

Original Research Article

Encapsulation of native strains of bioagents *Trichoderma asperellum* and *Pseudomonas putida* using different biopolymers

Abstract

Microencapsulation of biological control agents in biopolymer matrices is a valuable alternative to produce formulations with extended shelf-life. In this work, *Trichoderma asperellum* TAIK 1 conidia and *Pseudomonas putida* PIK1 bacterial cells were microencapsulated using different wall materials viz., maltodextrin, sodium alginate, carboxy methyl cellulose (CMC), gum arabic, and a cross linking agent gelatin with a hardening material calcium chloride. The encapsulation methods extrusion, emulsification and spray drying was performed using these wall materials. Spray drying process resulted in compact and smooth surface capsule with each individual cell in comparison to the other two methods, confirmed using scanning electron microscope (SEM). Further the viability of organisms were checked, among these the combination maltodextrin (2%) + gelatin (1.5%) + CaCl₂ (2%) resulted in 70% viability. And also effect of spray-drying inlet and outlet air temperatures were standardized for TAIK 1 and PIK1 spores. Highest conidial survival was shown in inlet and outlet temperature, feed rate/h (130⁰C, 60⁰C, feed rate 60ml/h for TAIK1) and (120⁰C, 50⁰C, feed rate 60ml/h for PIK1). Therefore, our study obtained the optimal process for encapsulation of bioagents TAIK1 and PIK1. Thus it can be used as sustainable tool for eco-friendly bio-control agents release.

Key words: *Trichoderma*, *Pseudomonas*, Encapsulation, Spray drying, Scanning electron microscope

Introduction

The successful implementation of biological control relies on two key factors viz., careful selection of suitable biological control agents (BCAs) and the effective delivery of these agents in their most functionally active form at the target site (Bressan, 2003; Copping and Menn, 2000). One innovative technology in modern formulations has opened new possibilities for developing eco-friendly and

effective biopesticides to control plant diseases is microencapsulation (Shishir et al., 2018). By encapsulating antagonistic microbes and their metabolites, their shelf life, viability, and biocontrol efficiency can be enhanced as they are protected from adverse environmental conditions (Chi et al., 2020). Microencapsulation is widely used in the food and pharmaceutical industries, there is limited information available on its application in bioagents. Therefore, significant advancements in cost-effective formulations, handling, transport, and timely delivery are needed to unlock the full potential of these beneficial microbes in disease management and enable the commercialization of products that are user-friendly for farmers.

Encapsulation refers to the process of enclosing active agents within a carrier material to create particles or capsules at a micro or nanoscale level. The active agents, also known as the core or internal phase, are coated with a wall material, membrane, or shell, called the carrier material or external phase (Devi, Sarmah, Khatun, & Maji, 2017). Encapsulation technology finds wide application in the food and pharmaceutical industries for the protection and delivery of bioactive compounds such as polyphenols, micronutrients, enzymes, and antioxidants. By creating protective barriers against factors like light, oxygen, pH, moisture, heat, and shear, encapsulation enhances the bioavailability, controlled release, and targeted delivery of these bioactive compounds. Additionally, encapsulation helps mask undesirable flavors, form solid particles, reduce evaporation or volatility loss, increase reactivity barriers, and improve the physical stability, biological activity, and shelf life of the encapsulated compounds (Aguiar et al., 2016; Jia et al., 2016; de Souza Simões et al., 2017).

The encapsulation process involves the production of microcapsules (1–1000 μm), submicron capsules (several hundred nanometers to less than 1 μm), and nanocapsules (1 to several hundred nanometers) using encapsulation techniques (Lević et al., 2015; O'Toole et al., 2012; Rodríguez et al., 2016). Moreover, microcapsules offer advantages in terms of enhanced bioavailability and sustained release of drugs, enabling precise targeting of active compounds (Yang et al., 2017; Yao, Chang, Ahmad, & Li, 2016; de Souza Simões et al., 2017). The objective of the present study is to standardize and develop a microencapsulated formulation of promising bioagents.

Materials and Methods

Bioagents

Native bioagents (BCAs)- *Trichoderma asperellum* strain *TAIK1* and *Pseudomonas putida* strain *PIK1* (Kannan et al., 2022) were collected from culture collections of the Department of Plant Pathology, ICAR- IIRR, Hyderabad, Telangana, India.

Development of micro-encapsulated formulation of potential bioagents

Core materials used in the study are TAIK 1 conidia and PIK 1 cells. The coating/wall materials used are maltodextrin, Carboxy methyl cellulose, Gaur gum, Sodium alginate, gelatin and hardening agents CaCl_2 . Spore suspensions were prepared with cfu/ml of 1.86×10^8 for PIK1 and 2.26×10^7 for TAIK1 maintaining $\text{OD}_{600\text{nm}} \sim 1.0$.

Extrusion

Extrusion process was done by mixing the microorganism with hydrocolloids followed by extrusion into a hardening solution. The size and shape of the beads could vary based on the distance of free-fall and the size of needle pore. The bioagents suspension was then mixed with wall materials and gelatin. Once prepared, the mixture was extruded by applying continuous pressure using a syringe (60 ml) equipped with a fine needle (0.2 mm diameter) into a CaCl_2 solution under continuous agitation. The syringe was maintained at a constant distance (5 ± 1 cm) to the surface of the CaCl_2 solution to create spherical beads. Alginate beads were maintained in the CaCl_2 solution for at least 1 h. After that, beads were sieved and washed several times with sterile distilled water to remove the excess of CaCl_2 . The combinations of wall materials, gelatin and CaCl_2 combinations were listed in (Table 1).

Emulsification method

Different concentration of wall materials was prepared and kept for continuous stirring in separate beakers. One ml of bioagents suspension was added after 1 h of stirring. Then, 1ml of gelatin was added. It was kept for continuous stirring for 5 h. Calcium chloride solution was prepared and incubated for 1 hour at 4°C . After 5 h, one ml of calcium chloride was added and the change in transparency was observed. The addition of calcium chloride was up to the point where transparency was obtained in the solution. The maximum limit of CaCl_2 used was 15ml. The solution was kept for continuous stirring overnight and product formation was investigated for each alginate concentration. The combinations of wall materials with gelatin and CaCl_2 were given below (Table 1).

Table 1: Combination of wall materials with gelatin and CaCl_2

Wall material	Cross linking agent	Hardening agent
Maltodextrin (1%)	Gelatin (1%)	CaCl_2 (1%)

Maltodextrin (1%)	Gelatin (1.5%)	CaCl ₂ (1%)
Maltodextrin (1%)	Gelatin (1%)	CaCl ₂ (2%)
Maltodextrin (2%)	Gelatin (1.5%)	CaCl ₂ (2%)
Maltodextrin (3%)	Gelatin (1.5%)	CaCl ₂ (2%)
Carboxy methyl cellulose (CMC) (1%)	Gelatin (1%)	CaCl ₂ (1%)
Carboxy methyl cellulose (CMC) (1%)	Gelatin (1.5%)	CaCl ₂ (1%)
Carboxy methyl cellulose (CMC) (1%)	Gelatin (1%)	CaCl ₂ (2%)
Carboxy methyl cellulose (CMC) (2%)	Gelatin (1.5%)	CaCl ₂ (2%)
Carboxy methyl cellulose (CMC) (3%)	Gelatin (1.5%)	CaCl ₂ (2%)
Sodium alginate (1%)	Gelatin (1%)	CaCl ₂ (1%)
Sodium alginate (1%)	Gelatin (1.5%)	CaCl ₂ (1%)
Sodium alginate (1%)	Gelatin (1%)	CaCl ₂ (2%)
Sodium alginate (2%)	Gelatin (1.5%)	CaCl ₂ (2%)
Sodium alginate (3%)	Gelatin (1.5%)	CaCl ₂ (2%)
Gum Arabic (1%)	Gelatin (1%)	CaCl ₂ (1%)
Gum Arabic (1%)	Gelatin (1.5%)	CaCl ₂ (1%)
Gum Arabic (1%)	Gelatin (1%)	CaCl ₂ (2%)

Gum Arabic (2%)	Gelatin (1.5%)	CaCl ₂ (2%)
Gum Arabic (3%)	Gelatin (1.5%)	CaCl ₂ (2%)

Spray Drying

From the above methods maltodextrin, gelatin and CaCl₂ combination was further used for spray drying method. One ml of spore suspension was added to maltodextrin (2%) and gelatin (1.5%), further continuously agitated. After uniform dispersion of all the components it was fed to the spray dryer (Spraymate, JISL pvt ltd). The basic principle of spray drying is atomization of liquid feed into the drying chamber in the form of tiny droplets containing bioactive compounds, supply of hot air into the drying chamber, formation of microcapsules in the drying chamber, and separation of microcapsules was done. The combinations of wall materials with inlet and outlet temperatures were listed below:

Table 2: Combinations of inlet and outlet temperatures for spray drying encapsulation for TAIK1 and PIK1 spores

Bioagents	Wall material	Feed flow rate (ml/h)	Inlet temperature	Outlet temperature
TAIK1	Maltodextrin (2%) + Gelatin (1.5%)	60	130 ⁰ C	60 ⁰ C
TAIK1	Maltodextrin (2%) + Gelatin (1.5%)	60	130 ⁰ C	55 ⁰ C
TAIK1	Maltodextrin (2%) + Gelatin (1.5%)	60	150 ⁰ C	60 ⁰ C
TAIK1	Maltodextrin (2%) + Gelatin (1.5%)	60	150 ⁰ C	55 ⁰ C
PIK1	Maltodextrin (2%) + Gelatin (1.5%)	60	120 ⁰ C	50 ⁰ C
PIK1	Maltodextrin (2%) +	60	120 ⁰ C	45 ⁰ C

	Gelatin (1.5%)			
PIK1	Maltodextrin (2%) + Gelatin (1.5%)	60	100 ⁰ C	50 ⁰ C
PIK1	Maltodextrin (2%) + Gelatin (1.5%)	60	100 ⁰ C	45 ⁰ C

Viability of encapsulated Cells

Enumeration of the free and microencapsulated TAIK1 and PIK1 was determined by the spread plate method. Serial dilutions (10^{-1} - 10^{-9}) of different products were made in 0.85% (w/v) sterile saline (NaCl) solution. Afterwards, 0.1 mL of each dilution was spread onto PDA and King's B plates. Colony forming units (CFU) were calculated after incubation at 25°C for 24 h. The survival rate of TAIK1 and PIK1 were calculated by dividing the surviving population with the initial population:

$$\text{Survival rate (\%)} = (N/N_0) \times 100$$

where N_0 is the number of TAIK1 and PIK1 cells per gram of dry matter before drying, and N is the number of TAIK1 and PIK1 cells per gram of dry matter in the capsules after drying. All of the tests were performed in triplicate.

SEM analysis

The samples adhered onto the aluminium studs mounted with double stick carbon adhesive, later coated with gold-palladium in an automated sputter coater (Leica Ultra Microtome EM UC7) and examined under a scanning electron microscope (Apreo LoVac) as per the standardized protocols at Central Instrumentation Lab, BITS pilani and Hyderabad Central University, Hyderabad, Telangana, India.

Results and Discussion

Determination of encapsulation process

Upon different combinations of wall materials with different concentrations among the bioagents used. Extrusion process resulted in formation of microcapsule with improper release of bioagents. Emulsification process resulted in compact capsule and also dissolved into culture media, allowed to release bioagents. Spray dryer method used for bioagents encapsulation resulted in dry powder formulation with individual cells encapsulated, further easily dissolved with moisture and releases bioagents into the respective media, retains its antagonistic activity. Spray drying is the most extensively implemented technique for the microencapsulation of bioactive compounds rather than extrusion and emulsification (Shishir et al 2018). Extrusion is a very old and simple method of

making capsules with hydrocolloids (King, 1995). Our results are in line with those reported by Rodríguez-Huezo et al. (2007).

Determination of optimum wall materials for microencapsulation

The effect of different wall materials on TAIK1 and PIK 1 viability after encapsulation process was investigated (Fig. 1). The results showed that cells of TAIK1 hardly survived when no wall material was used after spray drying. The viable counts remarkably increased upon using any kind of wall material, which suggested that wall material plays an important role in the microencapsulation process. The survival rates of TAIK1 $71.32 \pm 1.47\%$ and PIK1 $70.34 \pm 0.12\%$ were obtained, when maltodextrin were dissolved into culture media, respectively. Different ratios of maltodextrin/gelatin (w/w) were used to investigate the particle size and survival rate of TAIK1 and PIK 1. In this study, the size of a non-capsulated spore size of TAIK1 and PIK1 was over $4 \mu\text{m}$ and $5 \mu\text{m}$ respectively.

According to the above results, maltodextrin (2%) + gelatin (1.5%) + CaCl_2 (2%) is considered to be the best combination wall material for the microencapsulation process by emulsification and spray drying, since most of the core materials were embedded and the highest survival rate was obtained. A proper combination of wall materials can compensate for the defects of a single wall material, thereby improving the product quality and application range of the microcapsules (Tupuna et al. 2017). Furthermore, as the maltodextrin fraction in the wall materials increased from 2.0 to 3%, the particle size diminished, which could be due to the reduction in viscosity of the feeding solution (Fernandes et al., 2014). Liu and Liu (2009a) reported that the encapsulation of *M. anisopliae* in an alginate–clay matrix, with dextrin and hydroxypropyl methyl cellulose as additives, yielded in 80% of germination after six months of storage.

Determination of optimum spray drying conditions for microencapsulation

Effects of inlet/outlet temperature and flow rate on survival rate, powder recovery, as well as moisture content were investigated. As shown in Table 2, the survival rate of TAIK1 and PIK1 spores decreased when the inlet temperature was 150.0°C . The relatively higher maximum survival rates of $81.6 \pm 0.37\%$, $82.3 \pm 0.61\%$ were obtained under treatment (130.0°C , 60 mL h^{-1}) for TAIK1 and for PIK1 (120.0°C , 60 mL h^{-1}) respectively. Results showed that survival rate at the inlet temperature of 130.0°C appeared to be favourable as compared to those at 150.0°C , and 100.0°C . This is mainly because of the rapid drying at higher inlet temperatures, which resulted in a significant decrease in water activities, leading to the death of bacteria (Berninger et al., 2018). According to Jin and Custis (2011), increasing inlet/outlet temperature caused the death of conidia of *Trichoderma harzianum*; this also derived from the water loss. Lower temperatures resulted in higher moisture content, which is a critical factor in the shelf life of biological agents because water may influence dissolution of wall

materials, which could provide protection for bioagents against the external environment (Rouissi et al. 2013).

SEM

The SEM analysis resulted in the structure and size of capsules having 200 μm and 300 μm in emulsification method for TAIK1 and PIK1 cells, in case spray drying the size of capsules was 8 μm and 7 μm for TAIK1 and PIK1 cells. (Fig1,2) The microcapsules were heterogeneous spheres with a smooth surface and a few dents. No free cells and no obvious cracks were observed on the surface of microcapsules, which indicated that the dense surface was shaped by strong complex formations by the combination of gelatin and maltodextrin. A smooth surface with no cracks and pores is vital in protecting the core material and prolonging storage time (Aghbashlo et al., 2013).

Fig 1: SEM image of encapsulated TAIK1 spores [Maltodextrin (2%) + gelatin (1.5%) + CaCl₂ (2%)]

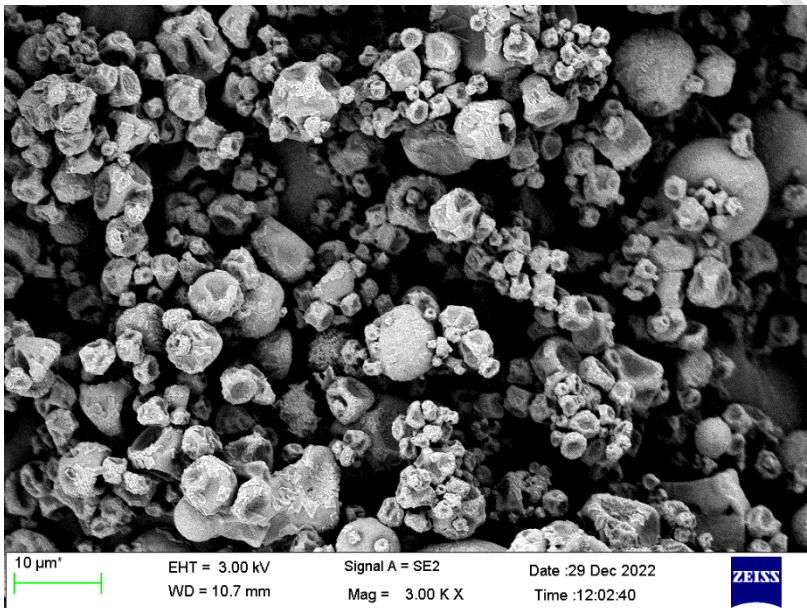
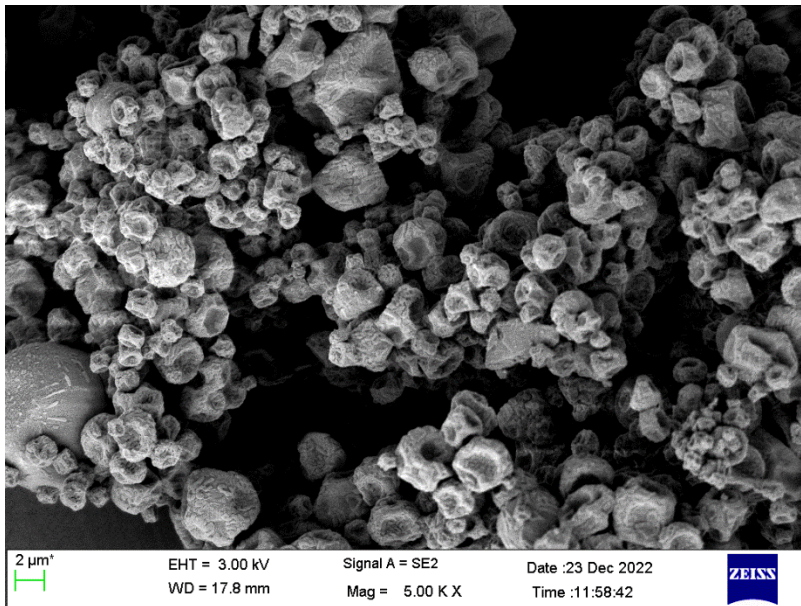


Fig 2: SEM image of encapsulated PIK1 spores [Maltodextrin (2%) + gelatin (1.5%) + CaCl₂ (2%)]



Conclusion

This study focused on standardizing the microencapsulation process of the spores of *Trichoderma asperellum* and bacterial cells of *Pseudomonas putida* using three different methods viz., extrusion, emulsification, and spray drying. The optimization of wall material, inlet/outlet temperatures, and feed flow rate was carried out. Accordingly, the best results in terms of survival and viability rates of the microcapsules were achieved using a combination of maltodextrin (2%), gelatin (1.5%), and CaCl₂ (2%) as the wall material, along with optimized spray drying conditions i.e., inlet temperature of 130.0°C and flow rate of 60 mL/h for TAIK1; and inlet temperature of 120.0°C and flow rate of 60 mL/h for PIK1. These microcapsules exhibited improved shelf life for *Trichoderma* and *Pseudomonas*. The encapsulation of the spores and cells are expected to protect them from various biotic and abiotic stress factors and promote promising potential for practical applications.

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