Adequacy of low-cost technique for soil bacterial alginate/zein encapsulation at laboratory scale

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ABSTRACT

In the contemporary context, the utilization of microorganisms across various disciplines has emphasized the growing significance of comprehending their fundamental mechanisms and enhancing delivery techniques, particularly in agriculture. Microencapsulation is one notably viable technique that establishes a controlled microenvironment, thus safeguarding microorganisms, offering superior handling, stability, and precisely controlled release. To evaluate a low-cost technique adaptation, this study explores the encapsulation of one plant growth-promoting bacteria isolated from burned soil, utilizing the ion gelation method facilitated by a simplified syringe pump model. The core materials comprise sodium alginate and LB medium as the support matrix, complemented by zein as the protective coat. The best result for capsule formation with viable bacteria was obtained with a 5% sodium alginate matrix compound in 2.5% Luria Bertani broth and 5% Zein for coating.

Additionally, in-depth insights into the chemical properties of the capsules and their components were obtained through ATR-FTIR spectroscopy, revealing vital interactions within these structures. In summary, this study represents a significant advancement in addressing contemporary agricultural challenges, especially those exacerbated by climate change. By introducing an economical and exploratory laboratory-scale technique for encapsulating soil bacteria, this research contributes to the development of sustainable agricultural practices. It underscores the potential of encapsulation in enhancing soil health, promoting plant vitality, and mitigating the adverse effects of climate change on agricultural ecosystems..

Keywords: *Alginate, Bacteria encapsulation, Low-cost Encapsulation.*

1. INTRODUCTION

In the last century, the application of microorganisms across diverse fields has underscored the growing importance of comprehending their underlying mechanisms and refining delivery techniques, with particular relevance in pharmacy and agriculture [1-4]. As one of the available techniques, micro-encapsulation is an innovative approach entailing the con-finement of substances within nanometer to millimetersized particles using a polymeric matrix [5]. This technique establishes a controlled microenvironment, protecting microorganisms, such as bacteria, and various bioactive molecules, including enzymes, hormones, and proteins [6].

Consequently, microencapsulation methods have been incre-asingly integrated into medicine, pharmacy, biotechnology, agriculture, and the food industry, offering advantages encompassing improved handling, enhanced stability, and precise, controlled release [5,7]

At the same time, the technique of spray drying micro-encapsulation has emerged as a prevalent choice in the food and pharmaceutical sectors due to its merits in speed, cost-efficiency, simplicity, and scalability [5,6,8-11]. It is essential to acknowledge its inherent limitations. These include challenges related to irregular capsule sizes, inconsistent particle production, loss of products, and suboptimal yields, particularly in laboratory-scale applications [12]. Consequently, researchers have explored alternative methods such as electrospinning, electrospraying, liposome delivery, and sharp-hole coagulation bath techniques, albeit these methods have exhibited constraints concerning equipment requirements, efficiency, and reproducibility [13].

Using sodium alginate as a biodegradable carrier system has been extensively investigated in various studies [7,14,15]. Sodium alginate is a natural anionic polymeric polysaccharide derived from brown algae species, including *Macrocystis pyrifera* [16] and certain bacterial strains like *Azotobacter vinelandii* and *Pseudomonas spp*. [17], finds widespread application in the food

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and pharmaceutical industries due to its safety and efficiency attributes. Additionally, zein, a natural plant protein characterized by its commendable biocompatibility and elevated levels of non-polar amino acids such as alanine, leucine, and proline [18,19], offers exciting prospects in microencapsulation. It is imperative to underscore the pressing challenges climate change poses, as indicated in the sixth assessment report by the Intergovernmental Panel on Climate Change [20]. The extensive negative impacts on terrestrial ecosystems, particularly the degradation of productive soil, have been classified as high or very high. In this context, innovative techniques like micro-encapsulation arise as an attractive and sustainable alternative in agriculture, where the role of microorganisms in preserving soil health and enhancing plant fitness is well-documented [21-24]. Consequently, developing alternative methods for selecting high-performing bacterial strains becomes paramount in this arena. Hence, the present study endeavors to delineate a cost-effective, laboratory-scale technique for encapsulating living soil bacteria utilizing the ion gelation method facilitated by readily available equipment. This approach can potentially serve as a valuable exploratory tool in addressing the evolving challenges of contemporary agriculture.

2. EXPERIMENTAL

2.1. Materials and equipment.

Only standard laboratory equipment, disposals, and reagents are required. An analog alternative could be used. Their company's names are given here as references. All laboratory glass, polypropylene plastic supplies, and deionized water used during the assay were previously sterilized by autoclaving. All the reagents used were of analytical reagent grade except for sodium alginate.

2.1.1. Materials and reagents

Sodium alginate (Alg-Na) for research use, MW 12-40 KDa (Sigma); Zein (Sigma); ethanol (Merck); calcium chloride anhydrous (Merck); sterilized deionized ultrapure water; agar-agar (VWR International); Luria Bertani (LB); Miller Broth (Himedia); glass Petri dishes (90x20 mm) and magnetic stirrer bar were used.

2.1.2. Equipment

Orbital shaker incubator with universal spring platform (Daihan Labtech Co., Ltd, Kr); Microbiological incubator (Memmert GmbH + Co., UK), Biological Safety Cabinet Class II (Haier Biomedical Co., Ltd, CN), microcentrifuge (Kubota Co., JP), refrigerated centrifuge (NuAire Inc., CH), KDS single-syringe pump series 100 (SigmaAldrich Co., US), water purification system Bio CB1905 UV (Adrona Co., LV), magnetic stirrer hot plate (Labnet International Inc., US). Fourier Transform Infrared Spectroscopy (FTIR) Shimadzu (IR Spirit-T) spectrometer equipped with a diamond attenuated total reflection (ATR) accessory.

2.2. Microorganism and growth conditions.

The bacterium strain S1_08 was isolated in previous research from bulk soil of a mixed forest plantation after a forest fire event from Purén city in the La Araucanía region of Chile and identified by 16S-rDNA sequence like a Bacillus spp. species (unpublished data). According to their texture, the soil in this area is classified as clay loam [25], whose major trait when assuring texture soil by hand is that it is easily molded and rolled into threads [26]. The S1_08 strain was isolated and replicated on LB, Miller broth medium for 24 hours at 25ºC under agitation at 125 rpm in an orbital shaker incubator. The final cell concentration reached ~1×10^6 cfu mL^{-1}, which was determined by the plate count technique [27].

2.3. Solutions and dispersions preparation.

A dispersion of sodium alginate was prepared at three different concentrations (1.4, 2, and 5%) in LB Miller broth medium at 2,5% w/v (according to manufacture protocol). The dispersion was homogenized for 24 hours in a stir plate at room temperature before the autoclaved sterilization process for 15 min at 1 atm and 121ºC. In parallel, a solution of $CaCl_2$ at 2% w/v was prepared and homogenized for 24 hours in a stir plate at room temperature before sterilization by autoclaving, as previously described. Meanwhile, due to its high hydrophobic characteristics, the zein dispersion was prepared at 0.5%, 1%, 2%, 3%, and 5% w/v concentrations in a mixture of EtOH: H_2O (1:1) and homogenized in a stir plate for 24 hours at room temperature. Due to the utilization of EtOH, this dispersion was sterilized after its preparation through UV radiation exposition (254 nm) for 30 minutes.

2.4. Inoculation of the polymeric matrix.

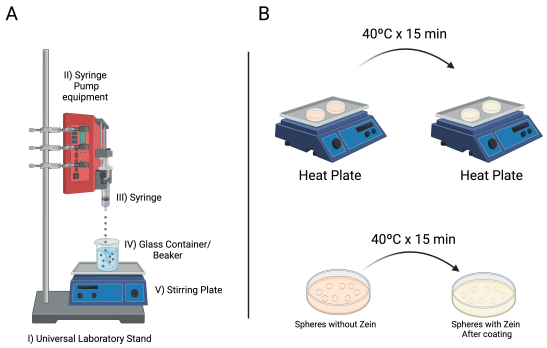
900 mL of the dispersion formed by sodium alginate and LB Miller broth (Alg-Na+LB) was inoculated with 100 mL of S1_08 bacterium strain. The inoculated dispersion was kept in constant agitation in an orbital shaker incubator at 152 rpm, 25ºC for 24 h. After 24 h of the inoculation process, an aliquot of 20 mL was extracted from the Alg-Na+LB+bacterium strain solution and utilized in the spheres manufacturing process.

2.5. Encapsulation and coating process.

The encapsulation process comprises two steps:

I) Spheres formation by an ionic exchange between sodium alginate and $CaCl_2$;
II) Spheres coating with zein solution (Figure 1). The process described above was realized under a biological safety cabinet class II to avoid contamination with other microorganisms.

Figure 1. Schematic representation of encapsulation and coating process.



A. Schematic representation of the tools system used to manufacture capsulated spheres, composed of a syringe pump and a stir plate as core tools.
B. Graphic representation of the zein coating process. The spheres were placed in a glass petri dish with enough zein until completely submerged. The plate was heated without exceeding 40ºC until the liquid evaporated, finally coating the spheres.

2.5.1. Spheres formation by an ionic exchange between sodium alginate and $CaCl_2$

The first step was performed by manually constructing our inoculated solution release system. Which consists of 5 elements:

i) universal laboratory stand,
ii) syringe pump equipment (with constant rate release),
iii) syringe,
iv) glass container/beaker, and
v) stirring plate (Figure 1A). The mixture within the syringe corresponds to Alg-Na+LB+ bacterium strain. The syringe pump was configured with a flow ratio of 30 mL/h, using a syringe of 10 mL with a diameter of 14.4 mm.

The receiver solution corresponds to $CaCl_2$ 2% w/v. The mixture was added to the $CaCl_2$ solution by dripping at a constant rate, forming the spheres at room temperature, and stirring constantly. Finally, capsules were collected from the $CaCl_2$ solution and prepared for the next step.

2.5.2. Spheres coating with zein dispersion.

This second step corresponds to the coating of the spheres with zein. To coat the spheres with zein, the newly formed spheres (60 units approx.) were immersed in zein dispersion (~10 mL) and heated at 40°C for 15 minutes. Finally, the EtOH was evaporated, obtaining Alg-Na+LB+bacterium strain spheres coated with zein.

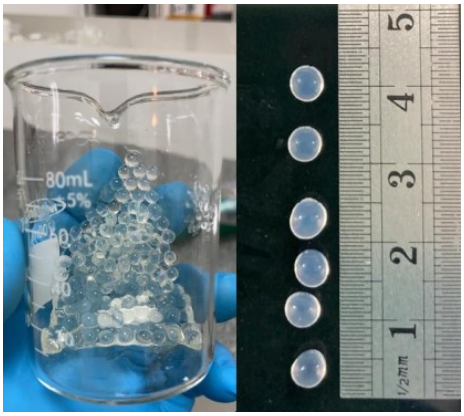
2.6. Bacterial viability assay.

In vitro survival assays were performed to evaluate the capbility of the bacteria to survive the encapsulating methodology and repopulation of the bacteria into the solid culture media. Briefly, between 10 and 12 coated spheres were loaded into a petri dish with an LB Miller agar medium and incubated at 25°C for 48 hrs. A qualitative analysis was performed based on the presence or absence of typical colony bacterial strains colonizing the agar medium outside the capsule.

2.7. ATR-FTIR measurements.

The chemical properties of the capsules and their components were analyzed with a Fourier transform infrared (FTIR) spectrophotometer equipped with a diamond attenuated total reflection (ATR) accessory. The spectra were obtained at 500-4000 cm^{-1} range at a resolution of 4 cm^{-1}. Each spectrum represents an average of 64 scans.

Figure 2. Representative photograph of capsule elaborated using a syringe pump.



3. RESULTS AND DISCUSSION

3.1. Capsule formation.

Different alginate concentrations described in step 1 of the experimental section, assessed in a 2% CaC_{l2} solution, optimal performance, well-defined, homogeneous shapes, and hand-pressed resistant spheres, were obtained at sodium alginate concentrations of 5% (Figure 2). The average size of the capsules corresponds to 4.89 ± 1.3 mm. Similar concentrations have been employed in various procedures involving sodium alginate, such as microencapsulation of oils [14], therapeutic drugs [28], vegetable extracts [15, 29,30], enzymes [31]; propolis extract [32] and probiotic bacteria [33]. Introducing LB Miller broth into the alginate dispersion enhanced the bacterial environment, ensuring the supply of necessary nutrients for optimal bacterial growth.

Zein coating process.

Previously selected capsules were assayed for coating with 0.5%, 1%, 2%, 3%, and 5% zein concentrations. Comprehensive details about this stage of the process can be found in the methodology section previously described. Ultimately, a 5% Zein solution was selected for coating, following a meticulous assessment of visual parameters designed to ensure coating uniformity and thickness. Following the coating process, the spheres displayed the characteristic color of zein, thereby confirming the successful formation of an external zein layer on the sodium alginate spheres (Figure 4A, Figure 4B).

3.2. Spheres FTIR characterization.

The FTIR spectra of sodium alginate (Alg-Na), zein, and sphere (Alg-Na+LB+Zein)- bacterium mixture, henceforth, sphere-bacterium mixture, are shown in Figure 3.

Sodium alginate exhibits characteristic peaks related to carbohydrates, with a broad peak at 3450 cm^{-1} corresponding to hydroxyl groups stretching (O-H) and sharp peaks at 1600 and 1420 cm^{-1} attributed to carbonyl (-COO-) [34].

Zein, classified as a prolamine protein, displays absorption bands related to its distinctive amide group, including peaks at 3294 cm^{-1} and 1534 cm^{-1}, representing stretching and bending modes of amide II (N-H) [35]. Additional peaks at 1650 cm^{-1} and 1517 cm^{-1} (C=O) are associated with vibrational stretching amide I [36] and at 2920 cm^{-1}, indicating hydrophobic group stretching (C-H) [37].

In the spectrum of the (Alg-Na+LB+Zein)-bacterium composite, a prominent new band at 3010 cm^{-1} emerges. Filip et al. [38] identified similar bands in Bacillus subtilis cell mass. They associated them with nucleic acid structures at 3300 cm^{-1} (N-H stretching), cell wall constituents at 3000-2800 cm^{-1} (C-H stretching), and proteinaceous structures at 1660, 1544, and 1235 cm^{-1} (corresponding to amide I, II and III respectively). This new broadband suggests an interaction between aliphatic groups in cell wall constituent and the hydrophobic groups in zein, particularly the stretching of (C-H) bonds. When zein is applied to the bacterium and alginate mixture, shifts are observed in the vibrational peak intensities of C=O, from 1684-1650 cm^{-1} and 1588-1517 cm^{-1}. These results indicate that the microstructure of the (Alg-Na, Zein)-hydrophobic and electrostatic interactions primarily influence bacterium composite.

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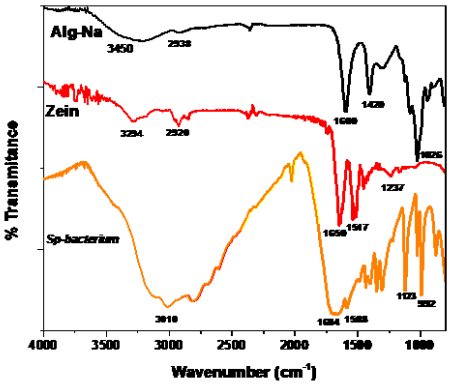
Figure 3. Fourier transforms infrared spectroscopy of individual polymers and the sphere-bacterium mixture.

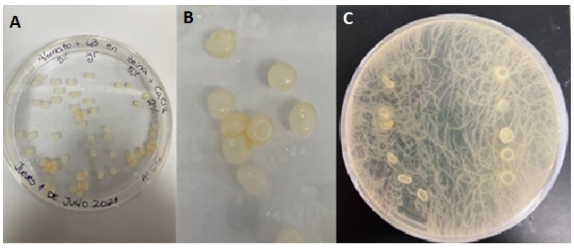


Black-line: Alg-Na sodium alginate.
Red-line: Zein.
Yellow-line: Sp-bacterium, sphere-bacterium mixture.

3.3. Bacterial survival assay

The critical aspect of this methodology lies in the viability of the S1_08 bacteria strain. Capsules formed with 5% Alg-Na and coated with 5% zein were placed onto a Petri dish with LB agar. Our findings illustrate the unimpeded growth of bacteria within zein-coated spheres (Figure 4C), confirming the effectiveness of the encapsulation process.

Figure 4. Sphere-bacterium mixture.



A. and B. Correspond to the final sphere coated with zein by our methodology. These spheres present the characteristic yellowish tonality through the presence of zein.
C. Representative Petri plate with typical growth of bacterial S1_08 strain encapsulated in spheres after 48 hours.

4. CONCLUSIONS

The research initially focused on the formation of capsules, and the uniformity and stability of these capsules were determined primarily through visual assessment. The choice of a 5% sodium alginate concentration for capsule formation and a 5% Zein dispersion for coating was made based on visual parameters that ensured coating uniformity. Using a basic model of a syringe pump allows us to make bacteria S1_08 strain cells successfully encapsulated using alginate-Na+LB as a supporting matrix and zein as a protective coat, making possible the viability of the bacterium after the encapsulation process.

Additionally, ATR-FTIR spectroscopy analysis provided a deeper understanding of the chemical properties of the capsules and their components, revealing critical interactions between the microcapsules' constituents.

This study provides valuable insights into encapsulation techniques and their potential applications in soil bacteria. By offering an exploratory and cost-effective laboratory-scale technique for encapsulation using the ion gelation method, this study contributes to developing different fields by optimizing resources.

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