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Encapsulation of Native Strains of Bioagents *Trichoderma asperellum* and *Pseudomonas putida* Using Different Biopolymers

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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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 viablility

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of organisms were checked, among these the combination maltoderxtrin (2%) + gelatin (1.5%) + $CaCl_2$ (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 (130^0C, 60^0C, feed rate 60ml/h for TAIK1) and (120^0C, 50^0C, 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.

Keywords: Trichoderma; pseudomonas; encapsulation; spray drying; scanning electron microscope.

1. INTRODUCTION

Soil is a crucial component for plant growth, as it provides water, nutrients, and mechanical support. Various factors, such as crop cultivation, microflora, nutrient addition, and water availability, significantly affect soil properties. Maintaining soil health is important, and one approach is the introduction of native organisms with multifaceted activities. In this study, two native bioagents (*Trichoderma asperellum*, *Pseudomonas putida*) were encapsulated and so, after their release can maintain soil health, plant growth, and reduces disease incidence by creating unfavourable conditions to the pathogens.

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 [1,2]. One innovative technology in modern formulations has opened new possibilities for developing eco-friendly and effective biopesticides to control plant diseases is microencapsulation [3]. 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 [4]. 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" [5]. "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" [6-8].

"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" [9-11]. "Moreover, microcapsules offer advantages in terms of enhanced bioavailability and sustained release of drugs, enabling precise targeting of active compounds" [12], (Yao, Chang, Ahmad, & Li, 2016); [8]. The objective of the present study is to standardize and develop a microencapsulated formulation of promising bioagents.

2. MATERIALS AND METHODS

2.1 Bioagents

Native bioagents (BCAs)- *Trichoderma asperellum* strain *TAIK1* and *Pseudomonas putida* strain *PIK1* [13] were collected from culture collections of the Department of Plant Pathology, ICAR- IIRR, Hyderabad, Telangana, India.

2.2 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 x 10^8 for PIK1 and 2.26 × 10^7 for TAIK1 maintaining OD_{600nm}~1.0.

2.2.1 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" [14]. 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).

2.2.2 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 [3]. The combinations of wall materials with gelatin and $CaCl_2$ were given (Table 1).

Table 1. Combination of wall materials for extrusion and emulsification methods

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%)

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	130^0C	60^0C
TAIK1	Maltodextrin (2%) + Gelatin (1.5%)	60	130^0C	55^0C
TAIK1	Maltodextrin (2%) + Gelatin (1.5%)	60	150^0C	60^0C
TAIK1	Maltodextrin (2%) + Gelatin (1.5%)	60	150^0C	55^0C
PIK1	Maltodextrin (2%) + Gelatin (1.5%)	60	120^0C	50^0C
PIK1	Maltodextrin (2%) + Gelatin (1.5%)	60	120^0C	45^0C
PIK1	Maltodextrin (2%) + Gelatin (1.5%)	60	100^0C	50^0C
PIK1	Maltodextrin (2%) + Gelatin (1.5%)	60	100^0C	45^0C

2.2.3 Spray drying

From the above methods matlodextrin, gelatin and $CaCl_2$ combination was further used for spray drying method. One ml of spore suspension was added to matlodextrin (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 Table 2.

2.3 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:

$$\text{Survival rate (\%)} = (N/No) \times 100$$

Where, No 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.

2.4 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.

3. RESULTS AND DISCUSSION

3.1 Determination of Encapsulation Process

Upon different combinations of wall materials with different concentrations among the bioagents used. Extrusion process resulted in the formation of microcapsules with the improper release of bioagents. Emulsification process resulted in a compact capsule and also dissolved into culture media, allowing to release of bioagents. The spray dryer method used for bioagent encapsulation resulted in dry powder formulation with individual cells encapsulated, further easily dissolved with moisture and releasing bioagents into the respective media, retaining its antagonistic activity. Spray drying is the most extensively implemented technique for

the microencapsulation of bioactive compounds rather than extrusion and emulsification [3]. Extrusion is a very old and simple method of making capsules with hydrocolloids [15]. Our results are in line with those reported by Rodríguez-Huezo et al. [11].

3.2 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 ± 1.47% and PIK1 70.34 ± 0.12% were obtained, when maltodextrin was 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 µm and 5 µm 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" [16]. "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" [17]. Liu and Liu [18] reported that "the encapsulation of *M. anisopliae* in an alginate–clay matrix, with dextrin and hydoxypropyl methyl cellulose as additives, yielded 80% of germination after six months of storage".

3.3 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 ± 0.37%, 82.3 ± 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" [19]. According to Jin and Custis [20], "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" [21].

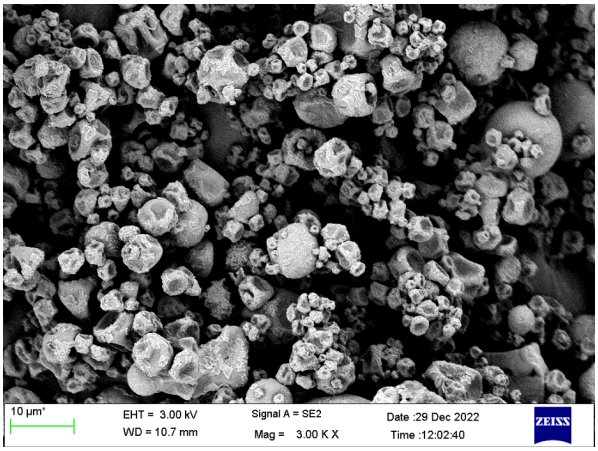

Fig. 1. SEM image of encapsulated TAIK1 spores [Maltodextrin (2%) + gelatin (1.5%) + $CaCl_2$ (2%)]-using spray drying method

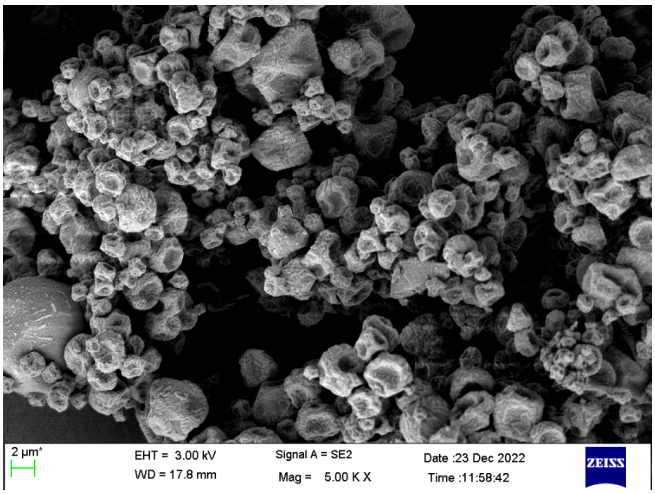

Fig. 2. SEM image of encapsulated PIK1 spores [Maltodextrin (2%) + gelatin (1.5%) + $CaCl_2$ (2%)]- using spray drying method

3.4 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. (Figs. 1,2) "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" [22,23].

4. 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., an 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 is expected to protect them from various biotic and abiotic stress factors and promote promising potential for practical applications.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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