



RESEARCH ARTICLE

Enhancing the viability of *Trichoderma harzianum* MF87546.1 through alginate-bentonite clay encapsulation: A physicochemical and biological study

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Abstract

The development of eco-friendly biocontrol solutions is critical to reducing reliance on synthetic fungicides, which contribute to environmental pollution, pathogen resistance and health hazards. This study investigates the encapsulation of *Trichoderma harzianum*, a beneficial fungal biocontrol agent, within alginate-bentonite (BNT) clay composite beads to enhance its stability and efficacy. Sodium alginate served as the primary matrix, while BNT clay was incorporated to improve mechanical strength and thermal resistance. The resulting microbeads exhibited uniform morphology, ensuring effective protection and sustained release of fungal conidia under varying environmental conditions. Structural and thermal properties were analysed using Fourier-transform infrared spectroscopy (FTIR), Scanning electron microscopy (SEM) and Thermogravimetric analysis (TGA), confirming the composite's durability and heat tolerance. Encapsulated conidia maintained high viability over six months, demonstrating the formulation's potential for long-term storage. The findings suggest that alginate-BNT encapsulation significantly improves the shelf life and field performance of microbial pesticides, offering a viable alternative to chemical treatments. By combining biopolymer science with clay-based enhancements, this research contributes to sustainable crop protection strategies. The proposed method aligns with green chemistry principles, providing a scalable solution for agricultural applications while minimising ecological impact.

Keywords: alginate-bentonite encapsulation; microbial pesticides; microencapsulation technology; sustainable agriculture; thermal stability; *Trichoderma harzianum*

Introduction

The devastating effects of plant diseases are readily visible in crops and the adverse impacts of synthetic chemicals used to manage these diseases are also increasingly evident. For many decades, synthetic chemicals have been used for controlling diseases and pests in modern agriculture, raising several issues such as pathogen resistance, biodiversity loss, environmental degradation and human health (1, 2). It has now become evident to preferentially employ other, safer methods of disease and pest control. Using biological control agents (BCAs) provides a more environmentally friendly approach to managing problems that synthetic chemicals fail to address, both *in vitro* and under open field conditions (3). *Trichoderma* is a notable antagonist and several of its species have been widely used in crop production and plant protection, because it produces a large number of hydrolytic enzymes: β -1,3-glucanase, chitinase and protease for the control of phytopathogens (4).

It is difficult for biocontrol agents to adapt to new environments because they are highly sensitive to high temperatures, moisture, salinity, synthetic chemicals and UV

radiation. Therefore, opting for an ideal manufacturing process and choosing a suitable carrier is the most important step to ensure the protection of active ingredients (spore and mycelium) from a challenging environment and maintain viability from manufacture until application at the target site (5, 6). To overcome these challenges and provide antagonists with a competitive advantage over phytopathogens and other mycoflora, improving the delivery and performance of biocontrol agents is critically needed. Biocontrol agents have been engineered to date into several types of formulations such as soluble powder (SP), wettable powder (WP), water-in-oil emulsions (EO), dispersible granules (WG), oil-in-water emulsions (EW) and encapsulated granules (CG) (7). Among potential reinforcing materials, bentonite (BNT) clay was selected in the present study due to its high swelling capacity, moisture retention ability and large surface area, which enhance mechanical stability and water-holding properties when combined with alginate matrices. Unlike inert carriers such as talc or kaolin that mainly act as fillers, BNT can improve bead integrity and create a protective microenvironment that reduces desiccation stress and supports sustained release of microbial

propagules.

Despite all the benefits, these formulations often fail under field conditions; therefore, an alternative method is needed to enhance their survival, population growth and performance in the field.

Utilising the microencapsulation technique is one approach that could be taken to overcome the challenges in biocontrol applications under greenhouse and field conditions (8, 9). Polymeric microparticle-based formulations of the *Trichoderma* fungus have attracted considerable interest from researchers, as they possess various beneficial properties upon encapsulation, such as biocompatibility and biodegradability, thereby enhancing viability, thermal resistance and degradability (10, 11). There are several biodegradable polymers, including starch and chitosan (12). Alginate facilitates the gradual release of nutrients into the soil through pore-assisted diffusion, as well as the erosion and degradation of capsules (13). For instance, employing alginate-based encapsulation of microorganisms can shield them from biotic and abiotic stresses while also extending their shelf life (1, 13). However, these biopolymer matrices often exhibit an unstable capsule structure or have a restricted loading capacity (12). Sodium alginate is a non-toxic, thermostable and biodegradable natural polymer that is widely utilised for encapsulating microbial cells, hormones, enzymes and herbal extracts (14).

The size, shape, biocompatibility and mechanical and chemical stability of alginate beads all affect their success (15). *Trichoderma* spores encapsulated in alginate have been the subject of numerous investigations. For example, a study used the dripping approach to successfully encapsulate *T. harzianum* conidia in alginate beads of different diameters (16).

Several methods are employed to develop *Trichoderma* formulations using fungal biomass though these bioproducts face challenges, including poor stability and viability, which can limit their ability to fully express their antagonistic potential against soil-borne plant pathogens (17, 18). Formulation must ensure the preservation and protection of the microorganism's spore viability (19). Therefore, microencapsulation is a method used to safeguard sensitive active ingredients by enclosing them within a matrix, thereby shielding them from the external environment (20). In this process, microorganisms are encapsulated to establish a controlled atmosphere that facilitates their release at specific times and locations, while also improving their survival during storage and simplifying delivery (12, 21). Despite these advances, few studies have explored integrating reinforcing clay materials into alginate-based encapsulation systems to improve physicochemical stability and biological performance, underscoring a clear need for optimised composite formulations.

Numerous studies have shown that microencapsulation enhances the efficient transport of nutrients, oxygen and metabolites, leading to sustained higher cell densities compared to commercial formulations (17, 22). Therefore, smaller beads (<1000 µm) are typically preferred over larger ones.

The microencapsulation process can be used to create these beads. In this context, the primary objective of this research was to develop an alginate-based encapsulation containing *T. harzianum* conidia. To assess conidia survivability using a statistical experimental design, microcapsules were created using

a sodium alginate matrix.

Materials and Methods

Preparation of Calcium-alginate-bentonite beads

A solution of BNT (2.5 g, Extra Pure, sourced from LOBA Chemie Pvt. Ltd.) was prepared by dissolving it in 250 mL of double-distilled water and stirring at 250 rpm for 24 hr. Subsequently, 7.5 g of alginate powder (purchased from Sigma-Aldrich) was added to the BNT solution and the mixture was stirred for an additional 4 hr. To this mixture, glycerol (7.5 mL) was added, forming an alginate-BNT clay solution. For comparison, alginate-only beads were prepared using the same protocol without BNT addition, while non-encapsulated fungal biomass served as a biocontrol agent. The solution was then carefully transferred into a pre-cooled, sterile 0.5 M calcium chloride (CaCl₂) aqueous solution (Sigma-Aldrich) while gently stirring. Using a syringe (5 mL), the mixture was dispensed to form beads. Calcium ions in CaCl₂ acted as a binding agent for alginate, resulting in the formation of calcium alginate-BNT clay beads. These water-insoluble beads were left to solidify at room temperature for 3 to 6 hr. Finally, the *Trichoderma* beads were thoroughly washed and rinsed multiple times with sterile water (1, 23).

Preparation of the *Trichoderma* culture

A *Trichoderma* strain was isolated from the surrounding soil of chickpea using the serial dilution technique (24). After isolation, the *Trichoderma* culture was transferred to potato dextrose agar (PDA) medium and incubated for 5 days. After full growth of *Trichoderma*, the conidial suspension was centrifuged at 10000 rpm for 15 min, after which the conidial pellet was rinsed with 0.85 % saline solution, following a standardised protocol (23).

Encapsulation of *Trichoderma* in Calcium-alginate-bentonite beads

A conidial suspension of *Trichoderma* [1.97×10^8 colony-forming units (CFU) mL⁻¹] was dissolved in a sodium alginate-BNT clay solution (250 mL) to prepare microcapsules. The BNT clay used in the study is a naturally occurring aluminosilicate predominantly composed of montmorillonite, characterised by a fine particle size (<75 µm) and a high cation-exchange capacity (CEC), which enhances water absorption and interactions with polymer matrices. These physicochemical properties facilitate improved gel strength and stability in alginate-based composites. The method for forming the microbeads followed a previously described protocol. The resulting beads were sieved to ensure uniform size and then rinsed with sterile water. To prepare them for storage, the beads were dried at 30 °C for 24 hr. Subsequently, they were kept at 4 °C in a sterilised beaker for future use (25) (Figs. 1 & 2).

Measurement of bead size

A total of five beads, consisting of calcium-alginate (Ca-alginate) with *Trichoderma* and Ca-alginate-BNT, were randomly selected from each replicate, making a total of twenty beads for analysis. The diameter of each bead was measured using a digital Vernier calliper. The sphericity factor (SF) of the beads was then determined using the following equation:

$$\text{Sphericity factor (SF)} = (d_{\text{max}} - d_{\text{min}}) / (d_{\text{max}} + d_{\text{min}}) \quad (26) \\ (\text{Eqn. 1})$$

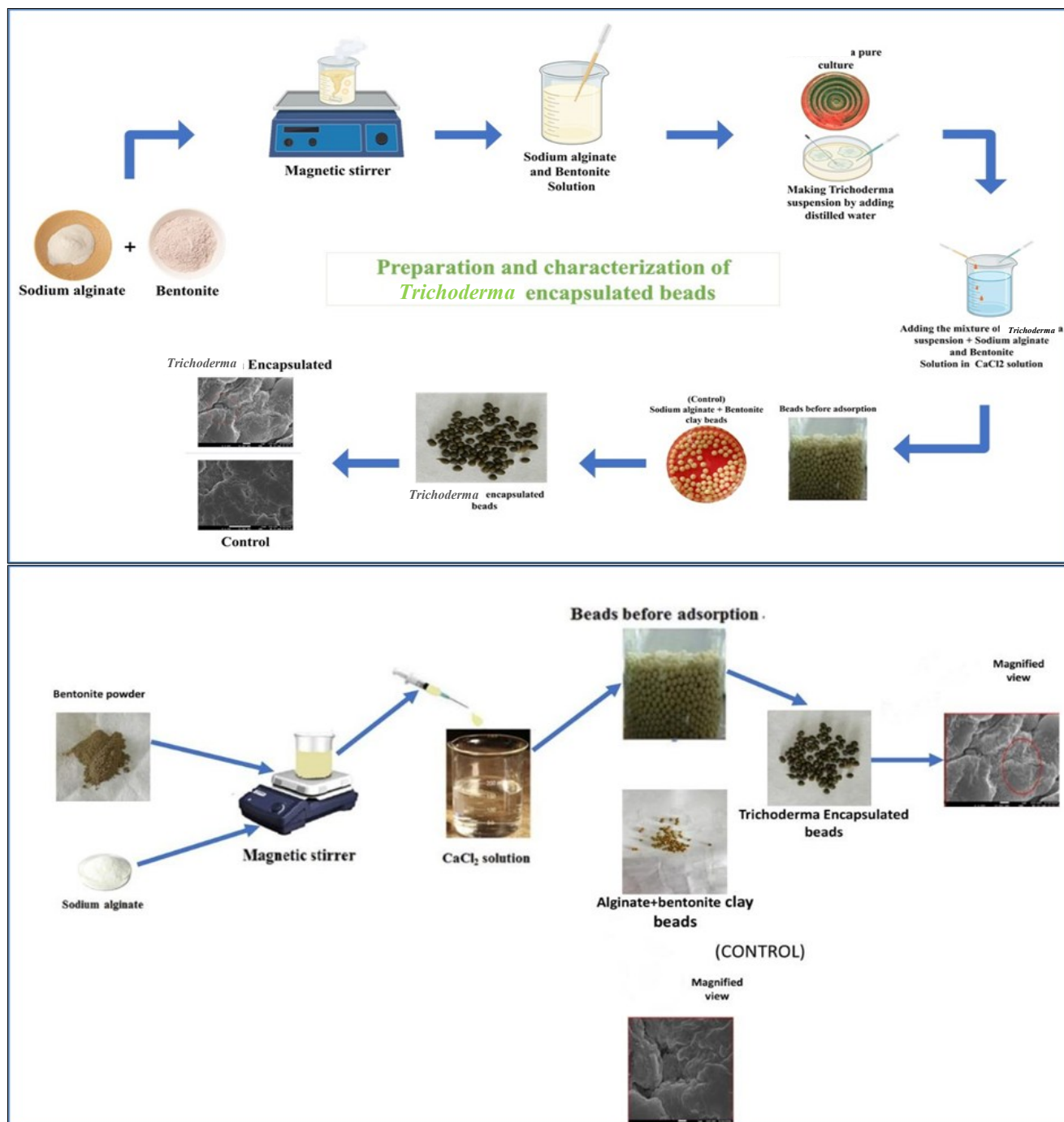


Fig. 1. Schematic representation of the formation of *Trichoderma* encapsulated beads.

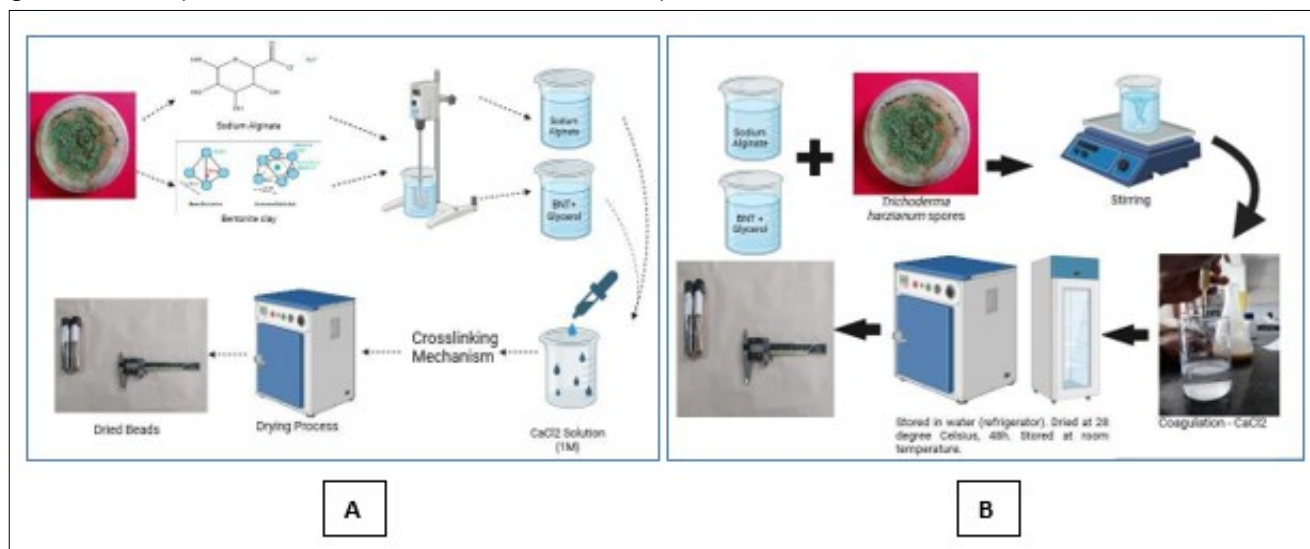


Fig. 2. Graphic representation of beads production (A) Control; (B) *Trichoderma harzianum* spores (2×10^8 cfu g^{-1}).

where,

d_{max} represents the largest diameter of the beads

dmin represents the smallest diameter

Fourier-transform infrared spectroscopy

Fourier-transform infrared spectroscopy (FTIR) was employed to assess the interactions between *Trichoderma* and the microparticles. Spectra were recorded using a PerkinElmer Spectrum Two equipped with an attenuated total reflectance (ATR) accessory. Dried bead samples were finely ground and directly placed on the ATR crystals without additional chemical treatment. The spectra were recorded between 4000 and 400 cm^{-1} . Each sample was subjected to 32 scans with a resolution of 8 cm^{-1} . The analysis focused on identifying shifts in hydroxyl (-OH), carboxylate (-COO) and silicate-related bands to evaluate potential interactions between alginate functional groups, BNT clay and fungal biomolecules within the encapsulated matrix.

Assessment of the viability of encapsulated cells

The spread plate technique was used to evaluate the viability of both free spores and encapsulated *Trichoderma* microcapsules. Colony-forming units per gram of encapsulated beads were quantified using a digital colony counter and denoted as log CFU g⁻¹. The experiment was repeated 3 times and the average CFU g⁻¹ of *T. harzianum* was calculated. Measurements were performed monthly over a six-month storage period (27).

Thermogravimetric analysis of encapsulated beads

Thermogravimetric analysis (TGA) was conducted using a TA instrument to evaluate the thermal stability of the samples. Additionally, the surface morphology of the samples was examined using scanning electron microscopy (SEM). Each characterisation analysis was performed in quadruplicate to ensure reliability.

Experimental design and treatment structure

The study was conducted under a completely randomised design (CRD) with treatments including Ca-alginate beads and Ca-alginate-BNT composite beads containing *T. harzianum*. Storage temperatures (4 °C and 26 °C) were considered an additional factor for comparison in the viability assessment. All treatments were

prepared in triplicate and measurements were recorded as mean $\pm$ standard deviation. Statistical comparison among treatments was performed using analysis of variance (ANOVA), followed by mean separation where applicable.

Results

Drying and measurement of encapsulated beads

In the current research, the mean bead size was calculated using the freeze-drying and airflow-drying methods. The encapsulated beads were dried for 24 hr at 28 °C. *Trichoderma harzianum* (MF87546.1) encapsulated beads had an average diameter of 2.4 ± 0.50 mm, a mean weight of 5.40 ± 0.45 mg and a moisture content of 5.35 ± 2.2 %. All measurements were performed in triplicate ($n = 3$) and data were expressed as mean $\pm$ standard deviation. Statistical differences among treatments were analysed using one-way ANOVA and significance was determined at $p < 0.05$. Their shape was assessed using the SF, where a value of zero indicates a perfect sphere, while higher values approach unity as the shape becomes more elongated. Another study investigated the mean size of Ca-alginate beads containing *T. asperellum*, at 2.5 and 1.1 mm, which are larger than the size reported in the present research (28). This might be due to various factors, such as the chemical viscosity of the alginate, the concentration of calcium chloride, etc (9, 28).

Fourier-transform infrared spectroscopy analysis

Fourier transform infrared spectroscopy (FTIR) was used to identify the functional groups present in Ca-alginate beads, BNT clay and Ca-alginate-BNT *Trichoderma* beads, as illustrated in Fig. 3. A broad peak was detected at about 3339 cm^{-1} (1.42 % transmittance), corresponding to the O-H stretching vibration, which is characteristic of water or hydroxyl groups. A similar peak at 3350 cm^{-1} (1.47 % transmittance) was also noted, indicating the presence of O-H stretching. Additional peaks at 3693 cm^{-1} (3.85 % transmittance) and 3603 cm^{-1} (7.79 % transmittance) were observed, which are slightly higher in intensity, suggesting reduced hydrogen bonding in these samples compared to the others. A peak at 2107 cm^{-1} (96.09 % transmittance) in the *Trichoderma* sample indicated the presence of a $\text{C}\equiv\text{C}$ or $\text{C}\equiv\text{N}$ triple bond stretching vibration, which was absent in

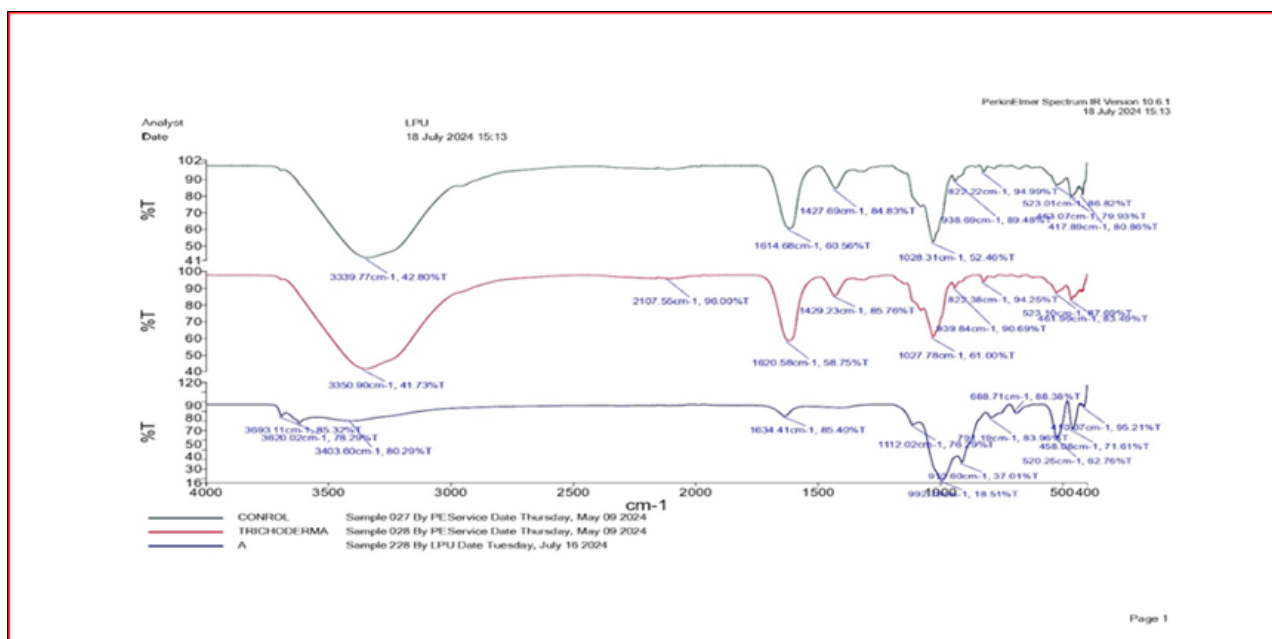


Fig. 3. FTIR analysis of beads (A) Bentonite clay; (B) *Trichoderma*; (C) Control.

both the control and BNT clay samples. The broad peaks between 3400 and 3600 cm^{-1} observed in all three samples suggest the presence of hydroxyl groups, possibly due to water or alcohol functional groups. Variations in peak position and intensity across the samples may reflect differences in hydrogen bonding environments. The distinct peak at 2107 cm^{-1} in the *Trichoderma* sample indicates the presence of triple bonds, likely arising from nitriles or alkynes, which are absent in the control or BNT clay. The peaks and functional groups detected in the FTIR spectrum are closely associated with the chemical structure of the cell wall of this fungus and these findings support those reported previously (29,30).

Thermogravimetric analysis

Thermogravimetric analysis was conducted to evaluate the thermal behaviour of both *Trichoderma*-encapsulated beads and the control, as illustrated in Fig. 4. The thermal decomposition of polysaccharides typically occurs in multiple stages. It begins with the removal of structural water (dehydration) and the release of physically absorbed moisture. This is followed by depolymerisation, which involves the cleavage of C–O and C–C bonds within the polymeric units. As a result, gases such as carbon monoxide (CO), carbon dioxide (CO₂) and water (H₂O) are formed, along with the development of polynuclear aromatic and graphitic carbon structures. Based on the mass-loss profile, the three major degradation phases can be distinguished. The first phase (room temperature to 100 °C) corresponds to evaporation of surface-bound and absorbed moisture. The second phase (250 – 350 °C) is depolymerisation of the alginate backbone through cleavage of C–O and C–C bonds, which is typically associated with the main derivative thermogravimetry (DTG) peak reported for alginate-based systems. The third phase (350 – 450 °C) reflects decomposition of organic residues, including fungal biomass and remaining encapsulation components. In the present

study, the initial decomposition temperature of sodium alginate beads was 246 °C, whereas that of Ca-alginate-BNT beads was slightly higher at 264 °C. The final decomposition temperatures for alginate and Ca-alginate-BNT beads were 542 °C and 669 °C, respectively, with corresponding residues of 31.8 % and 35.9 %. These results suggest that Ca-alginate-BNT beads exhibit superior thermal stability compared to the alginate beads.

Scanning Electron Microscopy analysis

Scanning electron microscopy was used to analyse the cross-sectional view of encapsulated beads of *T. harzianum*, which showed the presence of *T. harzianum* spores. The images also demonstrated that Ca-alginate beads possess a homogeneous, interpenetrating alginate network, whereas in Ca-alginate BNT, BNT clay particles were uniformly distributed within the alginate matrix, as illustrated in Fig. 5. Encapsulated conidia of *Trichoderma* are crucial for drying and storage. This analysis also showed that there are no cracks on the bead's surface, suggesting that the bead's compact, smooth surface was formed through a strong interaction between alginate and BNT clay particles. The compact, smooth surface, free of pores and cracks, is crucial for protecting the active ingredient of *Trichoderma* (conidia) and enabling longer storage.

The results in Table 1 clearly illustrate that cold storage (–4 °C) effectively maintained higher microbial viability over six months, with CFU counts gradually declining from 9.14×10^8 to 4.11×10^8 . In contrast, at room temperature ($26 \pm 2^\circ\text{C}$), CFU counts declined much faster, dropping significantly from 8.80×10^8 to 0.50×10^8 by the sixth month. Notably, significant differences in viability were observed after the fourth month, with room-temperature storage leading to a dramatic reduction in microbial counts. By the fifth month, room-temperature storage rendered the microorganisms almost nonviable, with a CFU count of just 0.95×10^8 . The enhanced preservation under cold conditions is

Table 1. Assessment of bead viability

Storage period (in months)	Colony count in different storage conditions (CFU $\text{g}^{-1} \times 10^8$)	
	Cold storage (–4 °C)	Room temperature ($26 \pm 2^\circ\text{C}$)
1	9.14 ± 0.11^f	8.80 ± 0.005^f
2	7.85 ± 0.03^e	7.29 ± 0.020^e
3	6.33 ± 0.05^d	6.09 ± 0.090^d
4	5.84 ± 0.07^c	3.02 ± 0.422^c
5	4.81 ± 0.57^b	0.95 ± 0.002^b (no viability)
6	4.11 ± 0.55^a	0.50 ± 0.006^a (no viability)

Values are expressed as mean $\pm$ standard deviation ($n = 3$). Different superscript letters within a column indicate significant differences at $p < 0.05$.

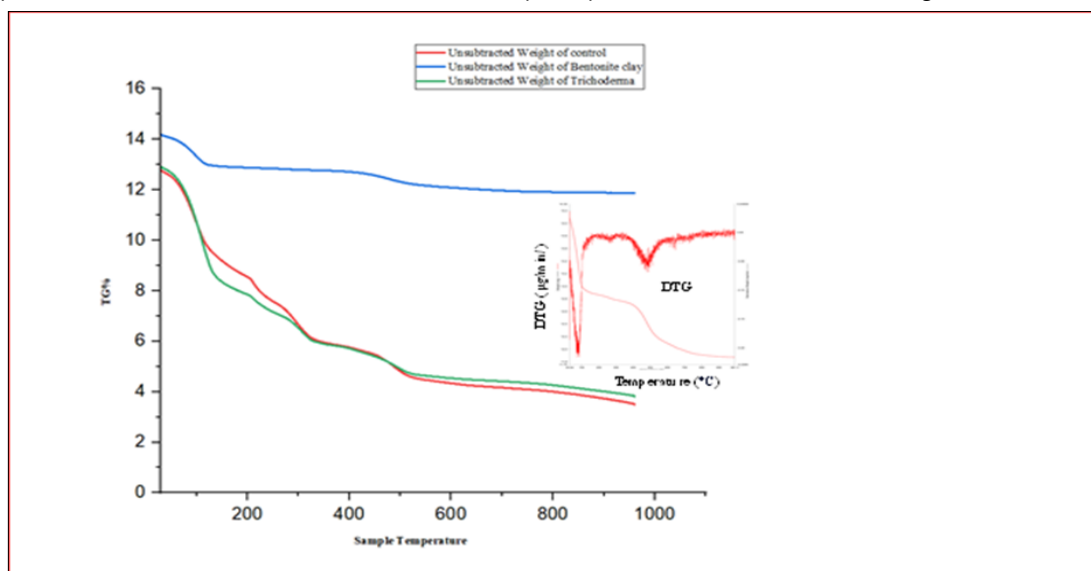


Fig. 4. TGA and DTG thermogram of Ca-alginate, Bentonite clay and Ca-alginate-bentonite, *Trichoderma* beads.

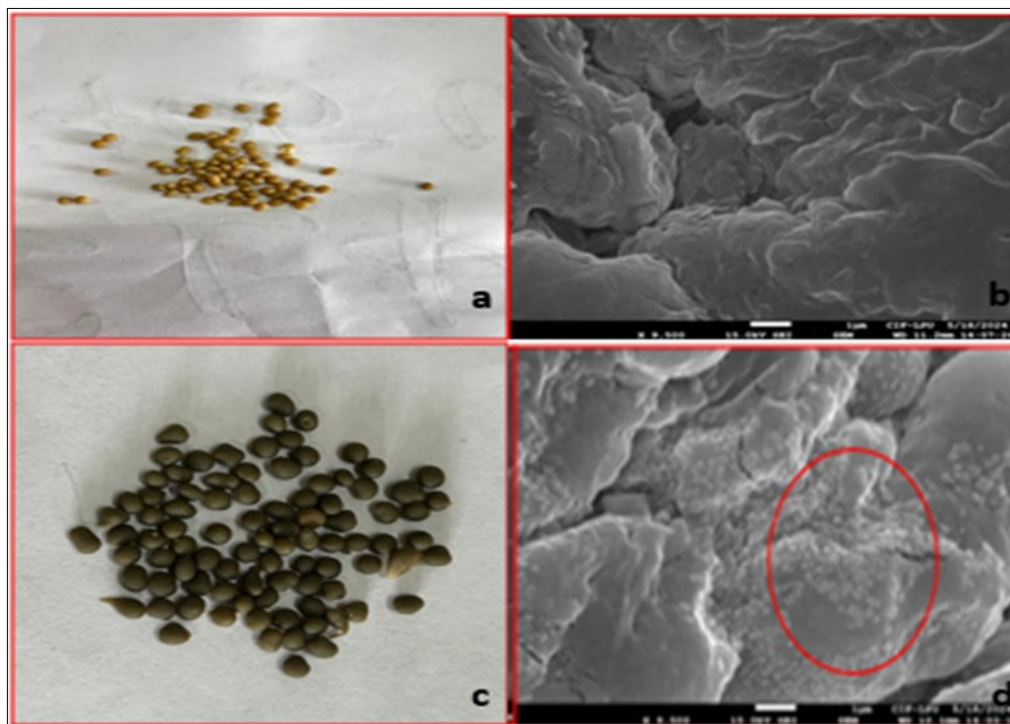


Fig. 5. Observation of sodium alginate bentonite clay encapsulated *Trichoderma* using SEM (A) Control; (B) Scanning view of control; (C) Encapsulated *Trichoderma*; (D) Scanning view of *Trichoderma* beads.

consistent with the structural and physicochemical characteristics observed by FTIR, TGA and SEM. FTIR indicates interaction between the alginate and BNT functional groups, TGA demonstrates improved thermal stability of the composite beads and SEM reveals a compact, crack-free morphology, all of which likely contribute to reduced desiccation stress and prolonged microbial survival during storage.

Discussion

These findings align with previous studies demonstrating the beneficial impact of Ca^{2+} ions on sporulation rates (29). A further advantage of encapsulating *Trichoderma* in alginate is that it does not negatively impact the hydrolytic enzymatic activities involved in biocontrol mechanisms. For example, encapsulation of *T. harzianum* in alginate beads was reported to improve photostability against UV radiation, while chitinase and cellulase activities were either preserved or enhanced following encapsulation (1). The encapsulated spores also exhibited strong *in vitro* antagonistic potential against *Sclerotinia sclerotiorum* (31). Another study investigated the effects of various sodium alginate blends with other polymers during the process of ionic gelation encapsulation of *Trichoderma* (13). The results of the present study emphasised the interactions between the alginate matrix and various polymers, with a combination of alginate and starch exhibiting the highest conidial viability during drying. The formulation with the highest spore viability (2.4×10^6 CFU g^{-1}) after 10 months of storage at 28 ± 2 °C contained 2 % sodium polyphosphate and 2 % glycerol. By submerging alginate capsules in a 2 % sodium citrate solution, the encapsulated conidia could be released. These results confirmed that when *Trichoderma* spores are enclosed in alginate beads, they retain exceptional vitality throughout storage. It also highlights the fact that encapsulation method, particularly under cold storage, is more effective than room-temperature storage in preserving microbial viability. Other studies confirmed that spores of the fungus encapsulated in

calcium alginate enhanced survival (32, 33).

In addition, the beads are an ideal option for developing biocontrol formulations because they are inexpensive to produce, eco-friendly, non-hazardous and capable of providing enhanced sporulation rates, protection from UV light and increased biocontrol enzymatic activity. Clays have long been used as microbial inoculants and are widely incorporated into biological formulations, including dust, WP, granules and tablets (34). They are excellent desiccants, providing ideal storage conditions for dried inoculants due to their large surface area, pore-size distribution and overall porosity. Additionally, clays help regulate moisture levels, ensuring optimal hydration of microbial formulations (35). Moreover, clays can absorb or disperse suspending agents, enhancing the survival of rhizobia in the soil for up to 60 days (34). This effect is attributed to the formation of protective microhabitats that keep bacteria accessible while shielding them from predators (36).

Fourier transform infrared spectroscopy was used to identify the functional groups present in Ca-alginate beads, BNT clay and Ca-alginate-BNT *Trichoderma* beads, as illustrated in Fig. 3. Variations in peak positions and intensities across the samples may reflect differences in hydrogen-bonding environments. The distinct peak at 2107 cm^{-1} in the *Trichoderma* sample indicates the presence of triple bonds, likely from nitriles or alkynes, which are absent in the control or BNT clay. The peaks and functional groups detected in the FTIR spectrum are closely associated with the chemical structure of the cell wall of this fungus and these findings support those reported by previous research (29, 30).

The TGA curve of *Trichoderma*-encapsulated beads (Fig. 4) was obtained under a nitrogen atmosphere at a heating rate of $10^\circ\text{C min}^{-1}$, covering a temperature range from room temperature to 600°C . An initial mass loss of approximately 5 % was observed between room temperature and 100°C , which was attributed to the evaporation of absorbed moisture. From 100°C to 250°C , the bead

mass remained relatively stable, indicating good thermal stability within this temperature range. Although degradation at higher temperatures (250–450 °C) reflects polymer decomposition behavior, the stability observed below 100–150 °C is more relevant to practical agricultural handling, storage and transport conditions, where moderately elevated but not extreme temperatures are typically encountered. This is likely due to the degradation of the polymer matrix. A second decomposition phase at 400 °C resulted in an additional 30 % mass loss, attributable to the breakdown of *Trichoderma* spores and residual encapsulation materials. By 600 °C, approximately 45 % of the original mass remained, indicating the presence of inorganic materials. The initial mass loss of 5 % corresponds to the moisture content of the encapsulated beads, which is consistent with moisture levels observed in similar encapsulation systems (3–7 %). The stable temperature range between 100 °C and 250 °C indicates that the encapsulation matrix effectively protects *Trichoderma* spores at moderate temperatures, suggesting that the beads are well-suited for applications requiring thermal resistance. The decomposition events at 300 °C and 400 °C highlight the thermal limits of both the encapsulation materials and the spores, with temperatures that align with those reported for other polymer-based systems. The final residue of 45 % indicates a significant amount of inorganic material, which contributes to the thermal stability of the beads. However, this high residue level may impact the biodegradability and environmental implications of the beads. Overall, TGA analysis demonstrates that *Trichoderma*-encapsulated beads exhibit good thermal stability up to 250 °C, with staged decomposition observed up to 450 °C. Scanning electron microscopy was used to analyse the cross-sectional view of encapsulated *T. harzianum* beads, revealing the presence of *T. harzianum* spores. The images also demonstrated that Ca-alginate beads possess a homogeneous, interpenetrating alginate network, while in Ca-alginate BNT, BNT clay particles were uniformly distributed within the alginate matrix, as illustrated in Fig. 5. The analysis also revealed the absence of surface cracks in the beads, suggesting the formation of a compact and smooth structure due to strong interactions between alginate and BNT clay particles. Such a dense, crack-free, and non-porous surface is crucial for protecting the active ingredient (*Trichoderma* conidia) and enhancing storage stability. The findings from the present study align with previous studies (28, 29). These results suggest their potential for use in applications involving moderate heat exposure, although further optimisation may be required to enhance biodegradability.

Conclusion

The results indicate that Ca-alginate-BNT serves as an effective matrix for encapsulating *Trichoderma harzianum* and other BCAs. Scanning electron microscopy images revealed distinct differences in surface characteristics between the control and *Trichoderma*-loaded Ca-alginate-BNT beads. These observations further support the presence of strong interactions between the functional groups of BNT clay and alginate, as also indicated by FTIR analysis. Thermal analysis also confirmed that the uniform distribution of BNT clay particles within the alginate matrix produced a more rigid and stable bead structure. These findings pave the way for future research on the formulation and application of BCAs using Ca-alginate-BNT matrices.

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Authors' contributions

VK and SR contributed to the conceptualisation and to writing the original draft. AK and DM contributed to the preparation of the figures and to editing the manuscript. KA contributed to reviewing and editing the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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