decreased rapidly, with only 0.37×10^6 cfu/g remaining at the end of six months, indicating poor preservation ability.

Fly ash capsules showed the poorest performance overall, with an initial cfu count of 17.55×10^6 cfu/g, which declined drastically to just 0.27×10^6 cfu/g by day 180. The Control group, with no spores, showed no growth throughout the experimental period, confirming that the fungal spores did not survive in the absence of a carrier.

Among the carrier materials tested, sorghum grain capsules and Maize grain capsules were the most effective in preserving *Metarhizium anisopliae* spores at room temperature for six months. These carriers maintained relatively high CFU counts compared to others, including talc, charcoal and kaolin. In contrast, fly Ash and lignite capsules were the least effective, with significant reductions in spore viability. Therefore, sorghum and maize grains appear to be optimal choices for long-term storage of *Metarhizium anisopliae* in formulations.

Similar findings by Rana *et al.* (2011) [6] found that cereal-based carriers, including sorghum and maize, were effective in maintaining the viability of *Metarhizium anisopliae* during storage. Sorghum and maize grains are rich in nutrients that support fungal growth, making them suitable for long-term storage of fungal spores. Singh *et al.* (2014) [8] also reported that maize was one of the most effective carriers for *M. anisopliae*, as it provided a stable environment for fungal spores during prolonged storage periods. Kumar *et al.* (2017) also highlighted that fly ash was a poor carrier for entomopathogenic fungi, as it resulted in rapid desiccation and poor spore retention, mirroring the findings of the current study.

Table 1: Month wise CFU counts of capsule formulations

Carrier	Initial	Colony forming units (1×10^6 cfu/g) (Mean $\pm$ S.D.)					
	0(days)	30	60	90	120	150	180
Talc powder capsule	17.60 $\pm$ 0.77a	14.50 $\pm$ 0.50b	8.59 $\pm$ 0.55c	6.69 $\pm$ 0.42d	3.30 $\pm$ 0.28e	1.86 $\pm$ 0.26f	1.05 $\pm$ 0.01f
Charcoal capsule	18.60 $\pm$ 0.81a	14.73 $\pm$ 0.62b	9.19 $\pm$ 0.59c	7.59 $\pm$ 0.48d	3.34 $\pm$ 0.28e	2.13 $\pm$ 0.29f	1.17 $\pm$ 0.17g
Kaolin capsule	19.40 $\pm$ 0.84a	14.30 $\pm$ 0.50b	9.50 $\pm$ 0.62c	7.27 $\pm$ 0.45d	3.56 $\pm$ 0.30e	2.30 $\pm$ 0.32f	1.24 $\pm$ 0.01g
Lignite capsule	18.63 $\pm$ 0.87a	14.10 $\pm$ 0.49b	9.19 $\pm$ 0.59c	4.59 $\pm$ 0.28d	2.80 $\pm$ 0.23e	1.84 $\pm$ 0.25f	0.37 $\pm$ 0.00g
Sorghum grain capsule	19.09 $\pm$ 0.83a	14.60 $\pm$ 0.51b	10.19 $\pm$ 0.66c	7.79 $\pm$ 0.48d	4.19 $\pm$ 0.36e	2.96 $\pm$ 0.41f	1.33 $\pm$ 0.01g
Maize grain capsule	18.90 $\pm$ 0.82a	14.70 $\pm$ 0.51b	9.69 $\pm$ 0.63c	7.09 $\pm$ 0.44d	3.96 $\pm$ 0.34e	2.64 $\pm$ 0.37f	1.24 $\pm$ 0.17g
Fly Ash capsule	17.55 $\pm$ 0.77a	13.10 $\pm$ 0.46b	8.29 $\pm$ 0.54c	3.97 $\pm$ 0.24d	2.06 $\pm$ 0.17e	0.96 $\pm$ 0.13f	0.27 $\pm$ 0.00f
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00

*Average of three replication

* Means values shared by the same letter within a column are not significantly different at $P=0.05$ according to Duncan's test

Similarly, Pandey and Kanaujia (2004) [4] worked on dust formulations of the entomopathogenic fungus *Beauveria bassiana* by mixing different diluents, including bentonite, attapulgit, clay, talc, walnut shell powder, maize flour, soybean flour and sorghum flour. The formulations were evaluated for their ability to retain viability, measured in terms of cfu/gm, after storage for 1 to 5 months at temperatures of 0 °C, 5 °C, 10 °C and 15 °C. The study revealed that viability decreased with longer storage durations and higher storage temperatures.

Prasad and Rangeshwaran (2000) [5] conducted a study on three carrier materials talc, kaolin and bentonite to assess their impact on the shelf life of *Metarhizium anisopliae*. They concluded that kaolin and talc were the most effective carriers for *M. anisopliae*. Sarode *et al.* (1998) [7] conducted experiments to evaluate talc and other substrates as carriers for *Metarhizium anisopliae*. They concluded that talc powder could be safely stored at room temperature for up to 8 months without a noticeable decrease in CFU count.

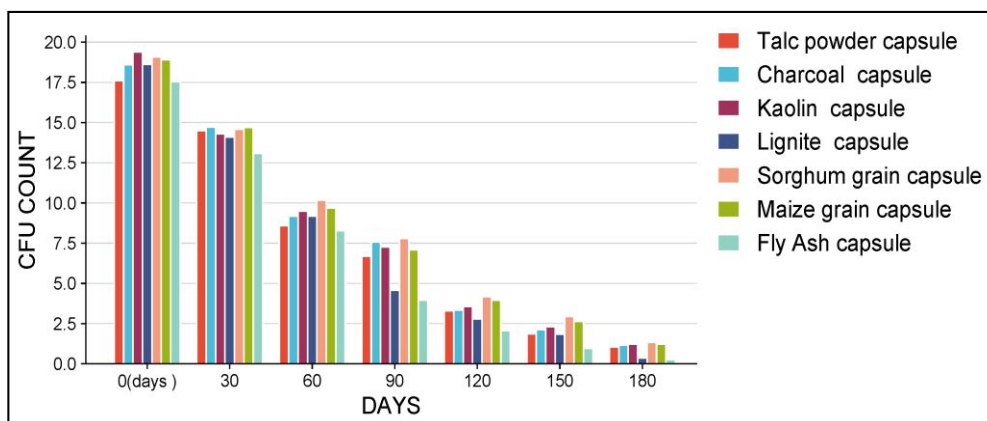


Fig 1: Shelf life of carrier formulations

Conclusions

The study on CFU counts of capsule formulations stored at room temperature for up to 180 days showed a decline in microbial viability across all formulations. In talc powder capsules dropped from 17.60×10^6 to 1.05×10^6 CFU/g, charcoal capsules from 18.60×10^6 to 1.17×10^6 CFU/g and in kaolin capsules from 19.40×10^6 to 1.24×10^6 CFU/g. Lignite capsules showed the steepest decline, from 18.63×10^6 to 0.37×10^6 CFU/g. Sorghum grain and maize grain capsules retained relatively higher counts, with values of 1.33 and 1.24×10^6 CFU/g, respectively, by day 180. Fly ash capsules experienced the greatest loss, dropping to 0.27×10^6 CFU/g. The control group showed no microbial growth.

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