Immobilized *Bacillus subtilis* by ionic gelation as biocontrol alternative of *Fusarium oxysporum* f.sp. *lycopersici*

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**ABSTRACT**

For farmers the use of agrochemicals is the preferred method to control pests and diseases. Considering the market demand for biological control products, the encapsulation could be a competent alternative to current commercial formulations for cellular viability and controlled release. The purpose of this study was to use ionic gelation with sodium alginate, starch and maltodextrin to immobilize *Bacillus subtilis* and to evaluate the biocontrol effect against *Fusarium oxysporum* f. sp. *lycopersici in vitro*. The matrix with a concentration of 2% sodium alginate, 1% starch, and 1% maltodextrin is a suitable method for cellular viability and biological control activity against *Fusarium oxysporum* f. sp. *lycopersici*, with a reduction of mycelial growth of 49.6% and a survival rate for *Bacillus subtilis* of 98.05% (*p* < 0.0001). The use of immobilized bacteria as biological control agents are sustainable and effective bio-inputs that could be used at industrial scale and benefit the tomato crops against attack by *Fusarium oxysporum* f. sp. *lycopersici*.

**Key words**: *Bacillus subtilis*, Bacterial immobility; Biological control, *Fusarium oxysporum* f. sp. *lycopersici*, Ionic gelation, Maltodextrin.

**INTRODUCTION**

The natural ability to reduce the density of a pathogen or parasite and/or its illness-producing activities through manipulation of the environment, the host, or pathogen antagonists is called biological control (Baker and Cook, 1982; Kamal et al., 2015). The *Bacillus subtilis*, a Gram positive bacillus, is a most important biological control agent (BCA) of its genus and is known to produce enzymes and secondary metabolites, grouped into surfactin, fengycin, and iturin families (Ongena and Jacques, 2008; Cao et al., 2012). Iturins are cyclic lipopeptides with three homologs (A-C) composed of 7 amino acids (l-Asn, d-Tyr, d-Asn, l-Gln, l-Pro, d-Asn, and l-Ser) and a chain of fatty acids. Each molecule differs in composition, amino acid position, and fatty acid chain length (Ongena and Jacques, 2008). There is evidence that iturin A shows specific activity against the plasma membranes of fungi such as *Rhizoctonia solani*, *Glomerella cingulata*, *Sclerotium rolfsii*, *Botrytis cinerea*, *Aspergillus spp.*, *Fusarium oxysporum*, *Fusarium graminearum*, *Phomopsis persea*, *Fusiciocum aromaticum*, and *Colletotrichum gloeosporioides* (Ongena and Jacques, 2008; Ma et al., 2015).

Currently, the use of agrochemicals is the preferred method for stopping pests and illnesses in crops. However, their frequent and inappropriate use has produced microbial resistances and soil and groundwater toxicity. As a result, the development of encapsulation methodologies, already successful in the pharmaceutical and health industries, is a newly considered alternative in the agricultural sector.

Microencapsulation (ME) technology traps cells and compounds and/or immobilizes with natural polymer wall materials, creating microspheres with an average size between 1-1000 µm (Rathore et al., 2013). Cellular conservation and viability, protection against degradation from the environmental medium (heat, air, light, humidity), gradual release of the encapsulated material, reduction of hygroscopy, stabilization of active ingredients, low costs, and scalable production are some of the advantages of ME (Burgain et al., 2011; Rathore et al., 2013).

Among developed methods for microencapsulation are extrusion, emulsification, gelation, liposomes, and spray drying. Ionic gelation is a process that involves immobilizing cells by cross-linking units of L-guluronic acid from sodium alginate and divalent calcium ions, inducing the formation of a hydrogel (Vemmer and Patel, 2013; Paques et al., 2014). This process, called gelation, results in microcapsule resistance to pH between 3 and 10, nutrient and oxygen transport and, consequently, provides stability and conservation of the encapsulated cells (Krasaekoopt et al., 2003). Due to its simplicity, non-toxicity, and low cost, this method has been used extensively to immobilize lactic acid bacteria (LAB) (Mirzaei et al., 2012).

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Mixtures of alginate with other polymers, such as starch, can lead to improvements in stability, integrated structures, and pearl yield; they have been reported as an effective method for probiotic encapsulation (Sultana et al., 2000; Homayouni et al., 2008). Additionally, the use of maltodextrins, derived from the hydrolysis of potato, rice, or corn starches, are linked to low viscosity, high solid content, good solubility, and the ability to produce films (Lopera et al., 2009; Yufeng et al., 2014).

The objective of this study was to immobilize *Bacillus subtilis* (Bs) through ionic gelation, with sodium alginate, starch, and maltodextrin to optimize the production of capsules with high effectiveness in cellular viability, and demonstrate its biological control efficiency against *Fusarium oxysporum* f.sp. *lycopersici* (Foxl).

**MATERIALS AND METHODS**

Isolation of *Bacillus subtilis* and *Fusarium oxysporum f.sp. lycopersici*: Strains of *Bacillus subtilis* (Bs) and *Fusarium oxysporum f.sp. lycopersici* (Foxl) were obtained from the strain collection of “Universidad Colegio Mayor de Cundinamarca”. Bacteria were recovered in Brain-heart infusion agar (BHI) (OxoidTM) and blood agar (OxoidTM), incubated at 37°C for 24 h for phenotypic identification. *Fusarium oxysporum f.sp. lycopersici* was grown in potato dextrose agar (PDA) (OxoidTM) for 7 days at 28°C.

**Bacterial Immobilization: ionic gelation:** The matrices of wall material included were: sodium alginate (alg) (Sigma Aldrich), corn starch (cs) and maltodextrin (mtx). Solutes were mixed according to a randomized experimental design using response surface methodology (RSM) (Table 1).

All materials and laboratory equipment were sterilized. A 1:1 volume of wall material and an inoculum of *3.1 x 10^6* CFU/mL Bs was homogenized under constant agitation at 300rpm in an orbital agitator for 15min.

Bacteria was immobilized according to the methods described by Sandoval et al., 2010. The resulting capsules were stored in sterile 1% peptone water and refrigerated at 4°C.

**Capsule size and morphology:** The size and morphology of the capsules were characterized with bright-field microscopy using a Carl Zeiss microscope and an AxioCam ICC5 camera (Germany). Their average size was determined by measuring the diameter of 50 capsules with a 2.5X objective, using ZEN2 software from the same company.

**Encapsulation efficiency of *Bacillus subtilis***: Pearls were suspended in a 1:9 (m/v) volume of 0.1 M phosphate buffer

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**Table 1:** Experimental design and response values for encapsulation matrix formation.

| No | Matrix | Decoded Values | Response Variables |
|----|--------|----------------|--------------------|
|    | Alg %  | Mtx % | CS % | Particle size (µm) | Encapsulation yield (%) |
| 1  | 2      | -     | -    | 2364.13           | 86.68                 |
| 2  | 1      | -     | 0.5  | 2363.01           | 90.72                 |
| 3  | 1      | 0.5   | -    | 2313.06           | 94.23                 |
| 4  | 2      | 1     | -    | 2200.94           | 90.94                 |
| 5  | 2      | -     | 1    | 2231.06           | 96.07                 |
| 6  | 5      | -     | 0.5  | 2788.85           | 66.14                 |
| 7  | 5      | 0.5   | -    | 2765.18           | 55.40                 |
| 8  | 1      | 0.5   | 1    | 2757.66           | 97.98                 |
| 9  | 1      | 1     | 0.5  | 2557.66           | 98.05                 |
| 10 | 2      | 0.5   | 0.5  | 2492.81           | 98.05                 |
| 11 | 2      | 0.5   | 0.5  | 2492.81           | 98.05                 |
| 12 | 2      | 1     | 1    | 2492.81           | 98.05                 |
| 13 | 2      | 1     | 0.5  | 2492.81           | 98.05                 |
| 14 | 5      | 1     | 0.5  | 3297.14           | 66.14                 |
| 15 | 5      | 0.5   | 1    | 2669.79           | 66.14                 |

**Table 2:** Analysis of variance for the responses.

| Factor | Sum of squares | Mean Square | F statistic | Pr>F |
|--------|----------------|-------------|-------------|------|
| alg    | 195.95         | 4898.81     | 41.63       | <.0001|
| mtx    | 5157.19        | 1289.29     | 10.96       | <.0001|
| cs     | 2750.55        | 687.63      | 5.84        | 0.001 |

| Factor | Sum of squares | Mean Square | F value | Pr>F |
|--------|----------------|-------------|---------|------|
| alg    | 2236275        | 559069      | 41.17   | <.0001|
| mtx    | 622056         | 155514      | 11.45   | <.0001|
| cs     | 683342         | 170835      | 12.58   | <.0001|
solution (PBS) pH 7.0 (Sigma Aldrich®) and homogenized in an orbital agitator at 300 rpm for 30 min. Afterwards, 20 µl of the solution was recovered in BHI agar plates and incubated at 37°C for 24h to perform a cell count. Encapsulation efficiency (EY) was calculated according with the formula (Martín et al., 2013):

\[ EY = \left( \frac{N}{N_0} \right) \times 100 \quad \text{Eq.1.} \]

where N is the number of viable trapped cells freed from the capsules and \( N_0 \) is the number of free cells added to the biopolymer during particle production.

**Antagonistic action toward Fusarium oxysporum f.sp. lycopersici in Vitro:** Seven (7) treatments were evaluated in triplicate using dual confrontation assays in PDA. Each petri dish was inoculated with 5 mm Fox1 loops grown over the previous 7 days. At 5 mm distance were inoculated 1G of each treatment separately. The dishes were incubated for 7 days; every 24 h, the radial diameter of the pathogen and the halo of inhibition was measured using a caliper.

**Statistical analysis:** Particle size and encapsulation yield were analyzed using the response surface method (RSM). For the antagonism tests, an analysis of variance and the SNK (Student-Newman-Keuls). All analyses were carried out using Statistical Analysis Software (SAS).

**RESULTS AND DISCUSSION**

**Morphology and Encapsulation yield:** Microscopic analysis showed spherical pearls with a delimited outline, with both rough and smooth surfaces and detached between them, however other pearls have an irregular and tear-drop shape. Opalescent zones indicated the presence of bacteria in the interior of the capsule (Fig 1A-1C). The Fig 2 show the effect of each wall material on minimizing particle size, with an F-statistic of 41.17 for alginate, 11.45 for maltodextrin, and 12.58 for starch, and \( p < 0.0001 \) for the three factors, as shown in Table 2. The estimated value of minimum response for the particle size variable ranges from 2140 µm to 2700 µm.
Table 3: Antagonism Tests for Bacillus Subtilis Vs. Fusarium oxysporum f. sp. lycopersici.

| Treatment          | Phytopathogen growth (mm) | Halo of Inhibition (mm) | PIRG (%) |
|--------------------|---------------------------|------------------------|----------|
| Matrix No14        | 42.3 \( ^a \)           | 0 \( ^c \)             | 4.5      |
| Matrix No1          | 34.3 \( ^b \)           | 4.3 \( ^b \)           | 22.5     |
| Matrix No13         | 22.3 \( ^c \)           | 7.6 \( ^c \)           | 49.6     |
| Solution Bs1        | 31.6 \( ^b \)           | 5.0 \( ^b \)           | 24       |
| Solution Bs2        | 31.3 \( ^b \)           | 4.6 \( ^b \)           | 24.8     |
| Negative Control    | 44.3 \( ^a \)           | 0 \( ^c \)             | 0        |
| Ext-se              | 33.6 \( ^b \)           | 4.6 \( ^b \)           | 19.2     |

Matrix 14: \( Bs \) in 5% alg + 1% mtx + 0.5% cs; Matrix 1: \( Bs \) in 2% alg; Matrix 13: \( Bs \) in 2% alg + 1% mtx + 1% cs; Solution Bs1: 3.1 \( \times 10^6 \) CFU/mL; Solution Bs2: 3.1 \( \times 10^5 \) CFU/mL. Negative Control: Distilled water; Ext-se: Culture medium extract.

Averages with the same superscript letter are statistically equal with \( p < 0.05 \).
material (Rokka and Rantamäki, 2010). However, our study demonstrates that the use of alginate (5%)/starch (5%) or alginate (5%)/maltodextrin (0.5%) combination produces encapsulation yields of 66.14% and 55.40%, respectively. This suggests that the conformation of the alginate net-hydrogel produces synergetic characteristics with greater cellular protection for Bs when the three materials are combined, provided that the concentration of alginate is less than 5%.

Our study produced pearls that were between 2140 μm and 2700 μm in size, similar to the ranges reported by Burgain et al. (2011) for particle size using ionic gelation. Despite the implication that smaller capsules contain higher cellular concentrations and better distributions, our results show that cellular immobility in the three matrices, with generally superficial distribution in larger pearls and concentric distributions in smaller pearls, preserve Bs protection at the interior without differing in EY. Additionally, we observed that at 1% maltodextrin concentration, regardless of the alginate and starch concentrations, capsules were irregular and drop-shaped, associated with dragging forces from the impact of the drops with the calcium chloride bath, as described by (Cai et al., 2014), and with crosslinking and the structural conformation of maltodextrin.

**Biocontrol effect of Bacillus subtilis on Fusarium oxysporum f.sp. lycopersici:** The antagonism of immobilized Bs was analyzed with the percent inhibition of radial growth (PIRG) formula, described by Ezziyyani et al.(2004):

\[
PIRG = \frac{(R1 - R2)}{R1} \times 100
\]

Where R1 is the initial pathogen radius and R2 is the radius of the attacked pathogen.

According with the results the PIRG and halo formation, the SNK test demonstrates that the best treatment is matrix No 13, which corresponds to Bs encapsulated at 2% alg + 1% cs + 1% mtx (p<0.001) followed by those treatments marked with b and a as shown in Table 3. The Fig 3 shows the antagonism tests for the Bs treatments in solution, and Fig 4 shows the treatments with immobilized Bs 72 h after confrontation with Foxl.

The global market for biological and synthetic pesticides primarily consists of liquid-based formulations, water-soluble granules, soluble powders, and pellets. Selection depends on ease of use, preferences in application, and culture conditions (Thakore, 2006). Therefore, success of any “bio” product should coincide with the conditions for growth and metabolism of the microorganism and/or the activity of its metabolites and the interaction of these products with the ecosystem.

Biocontrol strategies for the management of disease by *Fusarium* includes biocides, fungicides with agents as *Pseudomonas fluorescens*, *Trichoderma harzianum Trichoderma viride*, and eco-friendly practices combined with soil amendments (Jayasekhar, Manonmani, & Justin, 2008; Kala, Gangopadhyay, & Godara, 2016). However, few studies have investigated capsules formed from the combination of alginate and maltodextrin; our results illustrate that the use of encapsulation that includes 1% maltodextrin produces a Bs survival rate of 98.05% (p<0.001), with an inhibitory effect on fungal growth of 49.6% (p<0.001). As shown by Ma et al. (2015), maltodextrin is an efficient encapsulation material, especially at concentrations greater than 80% for spray drying methods, producing a more prolonged lifespan when compared with soluble powders of *B. subtilis* B99-2, and greater efficiency in controlling tomato rot in *in vivo* tests (Ma et al., 2015).

**CONCLUSION**

The advantages of formulations bacteria immobilized depend on selection the wall materials. Its physical and mechanical properties support the efficiency and biological viability of *Bacillus subtilis* as control agent immobilized. The results revealed that Bs encapsulated with a wall of 2% sodium alginate, 1% starch and 1% maltodextrin, is a suitable method for protection, cellular viability, and biological control activity against *Fusarium oxysporum f.sp. lycopersici*. The response time was 72h for *in vitro* application, the growth reduction was 49.6%, and the survival rate was 98.05%; it was better than other types of formulations, such as pellets. Consequently, the use of local microorganisms with the potential for biological control activities and promoting plant growth should involve the integration of technological fields that favor industrial-level scalability and translating research results to the farmer.

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