

UNIVERSIDADE FEDERAL DE SÃO CARLOS – UFSCar
CENTRO DE CIÊNCIAS EXATAS E DE TECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA QUÍMICA – PPGEQ
Embrapa Instrumentação

Tese de Doutorado

**PLATAFORMA DE ENCAPSULAMENTO DE MICRORGANISMOS EM MATRIZ
PVA PARA USO NA AGRICULTURA COMO INOCULANTE MICROBIANO**

Doutoranda: Ludimila Araújo Lodi

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Orientadora: Dra. Cristiane Sanchez Farinas (Embrapa Instrumentação/PPGEQ-UFSCar)

São Carlos/SP

Abril de 2024

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Tese de Doutorado apresentada ao programa de Pós- Graduação de Engenharia Química da
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“A vida não é fácil para nenhum de nós. Mas e daí? Nós devemos ter persistência e, acima de tudo, confiança em nós mesmos. Devemos acreditar que somos talentosos em alguma coisa, e que essa coisa, a qualquer custo, deve ser alcançada.”

Marie Curie

RESUMO

Este estudo teve como objetivo o desenvolvimento de novas formulações para inoculantes microbianos, focando no encapsulamento de uma cepa de *Bacillus megaterium*. Foram produzidos filmes e micropartículas a partir de formulações à base de Álcool Polivinílico (PVA), usando as técnicas de casting, emulsão e spray-dryer. As formulações que demonstraram melhor efeito na proteção a bactéria encapsulada contra estresses como contato com calor, luz UV e agroquímicos foram aplicadas no revestimento de sementes de soja (filmes), em solo com alta salinidade (micropartículas de emulsão) e como inoculante sólido em experimentos de casa de vegetação com soja como planta modelo (micropartículas de spray dryer). Inicialmente, filmes biodegradáveis de PVA reticulados com ácido cítrico foram desenvolvidos. Na concentração de 30% (m/v), o ácido cítrico reduziu o inchaço e a solubilidade dos filmes, aumentando sua resistência à degradação e isso influenciou em uma liberação controlada da bactéria encapsulada. No revestimento de sementes de soja, os filmes promoveram taxas de germinação comparáveis ao tratamento com polímero comercial e superiores às sementes não tratadas evidenciando seu potencial para aplicação, sem afetar as sementes. Em seguida, micropartículas à base de PVA/amido catiônico foram desenvolvidas a partir de uma metodologia simples de reticulação/emulsão, investigando a ação dos agentes reticulantes ácido cítrico (CA) e Trimetafosfato de sódio (STMP) e da bentonita como agente de modificação superficial. A presença de STMP/bentonita influenciou no aumento da estimativa de vida útil dessa formulação e ao ser aplicada em ensaios com solo de alta salinidade (200 mM), as micropartículas mostraram-se um ambiente propício para o desenvolvimento da bactéria, apresentando uma viabilidade celular final maior do que a inoculada, mesmo após 7 dias de contato com a alta salinidade. Por fim, micropartículas de PVA/Amido catiônico por spray-dryer foram produzidas, investigando a adição de óxido de zinco (ZnO) à matriz. Quando aplicada como um inoculante sólido em ensaios de casa de vegetação, a presença de zinco e da bactéria na formulação promoveu um aumento significativo na produção de biomassa aérea e radicular das plantas de soja, com maior absorção de fósforo e zinco. Portanto, os resultados deste estudo destacam que as formulações a base de PVA propostas representam estratégias promissoras para o encapsulamento e controle da liberação de microrganismos, bem como para aplicações eficientes na agricultura.

Palavras-chaves: *Bacillus*; biofertilizantes; amido catiônico; ácido cítrico; trimetafosfato de sódio; zinco; estresses; emulsão; spray-dryer.

ABSTRACT

This study aimed to develop novel microbial inoculants for the encapsulation of *Bacillus megaterium*. Films and microparticles were prepared from polyvinyl alcohol (PVA)-based formulations using casting, emulsion, and spray drying techniques. The materials that best protected the encapsulated bacteria from stresses such as heat, UV light, and agrochemicals were applied in different ways: as a coating on soybean seeds (films), in soils with high salinity (emulsion microparticles), and as a solid inoculant in greenhouse experiments using soybeans with a model plant (spray-dried microparticles). Initially, biodegradable PVA films were developed that were cross-linked with citric acid. At a concentration of 30% (w/v), citric acid reduced the swelling and solubility of the films, increased their resistance to degradation, and influenced the controlled release of the encapsulated bacteria. The films promoted similar germination rates of soybean seeds treated with commercial polymers and outperformed the untreated seeds. This proves their application potential. Microparticles based on PVA/cationic starch were developed using a simple crosslinking/emulsion method, and the effect of the cross-linking agents citric acid (CA), sodium trimetaphosphate (STMP), and bentonite as surface modifiers was investigated. The presence of STMP/bentonite increased the estimated shelf life of this formulation. In high salinity soil tests (200 mM), these microparticles provided a favorable environment for bacterial development, with final cell viability higher than that of the inoculum, even after 7 days of high salinity. Finally, zinc oxide (ZnO) was added to the PVA/cationic starch formulation to produce microparticles by spray drying. The presence of zinc and bacteria in the formulation significantly promoted the production of biomass in the aerial and root zones of soybean plants and enhanced the uptake of phosphorus and zinc when applied as a solid inoculant in greenhouse tests. The results show that the proposed formulations and methods to produce novel microbial inoculants are promising strategies for efficient agricultural applications.

Keywords: *Bacillus*; Biofertilizers; Cationic starch; Citric acid; Sodium Trimetaphosphate; Zinc; Stress; Emulsion; Spray dryer.

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Introduction

Contemporary agriculture is facing a critical challenge: increasing food production to meet the growing global population in the coming decades. As the world's population continues to rise, it is increasingly imperative to expand agricultural production to ensure an adequate supply of food in terms of quantity and quality (Fróna et al., 2019). This increase in production is closely linked to the need to enhance the productivity of arable land, which demands an excessive use of chemical fertilizers. Therefore, there is a growing interest in developing products and technologies that are more effective, sustainable, and environmentally friendly. In this context, microbial inoculants have emerged as a promising alternative to address this challenge (Alori and Babalola, 2018; Bargaz et al., 2018; Timmusk et al., 2017).

Microbial inoculants consist of beneficial microorganisms that promote plant growth and development (Alori and Babalola, 2018). These microorganisms, such as fungi and bacteria naturally present in the soil rhizosphere, colonize plant roots, enhancing their development through various actions, such as nutrient availability, nitrogen fixation, phytohormone production, and protection against pathogens and environmental stresses (Bargaz et al., 2018; Herrmann and Bucksch, 2014; Stamenković et al., 2018). Among them, *Bacillus* bacteria have been highlighted for their positive effects on plant growth, phosphate solubilization in the soil, and disease control, showing inhibitory effects against various phytopathogens (Ma et al., 2015; Oliveira et al., 2021; Saeid et al., 2018).

However, the practical application of *Bacillus* strains in the field may face challenges due to adverse environmental conditions, leading to a loss of cell viability. Factors such as competition with other soil microorganisms, exposure to agrochemicals, water stress, salinity, soil pH, heat, and UV radiation contribute to the reduced survival of these microorganisms (Koza et al., 2022; Rahman et al., 2021). Therefore, it is essential to develop formulations that protect and ensure the viability of these microorganisms from storage to application (Berninger et al., 2018).

An effective formulation must provide physical and/or chemical protection to the microorganism to preserve cell viability during storage and protect them against adverse conditions during field application (Bashan et al., 2014). The choice of formulation is crucial to ensure product quality (Stamenković et al., 2018). Inoculants containing encapsulated microorganisms offer several advantages over traditional ones, especially over liquid

formulations, such as controlled release of the microorganism, greater protection against environmental stresses, and a longer shelf life. However, these formulations tend to be more expensive in terms of production costs compared to conventional inoculants, necessitating the search for methodologies and materials to make this process more viable (Rojas-Sánchez et al., 2022; Sammauria et al., 2020).

Various techniques are reported in the literature for obtaining encapsulation structures that can be applied as films for seed coating or as solid microparticles for seed coating with an adhesive agent or dispersion in the soil (Ayyaril et al., 2023). Encapsulated films are typically produced using the casting technique, which involves spreading the film solution onto a flat surface and allowing it to dry. For microparticle production, simple techniques such as dripping in a coagulant solution, usually calcium chloride (CaCl_2), are commonly used for particle formation (Brondi et al., 2022). However, the instability and inhomogeneity of the products formed have led to the adoption of emulsion and spray drying techniques for production. Both techniques are easier to scale up industrially and ensure more homogeneous products with better physical characteristics on a micrometric scale, allowing for improved diffusion of nutrients and oxygen to the microorganism during application (Ayyaril et al., 2023; Zabot et al., 2022).

Another important factor to consider is the selection of efficient matrices that ensure homogeneity and standardization during the encapsulation process. Various natural polymers (such as starch, cellulose, alginate, chitosan) and synthetic polymers (including polyvinyl alcohol, polyethylene glycol, polyurethanes, polyamides) have been evaluated as potential matrices for encapsulating microorganisms and enzymes in the past decade (Chanratana et al., 2018; Gamage et al., 2022; Klačić et al., 2018; Krasňan et al., 2016; Lal et al., 2021; Poraj-Kobielska et al., 2015; Wilpiszewska et al., 2019; Yang et al., 2019). This work focuses on the use of synthetic polymers Polyvinyl alcohol (PVA) and later, Cationic starch (CS) as potential polymers for microorganism encapsulation.

PVA is a water-soluble, non-toxic, and biodegradable polymer that exhibits high compatibility with living tissues and cells. It offers advantages over other polymers, including lower cost, greater durability, and chemical stability (Havstad, 2020; Pham and Tho Bach, 2014). Several studies have demonstrated promising results using PVA for encapsulating various materials such as enzymes and microorganisms (Chandran et al., 2016; Huang et al., 2015; Li et al., 2020; Porto et al., 2019; Shinde et al., 2018). Moreover, PVA can be partially or totally degraded by various microorganisms, including *Bacillus* bacteria (Patil and Bagde,

2015; Ullah et al., 2019), making it a suitable vehicle for delivering beneficial microorganisms and promoting sustainable agricultural practices (Bian et al., 2020, 2019; Ullah et al., 2019).

Cationic starch (CS) is a synthetic polymer derived from chemical modifications of natural starch, characterized by positively charged groups in its structure. This derivative is highly soluble in water and exhibits excellent film-forming properties, offering new opportunities for interactions with other materials in innovative applications (Nornberg et al., 2022). Compared to natural starch, CS presents enhanced characteristics, overcoming limitations such as insolubility in water at room temperature, retrogradation, lack of mechanical strength, thermal instability, and lower resistance to pH variations and shear forces (Goswami and Mahanta, 2021). CS has been extensively studied for immobilizing enzymes and phenolic compounds and for the controlled release of drugs (Chatterjee et al., 2023; El-Naggar et al., 2021; Mohamed et al., 2016; Simanaviciute et al., 2017).

Both PVA and CS are hydrophilic materials, necessitating the use of crosslinking agents to modify the encapsulation matrix for controlling characteristics like the controlled release of the encapsulated material. Various crosslinking agents have been explored in the literature for this purpose, including citric acid, sodium trimetaphosphate, boric acid, glutaraldehyde, among others (Bates et al., 2020; Chang et al., 2019; Gadhav et al., 2019; Liu et al., 2017; Yu et al., 2021). These agents aim to enhance the stability of polymer bonds and their properties.

In this study, we investigated the use of two crosslinking agents, citric acid (CA) and sodium trimetaphosphate (STMP), to strengthen the bonds of the developed matrices. Citric acid is a widely available metabolic agent known for improving the mechanical properties and stability of polymeric materials (Jose and Al-Harhi, 2017; Salihu et al., 2021). Recent research has shown its potential in fruit and seed coatings, enhancing characteristics like mechanical resistance and thermal stability, leading to positive outcomes in product storage and development (Dudeja et al., 2023; Wardak et al., 2024).

STMP, on the other hand, is a safe crosslinking agent extensively studied in medical applications for its compatibility with starch and PVA (Jeong et al., 2021; Pan et al., 2021; Riahi et al., 2017). Although less common in agricultural products, recent studies have explored its potential to improve the physical and mechanical properties of polymeric matrices (Chandak et al., 2022; Joglekar et al., 2020; Noulis et al., 2023). Both citric acid and STMP are considered

biocompatible, sustainable, and promising agents for formulations intended for microbial encapsulation.

The objective of this study was to develop effective microbial inoculants for agricultural use, focusing on encapsulating a strain of *Bacillus megaterium*. Film and microparticle inoculants were created using PVA-based formulations and the techniques casting, emulsion, and spray-drying. The formulations underwent a thorough analysis of their physicochemical and morphological characteristics, as well as an evaluation of the release profile of the encapsulated bacteria and their ability to withstand adverse conditions. The top-performing formulations were then further tested in experiments, including coating soybean seeds with films, applying them to soil with high salinity using emulsion microparticles, and using them as solid inoculants in greenhouse trials with soybeans as the test plant for spray-dried microparticles.

CHAPTER 1

1. Literature Review

1.1 Microbial Inoculants

Microbial inoculants are products based on beneficial microorganisms that, when applied to seeds, soil, or plant leaves, enhance crop growth and development (Alori and Babalola, 2018). The use of these microorganisms has been extensively studied in recent years, focusing on their isolation and application benefits (Abhilash et al., 2016; Loján et al., 2017; Sammauria et al., 2020; Stamenković et al., 2018).

The most utilized microorganisms in agriculture as biological inputs are bacteria such as *Azospirillum* sp., *Bacillus* sp., *Pseudomonas* sp., *Rhizobium* sp., and fungi like *Penicillium* sp. and *Trichoderma* sp. These plant growth-promoting microorganisms (PGPM) are typically found in the soil rhizosphere and play a crucial role in enhancing plant growth and development by colonizing plant roots (Abhilash et al., 2016b; Bargaz et al., 2018; Hashem et al., 2019; Stamenković et al., 2018).

Bacteria from the *Bacillus* genus have shown promise in increasing crop productivity, reducing the reliance on chemical fertilizers and pesticides in agriculture (Radhakrishnan et al., 2017). They offer various benefits to plants, including promoting growth by solubilizing essential nutrients and providing biological control against pathogens (Ahmad et al., 2021; de Sousa et al., 2021; Khan et al., 2022; Mumtaz et al., 2017; Saeid et al., 2018; Zhang et al., 2023).

Despite the advantages of microbial inoculants over traditional agrochemicals, they face legal challenges before widespread commercialization. The lack of globally accepted standards for their marketing is a significant hurdle, as requirements vary based on local regulations (Kumari et al., 2023). For example, in India, a minimum viable cell concentration of $5 * 10^7 CFU/g$ or $1 * 10^8 CFU/mL$ has been established, with only the microorganisms *Rhizobium*, *Azotobacter*, *Azospirillum*, phosphate-solubilizing bacteria, and mycorrhizal fungi being allowed in these products. In China, permitted microorganisms include rhizobia, organic and inorganic phosphate-solubilizing bacteria, nitrogen-fixing bacteria, silicon-solubilizing bacteria, and various consortia containing multiple microorganisms, with an initial concentration of $0.1 - 0.2 * 10^9 CFU/g$ or $0.5 - 1.5 * 10^7 CFU/mL$ (Kumari et al.,

2023). In Brazil, for an inoculant to be commercialized, it must meet the specifications of the Ministry of Agriculture, Livestock, and Supply (MAPA), which stipulates a minimum cell concentration of $1 * 10^9 CFU/g$ or mL , with the recommendation that this concentration be maintained for at least six months of storage. The most common microorganisms for inoculant production in Brazil are *Bradyrhizobium* sp., *Azospirillum* sp., *Trichoderma* sp., and *Bacillus* sp. (Brasil, 2020). On the other hand, European Union member countries, as well as the United States, do not establish specific regulations to ensure the quality and homogeneity of microbial inoculants (Kumari et al., 2023). Despite the absence of global standards, it is essential to prioritize factors like shelf life, cell viability, and product safety during the formulation and production of microbial inoculants to ensure their quality and effectiveness.

1.1.1 Types of Formulations

An effective microbial inoculant must create a favorable environment for the microorganism, ensuring its physical and/or chemical protection to prevent rapid reduction in cell viability during storage and potential losses during soil application. It should also contain a reliable source of live cells for interaction with plants and soil microbiota (Bashan et al., 2014; Herrmann and Lesueur, 2013; Vassilev et al., 2020). Therefore, selecting the right inoculant formulation is crucial for developing an ideal product, as issues related to its commercial use often stem from the quality, viability, and stability of the product. Understanding the inoculant production process, including the physiology of the microorganism and the plant, as well as the desired effects on crops, is essential (Stamenković et al., 2018).

Microbial inoculant formulations can be liquid, solid (organic and inorganic), or encapsulated, as illustrated in Figure 1. In liquid formulations, microorganisms are added to a carrier medium typically composed of culture medium and water (Bashan et al., 2014).

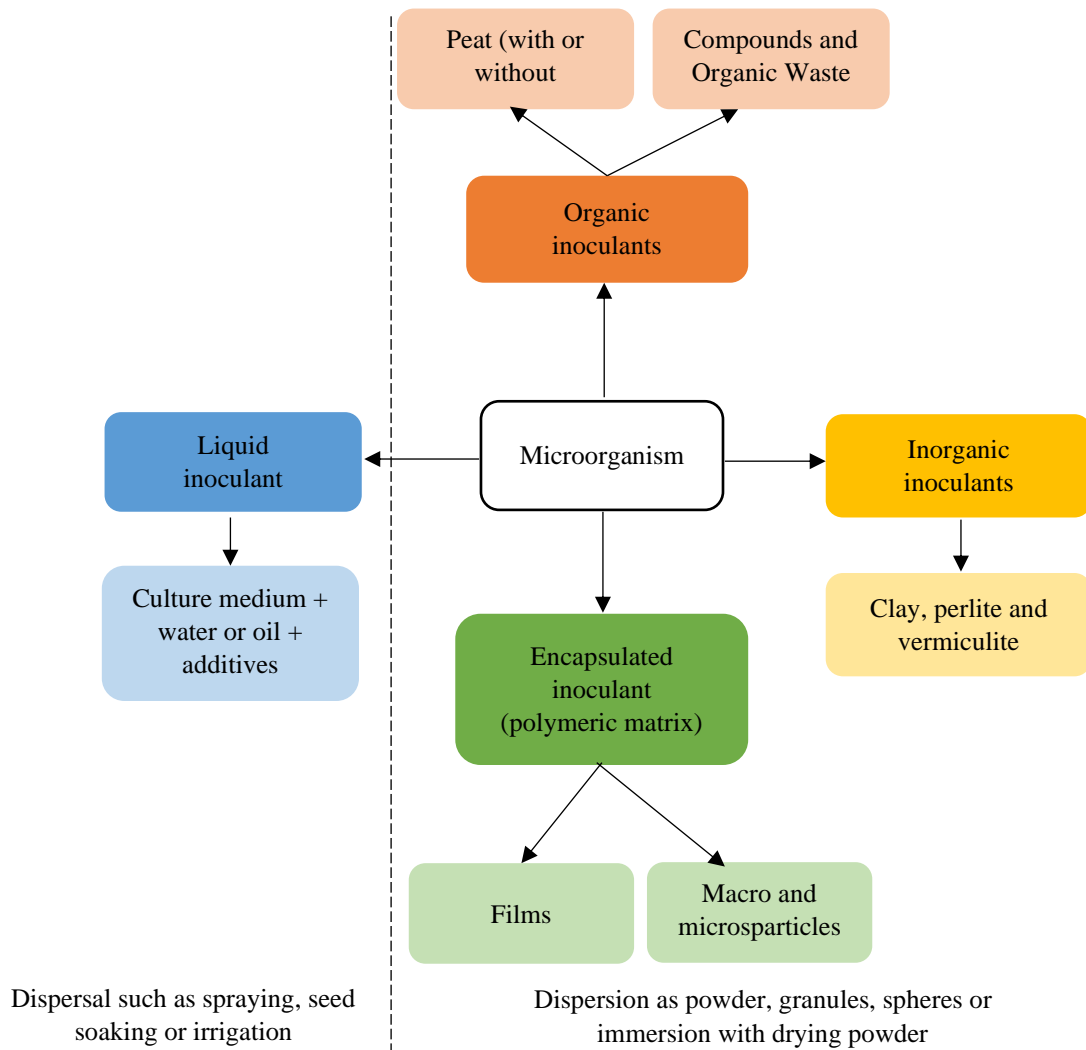


Figure 0.1. Different types of inoculants, their formulations and how they are applied, adapted from Bashan et al., (2014).

Liquid inoculants are commonly used due to their compatibility with field spraying equipment and ease of handling and application for farmers. However, they often exhibit low survival rates of microorganisms post-application, necessitating multiple applications to maintain the required cell viability for optimal crop growth. On the other hand, solid formulations face challenges such as low microorganism survival during storage, application uniformity issues, and higher contamination risks compared to liquid and encapsulated formulations (Bashan et al., 2014; John et al., 2011; Mahanty et al., 2017).

Encapsulated inoculants offer several advantages over other formulations, including controlled release of microorganisms (Helal et al., 2023; Zhan et al., 2020), protection against natural competition in the plant's rhizosphere (Rojas-Sánchez et al., 2022; Vassilev et al., 2020),

and enhanced resilience to adverse stress conditions (Lopes et al., 2024; Riseh et al., 2021; Souza-Alonso et al., 2021). The encapsulation structure acts as a protective barrier, providing greater protection and potentially serving as a long-term carbon source for the microorganism, depending on the matrix material used (Bian et al., 2020, 2019; Liu et al., 2019; Ullah et al., 2019). Encapsulated inoculants offer significant benefits, requiring less storage space, higher cell concentration, longer shelf life, and improved viability during storage and application, reducing the need for frequent reapplications, especially in challenging conditions like heavy rainfall, water scarcity, or high temperatures during planting.

1.1.2 Methods for Encapsulating Microorganisms

Various techniques are described in the literature for obtaining encapsulation structures, which vary depending on the specific product. This study primarily explores different methodologies for producing microbial inoculants using polymeric films to coat seeds and microparticles for solid encapsulated products in the field.

1.1.2.1 Encapsulation in Films for Seed Coating

Seed coating is a widely used commercial technique dating back to the 1960s, involving the application of materials onto germinating seeds to modify their physical properties and incorporate various compounds to enhance quality, yield, and performance (Ma, 2019; Pedrini et al., 2017). This method allows for better control of seed surface properties, impacting nutrient absorption, water uptake, pest resistance, and tolerance to environmental stresses like salinity and temperature fluctuations, while also extending storage life (Ma, 2019; Zvinavashe et al., 2019). In addition to microorganisms, seed coatings may include protective agents (insecticides, herbicides, fungicides), micronutrients (phosphorus, potassium, copper, zinc), growth stimulants, and marker substances (visible dyes, invisible dyes, chips) (Ma, 2019).

The primary seed coating technique involves applying a thin layer of polymer or polymer blend onto the seed surface, enhancing surface characteristics, development, and productivity without altering seed size or shape. Thin films are typically prepared using the casting method, where a polymer solution is dried in an oven (Butt, 2022; Javed et al., 2022). Pelletizing and encrusting are alternative methods that involve more significant structural changes to the seeds. Pelleting coats seeds with inert materials like talc, bentonite, or calcium peroxide to modify seed size, shape, and weight, facilitating easier sowing. Encrustation adds

adhesive and inert materials to create a smoother, more uniform seed surface, offering a cost-effective alternative to pelleting (Ma, 2019).

Recent research has focused on biodegradable polymeric matrices such as starch, chitosan, and alginate for seed coating, as the industry increasingly embraces coating with biodegradable films to enhance crop productivity for key crops like soybeans, wheat, corn, and canola (Accinelli et al., 2016; Jarecki and Wietecha, 2021; Paravar et al., 2023; Vercelheze et al., 2019).

1.1.2.2 Encapsulation in Microparticles

Encapsulation of microorganisms in macro (1 - 4 mm) and microparticles (1 - 1000 μm) is a technique that involves physically confining whole cells in a limited space to preserve their viability (Bashan et al., 2014; Karel et al., 1985). This technology is commonly used for encapsulating pharmaceutical, food, and enzyme products (Chaudhary et al., 2020) and has recently been applied to immobilizing microorganisms (Fernández et al., 2024; Kapishon et al., 2023; Lopes et al., 2024). Natural and synthetic polymers are widely used to produce stable particles for microorganism encapsulation, and various production techniques have been studied to enhance protection and viability (Chaudhary et al., 2020; Rathore et al., 2013; Vemmer and Patel, 2013).

The extrusion technique, commonly used for macroparticle production, involves dripping a polymer solution inoculated with microorganisms into a coagulant solution, typically calcium chloride (CaCl_2) (Brondi et al., 2022). Despite being a simple and cost-effective method, it has drawbacks such as particle size inhomogeneity, the requirement for low to moderate viscosity polymer solutions, and the formation of unstable particles due to rapid surface hardening (Ayyaril et al., 2023; Zabot et al., 2022).

The emulsion technique, an efficient alternative to extrusion, provides better stability to formed particles (Kotliarevski et al., 2021; Pupa et al., 2021). However, this method is less explored in cell encapsulation due to the presence of toxic solvents or aggressive drying required for homogeneity (Hwang et al., 2022). In the emulsion technique, the inoculated polymeric suspension (dispersed phase) is dispersed in an oil phase (continuous phase) and homogenized to form a water-in-oil emulsion using a surfactant and stirring. Microparticles are formed by cooling or adding a hardening/cross-linking agent to the emulsion, with particle size

influenced by stirring speed. The microspheres produced can be kept wet at refrigeration temperature or dried after undergoing a drying process, followed by collection through filtration or centrifugation (Martín et al., 2015).

Another established technique is spray drying, widely used in the food industry since the 1950s. In this method, an inoculated polymer suspension is atomized in hot air, leading to rapid water evaporation and producing microencapsulated dry powder. This method offers high-speed production, reproducibility, ease of operation, and scalability (Saber-Riseh et al., 2021). Proper temperature and process time adjustments are crucial to maintain cell viability. Recent studies have shown successful microencapsulation of fungi and bacteria using spray drying, preserving around 90% of their viability (Braga et al., 2019; Seth et al., 2017; Strobel et al., 2018), highlighting its effectiveness for microbial encapsulation.

1.2 Polymeric Matrices for Encapsulation

The industry has been searching for efficient matrices that ensure homogeneity and standardization during the encapsulation process. Various natural (starch, cellulose, alginate, chitosan, etc.) and synthetic (polyvinyl alcohol, polyethylene glycol, polyurethanes, polyamides, etc.) polymers have been evaluated as potential matrices for encapsulating microorganisms and enzymes over the past decade (Chanratana et al., 2018; Gamage et al., 2022; Klačić et al., 2018; Krasňan et al., 2016; Lal et al., 2021; Poraj-Kobielska et al., 2015; Wilpiszewska et al., 2019; Yang et al., 2019). In this work, we will focus on the use of the synthetic polymers polyvinyl alcohol (PVA) and cationic starch (CS) as potential polymers for encapsulating microorganisms.

1.2.1 Polyvinyl Alcohol (PVA)

PVA is a synthetic semi-crystalline polymer derived from the hydrolysis of Polyvinyl Acetate, as shown in Figure 2 (Pham and Tho Bach, 2014; Rynkowska et al., 2019). It is a water-soluble, non-toxic, biodegradable polymer with high biocompatibility with living tissues and cells (Havstad, 2020). PVA is known for its adhesive and oxygen barrier properties, flexibility, good tensile strength, and abrasion resistance (Alvarado et al., 2019; Singh et al., 2021), being one of the most versatile synthetic polymers used in various industries such as pharmaceuticals, chemicals, food, and cosmetics (Khana et al., 2015). Moreover, its partial or total degradation by various microorganisms, including the bacterium *Bacillus* sp., makes it a

promising choice as a carrier for beneficial microorganisms in sustainable agricultural practices (Bian et al., 2020, 2019; Liu et al., 2019; Ullah et al., 2019).

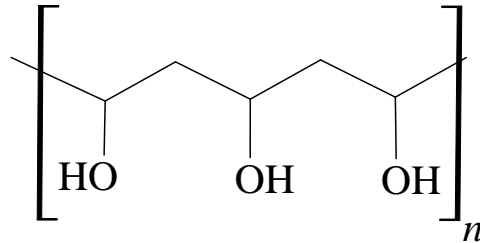


Figure 0.2. Structural formula of Polyvinyl Alcohol (PVA).

Several studies have demonstrated the promising use of PVA for encapsulating various materials, including enzymes and microorganisms. Here, we summarize key research on the encapsulation/immobilization of microorganisms in PVA-based matrices, either alone or in combination with other polymers, for agricultural applications.

De Gregorio et al. (2017) immobilized *Pantoea agglomerans* ISIB55 and *Burkholderia caribensis* ISIB40 bacteria in nanofibers within a PVA matrix for coating soybean seeds. The study showed enhanced survival of the bacteria during seed storage and successful colonization in plant roots. Coating with *P. agglomerans* increased germination, root length, and dry weight, while *B. caribensis* coating led to more leaves and aerial part dry weight.

Mukiri et al. (2022) studied the encapsulation of *Methylobacterium aminovorans* in PVA nanofibers for coating peanut seeds, resulting in improved germination, root and shoot length, seedling vigor, dry mass production, and plant biomass.

Zheng et al. (2022) encapsulated *Bacillus pumilus* in PVA/Sodium alginate microspheres for a pot trial with *Ipomoea Nil* under drought and salt stress, showing increased root and stem length, plant dry weight, bacterial population, and soil enzyme activities.

Lopes et al. (2024) developed an encapsulation strategy using PVA/starch-based matrix to enhance *Bacillus megaterium* cell viability, showing improved viability under heat stress compared to free bacteria. These studies demonstrate the potential of PVA-based formulations for encapsulating microorganisms with agricultural benefits, enhancing bacterial survival, colonization, and growth parameters. PVA encapsulation technologies offer a promising approach for developing efficient microbial inoculants.

1.2.2 Cationic Starch (CS)

The chemical modification of natural starch has been extensively studied in various industries to enhance its properties. During this process, functional groups are introduced into the starch molecules while preserving its granular structure and size distribution. Cationic starch (CS) is an example of such modification, where natural starch is chemically altered to create a synthetic polymer (Goswami and Mahanta, 2021). Cationic starch overcomes the drawbacks of natural starch, like low solubility in water, weak mechanical properties, and thermal instability. This modification introduces positively charged groups into the starch structure (Figure 3), resulting in improved water solubility and excellent film-forming properties. These enhancements broaden the potential applications of cationic starch, particularly when combined with other materials (Nornberg et al., 2022).

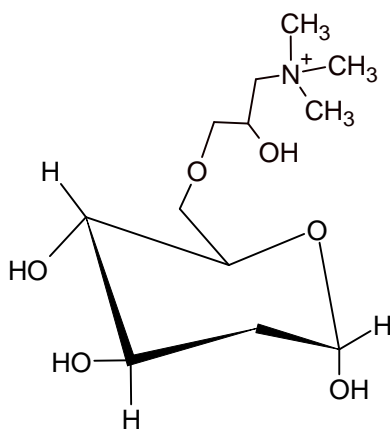


Figure 3. Structural formula of Cationic Starch (CS).

Cationic starch has been widely studied for its diverse applications, including as a flocculant in water treatment, an additive in industries such as textiles, paper, and cosmetics, and for controlled drug release in medicine. Its cost-effectiveness, biocompatibility, and rapid degradation make it a versatile material (Chatterjee et al., 2023; Goswami and Mahanta, 2021; Nornberg et al., 2022). Recent research has also explored its use in immobilizing microorganisms, enzymes, and phenolic compounds, as well as in controlled pesticide release and film coatings for packaging (El-Naggar et al., 2021; Kurabetta et al., 2023; Lima-Tenório et al., 2023; Mohamed et al., 2016; Petry et al., 2024; Simanaviciute et al., 2017; Wang et al., 2022). Here are some key studies on the agricultural applications of cationic starch.

Wang et al. (2022) investigated the controlled release of the herbicide Alachlor using a matrix of Alginate/Bentonite with cationic starch as a modifying agent. The addition of cationic starch improved adsorption capacity and reduced permeability, resulting in a slower release of the pesticide. The release profile showed effective control, with a significantly longer time to release 50% of the active ingredient compared to the pure alginate hydrogel.

Lima-Tenório et al. (2023) studied the encapsulation of *Azospirillum brasilense* AbV5/6 in microparticles made of Starch/Cationic Starch/Chitosan cross-linked with sodium tripolyphosphate. The encapsulation maintained the viability of *A. brasilense* for 60 days and promoted corn growth in greenhouse experiments. The use of microparticles led to increased plant mass and root length under controlled conditions, as well as improved chlorophyll levels in corn plants in the greenhouse.

Petry et al. (2024) evaluated a PVA/Cationic Starch matrix for coating acerola fruits and optimized the concentration of sorbitol as a plasticizing agent. The coated fruits showed reduced moisture content, preserved color, and slowed ripening, extending their shelf life by one day compared to the control when stored at room temperature.

In conclusion, these studies demonstrate the potential of cationic starch in agricultural applications. Its use in herbicide release control, microorganism encapsulation, and fruit coating shows promising results in enhancing plant growth and product quality. Cationic starch modification offers a valuable approach for improving agricultural practices and product preservation.

1.2.3 Crosslinking Agents

In the context of using both PVA and cationic starch, which are known for their hydrophilic properties, crosslinking agents are commonly employed to create an encapsulation matrix that facilitates the controlled release of the encapsulated material (Rynkowska et al., 2019). Various crosslinking agents have been studied in the literature to enhance the stability of polymer bonds and their properties, including citric acid, sodium trimetaphosphate, boric acid, glutaraldehyde, among others (Bates et al., 2020; Chang et al., 2019; Gadhav et al., 2019; Liu et al., 2017; Yu et al., 2021). In this study, citric acid and sodium trimetaphosphate were explored as crosslinking agents for PVA-based matrices.

Citric acid, a non-toxic and readily available metabolic product, possesses a simple reaction mechanism. Its multi-carboxyl structure (Figure 4) allows it to serve as a cross-linker, enhancing the intermolecular bonding of the mixture by introducing covalent bonds that complement the natural intermolecular hydrogen bonds. This reinforcement improves mechanical properties and stability in water (Jose and Al-Harathi, 2017). Recent research has highlighted the use of citric acid as a crosslinker in controlled release applications in medical fields (Ghorpade et al., 2019; Mali et al., 2023), packaging film production (Jiang et al., 2023; Sornsumdaeng et al., 2021; Wen et al., 2021; Zhang et al., 2023), and coatings for fruits and seeds (Amelia et al., 2024; Wardak et al., 2024). Citric acid has demonstrated its ability to enhance matrix characteristics, influencing compound release, improving mechanical and thermal properties, and reducing swelling and solubility.

Sodium trimetaphosphate (STMP) (Figure 4) is a safe crosslinking agent compatible with starch and PVA, extensively studied for medical applications (Jeong et al., 2021; Pan et al., 2021; Riahi et al., 2017). It facilitates the formation of intra- and intermolecular bonds at random sites within the polymer of interest through esterification reactions. This process occurs in an alkaline environment, activating the hydroxyl groups of the polymer. The esterification reaction leads to crosslinking of the material, decreasing its interaction with water and enhancing the structural integrity of the matrix by forming a robust polymer network (Cagnin et al., 2021). While STMP is not commonly used as a crosslinking reagent in agricultural products, recent studies have explored its potential to enhance the physical and mechanical properties of various biodegradable polymeric matrices (Cagnin et al., 2021; Chandak et al., 2022; Joglekar et al., 2020; Noulis et al., 2023).

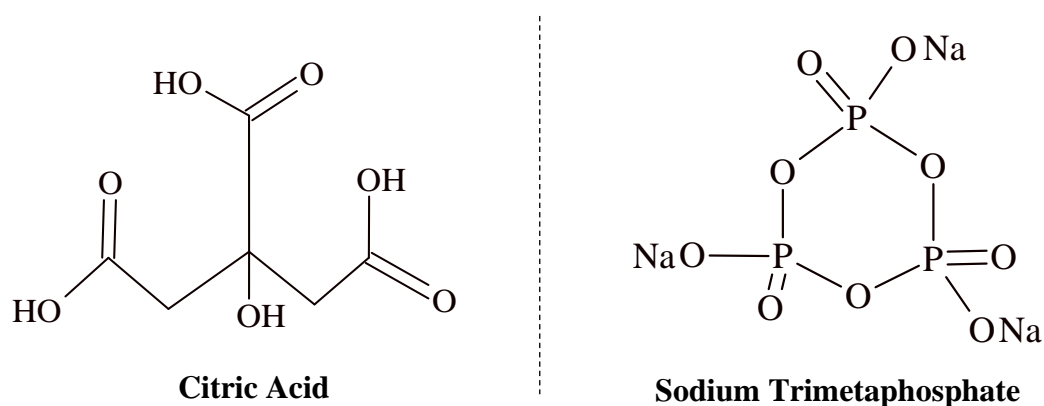


Figure 4. Chemical structures of the crosslinkers citric acid (CA) and sodium trimetaphosphate (STMP).

Both citric acid and STMP are considered biocompatible, sustainable, and promising agents for use in formulations intended for microbial encapsulation. Figure 5 illustrates the possible bonds in a PVA-based matrix with or without the presence of cationic starch, in the presence of the cross-linking agent's citric acid and STMP. Both PVA and cationic starch contain -OH groups in their structures, facilitating the formation of bonds between the molecules themselves (PVA-PVA; CS-CS) and between PVA and cationic starch (PVA-CS). Additionally, citric acid and STMP can form hydrogen bridges between the molecules of these polymers, enhancing the stability of these bonds. These interactions can create a more cohesive and stable network, enhancing the effectiveness of encapsulation and the protection of the encapsulated cells.

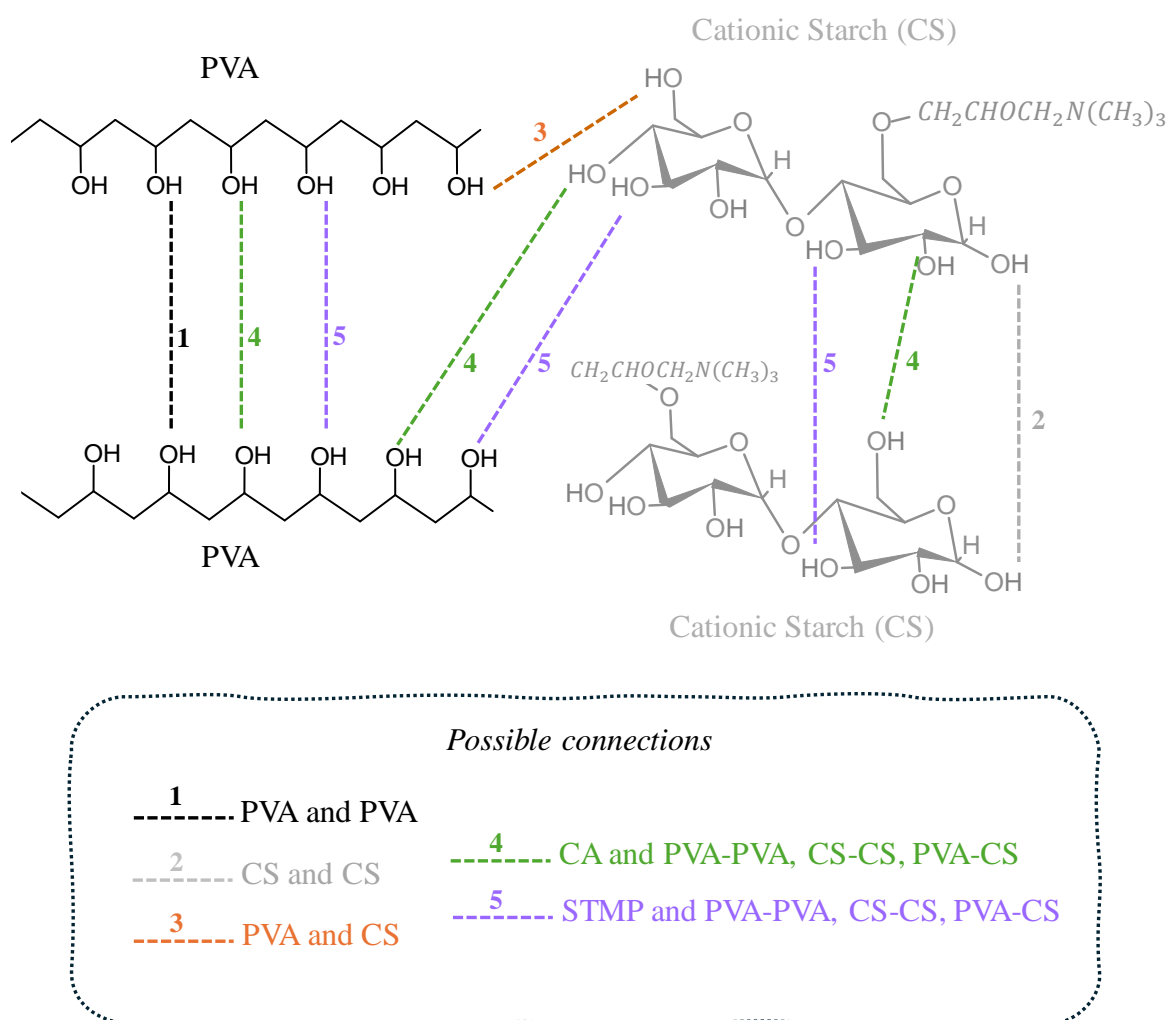


Figure 5. Schemes illustrating the possible interactions that occur in the matrix in matrices based on PVA and PVA/Cationic-starch. PVA (polyvinyl alcohol), CS (cationic starch), CA (citric acid), STMP (sodium trimetaphosphate).

1.3 Microorganisms

Bacteria belonging to the genus *Bacillus* are gram-positive, spore-forming, and rod-shaped microorganisms. They are naturally present in soil and vegetation and, along with other plant growth-promoting bacteria such as *Acetobacter*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Klebsiella*, *Pseudomonas*, and *Serratia*, play a crucial role in agriculture (Radhakrishnan et al., 2017). *Bacillus* species have been extensively studied for their ability to enhance plant growth and development by promoting nitrogen fixation, phosphorus and potassium solubilization, and disease suppression through indirect mechanisms (Cho and Chung, 2020; Khan et al., 2022). They also exhibit extended shelf life in bioformulations, efficient colonization of plant tissues, and antifungal properties (Shahid et al., 2021).

In this study, *Bacillus megaterium* was encapsulated in PVA-based matrices using various production techniques. This versatile microorganism is found in diverse habitats such as soil, seawater, rice fields, honey, fish, and dried food. *Bacillus megaterium* can utilize 62 different carbon sources for growth and produce various industrial products like enzymes, proteins, and vitamins. It is known for its wide temperature tolerance range from 3°C to 45°C (Goswami and Mahanta, 2021). The ability of *Bacillus megaterium* to solubilize phosphorus (P) and potassium (K) in soil, promote plant growth, regulate soil bacterial community structure, and inhibit pathogens has been documented in the literature (Zhao et al., 2021).

Despite the numerous benefits *Bacillus* strains offer for plant growth, their application as microbial inoculants in the field are limited due to cell viability loss under adverse environmental conditions. Factors such as competition with other soil microorganisms, exposure to agrochemicals, heat, high temperatures, salinity, water stress, and UV radiation can impact the survival of these microorganisms in the field. Therefore, it is crucial to develop formulations that ensure the protection and viability of *Bacillus* strains from storage to field application, facilitating successful microbial colonization in plants and soil.

1.4 Exposure of Microorganisms to Field Stresses

To ensure sustainable agricultural production and food quality, it is important to address challenges that affect cultivation, such as the decrease in arable land and adverse environmental factors. However, it is crucial to consider the stresses that microorganisms face in the field, including the physicochemical conditions of the soil, competition with native microbiota,

exposure to agrochemicals, water scarcity, salinity, heat, and UV radiation (Inbaraj, 2021; Koza et al., 2022; Kumar et al., 2022). Figure 6 illustrates these challenges.

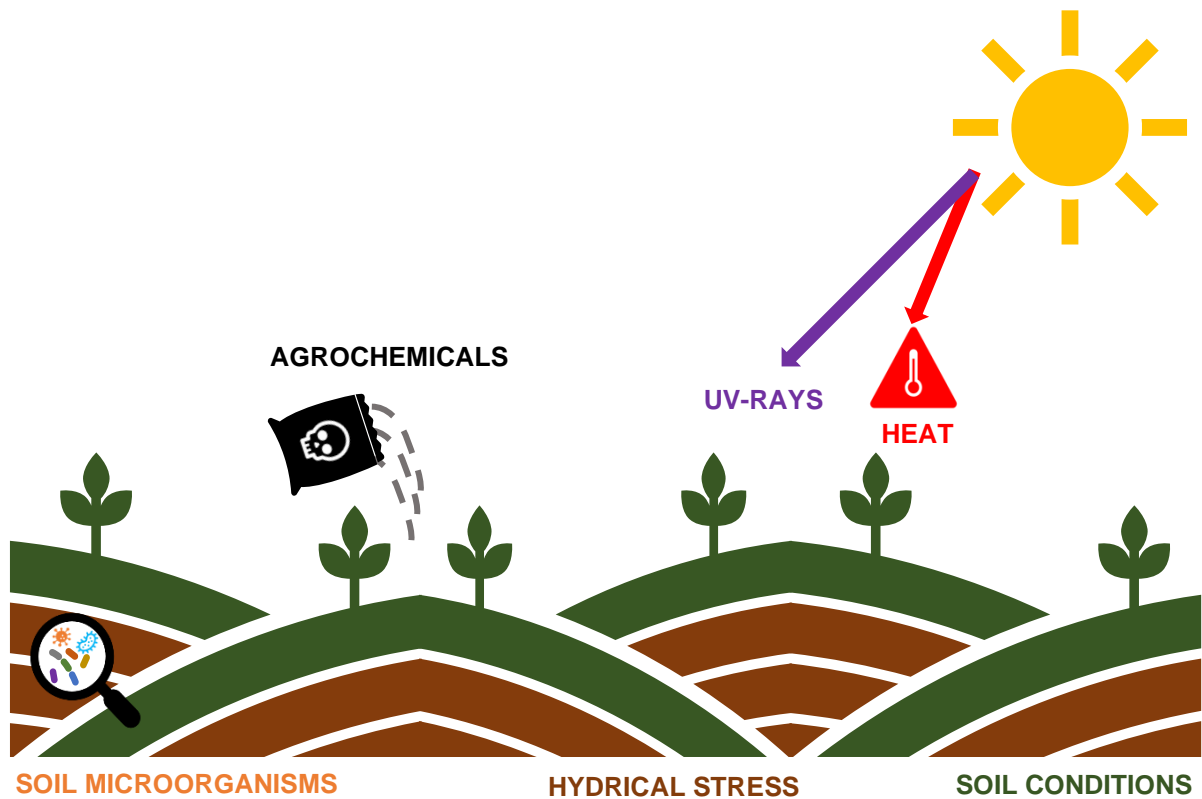


Figure 6. Representation of the main stresses that microorganisms are exposed to when applied to the soil.

The application of agrochemical substances, such as fungicides and insecticides, in soil and seed treatments is a common practice in modern agriculture to control pests and diseases. However, these products can harm the survival and effectiveness of microorganisms in the agricultural environment. Therefore, it is crucial to develop formulations that enhance the resistance and viability of these microorganisms, particularly *Bacillus*, which has biocontrol potential (Meftaul et al., 2020). While microorganisms are sensitive to specific environmental conditions, recent studies have explored the combined use of microorganisms and pesticides as a strategy to reduce reliance on agrochemicals in soil, while improving disease control in plants (Guimarães et al., 2018; Ons et al., 2020; Park et al., 2021).

Moreover, high UV exposure, heat stress, and water stress in the soil pose significant threats to agricultural production. These stresses can have adverse effects on the viability of microorganisms and various stages of plant development (Ahmad et al., 2022). However,

research on the survival of microorganisms exposed to UV light and high temperatures for agricultural applications is limited, with most studies focusing on cells for food processing or the elimination of *Bacillus* as contaminants. Therefore, there is a substantial knowledge gap regarding the behavior of microorganisms under the mentioned stresses (Cramer et al., 2018; Wen et al., 2022).

The lack of data on how *Bacillus* respond to the mentioned stresses underscores the importance of gaining a better understanding of their behavior. This highlights the urgent need to study formulations that ensure the protection and viability of these microorganisms at all stages, from storage to application in the field.

1.5 Objectives

1.5.1 General Objective

This study focused on developing innovative formulations of microbial inoculants for agricultural use, specifically focusing on encapsulating *Bacillus megaterium*. The primary goal was to create effective formulations using Polyvinyl Alcohol (PVA) based matrices and the techniques casting, emulsion, and spray-drying to protect and maintain the viability of the encapsulated microorganism against stresses. These formulations were designed to promote the growth and development of crops, with a particular emphasis on soybean seeds.

1.5.2 Specific objectives

To achieve the overall objective, the following steps were undertaken:

1. Production of *films using the casting method* from PVA-based formulations with the addition of citric acid as a crosslinking agent. Morphological, physicochemical, mechanical, and thermal characterizations of the matrices were conducted, along with assessing the release profile of *Bacillus megaterium*, protection against stress exposure, and cell viability during a 6-month storage period. The film with the best performance was evaluated for soybean seed coating application and its impact on seed germination over a 6-month storage period.
2. Production of *microparticles using the emulsion method*, exploring the use of citric acid and sodium trimetaphosphate as cross-linking agents and bentonite as a surface

modification agent in PVA/cationic starch-based formulations. Morphological, physicochemical characterizations, release of bacteria, and protection under stress conditions were evaluated. An adapted Accelerated Shelf-Life Test (ASLT) methodology was applied to estimate the shelf life of the formulations.

3. Production of *microparticles using the spray-dryer method*, investigating the addition of zinc oxide to PVA/cationic starch-based formulations. Morphological, physicochemical characterizations, release of encapsulated bacteria, and protection under stress conditions were assessed. An adapted ASLT methodology was used to estimate the shelf life of the formulations. The performance of the zinc formulation was evaluated in a greenhouse trial with soybeans to analyze its impact on plant growth and development.

1.6 Thesis Structure

The research studies in this doctoral thesis were structured into chapters. The focus of this work was to create an encapsulation platform for the *Bacillus megaterium* strain using Polyvinyl Alcohol (PVA) as a polymer for agricultural applications to enhance the protection of the encapsulated bacteria, particularly under environmental stress conditions. **Chapter 1** provides a comprehensive literature review, essential background information, and research objectives.

Chapter 2 details the production of biodegradable films to encapsulate *Bacillus megaterium* using a PVA matrix cross-linked with citric acid at varying concentrations (0 to 30% w/v). The chapter discusses the morphological, physical, chemical, thermal, and mechanical properties of the films, as well as the performance of the encapsulated bacteria, including release, protection against stress factors like heat and exposure to agrochemicals, and viability after storage. The study also examines the coating of soybean seeds and the impact of the formulations on seed germination over six months. The results were compiled in an article titled “*PVA-based Biodegradable Films for the Distribution of Bacillus megaterium as a Seed Coating.*”

Based on the findings in **Chapter 2**, citric acid was found to enhance the properties of PVA-based films for seed coating. However, initial experiments on microparticle production revealed that both PVA and the PVA/citric acid combination did not have the ideal characteristics, especially in terms of viscosity, for forming particles via emulsion. To address this viscosity issue, preliminary tests were conducted with PVA in combination with citrus pectin, carboxymethylcellulose, starch, and cationic starch to produce emulsion microparticles. Only cationic starch exhibited the necessary characteristics for microparticle formation, even without a cross-linking agent, using a PVA/cationic starch composite matrix.

Chapter 3 describes a simple emulsion method for microencapsulating *Bacillus megaterium* using a PVA-based matrix with cationic starch. The study explores the use of citric acid and sodium trimetaphosphate as cross-linking agents and bentonite as a surface modifier. The resulting microparticles were analyzed for physicochemical and morphological characteristics, bacterial release, and viability profiles under various stress conditions. An accelerated shelf-life test (ASLT) was used to estimate cell viability during storage. The results were compiled in an article titled “*Microencapsulation of Bacillus megaterium in PVA/Cationic Starch-based Matrices.*”

Chapter 4 presents the results of producing microspheres using the spray drying method to encapsulate *Bacillus megaterium* in a PVA/cationic starch matrix. The study also explores the inclusion of zinc oxide in the formulation to enhance plant growth and zinc availability. Zinc oxide, an essential micronutrient, has been extensively studied in UV protection formulations. Like bentonite, zinc oxide modifies the matrix surface and provides increased protection to encapsulated microorganisms. The physicochemical and morphological properties of the formulations, bacterial release profiles, and protection under various stress conditions were evaluated. The shelf-life of the materials was determined using the ASLT methodology. A greenhouse test was conducted to assess the performance of the ZnO-containing matrix in growing soybeans. The results were compiled in an article titled “*Spray-Dryer Microencapsulation of Bacillus megaterium in PVA/Cationic Starch/Zinc Oxide for Promoting Growth and Zinc Availability in Soybean Plants.*”

Finally, **Chapter 5** presents the conclusions and prospects of this work.

CHAPTER 2

Biodegradable PVA-based films for delivery of *Bacillus megaterium* as seed coating

Abstract

The synthetic polymer Polyvinyl alcohol (PVA) is known for its water solubility, non-toxicity, biodegradability, and high biocompatibility with cells. These characteristics, along with its ability to be degraded by various microorganisms, including *Bacillus* sp., make it a promising candidate as a vehicle for beneficial microorganisms in sustainable agricultural practices. In this study, we developed biodegradable films made of PVA crosslinked with citric acid (in concentrations ranging from 0 to 30%) to deliver encapsulated a strain of *Bacillus megaterium* as seed coating. Physical-chemical and morphological analyses showed promising compatibility between PVA and citric acid, with the latter improving the thermal behavior of the films. The addition of 30% citric acid (F-30CA) significantly reduced the swelling and solubility of the films, thereby increasing their resistance to degradation, a crucial quality for seed coating. We observed that the F-30CA matrix allowed for the controlled release of *B. megaterium*, maintaining its cell viability during six months of storage and after exposure to adverse conditions. After exposure to heat (55°C/2 h) and insecticide (2 h), the PVA film with citric acid maintained cell viability in the same order as the initial concentration (10^6 CFU/g), whereas this was not observed in free bacteria, which experienced a decrease of 2 to 4 orders of magnitude in cell viability (10^9 to 10^5 CFU/mL). The F-30CA film with encapsulated *B. megaterium* also promoted soybean seed germination rates comparable to treatment with commercial polymer and superior to untreated seeds, demonstrating its viability for agricultural applications. The PVA film crosslinked with citric acid showed promising potential for encapsulating *Bacillus megaterium* and seed coating, benefiting microorganism protection and seed germination performance.

Keywords: Polyvinyl Alcohol, *Bacillus megaterium*, Seed coating, Seed germination, Stress exposure.

1. Introduction

Microbial inoculants are bio-based products that utilize beneficial microorganisms to improve soil quality, plant growth, and crop yield (Alori and Babalola, 2018). These microorganisms, including fungi and bacteria naturally present in the soil rhizosphere, promote plant growth by colonizing crop roots, providing nutrients, producing phytohormones, and protecting against pathogens and adverse environmental conditions (Bargaz et al., 2018; Stamenković et al., 2018). Among them, *Bacillus* bacteria promote several beneficial effects on plants, such as promoting growth (de Sousa et al., 2021; Gohil et al., 2022; Zhang et al., 2023), solubilizing insoluble phosphorus (Iqbal et al., 2024; Prakash and Arora, 2019; Silva et al., 2023) and acting as biocontrol agent (Jan et al., 2023; Miljaković et al., 2020; Zhang et al., 2023). Their incorporation into seed coating matrices can promote a more uniform cell distribution during application, leading to better field efficiency. It can facilitate direct contact between beneficial microorganisms and emerging seedlings, helping in the early establishment of beneficial microbial communities in the rhizosphere. This approach is also a simple alternative for farmers who do not want to invest in specialized equipment for microbial products (Paravar et al., 2023). Biodegradable films with added microorganisms are being explored in the seed treatment industry to increase agricultural productivity. These films have shown promising results for soybean, wheat, maize, and canola crops (Accinelli et al., 2018; Jarecki and Wietecha, 2021; Paravar et al., 2023; Vercelheze et al., 2019).

For a microbial inoculant to be effective, it needs to be protected and maintained during storage and field application (Inbaraj, 2021; Koza et al., 2022; Kumar et al., 2022). The film must create a favorable environment that supports the growth and development of microorganisms and seeds. In this sense, one challenge is developing coatings with lower water solubility to ensure the long-term preservation of the seeds integrity but still capable of permeating water in certain conditions to allow seed germination (Pirzada et al., 2020). In this scenario, Polyvinyl alcohol (PVA) becomes a good candidate. PVA is widely used in various industrial sectors due to its water-soluble, non-toxic, biodegradable properties and high biocompatibility with cells, besides its adhesive properties, oxygen barrier, flexibility, and good tensile and abrasion resistance (Alvarado et al., 2019; Havstad, 2020; Singh et al., 2021). Furthermore, recent studies have shown the ability of microorganisms to biodegrade PVA partially or completely (Mohamed et al., 2022; Pantelic et al., 2021; Ullah et al., 2019), which can significantly reduce the environmental impact caused by the residual accumulation of this

type of plastic in the agricultural environment. These characteristics enhance the motivation for using PVA as a delivery vehicle for beneficial microorganisms in more sustainable agricultural practices. Such characteristics intensify the motivation for using PVA as a delivery carrier of beneficial microorganisms for more sustainable agricultural practices. PVA can be crosslinked with citric acid (CA), a non-toxic organic compound, reducing its solubility. Citric acid is a multi-carboxylic structure which reinforces intermolecular bonds, improving the mechanical properties and stability of polymeric matrices (Jose and Al-Harthi, 2017; Salihu et al., 2021). Studies have shown that this reaction increases thermal and mechanical strength and decreases the solubility and swelling of matrices such as carboxymethylcellulose-poly(ethylene glycol) and PVA/starch/graphene nanocomposites in water (Ghorpade et al., 2019; Jose and Al-Harthi, 2017).

Here, we propose the development of biodegradable films based on PVA crosslinked with citric acid to deliver encapsulated *Bacillus megaterium* as seed coating. We investigated the films physical-chemical, morphological, and mechanical characteristics, as well as their barrier properties. Additionally, we examined their effectiveness in protecting bacteria under adverse field conditions, such as heat exposure and contact with insecticide. The developed formulations were evaluated for their potential in coating soybean seeds to investigate their impact on seed germination and cell viability during a six-month storage period.

2. Materials and methods

2.1 Materials and Microorganisms

The films were produced with Polyvinyl Alcohol (PVA – molecular weight 89.000-98.000, 99+% hydrolyzed, Sigma Aldrich) as the polymeric base and crosslinked with anhydrous citric acid (CA - Vetec). The bacterium used was *Bacillus megaterium* (CNPMS B119) from the Multifunctional Microorganisms Collection of Embrapa Maize and Sorghum (Sousa et al., 2021).

2.2 Film Preparation

PVA solution (10% by weight - wt%) was initially prepared in a water bath at 80-90 °C until complete dissolution (Schlieker and Vorlop, 2006). The solution was cooled to 30 °C, and then citric acid at concentrations of 0 (F-0CA), 10 (F-10CA), 20 (F-20CA), and 30 (F-30CA) (wt% polymer dry weight) was added under constant stirring for 15 min at 25 °C (Shi et al.,

2008b). The solutions were spread on Teflon plates for film formation and dried in ovens with air circulation for 24 h at 25 °C.

2.3 Characterizations

2.3.1 Morphological and Physicochemical Analysis

The film samples were fractured in liquid nitrogen. Scanning Electron Microscopy (SEM) observed the fracture surface using a JEOL (JSM-6510) at an accelerating voltage of 5 kV, a working distance of 10 mm, and a secondary electron detector. Fourier Transform Infrared Spectroscopy (FTIR) analyzed the polymer blends molecular structure using a Bruker Vertex 70 model. The FTIR was performed in the 4000-500 cm⁻¹ with a resolution of 4 cm⁻¹. Moreover, to identify the phases present in the material, X-ray diffraction (XRD) analyses of the films produced were carried out using a LabX XRD-6000 diffractometer (Shimadzu, Japan). The diffractometer was operated with Cu-K α radiation ($\lambda = 1.54056 \text{ \AA}$), a voltage of 30 kV, and a current of 30 mA. The diffractograms were recorded for 2θ from 4° to 70°, and a continuous scanning speed of 1°/min was used. The degree of crystallinity (% X_c) was calculated based on the proportions of crystalline and amorphous areas, as determined by Equation 1.

$$\%X_c = \frac{\text{crystalline area}}{\text{crystalline area} + \text{amorphous area}} * 100 \quad (1)$$

2.3.2 Thermal analysis

Thermogravimetric analysis (TGA - TA Instruments, model TGA Q500) was used to evaluate the thermal behavior and stability of the films. Samples were heated in a nitrogen atmosphere at 10 °C/min from 30 to 600 °C.

2.3.3 Swelling and solubility degrees

Swelling and solubility analyses were carried out in the same experiment (Bigi et al., 2004; Gontard et al., 1992). The films were cut (2x2 cm) and dried in an oven at 100 °C for 24 h, and their weight was recorded (m_0). Samples in triplicate were placed in a Falcon tube containing 50 mL of sterile distilled water and placed in an orbital incubator at 25 °C with gentle shaking. Time points of 1, 2, 4, and 24 h were observed for both analyses to determine the swelling. The films were carefully removed from the water with tweezers, and their surface was

lightly dried on absorbent paper to remove excess water and weighed again (m_1). The swelling degree of the materials could be determined from the relationship between wet and initial weight as a function of immersion time (Equation 2).

$$\text{Degree of Swelling (\%)} = \frac{m_1 - m_0}{m_0} * 100 \quad (2)$$

After weighing the wet films, the samples were dried again in an oven at 100 °C for 24 h, and their dry weight (m_2) was recorded. The materials solubility was calculated from the difference between the initial and final weights using Equation (3).

$$\text{Degree of Solubility (\%)} = \frac{m_0 - m_2}{m_0} * 100 \quad (3)$$

2.4 Preparation of the Inoculum and the Encapsulation Process

To reactivate the stocks of *B. megaterium* (-80°C), the bacteria were inoculated on a Petri dish containing Soybean Tryptone Broth (TSB) and bacteriological agar at 30 °C for 12h. Then, the bacteria colony was incubated in a liquid TSB medium with constant agitation at 250 rpm, 30°C for 72 h to obtain cells used for film inoculation. A volume equivalent to 10⁹ CFU/g of dry polymer weight was added to the polymeric solutions (described in section 2.2) at 25 °C for encapsulation. Inoculation solutions were placed on Teflon plates and dried in an oven with air circulation at 25 °C for 24 h to form a film.

2.5 Microorganism Release Profile from the Matrices

The release profile of *B. megaterium* encapsulated in the films was evaluated. For this, 1g of film was placed in 50 mL of 0.85% NaCl saline solution and incubated in a rotary incubator at 30 °C and 250 rpm. Samples of 1 mL were taken after 2, 24, and 48 h, and serial dilutions of these samples were inoculated onto TSB medium and bacteriological agar. Biological and analytical duplicates of each dilution were used in this assay. The inoculated plates were then incubated at 25 °C for 24 h for colony counting.

2.6 Exposure to Stress Conditions

2.6.1 Thermal Exposure

Bacillus megaterium, in both free form (10^9 CFU/mL) and encapsulated form (10^9 CFU/g), was placed in Falcon tubes and exposed to 55 °C for 24 h in an oven with air circulation. After exposure, 5 mL of saline solution (0.85% NaCl) was added to the samples, and the encapsulated bacteria were released for 2 h at 30°C with agitation at 250 rpm. Both samples were then serially diluted and plated on TSB and bacteriological agar. Biological and analytical triplicates were prepared for each dilution. The inoculated plates were incubated at 25°C for 24 h to enumerate the colonies.

2.6.2 Insecticide Exposure

Bacillus megaterium, in both free form (10^9 CFU/mL) and encapsulated form (10^9 CFU/g), was placed in Falcon tubes and incubated in 5 mL Thiamethoxam ($1600 \mu\text{g mL}^{-1}$) solution for 24 h at 30 °C (Shahid and Khan, 2018). After exposure, both samples were then serially diluted and plated and plated on TSB and bacteriological agar. Biological and analytical triplicates were prepared for each dilution. The inoculated plates were incubated at 25°C for 24 h to enumerate the colonies.

2.7 Viability of Free and Encapsulated Bacteria in the Films

Two milliliters of *B. megaterium* solutions were stored in Eppendorf tubes and kept in a refrigerator at 4 °C to evaluate their viability over six months. Meanwhile, encapsulated films were stored in a controlled environment (45% relative humidity and 25 °C) in ten milliliter Falcon tubes (with one gram equivalent to 10^9 CFU/g) in triplicate for the same duration. Afterward, the encapsulated bacteria were incubated in saline solution (0.85% NaCl) and released for 2 h at 30 °C. After encapsulation, free and released bacteria were sampled and subjected to serial dilutions, which were then inoculated into TSB and bacteriological agar. The samples were then incubated for 24 h at 25 °C for colony counting.

2.8 Coating and Viability of Soybean Seeds

The seed coating protocol was based on commercial guidelines and involved adapting the process by adding the seeds to be coated into a portable drum coater to simulate industrial processes (Accinelli et al., 2018, 2016; Brasil, 2009; Prasad et al., 2020; Teixeira et al., 2021). The entire procedure was conducted under continuous agitation. Initially, 1 kg of seeds was introduced into the equipment, adding 2.5 mL of fungicide (Maxin, Syngenta) and 2.5 mL of insecticide (Cropstar, Bayer) to the seeds until they dried. Subsequently, 4 mL of commercial

polymer (Azul Labfix – Laborsan Agro Brasil LTDA) or F-0CA or F-30CA polymer solution inoculated with *B. megaterium* (2.40×10^5 CFU/seed) were incorporated into the seeds, followed by the addition of 2g of drying powder (Labsec SuperFluid Graf – Laborsan Agro), being stirred for about 5 minutes. The coated seeds were dried in air-circulating ovens for 24 h at 25 °C. Table 1 describes the treatments and their respective codes.

Table 0-1. Treatments used in the coating of soybean seeds and their respective codifications.

Code	Treatment
Control	No Treatment
Agrochemicals	Only fungicides and insecticides
Commercial polymer	Fungicide, insecticide, and commercial polymer
F-0CA	Fungicide, insecticide, and encapsulated F-0CA
F-30CA	Fungicide, insecticide, and encapsulated F-30CA

After these steps, a portion of the coated seeds was divided into 50 mL Falcon tubes (100 seeds in triplicate for each treatment) and stored in a controlled environment with 45% relative humidity and a temperature of 25 °C for six months. After these steps, a portion of the seeds coated with F-0CA and F-30CA were stored in 50 mL Falcon tubes (100 seeds in triplicate for each treatment) and a controlled environment with a relative humidity (RH) of 45% and a temperature of 25 °C for six months. At zero, three, and six months, the encapsulated bacteria in the seed coat were then released (100 seeds/100 mL of 0.85% NaCl saline solution) in a rotating incubator at 250 rpm for 2 h, 30 °C. The solution with the bacteria released in the medium was then subjected to serial dilutions, which were inoculated into TSB and bacteriological agar. The samples were then incubated for 24 h. at 25 °C to count colonies.

2.8.1 Germination of Soybean Seeds Coated

Additionally, the germination of soybean seeds coated with different treatments was assessed to investigate the effect of matrices inoculated with *B. megaterium* on initial development over six months. For each treatment, 200 seeds were randomly selected, placed between sheets of germination paper (15 seeds per sheet), rolled, and moistened with sterile distilled water. Secured with rubber bands, these rolls were positioned vertically in a plastic container, with sterile water added daily to maintain a constant depth of 2 cm. The number of normal seedlings was recorded after eight days of incubation in a germination chamber at 25 °C, 70% relative humidity, and a 12 h photoperiod. The germinated seeds (GS) percentage

was calculated according to Equation 4. The aerial parts and roots of the germinated seeds were separated and dried in an air-circulating oven at 60 °C for 48 h, and the dry weight was recorded. These experimental data were verified by analysis of variance and Tukeys test at a confidence level of 95%, using Origin 9.8.0.200 software.

$$Gs = \frac{\text{number of germinated seeds}}{\text{total number of seeds}} * 100 \quad (4)$$

3. Results and Discussion

3.1 Morphological, Physicochemical, and Thermal Characterizations

The fracture areas of the PVA-based films, using different concentrations of citric acid (CA) as a crosslinking agent, were examined by SEM (Figure 1). The F-0CA (Figure 1a-b) film demonstrated small pores on its surface and a rough texture along the fracture. The F-10CA (Figure 1c-d) and F-20CA (Figure 1e-f) films exhibited streaks and a rougher surface, and the F-30CA (Figure 1g-h) film had a more compact and dense structure, with streaks but without the same roughness observed in the other films. Despite the surface defects in the citric acid-containing films, unlike F-0CA, the absence of pores in these samples suggests adequate compatibility between PVA and citric acid, which is consistent with previous studies (Jose and Al-Harathi, 2017; Salihu et al., 2021; Wahid et al., 2018; Wang et al., 2014).

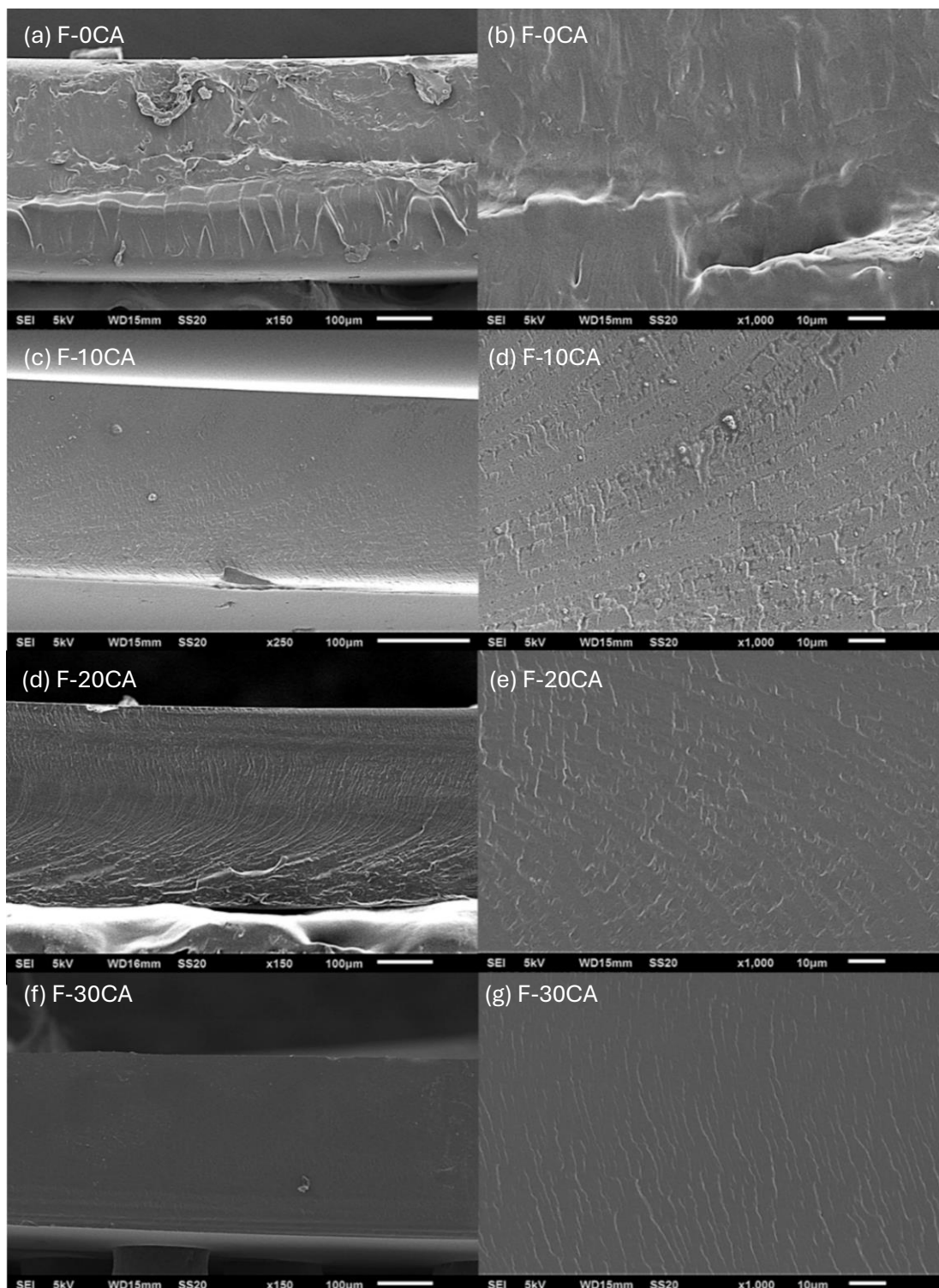


Figure 1. Fractures of PVA:CA films by Scanning Electron Microscopy (SEM) micrographs. (a) - (b) F-0CA, (c) - (d) F-10CA, (e) - (f) F-20CA, and (g) - (h) F-30CA

The XRD patterns of the PVA-based films are illustrated in Figure 2a. The relative intensities of PVA characteristic peaks at observed 19.4° and 41° (2θ) increase with the CA amount in their composition (Wahid et al., 2018). The crystallinity degree of the films (Table

2) was measured from the diffractograms by the ratio of the peak area to the total curve area. The materials crystallinity decreased from 63.58% (F-0CA) to 48.38% (F-30CA) as the concentration of CA increased in the matrix, suggesting that CA influenced the crystalline ordering of the film. A similar crystallinity value was observed for the PVA powder (44.54%), a natural semi-crystalline material.

The FTIR spectra are shown in Figure 2b. The PVA and CA bands generally overlap with the citric acid addition to the matrices. For example, although band overlap is also observed in the region between 1817 – 1510 cm⁻¹, there is a shift of the peak from 1654 cm⁻¹ (attributed to C-H deformation, related to PVA) to ~1724 cm⁻¹, which can be attributed to the increase in the concentration of COO- groups linked to citric acid (Das et al., 2011; Jose and Al-Harhi, 2017).

Table 0-2. XRD obtained degrees of crystallization (X_c) of the PVA (powder) and films.

Formulations	% X_c
PVA (powder)	44.54
F-0CA	63.58
F-10CA	62.98
F-20CA	60.55
F-30CA	48.38

The thermal analysis of pure PVA and the formulations confirm the crosslink, as seen in Figure 2c-d, showing that CA modifies the thermal behavior of the PVA matrix. Four main thermal events exist in the TGA and DTG curves with and without CA matrices. The first thermal event is associated with the water loss centered at around 100 °C up to 200 °C. The second thermal event related to matrices containing CA can be attributed to the beginning of the thermal decomposition of citric acid. However, a shift in the center of the event from 190 °C to 220 °C is observed. The subsequent thermal event centered at 365 °C is evident only for F-0CA and is related to the initial thermal decomposition of the PVA polymer chains. This behavior indicates an increase in the thermal stability of matrices containing CA since the following thermal decomposition event in all films is centered at 348 °C. The final thermal event at 425 °C corroborates the increase in thermal stability, as the amount of carbonaceous material formed before complete decomposition is significantly greater for matrices containing CA. These thermal events were also observed by other authors when investigating matrices

based on PVA/starch/graphene nanocomposites (Jose and Al-Harhi, 2017) and starch/polyvinyl alcohol/citric acid (Wu et al., 2017).

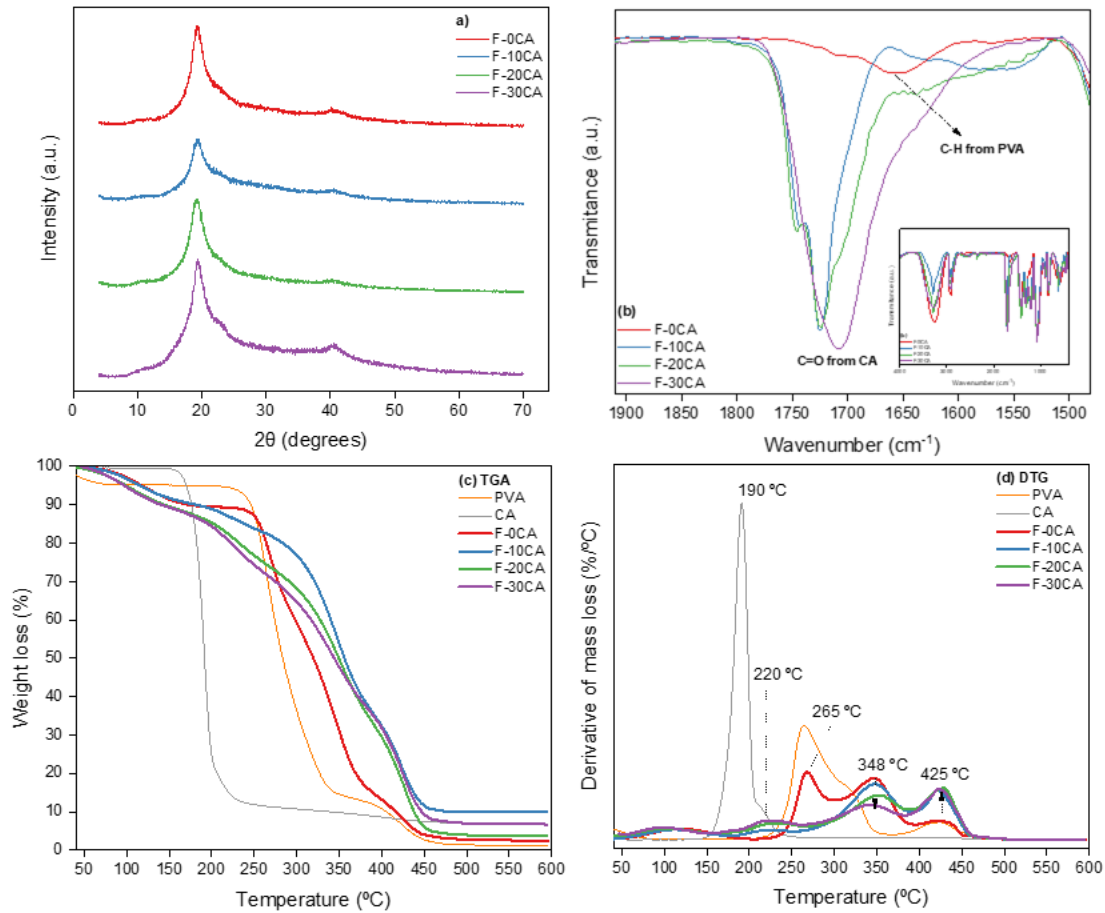


Figure 2. Physicochemical characterizations of the films. (a) X-ray diffraction (XRD); (b) Fourier Transform Infrared Spectroscopy (FTIR); Thermograms (c) weight loss (TGA); (d) weight loss derivative (DTG).

The assessment of PVA-based film behavior in solution included the analysis of swelling degree (Figure 3a) and solubility (Figure 3b). The films F-0CA and F-10CA were more susceptible to swelling than the others after the first hour, while F-30CA showed the lowest swelling over time. This reduction can be attributed to potentially stronger interactions between PVA chains and CA. Concerning solubility, all PVA-based films showed similar profiles during the initial 24 h. However, after 48 h, the F-30CA film exhibited the lowest solubility (15.54%) compared with F-0CA (23.39%). All CA-containing materials demonstrated solubility below 20% during 48 h.

Increasing citric acid content in the matrix decreased swelling and solubility, slowing down solubilization. It improved plasticity and formed crosslinks, reducing swelling by up to 70%, especially in F-30CA. Previous studies showed similar reductions in swelling with citric acid concentrations of 20-30% (Uranga et al., 2020; Wilpiszewska et al., 2019). Citric acid replaces water molecules in hydrogen bonds between polymer chains, enhancing barrier properties. These findings are crucial for developing coatings with low water solubility to maintain seed coating integrity over time despite challenges with rapid dissolution of hydrophilic films (Pirzada et al., 2020).

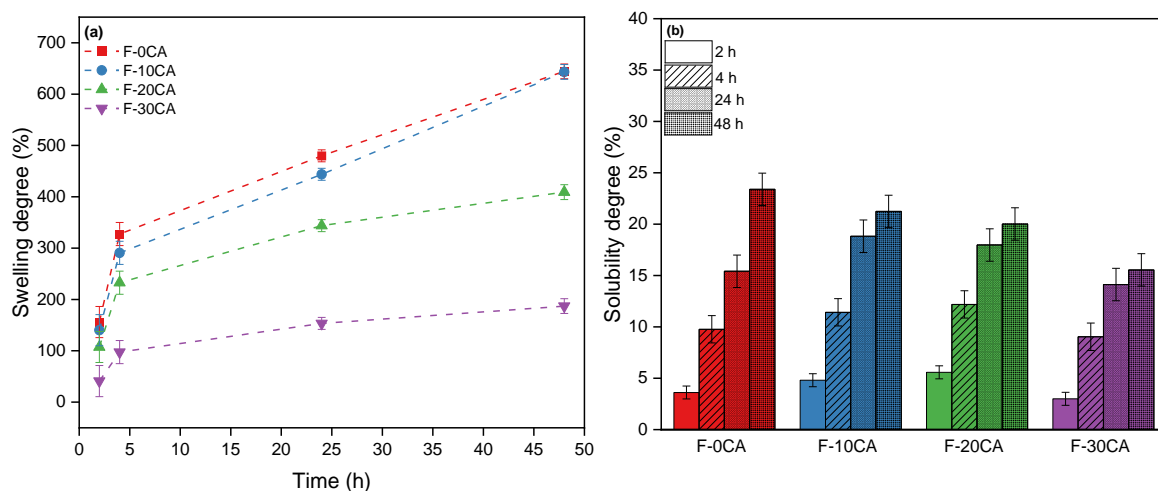


Figure 3. Degrees of Swelling (a) and solubility (b) profiles of the films.

3.2 Application of PVA-based films for microbial encapsulation

Based on the characterization results of the PVA-based films, the F-0CA and F-30CA matrices were chosen to encapsulate the *Bacillus megaterium* strain. Both matrices exhibited contrasting results in characterizations, especially concerning solubility, and swelling degrees, as shown in Figure 3, respectively. Consequently, these matrices can be used as models to investigate their effects on release assays, stress exposure, and seed coating.

A release assay of the encapsulated microorganism in the F-0CA and F-30CA matrices was performed to observe its behavior and release profile over time. Figure 4 illustrates the *B. megaterium* release over 48 h from formulations F-0CA and F-30CA. The results show that F-30CA exhibited slower bacterial release compared to F-0CA. Both formulations released around 10^6 CFU/g within 2 h. However, after 24 h, the F-0CA matrix had already released

approximately 300 times more cells ($1.38 \times 10^9 \text{ CFU/g}$) than F-30CA ($4.38 \times 10^6 \text{ CFU/g}$). This pattern was maintained over the 48 h period.

The higher release observed in the F-0CA matrix may be attributed to the increased swelling showed in Figure 3a. This swelling facilitates the separation of polymeric chains, resulting in a faster release of the encapsulated microorganism. On the other hand, the F-30CA formulation showed a lower swelling (Figure 3a), which is associated with a slower release of the microorganism. This slower release can help to maintain cell viability over time and facilitate a controlled release of the bacteria to the plant. These results highlight the effect of the crosslinking agent CA in improving the control of microorganism release in the PVA-based matrix. This behavior is especially relevant to the development of coating matrices for seeds. A suitable matrix should protect the microorganism during storage and allow for controlled release and growth while maximizing seed germination and healthy development (Ma, 2019; Pirzada et al., 2020).

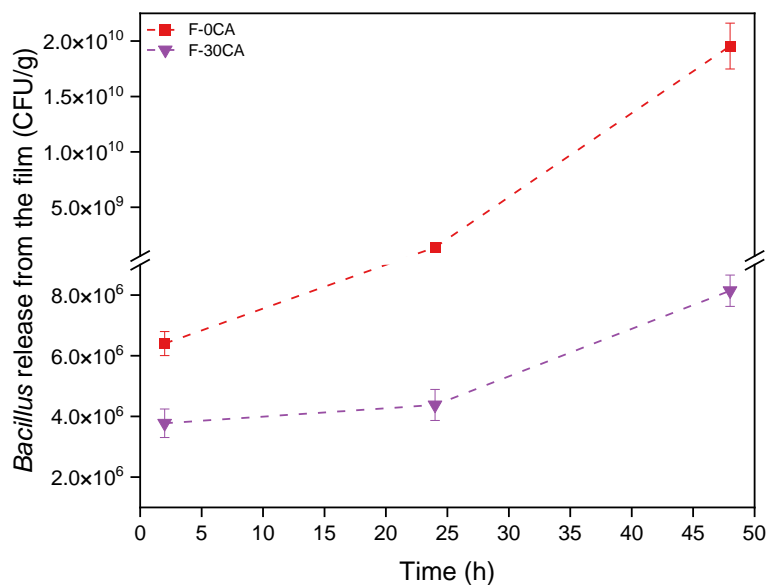


Figure 4. Release profiles of *B. megaterium* from matrices F-0CA and F-30CA in saline solution over 48 h.

Figure 5 compares the survival of the free and encapsulated cells of *B. megaterium* in the PVA matrices before and after exposure to stresses. The applied thermal stress ($55 \text{ }^\circ\text{C}/2 \text{ h}$) significantly affected the free microorganism, showing a reduction of two orders of magnitude (10^9 to 10^7 CFU/mL) after exposure (Figure 5a). On the other hand, the evaluated PVA-based matrices protected the bacteria, especially when the matrix contained CA in its

composition. After exposure to heat, both matrix F-30CA and F-0CA maintained cell viability in the same order of magnitude (10^6 CFU/g), with the viability loss being more significant for F-0CA (6.1×10^6 to 1.4×10^6 CFU/g), while F-30CA decreased slightly from (3.1×10^6 to 1.6×10^6 CFU/g). The better performance of matrix F-30CA after heat exposure can be attributed to its higher thermal stability, as evidenced by Figures 2c-d, which likely provided more effective protection even under severe stress conditions.

After exposure to the insecticide (Figure 5b), matrix F-30CA maintained cell viability at 10^6 UFC/g (decreasing from 8.1×10^6 to 4.4×10^6 CFU/g), while F-0CA experienced a reduction of one order of magnitude after stress exposure (1.46×10^7 to 6.0×10^6 CFU/g). Free bacteria, on the other hand, exhibited a decrease of four orders of magnitude (5.3×10^9 to 1.4×10^5 CFU/mL). These results emphasize the beneficial effect of adding CA to the formulation to protect microorganisms in the PVA matrix, especially considering that previous studies have shown that *Bacillus* genus bacteria can be harmed by exposure to agrochemical agents used in seed coating (Ons et al., 2020; Shripad Kulkarni, 2017). Pesticides, including insecticides, fungicides, and herbicides, can impact microbial communities by introducing antibiotics and harmful additives like alcohol derivatives and formaldehyde (Muturi et al., 2017). These components can damage or eliminate vulnerable microbial species. For instance, recent studies have shown that thiamethoxam treatment can lead to notable alterations in the bacterial composition of the soil (Akter et al., 2023; Wu et al., 2021; Zhang et al., 2021).

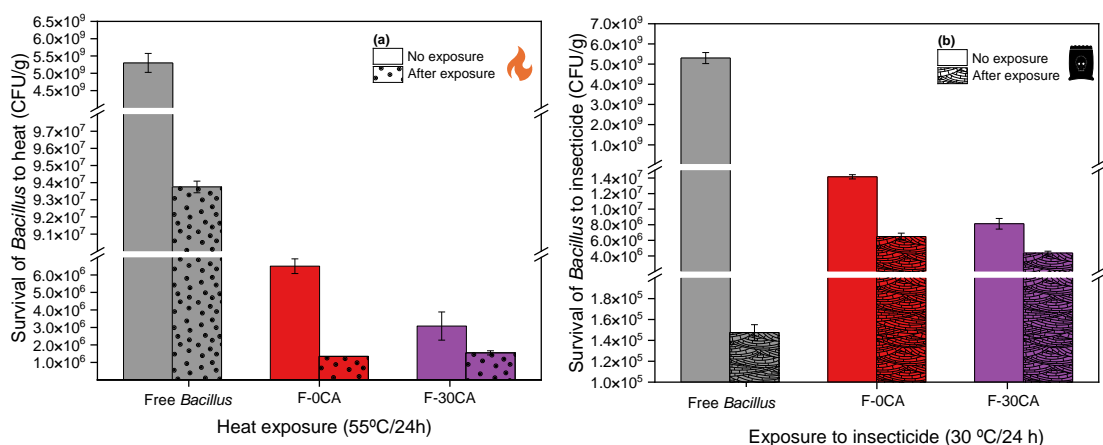


Figure 5. Survival of *B. megaterium* before and after exposure to thermal stress and contact with the insecticide.

Figure 6 illustrates the viability of the microorganism, both in its free form and its encapsulation in the films (Figure 6a), and the viability of the cells applied to seed coatings

(Figure 6b). The films and the coated seeds were stored for six months under controlled temperature and humidity conditions (25 °C/45% RH), and the free bacteria were kept in the refrigerator (4°C). The free-form microorganism showed a significant decrease in viability during the storage period, decreasing the cell concentration from 10^8 to 10^6 over six months (Fig. 6a). Conversely, for both PVA-based films, the initial viability (around 10^6 CFU/g), increased over the six-month storage period. The F-30CA film exhibited a significantly higher final cell concentration (2.82×10^7 CFU/g) compared to the F-0CA film (1.32×10^7 CFU/g), indicating that the citric acid matrix was more effective in protecting the bacteria. The increase in cell concentration may be the result of the aging of the matrix, which influences the release of more cells during the process. In this context, the ability of the *Bacillus* genus to degrade PVA and use it as a carbon source is also relevant (Bian et al., 2020; Patil and Bagde, 2015; Ullah et al., 2019).

The cell viability of the seed coatings (Figure 6b) exhibited a similar pattern to the films (Figure 6a). The results indicate that the F-30CA coating outperforms F-0CA over the six months, releasing more bacteria during storage. Although initially inoculated with 10^5 CFU/seed, the initial cell count at time 0 months was 10^2 CFU/seed. It may be explained by the more controlled release profile of cells in PVA-based matrices, as observed in the solubility (Figure 3) and bacterial release (Figure 4) assays. However, noteworthy, both matrices maintain cell viability at around 10^2 CFU/seed over the six months, demonstrating a gradual increase over time. It suggests that the matrices, especially F-30CA, can maintain cell viability over the six-month storage period.

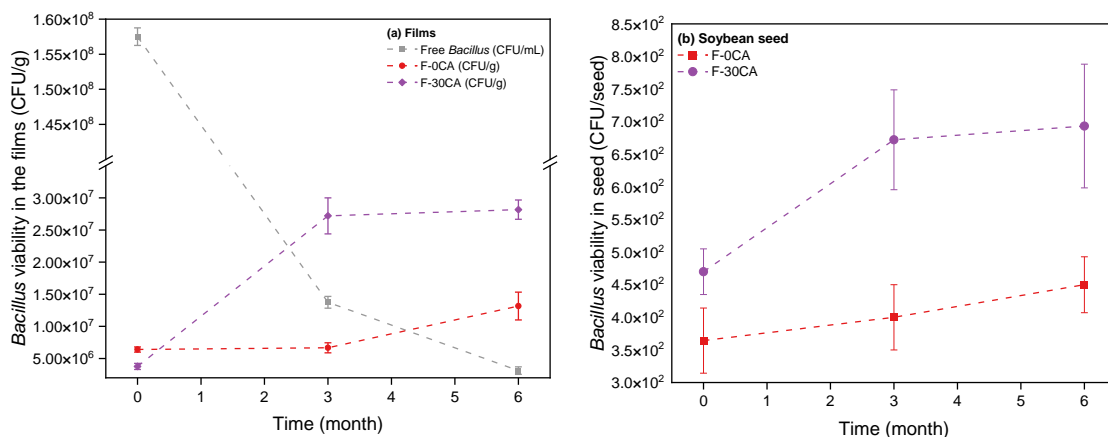


Figure 6. Microorganism viability during six months. (a) Free and encapsulated microorganisms in the films; (b) Encapsulated microorganisms as seed coatings.

3.3 Germination rate

Figure 7 displays seeds with various treatments, including a micrograph of the coating and a germination model. Incorporating beneficial microorganisms into seed coating matrices ensures even distribution of cells and enhances effectiveness in the field. Therefore, it is crucial to study the impact of inoculated films on seed growth to ensure they do not hinder germination rates, which could affect their viability compared to untreated seeds.

The impact on germination rates of soybean seeds treated with different coatings are presented in Table 3. The germination rates of seeds treated with F-0CA and F-30CA were not adversely affected compared to untreated soybean seeds (control). Throughout the six-month storage period, all coatings showed similar germination rates. However, as storage time progressed, there were statistical differences between the treatments, leading to a decline in germination for all materials. By the end of the six months, seeds coated with F-30CA exhibited a 12% reduction in germination, while seeds treated with F-0CA and untreated seeds (control) experienced reductions of 17% and 25%, respectively. The treatment with the commercial polymer initially had the lowest germination rate but maintained stable germination over the six months (60 - 65%). Notably, the similarity in germination rates between F-30CA and the commercial polymer underscores the efficacy of the proposed PVA-based matrix with citric acid. Few studies have examined soybean seed treatments using coating films containing microorganisms, making it difficult to compare their effectiveness with other materials.

Yusnawan et al. (2019) examined the impact of coating soybean seeds with different isolates of *Trichoderma virens* on germination. They found that, despite the agricultural interest in the fungus, five out of seven isolates tested inhibited seed germination without improvement in the final germination rate compared to untreated seeds. On the other hand, Teixeira et al. (2021) investigated the use of *Bacillus velezensis* isolate in soybean seed coating, observing an approximately 10% increase in germination rate compared to the control (55.5% to 64%), thus highlighting the positive impact of the *Bacillus* genus on soybean culture.

Table 0-3. The germination rate of control treatments and formulated films.

Code	Germinated seeds (%)		
	0 months	3 months	6 months
Control	77.95 ± 5.18 ^{Aa}	59.49 ± 7.89 ^{Ab}	53.33 ± 5.83 ^{Bb}
Agrochemical	72.31 ± 9.25 ^{Aa}	61.33 ± 9.6 ^{Ab}	63.59 ± 9.63 ^{ABab}
Commercial polymers	65.55 ± 18.52 ^{Aa}	60.00 ± 8.33 ^{Aa}	64.67 ± 6.40 ^{Aa}
F-OCA	73.89 ± 1.85 ^{Aa}	61.67 ± 3.33 ^{Ab}	57.22 ± 8.15 ^{ABb}
F-30CA	70.26 ± 5.55 ^{Aa}	55.55 ± 7.78 ^{Ab}	58.33 ± 5.56 ^{ABb}

A, B: Different uppercase letters within columns indicate statistical difference by Tukey Test (P<0.05); a, b: Different lowercase letters within rows indicate statistical difference by Tukey Test (P<0.05); Equal letters do not differ significantly from each other.

Few studies have examined soybean seed treatments using coating films containing microorganisms, making it difficult to compare their effectiveness with other materials. Yusnawan et al. (2019) investigated the impact of coating soybean seeds with different isolates of *Trichoderma virens*. They found that five out of seven isolates inhibited germination and did not improve the final rate compared to untreated seeds. In contrast, Teixeira et al. (2021) studied *Bacillus velezensis* seed coating and observed an increase of approximately 10% in the germination rate (from 55.5% to 64%). Both studies applied the microorganisms directly to the seeds without a polymeric matrix, highlighting the importance of an effective polymeric matrix to protect both the microorganisms and the seeds. The combination of *Bacillus* with an efficient polymeric matrix, as observed in this study, demonstrates the benefits of this approach for improving seed germination and protection.

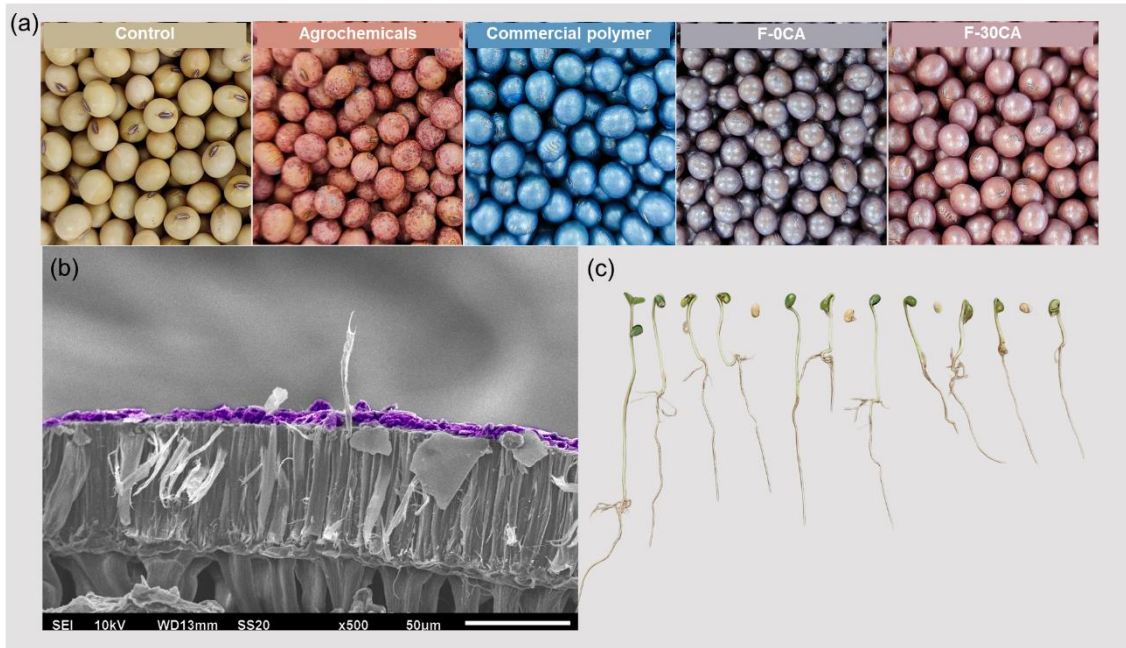


Figure 7. Representation of coated and germinated seeds. (a) Seeds coated with different treatments, including control (untreated seed), agrochemicals (fungicide and insecticide), commercial polymer (fungicide, insecticide, and commercial polymer), F-0CA (fungicide, insecticide, F-0CA, and *Bacillus megaterium*), and F-30CA (fungicide, insecticide, F-30CA, and *Bacillus megaterium*); (b) Micrograph of the surface coating on the seed; (c) Germinated seeds.

4. Conclusions

This study highlighted the improvements achieved in the PVA-based films by adding citric acid as a crosslinking agent. Citric acid helped to increase thermal stability, reducing swelling and solubility of the matrices, which is essential to ensure the integrity of seed coatings for extended periods. Specifically, the PVA matrix with 30% citric acid (F-30CA) showed a pattern of controlled release of *Bacillus megaterium* and enhanced preservation of cell viability during storage for six months and under adverse conditions such as exposure to heat and agrochemicals. When analyzing the germination of coated soybean seeds, the treatment with citric acid produced similar results to the treatment with a commercial polymer and better than untreated seeds (control), confirming the positive effect of the proposed formulation on soybean seeds. The promising results of this study suggest that these PVA-based matrices have the potential to be applied in practice in seed coating strategies, offering benefits for both microorganism protection and agronomic performance of crops.

References

- Accinelli, C., Abbas, H.K., Little, N.S., Kotowicz, J.K., Mencarelli, M., Shier, W.T., 2016. A liquid bioplastic formulation for film coating of agronomic seeds. **Crop Protection** 89, 123–128. <https://doi.org/10.1016/j.cropro.2016.07.010>
- Accinelli, C., Abbas, H.K., Shier, W.T., 2018. A bioplastic-based seed coating improves seedling growth and reduces production of coated seed dust. **J Crop Improv** 32, 318–330. <https://doi.org/10.1080/15427528.2018.1425792>
- Akter, S., Hulugalle, N.R., Jasonsmith, J., Strong, C.L., 2023. Changes in soil microbial communities after exposure to neonicotinoids: A systematic review. **Environ Microbiol Rep.** <https://doi.org/10.1111/1758-2229.13193>
- Alori, E.T., Babalola, O.O., 2018. Microbial inoculants for improving crop quality and human health in Africa. **Front Microbiol** 9, 1–12. <https://doi.org/10.3389/fmicb.2018.02213>
- Alvarado, Y., Muro, C., Illescas, J., Riera, F., 2019. Polymer nanoparticles for the release of complex molecules, in: Holban, A.-M., Grumezescu, A.M. (Eds.), **Materials for Biomedical Engineering**. Elsevier, pp. 135–163. <https://doi.org/10.1016/B978-0-12-818433-2.00005-4>
- Bargaz, A., Lyamlouli, K., Chtouki, M., Zeroual, Y., Dhiba, D., 2018. Soil Microbial Resources for Improving Fertilizers Efficiency in an Integrated Plant Nutrient Management System. **Front Microbiol** 9. <https://doi.org/10.3389/fmicb.2018.01606>
- Bian, H., Wang, G., Cao, M., Wang, Z., Cui, J., 2020. Improved biodegradation of polyvinyl alcohol by hybrid nanoflowers of degrading enzymes from *Bacillus niacini*. **Korean Journal of Chemical Engineering** 37, 1020–1028. <https://doi.org/10.1007/s11814-020-0547-9>
- Bigi, A., Panzavolta, S., Rubini, K., 2004. Relationship between triple-helix content and mechanical properties of gelatin films. **Biomaterials** 25, 5675–5680. <https://doi.org/10.1016/j.biomaterials.2004.01.033>
- Brasil, 2009. Ministério da Agricultura e Reforma Agrária. Análise de sementes revestidas, in: Regras Para Análise de Sementes. Mapa/ACS, Brasília, pp. 325–334.

- Das, P., Ray, S.K., Kuila, S.B., Samanta, H.S., Singha, N.R., 2011. Systematic choice of crosslinker and filler for pervaporation membrane: A case study with dehydration of isopropyl alcohol-water mixtures by polyvinyl alcohol membranes. **Sep Purif Technol** 81, 159–173. <https://doi.org/10.1016/j.seppur.2011.07.020>
- Sousa, S.M., de Oliveira, C.A., Andrade, D.L., de Carvalho, C.G., Ribeiro, V.P., Pastina, M.M., Marriel, I.E., de Paula Lana, U.G., Gomes, E.A., 2021. Tropical *Bacillus* Strains Inoculation Enhances Maize Root Surface Area, Dry Weight, Nutrient Uptake and Grain Yield. **J Plant Growth Regul** 40, 867–877. <https://doi.org/10.1007/s00344-020-10146-9>
- Ghorpade, V.S., Dias, R.J., Mali, K.K., Mulla, S.I., Yadav, A.V., Dias, R.J., Mali, K.K., Pargaonkar, S.S., Shinde, P.V., Dhane, N.S., Mulla, S.I., 2019. Citric acid crosslinked carboxymethylcellulose-polyvinyl alcohol hydrogel films for extended release of water soluble basic drugs. **J Drug Deliv Sci Technol** 52, 421–430. <https://doi.org/10.1016/j.jddst.2019.05.013>
- Gohil, R.B., Raval, V.H., Panchal, R.R., Rajput, K.N., 2022. Plant Growth-Promoting Activity of *Bacillus* sp. PG-8 Isolated From Fermented Panchagavya and Its Effect on the Growth of *Arachis hypogea*. **Frontiers in Agronomy** 4. <https://doi.org/10.3389/fagro.2022.805454>
- Gontard, N., Guilbert, S., Cuq, J.-L., 1992. Edible Wheat Gluten Films: Influence of the Main Process Variables on Film Properties using Response Surface Methodology. **J Food Sci** 191–199.
- Havstad, M.R., 2020. Biodegradable plastics, in: Letcher, T.M. (Ed.), *Plastic Waste and Recycling*. **Academic Press**, pp. 97–129. <https://doi.org/10.1016/B978-0-12-817880-5.00005-0>
- Inbaraj, M.P., 2021. Plant-Microbe Interactions in Alleviating Abiotic Stress—A Mini Review. **Frontiers in Agronomy**. <https://doi.org/10.3389/fagro.2021.667903>
- Iqbal, Z., Ahmad, M., Raza, M.A., Hilger, T., Rasche, F., 2024. Phosphate-Solubilizing *Bacillus* sp. Modulate Soil Exoenzyme Activities and Improve Wheat Growth. **Microb Ecol** 87. <https://doi.org/10.1007/s00248-023-02340-5>
- Jan, F., Arshad, H., Ahad, M., Jamal, A., Smith, D.L., 2023. In vitro assessment of *Bacillus subtilis* FJ3 affirms its biocontrol and plant growth promoting potential. **Front Plant Sci** 14. <https://doi.org/10.3389/fpls.2023.1205894>

- Jarecki, W., Wietecha, J., 2021. Effect of seed coating on the yield of soybean *Glycine max (L.) Merr.* **Plant Soil Environ** 67, 468–473. <https://doi.org/10.17221/246/2021-PSE>
- Jose, J., Al-Harhi, M.A., 2017. Citric acid crosslinking of poly(vinyl alcohol)/starch/graphene nanocomposites for superior properties. **Iranian Polymer Journal** (English Edition) 26, 579–587. <https://doi.org/10.1007/s13726-017-0542-0>
- Koza, N.A., Adedayo, A.A., Babalola, O.O., Kappo, A.P., 2022. Microorganisms in Plant Growth and Development: Roles in Abiotic Stress Tolerance and Secondary Metabolites Secretion. **Microorganisms**. <https://doi.org/10.3390/microorganisms10081528>
- Kumar, P., Singh, S., Pranaw, K., Kumar, S., Singh, B., Poria, V., 2022. Bioinoculants as mitigators of multiple stresses: A ray of hope for agriculture in the darkness of climate change. **Heliyon**. <https://doi.org/10.1016/j.heliyon.2022.e11269>
- Ma, Y., 2019. Seed coating with beneficial microorganisms for precision agriculture. **Biotechnol Adv** 37, 107423. <https://doi.org/10.1016/j.biotechadv.2019.107423>
- Miljaković, D., Marinković, J., Balešević-Tubić, S., 2020. The significance of *Bacillus* sp. In disease suppression and growth promotion of field and vegetable crops. **Microorganisms**. <https://doi.org/10.3390/microorganisms8071037>
- Mohamed, H., Shah, A.M., Nazir, Y., Naz, T., Nosheen, S., Song, Y., 2022. Biodegradation of Poly(vinyl alcohol) by an *orychophragmus* rhizosphere-associated fungus *Penicillium brevicompactum* OVR-5, and its proposed PVA biodegradation pathway. **World J Microbiol Biotechnol** 38. <https://doi.org/10.1007/s11274-021-03197-x>
- Muturi, E.J., Donthu, R.K., Fields, C.J., Moise, I.K., Kim, C.H., 2017. Effect of pesticides on microbial communities in container aquatic habitats. **Sci Rep** 7. <https://doi.org/10.1038/srep44565>
- Ons, L., Bylemans, D., Thevissen, K., Cammue, B.P.A., 2020. Combining biocontrol agents with chemical fungicides for integrated plant fungal disease control. **Microorganisms** 8, 1–19. <https://doi.org/10.3390/microorganisms8121930>
- Pantelic, B., Ponjavic, M., Jankovic, V., Aleksic, I., Stevanovic, S., Murray, J., Fournet, M.B., Nikodinovic-Runic, J., 2021. Upcycling biodegradable PVA/starch film to a bacterial biopigment and biopolymer. **Polymers (Basel)** 13. <https://doi.org/10.3390/polym13213692>

- Paravar, A., Piri, R., Balouchi, H., Ma, Y., 2023. Microbial seed coating: An attractive tool for sustainable agriculture. **Biotechnology Reports**. <https://doi.org/10.1016/j.btre.2023.e00781>
- Patil, R., Bagde, U.S., 2015. Enrichment and isolation of microbial strains degrading bioplastic Polyvinyl Alcohol and time course study of their degradation potential. **African Journal of Biotechnology** 14, 2216–2226. <https://doi.org/10.5897/AJB2011>
- Pirzada, T., de Farias, B. V., Mathew, R., Guenther, R.H., Byrd, M. V., Sit, T.L., Pal, L., Opperman, C.H., Khan, S.A., 2020. Recent advances in biodegradable matrices for active ingredient release in crop protection: Towards attaining sustainability in agriculture. **Curr Opin Colloid Interface Sci**. <https://doi.org/10.1016/j.cocis.2020.05.002>
- Prakash, J., Arora, N.K., 2019. Phosphate-solubilizing *Bacillus* sp. enhances growth, phosphorus uptake and oil yield of *Mentha arvensis* L. 3 **Biotech** 9. <https://doi.org/10.1007/s13205-019-1660-5>
- Prasad, R.D., Chandrika, K.S.V.P., Godbole, V., 2020. A novel chitosan biopolymer based *Trichoderma* delivery system: Storage stability, persistence and bio efficacy against seed and soil borne diseases of oil seed crops. **Microbiol Res** 237. <https://doi.org/10.1016/j.micres.2020.126487>
- Radhakrishnan, R., Hashem, A., Abd Allah, E.F., 2017. *Bacillus*: A biological tool for crop improvement through bio-molecular changes in adverse environments. **Front Physiol** 8. <https://doi.org/10.3389/fphys.2017.00667>
- Salihu, R., Abd Razak, S.I., Ahmad Zawawi, N., Rafiq Abdul Kadir, M., Izzah Ismail, N., Jusoh, N., Riduan Mohamad, M., Hasraf Mat Nayan, N., 2021. Citric acid: A green cross-linker of biomaterials for biomedical applications. **Eur Polym J**. <https://doi.org/10.1016/j.eurpolymj.2021.110271>
- Schlieker, M., Vorlop, K.-D., 2006. A Novel Immobilization Method for Entrapment: LentiKats®, in: Guisan, J.M. (Ed.), Immobilization of Enzymes and Cells. Methods in BiotechnologyTM. **Humana Press**, pp. 333–343. https://doi.org/10.1007/978-1-59745-053-9_29
- Shahid, M., Khan, M.S., 2018. Cellular destruction, phytohormones and growth modulating enzymes production by *Bacillus subtilis* strain BC8 impacted by fungicides. **Pestic Biochem Physiol** 149, 8–19. <https://doi.org/10.1016/j.pestbp.2018.05.001>

- Shi, R., Bi, J., Zhang, Z., Zhu, A., Chen, D., Zhou, X., Zhang, L., Tian, W., 2008. The effect of citric acid on the structural properties and cytotoxicity of the Polyvinyl Alcohol/Starch films when molding at high temperature. **Carbohydr Polym** 74, 763–770. <https://doi.org/10.1016/j.carbpol.2008.04.045>
- Shripad Kulkarni, H.B., 2017. Studies on Compatibility of *Bacillus subtilis* (Ehrenberg) Cohn. with Chemical Fungicides. **Int J Curr Microbiol Appl Sci** 6, 578–586. <https://doi.org/10.20546/ijcmas.2017.603.067>
- Silva, L.I. da, Pereira, M.C., Carvalho, A.M.X. de, Buttrós, V.H., Pasqual, M., Dória, J., 2023. Phosphorus-Solubilizing Microorganisms: A Key to Sustainable Agriculture. **Agriculture (Switzerland)**. <https://doi.org/10.3390/agriculture13020462>
- Singh, R., Gautam, S., Sharma, B., Jain, P., Chauhan, K.D., 2021. Biopolymers and their classifications, in: **Biopolymers and Their Industrial Applications**. Elsevier Inc., pp. 21–44. <https://doi.org/10.1016/b978-0-12-819240-5.00002-x>
- Stamenković, S., Beškoski, V., Karabegović, I., Lazić, M., Nikolić, N., 2018. Microbial fertilizers: A comprehensive review of current findings and future perspectives. **Spanish Journal of Agricultural Research** 16, e09R01. <https://doi.org/10.5424/sjar/2018161-12117>
- Teixeira, G.M., Mosela, M., Nicoletto, M.L.A., Ribeiro, R.A., Hungria, M., Youssef, K., Higashi, A.Y., Mian, S., Ferreira, A.S., Gonçalves, L.S.A., Pereira, U. de P., de Oliveira, A.G., 2021. Genomic Insights Into the Antifungal Activity and Plant Growth-Promoting Ability in *Bacillus velezensis* CMRP 4490. **Front Microbiol** 11. <https://doi.org/10.3389/fmicb.2020.618415>
- Ullah, M., Li, H., Sun, S.W., Weng, C.H., Zhang, H., Zhu, H., 2019. Polyvinyl alcohol degradation by *Bacillus cereus* RA23 from oil sludge sample. **3 Biotech** 9. <https://doi.org/10.1007/s13205-019-1882-6>
- Uranga, J., Nguyen, B.T., Si, T.T., Guerrero, P., De la Caba, K., 2020. The effect of cross-linking with citric acid on the properties of agar/fish gelatin films. **Polymers (Basel)** 12. <https://doi.org/10.3390/polym12020291>
- Vercelheze, A.E.S., Marim, B.M., Oliveira, A.L.M., Mali, S., 2019. Development of biodegradable coatings for maize seeds and their application for *Azospirillum brasilense*

- immobilization. **Appl Microbiol Biotechnol** 103, 2193–2203. <https://doi.org/10.1007/s00253-019-09646-w>
- Wahid, M.N.A., Abd Razak, S.I., Abdul Kadir, M.R., Hassan, R., Nayan, N.H.M., Mat Amin, K.A., 2018. Influence of citric acid on the physical and biomineralization ability of freeze/thaw poly(vinyl alcohol) hydrogel. **J Biomater Appl** 33, 94–102. <https://doi.org/10.1177/0885328218771195>
- Wang, S., Ren, J., Li, W., Sun, R., Liu, S., 2014. Properties of polyvinyl alcohol/xylan composite films with citric acid. **Carbohydr Polym** 103, 94–99. <https://doi.org/10.1016/j.carbpol.2013.12.030>
- Wu, C., Wang, Z., Ma, Y., Luo, J., Gao, X., Ning, J., Mei, X., She, D., 2021. Influence of the neonicotinoid insecticide thiamethoxam on soil bacterial community composition and metabolic function. **J Hazard Mater** 405. <https://doi.org/10.1016/j.jhazmat.2020.124275>
- Wu, Y.H., Yu, D.G., Li, H.P., Wu, X.Y., Li, X.Y., 2017. Medicated structural PVP/PEG composites fabricated using coaxial electrospinning. **E-Polymers** 17, 39–44. <https://doi.org/10.1515/epoly-2016-0244>
- Yusnawan, E., Inayati, A., Baliadi, Y., 2019. Effect of soybean seed treatment with *Trichoderma virens* on its growth and total phenolic content, in: AIP Conference Proceedings. American Institute of Physics Inc. <https://doi.org/10.1063/1.5115604>
- Zhang, H., Zhang, Z., Song, J., Mei, J., Fang, H., Gui, W., 2021. Reduced bacterial network complexity in agricultural soils after application of the neonicotinoid insecticide thiamethoxam. **Environmental Pollution** 274. <https://doi.org/10.1016/j.envpol.2021.116540>
- Zhang, N., Wang, Z., Shao, J., Xu, Z., Liu, Y., Xun, W., Miao, Y., Shen, Q., Zhang, R., 2023. Biocontrol mechanisms of *Bacillus*: Improving the efficiency of green agriculture. **Microb Biotechnol** 16, 2250–2263. <https://doi.org/10.1111/1751-7915.14348>

CHAPTER 3

Microencapsulation of *Bacillus megaterium* in PVA/Cationic Starch-based matrices

Abstract

The increasing demand for effective agricultural bio-based products drives research into technologies to encapsulate beneficial microorganisms, such as strain of *Bacillus* bacteria, known for promoting plant growth and controlling pathogens. This study proposes an efficient methodology to encapsulate *Bacillus megaterium* in microparticles using a simple cross-linking/emulsion of matrices based on polyvinyl alcohol (PVA) and Cationic Starch (CS). Citric acid (CA) and Sodium Trimetaphosphate (STMP) were employed as cross-linking agents, while bentonite was added to modify the materials. The resulting microparticles exhibited various properties influenced by the cross-linking agents and bentonite. Matrices with STMP and STMP/Bentonite provided controlled release of bacteria and high protection against adverse conditions, maintaining up to 95% of the initial cell viability after exposure to heat (55 °C/24 h) and up to 98% after exposure to UV light (180 min) or pesticides (30 °C/24 h). Conversely, matrices with CA and CA/Bentonite, as well as the free bacteria, were significantly more affected by bacterial survival throughout these assays. In a soil test with high salinity (200 mM), matrices with STMP showed the best results in protecting encapsulated bacteria, with an increase of approximately 10% in the growth of *B. megaterium* for both formulations. Loss of bacterial viability was observed throughout the experiment for the other evaluated formulations and the free bacteria. Matrices with STMP showed an estimated shelf life of over 38 months when stored at 30 and 45 °C temperatures, while the matrix with STMP/Bentonite reached 358 months at 15 °C. These results indicate that the PVA/Cationic starch matrices with STMP can be a potential alternative to develop more effective agricultural bio-based products.

Keywords: Oil-in-water Emulsion; Accelerated Shelf-Life Testing; Stress Tests; Controlled Release; Soil Salinity.

1. Introduction

Using beneficial microorganisms is a promising alternative to increase agricultural productivity effectively and sustainably. This becomes even more relevant in the face of contemporary agriculture's primary challenge of increasing food production to meet the growing demands of the global population (Alori & Babalola, 2018; Bargaz et al., 2018; Fróna et al., 2019; Timmusk et al., 2017). In this context, bacteria of the *Bacillus* genus stand out for their positive effects on plant growth and development, providing essential macro and micronutrients (Ahmad et al., 2021; Mumtaz et al., 2017; Oliveira et al., 2021; Saeid et al., 2018) and exerting biological control against pathogens (Khan et al., 2022; Radhakrishnan et al., 2017; Zhang et al., 2023).

However, the application of *Bacillus* strains in the field can be challenging due to various environmental adverse factors, such as competition with other soil microorganisms, exposure to agrochemicals, heat, UV radiation, hydric stress, and high soil salinities, affecting their survival (Berninger et al., 2018). Therefore, the encapsulation of microorganisms in polymeric matrices has emerged as an effective strategy to preserve their viability from storage to application in the soil (Oliveira et al., 2021; Vassilev et al., 2020; Zheng et al., 2022).

Polymers such as Polyvinyl Alcohol (PVA) and Starch (ST) and their derivatives have been investigated in recent decades as potential matrices for microbial encapsulation (David et al., 2021; Gamage et al., 2022; Klaic et al., 2018; Lopes-Assad et al., 2006). PVA is a synthetic polymer recognized for its biodegradability and high compatibility with cells (Havstad, 2020; Pham & Tho Bach, 2014; Rynkowska et al., 2019), demonstrating effectiveness in encapsulating enzymes, and microorganisms (Lal et al., 2021; Poraj-Kobielska et al., 2015; Sonjan et al., 2021; Yang et al., 2019). Meanwhile, cationic starch (CS) is a synthetic polymer developed to overcome the limitations of starch, such as insolubility in water at room temperature, low mechanical resistance, and thermal instability (Goswami & Mahanta, 2021). CS has positively charged groups in its structure, giving it high solubility in water and excellent film forming properties, opening new possibilities for bonding with other materials (Nornberg et al., 2022). Given these remarkable properties, matrices based on PVA/CS present a potential alternative for encapsulating microorganisms for agricultural applications.

With the selection of an efficient matrix for encapsulation, there is a need to develop effective and feasible methods for the encapsulation of beneficial microorganisms. Among the

different production methodologies, emulsion has not been commonly explored in cell encapsulation due to the toxic solvents or aggressive drying required for homogeneity, despite being industrially scalable (Hwang et al., 2022). To overcome this, cross-linking agents such as citric acid (CA) and Sodium Trimetaphosphate (STMP) can be applied to strengthen the bonds between PVA/CS polymers before the emulsion process. CA is a non-toxic metabolic agent, widely available and simple to react, that can improve mechanical properties and stability in water (Chi et al., 2024; Jose & Al-Harhi, 2017; Wilpiszewska et al., 2019). STMP is also recognized as a safe cross-linking agent compatible with starch and PVA, extensively investigated for medical applications (Jeong et al., 2021; Pan et al., 2021; Riahi et al., 2017). Additionally, the addition of bentonite clay after the cross-linking process can act as a surface modification agent due to its ease of chemical functionalization with various polymers (Baigorria et al., 2020) and efficiency in controlled release of compounds (Batista et al., 2017; Flores-Céspedes et al., 2023; Helal et al., 2023; Y. Zhang et al., 2020).

To the best of the authors knowledge, these materials have not been studied together to produce emulsion microcapsules. Overcoming the challenges of emulsion encapsulation and exploring different matrices and crosslinking agents are essential for developing effective carriers for microorganisms in agriculture, ensuring protection under stress conditions. Therefore, this study aimed to microencapsulate *Bacillus megaterium* through a simple cross-linking followed by an emulsion process for application in agriculture. PVA and cationic starch (CS) were used as base matrices, Citric Acid (CA) and Sodium Trimetaphosphate (STMP) acted as cross-linking agents. At the same time, bentonite played the role of surface modification agent for the cross-linked matrices. The produced microparticles were evaluated for their physicochemical and morphological characteristics, bacterial release profiles, and cell viability during encapsulation and after exposure to conditions such as heat, UV, pesticides, and soil salinity. An Adapted Accelerated Shelf-Life Test (ASLT) methodology was also applied to estimate cell viability during storage.

2. Materials and methods

2.1 Materials

For the base of the matrix, Polyvinyl Alcohol (PVA - Molecular Weight 89,000-98,000, hydrolyzed to 99+%) and cationic Starch (CS - Ingredient) were used. Citric Acid (CA), Sodium Trimetaphosphate (STMP), and Sodium Hydroxide (NaOH) from Sigma Aldrich were used in

the cross-linking process. Bentonite (Bent - Buntech) was used to modify the surface of the matrices. Tween 80 (Sigma Aldrich) and soybean oil (Liza, Brazil) were used for the emulsion process.

2.2 Preparation and Microorganism Encapsulation

The bacterium utilized in this study was a strain of *Bacillus megaterium* (CNPMS B119) obtained from the Multifunctional Microorganism Collection at Embrapa Maize and Sorghum (de Sousa et al., 2021). To reactivate the cells stored at -80°C, the bacteria were inoculated on Soy Tryptone broth (TSB) and bacteriological agar in a Petri dish and then incubated at 30 °C for 12 h. Subsequently, the inoculum was transferred to a TSB liquid medium and stirred at 250 rpm, at 30 °C, for 72 h to produce the cells used for inoculation.

This study evaluated five formulations based on PVA and Cationic Starch (CS): F (PVA/CS), FCA (PVA/CS/Citric Acid), FCA-Bent (PVA/CS/Citric Acid/Bentonite), FSTMP (PVA/CS/STMP), and FSTMP-Bent (PVA/CS/STMP/Bentonite).

PVA (10% w/v) and CS (10% w/v) were dissolved in 50 mL of distilled water heated to 80 °C for 15 min. After cooling to 30 °C, a volume of cell suspension containing 10⁹ CFU/g of dry polymer weight was added and stirred for 5 minutes, resulting in the base formulation named as F. To investigate the effect of citric acid (CA) as a cross-linking agent, 5 mL of CA (2M) was added to the base matrix and mixed for another 5 min, producing the formulation FCA. Additionally, 5 g of bentonite was added to FCA and stirred for another 5 min to disperse the clay, forming the formulation FCA-Bent. Similarly, STMP was investigated as a cross-linking agent by adding 1 mL of a NaOH solution (30% w/v) and 5 mL of STMP (15% w/v) to the base formulation, resulting in the formulation FSTMP. 5g of bentonite was also added to FSTMP, forming the formulation FSTMP-Bent. The resulting polymeric solutions were then carefully added to 300 mL of a soybean oil solution containing 1.5% Tween 80, stirred at 400 rpm for 1 h, and rested for another hour to stabilize the formed microparticles. Subsequently, the microparticles were separated from the oil, placed on filter paper, and dried at 30 °C for 12 h. Finally, the microparticles were washed with distilled water and dried at 30 °C for 6 h. Figure 1 provides a summarized diagram of this procedure.

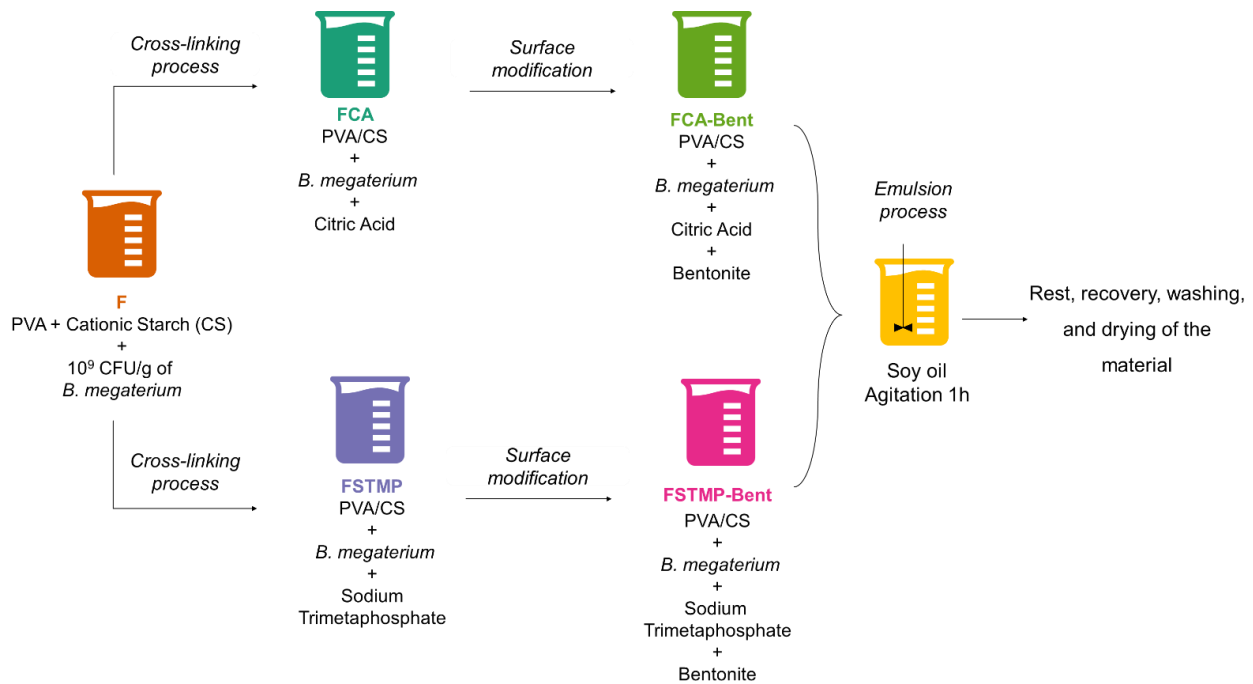


Figure 1. A simplified scheme for preparing materials and encapsulating *B. megaterium* using the cross-link/emulsion methodology. PVA (Polyvinyl Alcohol), CS (Cationic Starch), CA (Citric Acid), STMP (Sodium Trimetaphosphate) and Bent (Bentonite).

2.3 Characterizations

2.3.1 Morphological and Physicochemical Analysis

The morphology of the microparticles was observed using a Scanning Electron Microscopy (SEM), using a JEOL (JSM-6510) at an accelerating voltage of 5 kV, a working distance of 10 mm, and a secondary electron detector. The identification of the main phases of the formulations was carried out through X-Ray Diffraction analysis employing a LabX XRD-6000 diffractometer (Shimadzu, Japan), operated with Cu-K α radiation ($\lambda = 1.54056 \text{ \AA}$), a voltage of 30 kV, and a current of 30 mA. The diffractograms were recorded for 2θ from 4° to 70° , and a continuous scanning speed of $1^\circ/\text{min}$ was used. The molecular structure was studied by Fourier-Transform Infrared Spectroscopy (FTIR) was performed in the $4000\text{-}500 \text{ cm}^{-1}$ with a resolution of 4 cm^{-1} using a Bruker Vertex 70 model equipped with an Attenuated Total Reflectance (ATR) accessory.

2.3.2 Degrees of swelling and solubility

Swelling and solubility analyses were performed in the same experiment (Bigi et al., 2004; Gontard et al., 1992; Lopes et al., 2024). 1 g of the microparticles was dried in an oven

at 100 °C for 24 h, and its initial weight was recorded (m_0). Subsequently, triplicate samples were placed in Falcon tubes containing 50 mL of sterile distilled water and kept in an orbital incubator at 25 °C with gentle agitation for periods 2, 24, and 48 h). The particles were carefully removed from the water to determine swelling, and their wet mass was recorded (m_1). The material swelling degree was calculated based on the relationship between wet and initial weight over the immersion time (Equation 1). The material was dried again to obtain the dry weight (m_2). Material solubility was calculated as the difference between the initial and final weights, as described in Equation (2).

$$\text{Degree of swelling (\%)} = \frac{m_1 - m_0}{m_0} * 100 \quad (1)$$

$$\text{Degree of solubility (\%)} = \frac{m_0 - m_2}{m_0} * 100 \quad (2)$$

2.3 Encapsulated Material Assays

2.3.1 Initial Viability of Matrices and Bacteria Release Profile

To evaluate cellular viability during encapsulation, 0.1 g of microparticles were mechanically ruptured in 5 mL of saline solution (NaCl 0.85%) using an agate mortar to release encapsulated cells and obtain an aliquot. The release profile of encapsulated *B. megaterium* was investigated by adding 0.1 g of material to 5 mL of saline solution (NaCl 0.85%) in a rotary incubator at 30 °C, 250 rpm. Samples of 1 mL were collected at 2, 24, and 48 h. In both cases, aliquots of the solutions and their serial dilutions were inoculated on TSB and bacteriological agar media, followed by incubation at 25 °C for 24 h. Biological and analytical duplicates were employed for each dilution in this assay.

2.2.1 Exposure to Conditions of Stress

2.4.1 Thermal Exposure

Bacillus megaterium, both in free form and encapsulated form (9 log₁₀ CFU/mL or g), was subjected to heat treatment at 55 °C for 24 h in Falcon tubes in an oven with air circulation. After exposure, 5 mL of saline solution (0.85% NaCl) was added to the samples, and the free

bacteria were inoculated onto Petri dishes containing TSB medium and bacteriological agar. The encapsulated bacteria in formulations were then subjected to a 2 h release process at 30 °C with stirring at 250 rpm to release the cells from the matrix before inoculation. Biological and analytical triplicates were prepared for each sample and their respective serial dilutions, and the samples were inoculated onto Petri dishes containing TSB medium and bacteriological agar. The plates were then incubated at 25 °C for 24 h for colony counting.

2.5.2 UV Exposure

Bacillus megaterium, both in free form and encapsulated (9 log₁₀ CFU/mL or g), were subjected to UV radiation in a chamber equipped with six 15W UV-C lamps (Philips 15W G15T8) for durations of 20 and 180 min in Falcon tubes. After exposure, 5 mL of saline solution (0.85% NaCl) was added to the samples, and the free bacteria were inoculated onto Petri dishes containing TSB medium and bacteriological agar. The encapsulated formulations were agitated at 30 °C and 250 rpm for 2 h to release the cells from the matrix before inoculation. Biological and analytical triplicates were prepared for each sample and their respective serial dilutions, and the samples were inoculated onto Petri dishes containing TSB medium and bacteriological agar. The plates were then incubated at 25 °C for 24 h for colony counting.

2.5.3 Pesticide Exposure

B. megaterium, both in free form and encapsulated (9 log₁₀ CFU/mL or g), was incubated in 5 mL of an insecticide Thiamethoxam (1600 µg/mL) for 24 h at 30 °C (Shahid and Khan, 2018). Biological and analytical triplicates were prepared for each sample and their respective serial dilutions, and the samples were inoculated onto Petri dishes containing TSB medium and bacteriological agar. The plates were then incubated at 25 °C for 24 h for colony counting.

2.6 Accelerated Shelf-Life Test (ASLT)

We conducted an Accelerated Shelf-Life Test (ASLT) to estimate the validity of the formulations as a screening process between the matrices (Lopes et al., 2024). The free microorganisms and encapsulated material (9 log₁₀ CFU/mL or g) were stored in chambers under controlled conditions, maintaining temperatures of 15, 30, and 45 °C and a relative humidity of 76 %. Free bacteria and formulations exposed to these conditions were collected on days 0, 7, 14, 21, and 28. Free bacteria were directly plated, and the formulations were added

to a saline solution (NaCl 0.85%) for release at 250 rpm, 30 °C, for 2 h. Then, an aliquot of this solution was plated, and Colony Forming Units (CFU) were counted using the plate count method. All assays were conducted in triplicate. The estimated time for the ASLT was assessed through first-order degradation kinetics, with the time calculated from Equation 3, where N_0 is the value at the beginning of the shelf-life, N_t is the value at the end of the shelf-life, and k_T is the calculated value of k for each temperature using the Arrhenius Equation. Tables, calculations, and graphs for determining the life estimation of *Bacillus megaterium* and matrices using the ASLT methodology are available for consultation in Tables S1 – S6 (Appendix A).

$$t = \frac{\ln N_0 - \ln N_t}{k_T} \quad (3)$$

2.7 Influence of Soil Salinity

Based on previous experimental assays, emulsions F, FSTMP, and FSTMP-Bent were pre-selected for application in soil with varying salinity levels. Clayey soil collected in São Carlos/SP - Brazil underwent basic chemical characterization (Appendix A - Table S7). The soil was sterilized and distributed into small plastic pots, identified, and weighed at a volume of 50 g per pot. A nutrient solution was added to each pot (Hoagland and Arnon, 1950). Soil moisture content was maintained at 60 %, and the pH was controlled between 5 and 6 when assessed in H₂O.

At each pot, the proper number of free bacteria and the formulations were added (~ 6 log₁₀ CFU/g or mL). Then, a saline solution was added according to the treatment, either 0 or 200 mM of NaCl (Akram et al., 2019; Aslam et al., 2018). Pots were arranged in a plastic organizer box containing a small water strip to maintain the desired humidity. To conclude this stage, the box was closed and placed inside an incubator at 30 °C for seven days. The objective was to assess how this abiotic condition influences the performance of these formulations in clayey soil. All treatments were conducted in duplicate, both analytically and biologically.

At the end of the seven days, 1 g of soil was collected from each treatment, and the present microorganisms were extracted in a saline solution (NaCl 0.85%) and subjected to agitation at 250 rpm at 30 °C for one h in a shaker incubator (Wu et al., 2012). Subsequently, 1 mL samples were diluted and inoculated in TSB and bacteriological agar. Biological and

analytical duplicates were prepared for each dilution, and the inoculated plates were incubated at 25 °C for 24 h for colony counting.

3. Results and discussion

3.1 Morphological and Physicochemical Characterizations

Figure 2 displays optical images and micrographs of microparticles from the cross-linking/emulsion for the five formulations based on PVA/Cationic starch matrices. The particles have an irregular shape and size distribution, as shown in Figure S1 (Appendix A). The particle size at the end of the process is influenced by the type of cross-linking agent used and the presence of bentonite in the formulation. The base formulation (F), without any crosslinking agent or surface modification, had an average particle size of 330 μm . However, formulations with crosslinking agents exhibited different average particle sizes: 736 μm (FCA) and 256 μm (FSTMP). When crosslinkers and bentonite were added, the particle sizes changed to 423 μm (FCA-Bent) and 202 μm (FSTMP-Bent). This indicates that the matrices could form smaller particles during emulsion when STMP and bentonite were present. However, it is crucial to note that emulsion involves the dispersion of one liquid phase into another, and various factors during production, such as material viscosity and stirring speed, can affect particle size. These factors make it challenging to achieve homogeneous sizes, as observed in this and other studies (Schroën et al., 2020; Zabet et al., 2022).

The XRD data for formulations without bentonite (F, FCA, and FSTMP) presented in Figure 3a predominantly show characteristic peaks of PVA observed at 19.4° and 41° (2 θ) (Wahid et al., 2018). It suggests that the cationic starch phase could be overlapped or present as an amorphous component in the PVA phase. On the other hand, a completely different XRD pattern is observed with the addition of bentonite (FCA-Bent and FSTMP-Bent). The characteristic peaks of PVA are no longer observed, indicating that the addition of bentonite hampers the packing of PVA chains acting as impurity nodes, preventing long-range molecular ordering in the material. The XRD patterns of FCA-Bent and FSTMP-Bent samples are predominantly composed of bentonite, specifically the montmorillonite phase (Tan et al., 2008). Such differences in XRD patterns among formulations may lead to different material properties and influence their efficiency regarding the desired application.

From the FTIR data, it is observed that the characteristic bands of PVA remain predominant in all formulations (Figure 3b) as observed for the –OH stretching vibrations around 3350 cm^{-1} attributed to physically and chemically bound water; C–H stretching at 2920 cm^{-1} and 2850 cm^{-1} , out-of-plane C–H at 1455 cm^{-1} ; and C–O stretching associated with a broadened band around 1090 cm^{-1} . All formulations present a new band at 1744 cm^{-1} that can be related to C=O groups in cross-linked PVA matrices or the dehydration process of the –OH group of PVA (Korbag and Mohamed Saleh, 2016; Suganthi et al., 2020). In this work, these bands probably indicate the –COOH group of citric acid for FCA and FCA-Bent samples, indicating the effective cross-linking of the PVA/CS matrices with this cross-linking agent (Taspika et al., 2019). The cross-linking of the matrix with SMTP in samples FSTMP and FSTMP-Bent can be verified by the band at 1295 cm^{-1} and is attributed to its phosphate groups (Samoila et al., 2019). It is also possible to identify the presence of bentonite due to the band at 450 cm^{-1} in samples FCA-Bent and FSTMP-Bent (El Ouardi et al., 2019). However, the other characteristic bands of STMP, bentonite, and the relative bands of cationic starch and citric acid may have overlapped with the PVA bands. Based on this, it is hypothesized that the main interactions between PVA/CS and the cross-linkers CA and STMP, as well as the addition of bentonite, are predominantly electrostatic, mainly between PVA/CS-CA and PVA/CS-STMP, with bentonite primarily acting as a filler with surface interactions between its dispersed particles and the cross-linked polymeric matrix. With all the information obtained through the characterization analysis and information in the literature, it was possible to construct a scheme inferring potential interactions occurring in the produced particles (Figure 4).

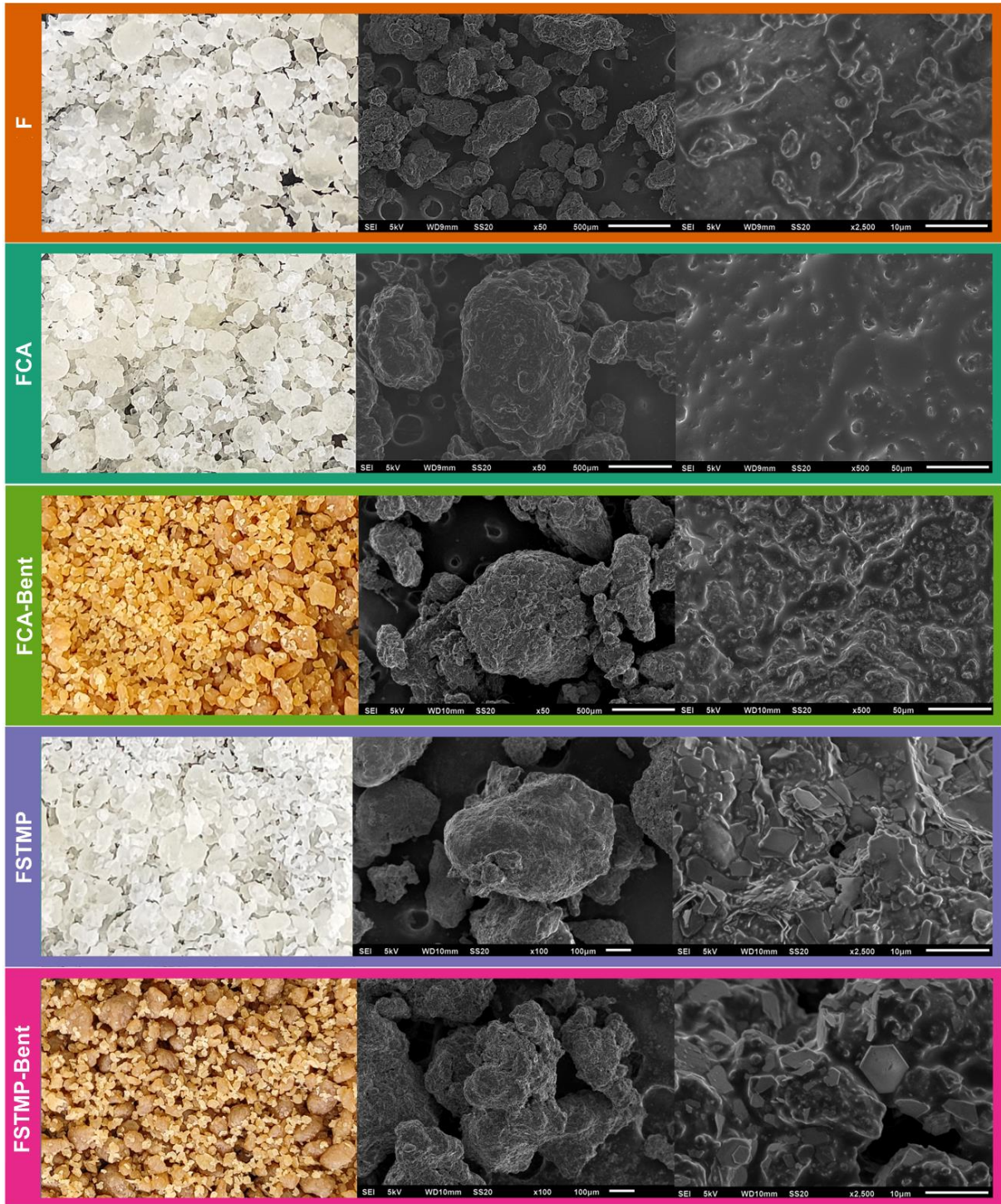


Figure 2. Photograph of the particles and their respective micrographs by scanning electron microscopy (SEM). F (PVA/CS); PVA (Polyvinyl Alcohol), CS (Cationic Starch), CA (Citric Acid), STMP (Sodium Trimetaphosphate) and Bent (Bentonite).

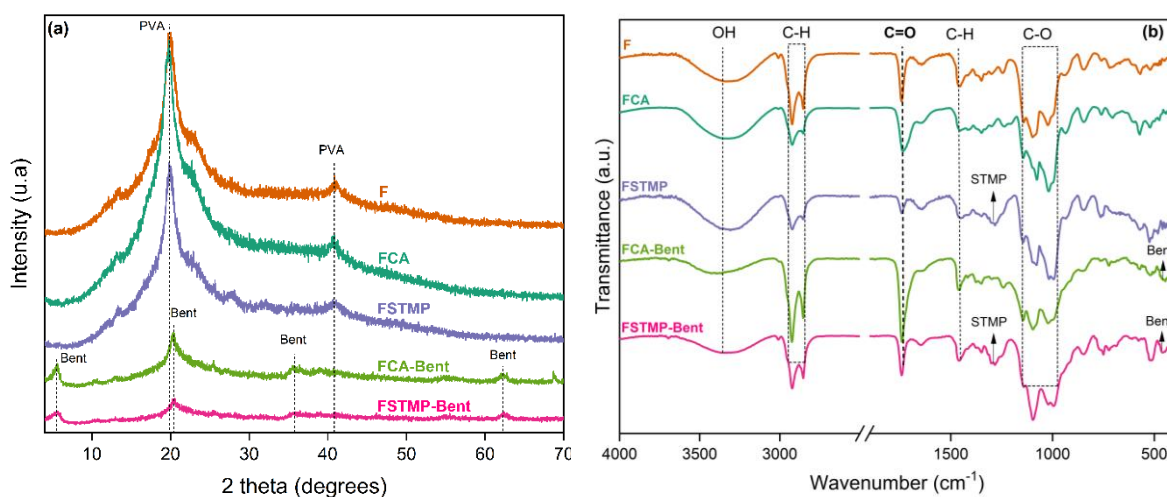


Figure 3. Physicochemical characterizations of the particles. (B) X-ray diffraction (XRD); (B) Fourier Transform Infrared Spectroscopy (FTIR). F (PVA/CS); PVA (Polyvinyl Alcohol), CS (Cationic Starch), CA (Citric Acid), STMP (Sodium Trimetaphosphate) and Bent (Bentonite).

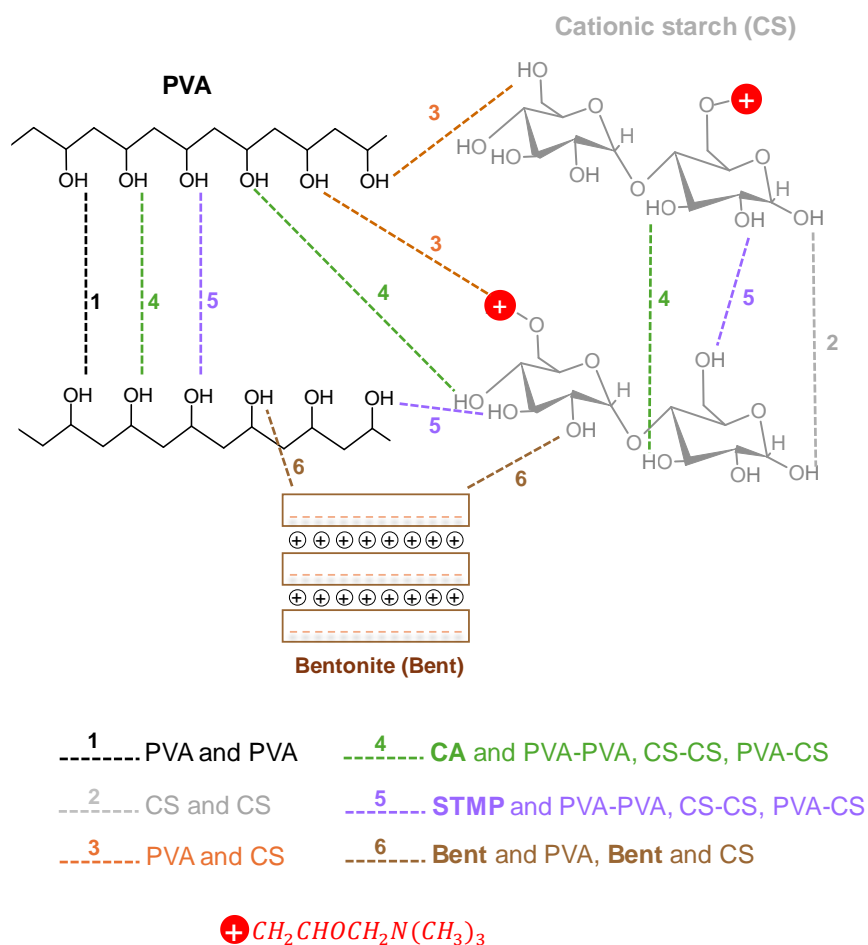


Figure 4. Schematics illustrating the possible interactions occurring in the matrix. PVA (Polyvinyl Alcohol), CS (Cationic Starch), CA (Citric Acid), STMP (Sodium Trimetaphosphate).

3.2 Degrees of Swelling and Solubility

Both the swelling (Figure 5a) and solubility (Figure 5b) of the materials are crucial characteristics of the encapsulating matrix, influencing the control of microorganism release and nutrients to the microorganism over time. After 2 h of water exposure, the formulations FCA, FSTMP-Bent, and FCA-Bent showed the lowest swelling degrees, ranging between 79% and 120%. This behavior persisted during the initial 24 h of testing, indicating that adding citric acid and STMP-Bent to the PVA/CS formulation could reduce the swelling of the materials compared to the other formulations.

As for the solubility of the materials, only citric acid significantly decreased the solubility of the formulations (FCA and FCA-Bent) in the initial 24 h of testing, presenting a maximum solubility of 18%. The initial reduction effect of swelling and solubility degrees in materials with citric acid was observed in other studies, where the authors concluded that citric acid helps improve the material's barrier properties by replacing water molecules in hydrogen bonds between polymer chains (Jose and Al-Harathi, 2017; Sharmin et al., 2022).

On the other hand, the matrices incorporating STMP in their composition (FSTMP and FSTMP-Bent) exhibited significantly higher solubilization percentages than others, solubilizing up to 54% of their composition. This phenomenon can be attributed to the cross-linking process with STMP, which, as Labelle et al. (2020) suggested, facilitates water molecule penetration into starch granules when present at lower levels in starch-based matrices. This, in turn, promotes more effective leaching of starch molecules into the aqueous phase, resulting in higher overall solubility.

Finally, it was observed that materials with bentonite (FCA-Bent and FSTMP-Bent), compared to those without (FCA and FSTMP), exhibited higher solubility, especially after 24 h. The addition of the mineral to the matrices may have increased their surface porosity, leading to faster particle integrity breakdown and, consequently, increased water absorption (Wang et al., 2022).

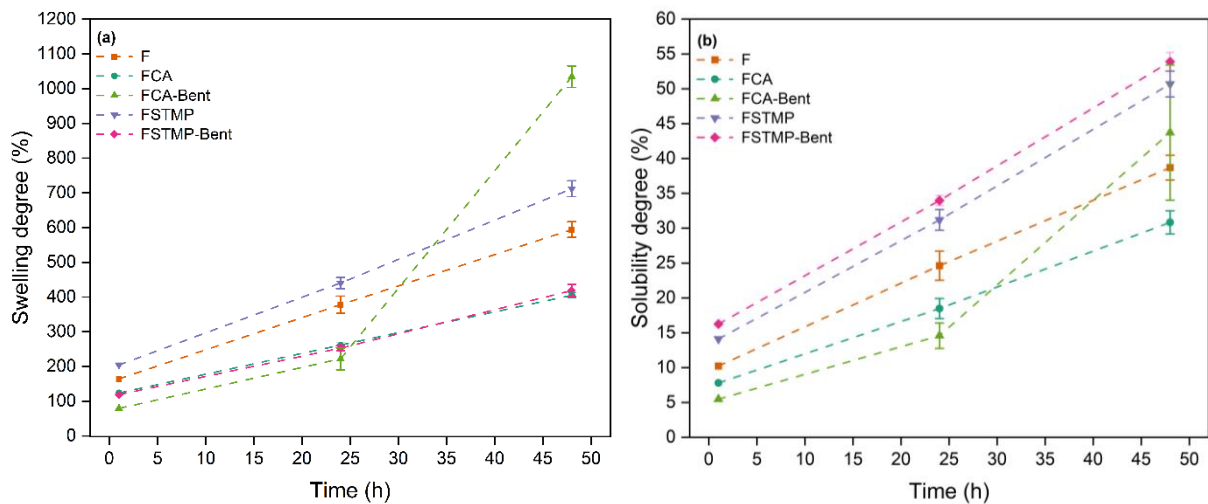


Figure 5. Degrees of Swelling (a) and solubility (b) profiles of the particles. F (PVA/CS); PVA (Polyvinyl Alcohol), CS (Cationic Starch), CA (Citric Acid), STMP (Sodium Trimetaphosphate) and Bent (Bentonite).

3.3 Cell Viability Assays

Originally, $9 \log_{10}$ CFU/g *Bacillus megaterium* was added to the formulations; however, all matrices showed higher initial cell viability than those inoculated, with 10 (F), 9.83 (FCA), 9.45 (FCA-Bent), 9.97 (FSMTP) and $9.60 \log_{10}$ CFU/g (FSTMP-Bent). The increase in initial viability suggests the potential for bacterial growth during cell release, as both PVA and starch and in some cases, citric acid (CA), can serve as a carbon source for *Bacillus* bacteria (Bian et al., 2020; Çalík et al., 1998; Patil and Bagde, 2015; Ullah et al., 2019; Yao et al., 2019). These results indicate high compatibility of the matrices with *Bacillus megaterium* and that the cross-linking/emulsion procedure is not detrimental to cell survival, offering a promising alternative for microbial encapsulation.

In the release assay of the encapsulated microparticles in saline solution (0.85% NaCl), the bacterial release profile over 48 h can be observed (Figure 6a). While matrices containing citric acid in their composition showed a release of up to 87% (FCA) and 98% (FCA-Bent), relative to the initial viability determined for each formulation (Table 1), it was observed that the formulations F, FSTMP, and FSMTP-Bent provided a more favorable environment for bacterial development and multiplication over time, releasing up to 110%, 103%, and 108%, respectively. This result is consistent with the higher solubility of these materials (Figure 5b), which possibly allowed the bacteria to use the matrix as a carbon source for their multiplication. The partial or total release of the microorganism highlights the materials versatility, indicating

their ability to provide a variety of products with distinct properties. However, when considering the application of these materials in the soil, it is crucial to examine the potential challenges that may impact field cultivation, such as exposure to heat, UV light, and contact with agrochemicals.

3.1 Exposure to Conditions of Stress

Exposure tests to stresses conditions commonly during field application, including heat (Figure 6b), UV light (Figure 6c), and pesticides (Figure 6d), were conducted to assess the behavior of *B. megaterium* in its free form and the protective efficacy of different encapsulation matrices. The base matrices with STMP in the composition showed significant protection for the bacteria, with viability of approximately 89% (F), 95% (FSTMP), and 94% (FSTMP-Bent). On the other hand, formulations with citric acid (FCA and FCA-Bent) showed total losses. Although *Bacillus megaterium* is known for its resistance to a wide range of temperatures (Goswami and Mahanta, 2021), exposure to 55°C for 24 h resulted in a decrease of approximately 27% compared to the initial value (Figure 6b).

After exposure to UV light (Figure 6c), it was observed that the presence of bentonite in the formulations provided better protection to the bacteria. FCA-Bent and FSTMP-Bent formulations maintained their total viability after 20 min of exposure and approximately 94% and 99%, respectively, after 180 min. This can be attributed to bentonites ability to absorb higher levels of UV light, potentially reflecting or dispersing light and acting as a physical filter. Previous studies have shown that adding 5 to 10% (w/v) bentonite improved UV light protection in film formulations and sunscreen production (Kim et al., 2020; Passaretti et al., 2019). Additionally, matrices F and FSTMP showed significant results, preserving 89% of encapsulated bacteria viability after 180 min of exposure, surpassing the survival observed for free bacteria, up to 81% in the same period.

Finally, regarding exposure to the chemical pesticide (Thiamethoxam – 30 °C/24 h), all encapsulated materials offered significant protection compared to free bacteria. Matrices F (99%), FSTMP, and FSTMP-Bent (98% each) deserve attention in protecting encapsulated *B. megaterium*. This result is encouraging, especially considering previous research indicating that bacteria of the *Bacillus* genus can be damaged by agrochemical exposure in agricultural environments (Ons et al., 2020; Shripad Kulkarni, 2017).

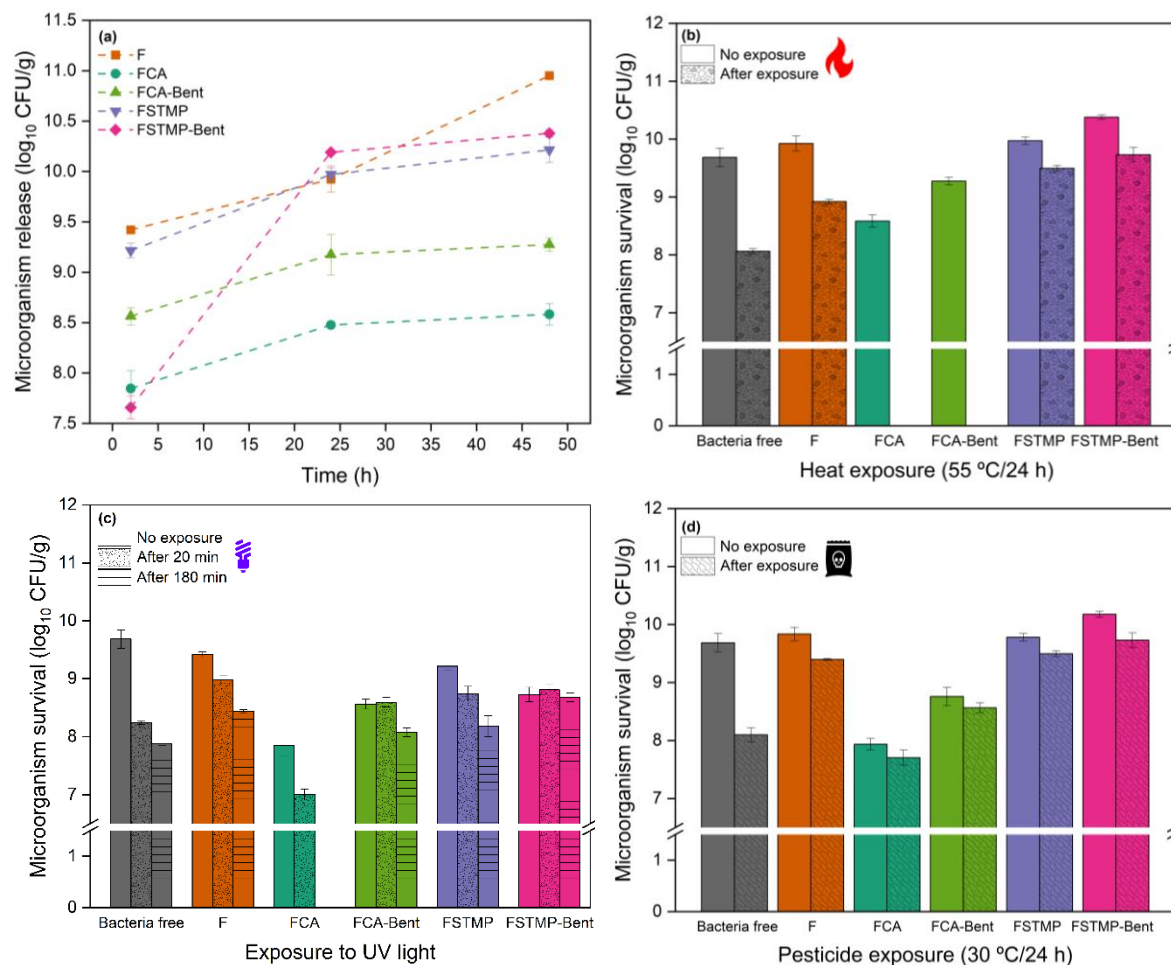


Figure 6. Tests with microorganisms. (a) Release profiles of *B. megaterium* in saline over 48 h; Survival of *B. megaterium* before and after exposure (b) to thermal stress (55 °C/24 h); (c) to UV light (20 and 180 min); (d) to contact with the pesticide (30 °C/24 h). F (PVA/CS); PVA (Polyvinyl Alcohol), CS (Cationic Starch), CA (Citric Acid), STMP (Sodium Trimetaphosphate) and Bent (Bentonite).

3.2 Accelerated Shelf-Life Test (ASLT)

Assessing the viability during storage is crucial when developing new matrices to encapsulate microorganisms. The lack of globally accepted standards for commercializing these products makes this evaluation even more critical, as requirements may vary according to local regulations. For instance, China and India only need inoculants with a minimum concentration of viable cells, set at 8 - 8.3 log₁₀ CFU/g and 7 log₁₀ CFU/g, respectively (Kumari et al., 2023). In Brazil, this value is 9 log₁₀ CFU/g. Despite regional variations, it is commonly recommended that these concentrations be maintained for at least six months of storage (180 days), following

local policies (Brasil, 2020; Kumari et al., 2023). The use of accelerated shelf-life testing (ASLT) can be a useful preliminary screening tool to compare the performance of different products under extreme storage conditions, such as temperature and humidity, to estimate their shelf life.

The results proved promising regarding the estimated shelf life of the matrices, especially when kept at 15 °C. Both for the F and FSTMP matrices, the shelf-life estimates exceeded 38 months. Remarkably, for FSTMP-Bent, the results were even more impressive, with an estimated shelf life of 358 months before total loss of viability. Lopes et al. (2024) also employed the ASLT methodology to estimate the shelf life of particles based on PVA and starch encapsulated with *Bacillus*, reinforced with starch and montmorillonite. They observed that the particles maintained high shelf-life estimates at 15°C (up to 39 months), suggesting that this temperature is effective for storing PVA/starch-based materials.

Formulations containing STMP demonstrated remarkable resistance, maintaining an estimated viability of up to 49 months (FSTMP) and 109 months (FSTMP-Bent), even under higher temperatures (45 °C). Under conditions close to room temperature in tropical countries (30 °C), the estimated shelf life of these matrices, especially FSTMP-Bent, tripled. These results are consistent with the low rates of viability loss observed for these matrices under adverse conditions. On the other hand, formulations containing citric acid showed the worst performance in preserving the microorganism during storage, with total viability loss occurring in just one month. This performance was even inferior to that of the free microorganism, which had an estimated shelf life of up to 6 months, depending on the storage temperature.

Finally, based on the estimates of viability decay of the materials to 9, 8, and 7 log₁₀ CFU/g, it was possible to determine which matrices would comply with regulations worldwide. Found that matrices F, FSTMP, and FSTMP-Bent would comply with standards for commercialization in China and India, as they had an initial concentration of encapsulated cells higher than that established for these countries (8 - 8.3 log₁₀ CFU/g in China and 7 log₁₀ CFU/g in India), along with an estimated shelf life exceeding 38 months when stored at 15 °C. However, only the FSTMP-Bent matrix met the standards established by Brazilian legislation, which requires the initial viability of products (9 log₁₀ CFU/g) to be maintained for at least six months of storage, achieved for temperatures of 15 and 30°C.

Table 0-1. Shelf-life estimate of free and encapsulated *B. megaterium* using the Accelerated Shelf-Life Test (ASLT) method.

Formulations / Months to lose viability	Bacteria free			F			FCA			FCA-Bent			FSTMP			FSTMP-Bent		
<i>Temperature of storage (°C)</i>	15	30	45	15	30	45	15	30	45	15	30	45	15	30	45	15	30	45
Total	6.6	5.6	4.8	37.9	23.5	15.3	1.0	1.0	1.0	10.6	5.8	3.3	60.3	53.8	48.5	358.2	118.5	43.5
9 log ₁₀ CFU	1.2	1.0	0.9	3.8	2.4	1.5	0.1	0.1	0.1	0.5	0.3	0.2	5.8	5.2	4.7	24.2	8.0	2.9
8 log ₁₀ CFU	1.8	1.5	1.3	11.4	7.1	4.6	0.2	0.2	0.2	1.6	0.9	0.5	11.8	10.6	9.6	61.3	20.3	7.5
7 log ₁₀ CFU	2.4	2.0	1.8	19.0	11.8	7.6	0.3	0.3	0.3	2.8	1.5	0.9	17.9	16.0	14.4	98.4	32.6	12.0

3.3 Influence of Soil Salinity

Given the favorable bacterial protection properties and the long estimated shelf-life, the formulations F, FSTMP, and FSTMP-Bent were chosen for a practical test in soil with high salinity. Soil salinity is a global challenge that impairs crop growth and productivity, especially in arid and semi-arid regions. Essential crops such as rice, wheat, and corn are affected when salinity concentrations in the soil reach 200 mM, compromising their growth and development processes and subjecting them to osmotic stress (Parida and Das, 2005). Figure 7 illustrates the influence of two soil salinity conditions (0 and 200 mM) before and after seven days, both in free *Bacillus* and encapsulated in pre-selected matrices. The experiment, lasting seven days, included the addition of a nutrient solution to the soil to ensure minimal survival conditions over time for both forms of bacteria.

Once again, the matrices FSTMP and FSTMP-Bent demonstrated more significant protective effects for encapsulated bacteria, with no cell losses observed after exposure to saline stress. There was even an increase in *B. megaterium* growth between 4% to 9% for FSTMP and 1% to 12% for FSTMP-Bent, compared to the inoculated values at the start of the experiment. In contrast, the formulation F showed a 23% loss in initial cell concentration after exposure to stress, indicating no significant difference regarding the salinity increase despite previous promising results for cell protection against adverse conditions. Finally, the survival of free bacteria was also affected, especially at 200 mM, with a reduction in viability of up to 14%.

The results indicate that the FSTMP and FSTMP-Bent formulations enhanced the survival of *Bacillus megaterium* in harsh environments, such as high salinity, providing increased protection for the microorganism. Sodium Trimetaphosphate (STMP) not only aids in cell maintenance but also serves as an additional phosphorus source for plants, potentially leading to higher crop yields (Du et al., 2021).

Research on cell survival in saline soils is limited, but some studies have explored various encapsulation formulations for microbes and their impact on crop growth under salt stress. For instance, Zhang et al. (2023) observed enhanced bacterial population, enzyme activities, and nutrient availability by microencapsulating *Bacillus pumilus* G5 in sodium alginate, trehalose, and kaolin, even in soils with 50 mM salinity and water stress. Bishe (2019) demonstrated that applying *Pantoea agglomerans* encapsulated in sodium alginate beads improved rice plant growth under 100 mM salt stress compared to free cells. Similarly,

Chanratana (2019) achieved significant growth promotion of tomato seeds in soils with 50 and 100 mM NaCl by immobilizing *Methylobacterium oryzae* CBMB20 in a cross-linked chitosan matrix.

These studies underscore the significance of effective formulations for protecting encapsulated microorganisms, which directly impacts their beneficial effects on various crops. Here, the FSTMP and FSTMP-Bent formulations emerge as promising candidates for agricultural applications based on the results obtained in this study. However, it is essential to continue research to understand the dynamics of these matrices under field conditions, thus contributing to significant advancements in the development of innovative bioinputs.

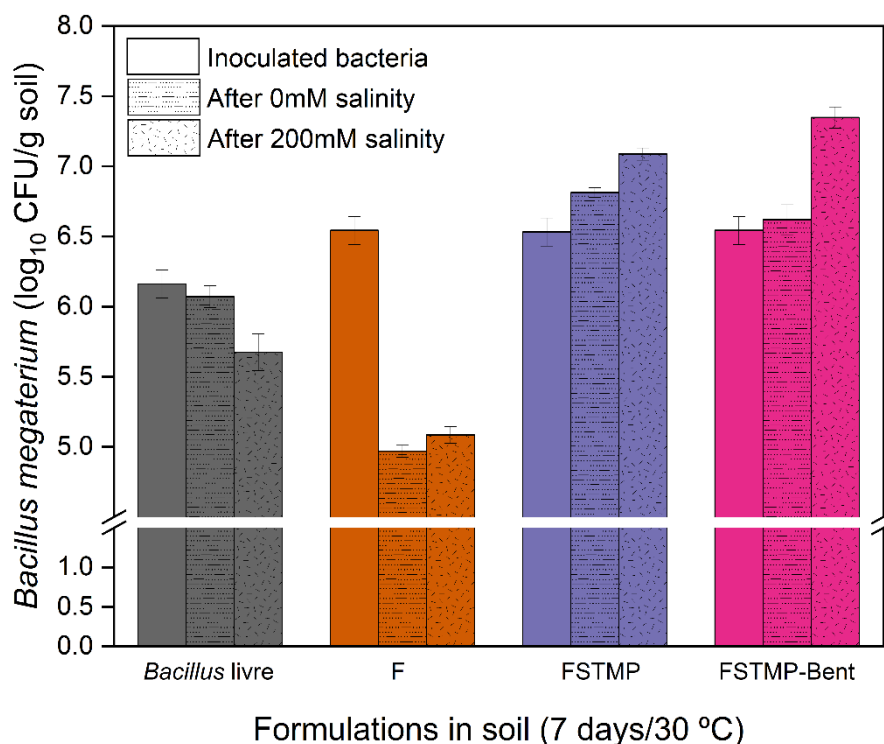


Figure 7. Influence of soil salinity on free and encapsulated microorganisms before and after 7 days at 30 °C. F (PVA/CS); PVA (Polyvinyl Alcohol), CS (Cationic Starch), STMP (Sodium Trimetaphosphate) and Bent (Bentonite).

4. Conclusions

This study describes a simple methodology for cross-linking/emulsion for the microencapsulation of *Bacillus megaterium* in a matrix based on PVA and cationic starch. The

presence of the cross-linker Sodium Trimetaphosphate (STMP) and STMP/Bentonite, with the latter acting as a surface modification agent, provided the formulations with controlled release of the bacteria, as well as greater protection against adverse conditions such as heat, UV light, pesticide exposure, and high soil salinities. Furthermore, the extended shelf-life estimate, exceeding 38 months until complete loss of viability of the materials, especially for those with STMP/Bentonite, demonstrated compliance with international regulations for the potential commercialization of these products.

References

- Ahmad, I., Ahmad, M., Hussain, A., & Jamil, M. (2021). Integrated use of phosphate-solubilizing *Bacillus subtilis* strain IA6 and zinc-solubilizing *Bacillus* sp. strain IA16: a promising approach for improving cotton growth. **Folia Microbiologica**, 66, 115–125. <https://doi.org/10.1007/s12223-020-00831-3>/Published
- Akram, W., Aslam, H., Ahmad, S. R., Anjum, T., Yasin, N. A., Khan, W. U., Ahmad, A., Guo, J., Wu, T., Luo, W., & Li, G. (2019). *Bacillus megaterium* strain A12 ameliorates salinity stress in tomato plants through multiple mechanisms. **Journal of Plant Interactions**, 14(1), 506–518. <https://doi.org/10.1080/17429145.2019.1662497>
- Alori, E. T., & Babalola, O. O. (2018). Microbial inoculants for improving crop quality and human health in Africa. **Frontiers in Microbiology**, 9, 1–12. <https://doi.org/10.3389/fmicb.2018.02213>
- Aslam, H., Ahmad, S. R., Anjum, T., & Akram, W. (2018). Native halotolerant plant growth promoting bacterial strains can ameliorate salinity stress on tomato plants under field conditions. **International Journal of Agriculture and Biology**, 20(2), 315–322. <https://doi.org/10.17957/IJAB/15.0491>
- Baigorria, E., Cano, L. A., Sanchez, L. M., Alvarez, V. A., & Ollier, R. P. (2020). Bentonite-composite polyvinyl alcohol/alginate hydrogel beads: Preparation, characterization and their use as arsenic removal devices. **Environmental Nanotechnology, Monitoring and Management**, 14. <https://doi.org/10.1016/j.enmm.2020.100364>
- Bargaz, A., Lyamlouli, K., Chtouki, M., Zeroual, Y., & Dhiba, D. (2018). Soil Microbial Resources for Improving Fertilizers Efficiency in an Integrated Plant Nutrient Management System. **Frontiers in Microbiology**, 9(July). <https://doi.org/10.3389/fmicb.2018.01606>

- Batista, D. P. C., De Oliveira, I. N., Ribeiro, A. R. B., Fonseca, E. J. S., Santos-Magalhães, N. S., De Sena-Filho, J. G., Teodoro, A. V., Grillo, L. A. M., De Almeida, R. S., & Dornelas, C. B. (2017). Encapsulation and release of: *Beauveria bassiana* from alginate-bentonite nanocomposite. **RSC Advances**, 7(42), 26468–26477. <https://doi.org/10.1039/c7ra02185b>
- Berninger, T., González López, Ó., Bejarano, A., Preininger, C., & Sessitsch, A. (2018). Maintenance and assessment of cell viability in formulation of non-sporulating bacterial inoculants. In **Microbial Biotechnology** (Vol. 11, Issue 2, pp. 277–301). John Wiley and Sons Ltd. <https://doi.org/10.1111/1751-7915.12880>
- Bhise, K. K., & Dandge, P. B. (2019). Alleviation of salinity stress in rice plant by encapsulated salt tolerant plant growth promoting bacteria *Pantoea agglomerans* strain KL and its root colonization ability. **Archives of Agronomy and Soil Science**, 65(14), 1955–1968. <https://doi.org/10.1080/03650340.2019.1584395>
- Bian, H., Wang, G., Cao, M., Wang, Z., & Cui, J. (2020). Improved biodegradation of polyvinyl alcohol by hybrid nanoflowers of degrading enzymes from *Bacillus niacini*. **Korean Journal of Chemical Engineering**, 37(6), 1020–1028. <https://doi.org/10.1007/s11814-020-0547-9>
- Bigi, A., Panzavolta, S., & Rubini, K. (2004). Relationship between triple-helix content and mechanical properties of gelatin films. **Biomaterials**, 25(25), 5675–5680. <https://doi.org/10.1016/j.biomaterials.2004.01.033>
- Brasil. (2020). Normas sobre especificações, garantias, registro, embalagem e rotulagem dos inoculantes destinados à agricultura. Ministério Da Agricultura e Pecuária (MAPA). <https://doi.org/https://www.gov.br/agricultura/pt-br/assuntos/insumos-agropecuarios/insumos-agricolas/fertilizantes/legislacao/in-sda-13-de-24-03-2011-inoculantes.pdf>
- Çalık, P., Çalık, G., & Özdamar, T. H. (1998). Oxygen transfer effects in serine alkaline protease fermentation by *Bacillus licheniformis*: Use of citric acid as the carbon source.
- Chanratana, M., Joe, M. M., Roy Choudhury, A., Anandham, R., Krishnamoorthy, R., Kim, K., Jeon, S., Choi, J., Choi, J., & Sa, T. (2019). Physiological response of tomato plant to chitosan-immobilized aggregated *Methylobacterium oryzae* CBMB20 inoculation under salinity stress. **3 Biotech**, 9(11). <https://doi.org/10.1007/s13205-019-1923-1>

- Chi, Y., Maitland, E., & Pascall, M. A. (2024). The effect of citric acid concentrations on the mechanical, thermal, and structural properties of starch edible films. **International Journal of Food Science and Technology**, 59(3), 1801–1813. <https://doi.org/10.1111/ijfs.16933>
- David, N. Ben, Mafi, M., Nyska, A., Gross, A., Greiner, A., & Mizrahi, B. (2021). *Bacillus subtilis* in PVA Microparticles for Treating Open Wounds. **ACS Omega**, 6(21), 13647–13653. <https://doi.org/10.1021/acsomega.1c00790>
- de Sousa, S. M., de Oliveira, C. A., Andrade, D. L., de Carvalho, C. G., Ribeiro, V. P., Pastina, M. M., Marriel, I. E., de Paula Lana, U. G., & Gomes, E. A. (2021). Tropical *Bacillus* Strains Inoculation Enhances Maize Root Surface Area, Dry Weight, Nutrient Uptake and Grain Yield. **Journal of Plant Growth Regulation**, 40(2), 867–877. <https://doi.org/10.1007/s00344-020-10146-9>
- Du, T. ming, Yang, H. sheng, & Niu, X. feng. (2021). Phosphorus-containing compounds regulate mineralization. In **Materials Today Chemistry** (Vol. 22). Elsevier Ltd. <https://doi.org/10.1016/j.mtchem.2021.100579>
- El Ouardi, M., Laabd, M., Abou Oualid, H., Brahmi, Y., Abaamrane, A., Elouahli, A., Ait Addi, A., & Laknifli, A. (2019). Efficient removal of p-nitrophenol from water using montmorillonite clay: insights into the adsorption mechanism, process optimization, and regeneration. **Environmental Science and Pollution Research**, 26(19), 19615–19631. <https://doi.org/10.1007/s11356-019-05219-6>
- Flores-Céspedes, F., Villafranca-Sánchez, M., & Fernández-Pérez, M. (2023). Alginate-Bentonite-Based Hydrogels Designed to Obtain Controlled-Release Formulations of Dodecyl Acetate. **Gels**, 9(5). <https://doi.org/10.3390/gels9050388>
- Fróna, D., Szenderák, J., & Harangi-Rákos, M. (2019). The challenge of feeding the world. In **Sustainability (Switzerland)** (Vol. 11, Issue 20). MDPI. <https://doi.org/10.3390/su11205816>
- Gamage, A., Liyanapathirana, A., Manamperi, A., Gunathilake, C., Mani, S., Merah, O., & Madhujith, T. (2022). Applications of Starch Biopolymers for a Sustainable Modern Agriculture. In **Sustainability (Switzerland)** (Vol. 14, Issue 10). MDPI. <https://doi.org/10.3390/su14106085>

- Gontard, N., Guilbert, S., & Cuq, J.-L. (1992). Edible Wheat Gluten Films: Influence of the Main Process Variables on Film Properties using Response Surface Methodology. **Journal of Food Science**, 191–199. doi.org/10.1111/j.1365-2621.1992.tb05453.x
- Goswami, B., & Mahanta, D. (2021). Starch and its Derivatives: Properties and Applications. In Inamuddin, Mohd Imran Ahamed, Rajender Boddula, & Tariq Altalhi (Eds.), **Polysaccharides: Properties and Applications** (pp. 253–281). Scrivener Publishing. <https://doi.org/10.1002/9781119711414.ch12>
- Havstad, M. R. (2020). Biodegradable plastics. In T. M. Letcher (Ed.), *Plastic Waste and Recycling* (Vol. 68, Issue 9, pp. 97–129). **Academic Press**. <https://doi.org/10.1016/B978-0-12-817880-5.00005-0>
- Helal, M. I. D., El-Mogy, M. M., Khater, H. A., Fathy, M. A., Ibrahim, F. E., Li, Y. C., Tong, Z., & Abdelgawad, K. F. (2023). A Controlled-Release Nanofertilizer Improves Tomato Growth and Minimizes Nitrogen Consumption. **Plants**, 12(10). <https://doi.org/10.3390/plants12101978>
- Hoagland, D. R., & Arnon, D. I. (1950). The Water-Culture Method for Growing Plants without Soil.
- Hwang, Y. H., Um, T., Ahn, G. N., Kim, D. P., & Lee, H. (2022). Robust and scalable production of emulsion-templated microparticles in 3D-printed milli-fluidic device. **Chemical Engineering Journal**, 431. <https://doi.org/10.1016/j.cej.2021.133998>
- Jeong, Y. J., Yao, Y., Mekonnen, T. H., & Yim, E. K. F. (2021). Changing Compliance of Poly(Vinyl Alcohol) Tubular Scaffold for Vascular Graft Applications Through Modifying Interlayer Adhesion and Crosslinking Density. **Frontiers in Materials**, 7. <https://doi.org/10.3389/fmats.2020.595295>
- Jose, J., & Al-Harhi, M. A. (2017). Citric acid crosslinking of poly(vinyl alcohol)/starch/graphene nanocomposites for superior properties. **Iranian Polymer Journal** (English Edition), 26(8), 579–587. <https://doi.org/10.1007/s13726-017-0542-0>
- Khan, A. R., Mustafa, A., Hyder, S., Valipour, M., Rizvi, Z. F., Gondal, A. S., Yousuf, Z., Iqbal, R., & Daraz, U. (2022). *Bacillus* spp. as Bioagents: Uses and Application for Sustainable Agriculture. In *Biology* (Vol. 11, Issue 12). **MDPI**. <https://doi.org/10.3390/biology11121763>

- Kim, D., Kim, D., Kim, J., Park, C., Roh, K. M., Kang, I. M., & Seo, S. M. (2020). Synthesis and Characterization of Montmorillonite-Supported TiO₂ Composites for enhanced UV Absorption. **Clays and Clay Minerals**, 68(6), 533–543. <https://doi.org/10.1007/s42860-020-00094-6>
- Klaic, R., Plotegher, F., Ribeiro, C., Zangirolami, T. C., & Farinas, C. S. (2018). A Fed-Batch Strategy Integrated with Mechanical Activation Improves the Solubilization of Phosphate Rock by *Aspergillus niger*. **ACS Sustainable Chemistry and Engineering**, 6(9), 11326–11334. <https://doi.org/10.1021/acssuschemeng.8b00885>
- Korbag, I., & Mohamed Saleh, S. (2016). Studies on the formation of intermolecular interactions and structural characterization of Polyvinyl alcohol/Lignin film. **International Journal of Environmental Studies**, 73(2), 226–235. <https://doi.org/10.1080/00207233.2016.1143700>
- Kumari, M., Swarupa, P., Kesari, K. K., & Kumar, A. (2023). Microbial Inoculants as Plant Biostimulants: A Review on Risk Status. In *Life* (Vol. 13, Issue 1). **MDPI**. <https://doi.org/10.3390/life13010012>
- Labelle, M. A., Ispas-Szabo, P., & Mateescu, M. A. (2020). Structure-Functions Relationship of Modified Starches for Pharmaceutical and Biomedical Applications. In *Starch/Staerke* (Vol. 72, Issues 7–8). **Wiley-VCH Verlag**. <https://doi.org/10.1002/star.202000002>
- Lal, S., Kumar, V., & Arora, S. (2021). Eco-friendly synthesis of biodegradable and high strength ternary blend films of PVA/starch/pectin: Mechanical, thermal and biodegradation studies. **Polymers and Polymer Composites**, 29(9), 1505–1514. <https://doi.org/10.1177/0967391120972881>
- Lopes, M. M., Lodi, L. A., Oliveira-Paiva, C. A. de, & Farinas, C. S. (2024). Emulsion/Cross-Linking Encapsulation of *Bacillus* in Starch/PVA-Based Microparticles for Agricultural Applications. **ACS Agricultural Science and Technology**. <https://doi.org/10.1021/acscitech.4c00029>
- Mumtaz, M. Z., Ahmad, M., Jamil, M., & Hussain, T. (2017). Zinc solubilizing *Bacillus* spp. potential candidates for biofortification in maize. **Microbiological Research**, 202, 51–60. <https://doi.org/10.1016/j.micres.2017.06.001>

- Nornberg, A. B., Martins, C. C., Cervi, V. F., Sari, M. H. M., Cruz, L., Luchese, C., Wilhelm, E. A., & Fajardo, A. R. (2022). Transdermal release of methotrexate by cationic starch/poly(vinyl alcohol)-based films as an approach for rheumatoid arthritis treatment. **International Journal of Pharmaceutics**, 611. <https://doi.org/10.1016/j.ijpharm.2021.121285>
- Oliveira, J. L., Fraceto, L. F., Bravo, A., & Polanczyk, R. A. (2021). Encapsulation Strategies for *Bacillus thuringiensis*: From Now to the Future. **Journal of Agricultural and Food Chemistry**, 69(16), 4564–4577. <https://doi.org/10.1021/acs.jafc.0c07118>
- Ons, L., Bylemans, D., Thevissen, K., & Cammue, B. P. A. (2020). Combining biocontrol agents with chemical fungicides for integrated plant fungal disease control. **Microorganisms**, 8(12), 1–19. <https://doi.org/10.3390/microorganisms8121930>
- Pan, Y., Li, P., Liang, F., Zhang, J., Yuan, J., & Yin, M. (2021). A Nano-Silver Loaded PVA/Keratin Hydrogel With Strong Mechanical Properties Provides Excellent Antibacterial Effect for Delayed Sternal Closure. **Frontiers in Bioengineering and Biotechnology**, 9. <https://doi.org/10.3389/fbioe.2021.733980>
- Parida, A. K., & Das, A. B. (2005). Salt tolerance and salinity effects on plants: A review. **Ecotoxicology and Environmental Safety**, 60(3), 324–349. <https://doi.org/10.1016/j.ecoenv.2004.06.010>
- Passaretti, M. G., Ninago, M. D., Di Anibal, C., Pacheco, C., Vega, D. A., Villar, M. A., & López, O. V. (2019). Composite films with UV barrier capacity to minimize flavored waters degradation. **Food Packaging and Shelf Life**, 21. <https://doi.org/10.1016/j.fpsl.2019.100334>
- Patil, R., & Bagde, U. S. (2015). Enrichment and isolation of microbial strains degrading bioplastic polyvinyl alcohol and time course study of their degradation potential. **African Journal of Biotechnology**, 14(27), 2216–2226. <https://doi.org/10.5897/AJB2011>
- Pham, D. Van, & Tho Bach, L. (2014). Immobilized bacteria by using PVA (Polyvinyl alcohol) crosslinked with Sodium sulfate. **International Journal of Science and Engineering**, 7(1). <https://doi.org/10.12777/ijse.7.1.41-47>
- Poraj-Kobielska, M., Peter, S., Leonhardt, S., Ullrich, R., Scheibner, K., & Hofrichter, M. (2015). Immobilization of unspecific peroxygenases (EC 1.11.2.1) in PVA/PEG gel and

- hollow fiber modules. **Biochemical Engineering Journal**, 98, 144–150. <https://doi.org/10.1016/j.bej.2015.02.037>
- Radhakrishnan, R., Hashem, A., & Abd Allah, E. F. (2017). *Bacillus*: A biological tool for crop improvement through bio-molecular changes in adverse environments. **Frontiers in Physiology**, 8(SEP). <https://doi.org/10.3389/fphys.2017.00667>
- Riahi, N., Liberelle, B., Henry, O., & De Crescenzo, G. (2017). Impact of RGD amount in dextran-based hydrogels for cell delivery. **Carbohydrate Polymers**, 161, 219–227. <https://doi.org/10.1016/j.carbpol.2017.01.002>
- Rynkowska, E., Fatyeyeva, K., Marais, S., Kujawa, J., & Kujawski, W. (2019). Chemically and thermally crosslinked PVA-based membranes: Effect on swelling and transport behavior. **Polymers**, 11(11). <https://doi.org/10.3390/polym11111799>
- Saeid, A., Prochownik, E., & Dobrowolska-Iwanek, J. (2018). Phosphorus solubilization by *Bacillus* species. **Molecules**, 23(11). <https://doi.org/10.3390/molecules23112897>
- Samoila, I., Dinescu, S., Pircalabioru, G. G., Marutescu, L., Fundueanu, G., Aflori, M., & Constantin, M. (2019). Pullulan/poly(vinyl alcohol) composite hydrogels for adipose tissue engineering. **Materials**, 12(19). <https://doi.org/10.3390/ma12193220>
- Schroën, K., de Ruiter, J., & Berton-Carabin, C. (2020). The importance of interfacial tension in emulsification: Connecting scaling relations used in large scale preparation with microfluidic measurement methods. In **ChemEngineering** (Vol. 4, Issue 4, pp. 1–22). MDPI AG. <https://doi.org/10.3390/chemengineering4040063>
- Sharmin, N., Rosnes, J. T., Prabhu, L., Böcker, U., & Sivertsvik, M. (2022). Effect of Citric Acid Cross Linking on the Mechanical, Rheological and Barrier Properties of Chitosan. **Molecules**, 27(16). <https://doi.org/10.3390/molecules27165118>
- Shripad Kulkarni, H. B. (2017). Studies on Compatibility of *Bacillus subtilis* (Ehrenberg) Cohn. with Chemical Fungicides. **International Journal of Current Microbiology and Applied Sciences**, 6(3), 578–586. <https://doi.org/10.20546/ijcmas.2017.603.067>
- Sonjan, S., Ross, G. M., Mahasaranon, S., Sinkangam, B., Intanon, S., & Ross, S. (2021). Biodegradable Hydrophilic Film of Crosslinked PVA/Silk Sericin for Seed Coating: The Effect of Crosslinker Loading and Polymer Concentration. **Journal of Polymers and the Environment**, 29(1), 323–334. <https://doi.org/10.1007/s10924-020-01867-9>

- Suganthi, S., Vignesh, S., Kalyana Sundar, J., & Raj, V. (2020). Fabrication of PVA polymer films with improved antibacterial activity by fine-tuning via organic acids for food packaging applications. **Applied Water Science**, 10(4). <https://doi.org/10.1007/s13201-020-1162-y>
- Tan, X. L., Hu, J., Zhou, X., Yu, S. M., & Wang, X. K. (2008). Characterization of Lin'an montmorillonite and its application in the removal of Ni²⁺ from aqueous solutions. **Radiochimica Acta**, 96(8), 487–495. <https://doi.org/10.1524/ract.2008.1515>
- Taspika, M., Permatasari, F. A., Nuryadin, B. W., Mayangsari, T. R., Aimon, A. H., & Iskandar, F. (2019). Simultaneous ultraviolet and first near-infrared window absorption of luminescent carbon dots/PVA composite film. **RSC Advances**, 9(13), 7375–7381. <https://doi.org/10.1039/C8RA09742A>
- Timmusk, S., Behers, L., Muthoni, J., Muraya, A., & Aronsson, A. C. (2017). Perspectives and challenges of microbial application for crop improvement. **Frontiers in Plant Science**, 8(February), 1–10. <https://doi.org/10.3389/fpls.2017.00049>
- Ullah, M., Li, H., Sun, S. W., Weng, C. H., Zhang, H., & Zhu, H. (2019). Polyvinyl alcohol degradation by *Bacillus cereus* RA23 from oil sludge sample. **3 Biotech**, 9(10). <https://doi.org/10.1007/s13205-019-1882-6>
- Vassilev, N., Vassileva, M., Martos, V., Garcia del Moral, L. F., Kowalska, J., Tylkowski, B., & Malusá, E. (2020). Formulation of Microbial Inoculants by Encapsulation in Natural Polysaccharides: Focus on Beneficial Properties of Carrier Additives and Derivatives. **Frontiers in Plant Science**, 11(March), 1–9. <https://doi.org/10.3389/fpls.2020.00270>
- Wahid, M. N. A., Abd Razak, S. I., Abdul Kadir, M. R., Hassan, R., Nayan, N. H. M., & Mat Amin, K. A. (2018). Influence of citric acid on the physical and biomineralization ability of freeze/thaw poly(vinyl alcohol) hydrogel. **Journal of Biomaterials Applications**, 33(1), 94–102. <https://doi.org/10.1177/0885328218771195>
- Wang, Q., Cao, L., Wang, Y., Qin, M., & Wang, Q. (2022). Shell/core structure zinc oxide/iron oxide: A new sunscreen material against blue light. **Materials Letters**, 322. <https://doi.org/10.1016/j.matlet.2022.132529>
- Wilpiszewska, K., Antosik, A. K., & Zdanowicz, M. (2019). The Effect of Citric Acid on Physicochemical Properties of Hydrophilic Carboxymethyl Starch-Based Films. **Journal of**

Polymers and the Environment, 27(6), 1379–1387. <https://doi.org/10.1007/s10924-019-01436-9>

Wu, Z., Guo, L., Qin, S., & Li, C. (2012). Encapsulation of *R. planticola* Rs-2 from alginate-starch-bentonite and its controlled release and swelling behavior under simulated soil conditions. **Journal of Industrial Microbiology and Biotechnology**, 39(2), 317–327. <https://doi.org/10.1007/s10295-011-1028-2>

Yang, Q., Yan, Y., Yang, X., Liao, G., Wang, D., & Xia, H. (2019). Enzyme immobilization in cage-like 3D-network PVA-H and GO modified PVA-H (GO@PVA-H) with stable conformation and high activity. **Chemical Engineering Journal**, 372, 946–955. <https://doi.org/10.1016/j.cej.2019.04.216>

Yao, K., Zhou, Q. xing, Liu, D. mei, Chen, S. min, & Yuan, K. (2019). Comparative proteomics of the metabolic pathways involved in L-lactic acid production in *Bacillus coagulans* BCS13002 using different carbon sources. **LWT**, 116. <https://doi.org/10.1016/j.lwt.2019.108445>

Zabot, G. L., Schaefer Rodrigues, F., Polano Ody, L., Vinicius Tres, M., Herrera, E., Palacin, H., Córdova-Ramos, J. S., Best, I., & Olivera-Montenegro, L. (2022). Encapsulation of Bioactive Compounds for Food and Agricultural Applications. In **Polymers** (Vol. 14, Issue 19). MDPI. <https://doi.org/10.3390/polym14194194>

Zhang, N., Wang, Z., Shao, J., Xu, Z., Liu, Y., Xun, W., Miao, Y., Shen, Q., & Zhang, R. (2023). Biocontrol mechanisms of *Bacillus*: Improving the efficiency of green agriculture. In **Microbial Biotechnology** (Vol. 16, Issue 12, pp. 2250–2263). John Wiley and Sons Ltd. <https://doi.org/10.1111/1751-7915.14348>

Zhang, W., Zheng, L., Lang, D., Zhang, X., Ma, X., Li, X., & Zhang, X. (2023). Eco-friendly bio-encapsulation from sodium alginate-trehalose-kaolin and its performance evaluation in improving plant growth under salt or/and drought conditions. **International Journal of Biological Macromolecules**, 225, 123–134. <https://doi.org/10.1016/j.ijbiomac.2022.12.009>

Zhang, Y., Zhou, Q., Tian, P., Li, Y., Duan, G., Li, D., Zhan, J., & Chen, F. (2020). Induced expression of CYP51 associated with difenoconazole resistance in the pathogenic *Alternaria sect.* on potato in China. **Pest Management Science**, 76(5), 1751–1760. <https://doi.org/10.1002/ps.5699>

Zheng, L., Ma, X., Lang, D., Zhang, X., Zhou, L., Wang, L., & Zhang, X. (2022). Encapsulation of *Bacillus pumilus* G5 from polyvinyl alcohol sodium alginate (PVA-SA) and its implications in improving plant growth and soil fertility under drought and salt soil conditions. **International Journal of Biological Macromolecules**, 209, 231–243. <https://doi.org/10.1016/j.ijbiomac.2022.04.017>

CHAPTER 4

Spray-Dryer Microencapsulation of *Bacillus megaterium* in PVA/Cationic Starch/Zinc Oxide for Promoting Growth and Zinc Availability in Soybean

Abstract

Zinc (Zn) is essential for plant development, and its deficiency can reduce agricultural productivity. Nutrient-solubilizing microorganisms are a promising alternative to increase zinc availability for plants. However, due to low bacterial tolerance to zinc, it is crucial to study efficient ways of solubilizing and making this nutrient available. Here, we developed a formulation using polyvinyl alcohol (PVA), cationic starch (CS) and zinc oxide (ZnO) for the microencapsulation of *Bacillus megaterium* through the spray-drying technique. Our results showed that the *B. megaterium* strain has a high potential for solubilizing zinc oxide. The PVACS-ZnO matrix provided a favorable environment for growth and development of *B. megaterium*, releasing cells in quantities exceeding those initially inoculated ($10 \log_{10}$ CFU/g). Additionally, it demonstrated efficacy in protecting the cells against adverse field conditions, maintaining bacterial viability after exposure to heat (50°C/2 h), up to 95% after UV light exposure (180 min), and 99% after exposure to fungicides and insecticides (30°C/2 h). In contrast, free bacteria were significantly affected, highlighting the protection provided to *Bacillus* by the proposed formulation. Accelerated Shelf-Life Tests (ASLT) indicated prolonged stability of PVACS-ZnO microspheres, with double estimated shelf-life for encapsulated bacteria (14 months) compared to free bacteria (6 months). In greenhouse experiments, the presence of zinc and *Bacillus megaterium* in the formulation significantly influenced the increase in aerial and root biomass production of soybean plants, as well as promoted greater absorption of phosphorus and zinc nutrients by soybean biomass. These findings indicate that PVASC-ZnO formulations offer a promising strategy for encapsulating microorganisms and enhancing zinc availability, resulting in an effective and environmentally friendly biofertilizer product.

Keywords: *Bacillus*, spray drying, biofertilizer, zinc solubilization, cationic starch.

1. Introduction

Zinc (Zn) is a micronutrient essential for healthy plant growth, acting as a cofactor in various enzymes and proteins. Its presence is crucial for photosynthesis, stress resistance, and pathogen defense, promoting crop growth and productivity (Ahmad et al., 2021; Zeng et al., 2019). Zinc deficiency negatively impacts the production and development of various crops, such as soybeans (*Glycine max L.*), cotton (*Gossypium hirsutum L.*) and maize (*Zea mays*) making them more susceptible to pathogen attacks (Khande et al., 2017; Pawlowski et al., 2019; Xu et al., 2021).

Zinc can be applied to the soil as a fertilizer from various sources, such as zinc oxide (ZnO), zinc sulfate (ZnSO₄), and chelated zinc (Zn-EDTA), either as part of multinutrient fertilizers like monoammonium phosphate (MAP), or through foliar spraying (ZnSO₄.7H₂O or Zn-EDTA). Zinc oxide (ZnO) is recognized as one of the most cost-effective options for supplying zinc as a fertilizer (Degryse et al., 2020). Recent studies have highlighted the potential of microbial strains, to solubilize zinc sources, thereby enhancing the growth of crops such as potatoes (*Solanum tuberosum*), wheat (*Triticum aestivum*), cotton (*Gossypium hirsutum L.*), and peppers (*Capsicum annuum L.*) (Ahmad et al., 2021; Ali et al., 2023; Bhatt and Maheshwari, 2020; Gupta et al., 2023). However, the direct application of microorganisms in the fields faces challenges, such as loss of cell viability due to exposure to agrochemicals, heat, and UV radiation. Therefore, it is crucial to develop formulations that ensure the protection and viability of these microorganisms (Berninger et al., 2018). In this context, microencapsulation emerges as a strategy to increase shelf-life, regulate release, and preserve microorganisms against adverse environmental influences (Rojas-Sánchez et al., 2022).

Spray drying microencapsulation is a promising technique for cell encapsulation widely used in various industries due to its efficiency, speed, cost-effectiveness, and scalability (Braga et al., 2019; Piñón-Balderrama et al., 2020). Studies have shown success in microencapsulating microorganisms for the food industry, maintaining up to 90% of their viability post-process (Seth et al., 2017; Strobel et al., 2018). However, research on microencapsulating microorganisms for agricultural applications remains limited.

The choice of encapsulation matrix has a significant impact on the preservation of cellular viability. Polymers such as Polyvinyl Alcohol (PVA) and Starch (ST) are considered promising for encapsulating microorganisms and enzymes due to their cellular compatibility

and their partial or total degradation by various microorganisms, including the *Bacillus* bacteria (Bian et al., 2020, 2019; Liu et al., 2019; Ullah et al., 2019). Another polymer of growing interest is cationic starch (CS), a synthetic material derived from the chemical modification of natural starch (ST), which has amine groups with positive charges in its structure (Goswami and Mahanta, 2021). Cationic starch is highly soluble in water and has excellent film-forming properties. Recent research on PVA and cationic starch-based films has shown promising characteristics in applications such as fruit coating (Petry et al., 2024) and controlled drug release (Nornberg et al., 2022), highlighting the matrix as a promising option for microorganism encapsulation.

The objective of this work is to evaluate the microencapsulation of strain as *Bacillus megaterium* using the spray dryer technique in PVA and cationic starch-based formulations, investigating the addition of zinc oxide (ZnO) to assess its effect on the growth and development of soybean seeds. To achieve this, we investigated: (1) the maximum concentration of ZnO to be added to the matrix; (2) the encapsulation of *Bacillus megaterium* in matrices with and without ZnO and its effect on bacteria protection under conditions of heat stress, UV light, and exposure to agrochemicals; (3) the estimation of the shelf life of the materials produced using Accelerated Shelf Life Testing (ASLT) methodology; and (4) the performance of the ZnO-containing matrix in a greenhouse trial and its effect on soybean cultivation.

2. Materials and Methods

2.1 Materials

Cationic Starch (CS - Ingredient), Polyvinyl Alcohol (PVA - Molecular Weight 89,000-98,000, hydrolyzed to 99+%), and Zinc oxide (ZnO) were obtained from Sigma Aldrich. The fungicide (Maxin) and insecticide (Cruiser 350 FS) used in these studies are Syngenta Brasil.

2.2 Microorganism Reactivation for Encapsulation

The bacterium utilized in this study was a strain of *Bacillus megaterium* (CNPMS B119) obtained from the Multifunctional Microorganism Collection at Embrapa Maize and Sorghum (Sousa et al., 2021). To reactivate the cells stored at -80°C, the bacteria were inoculated on Soy Tryptone broth (TSB) and bacteriological agar in a Petri dish and then incubated at 30 °C for 12 h. Subsequently, the inoculum was transferred to a TSB liquid medium and stirred at 250 rpm, at 30 °C, for 72 h to produce the cells used for inoculation.

2.3 Solubilization of Different Concentrations of Zinc Oxide by *Bacillus megaterium*

A preliminary test was conducted to determine the optimal concentration of ZnO to be added to the matrix of PVA/cationic starch, aiming to achieve maximum solubility of Zn with minimal toxicity to *Bacillus megaterium*. To investigate the solubility of ZnO and bacterial survival, a liquid culture was conducted for 10 days in TSB medium (100 mL, 250 rpm, 30 °C), starting with an initial count of *B. megaterium* of 10^9 CFU/mL and adding predefined quantities of ZnO (0.5%, 0.75%, or 1% w/v). Samples of 2 mL were collected on days 1, 2, 3, 5, and 10 to assess the solubilized Zn and bacterial growth. Solubilized zinc (Zn^{2+}) was quantified using Flame Atomic Absorption Spectroscopy (FAAS) on a PerkinElmer PinAAcle 900 T spectrometer (wavelength 213.86 nm, slit width 0.7 nm, synthetic air at 10 L/min, acetylene at 2.5 L/min). Bacterial growth was assessed by inoculating aliquots onto TSB and agar plates, then incubating for 24 h at 25 °C. All steps were performed in triplicate.

2.4 Microorganism Encapsulation

Two formulations were evaluated in this study: PVA/Cationic Starch (PVACS) and PVA/Cationic Starch/ZnO (PVACS-ZnO). Initially, PVA (10% w/v) and CS (10% w/v) were dissolved in 50 mL of distilled water and stirred in a water bath at 80 °C for 15 min to form PVACS. After the solution was cooled (25 – 30 °C), a cell suspension volume containing *Bacillus megaterium* ($10 \log_{10}$ CFU/g) was added under stirring for 5 min to ensure complete dispersion. The formulation containing ZnO followed the same base and inoculation procedure as PVACS. However, after determining the ideal amount of ZnO in the previous step (Section 2.2), 0.5% ZnO (w/v) was added to PVACS at room temperature and stirred for 5 min to ensure complete homogenization of the element, resulting in PVACS-ZnO. Both formulations were subsequently subjected to the microencapsulation and drying process using the Spray Dryer MSD 1.0 (LabMaq), operating with an outlet temperature of 70 °C, flow rate of 0.3 L/h, and gas pressure of 1.2 m³/h. At the end of the procedure, the resulting microcapsules were recovered and stored in Falcon tubes at 25 °C for future analyses.

2.5 Characterizations

2.5.1 Morphological and Physicochemical Analysis

The morphology of microparticles was observed using a Scanning Electron Microscope (SEM) with a JEOL (JSM-6510) at an accelerating voltage of 5 kV, a working distance of 10 mm, a secondary electron detector and equipped with an energy dispersive X-ray analysis system (EDS spectrometer - NSS ThermoScientific coupled or linked). The size of the

microspheres was determined from the SEM images using ImageJ software (National Institute of Health). Formulation phases were identified using X-ray diffraction with a LabX XRD-6000 diffractometer (Shimadzu, Japan), operated with Cu-K α radiation ($\lambda = 1.54056 \text{ \AA}$), a voltage of 30 kV, and a current of 30 mA. The diffractograms were recorded for 2θ from 4° to 70° , with a continuous scanning speed of $1^\circ/\text{min}$. The molecular structure was studied via Fourier Transform Infrared Spectroscopy (FTIR) on a Bruker Vertex 70 model with ATR accessory in the $4000\text{-}500 \text{ cm}^{-1}$ range with a resolution of 4 cm^{-1} .

2.6 Encapsulated Material Assays

2.6.1 Release Profile of Microorganisms from the Matrices

The release profile of encapsulated *B. megaterium* was investigated by adding 0.1 g of microspheres to 5 mL of 0.85% NaCl saline solution in a rotating incubator at 30°C and 250 rpm. Samples of 1 mL were collected at 2, 24, and 48 h. Aliquots of the solutions and their serial dilutions were inoculated into TSB medium and bacteriological agar, followed by incubation at 25°C for 24 h. Biological and analytical duplicates were employed for each dilution in this assay.

2.5.2 Exposure to Conditions of Stress

Both free *Bacillus* and encapsulated in PVACS and PVACS-ZnO were subjected to tests of thermal resistance, exposure to UV light, and contact with fungicides and insecticides, considering an initial concentration of bacteria of $9 \log_{10} \text{ CFU/g}$ or mL (Lopes et al., 2024; Shahid and Khan, 2018).

The materials were exposed to 50°C for 2, 24, and 48 h to assess heat resistance. They were placed in a chamber with six UV-C lamps of 15W (Philips 15W G15T8) for UV light exposure for 10, 30, and 180 min. After each exposure, free bacteria were diluted and plated, while encapsulated ones were released in 0.85% NaCl saline solution for 2 h at 30°C . Diluted samples were then inoculated onto TSB medium and bacteriological agar, with biological and analytical duplicates for each dilution. The inoculated plates were incubated at 25°C for 24 h, followed by colony counting.

To test resistance to the fungicide (Maxin) and insecticide (Cruiser 350 FS), free and encapsulated *Bacillus* were separately incubated in 5 mL of the respective solution for 2 h at 30°C . After exposure, 1 mL samples were diluted and inoculated onto TSB medium and

bacteriological agar. Again, biological and analytical duplicates were prepared for each dilution, and the inoculated plates were incubated at 25 °C for 24 h for colony counting.

2.5 Accelerated Shelf-Life Test (ASLT)

An estimate of the Shelf-Life of the studied matrices was conducted by adapting the Accelerated Shelf-Life Test (ASLT) described by Lopes et al. (2024). Both free-form and encapsulated *Bacillus* (109 CFU/mL or g) were stored in chambers under controlled temperature conditions (15, 30, and 45 °C) and relative humidity (%RH - 76%). The exposed formulations were conditioned for 28 days, and evaluations were performed every 7 days after the release of the bacteria in NaCl solution (0.85%). Release occurred at 250 rpm in an orbital shaker at 30 °C for 2 h. A solution was plated, and the plate-counting method counted Colony Forming Units (CFUs). All tests were performed in triplicate.

The estimated time for ASLT was evaluated using first-order degradation kinetics, calculated from Equation 1, where concentration refers to the quantities of cells at the beginning and end of the experiment, and k_T is the calculated k value for each temperature using the Arrhenius Equation.

$$t = \frac{\ln(\text{initial concentration}) - \ln(\text{final concentration})}{k_T} \quad (1)$$

2.6 Greenhouse application

At this stage, PVACS-ZnO was selected for soybean greenhouse application. 1 dm³ of autoclaved (30 min 121 bar) oxysol soil was placed in plastic pots (four replications each). The soil composition is described in Table S1 (Appendix B). Each pot was fertilized with 200 mg of N/dm³ applied as urea; and 185 mg of K applied as potassium sulphate. Two concentrations of P were also evaluated: 0 mg of P/dm³ and 150 mg of P/dm³ applied as monobasic potassium phosphate. Two sets of experiments were performed: with and without *B. megaterium* inoculation. The experiments were also compared with the control treatment without fertilization (Table 1).

Table 1. Experimental design of soybean greenhouse application.

<i>Code</i>	Description
<i>Control</i>	No fertilization
<i>PVACS-ZnO</i>	PVACS-ZnO without <i>B. megaterium</i> and without phosphorus
<i>PVACS-ZnO + P</i>	PVACS-ZnO without <i>B. megaterium</i> and phosphorus
<i>PVACS-ZnO + bacteria</i>	PVACS-ZnO with <i>B. megaterium</i> and without phosphorus
<i>PVACS-ZnO + bacteria + P</i>	PVACS-ZnO with <i>B. megaterium</i> and phosphorus addition

The greenhouse experiments were conducted at a room temperature of approximately 25°C and a humidity level of 30-40%. Artificial light supplementation was provided for 12 h during the day. Soybean seeds (*Glycine max (L.)*) were germinated in a germination chamber for 6 days before being transplanted into pots already fertilized with macronutrients. The plants were regularly watered to prevent drought stress, and micronutrient fertilization was applied after two weeks (Table S2 - Appendix B). Since a nitrogenous source was included, the *Bradyrhizobium* sp. strain was not added to the pots.

The plants were harvested after four weeks. Aboveground and roots were collected, dried at 60 °C for 24 h, weighted to measure the dry matter produced, and milled using a SOLAB knife mill. Soil samples of each pot were also collected, dried, and stored for further nutrient analysis. The nutrient composition (mainly focused on P and Zn) of aboveground, roots, and soil substrate was determined by the Colorimetric method extracted with ion exchange resin (P) and Extraction with DTPA and determination by atomic absorption spectrophotometry (Zn) (IAC, 2001). The absorption of P and Zn was assessed based on their accumulated concentration (%) in the shoot or dry root matter and the dry matter generated (mg). These experimental data were verified by analysis of variance and Tukey's test at a confidence level of 95%, using Origin 9.8.0.200 software.

3. Results and Discussion

3.1 Solubility and Survival of *B. megaterium* at Different Zinc Oxide Concentrations

Zinc plays a crucial role in the biochemistry of organisms, including bacterial cells, but high concentrations of this mineral can be toxic due to its antimicrobial activity (Hutchings et al., 2020). Therefore, this preliminary set of experiments aimed to determine the maximum

amount of zinc oxide (ZnO), a source of zinc in water-insoluble powder form, that could be added to the PVA/cationic starch (PVACS) matrix without adversely affecting the encapsulated bacteria. Figure 1 illustrates the percentage of solubilized zinc (Figure 1a) and bacterial growth at different concentrations of ZnO in the medium (Figure 1b).

Among the evaluated concentrations, the optimal concentration was established at 0.5% (w/v) of ZnO, as this condition showed an increasing profile of zinc solubilization over the 10 days and initial bacterial growth superior to that observed in the other conditions. In the literature, it is common to find maximum concentration of 0.1% (w/v) of zinc sources due to low bacterial tolerance, resulting in solubility below 1% of the added zinc (Mahanty et al., 2017; Mumtaz et al., 2017; Shreya et al., 2023; Suriyachadkun et al., 2022). Here, *B. megaterium* proved promising, resisting higher concentrations of ZnO than those described in the literature, with a solubility of up to 8% in a 10-day assay for the medium containing 0.5% ZnO and showing good development throughout the assay. It is important to note that the mechanisms leading to the solubilization of zinc by *Bacillus* are similar to those observed for phosphorus, involving the acidification of the medium through the production of organic acids and the synthesis of metal chelating agents known as siderophores (Bhatt et al. 2020; Upadhayay et al 2022).

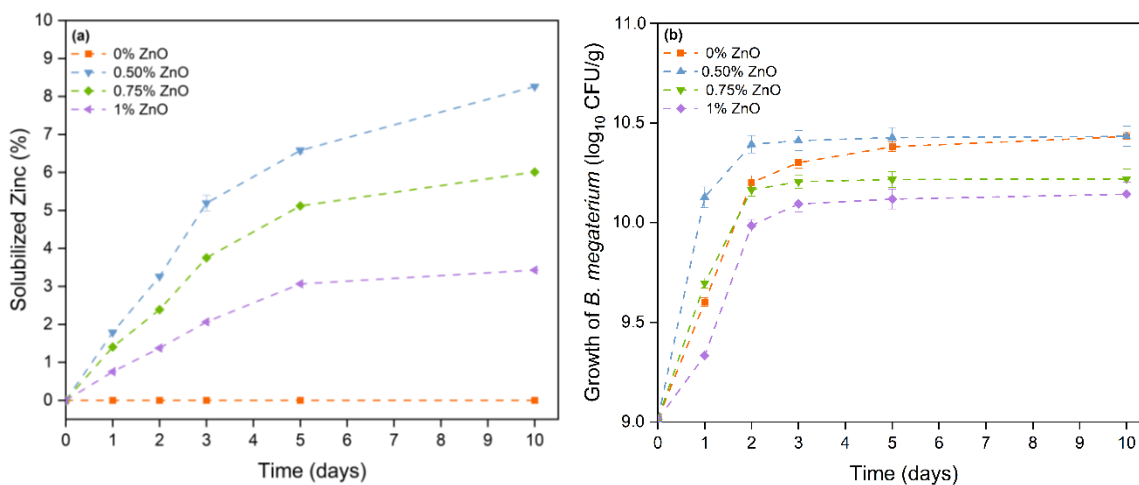


Figure 1. Zinc solubilized (a) and *Bacillus megaterium* growth (b) at different concentrations of ZnO (0 – 1% w/v) added to the Soy Tryptone broth (TSB) culture medium.

3.2 Physicochemical and Morphological Characterizations

Figure 2 shows the micrographs of spray-dried microcapsules of PVACS and PVACS-ZnO formulations. Both formulations exhibited a similar size distribution (Figure S1 – Appendix B), with average diameters of $5.52 \mu\text{m} \pm 3.23 \mu\text{m}$ (PVACS) and $5.61 \mu\text{m} \pm 3.72 \mu\text{m}$ (PVACS-ZnO). However, PVACS microspheres exhibited a smooth surface with only a few other microspheres adhering (Figures 2a-b). In contrast, PVACS-ZnO microspheres displayed dispersed ZnO particles on their surface (Figures 2c-d), as confirmed by energy dispersive X-ray analysis (Figure S2 - Appendix B).

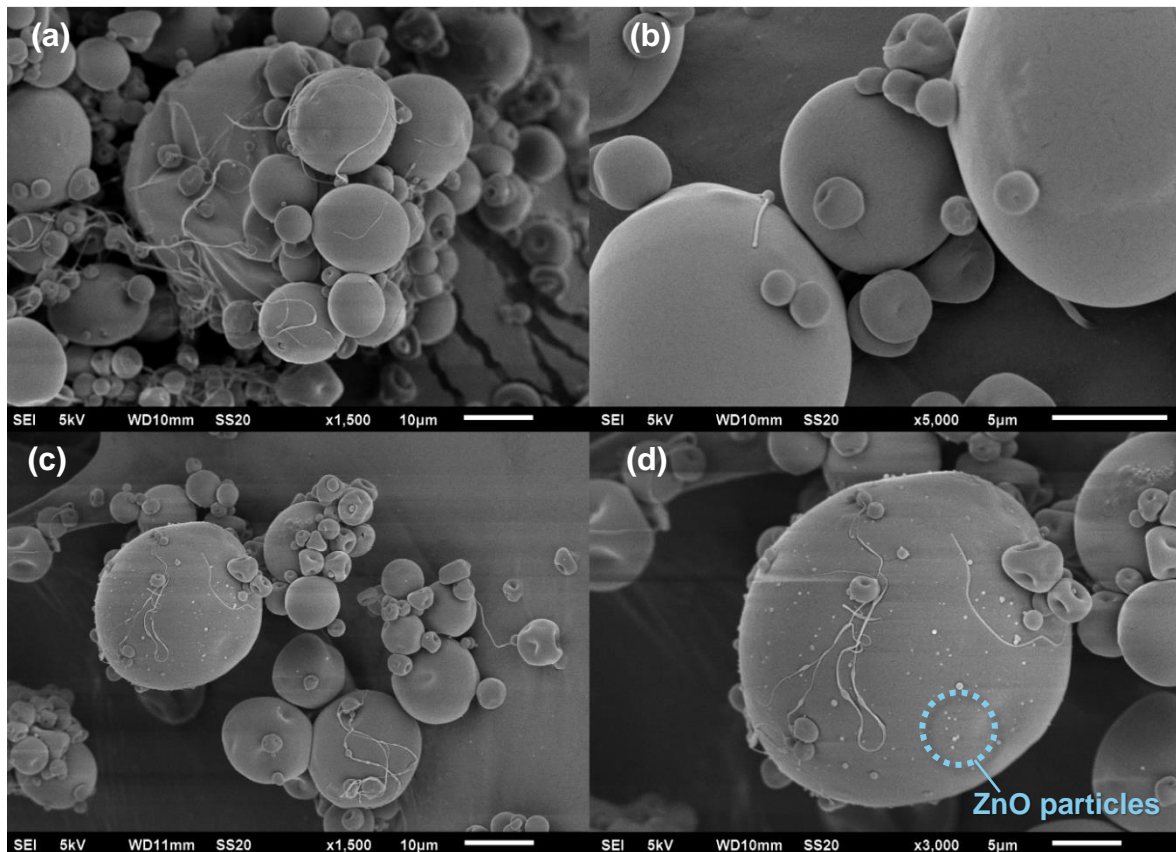


Figure 2. Microspheres by Scanning Electron Microscopy (SEM) micrographs. (a) and (b) PVACS; (c) and (d) PVACS-ZnO. PVACS (PVA/Cationic Starch) and PVACS-ZnO (PVA/Cationic Starch- Zinc oxide).

Regarding the physicochemical characteristics of the produced materials, as illustrated in Figure 3a, the XRD pattern of the PVACS matrix is predominantly amorphous. This suggests insufficient time for the orderly packing of the PVA and SC chains using the spray-drying method. However, in the PVACS-ZnO matrix, the peak at 19.4° 2θ corresponding to PVA could be identified. This behavior indicates that the ZnO particles may favor the formation of the polymer matrix ordered network, even under rapid drying conditions, although qualitatively less crystalline than casting drying (Souza Garcia et al., 2021). As expected, the PVACS-ZnO

sample also shows the characteristic peaks of ZnO (Grace et al., 2023), as confirmed by the EDS spectrometer (Figure S2 – Appendix B).

The FTIR results (Figure 3b) show the characteristic bands of PVA overlapped with the starch bands in the case of the PVACS sample and with ZnO in the case of the PVACS-ZnO sample (Petry et al., 2024). There was no significant change in the vibrational modes with the addition of ZnO, suggesting that it primarily acts as a filler in the matrix.

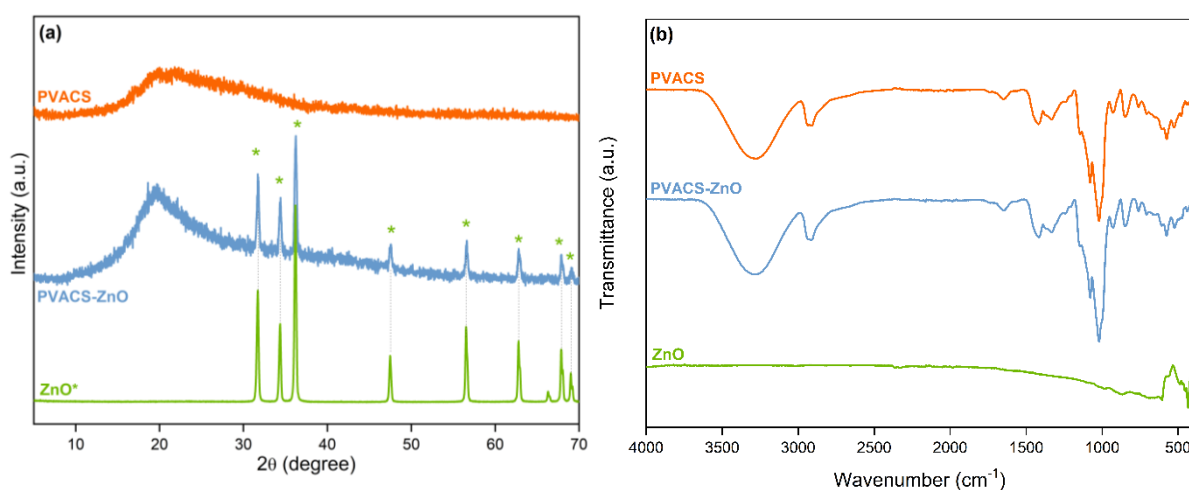


Figure 3. Physicochemical characterizations of the films. (a) X-ray diffraction (XRD); (b) Fourier Transform Infrared Spectroscopy (FTIR). PVACS (PVA/Cationic Starch) and PVACS-ZnO (PVA/Cationic Starch- Zinc oxide).

3.3 Encapsulated Microspheres

Figure 4 presents the results of tests conducted with the PVACS and PVACS-ZnO microspheres with *B. megaterium* encapsulated. Initially, we conducted an assay to investigate the release profile of the encapsulated bacteria over 48 h, as illustrated in Figure 4a. With an initial inoculation of *B. megaterium* at 10 log₁₀ CFU/g, we observed that within 2 h, the microspheres released approximately 98% of the inoculated bacteria for both PVACS and PVACS-ZnO, respectively, maintaining this trend up to 24 h. However, by 48 h, the release exceeded 100% of the initially inoculated bacteria, reaching 107% and 105% for PVACS and PVACS-ZnO, respectively. These findings suggest that the matrix may serve as a carbon source for the bacteria, aligning with previous studies highlighting the ability of *Bacillus* bacteria to utilize PVA, starch, and its derivatives as carbon sources for their growth and multiplication (Bian et al., 2020; Patil and Bagde, 2015; Ullah et al., 2019).

At this stage, we confirmed the tolerance of *Bacillus megaterium* to ZnO at the previously established concentration (0.5% ZnO), as the release of bacteria was not negatively affected by the presence of zinc compared to the formulation without the element. With the successful release and development of encapsulated bacteria in the matrix, it can be concluded that the spray drying process did not adversely affect the viability of the encapsulated bacteria. Recent studies have shown the effectiveness of microencapsulation of fungi and bacteria using a spray dryer, preserving up to 90% of cell viability (Braga et al., 2019; Seth et al., 2017; Strobel et al., 2018). This aligns with the results observed in this study, where approximately 98% cell viability was maintained.

To ensure the proper development of encapsulated microorganisms, it is crucial to consider the potential stresses that cells may encounter during field application, such as exposure to pesticides, heat, and UV light (Inbaraj, 2021; Koza et al., 2022b; Kumar et al., 2022). Thus, both free bacteria and encapsulated microspheres were exposed to stressful conditions, including UV light (Fig. 4b), heat (Fig. 4c), and agrochemicals (Fig. 4d).

Regarding exposure to UV light, the PVACS-ZnO formulation stood out, maintaining up to 95% bacterial survival over 180 minutes of exposure when compared to the control without exposure. Meanwhile, PVACS experienced a slightly more pronounced reduction, with survival of up to 93% over the same period. Free bacteria were the most affected by UV light exposure, with up to 88% survival during the experiment. The enhanced protection of the ZnO-containing material is attributed to its recognized ability to filter UV light, commonly used in products such as cosmetics (Bernstein et al., 2020; Elbrolesy et al., 2024; Mascarenhas-Melo et al., 2023; Q. Wang et al., 2022) and smart packaging (Alizadeh Sani et al., 2024; do Nascimento et al., 2024; Jayakumar et al., 2023; Roy et al., 2024) by the U.S. Food and Drug Administration (FDA). This is because Zn molecules can absorb, reflect, and retain UV light photons, primarily operating in photoprotection through the absorption of UV radiation (Channa et al., 2022), directly contributing to the barrier protection for *B. megaterium*.

Thermal stress is another significant factor affecting microorganisms in the field. The rise in temperature due to global warming threatens agricultural production, negatively impacting the viability of microorganisms and various plant developmental stages (Ahmad et al., 2022). Research on the survival of microorganisms under high temperatures for agricultural applications is limited, with most studies focusing on cells intended for food processing or the elimination of *Bacillus* considered contaminants (Cramer et al., 2018; Wen et al., 2022). Both

formulations maintained the initial viability of bacteria, even after exposure to 50°C, with the encapsulated material providing satisfactory protection to *B. megaterium* throughout the experiment. This contrasts with the observed result for free bacteria, which experienced a progressive loss of viability, with survival rates of 93%, 91%, and 88% after 2, 24, and 48 h, respectively.

Lastly, exposure to agrochemicals (fungicides and insecticides), commonly used in modern agriculture to combat pests and plant diseases and potentially affecting the viability of microorganisms (Meftaul et al., 2020), is presented in Figure 4d. Both formulations offered similar protection against the fungicide, with PVACS slightly reducing bacterial survival to 99%, while PVACS-ZnO maintained entire viability after contact with the solution. Regarding the insecticide, encapsulated *Bacillus* showed 97% and 99% survival rates for PVACS and PVACS-ZnO, respectively. The agrochemicals in this assay did not affect free bacteria survival.

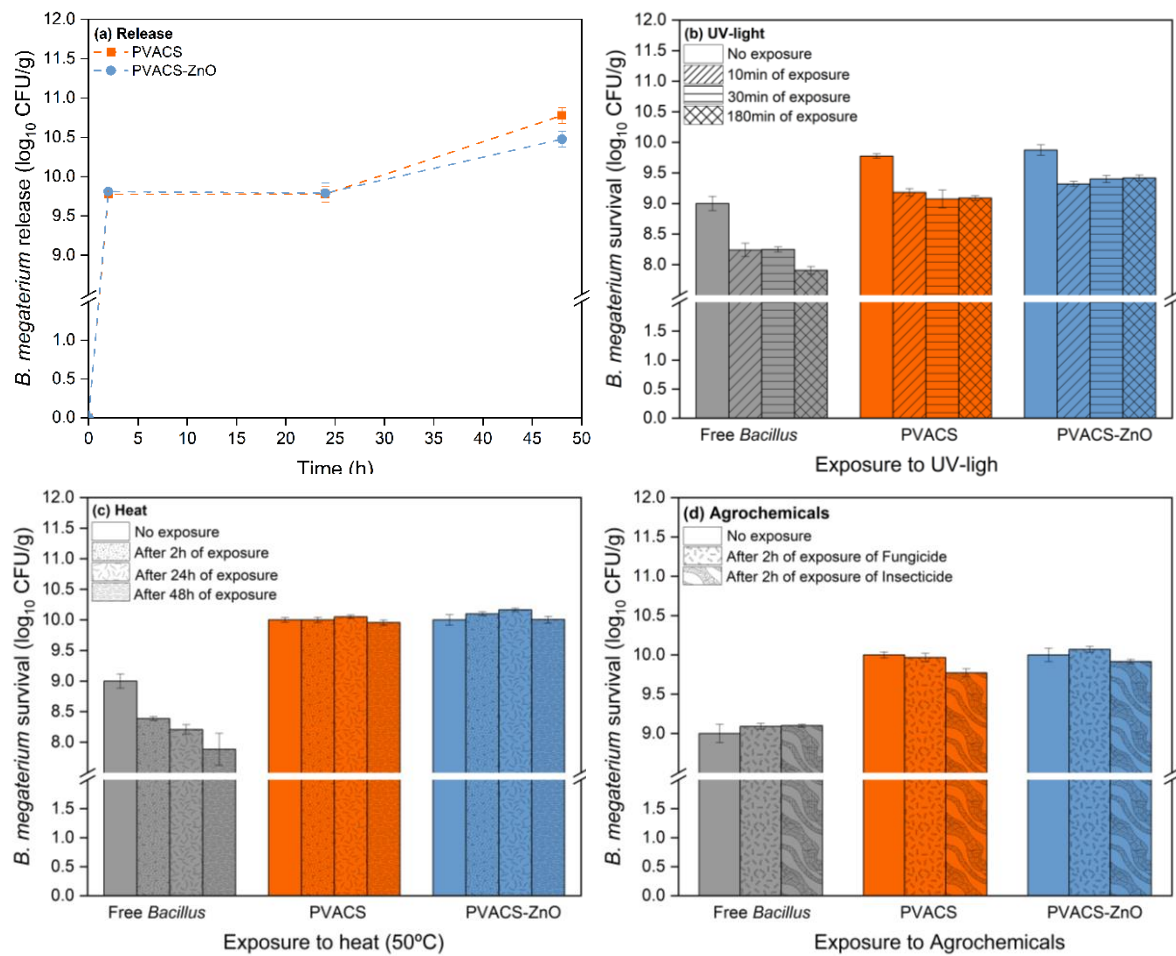


Figure 4. Tests with PVACS and PVACS-ZnO microspheres encapsulated with *Bacillus megaterium*. (a) Release profiles of bacteria in saline over 48 h; survival of bacteria before and after exposure (b) to UV light (10, 30, and 180 min); (c) to thermal stress (50 °C for 2, 24, and 48 h); (d) to agrochemicals (fungicide and insecticide).

48 h); and (d) to contact with the agrochemicals (Fungicide and Insecticide - 30 °C/2 h). PVACS (PVA/Cationic Starch) and PVACS-ZnO (PVA/Cationic Starch- Zinc oxide).

3.3.1 Accelerated Shelf-Life Test (ASLT)

In developing matrices for microorganism encapsulation, it is crucial to assess cell viability during storage, due to the lack of globally established standards for commercializing these products (Kumari et al., 2023). In this study, we employed the Accelerated Shelf-Life Test (ASLT) methodology, commonly used in the food industry, to estimate the shelf life of microspheres containing of strain *B. megaterium*, simplifying viability analysis through mathematical models (Lopes et al., 2024). Table 2 shows the estimated time for bacterial viability loss under various storage temperatures and high humidity conditions to accelerate particle degradation. The time needed for complete viability loss is presented in days for both free and encapsulated bacteria, offering insights into the formulations effectiveness in preserving bacterial viability. Tables and graphs related to the ASLT shelf-life estimation calculations are available in Tables S3, S4, and S5 (Appendix B).

Temperature plays a crucial role in storage, significantly affecting microorganism viability, as observed in this experiment, where there was a reduction in estimated shelf life for all matrices as storage temperature increased. Both free and encapsulated bacteria showed higher estimated shelf life at 15°C. Among the evaluated matrices, PVACS-ZnO stood out, achieving the best performance with up to 411.2 days (13.5 months) for complete viability loss. In the case of PVACS, it reached approximately 311.8 days (10.3 months) for total loss. In contrast, free *Bacillus megaterium* experienced total viability loss in 198 days (6.6 months). The studied matrices, especially PVACS-ZnO, contributed to doubling the shelf life of encapsulated microorganisms compared to free microorganisms, confirming the protection observed in stress tests (Figure 4) and highlighting the protective environment provided for encapsulated *Bacillus*.

Table 2. Free and encapsulated *B. megaterium* to achieve a complete loss of cell viability. PVACS (PVA/Cationic Starch) and PVACS-ZnO (PVA/Cationic Starch- Zinc oxide).

Formulations	Bacteria free			PVACS			PVACS-ZnO		
<i>Temperature of storage (°C)</i>	15	30	45	15	30	45	15	30	45
Days to lose viability	198	168	145	312	247	200	411	260	172

3.4 Greenhouse application

To investigate the effect of zinc in a greenhouse trial using soybean as a model plant, the agronomic efficiency of PVACS-ZnO was evaluated in a simple greenhouse trial. The experiment results are presented in Figures 5 and 6. The analysis included treatment *control* (no fertilization) and PVACS-ZnO without *B. megaterium*, both without P (*PVACS-ZnO*) and with phosphorus (P) addition (*PVACS-ZnO+P*), as well as PVACS-ZnO containing *B. megaterium*, again without P (*PVACS-ZnO+bacteria*) and with P addition (*PVACS-ZnO+ bacteria+P*).

In terms of composition, a significant increase in aerial biomass production was observed in treatments with bacteria (*PVACS-ZnO+bacteria* and *PVACS-ZnO+bacteria+P*) compared to their respective counterparts without the addition of the microorganism (*PVACS-ZnO* and *PVACS-ZnO+P*) (Fig. 5a). Treatment *PVACS-ZnO*, despite receiving fertilization excluding phosphorus, showed biomass production statistically equivalent to the unfertilized control, highlighting the crops high demand for phosphorus (Laira et al., 2023; Torres et al., 2024). However, when comparing treatments with P supplementation, such as *PVACS-ZnO+bacteria+P*, a considerably higher aboveground biomass production was evident than treatments without P supplementation, as seen in *PVACS-ZnO+bacteria*. The average values for root biomass production exhibited similar trends to those observed for aerial biomass production, with only the *PVACS-ZnO+bacteria+P* treatment showing statistical significance compared to the others (Fig 5b).

The same discussion applies to aerial biomass production and can be applied to phosphorus analysis (Fig 5c). This behavior may be related to the very low available nutrient concentration in the original soil (Table S1), which can harm the initial development of plants until microorganisms can develop, effectively occupying the entire rhizosphere and then

promoting greater availability of P and Zn. The delay in bioavailability slows down the development and loading of the plant with phosphorus and zinc (Dixon et al., 2020; Hamzah Saleem et al., 2022). Moreover, in the case of Zn (Fig 5d), as additional evidence of the synergistic effect between the microbiological activity of *B. megaterium* and a minimum concentration of readily available P., the *PVACS-ZnO+bacteria+P* sample demonstrated a significant increase in Zn uptake, for example, compared to the *PVACS-ZnO+P* sample, that is, without inoculation of the microorganism, but only with P supplementation.

The analysis of residual P (Figure 5e) and Zn (Figure 5f) in the soil substrate after greenhouse application showed, as expected, that the experiments with P supplementation still present a high content of this nutrient, around 40% (60 mg.dm³) of the dose initially applied (150 mg.dm³). The residual soil from the *PVACS-ZnO* sample is statistically equivalent to the *control* without nutritional supplementation or adding *B. megaterium*. However, the *PVACS-ZnO+bacteria* sample showed a subtle increase in available P concentration (~ 2.5 mg.dm³ to ~4 mg.dm³, control and *PVACS-ZnO+bacteria*, respectively, which indirectly suggests the microbial activity of *B. megaterium* it also contributes to an increase in the concentration of P in the residual soil. A different behavior is observed in the analysis of Zn in the residual soil (mg.dm³). The *PVACS-ZnO+bacteria+P* sample indicates a low Zn content in the residual soil, with values close to the *control*; this can be explained by its significant uptake in aerial biomass compared to the other samples. The *PVACS-ZnO* sample remains statistically equivalent to the *control*. The explanation is that although Zn was supplemented, the absence of *B. megaterium* and the delay in plant development due to the very low P content compromised the solubilization and uptake of this micronutrient. As further proof of the effectiveness of bacteria in Zn solubilization, a significant increase in residual Zn content is observed compared to the other samples, except for the *PVACS-ZnO+P* sample. In this last case, the release of biomolecules by soybean roots also synergizes Zn solubilization. However, it appears to be dependent on the concentration of available P (Bechtaoui et al., 2021).

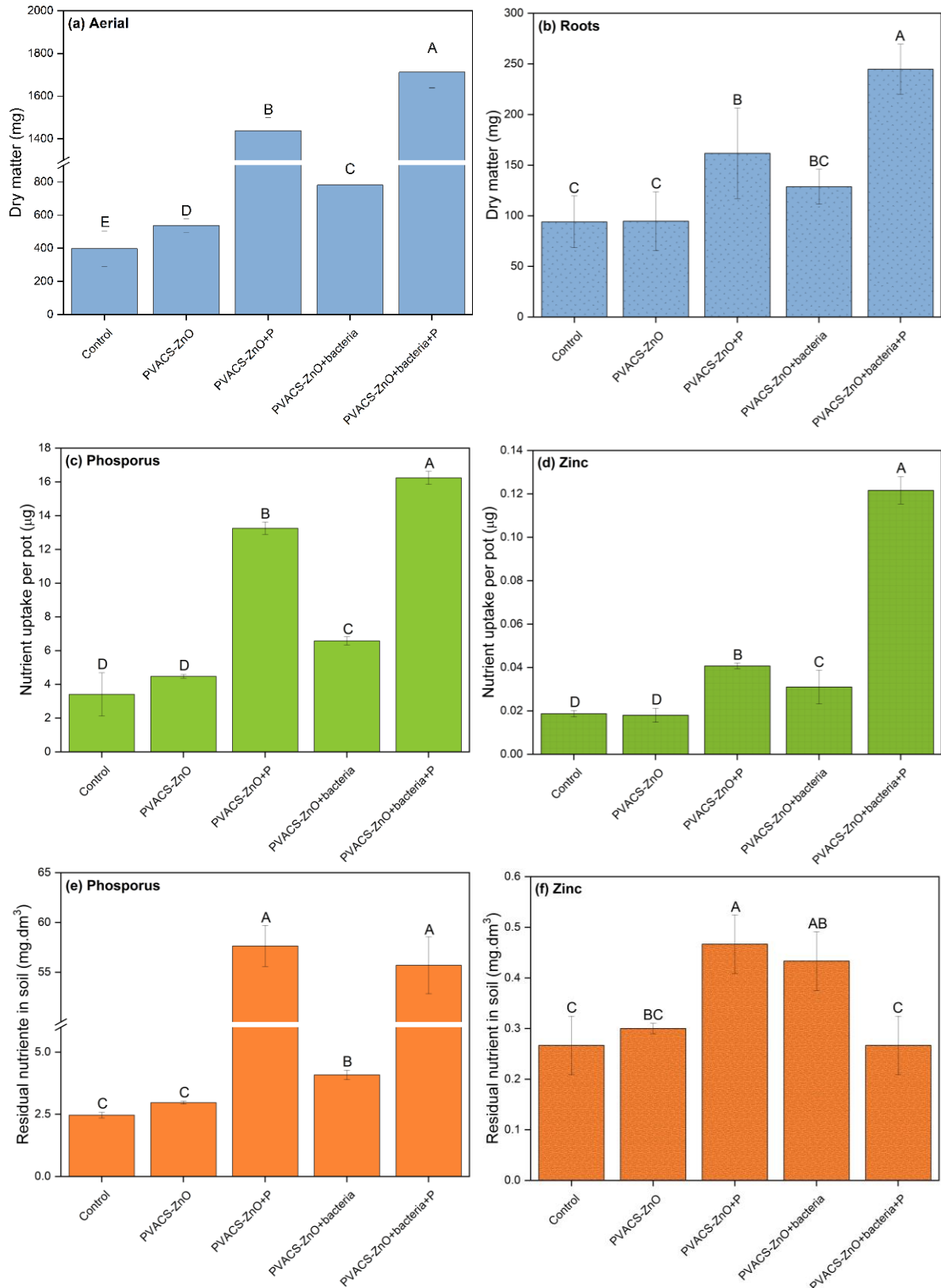


FIGURE 5. Results obtained from the greenhouse application trial. Dry matter production of aerial (a) and roots (b); Nutrient uptake per pot - P (c) and Zn (d); and Residual nutrient in soil - P (e) and Zn (f). Control (no fertilization); PVACS-ZnO: PVACS-ZnO without *B.*

megaterium and phosphorus; PVACS-ZnO+P: PVACS-ZnO without *B. megaterium* and with phosphorus; PVACS-ZnO+bacteria: PVACS-ZnO with *B. megaterium* without phosphorus; PVACS-ZnO+bacteria+P: PVACS-ZnO with *B. megaterium* and phosphorus. PVACS (PVA/Cationic Starch) and PVACS-ZnO (PVA/Cationic Starch- Zinc oxide). ^{A, B}: Different uppercase letters within the graphs indicate statistical difference by Tukeys Test ($P < 0.05$); Equal letters do not differ significantly.

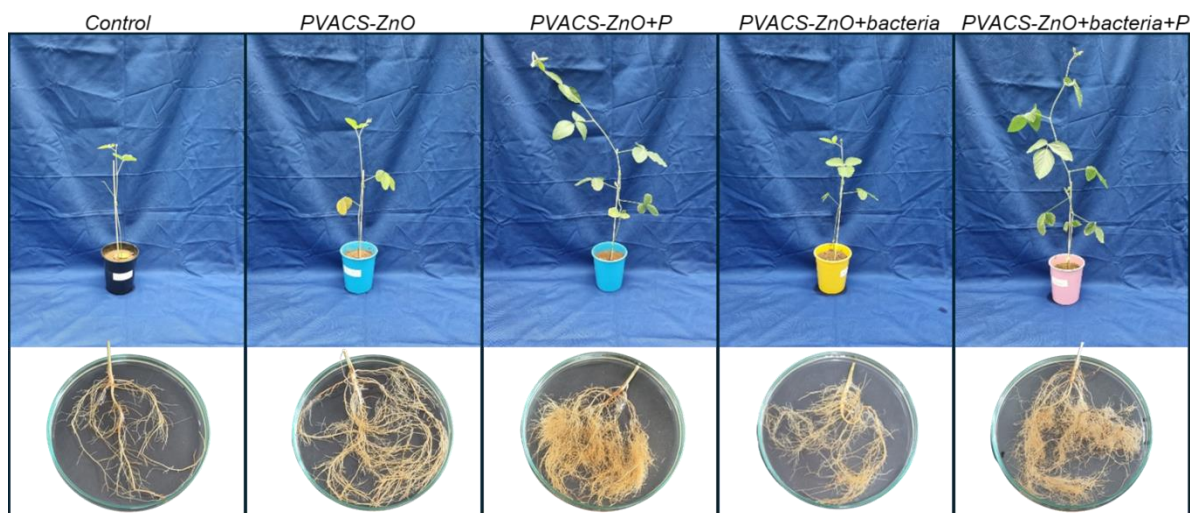


Figure 6. Pot with soybeans at the end of the experiment as follows: control (no fertilization); PVACS-ZnO: PVACS-ZnO without *B. megaterium* and phosphorus; PVACS-ZnO+P: PVACS-ZnO without *B. megaterium* and with phosphorus; PVACS-ZnO+bacteria: PVACS-ZnO with *B. megaterium* without phosphorus; PVACS-ZnO+bacteria+P: PVACS-ZnO with *B. megaterium* and phosphorus. PVACS (PVA/Cationic Starch) and PVACS-ZnO (PVA/Cationic Starch- Zinc oxide).

Overall, the greenhouse trial results demonstrate the effectiveness of the PVACS-ZnO with encapsulated *B. megaterium* in promoting plant growth, indicating its promising application in more sustainable agricultural practices. These favorable outcomes, coupled with the ease of production through atomization, the formulation ability to protect cells against stressful field conditions, and the satisfactory maintenance of viability during storage, highlight the material's potential as a novel and efficient biofertilizer.

4. Conclusions

In this study, the *Bacillus megaterium* strain demonstrated a high potential for solubilizing zinc by effectively solubilizing zinc oxide. PVA/cationic starch and zinc oxide microspheres (PVACS-ZnO), produced by spray drying, demonstrated superior cell protection under stressful conditions such as UV exposure, heat, and agrochemicals. Accelerated Shelf-Life Tests (ASLT) indicated an extended cell viability of PVACS-ZnO microspheres compared to free bacteria, doubling the estimated shelf-life of the bacteria, and highlighting their effectiveness in preserving bacterial viability during storage. In greenhouse experiments, the presence of zinc and *B. megaterium* in the formulation significantly influenced the increase in aerial and root biomass production of soybean plants, as well as promoting greater absorption of phosphorus and zinc nutrients by soybean biomass. The positive effect of zinc was corroborated by the observation of low zinc content in residual soil. These results suggest that both bacteria and zinc in the matrix benefited plant growth and nutrient absorption, indicating promising potential as a biofertilizer.

References

- Ahmad, I., Ahmad, M., Hussain, A., Jamil, M., 2021. Integrated use of phosphate-solubilizing *Bacillus subtilis* strain IA6 and zinc-solubilizing *Bacillus* sp. strain IA16: a promising approach for improving cotton growth. **Folia Microbiol (Praha)** 66, 115–125. <https://doi.org/10.1007/s12223-020-00831-3>/Published
- Ali, M., Ahmed, I., Tariq, H., Abbas, S., Zia, M.H., Mumtaz, A., Sharif, M., 2023. Growth improvement of wheat (*Triticum aestivum*) and zinc biofortification using potent zinc-solubilizing bacteria. **Front Plant Sci** 14. <https://doi.org/10.3389/fpls.2023.1140454>
- Alizadeh Sani, M., Khezerlou, A., Tavassoli, M., Abedini, A.H., McClements, D.J., 2024. Development of sustainable UV-screening food packaging materials: A review of recent advances. **Trends Food Sci Technol**. <https://doi.org/10.1016/j.tifs.2024.104366>
- Bechtaoui, N., Rabiou, M.K., Raklami, A., Oufdou, K., Hafidi, M., Jemo, M., 2021. Phosphate-Dependent Regulation of Growth and Stresses Management in Plants. **Front Plant Sci**. <https://doi.org/10.3389/fpls.2021.679916>
- Berninger, T., González López, Ó., Bejarano, A., Preininger, C., Sessitsch, A., 2018. Maintenance and assessment of cell viability in formulation of non-sporulating bacterial inoculants. **Microb Biotechnol**. <https://doi.org/10.1111/1751-7915.12880>

- Bernstein, E.F., Sarkas, H.W., Boland, P., Bouche, D., 2020. Beyond sun protection factor: An approach to environmental protection with novel mineral coatings in a vehicle containing a blend of skincare ingredients. **J Cosmet Dermatol** 19, 407–415. <https://doi.org/10.1111/jocd.13007>
- Bhatt, K., Maheshwari, D.K., 2020. Zinc solubilizing bacteria (*Bacillus megaterium*) with multifarious plant growth promoting activities alleviates growth in *Capsicum annuum L.* **3 Biotech** 10. <https://doi.org/10.1007/s13205-019-2033-9>
- Bian, H., Cao, M., Wen, H., Tan, Z., Jia, S., Cui, J., 2019. Biodegradation of polyvinyl alcohol using cross-linked enzyme aggregates of degrading enzymes from *Bacillus niacini*. **Int J Biol Macromol** 124, 10–16. <https://doi.org/10.1016/j.ijbiomac.2018.11.204>
- Bian, H., Wang, G., Cao, M., Wang, Z., Cui, J., 2020. Improved biodegradation of polyvinyl alcohol by hybrid nanoflowers of degrading enzymes from *Bacillus niacini*. **Korean Journal of Chemical Engineering** 37, 1020–1028. <https://doi.org/10.1007/s11814-020-0547-9>
- Braga, A.B.A.C., Costa, C.J.M., Pomella, A.W.V., Ribeiro, E.J., Santos, L.D., Zotarelli, M.F., 2019. Evaluation of lethality temperature and use of different wall materials in the microencapsulation process of *Trichoderma asperellum* conidias by spray drying. **Powder Technol** 347, 199–206. <https://doi.org/10.1016/j.powtec.2019.02.037>
- Channa, I.A., Ashfaq, J., Gilani, S.J., Shah, A.A., Chandio, A.D., Jumah, M.N. Bin, 2022. UV Blocking and Oxygen Barrier Coatings Based on Polyvinyl Alcohol and Zinc Oxide Nanoparticles for Packaging Applications. **Coatings** 12. <https://doi.org/10.3390/coatings12070897>
- Cramer, T.A., Kim, H.W., Chao, Y., Wang, W., Cheng, H.W., Kim, Y.H.B., 2018. Effects of probiotic (*Bacillus subtilis*) supplementation on meat quality characteristics of breast muscle from broilers exposed to chronic heat stress. **Poult Sci** 97, 3358–3368. <https://doi.org/https://doi.org/10.3382/ps/pey176>
- de Sousa, S.M., de Oliveira, C.A., Andrade, D.L., de Carvalho, C.G., Ribeiro, V.P., Pastina, M.M., Marriel, I.E., de Paula Lana, U.G., Gomes, E.A., 2021. Tropical *Bacillus* Strains Inoculation Enhances Maize Root Surface Area, Dry Weight, Nutrient Uptake and Grain Yield. **J Plant Growth Regul** 40, 867–877. <https://doi.org/10.1007/s00344-020-10146-9>

- Degryse, F., da Silva, R.C., Baird, R., Cakmak, I., Yazici, M.A., McLaughlin, M.J., 2020. Comparison and modelling of extraction methods to assess agronomic effectiveness of fertilizer zinc. **Journal of Plant Nutrition and Soil Science** 183, 248–259. <https://doi.org/10.1002/jpln.201900340>
- Dixon, M., Simonne, E., Obreza, T., Liu, G., 2020. Crop response to low phosphorus bioavailability with a focus on tomato. **Agronomy**. <https://doi.org/10.3390/agronomy10050617>
- do Nascimento, W.J., da Costa, J.C.M., Alves, E.S., de Oliveira, M.C., Monteiro, J.P., Souza, P.R., Martins, A.F., Bonafe, E.G., 2024. Zinc oxide nanoparticle-reinforced pectin/starch functionalized films: A sustainable solution for biodegradable packaging. **Int J Biol Macromol** 257. <https://doi.org/10.1016/j.ijbiomac.2023.128461>
- Elbrolesy, A., Elhussiny, F.A., Abdou, Y., Morsy, R., 2024. Facile Synthesis and Biophysical Characterization of Novel Zinc Oxide/Fe₃O₄ Hybrid Nanocomposite as a Potentially Active Agent in Sunscreens. **Arab J Sci Eng** 49, 1083–1093. <https://doi.org/10.1007/s13369-023-08082-3>
- Goswami, B., Mahanta, D., 2021. Starch and its Derivatives: Properties and Applications, in: Inamuddin, Mohd Imran Ahamed, Rajender Boddula, Tariq Altalhi (Eds.), **Polysaccharides: Properties and Applications**. Scrivener Publishing, Beverly, pp. 253–281. <https://doi.org/10.1002/9781119711414.ch12>
- Grace, M.A.L., Rao, K.V., Anuradha, K., Jayarani, A.J., Kumar, A.A., Rathika, A., 2023. X-ray analysis and size-strain plot of zinc oxide nanoparticles by Williamson-Hall, in: **Materials Today: Proceedings**. Elsevier Ltd, pp. 1334–1339. <https://doi.org/10.1016/j.matpr.2023.05.492>
- Gupta, S., Srivastava, P.K., Singh, R.P., 2023. Application of plant growth promoting microbes to enrich zinc in potato for nutritional security and sustainable agriculture. **Rhizosphere** 25. <https://doi.org/10.1016/j.rhisph.2023.100665>
- Hamzah Saleem, M., Usman, K., Rizwan, M., Al Jabri, H., Alsafran, M., 2022. Functions and strategies for enhancing zinc availability in plants for sustainable agriculture. **Front Plant Sci**. <https://doi.org/10.3389/fpls.2022.1033092>

- Hutchings, C., Rajasekharan, S.K., Reifen, R., Shemesh, M., 2020. Mitigating milk-associated bacteria through inducing zinc ions antibiofilm activity. **Foods** 9. <https://doi.org/10.3390/foods9081094>
- Inbaraj, M.P., 2021. Plant-Microbe Interactions in Alleviating Abiotic Stress—A Mini Review. **Frontiers in Agronomy**. <https://doi.org/10.3389/fagro.2021.667903>
- Jayakumar, A., Radoor, S., Shin, G.H., Siengchin, S., Kim, J.T., 2023. Active and intelligent packaging films based on PVA/Chitosan/Zinc oxide nanoparticles/Sweet purple potato extract as pH sensing and antibacterial wraps. **Food Biosci** 56. <https://doi.org/10.1016/j.fbio.2023.103432>
- Khande, R., Sharma, S.K., Ramesh, A., Sharma, M.P., 2017. Zinc solubilizing Bacillus strains that modulate growth, yield and zinc biofortification of soybean and wheat. **Rhizosphere** 4, 126–138. <https://doi.org/10.1016/j.rhisph.2017.09.002>
- Koza, N.A., Adedayo, A.A., Babalola, O.O., Kappo, A.P., 2022. Microorganisms in Plant Growth and Development: Roles in Abiotic Stress Tolerance and Secondary Metabolites Secretion. **Microorganisms**. <https://doi.org/10.3390/microorganisms10081528>
- Kumar, P., Singh, S., Pranaw, K., Kumar, S., Singh, B., Poria, V., 2022. Bioinoculants as mitigators of multiple stresses: A ray of hope for agriculture in the darkness of climate change. **Heliyon**. <https://doi.org/10.1016/j.heliyon.2022.e11269>
- Kumari, M., Swarupa, P., Kesari, K.K., Kumar, A., 2023. Microbial Inoculants as Plant Biostimulants: A Review on Risk Status. **Life**. <https://doi.org/10.3390/life13010012>
- Laira, M.D., Andrade, S.A.L., Silveira, N.M., Machado, E.C., Ribeiro, R. V., Zambrosi, F.C.B., 2023. High post-flowering phosphorus status promotes the tolerance of soybean to terminal heat stress. **Environ Exp Bot** 215. <https://doi.org/10.1016/j.envexpbot.2023.105501>
- Liu, Ping, Chen, W., Liu, C., Tian, M., Liu, Pengju, 2019. A novel poly (vinyl alcohol)/poly (ethylene glycol) scaffold for tissue engineering with a unique bimodal open-celled structure fabricated using supercritical fluid foaming. **Sci Rep** 9, 9534. <https://doi.org/10.1038/s41598-019-46061-7>
- Lopes, M.M., Lodi, L.A., Oliveira-Paiva, C.A. de, Farinas, C.S., 2024. Emulsion/Cross-Linking Encapsulation of *Bacillus* in Starch/PVA-Based Microparticles for Agricultural

Applications. **ACS Agricultural Science and Technology**.
<https://doi.org/10.1021/acsagscitech.4c00029>

Mahanty, T., Bhattacharjee, S., Goswami, M., Bhattacharyya, P., Das, B., Ghosh, A., Tribedi, P., 2017. Biofertilizers: a potential approach for sustainable agriculture development. **Environmental Science and Pollution Research** 24, 3315–3335.
<https://doi.org/10.1007/s11356-016-8104-0>

Mascarenhas-Melo, F., Mathur, A., Murugappan, S., Sharma, A., Tanwar, K., Dua, K., Singh, S.K., Mazzola, P.G., Yadav, D.N., Rengan, A.K., Veiga, F., Paiva-Santos, A.C., 2023. Inorganic nanoparticles in dermopharmaceutical and cosmetic products: Properties, formulation development, toxicity, and regulatory issues. **European Journal of Pharmaceutics and Biopharmaceutics**. <https://doi.org/10.1016/j.ejpb.2023.09.011>

Md Meftaul, I., Venkateswarlu, K., Dharmarajan, R., Annamalai, P., Megharaj, M., 2020. Pesticides in the urban environment: A potential threat that knocks at the door. **Science of the Total Environment**. <https://doi.org/10.1016/j.scitotenv.2019.134612>

Mumtaz, M.Z., Ahmad, M., Jamil, M., Hussain, T., 2017. Zinc solubilizing *Bacillus* spp. potential candidates for biofortification in maize. **Microbiol Res** 202, 51–60.
<https://doi.org/10.1016/j.micres.2017.06.001>

Nornberg, A.B., Martins, C.C., Cervi, V.F., Sari, M.H.M., Cruz, L., Luchese, C., Wilhelm, E.A., Fajardo, A.R., 2022. Transdermal release of methotrexate by cationic starch/poly(vinyl alcohol)-based films as an approach for rheumatoid arthritis treatment. **Int J Pharm** 611.
<https://doi.org/10.1016/j.ijpharm.2021.121285>

Patil, R., Bagde, U.S., 2015. Enrichment and isolation of microbial strains degrading bioplastic polyvinyl alcohol and time course study of their degradation potential. **African Journal of Biotechnology** 14, 2216–2226. <https://doi.org/10.5897/AJB2011>

Pawlowski, M.L., Helfenstein, J., Frossard, E., Hartman, G.L., 2019. Boron and zinc deficiencies and toxicities and their interactions with other nutrients in soybean roots, leaves, and seeds. **J Plant Nutr** 42, 634–649. <https://doi.org/10.1080/01904167.2019.1567782>

Petry, J.M., Pellá, M.C.G., Silva, O.A., Caetano, J., Dragunski, D.C., 2024. Plasticizer concentration effect on films and coatings based on poly(vinyl alcohol) and cationic starch blends. **Food Chem** 438. <https://doi.org/10.1016/j.foodchem.2023.137977>

- Piñón-Balderrama, C.I., Leyva-Porras, C., Terán-Figueroa, Y., Espinosa-Solís, V., Álvarez-Salas, C., Saavedra-Leos, M.Z., 2020. Encapsulation of active ingredients in food industry by spray-drying and nano spray-drying technologies. **Processes**. <https://doi.org/10.3390/PR8080889>
- Rojas-Sánchez, B., Guzmán-Guzmán, P., Morales-Cedeño, L.R., Orozco-Mosqueda, Ma. del C., Saucedo-Martínez, B.C., Sánchez-Yáñez, J.M., Fadiji, A.E., Babalola, O.O., Glick, B.R., Santoyo, G., 2022. Bioencapsulation of Microbial Inoculants: Mechanisms, Formulation Types and Application Techniques. **Applied Biosciences** 1, 198–220. <https://doi.org/10.3390/applbiosci1020013>
- Roy, S., Ramakrishnan, R., Goksen, G., Singh, S., Łopusiewicz, Ł., 2024. Recent progress on UV-light barrier food packaging films – a systematic review. **Innovative Food Science and Emerging Technologies**. <https://doi.org/10.1016/j.ifset.2023.103550>
- Seth, D., Mishra, H.N., Deka, S.C., 2017. Effect of microencapsulation using extrusion technique on viability of bacterial cells during spray drying of sweetened yoghurt. **Int J Biol Macromol** 103, 802–807. <https://doi.org/10.1016/j.ijbiomac.2017.05.099>
- Shahid, M., Khan, M.S., 2018. Cellular destruction, phytohormones and growth modulating enzymes production by *Bacillus subtilis* strain BC8 impacted by fungicides. **Pestic Biochem Physiol** 149, 8–19. <https://doi.org/10.1016/j.pestbp.2018.05.001>
- Shreya, D., Amaresan, N., Supriya, N.R., 2023. Zinc solubilizing *Bacillus* sp (SS9) and *Enterobacter* sp. (SS7) promote mung bean (*Vigna radiata L.*) growth, nutrient uptake and physiological profiles. **Lett Appl Microbiol** 76. <https://doi.org/10.1093/lambio/ovac063>
- Souza Garcia, D., Inês Bruno Tavares, M., Inês Bruno Tavares, M., 2021. Study of the Interactions of the Nanocomposite Formed By Poly (Vinyl Alcohol), Clay and Zinc Oxide Nanoparticles. <https://doi.org/10.37118/ijdr.20761.01.2021>
- Strobel, S.A., Allen, K., Roberts, C., Jimenez, D., Scher, H.B., Jeoh, T., 2018. Industrially-Scalable Microencapsulation of Plant Beneficial Bacteria in Dry Cross-Linked Alginate Matrix. **Industrial Biotechnology** 14, 138–147. <https://doi.org/10.1089/ind.2017.0032>
- Suriyachadkun, C., Chunchart, O., Srithaworn, M., Tangchitharoenkhul, R., Tangjitjareonkun, J., 2022. Zinc-Solubilizing *Streptomyces* spp. as Bioinoculants for

- Promoting the Growth of Soybean (*Glycine max* (L.) Merrill). **J Microbiol Biotechnol** 32, 1435–1446. <https://doi.org/10.4014/jmb.2206.06058>
- Torres, P., Altier, N., Beyhaut, E., Fresia, P., Garaycochea, S., Abreo, E., 2024. Phenotypic, genomic and in planta characterization of *Bacillus* sensu lato for their phosphorus biofertilization and plant growth promotion features in soybean. **Microbiol Res** 280. <https://doi.org/10.1016/j.micres.2023.127566>
- Ullah, M., Li, H., Sun, S.W., Weng, C.H., Zhang, H., Zhu, H., 2019. Polyvinyl alcohol degradation by *Bacillus cereus* RA23 from oil sludge sample. **3 Biotech** 9. <https://doi.org/10.1007/s13205-019-1882-6>
- Upadhayay, V.K., Singh, A.V., Khan, A., Sharma, A., 2022. Contemplating the role of zinc-solubilizing bacteria in crop biofortification: An approach for sustainable bioeconomy. **Frontiers in Agronomy**. <https://doi.org/10.3389/fagro.2022.903321>
- Wang, Qian, Cao, L., Wang, Y., Qin, M., Wang, Qianqian, 2022. Shell/core structure zinc oxide/iron oxide: A new sunscreen material against blue light. **Mater Lett** 322. <https://doi.org/10.1016/j.matlet.2022.132529>
- Wen, J., Smelt, J.P.P., Vischer, N.O.E., Vos, A.L., Setlow, P., Brul, S., 2022. Heat Activation and Inactivation of Bacterial Spores: Is There an Overlap? **Appl Environ Microbiol** 88.
- Xu, J., Wang, X., Zhu, H., Yu, F., 2021. Maize Genotypes With Different Zinc Efficiency in Response to Low Zinc Stress and Heterogeneous Zinc Supply. **Front Plant Sci** 12. <https://doi.org/10.3389/fpls.2021.736658>
- Zeng, H., Zhang, Xin, Ding, M., Zhang, Xiajun, Zhu, Y., 2019. Transcriptome profiles of soybean leaves and roots in response to zinc deficiency. **Physiol Plant** 167, 330–351. <https://doi.org/10.1111/ppl.12894>

CHAPTER 5

Conclusions

The study successfully developed new microbial inoculants encapsulated with *Bacillus megaterium* in PVA-based matrices using casting, emulsion, and spray-dryer techniques. These techniques enabled the production of films and microparticles with promising potential for agricultural applications. Analysis of different formulations and cross-linking agents resulted in inoculants with specific characteristics for various applications, influencing the success of application trials.

In Chapter 2, “*Biodegradable PVA-based films for Bacillus megaterium seed coating*”, citric acid as a crosslinking agent at higher concentrations (30% m/v) reduced film swelling and solubility, enhancing resistance to degradation for seed coating. These films exhibited controlled release of bacteria, increased protection against stressors, and maintained viability after storage, promoting germination rates comparable to commercial treatments on soybean seeds.

In Chapter 3, “*Microencapsulation of Bacillus megaterium in PVA/Cationic Starch-based matrices*”, sodium trimetaphosphate (STMP) and bentonite as crosslinking and surface modification agents, respectively, improved protection against stress conditions and extended shelf life. The formulation showed higher cell viability in high salinity soil, indicating a favorable environment for bacterial protection and development.

In Chapter 4, “*Spray-Dryer Microencapsulation of Bacillus megaterium in PVA/Cationic Starch/Zinc Oxide*”, microparticles produced by spray-dryer in a PVA/Cationic Starch/Zinc Oxide matrix enhanced growth and development of *B. megaterium*, protecting it against stress conditions. The presence of zinc and the bacterium in the formulation significantly increased soybean plant biomass production and nutrient absorption.

Overall, the formulations and methodologies proposed in this study offer promising strategies for encapsulating and controlling the release of microorganisms, providing protection against stress conditions, extended shelf life, and positive effects on soybean seeds and plant growth. These results demonstrate the potential of these materials for efficient agricultural applications.

Suggestions for future work

- Evaluate the agronomic efficacy of the inoculants produced in field trials during different soybean sowing seasons.
- Emphasize spray-dryer production due to the better homogenization and industrial scalability offered by this technique:
 - Investigate the addition of other microorganisms of agricultural interest.
 - Explore the addition of nutrients and micronutrients, individually or in consortium, to develop a multinutrient microbial inoculant.

Referências

- Abhilash, P.C., Dubey, R.K., Tripathi, V., Gupta, V.K., Singh, H.B., 2016a. Plant Growth-Promoting Microorganisms for Environmental Sustainability. **Trends Biotechnol** 34, 847–850. <https://doi.org/10.1016/j.tibtech.2016.05.005>
- Abhilash, P.C., Dubey, R.K., Tripathi, V., Gupta, V.K., Singh, H.B., 2016b. Plant Growth-Promoting Microorganisms for Environmental Sustainability. **Trends Biotechnol.** <https://doi.org/10.1016/j.tibtech.2016.05.005>
- Accinelli, C., Abbas, H.K., Little, N.S., Kotowicz, J.K., Mencarelli, M., Shier, W.T., 2016. A liquid bioplastic formulation for film coating of agronomic seeds. **Crop Protection** 89, 123–128. <https://doi.org/10.1016/j.cropro.2016.07.010>
- Ahmad, I., Ahmad, M., Hussain, A., Jamil, M., 2021. Integrated use of phosphate-solubilizing *Bacillus subtilis* strain IA6 and zinc-solubilizing *Bacillus* sp. strain IA16: a promising approach for improving cotton growth. **Folia Microbiol (Praha)** 66, 115–125. <https://doi.org/10.1007/s12223-020-00831-3/Published>
- Ahmad, M., Imtiaz, M., Shoib Nawaz, M., Mubeen, F., Imran, A., 2022. What Did We Learn From Current Progress in Heat Stress Tolerance in Plants? Can Microbes Be a Solution? **Front Plant Sci.** <https://doi.org/10.3389/fpls.2022.794782>
- Alori, E.T., Babalola, O.O., 2018. Microbial inoculants for improving crop quality and human health in Africa. **Front Microbiol** 9, 1–12. <https://doi.org/10.3389/fmicb.2018.02213>
- Alvarado, Y., Muro, C., Illescas, J., Riera, F., 2019. Polymer nanoparticles for the release of complex molecules, in: Holban, A.-M., Grumezescu, A.M. (Eds.), **Materials for Biomedical Engineering.** Elsevier, pp. 135–163. <https://doi.org/10.1016/B978-0-12-818433-2.00005-4>
- Ayyaril, S.S., Shanableh, A., Bhattacharjee, S., Rawas-Qalaji, M., Cagliani, R., Shabib, A.G., Imran khan, M., 2023. Recent progress in micro and nano-encapsulation techniques for environmental applications: A review. **Results in Engineering.** <https://doi.org/10.1016/j.rineng.2023.101094>

- Bargaz, A., Lyamlouli, K., Chtouki, M., Zeroual, Y., Dhiba, D., 2018. Soil Microbial Resources for Improving Fertilizers Efficiency in an Integrated Plant Nutrient Management System. **Front Microbiol** 9. <https://doi.org/10.3389/fmicb.2018.01606>
- Bashan, Y., de-Bashan, L.E., Prabhu, S.R., Hernandez, J.P., 2014. Advances in plant growth-promoting bacterial inoculant technology: Formulations and practical perspectives (1998-2013). **Plant Soil** 378, 1–33. <https://doi.org/10.1007/s11104-013-1956-x>
- Bates, N.M., Puy, C., Journey, P.L., McCarty, O.J.T., Hinds, M.T., 2020. Evaluation of the Effect of Crosslinking Method of Poly(Vinyl Alcohol) Hydrogels on Thrombogenicity. **Cardiovasc Eng Technol** 11, 448–455. <https://doi.org/10.1007/s13239-020-00474-y>
- Berninger, T., González López, Ó., Bejarano, A., Preininger, C., Sessitsch, A., 2018. Maintenance and assessment of cell viability in formulation of non-sporulating bacterial inoculants. **Microb Biotechnol**. <https://doi.org/10.1111/1751-7915.12880>
- Bian, H., Cao, M., Wen, H., Tan, Z., Jia, S., Cui, J., 2019. Biodegradation of polyvinyl alcohol using cross-linked enzyme aggregates of degrading enzymes from *Bacillus niacini*. **Int J Biol Macromol** 124, 10–16. <https://doi.org/10.1016/j.ijbiomac.2018.11.204>
- Bian, H., Wang, G., Cao, M., Wang, Z., Cui, J., 2020. Improved biodegradation of polyvinyl alcohol by hybrid nanoflowers of degrading enzymes from *Bacillus niacini*. **Korean Journal of Chemical Engineering** 37, 1020–1028. <https://doi.org/10.1007/s11814-020-0547-9>
- Braga, A.B.A.C., Costa, C.J.M., Pomella, A.W.V., Ribeiro, E.J., Santos, L.D., Zotarelli, M.F., 2019. Evaluation of lethality temperature and use of different wall materials in the microencapsulation process of *Trichoderma asperellum* conidias by spray drying. **Powder Technol** 347, 199–206. <https://doi.org/10.1016/j.powtec.2019.02.037>
- Brasil, 2020. Normas sobre especificações, garantias, registro, embalagem e rotulagem dos inoculantes destinados à agricultura. Ministério da Agricultura e Pecuária (MAPA). <https://doi.org/https://www.gov.br/agricultura/pt-br/assuntos/insumos-agropecuarios/insumos-agricolas/fertilizantes/legislacao/in-sda-13-de-24-03-2011-inoculantes.pdf>

- Brondi, M., Florencio, C., Mattoso, L., Ribeiro, C., Farinas, C., 2022. Encapsulation of *Trichoderma harzianum* with nanocellulose/carboxymethyl cellulose nanocomposite. **Carbohydr Polym** 295. <https://doi.org/10.1016/j.carbpol.2022.119876>
- Butt, M.A., 2022. Thin-Film Coating Methods: A Successful Marriage of High-Quality and Cost-Effectiveness—A Brief Exploration. **Coatings**. <https://doi.org/10.3390/coatings12081115>
- Cagnin, C., Simões, B.M., Yamashita, F., Andrello, A.C., de Carvalho, G.M., Grossmann, M.V.E., 2021. Hydrogels of starch/carboxymethyl cellulose crosslinked with sodium trimetaphosphate via reactive extrusion. **J Appl Polym Sci** 138. <https://doi.org/10.1002/app.50194>
- Chandak, A., Dhull, S.B., Punia Bangar, S., Rusu, A.V., 2022. Effects of Cross-Linking on Physicochemical and Film Properties of Lotus (*Nelumbo nucifera G.*) Seed Starch. **Foods** 11. <https://doi.org/10.3390/foods11193069>
- Chandran, Sharmila, Ravichandran, V., Chandran, Selvi, Chemmanda, J., Chandarshekar, B., 2016. Biosynthesis of PVA encapsulated silver nanoparticles. **Journal of Applied Research and Technology** 14, 319–324. <https://doi.org/10.1016/j.jart.2016.07.001>
- Chang, S.Y., Ma, A.W.K., Lai, H.M., 2019. New Insight into the Preparation of Starch-Based Spherical Microgels with Tunable Volume. **Starch/Staerke** 71. <https://doi.org/10.1002/star.201800288>
- Chanratana, M., Han, G.H., Melvin Joe, M., Roy Choudhury, A., Sundaram, S., Halim, M.A., Sa, T., 2018. Evaluation of chitosan and alginate immobilized *Methylobacterium oryzae* CBMB20 on tomato plant growth. **Arch Agron Soil Sci** 64, 1489–1502. <https://doi.org/10.1080/03650340.2018.1440390>
- Chatterjee, S., Mahmood, S., Hilles, A.R., Thomas, S., Roy, S., Provaznik, V., Romero, E.L., Ghosal, K., 2023. Cationic starch: A functionalized polysaccharide based polymer for advancement of drug delivery and health care system - A review. **Int J Biol Macromol**. <https://doi.org/10.1016/j.ijbiomac.2023.125757>
- Chaudhary, T., Dixit, M., Gera, R., Shukla, A.K., Prakash, A., Gupta, G., Shukla, P., 2020. Techniques for improving formulations of bioinoculants. **3 Biotech**. <https://doi.org/10.1007/s13205-020-02182-9>

- Cho, W. Il, Chung, M.S., 2020. *Bacillus* spores: a review of their properties and inactivation processing technologies. **Food Sci Biotechnol**. <https://doi.org/10.1007/s10068-020-00809-4>
- Cramer, T.A., Kim, H.W., Chao, Y., Wang, W., Cheng, H.W., Kim, Y.H.B., 2018. Effects of probiotic (*Bacillus subtilis*) supplementation on meat quality characteristics of breast muscle from broilers exposed to chronic heat stress. **Poult Sci** 97, 3358–3368. <https://doi.org/https://doi.org/10.3382/ps/pey176>
- De Gregorio, P.R., Michavila, G., Muller, L.R., De Souza Borges, C., Pomares, M.F., De Sá, E.L.S., Pereira, C., Vincent, P.A., 2017. Beneficial rhizobacteria immobilized in nano-bers for potential application as soybean seed bioinoculants. **PLoS One** 12. <https://doi.org/10.1371/journal.pone.0176930>
- de Sousa, S.M., de Oliveira, C.A., Andrade, D.L., de Carvalho, C.G., Ribeiro, V.P., Pastina, M.M., Marriel, I.E., de Paula Lana, U.G., Gomes, E.A., 2021. Tropical *Bacillus* Strains Inoculation Enhances Maize Root Surface Area, Dry Weight, Nutrient Uptake and Grain Yield. **J Plant Growth Regul** 40, 867–877. <https://doi.org/10.1007/s00344-020-10146-9>
- Dixon, M., Simonne, E., Obreza, T., Liu, G., 2020. Crop response to low phosphorus bioavailability with a focus on tomato. **Agronomy**. <https://doi.org/10.3390/agronomy10050617>
- Dudeja, I., Mankoo, R.K., Singh, A., Kaur, J., 2023. Citric acid: An ecofriendly cross-linker for the production of functional biopolymeric materials. **Sustain Chem Pharm**. <https://doi.org/10.1016/j.scp.2023.101307>
- El-Naggar, M.E., Abdel-Aty, A.M., Wassel, A.R., Elaraby, N.M., Mohamed, S.A., 2021. Immobilization of horseradish peroxidase on cationic microporous starch: Physico-biochemical characterization and removal of phenolic compounds. **Int J Biol Macromol** 181, 734–742. <https://doi.org/10.1016/j.ijbiomac.2021.03.171>
- Fernández, M., Martínez, R.D., Ferraris, G.N., Pagnussat, L.A., Creus, C.M., 2024. Enhancement of late-sown maize production with immobilized bacteria in chitosan/starch beads in different crop management conditions. **Biol Fertil Soils**. <https://doi.org/10.1007/s00374-024-01795-z>

- Fróna, D., Szenderák, J., Harangi-Rákos, M., 2019. The challenge of feeding the world. **Sustainability (Switzerland)**. <https://doi.org/10.3390/su11205816>
- Gadhve, R. V., Mahanwar, P.A., Gadekar, P.T., 2019. Effect of glutaraldehyde on thermal and mechanical properties of starch and polyvinyl alcohol blends. **Des Monomers Polym** 22, 164–170. <https://doi.org/10.1080/15685551.2019.1678222>
- Gamage, A., Liyanapathiranage, A., Manamperi, A., Gunathilake, C., Mani, S., Merah, O., Madhujith, T., 2022. Applications of Starch Biopolymers for a Sustainable Modern Agriculture. **Sustainability (Switzerland)**. <https://doi.org/10.3390/su14106085>
- Ghorpade, V.S., Dias, R.J., Mali, K.K., Mulla, S.I., Yadav, A.V., Dias, R.J., Mali, K.K., Pargaonkar, S.S., Shinde, P.V., Dhane, N.S., Mulla, S.I., 2019. Citric acid crosslinked carboxymethylcellulose-polyvinyl alcohol hydrogel films for extended release of water soluble basic drugs. **J Drug Deliv Sci Technol** 52, 421–430. <https://doi.org/10.1016/j.jddst.2019.05.013>
- Goswami, B., Mahanta, D., 2021. Starch and its Derivatives: Properties and Applications, in: Inamuddin, Mohd Imran Ahamed, Rajender Boddula, Tariq Altalhi (Eds.), **Polysaccharides: Properties and Applications**. Scrivener Publishing, Beverly, pp. 253–281. <https://doi.org/10.1002/9781119711414.ch12>
- Guimarães, G.G.F., Klaic, R., Giroto, A.S., Majaron, V.F., Avansi, W., Farinas, C.S., Ribeiro, C., 2018. Smart Fertilization Based on Sulfur-Phosphate Composites: Synergy among Materials in a Structure with Multiple Fertilization Roles. **ACS Sustain Chem Eng** 6, 12187–12196. <https://doi.org/10.1021/acssuschemeng.8b02511>
- Hamzah Saleem, M., Usman, K., Rizwan, M., Al Jabri, H., Alsafran, M., 2022. Functions and strategies for enhancing zinc availability in plants for sustainable agriculture. **Front Plant Sci**. <https://doi.org/10.3389/fpls.2022.1033092>
- Hashem, A., Tabassum, B., Fathi Abd_Allah, E., 2019. Bacillus subtilis: A plant-growth promoting rhizobacterium that also impacts biotic stress. **Saudi J Biol Sci**. <https://doi.org/10.1016/j.sjbs.2019.05.004>
- Havstad, M.R., 2020. Biodegradable plastics, in: Letcher, T.M. (Ed.), Plastic Waste and Recycling. **Academic Press**, pp. 97–129. <https://doi.org/10.1016/B978-0-12-817880-5.00005-0>

- Helal, M.I.D., El-Mogy, M.M., Khater, H.A., Fathy, M.A., Ibrahim, F.E., Li, Y.C., Tong, Z., Abdelgawad, K.F., 2023. A Controlled-Release Nanofertilizer Improves Tomato Growth and Minimizes Nitrogen Consumption. **Plants** 12. <https://doi.org/10.3390/plants12101978>
- Herrmann, H., Bucksch, H., 2014. Chain Structure, Dictionary Geotechnical Engineering/Wörterbuch GeoTechnik. https://doi.org/10.1007/978-3-642-41714-6_31317
- Herrmann, L., Lesueur, D., 2013. Challenges of formulation and quality of biofertilizers for successful inoculation. **Appl Microbiol Biotechnol** 97, 8859–8873. <https://doi.org/10.1007/s00253-013-5228-8>
- Hoagland, D.R., Arnon, D.I., 1950. The Water-Culture Method for Growing Plants without Soil. Berkeley.
- Huang, Z., Chen, G., Zeng, G., Chen, A., Zuo, Y., Guo, Z., Tan, Q., Song, Z., Niu, Q., 2015. Polyvinyl alcohol-immobilized *Phanerochaete chrysosporium* and its application in the bioremediation of composite-polluted wastewater. **J Hazard Mater** 289, 174–183. <https://doi.org/10.1016/j.jhazmat.2015.02.043>
- Hwang, Y.H., Um, T., Ahn, G.N., Kim, D.P., Lee, H., 2022. Robust and scalable production of emulsion-templated microparticles in 3D-printed milli-fluidic device. **Chemical Engineering Journal** 431. <https://doi.org/10.1016/j.cej.2021.133998>
- Inbaraj, M.P., 2021. Plant-Microbe Interactions in Alleviating Abiotic Stress—A Mini Review. **Frontiers in Agronomy**. <https://doi.org/10.3389/fagro.2021.667903>
- Instituto Agronômico de Campinas (IAC), 2001. Análise química para avaliação da fertilidade de solos tropicais. Instituto Agronômico.
- Jarecki, W., Wietecha, J., 2021. Effect of seed coating on the yield of soybean *Glycine max (L.) Merr.* **Plant Soil Environ** 67, 468–473. <https://doi.org/10.17221/246/2021-PSE>
- Javed, T., Afzal, I., Shabbir, R., Ikram, K., Saqlain Zaheer, M., Faheem, M., Haider Ali, H., Iqbal, J., 2022. Seed coating technology: An innovative and sustainable approach for improving seed quality and crop performance. **Journal of the Saudi Society of Agricultural Sciences**. <https://doi.org/10.1016/j.jssas.2022.03.003>
- Jeong, Y.J., Yao, Y., Mekonnen, T.H., Yim, E.K.F., 2021. Changing Compliance of Poly(Vinyl Alcohol) Tubular Scaffold for Vascular Graft Applications Through Modifying Interlayer

- Adhesion and Crosslinking Density. **Front Mater** 7. <https://doi.org/10.3389/fmats.2020.595295>
- Jiang, S., Qiao, C., Liu, R., Liu, Q., Xu, J., Yao, J., 2023. Structure and properties of citric acid cross-linked chitosan/poly(vinyl alcohol) composite films for food packaging applications. **Carbohydr Polym** 312. <https://doi.org/10.1016/j.carbpol.2023.120842>
- Joglekar, M.M., Ghosh, D., Anandan, D., Yatham, P., Jayant, R.D., Nambiraj, N.A., Jaiswal, A.K., 2020. Crosslinking of gum-based composite scaffolds for enhanced strength and stability: A comparative study between sodium trimetaphosphate and glutaraldehyde. **J Biomed Mater Res B Appl Biomater** 108, 3147–3154. <https://doi.org/10.1002/jbm.b.34640>
- John, R.P., Tyagi, R.D., Brar, S.K., Surampalli, R.Y., Prévost, D., 2011. Bio-encapsulation of microbial cells for targeted agricultural delivery. **Crit Rev Biotechnol** 31, 211–226. <https://doi.org/10.3109/07388551.2010.513327>
- Jose, J., Al-Harathi, M.A., 2017. Citric acid crosslinking of poly(vinyl alcohol)/starch/graphene nanocomposites for superior properties. **Iranian Polymer Journal** (English Edition) 26, 579–587. <https://doi.org/10.1007/s13726-017-0542-0>
- Kapishon, V., Lorrain, M.J., Leung, A.C.W., Gélinas, A.M., Wu, M., Sarrazin, M., Hrapovic, S., Pagé, A., Monteil-Rivera, F., Hemraz, U.D., 2023. Encapsulation of Plant Growth-Promoting Bacteria in Poly(itaconic acid) Microspheres by Spray Drying. **ACS Agricultural Science and Technology** 3, 1185–1193. <https://doi.org/10.1021/acsagscitech.3c00356>
- Karel, S.F., Libicki, S.B., Robertson, C.R., 1985. The immobilization of whole cells: Engineering principles. **Chem Eng Sci** 40, 1321–1354. [https://doi.org/10.1016/0009-2509\(85\)80074-9](https://doi.org/10.1016/0009-2509(85)80074-9)
- Khan, A.R., Mustafa, A., Hyder, S., Valipour, M., Rizvi, Z.F., Gondal, A.S., Yousuf, Z., Iqbal, R., Daraz, U., 2022. *Bacillus* spp. as Bioagents: Uses and Application for Sustainable Agriculture. **Biology** (Basel). <https://doi.org/10.3390/biology11121763>
- Khana, K., Nthoiw, M., Diaz, C.A., Chaudhari, Y., 2015. Vinyl Alcohol Polymers, in: Olabisi, O., Adewale, K. (Eds.), Handbook of Thermoplastics. **CRC Press**, Boca Rato, pp. 53–88.

- Kim, Daeyoung, Kim, Daniel, Kim, J., Park, C., Roh, K.M., Kang, I.M., Seo, S.M., 2020. Synthesis and Characterization of Montmorillonite-Supported TiO₂ Composites for Enhanced UV Absorption. *Clays Clay Miner* 68, 533–543. <https://doi.org/10.1007/s42860-020-00094-6>
- Klaic, R., Plotegher, F., Ribeiro, C., Zangirolami, T.C., Farinas, C.S., 2018. A Fed-Batch Strategy Integrated with Mechanical Activation Improves the Solubilization of Phosphate Rock by *Aspergillus niger*. *ACS Sustain Chem Eng* 6, 11326–11334. <https://doi.org/10.1021/acssuschemeng.8b00885>
- Kotliarevski, L., Mani, K.A., Feldbaum, R.A., Yaakov, N., Belausov, E., Zelinger, E., Ment, D., Mechrez, G., 2021. Single-Conidium Encapsulation in Oil-in-Water Pickering Emulsions at High Encapsulation Yield. *Front Chem* 9, 1–11. <https://doi.org/10.3389/fchem.2021.726874>
- Koza, N.A., Adedayo, A.A., Babalola, O.O., Kappo, A.P., 2022. Microorganisms in Plant Growth and Development: Roles in Abiotic Stress Tolerance and Secondary Metabolites Secretion. *Microorganisms*. <https://doi.org/10.3390/microorganisms10081528>
- Krasňan, V., Stloukal, R., Rosenberg, M., Rebroš, M., 2016. Immobilization of cells and enzymes to LentiKats®. *Appl Microbiol Biotechnol* 100, 2535–2553. <https://doi.org/10.1007/s00253-016-7283-4>
- Kumar, P., Singh, S., Pranaw, K., Kumar, S., Singh, B., Poria, V., 2022. Bioinoculants as mitigators of multiple stresses: A ray of hope for agriculture in the darkness of climate change. *Heliyon*. <https://doi.org/10.1016/j.heliyon.2022.e11269>
- Kumari, M., Swarupa, P., Kesari, K.K., Kumar, A., 2023. Microbial Inoculants as Plant Biostimulants: A Review on Risk Status. *Life*. <https://doi.org/10.3390/life13010012>
- Kurabetta, L.K., Masti, S.P., Eelager, M.P., Gunaki, M.N., Madihalli, S., Hunashyal, A.A., Chougale, R.B., Kumar S.K., P., Kadapure, A.J., 2023. Physicochemical and antioxidant properties of tannic acid crosslinked cationic starch/chitosan based active films for ladyfinger packaging application. *Int J Biol Macromol* 253. <https://doi.org/10.1016/j.ijbiomac.2023.127552>

- Laira, M.D., Andrade, S.A.L., Silveira, N.M., Machado, E.C., Ribeiro, R. V., Zambrosi, F.C.B., 2023. High post-flowering phosphorus status promotes the tolerance of soybean to terminal heat stress. **Environ Exp Bot** 215. <https://doi.org/10.1016/j.envexpbot.2023.105501>
- Lal, S., Kumar, V., Arora, S., 2021. Eco-friendly synthesis of biodegradable and high strength ternary blend films of PVA/starch/pectin: Mechanical, thermal and biodegradation studies. **Polymers and Polymer Composites** 29, 1505–1514. <https://doi.org/10.1177/0967391120972881>
- Li, Y., Zhu, C., Fan, D., Fu, R., Ma, P., Duan, Z., Li, X., Lei, H., Chi, L., 2020. Construction of porous sponge-like PVA-CMC-PEG hydrogels with pH-sensitivity via phase separation for wound dressing. **International Journal of Polymeric Materials and Polymeric Biomaterials** 69, 505–515. <https://doi.org/10.1080/00914037.2019.1581200>
- Lima-Tenório, M.K., Furmam-Cherobim, F., Karas, P.R., Hyeda, D., Takahashi, W.Y., Pinto Junior, A.S., Galvão, C.W., Tenório-Neto, E.T., Etto, R.M., 2023. Azospirillum brasilense AbV5/6 encapsulation in dual-crosslinked beads based on cationic starch. **Carbohydr Polym** 308. <https://doi.org/10.1016/j.carbpol.2023.120631>
- Liu, B., Xu, H., Zhao, H., Liu, W., Zhao, L., Li, Y., 2017. Preparation and characterization of intelligent starch/PVA films for simultaneous colorimetric indication and antimicrobial activity for food packaging applications. **Carbohydr Polym** 157, 842–849. <https://doi.org/10.1016/j.carbpol.2016.10.067>
- Liu, Ping, Chen, W., Liu, C., Tian, M., Liu, Pengju, 2019. A novel poly (vinyl alcohol)/poly (ethylene glycol) scaffold for tissue engineering with a unique bimodal open-celled structure fabricated using supercritical fluid foaming. **Sci Rep** 9, 9534. <https://doi.org/10.1038/s41598-019-46061-7>
- Loján, P., Demortier, M., Velivelli, S.L.S., Pfeiffer, S., Suárez, J.P., de Vos, P., Prestwich, B.D., Sessitsch, A., Declerck, S., 2017. Impact of plant growth-promoting rhizobacteria on root colonization potential and life cycle of *Rhizophagus irregularis* following co-entrapment into alginate beads. **J Appl Microbiol** 122, 429–440. <https://doi.org/10.1111/jam.13355>
- Lopes, M.M., Lodi, L.A., Oliveira-Paiva, C.A. de, Farinas, C.S., 2024. Emulsion/Cross-Linking Encapsulation of *Bacillus* in Starch/PVA-Based Microparticles for Agricultural Applications. **ACS Agricultural Science and Technology**. <https://doi.org/10.1021/acsagscitech.4c00029>

- Ma, X., Wang, X., Cheng, J., Nie, X., Yu, X., Zhao, Y., Wang, W., 2015. Microencapsulation of *Bacillus subtilis* B99-2 and its biocontrol efficiency against *Rhizoctonia solani* in tomato. **Biological Control** 90, 34–41. <https://doi.org/10.1016/j.biocontrol.2015.05.013>
- Ma, Y., 2019. Seed coating with beneficial microorganisms for precision agriculture. **Biotechnol Adv** 37, 107423. <https://doi.org/10.1016/j.biotechadv.2019.107423>
- Mahanty, T., Bhattacharjee, S., Goswami, M., Bhattacharyya, P., Das, B., Ghosh, A., Tribedi, P., 2017. Biofertilizers: a potential approach for sustainable agriculture development. **Environmental Science and Pollution Research** 24, 3315–3335. <https://doi.org/10.1007/s11356-016-8104-0>
- Mali, K.K., Ghorpade, V.S., Dias, R.J., Dhawale, S.C., 2023. Synthesis and characterization of citric acid crosslinked carboxymethyl tamarind gum-polyvinyl alcohol hydrogel films. **Int J Biol Macromol** 236. <https://doi.org/10.1016/j.ijbiomac.2023.123969>
- Martín, M.J., Lara-Villoslada, F., Ruiz, M.A., Morales, M.E., 2015. Microencapsulation of bacteria: A review of different technologies and their impact on the probiotic effects. **Innovative Food Science and Emerging Technologies** 27, 15–25. <https://doi.org/10.1016/j.ifset.2014.09.010>
- Md Meftaul, I., Venkateswarlu, K., Dharmarajan, R., Annamalai, P., Megharaj, M., 2020. Pesticides in the urban environment: A potential threat that knocks at the door. **Science of the Total Environment**. <https://doi.org/10.1016/j.scitotenv.2019.134612>
- Mohamed, H.M., El-Homasy, R.F., Abd-Ellatef, A.E.H., Salh, F.M., Hussein, M.Y., 2016. Identification of Yeast Strains Isolated from Agricultural Soils for Releasing Potassium-bearing Minerals. **Geomicrobiol J** 34, 261–266. <https://doi.org/10.1080/01490451.2016.1186762>
- Mukiri, C., Raja, K., Senthilkumar, M., Subramanian, K.S., Govindaraju, K., Pradeep, D., Ranjan, S., 2022. Immobilization of beneficial microbe *Methylobacterium aminovorans* in electrospun nanofibre as potential seed coatings for improving germination and growth of groundnut *Arachis hypogaea*. **Plant Growth Regul** 97, 419–427. <https://doi.org/10.1007/s10725-021-00737-1>

- Mumtaz, M.Z., Ahmad, M., Jamil, M., Hussain, T., 2017. Zinc solubilizing *Bacillus* spp. potential candidates for biofortification in maize. **Microbiol Res** 202, 51–60. <https://doi.org/10.1016/j.micres.2017.06.001>
- Nornberg, A.B., Martins, C.C., Cervi, V.F., Sari, M.H.M., Cruz, L., Luchese, C., Wilhelm, E.A., Fajardo, A.R., 2022. Transdermal release of methotrexate by cationic starch/poly(vinyl alcohol)-based films as an approach for rheumatoid arthritis treatment. **Int J Pharm** 611. <https://doi.org/10.1016/j.ijpharm.2021.121285>
- Noulis, K., Frangopoulos, T., Arampatzidou, A., Tsekmes, L., Marinopoulou, A., Goulas, A., Karageorgiou, V., 2023. Sodium Trimetaphosphate Crosslinked Starch Films Reinforced with Montmorillonite. **Polymers (Basel)** 15. <https://doi.org/10.3390/polym15173540>
- Oliveira, J.L., Fraceto, L.F., Bravo, A., Polanczyk, R.A., 2021. Encapsulation Strategies for *Bacillus thuringiensis*: From Now to the Future. **J Agric Food Chem** 69, 4564–4577. <https://doi.org/10.1021/acs.jafc.0c07118>
- Ons, L., Bylemans, D., Thevissen, K., Cammue, B.P.A., 2020. Combining biocontrol agents with chemical fungicides for integrated plant fungal disease control. **Microorganisms** 8, 1–19. <https://doi.org/10.3390/microorganisms8121930>
- Pan, Y., Li, P., Liang, F., Zhang, J., Yuan, J., Yin, M., 2021. A Nano-Silver Loaded PVA/Keratin Hydrogel With Strong Mechanical Properties Provides Excellent Antibacterial Effect for Delayed Sternal Closure. **Front Bioeng Biotechnol** 9. <https://doi.org/10.3389/fbioe.2021.733980>
- Paravar, A., Piri, R., Balouchi, H., Ma, Y., 2023. Microbial seed coating: An attractive tool for sustainable agriculture. **Biotechnology Reports**. <https://doi.org/10.1016/j.btre.2023.e00781>
- Park, B.R., Son, H.J., Park, J.H., Kim, E.S., Heo, S.J., Youn, H.R., Koo, Y.M., Heo, A.Y., Choi, H.W., Sang, M.K., Lee, S.W., Choi, S.H., Hong, J.K., 2021. Chemical fungicides and *Bacillus siamensis* h30-3 against fungal and oomycete pathogens causing soil-borne strawberry diseases. **Plant Pathol J (Faisalabad)** 37, 79–85. <https://doi.org/10.5423/PPJ.NT.12.2020.0232>
- Passaretti, M.G., Ninago, M.D., Di Anibal, C., Pacheco, C., Vega, D.A., Villar, M.A., López, O. V., 2019. Composite films with UV barrier capacity to minimize flavored waters degradation. **Food Packag Shelf Life** 21. <https://doi.org/10.1016/j.fpsl.2019.100334>

- Patil, R., Bagde, U.S., 2015. Enrichment and isolation of microbial strains degrading bioplastic Polyvinyl Alcohol and time course study of their degradation potential. **African Journal of Biotechnology** 14, 2216–2226. <https://doi.org/10.5897/AJB2011>
- Pedrini, S., Merritt, D.J., Stevens, J., Dixon, K., 2017. Seed Coating: Science or Marketing Spin? **Trends Plant Sci** 22, 106–116. <https://doi.org/10.1016/j.tplants.2016.11.002>
- Petry, J.M., Pellá, M.C.G., Silva, O.A., Caetano, J., Dragunski, D.C., 2024. Plasticizer concentration effect on films and coatings based on Poly(vinyl alcohol) and Cationic starch blends. **Food Chem** 438. <https://doi.org/10.1016/j.foodchem.2023.137977>
- Pham, D. Van, Tho Bach, L., 2014. Immobilized bacteria by using PVA (Polyvinyl alcohol) crosslinked with Sodium sulfate. **International Journal of Science and Engineering** 7. <https://doi.org/10.12777/ijse.7.1.41-47>
- Poraj-Kobielska, M., Peter, S., Leonhardt, S., Ullrich, R., Scheibner, K., Hofrichter, M., 2015. Immobilization of unspecific peroxygenases (EC 1.11.2.1) in PVA/PEG gel and hollow fiber modules. **Biochem Eng J** 98, 144–150. <https://doi.org/10.1016/j.bej.2015.02.037>
- Porto, M.D.A., dos Santos, J.P., Hackbart, H., Bruni, G.P., Fonseca, L.M., da Rosa Zavareze, E., Dias, A.R.G., 2019. Immobilization of α -amylase in ultrafine Polyvinyl Alcohol (PVA) fibers via electrospinning and their stability on different substrates. **Int J Biol Macromol** 126, 834–841. <https://doi.org/10.1016/j.ijbiomac.2018.12.263>
- Pupa, P., Apiwatsiri, P., Sirichokchatchawan, W., Pirarat, N., Muangsin, N., Shah, A.A., Prapasarakul, N., 2021. The efficacy of three double-microencapsulation methods for preservation of probiotic bacteria. *Sci Rep* 11, 1–9. <https://doi.org/10.1038/s41598-021-93263-z>
- Radhakrishnan, R., Hashem, A., Abd Allah, E.F., 2017. *Bacillus*: A biological tool for crop improvement through bio-molecular changes in adverse environments. **Front Physiol** 8. <https://doi.org/10.3389/fphys.2017.00667>
- Rahman, N.S.N.A., Hamid, N.W.A., Nadarajah, K., 2021. Effects of abiotic stress on soil microbiome. **Int J Mol Sci**. <https://doi.org/10.3390/ijms22169036>
- Rathore, S., Desai, P.M., Liew, C.V., Chan, L.W., Heng, P.W.S., 2013. Microencapsulation of microbial cells. **J Food Eng** 116, 369–381. <https://doi.org/10.1016/j.jfoodeng.2012.12.022>

- Riahi, N., Liberelle, B., Henry, O., De Crescenzo, G., 2017. Impact of RGD amount in dextran-based hydrogels for cell delivery. **Carbohydr Polym** 161, 219–227. <https://doi.org/10.1016/j.carbpol.2017.01.002>
- Riseh, R.S., Ebrahimi-Zarandi, M., Tamanadar, E., Pour, M.M., Thakur, V.K., 2021. Salinity stress: Toward sustainable plant strategies and using plant growth-promoting rhizobacteria encapsulation for reducing it. **Sustainability** (Switzerland). <https://doi.org/10.3390/su132212758>
- Rojas-Sánchez, B., Guzmán-Guzmán, P., Morales-Cedeño, L.R., Orozco-Mosqueda, Ma. del C., Saucedo-Martínez, B.C., Sánchez-Yáñez, J.M., Fadiji, A.E., Babalola, O.O., Glick, B.R., Santoyo, G., 2022. Bioencapsulation of Microbial Inoculants: Mechanisms, Formulation Types and Application Techniques. **Applied Biosciences** 1, 198–220. <https://doi.org/10.3390/applbiosci1020013>
- Rynkowska, E., Fatyeyeva, K., Marais, S., Kujawa, J., Kujawski, W., 2019. Chemically and thermally crosslinked PVA-based membranes: Effect on swelling and transport behavior. **Polymers** (Basel) 11. <https://doi.org/10.3390/polym11111799>
- Saberi-Riseh, R., Moradi-Pour, M., Mohammadinejad, R., Thakur, V.K., 2021. Biopolymers for biological control of plant pathogens: Advances in microencapsulation of beneficial microorganisms. **Polymers** (Basel). <https://doi.org/10.3390/polym13121938>
- Saeid, A., Prochownik, E., Dobrowolska-Iwanek, J., 2018. Phosphorus solubilization by *Bacillus* species. **Molecules** 23. <https://doi.org/10.3390/molecules23112897>
- Salihu, R., Abd Razak, S.I., Ahmad Zawawi, N., Rafiq Abdul Kadir, M., Izzah Ismail, N., Jusoh, N., Riduan Mohamad, M., Hasraf Mat Nayan, N., 2021. Citric acid: A green cross-linker of biomaterials for biomedical applications. **Eur Polym J**. <https://doi.org/10.1016/j.eurpolymj.2021.110271>
- Sammauria, R., Kumawat, S., Kumawat, P., Singh, J., Jatwa, T.K., 2020. Microbial inoculants: potential tool for sustainability of agricultural production systems. **Arch Microbiol**. <https://doi.org/10.1007/s00203-019-01795-w>
- Seth, D., Mishra, H.N., Deka, S.C., 2017. Effect of microencapsulation using extrusion technique on viability of bacterial cells during spray drying of sweetened yoghurt. **Int J Biol Macromol** 103, 802–807. <https://doi.org/10.1016/j.ijbiomac.2017.05.099>

- Shahid, I., Han, J., Hanoq, S., Malik, K.A., Borchers, C.H., Mehnaz, S., 2021. Profiling of Metabolites of *Bacillus* spp. and Their Application in Sustainable Plant Growth Promotion and Biocontrol. **Front Sustain Food Syst** 5. <https://doi.org/10.3389/fsufs.2021.605195>
- Shahid, M., Khan, M.S., 2018. Cellular destruction, phytohormones and growth modulating enzymes production by *Bacillus subtilis* strain BC8 impacted by fungicides. **Pestic Biochem Physiol** 149, 8–19. <https://doi.org/10.1016/j.pestbp.2018.05.001>
- Shinde, P., Musameh, M., Gao, Y., Robinson, A.J., Kyrtziz, I. (Louis), 2018. Immobilization and stabilization of alcohol dehydrogenase on Polyvinyl Alcohol fibre. **Biotechnology Reports** 19. <https://doi.org/10.1016/j.btre.2018.e00260>
- Simanaviciute, D., Liudvinaviciute, D., Klimaviciute, R., Rutkaite, R., 2017. Cross-linked cationic starch derivatives for immobilization of chlorogenic acid. **Eur Polym J** 93, 833–842. <https://doi.org/10.1016/j.eurpolymj.2017.02.022>
- Singh, R., Gautam, S., Sharma, B., Jain, P., Chauhan, K.D., 2021. Biopolymers and their classifications, in: **Biopolymers and Their Industrial Applications**. Elsevier Inc., pp. 21–44. <https://doi.org/10.1016/b978-0-12-819240-5.00002-x>
- Sornsumdaeng, K., Seeharaj, P., Prachayawarakorn, J., 2021. Property improvement of biodegradable citric acid-crosslinked rice starch films by calcium oxide. **Int J Biol Macromol** 193, 748–757. <https://doi.org/10.1016/j.ijbiomac.2021.10.157>
- Souza-Alonso, P., Rocha, M., Rocha, I., Ma, Y., Freitas, H., Oliveira, R.S., 2021. Encapsulation of *Pseudomonas libanensis* in alginate beads to sustain bacterial viability and inoculation of *Vigna unguiculata* under drought stress. **3 Biotech** 11. <https://doi.org/10.1007/s13205-021-02818-4>
- Stamenković, S., Beškoski, V., Karabegović, I., Lazić, M., Nikolić, N., 2018. Microbial fertilizers: A comprehensive review of current findings and future perspectives. **Spanish Journal of Agricultural Research** 16, e09R01. <https://doi.org/10.5424/sjar/2018161-12117>
- Strobel, S.A., Allen, K., Roberts, C., Jimenez, D., Scher, H.B., Jeoh, T., 2018. Industrially-Scalable Microencapsulation of Plant Beneficial Bacteria in Dry Cross-Linked Alginate Matrix. **Industrial Biotechnology** 14, 138–147. <https://doi.org/10.1089/ind.2017.0032>
- Teixeira, G.M., Mosela, M., Nicoletto, M.L.A., Ribeiro, R.A., Hungria, M., Youssef, K., Higashi, A.Y., Mian, S., Ferreira, A.S., Gonçalves, L.S.A., Pereira, U. de P., de Oliveira,

- A.G., 2021. Genomic Insights Into the Antifungal Activity and Plant Growth-Promoting Ability in *Bacillus velezensis* CMRP 4490. **Front Microbiol** 11. <https://doi.org/10.3389/fmicb.2020.618415>
- Timmusk, S., Behers, L., Muthoni, J., Muraya, A., Aronsson, A.C., 2017. Perspectives and challenges of microbial application for crop improvement. **Front Plant Sci** 8, 1–10. <https://doi.org/10.3389/fpls.2017.00049>
- Torres, P., Altier, N., Beyhaut, E., Fresia, P., Garaycochea, S., Abreo, E., 2024. Phenotypic, genomic and in planta characterization of *Bacillus* sensu lato for their phosphorus biofertilization and plant growth promotion features in soybean. **Microbiol Res** 280. <https://doi.org/10.1016/j.micres.2023.127566>
- Ullah, M., Li, H., Sun, S.W., Weng, C.H., Zhang, H., Zhu, H., 2019. Polyvinyl alcohol degradation by *Bacillus cereus* RA23 from oil sludge sample. **3 Biotech** 9. <https://doi.org/10.1007/s13205-019-1882-6>
- Vassilev, N., Vassileva, M., Martos, V., Garcia del Moral, L.F., Kowalska, J., Tylkowski, B., Malusá, E., 2020. Formulation of Microbial Inoculants by Encapsulation in Natural Polysaccharides: Focus on Beneficial Properties of Carrier Additives and Derivatives. **Front Plant Sci** 11, 1–9. <https://doi.org/10.3389/fpls.2020.00270>
- Vemmer, M., Patel, A. V., 2013. Review of encapsulation methods suitable for microbial biological control agents. **Biological Control** 67, 380–389. <https://doi.org/10.1016/j.biocontrol.2013.09.003>
- Vercelheze, A.E.S., Marim, B.M., Oliveira, A.L.M., Mali, S., 2019. Development of biodegradable coatings for maize seeds and their application for *Azospirillum brasilense* immobilization. **Appl Microbiol Biotechnol** 103, 2193–2203. <https://doi.org/10.1007/s00253-019-09646-w>
- Wang, X., Hou, X., Zou, P., Huang, A., Zhang, M., Ma, L., 2022. Cationic starch modified bentonite-alginate nanocomposites for highly controlled diffusion release of pesticides. **Int J Biol Macromol** 213, 123–133. <https://doi.org/10.1016/j.ijbiomac.2022.05.148>
- Wardak, M.H., Nkede, F.N., Van, T.T., Meng, F., Tanaka, Fumina, Tanaka, Fumihiko, 2024. Development of edible films and partial coating, a novel coating technique for tomato fruits,

- using citric acid-crosslinked starch and cellulose nanofiber. **Prog Org Coat** 187. <https://doi.org/10.1016/j.porgcoat.2023.108127>
- Wen, J., Smelt, J.P.P., Vischer, N.O.E., Vos, A.L., Setlow, P., Brul, S., 2022. Heat Activation and Inactivation of Bacterial Spores: Is There an Overlap? **Appl Environ Microbiol** 88.
- Wen, L., Liang, Y., Lin, Z., Xie, D., Zheng, Z., Xu, C., Lin, B., 2021. Design of multifunctional food packaging films based on carboxymethyl chitosan/polyvinyl alcohol crosslinked network by using citric acid as crosslinker. **Polymer (Guildf)** 230. <https://doi.org/10.1016/j.polymer.2021.124048>
- Wilpiszewska, K., Antosik, A.K., Zdanowicz, M., 2019. The Effect of Citric Acid on Physicochemical Properties of Hydrophilic Carboxymethyl Starch-Based Films. **J Polym Environ** 27, 1379–1387. <https://doi.org/10.1007/s10924-019-01436-9>
- Yang, Q., Yan, Y., Yang, X., Liao, G., Wang, D., Xia, H., 2019. Enzyme immobilization in cage-like 3D-network PVA-H and GO modified PVA-H (GO@PVA-H) with stable conformation and high activity. **Chemical Engineering Journal** 372, 946–955. <https://doi.org/10.1016/j.cej.2019.04.216>
- Yu, D., Feng, Y.Y., Xu, J.X., Kong, B.H., Liu, Q., Wang, H., 2021. Fabrication, characterization, and antibacterial properties of citric acid crosslinked PVA electrospun microfibre mats for active food packaging. **Packaging Technology and Science** 34, 361–370. <https://doi.org/10.1002/pts.2566>
- Yusnawan, E., Inayati, A., Baliadi, Y., 2019. Effect of soybean seed treatment with *Trichoderma virens* on its growth and total phenolic content, in: AIP Conference Proceedings. **American Institute of Physics Inc.** <https://doi.org/10.1063/1.5115604>
- Zabot, G.L., Schaefer Rodrigues, F., Polano Ody, L., Vinícius Tres, M., Herrera, E., Palacin, H., Córdova-Ramos, J.S., Best, I., Olivera-Montenegro, L., 2022. Encapsulation of Bioactive Compounds for Food and Agricultural Applications. **Polymers (Basel)**. <https://doi.org/10.3390/polym14194194>
- Zhan, Y., Yu, Y., Lin, J., 2020. Impact of application mode on the control of phosphorus release from sediments using zirconium-modified bentonite as geo-engineering material. **Science of the Total Environment** 712. <https://doi.org/10.1016/j.scitotenv.2019.135633>

- Zhang, N., Wang, Z., Shao, J., Xu, Z., Liu, Y., Xun, W., Miao, Y., Shen, Q., Zhang, R., 2023. Biocontrol mechanisms of *Bacillus*: Improving the efficiency of green agriculture. **Microb Biotechnol** 16, 2250–2263. <https://doi.org/10.1111/1751-7915.14348>
- Zhang, W., Roy, S., Assadpour, E., Cong, X., Jafari, S.M., 2023. Cross-linked biopolymeric films by citric acid for food packaging and preservation. **Adv Colloid Interface Sci.** <https://doi.org/10.1016/j.cis.2023.102886>
- Zhao, Y., Mao, X., Zhang, M., Yang, W., Di, H.J., Ma, L., Liu, W., Li, B., 2021. The application of *Bacillus Megaterium* alters soil microbial community composition, bioavailability of soil phosphorus and potassium, and cucumber growth in the plastic shed system of North China. **Agric Ecosyst Environ** 307. <https://doi.org/10.1016/j.agee.2020.107236>
- Zheng, L., Ma, X., Lang, D., Zhang, Xiaojia, Zhou, L., Wang, L., Zhang, Xinhui, 2022. Encapsulation of *Bacillus pumilus* G5 from polyvinyl alcohol sodium alginate (PVA-SA) and its implications in improving plant growth and soil fertility under drought and salt soil conditions. **Int J Biol Macromol** 209, 231–243. <https://doi.org/10.1016/j.ijbiomac.2022.04.017>
- Zvinavashe, A.T., Lim, E., Sun, H., Marelli, B., 2019. A bioinspired approach to engineer seed microenvironment to boost germination and mitigate soil salinity. **Proc Natl Acad Sci U S A** 116, 25555–25561. <https://doi.org/10.1073/pnas.1915902116>

APPENDIX A

Supporting Information - Microencapsulation of *Bacillus megaterium* in PVA/Cationic Starch-based matrices

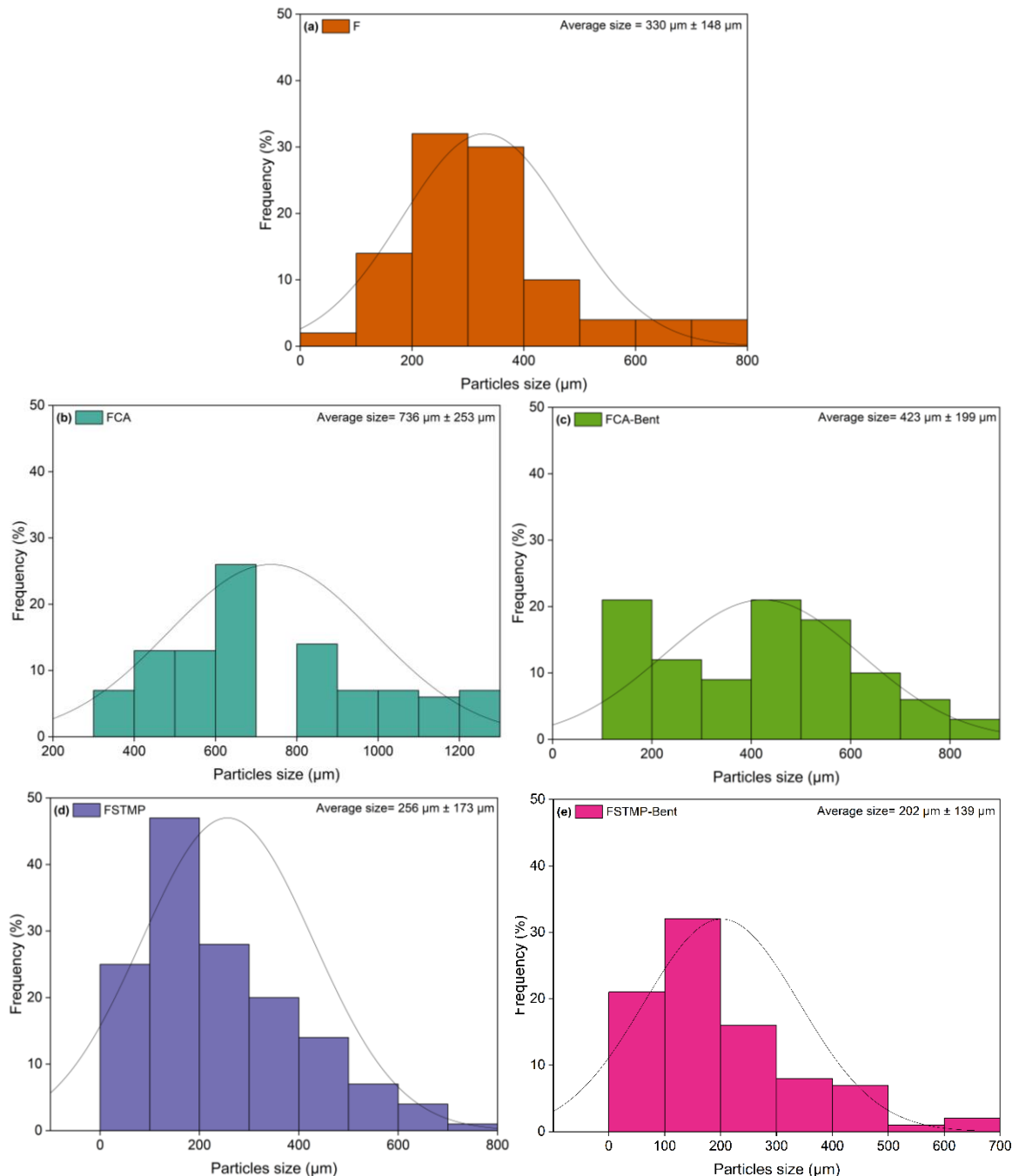
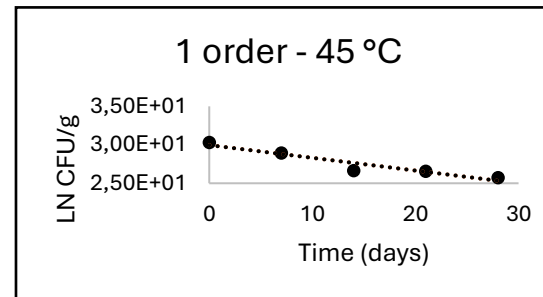
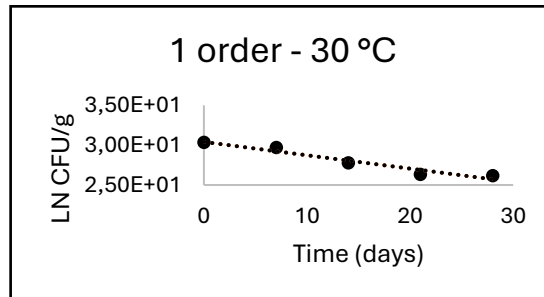
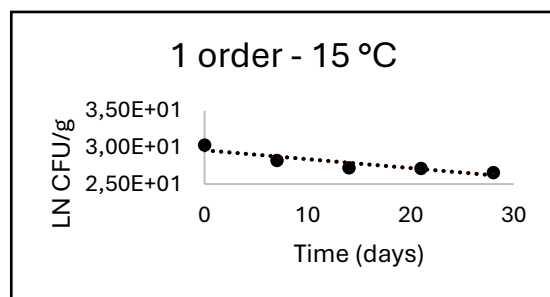


Figure S1. Histogram with the size distribution of microparticles obtained by crosslink/emulsion in oil. (a) F (PVA/CS), (b) FCA (PVA/CS/CA), (c) FCA-Bent (PVA/CS/CA/B), (d) FSTMP (PVA/CS/STMP), and (e) FSTMP-Bent (PVA/CS/STMP/B)

Table S1. Tables, calculations, and graphs for determining the life estimation of *Bacillus megaterium* using the ASLT methodology.

Bacteria free	15°C				30°C				45°C			
Time (days)	N	error	Ln N	1/N	N	error	Ln N	1/N	N	error	Ln N	1/N
0	1.5E+13	2.8E+12	3.0328E+01	6.74E-14	1.5E+13	2.8E+12	3.03E+01	6.74E-14	1.5E+13	2.8E+12	3.03E+01	6.74E-14
7	1.8E+12	5.3E+11	2.8237E+01	5.45E-13	8.0E+12	8.7E+11	2.97E+01	1.25E-13	3.7E+12	3.9E+11	2.89E+01	2.70E-13
14	6.8E+11	1.9E+11	2.7250E+01	1.46E-12	1.2E+12	4.1E+11	2.78E+01	8.70E-13	3.8E+11	1.1E+11	2.67E+01	2.64E-12
21	6.2E+11	7.6E+10	2.7148E+01	1.62E-12	2.7E+11	7.8E+10	2.63E+01	3.73E-12	3.4E+11	1.4E+11	2.66E+01	2.94E-12
28	3.6E+11	2.8E+10	2.6600E+01	2.80E-12	2.3E+11	1.5E+10	2.62E+01	4.32E-12	1.5E+11	1.8E+10	2.57E+01	6.67E-12

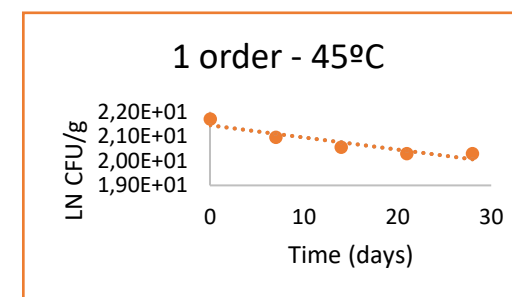
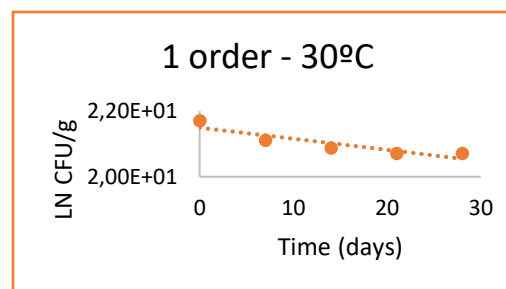
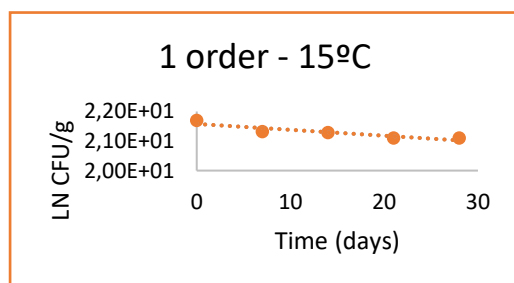


T (°C)	T (K)	1/T (1/K)	k (1/day)	R ²	ln k	ln k0 (1/day)	k0	Ea/R	R (J/mol.K)	Ea (J/mol)
15	288.15	0.00347041	0.122	0.841	-2.103					
30	303.15	0.0032987	0.167	0.942	-1.788	1.219356908	3.38501016	943.12997	8.3144	7841.559788
45	318.15	0.00314317	0.165	0.912	-1.800					

	15 °C	30°C	45°C	N ₀ = 1.225E+11
k (1/days)	0.020	0.033	0.050	
t (days)	198	168	145	Total loss of viability
t (days)	36	30	26	At 10 ⁹ CFU/g
t (days)	54	46	40	At 10 ⁸ CFU/g
t (days)	72	61	53	At 10 ⁷ CFU/g

Table S2. Tables, calculations, and graphs for determining the life estimation of F1 (PVA/SC) using the ASLT methodology.

F	15°C				30°C				45°C				
Time (days)	N	error	Ln N	1/N	N	error	Ln N	1/N	N	error	Ln N	1/N	
0	2.65E+09		2.50E+08	2.17E+01	3.77E-10	2.7E+09	2.5E+08	2.17E+01	3.77E-10	2.7E+09	2.5E+08	2.17E+01	3.77E-10
7	1.80E+09		5.13E+08	2.13E+01	5.56E-10	1.5E+09	9.4E+07	2.11E+01	6.84E-10	1.3E+09	1.5E+08	2.09E+01	8.00E-10
14	1.75E+09		2.50E+08	2.13E+01	5.71E-10	1.2E+09	5.8E+08	2.09E+01	8.60E-10	8.4E+08	8.4E+08	2.05E+01	1.19E-09
21	1.46E+09		2.88E+08	2.11E+01	6.84E-10	9.9E+08	2.6E+08	2.07E+01	1.01E-09	6.5E+08	6.5E+08	2.03E+01	1.54E-09
28	1.46E+09		2.88E+08	2.11E+01	6.84E-10	9.9E+08	2.6E+08	2.07E+01	1.01E-09	6.5E+08	6.5E+08	2.03E+01	1.54E-09

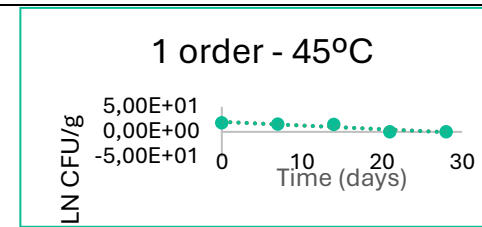
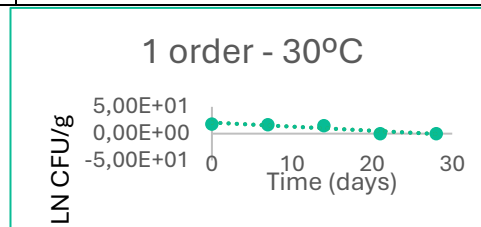
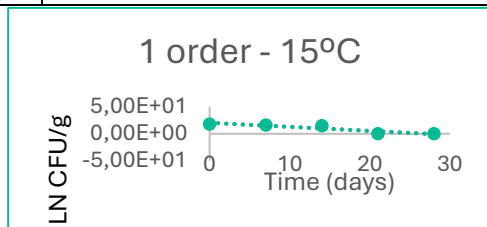


T (°C)	T (K)	1/T (1/K)	k (1/day)	R ²	ln k	ln k ₀ (1/day)	k ₀	Ea/R	R (J/mol.K)	Ea (J/mol)
15	288.14	0.003470535	0.020	0.826	-3.915					
30	303.14	0.003298806	0.034	0.825	-3.387	5.755341182	315.8732993	2781.642509	8.3144	23127.68847
45	318.14	0.00314327	0.049	0.859	-3.006					

	15 °C	30°C	45°C	N ₀ = 1.03E+10
k (1/days)	0.020	0.033	0.050	
t (days)	1137.0	705.2	457.5	Total loss of viability
t (days)	114.8	71.2	46.2	At 10 ⁹ CFU/g
t (days)	341.9	212.1	137.6	At 10 ⁸ CFU/g
t (days)	569.1	353.0	229.0	At 10 ⁷ CFU/g

Table S3. Tables, calculations, and graphs for determining the life estimation of FCA (PVA/CS/CA) using the ASLT methodology.

FCA	15°C				30°C				45°C			
Time (days)	N	error	Ln N	1/N	N	error	Ln N	1/N	N	error	Ln N	1/N
0	7.75E+07	3.00E+07	1.82E+01	1.29E-08	7.8E+07	3.0E+07	1.82E+01	1.29E-08	7.8E+07	3.0E+07	1.82E+01	1.29E-08
7	1.05E+07	0.00E+00	1.62E+01	9.52E-08	2.3E+07	2.5E+06	1.69E+01	4.44E-08	5.2E+06	8.5E+05	1.55E+01	1.91E-07
14	3.17E+06	4.17E+05	1.50E+01	3.16E-07	3.5E+06	0.0E+00	1.51E+01	2.86E-07	2.5E+06	0.0E+00	1.47E+01	4.00E-07
21	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.0E+00	0.0E+00	0.00E+00	0.00E+00	0.0E+00	0.0E+00	0.00E+00	0.00E+00
28	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.0E+00	0.0E+00	0.00E+00	0.00E+00	0.0E+00	0.0E+00	0.00E+00	0.00E+00

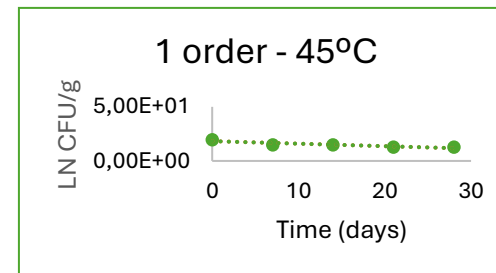
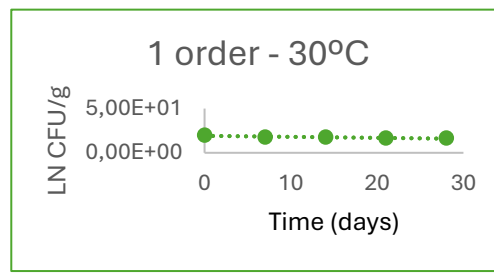
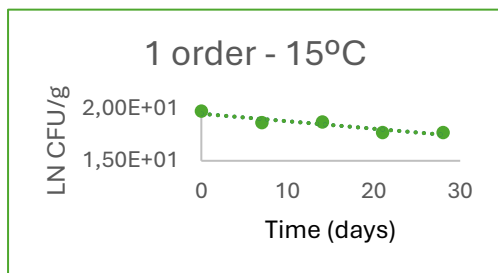


T (°C)	T (K)	1/T (1/K)	k (1/day)	R ²	ln k	ln k0 (1/day)	k0	Ea/R	R (J/mol.K)	Ea (J/mol)
15	288.14	0.003470535	0.750	0.837	-0.288					
30	303.14	0.003298806	0.761	0.833	-0.273	-0.415462	0.660034	-38.76618	8.3144	-322.31755
45	318.14	0.00314327	0.740	0.843	-0.301					

	15 °C	30°C	45°C	N ₀ = 7.09E+9
k (1/days)	0.755	0.750	0.746	
t (days)	30	30	30	Total loss of viability
t (days)	3	3	3	At 10 ⁹ CFU/g
t (days)	6	6	6	At 10 ⁸ CFU/g
t (days)	9	9	9	At 10 ⁷ CFU/g

Table S4. Tables, calculations, and graphs for determining the life estimation of FCA-Bent (PVA/SC/CA/B) using the ASLT methodology.

FCA-Bent	15°C				30°C				45°C			
Time (days)	N	error	Ln N	1/N	N	error	Ln N	1/N	N	error	Ln N	1/N
0	3.75E+08	7.50E+07	1.97E+01	2.67E-09	3.8E+08	7.5E+07	1.97E+01	2.67E-09	3.8E+08	7.5E+07	1.97E+01	2.67E-09
7	1.25E+08	2.33E+07	1.86E+01	8.00E-09	5.4E+07	1.1E+07	1.78E+01	1.86E-08	3.3E+06	3.3E+05	1.50E+01	3.00E-07
14	1.33E+08	7.78E+06	1.87E+01	7.50E-09	4.0E+07	1.3E+07	1.75E+01	2.50E-08	3.0E+06	0.0E+00	1.49E+01	3.33E-07
21	4.88E+07	2.13E+07	1.77E+01	2.05E-08	1.4E+07	6.0E+06	1.64E+01	7.27E-08	3.5E+05	7.5E+04	1.28E+01	2.86E-06
28	4.88E+07	2.13E+07	1.77E+01	2.05E-08	1.4E+07	6.0E+06	1.64E+01	7.27E-08	3.5E+05	7.5E+04	1.28E+01	2.86E-06

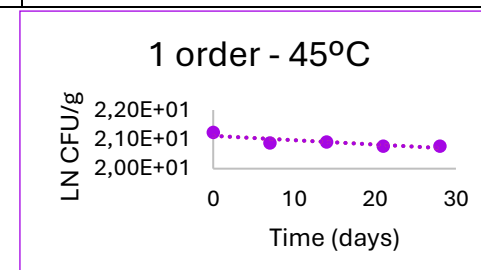
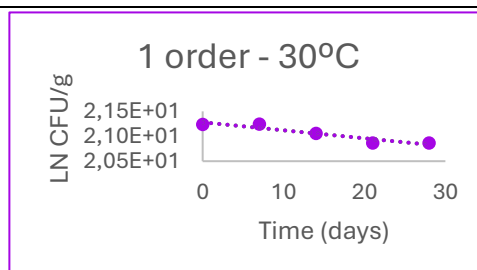
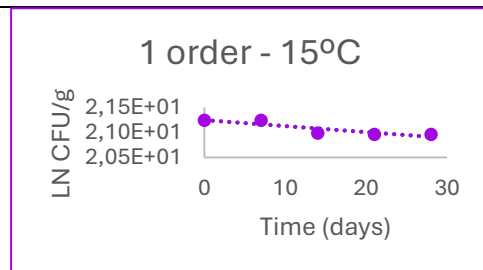


T (°C)	T (K)	1/T (1/K)	k (1/day)	R ²	ln k	ln k0 (1/day)	k0	Ea/R	R (J/mol.K)	Ea (J/mol)
15	288.14	0.003470535	0.072	0.876	-2.635					
30	303.14	0.003298806	0.114	0.866	-2.172	9.68855323	16131.88827	3564.703939	8.3144	29638.37443
45	318.14	0.00314327	0.232	0.809	-1.463					

	15 °C	30°C	45°C	N ₀ = 2.88E+9
k (1/days)	0.068	0.126	0.220	
t (days)	319	173	99	Total loss of viability
t (days)	15	8	5	At 10 ⁹ CFU/g
t (days)	49	27	15	At 10 ⁸ CFU/g
t (days)	83	45	26	At 10 ⁷ CFU/g

Table S5. Tables, calculations, and graphs for determining the life estimation of FSTMP (PVA/CS/STMP) using the ASLT methodology.

FSTMP	15°C				30°C				45°C			
Time (days)	N	error	Ln N	1/N	N	error	Ln N	1/N	N	error	Ln N	1/N
0	1.68E+09	3.13E+08	2.12E+01	5.97E-10	1.7E+09	3.1E+08	2.12E+01	5.97E-10	1.7E+09	3.1E+08	2.12E+01	5.97E-10
7	1.68E+09	2.38E+08	2.12E+01	5.97E-10	1.7E+09	1.1E+08	2.12E+01	5.93E-10	1.2E+09	1.6E+08	2.09E+01	8.60E-10
14	1.30E+09	1.67E+08	2.10E+01	7.69E-10	1.4E+09	1.3E+08	2.11E+01	7.14E-10	1.2E+09	4.7E+08	2.09E+01	8.33E-10
21	1.27E+09	2.78E+08	2.10E+01	7.89E-10	1.2E+09	2.6E+08	2.09E+01	8.66E-10	1.1E+09	1.3E+08	2.08E+01	9.52E-10
28	1.27E+09	2.78E+08	2.10E+01	7.89E-10	1.2E+09	2.6E+08	2.09E+01	8.66E-10	1.1E+09	1.3E+08	2.08E+01	9.52E-10

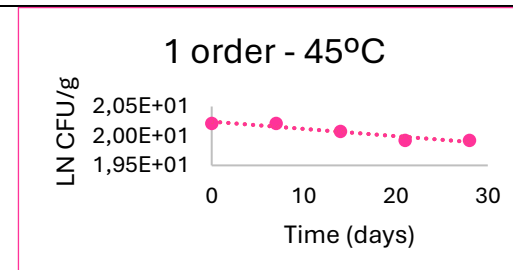
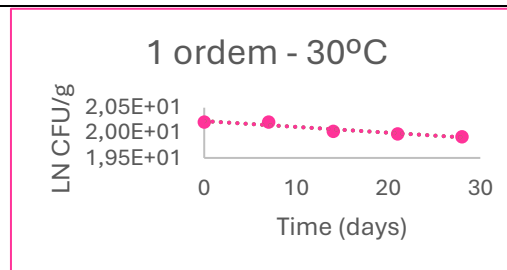
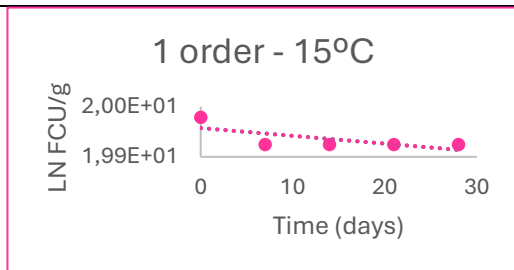


T (°C)	T (K)	1/T (1/K)	k (1/day)	R ²	ln k	ln k ₀ (1/day)	k ₀	Ea/R	R (J/mol.K)	Ea (J/mol)
15	288.14	0.003470535	0.012	0.795	-4.425					
30	303.14	0.003298806	0.016	0.894	-4.132	-2.059743	0.127486665	664.9176165	8.3144	5528.39103
45	318.14	0.00314327	0.015	0.727	-4.213					

	15 °C	30°C	45°C	N ₀ = 9.25E+9
k (1/days)	0.013	0.014	0.016	
t (days)	1809	1614	1455	Total loss of viability
t (days)	175	156	141	At 10 ⁹ CFU/g
t (days)	357	318	287	At 10 ⁸ CFU/g
t (days)	538	480	433	At 10 ⁷ CFU/g

Table S6. Tables, calculations, and graphs for determining the life estimation of FSTMP-Bent (PVA/CS/STMP/B) using the ASLT methodology.

FSTMP-Bent	15°C				30°C				45°C			
Time (days)	N	error	Ln N	1/N	N	error	Ln N	1/N	N	error	Ln N	1/N
0	4.75E+08	1.25E+08	2.00E+01	2.11E-09	6.0E+08	5.0E+07	2.02E+01	1.67E-09	6.0E+08	1.0E+08	2.02E+01	1.67E-09
7	4.50E+08	5.00E+07	1.99E+01	2.22E-09	6.0E+08	5.0E+07	2.02E+01	1.67E-09	6.0E+08	5.0E+07	2.02E+01	1.67E-09
14	4.50E+08	0.00E+00	1.99E+01	2.22E-09	5.0E+08	0.0E+00	2.00E+01	2.00E-09	5.3E+08	1.3E+08	2.01E+01	1.90E-09
21	4.50E+08	1.00E+08	1.99E+01	2.22E-09	4.8E+08	1.3E+08	2.00E+01	2.11E-09	4.5E+08	1.0E+08	1.99E+01	2.22E-09
28	4.50E+08	1.00E+08	1.99E+01	2.22E-09	4.5E+08	5.0E+07	1.99E+01	2.22E-09	4.5E+08	1.0E+08	1.99E+01	2.22E-09



T (°C)	T (K)	1/T (1/K)	k (1/day)	R ²	ln k	ln k ₀ (1/day)	k ₀	Ea/R	R (J/mol.K)	Ea (J/mol)
15	288.14	0.003470535	0.002	0.500	-6.473					
30	303.14	0.003298806	0.012	0.915	-4.460	16.16847615	10516711.84	6439.732372	8.3144	53542.51083
45	318.14	0.00314327	0.012	0.899	-4.396					

	15 °C	30°C	45°C	N ₀ = 4.50E+9
k (1/days)	0.002	0.006	0.017	
t (days)	10745	3556	1306	Total loss of viability
t (days)	727	241	88	At 10 ⁹ CFU/g
t (days)	1840	609	224	At 10 ⁸ CFU/g
t (days)	2953	977	359	At 10 ⁷ CFU/g

Table S7. Chemical, physical, and additional analyses of the soil used in the greenhouse trial.

Analysis	Quantified	Unit	Method
<i>Chemical analysis</i>			
pH CaCl ₂	4.95	-	pH em CaCl ₂ 0,01 mol/L
Organic matter	37.7	g/dm ³	Sodium Dichromate
Phosphorus (P)	6.4	mg/dm ³	Colorimetric method extracted with ion exchange resin
Calcium (Ca)	25.5	mmolc/dm ³	Extraction in 1 mol/L KCl and determination in an atomic absorption spectrophotometer*
Magnesium (Mg)	12.4	mmolc/dm ³	
Potassium (K)	0.85	mmolc/dm ³	Extraction with ion exchange resin and determination by atomic flame emission
Potential acidity (H+Al)	41.3	mmolc/dm ³	Extracted with SMP Buffer *
<i>Additional analyzes</i>			
Sum of exchangeable bases (SB)	38.8	mmolc/dm ³	-
Cation Exchange Capacity (CTC)	80.1	mmolc/dm ³	-
CTC saturation by bases (V)	48	%	-
<i>Micronutrient Analysis</i>			
Boron (B)	0.25	mg/dm ³	Extraction with hot water and determination by colorimetry
Copper (Cu)	04.6	mg/dm ³	Extraction with DTPA** and determination by atomic absorption spectrophotometry*
Iron (Fe)	11.7		
Manganese (Mn)	63.1		
Zinc (Zn)	3.1		

* Manual de análises químicas para avaliação de solos tropicais (Intituto Agrônômico de Campinas - IAC, 2001) **DPTA - Pentasodium diethylenetriaminepentaacetate – Sequestrant

APPENDIX 2

Supporting Information 2 - Spray-Dryer Microencapsulation of *Bacillus megaterium* in PVA/Cationic Starch/Zinc Oxide for Promoting Growth and Zinc Availability in Soybean

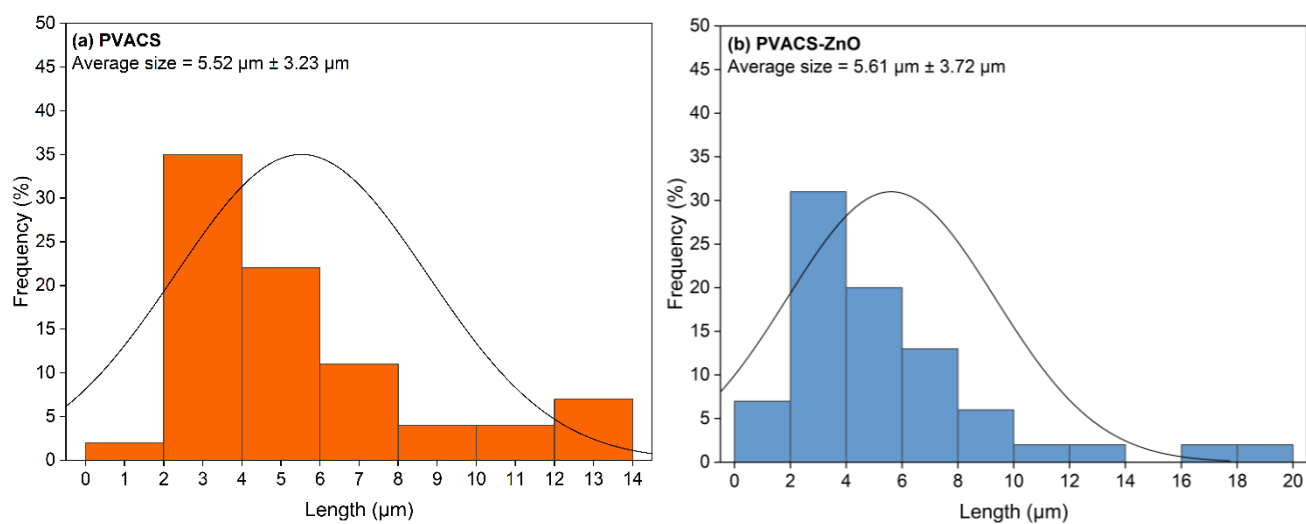


Figure S1. Histogram with the size distribution of microparticles obtained by spray dryer drying. (a) PVASC and (b) PVASC-ZnO.

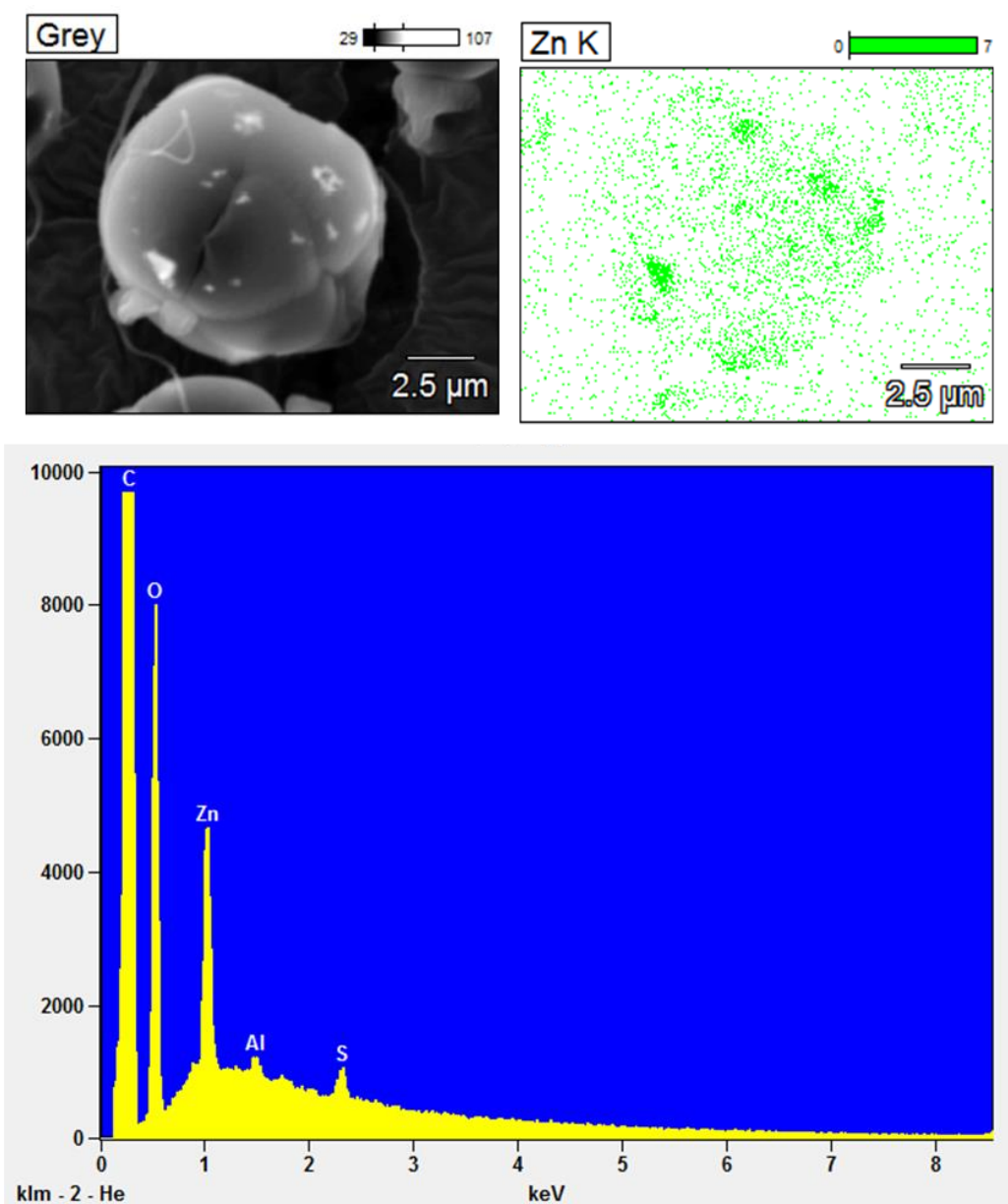


Figure S2. SEM images and energy dispersive spectrometer (EDS) of PVASC-ZnO (PVA/Cationic Starch-Zinc oxide).

Table S1. Chemical, physical, and additional analyses of the soil used in the greenhouse trial.

Analysis	Quantified	Unit	Method
<i>Chemical analysis</i>			
ph water	5,2		
pH CaCl ₂	4,4	-	pH em CaCl ₂ 0,01 mol/L
Organic matter	13	g/dm ³	Sodium Dichromate
Phosphorus (P)	4	mg/dm ³	Colorimetric method extracted with ion exchange resin
Calcium (Ca)	9	mmolc/dm ³	Extraction in 1 mol/L KCl and determination in an atomic absorption
Magnesium (Mg)	2	mmolc/dm ³	spectrophotometer*
Potassium (K)	0,3	mmolc/dm ³	Extraction with ion exchange resin and determination by atomic flame emission
Potential acidity (H+Al)	34	mmolc/dm ³	Extracted with SMP Buffer *
<i>Additional analyzes</i>			
Sum of exchangeable bases (SB)	11	mmolc/dm ³	-
Cation Exchange Capacity (CTC)	45	mmolc/dm ³	-
CTC saturation by bases (V)	25	%	-
<i>Micronutrient Analysis</i>			
Boron (B)	0,16	mg/dm ³	Extraction with hot water and determination by colorimetry
Copper (Cu)	0,8	mg/dm ³	
Iron (Fe)	16	mg/dm ³	
Manganese (Mn)	2,2	mg/dm ³	Extraction with DTPA** and determination by atomic absorption spectrophotometry*
Zinc (Zn)	0,6	mg/dm ³	
<i>Physical analysis</i>			
Total sand	608		-
Clay	367	g/Kg	-
Silt	25		-
Texture Classification	Clayey		-

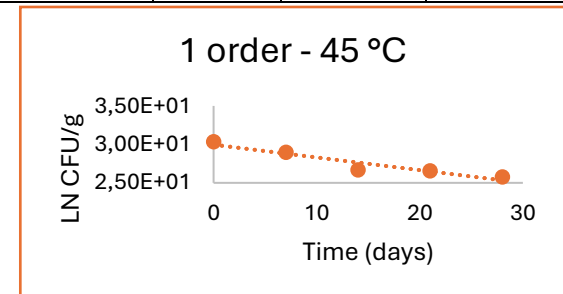
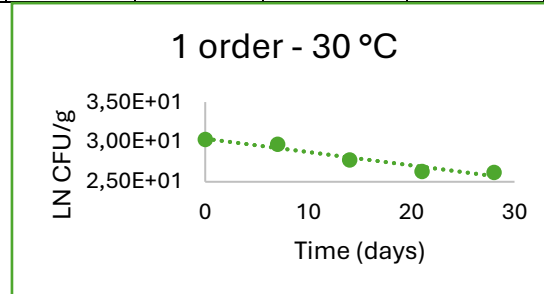
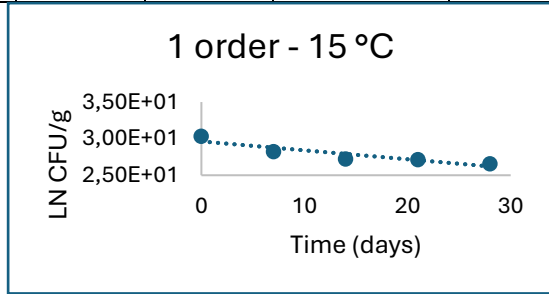
* Manual de análises químicas para avaliação de solos tropicais. Intituto Agronômico de Campinas (IAC), 2001 - Análise química para avaliação da fertilidade de solos tropicais.; **DTPA - Pentasodium diethylenetriaminepentaacetate – Sequestrant

Table S2. Micronutrient fertilization was used in the greenhouse trial.

Nutrient	Recommended dose (mg/1kg)	Established dose (mg/1kg)	Source of supplementation	Content (%)	Dose used (mg/ 1kg)
B	0.5 - 0.8	0.7	Boric acid	17	4.117647
Cu	1.3 - 1.5	1.4	Copper sulfate	24	5.833333
Mn	3.0 - 4.0	3.5	Manganese(II) chloride tetrahydrate	25	14
Zn	4.0 - 5.0	4.5	Zinc oxide	80	5.625
Mo	0.1 - 0.15	0.12	Ammonium molybdate	39	0.307692

Table S3. Tables, calculations, and graphs for determining the life estimation of *Bacillus megaterium* using the ASLT methodology.

Bacteria free	15°C				30°C				45°C			
Time (days)	N	error	Ln N	1/N	N	error	Ln N	1/N	N	error	Ln N	1/N
0	1.5E+13	2.8E+12	3.0328E+01	6.74E-14	1.5E+13	2.8E+12	3.03E+01	6.74E-14	1.5E+13	2.8E+12	3.03E+01	6.74E-14
7	1.8E+12	5.3E+11	2.8237E+01	5.45E-13	8.0E+12	8.7E+11	2.97E+01	1.25E-13	3.7E+12	3.9E+11	2.89E+01	2.70E-13
14	6.8E+11	1.9E+11	2.7250E+01	1.46E-12	1.2E+12	4.1E+11	2.78E+01	8.70E-13	3.8E+11	1.1E+11	2.67E+01	2.64E-12
21	6.2E+11	7.6E+10	2.7148E+01	1.62E-12	2.7E+11	7.8E+10	2.63E+01	3.73E-12	3.4E+11	1.4E+11	2.66E+01	2.94E-12
28	3.6E+11	2.8E+10	2.6600E+01	2.80E-12	2.3E+11	1.5E+10	2.62E+01	4.32E-12	1.5E+11	1.8E+10	2.57E+01	6.67E-12

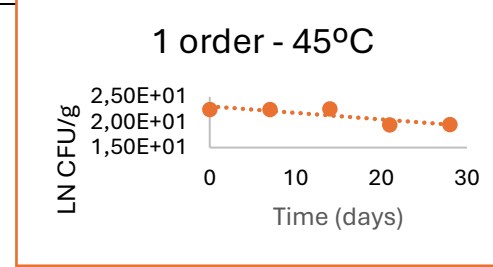
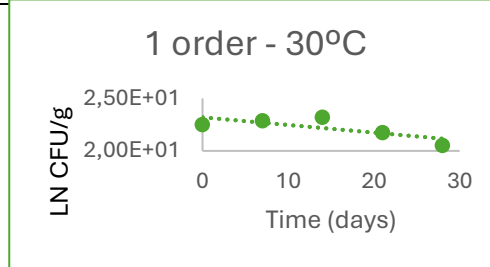
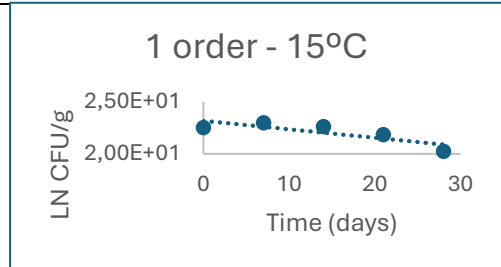


T (°C)	T (K)	1/T (1/K)	k (1/day)	R ²	ln k	ln k ₀ (1/day)	k ₀	Ea/R	R (J/mol.K)	Ea (J/mol)
15	288.15	0.00347041	0.122	0.841	-2.103					
30	303.15	0.0032987	0.167	0.942	-1.788	1.219356908	3.38501016	943.12997	8.3144	7841.559788
45	318.15	0.00314317	0.165	0.912	-1.800					

	15 °C	30°C	45°C	Total loss of viability (N _t = 0)	
k (1/days)	0.128	0.151	0.175		N ₀ = 1.225E+11
t (days)	199.1	169.3	146.2		
t (months)	6.5	5.6	4.8		

Table S4. Tables, calculations, and graphs for determining the life estimation of PVACS using the ASLT methodology.

PVACS	15°C				30°C				45°C			
Time (days)	N	error	Ln N	1/N	N	error	Ln N	1/N	N	error	Ln N	1/N
0	6.00E+09	3.33E+08	2.25E+01	1.67E-10	6.0E+09	5.0E+08	2.25E+01	1.67E-10	6.0E+09	5.0E+08	2.25E+01	1.67E-10
7	1.03E+10	3.67E+09	2.30E+01	9.68E-11	8.5E+09	2.0E+09	2.29E+01	1.18E-10	6.0E+09	1.5E+09	2.25E+01	1.67E-10
14	7.00E+09	2.33E+09	2.26E+01	1.43E-10	1.2E+10	2.5E+09	2.32E+01	8.33E-11	7.3E+09	1.8E+09	2.26E+01	1.38E-10
21	2.98E+09	2.78E+07	2.18E+01	3.35E-10	2.7E+09	3.1E+08	2.18E+01	3.67E-10	2.9E+08	1.9E+07	1.95E+01	3.48E-09
28	7.17E+08	3.50E+08	2.02E+01	1.40E-09	8.5E+08	1.0E+08	2.05E+01	1.18E-09	3.0E+08	0.0E+00	1.95E+01	3.33E-09

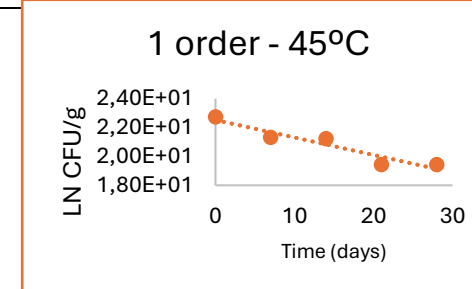
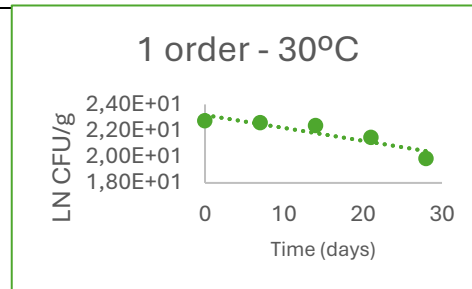
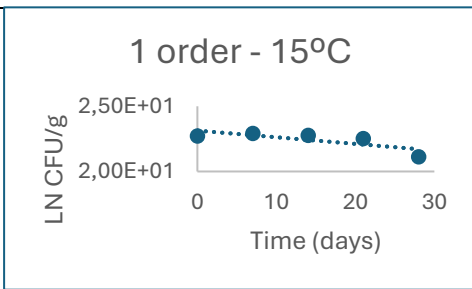


T (°C)	T (K)	1/T (1/K)	k (1/day)	R ²	ln k	ln k0 (1/day)	k0	Ea/R	R (J/mol.K)	Ea (J/mol)
15	288.14	0.0035	0.081	0.692	-2.508					
30	303.14	0.0033	0.073	0.567	-2.622	2.123885539	8.36357142	1367.0366	8.3144	11366.0887
45	318.14	0.0031	0.129	0.734	-2.049					

	15 °C	30°C	45°C	Total loss of viability (N _t = 0)
k (1/days)	0.073	0.092	0.114	
t (days)	311.8	246.6	199.4	
t (months)	10.3	8.1	6.6	

Table S5. Tables, calculations, and graphs for determining the life estimation of PVACS-ZnO using the ASLT methodology.

PVACS-ZnO	15°C				30°C				45°C			
Time (days)	N	error	Ln N	1/N	N	error	Ln N	1/N	N	error	Ln N	1/N
0	7.67E+09	1.56E+09	2.27E+01	1.30E-10	7.7E+09	1.6E+09	2.27E+01	1.30E-10	7.7E+09	1.6E+09	2.27E+01	1.30E-10
7	9.33E+09	2.33E+09	2.29E+01	1.07E-10	6.6E+09	7.3E+08	2.26E+01	1.52E-10	1.8E+09	1.3E+08	2.13E+01	5.56E-10
14	8.00E+09	1.67E+09	2.28E+01	1.25E-10	5.6E+09	1.6E+09	2.24E+01	1.79E-10	1.7E+09	3.2E+08	2.12E+01	5.94E-10
21	1.13E+10	1.12E+10	2.25E+01	8.88E-11	2.1E+09	2.6E+08	2.15E+01	4.72E-10	2.8E+08	2.9E+07	1.94E+01	3.59E-09
28	2.87E+09	2.76E+09	2.11E+01	3.49E-10	4.4E+08	8.7E+07	1.99E+01	2.27E-09	2.8E+08	2.9E+07	1.94E+01	3.59E-09



T (°C)	T (K)	1/T (1/K)	k (1/day)	R ²	ln k	ln k ₀ (1/day)	k ₀	Ea/R	R (J/mol.K)	Ea (J/mol)
15	288.14	0.00347	0.051	0.600	-2.979					
30	303.14	0.0033	0.098	0.829	-2.320	6.350109651	572.555487	2669.5003	8.3144	22195.2935
45	318.14	0.00314	0.121	0.908	-2.112					

	15 °C	30°C	45°C	N ₀ = 4.83E+9
k (1/days)	0.054	0.086	0.130	Total loss of viability (N _t = 0)
t (days)	411.2	260.0	171.6	
t (months)	13.5	8.5	5.6	

