

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

CAMILA CRISTINA VIEIRA VELLOSO

**Novas abordagens para aumentar a sobrevivência e eficiência
de cepas de *Bacillus* solubilizadoras de fosfato**

São Carlos/SP

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Camila Cristina Vieira Velloso*

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**New approaches to increase the survival and efficiency of
phosphate-solubilizing *Bacillus* strains**

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Thesis presented as part of the requirements to
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Development of Chemical Processes.

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“O universo não seria grande coisa se não fosse o lar das pessoas que você ama.”

“It would not be much of a universe if it wasn't home to the people you love.”

- Stephen Hawking

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RESUMO

NOVAS ABORDAGENS PARA AUMENTAR A SOBREVIVÊNCIA E EFICIÊNCIA DE CEPAS DE *BACILLUS* SOLUBILIZADORAS DE FOSFATO. Em um contexto em que o crescimento populacional global e os impactos exacerbados das mudanças climáticas impõem desafios críticos à segurança alimentar e à sustentabilidade ambiental, a adoção de práticas agrícolas sustentáveis surge como uma necessidade premente. Nesse cenário, as espécies do gênero *Bacillus*, notáveis por sua resiliência e capacidade de promover o crescimento vegetal através, por exemplo, da solubilização de nutrientes como o fósforo, são exploradas como inoculantes microbianos, devido ao seu potencial em aumentar a produtividade agrícola. Entretanto, a variabilidade das condições ambientais destaca desafios quanto à sobrevivência e eficiência desses inoculantes, demandando inovações estratégicas. A presente pesquisa propõe uma metodologia integrada visando potencializar a sobrevivência e a eficácia de cepas de *Bacillus* solubilizadoras de fósforo, estabelecendo assim, uma alternativa sustentável e ecologicamente responsável aos fertilizantes químicos convencionais. Esta abordagem foi delineada através de três eixos estratégicos: 1) a otimização das condições de cultivo para aumentar a resistência dos microrganismos a estresses ambientais; 2) a encapsulação em matrizes biopoliméricas para assegurar a liberação controlada e a proteção dos inoculantes; 3) a aplicação da técnica de Evolução Adaptativa de Laboratório (ALE) para selecionar cepas resistentes à salinidade e seca, com capacidades promotoras de crescimento melhoradas. Ajustes nos parâmetros do processo de cultivo, como pH e temperatura, juntamente com a escala de produção, resultaram em um aumento significativo na produção de esporos microbianos resistentes, atingindo uma concentração de $1.31 \cdot 10^{12}$ UFC/mL em um biorreator de coluna de bolhas, com uma eficiência de esporulação de 100%, resistência ao calor de 78.4%, e uma eficiência energética de $4.83 \cdot 10^{10}$ UFC/J. A técnica de encapsulação, empregando matrizes biopoliméricas baseadas em amido com aditivos como maltodextrina, celulose e bentonita, demonstrou proteger os inoculantes contra estressores ambientais, tais como UV e temperaturas extremas. Diferentes perfis de liberação foram observados entre os filmes modificados, com variações nas taxas de liberação inicial e prolongada, demonstrando a influência das propriedades dos materiais na difusão dos microrganismos. Enquanto a adição de maltodextrina acelerou a liberação de microrganismos, a celulose e a bentonita aumentaram a retenção e permitiram uma liberação gradual. A aplicação de ALE resultou no desenvolvimento de cepas de *Bacillus* com maior tolerância ao estresse salino e à hídrico, capazes de solubilizar fósforo, produzir exopolissacarídeos e formar biofilmes. Esta pesquisa oferece uma contribuição significativa para o avanço de práticas agrícolas sustentáveis, ao melhorar a viabilidade e a funcionalidade de inoculantes microbianos, representando uma alternativa frente aos desafios contemporâneos de segurança alimentar e sustentabilidade ambiental.

ABSTRACT

NEW APPROACHES TO INCREASE THE SURVIVAL AND EFFICIENCY OF PHOSPHATE-SOLUBILIZING *BACILLUS* STRAINS. In a context where global population growth and the exacerbated impacts of climate change pose critical challenges to food security and environmental sustainability, the adoption of sustainable agricultural practices emerges as an urgent necessity. In this scenario, species of the genus *Bacillus*, notable for their resilience and ability to promote plant growth through, for example, the solubilization of nutrients such as phosphorus, are explored as microbial inoculants, due to their potential to increase agricultural productivity. However, the variability of environmental conditions highlights challenges regarding the survival and efficiency of these inoculants, demanding strategic innovations. This research proposes an integrated methodology aiming to enhance the survival and efficacy of phosphorus-solubilizing *Bacillus* strains, thus establishing a sustainable and ecologically responsible alternative to conventional chemical fertilizers. This approach was outlined through three strategic axes: 1) optimizing cultivation conditions to increase the microorganisms' resistance to environmental stresses; 2) encapsulation in biopolymeric matrices to ensure controlled release and protection of the inoculants; 3) the application of Laboratory Adaptive Evolution (ALE) to select strains with improved salinity and drought resistance and growth-promoting capabilities. Adjustments in the cultivation process parameters, such as pH and temperature, along with production scale, resulted in a significant increase in the production of resistant microbial spores, reaching a concentration of $1.31 \cdot 10^{12}$ CFU/mL in a bubble column bioreactor, with a sporulation efficiency of 100%, heat resistance of 78.4%, and an energy efficiency $4.83 \cdot 10^{10}$ CFU/J. The encapsulation technique, employing starch-based biopolymeric matrices with additives like maltodextrin, cellulose, and bentonite, demonstrated protection of the inoculants against environmental stressors, such as UV and extreme temperatures. Different release profiles were observed among the modified films, with variations in initial and prolonged release rates, demonstrating the influence of material properties on the diffusion of microorganisms. While the addition of maltodextrin accelerated the release of microorganisms, cellulose and bentonite increased retention and allowed for gradual release. The application of ALE resulted in the development of *Bacillus* strains with greater tolerance to saline and water stress, capable of solubilizing phosphorus, producing exopolysaccharides, and forming biofilms. This research offers a significant contribution to the advancement of sustainable agricultural practices by improving the viability and functionality of microbial inoculants, representing an alternative in the face of contemporary challenges of food security and environmental sustainability.

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Velloso, C. C. V.; Lopes, M. M.; Badino, A. C.; Farinas, C. S. Exploring the roles of starch for microbial encapsulation through a systematic mapping review. *Carbohydrate Polymers*, 306, 120574, 2023. <https://doi.org/10.1016/j.carbpol.2023.120574>.

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INTRODUCTION

As the global population is projected to reach 9.8 billion by 2050, the pressing need to sustainably enhance agricultural productivity becomes increasingly evident (SCHNEIDER et al., 2011; UNITED NATIONS, 2019). This challenge is compounded by the impacts of global climate change, which threaten food security and environmental sustainability (BOGATI; WALCZAK, 2022; MUKHOPADHYAY et al., 2021; ULLAH; BANO; KHAN, 2021). In this context, microbial inoculants, leveraging beneficial bacteria and fungi to promote plant growth, emerged as a promising alternative to reduce fertilizer application by improving nutrient absorption, especially phosphorus and nitrogen, as well as promoting phytohormone production and resistance to environmental stresses (KUMAR et al., 2020; SANTOS; NOGUEIRA; HUNGRIA, 2019; SHAH et al., 2021; VELLOSO et al., 2021).

The significance of these technologies is further underscored by the burgeoning fertilizer market, valued at USD 202 billion in 2023 and anticipated to grow at a compound annual growth rate (CAGR) of 2.7% through 2032 (GLOBAL MARKET INSIGHTS INC, 2024; MORE, 2022; SPHERICAL INSIGHTS, 2023). This growth mirrors the ongoing demand for solutions that augment agricultural productivity in response to demographic expansion. Concurrently, the microbial inoculant market, projected to surpass USD 2.5 billion by 2032 with a CAGR of 8.5% (GLOBAL MARKET INSIGHTS INC, 2024; MORE, 2022; SPHERICAL INSIGHTS, 2023), signifies a shift towards more sustainable agricultural practices and a move away from synthetic fertilizers towards eco-friendly alternatives.

However, despite decades of research on microbial inoculants, significant hurdles to their widespread adoption remain, including reliability concerns and the challenge of evaluating success in field conditions (KAMINSKY et al., 2019; O'CALLAGHAN, 2016; O'CALLAGHAN; BALLARD; WRIGHT, 2022). The primary obstacle in developing effective inoculants lies in the extensive and diverse developmental stages these products must undergo. They require distinct microbial characteristics at different phases—capture and refinement, production, establishment, function, and downstream impacts—to express desired traits like nutrient solubilization effectively in field conditions (KAMINSKY et al., 2019). Unlike metabolite additives that do not require viable organisms, microbial inoculants must survive formulation and storage processes and thrive under varying environmental conditions. Ideally, after mass production, a microbial inoculant should remain viable for extended storage periods, necessitating tolerance to additives, agrochemicals used as seed coatings, and adverse environmental conditions encountered during production, formulation, storage, transportation, and field application (KAMINSKY et al., 2019; O'CALLAGHAN, 2016; O'CALLAGHAN; BALLARD; WRIGHT, 2022; SANTOS; NOGUEIRA; HUNGRIA, 2019).

Among the diverse microbial candidates, *Bacillus* species stand out for their robustness, spore-forming capability, and a wide array of plant growth-promoting traits, making them ideally suited for agricultural use (DE SOUSA et al., 2020; TIWARI; PRASAD; LATA, 2019; VELLOSO et al., 2021). For instance, strains *Bacillus* CNPMS B116 and CNPMS B119, isolated from the rhizosphere, and *Bacillus* CNPMS B2084, from the leaf endosphere of phosphorus-efficient maize genotypes, have demonstrated remarkable phosphate solubilization, auxin, siderophore, exopolysaccharide, phosphatase, and phytase production, and nitrogen fixation capabilities in vitro. Additionally, these strains have shown to enhance the growth of maize seedlings under hydroponic water stress, millet in greenhouse conditions, and maize and soybean in field trials (DE SOUSA et al., 2020; OLIVEIRA et al., 2009, 2020; RIBEIRO et al., 2018; VELLOSO et al., 2020). In 2019, Bioma Company, in collaboration with Embrapa Maize and Sorghum, launched BiomaPhos®, a liquid microbial inoculant comprising a consortium of *Bacillus* strains B119 and B2084, with a shelf life of six months. Other P-solubilizing microbial products in the market include JumpStart™, utilizing *Penicillium bilaiae*, and QuickRoots®, composed of *B. amyloliquefaciens* and *Trichoderma virens*.

Addressing these challenges, significant efforts have been directed towards extending microbial inoculants' shelf life and enhancing the survival rates of microbial inoculants throughout the production process and in field applications. These efforts encompass both upstream and downstream processes (O'CALLAGHAN; BALLARD; WRIGHT, 2022; SCHOEBITZ; LÓPEZ; ROLDÁN, 2013; VASSILEVA et al., 2021). Upstream processes focus on selecting fermentation models, optimizing culture medium components, adjusting process parameters, and designing bioreactor operation to achieve high biomass production, spore yield, and/or metabolic activity of microbial inoculants (VASSILEVA et al., 2021). Additionally, there is potential to enhance the adaptation of microorganisms to stressful conditions and improve plant growth promoting characteristics, such as using Adaptive Laboratory Evolution (ALE) technique or other genetic modification tool (MAUDE et al., 2024; SARAPAT et al., 2020; TOLIBIA et al., 2022). Meanwhile, downstream processes aim to develop strategic formulations that provide physical protection and create favorable conditions for the survival and proliferation of microorganisms (BRONDI et al., 2022; LOPES; OLIVEIRA-PAIVA; FARINAS, 2023).

Optimizing cultivation conditions is a crucial strategy for improving the viability of microbial inoculants. The literature underscores that fine-tuning bioprocess parameters such as pH, temperature, and aeration conditions can significantly enhance the production of microbial spores (LEGUÉRINEL; COUVERT; MAFART, 2007; LEVINSON; HYATT, 1964; MAFART; COUVERT; LEGUÉRINEL, 2001; SALAZAR-MAGALLON et al., 2016). These adjustments ensure the microorganisms' resistance against adverse environmental conditions. However, despite

advances, challenges persist in pinpointing optimal conditions that maximize viability while ensuring the functionality of the microorganisms (KAMINSKY et al., 2019). Despite recent studies in the realm of microbial inoculants production and formulation, additional research is warranted to further refine upstream operational productivity through alternative fermentation strategies, such as continuous mode fermentation or diverse bioreactor configurations (VASSILEVA et al., 2021). Furthermore, exploring the varying responses of different microbial strains to adjustments in bioprocess parameters is an avenue necessitating further investigation (XU ZHOU et al., 2017).

The encapsulation of microorganisms in biopolymeric matrices represents another promising approach to enhancing the effectiveness of microbial inoculants (BRONDI et al., 2022; VELLOSO et al., 2023b). This technique facilitates the controlled release of microorganisms, protecting them from environmental stressors and ensuring their availability in the rhizosphere at critical stages of plant growth (LOPES; OLIVEIRA-PAIVA; FARINAS, 2023; ZHENG et al., 2022). The literature highlights the success of this approach in extending the shelf life of microorganisms and in improving their effectiveness under field conditions (BALLA et al., 2022; SANTOS; NOGUEIRA; HUNGRIA, 2019; SCHOEBITZ; LÓPEZ; ROLDÁN, 2013). Scaling up the production and application of encapsulated bioformulations, precise control over additive proportions to maximize efficacy, expanding target species beyond cereals, legumes, and some vegetables, investigating the impact of polymer bead inoculation on plant microbiomes with a focus on function and structure, and cost-benefit analysis for commercialization strategies are critical challenges that require an integrated approach to economically viabilize and broaden the reach of these agricultural innovations (BALLA et al., 2022; O'CALLAGHAN; BALLARD; WRIGHT, 2022).

Lastly, ALE is used as an innovative method to increase the stress tolerance (GAN et al., 2022; PAPADOPOULOU et al., 2023) and plant growth-promoting capabilities of microbial strains (MAUDE et al., 2024; SARAPAT et al., 2020). There are few studies in the literature that apply this approach to improving microorganisms for application in agriculture. Moreover, several challenges remain, including the need to decipher the genetic and physiological mechanisms that underpin these adaptations (WANG et al., 2023) and the issue of maintaining these traits over multiple generations once transferred to field conditions. Additionally, a thorough evaluation of the long-term ecological impacts of these adapted strains is essential to verify their safety and sustainability in agricultural settings.

This thesis integrates these strategies—cultivation parameter optimization, microbial encapsulation, and adaptive laboratory evolution—to advance the development of robust, efficient phosphate-solubilizing *Bacillus* strains. By tackling the viability and functionality issues of microbial inoculants, this research aims to contribute significantly to sustainable agricultural practices,

providing scalable solutions to meet the intertwined challenges of global food security and environmental sustainability.

GENERAL CONSIDERATIONS

Goals and Overview

The general goal of this thesis was to develop new approaches to increase the survival and efficiency of phosphate-solubilizing *Bacillus* strains under stress conditions by optimizing sporulation, encapsulation, and adaptation processes.

Specific Goals

✓ *Analyze publication trends and identify the main strategies for optimizing the production of Bacillus spores and their resistance.* This objective aimed to comprehend the theoretical foundation and current practices in *Bacillus* spore production, evaluating factors that influence their sporulation and resistance, and identifying existing gaps.

✓ *Investigate the role of starch as a renewable and biodegradable biopolymer for the encapsulation of microorganisms.* This involved exploring how starch-based matrices could be utilized to encapsulate microbes effectively, ensuring their survival in adverse environments.

✓ *Optimize cultivation conditions to produce heat-resistant Bacillus megaterium spores.* This objective sought to determine and validate the most favorable cultivation practices in bioreactors to maximize the yield and heat resistance of *B. megaterium* spores, enhancing their application as microbial inoculants.

✓ *Modify properties of starch-based films with additives (maltodextrin, cellulose, and bentonite) for application as coating systems.* The goal here was to adjust the physicochemical properties of starch-based films to improve their utility as eco-friendly coatings that can protect and sustainably release beneficial microbes.

✓ *Investigate the release behavior of beneficial microorganisms from modified starch-based films, evaluating the effectiveness of additives (maltodextrin, cellulose, and bentonite) in protecting against adverse environmental conditions, such as UV and extreme temperatures.* This objective focused on understanding how the incorporation of various additives into starch-based films influences the release patterns and environmental resilience of encapsulated microorganisms.

✓ *Increase the resistance of phosphate-solubilizing Bacillus strains to drought and salinity stresses, while maintaining or improving their growth-promoting capabilities, through the adaptive laboratory evolution (ALE) technique.* This involved using ALE to adapt *Bacillus* strains genetically and phenotypically, enhancing their tolerance to environmental stresses without compromising their ability to promote plant growth.

Chapter Overview

This thesis is structured into seven chapters, each dedicated to exploring different facets of enhancing the viability and efficiency of phosphate-solubilizing *Bacillus* strains.

Chapter 1 sets the stage with an in-depth introduction to the *Bacillus* spore production process and its crucial role across various industries, including agriculture, food, waste management, and bioremediation. Through a detailed systematic mapping study covering publications from 2000 to 2024, this chapter underscores the interest in the areas of *Bacillus* sporulation and resistance. It delves into the impact of various cultivation and operational parameters, such as culture medium, temperature, pH, water activity, agitation, and aeration, on spore production and resilience. The chapter emphasizes the necessity for tailored approaches due to the differential effects of these factors on the sporulation of distinct *Bacillus* species. Additionally, it outlines optimization strategies aimed at enhancing sporulation efficiency.

Chapter 2 examines the potential of starch, a renewable and biodegradable biopolymer, for microbial encapsulation, providing protection and sustaining microbial viability under stress. This chapter presents a systematic mapping of the growing interest in starch-based encapsulation systems over recent years. It reviews prevalent encapsulation techniques, including ionotropic gelation, emulsification, and spray drying, and discusses starch's roles within the encapsulation matrix. This exploration offers insights for the development of novel starch-based microbial encapsulation systems. The content of this chapter is an adaptation of the scientific article entitled: “Exploring the roles of starch for microbial encapsulation through a systematic mapping review” by Velloso, C. C. V.; Lopes, M. M.; Badino, A. C.; Farinas, C. S., published in Carbohydrate Polymers (DOI: 10.1016/j.carbpol.2023.120574).

Chapter 3 focuses on identifying the optimal cultivation conditions for producing heat-resistant *Bacillus megaterium* B119 spores in bioreactors. This chapter discusses the ideal nutrient media, temperature, and initial pH based on experiments conducted in shake flasks and validated through bench-scale bioreactor trials. It underscores the key aspects of generating highly resistant spores to enhance microorganism survival rates during inoculant production and application, thereby augmenting field efficacy. The content of this chapter is an adaptation of the scientific article entitled: “High yield of heat-resistant spores of *Bacillus megaterium* in bioreactors” by Velloso, C. C. V.; Camargo, B. C. P.; Sousa, M. D. B.; Buffo, M. M.; de Oliveira Paiva, C. A.; Farinas, C. S., Badino, A. C., published in Biochemical Engineering Journal (DOI: 10.1016/j.bej.2023.109030).

Chapter 4 delves into the enhancement of starch-based films with additives like maltodextrin, cellulose, glycerol, and bentonite to bolster their physicochemical attributes for potential application

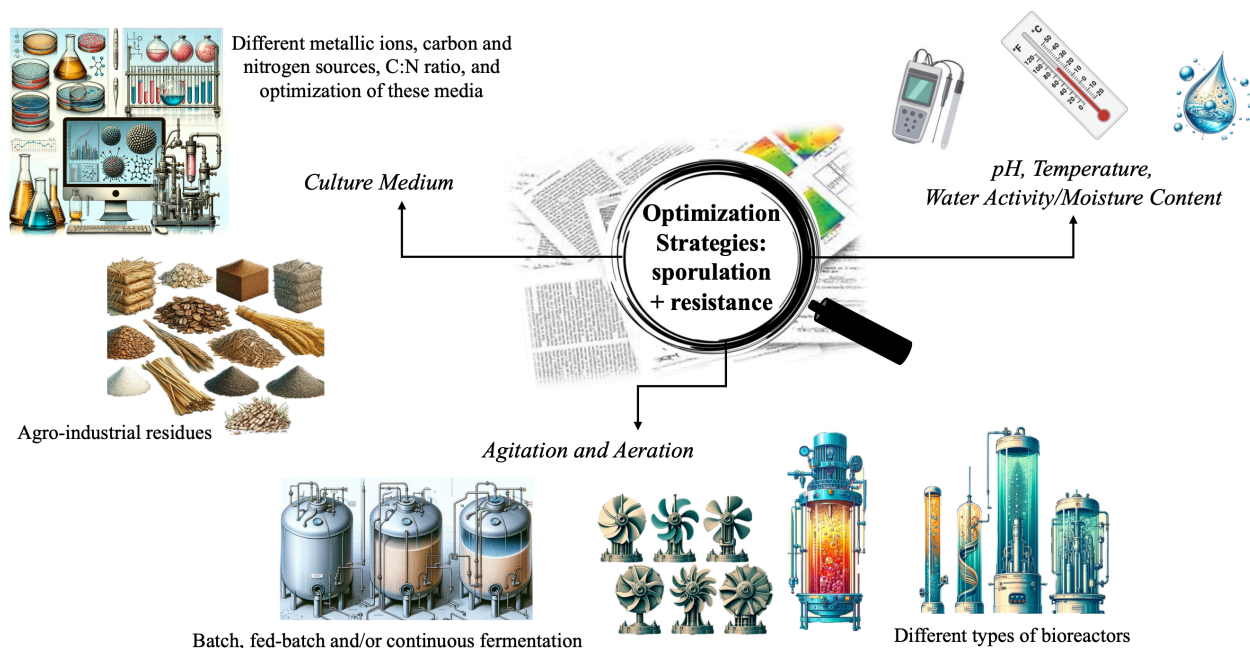
as eco-friendly coatings. It examines how these additives influence film properties such as hydrophobicity, water vapor permeability, viscosity, and mechanical strength, showcasing the versatility of customized starch-based films in seed coating applications.

Chapter 5 explores the targeted release of beneficial microbes, specifically *Priestia megaterium* (formerly known as *B. megaterium*), from modified starch-based films for use in agriculture. It assesses the films' bacteria release kinetics, physicochemical and morphological properties, and their protective efficacy against UV exposure and temperature extremes. The chapter evaluates how different additives can fine-tune film characteristics for controlled microbial release and enhanced survival under challenging conditions commonly found during field application.

Chapter 6 addresses the adaptive laboratory evolution (ALE) of P-solubilizing strains, *B. subtilis* NRRL B-354 and *B. megaterium* NRRL B-14308, to bolster their drought and salt tolerance while preserving or enhancing their plant growth-promoting capabilities. This chapter details the ALE process to acclimate these strains to desiccation and salinity, assessing the in vitro performance of both wild-type and adapted strains in biofilm formation, exopolysaccharide production, and phosphorus solubilization. Favorable strains undergo whole genome sequencing to pinpoint the genetic alterations responsible for the phenotypic changes observed, aiming to elucidate the molecular mechanisms behind the enhanced stress resistance of these *Bacillus* strains. This chapter aims to enhance our understanding of these strains' utility as agricultural inoculants under adverse environmental conditions.

Chapter 7 synthesizes the key findings and contributions of the thesis, highlighting the advancements made in optimizing *Bacillus* spore production, encapsulation, and adaptation to environmental stresses. It underscores the significance of these developments in improving the viability and efficiency of phosphate-solubilizing *Bacillus* strains for agricultural applications. The chapter also discusses the implications of this research for sustainable agriculture and environmental sustainability. Furthermore, it outlines future research directions, including exploring microbial consortia, assessing the genetic stability of strains, studies on the cost-effectiveness and ecological impact of polymeric films, and the assessment of the long-term ecological impact of ALE-strains. This final chapter emphasizes the potential of *Bacillus*-based technologies in addressing the challenges of modern agriculture and contributing to global food security.

1. *Bacillus* sporulation and resistance: insights from a systematic mapping study



1.1. Abstract

Bacillus spore production holds significant applications across sectors such as agriculture, food industry, waste treatment, and bioremediation. An analysis of publication trends from 2000 to 2024 demonstrates an ongoing interest in *Bacillus* sporulation and resistance, particularly highlighting its application in biological control and plant growth promotion in agriculture, as well as its relevance in food safety and probiotic potential within the food sector. This review details how cultivation and operational conditions, such as culture medium, temperature, pH, water activity, agitation, and/or aeration, influence spore production and resilience. These factors' influence on *Bacillus* sporulation varies by species, underlining the need for species-specific approaches. Strategies for optimization, such as employing agro-industrial residues, adjusting cultivation parameters in a phased manner, and leveraging predictive models to boost sporulation, are highlighted. This systematic analysis provides valuable insights for advancing *Bacillus* spore production bioprocesses and utilizing *Bacillus* spores as biological indicators for sterilization, offering a comprehensive resource for both academic and industrial stakeholders.

Keywords: bioprocess development, *Bacillus* spore resistance, operational conditions, culture conditions, strategies for sporulation.

1.2. Introduction

The *Bacillus* genus encompasses a group of Gram-positive bacteria distinguished by their ability to form spores. This trait not only grants them extraordinary resilience under adverse

environmental conditions but also underlines their significant role in ecological and biotechnological processes. Found ubiquitously in soil and water, *Bacillus* species contribute extensively to both natural ecosystems and human-driven applications, owing to their spore formation—a sophisticated survival mechanism enabling endurance through extreme conditions (BIERMANN et al., 2023; BRESSUIRE-ISOARD; BROUSSOLLE; CARLIN, 2018; XU ZHOU et al., 2017).

The versatility of *Bacillus* species is showcased through their pivotal roles across multiple sectors. In agriculture, they are harnessed as plant growth-promoting bacteria and biological control agents (BCA), offering an eco-friendly alternative to agrochemicals by targeting a broad range of pests and supporting plant growth (KHAN et al., 2022; PATANI et al., 2024; TIWARI; PRASAD; LATA, 2019; VALICENTE, 2019). In the realm of food technology, *Bacillus*-based fermentation processes and probiotic formulations enhance food safety and nutritional value, underscoring their importance in the food industry (WILLIAMS; WEIR, 2024; ZHAO et al., 2024). Furthermore, their capacity for pollutant biodegradation positions *Bacillus* species as key agents in waste management and environmental remediation efforts, aligning with the goals of sustainability and pollution reduction (BU et al., 2020; ESRINGÜ et al., 2014; NJOKU; AKINYEDE; OBIDI, 2020).

The exceptional resistance of *Bacillus* spores to harsh conditions such as high temperatures, UV radiation, and desiccation renders them invaluable in scenarios where stability and longevity are crucial, such as production, storage, and transportation (ELISASHVILI; KACHLISHVILI; CHIKINDAS, 2019). This resilience not only facilitates their use in challenging industrial, agricultural, and environmental applications but also makes them ideal biological indicators for sterilization efficacy, offering a reliable measure for ensuring the inactivation of less-resistant microorganisms (CHO; CHUNG, 2020; PRIBIL et al., 2007; SELLA et al., 2009a). Understanding sporulation and factors affecting spore robustness is key to optimizing production and improving sterilization processes.

This review delves into the influence of cultivation and operating conditions, including culture medium, temperature, pH, water activity, agitation, and/or aeration, on *Bacillus* sporulation and spore resistance. It enriches the work initiated by other reviews that have dissected the genetic underpinnings of sporulation (ERRINGTON, 1993; MEESKE et al., 2016; SHI et al., 2020) and the dynamics of bacterial germination (CHRISTIE; SETLOW, 2020; PETER, 2014), which fall outside the scope of this study. Employing a systematic mapping approach, this analysis offers a meticulous and expansive evaluation of current research on *Bacillus* sporulation. This strategy identifies gaps, research trends, and opportunities for innovation, providing a solid foundation for future studies and industrial applications. By systematically compiling and analyzing the available information, this work contributes not only to academic advancement but also to the development of more efficient

and sustainable biotechnological processes in the industry, promoting significant advances in both science and applied technology.

1.3. Methodology

The first step was to create a protocol that outlined the study's key topics, questions, keywords, search strings, and databases for finding primary literature. It also set the inclusion and exclusion criteria for selecting studies and described how to extract and analyze data from the articles chosen. Following this, 20 relevant articles related to the influence of cultivation and operating conditions on *Bacillus* sporulation and spore resistance were selected. The next step involved analyzing these articles to extract frequently used words in abstracts, keywords, and terms, which helped create a search string using Boolean operators (Fig. 1.1). This string was then applied to searches within several databases, including Web of Science, Scopus, and Compendex-Engineering Village. After removing duplicates and excluding articles published before the year 2000, a total of 1,265 articles were grouped into a spreadsheet for further analysis. The titles and abstracts of these articles were scrutinized based on predefined inclusion and exclusion criteria. Articles excluded and those that met the inclusion criteria were systematically organized into separate spreadsheets (Table 1.2S and Table 1.3S, respectively). Finally, 150 articles that fulfilled the inclusion criteria were selected for the data extraction phase, the details of which were also tabulated in a spreadsheet (Table 1.4S). This phase involved collecting basic information such as title, authors, year of publication, and abstract, in addition to specific data on the evaluated *Bacillus* species, strategies employed to enhance spore production, general and specific application areas, culture flask type and volume (whether reactor or Erlenmeyer flask), culture conditions, evaluated cultivation and operational conditions, and principal findings.

This methodology represents a comprehensive step-by-step approach to systematic mapping. Each stage of the process was meticulously documented in spreadsheets. The methodology adhered to established guidelines (CEE, 2013; HIGGINS et al., 2021; PULLIN; STEWART, 2006) as showed in Figure 1.1. Data analysis was conducted using statistical R software to create graphs and figures, and network maps were generated using VOSviewer software.

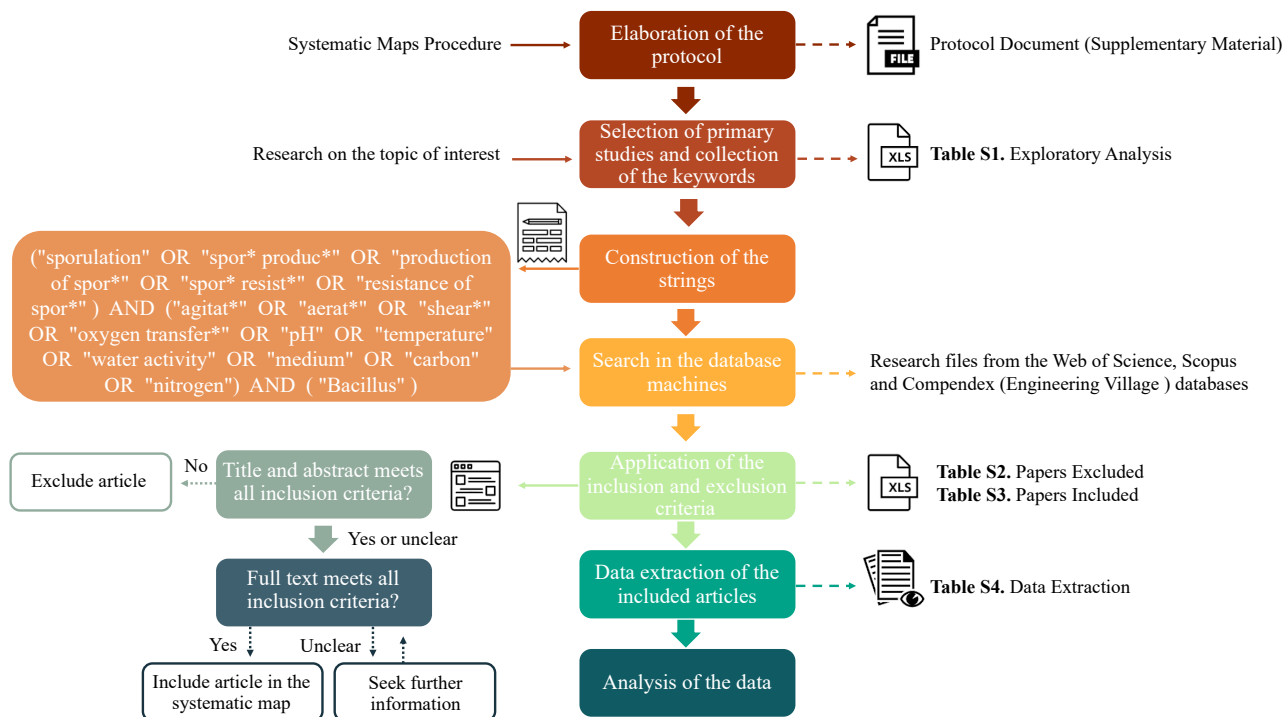


Figure 1.1. Flow diagram of the methodology used to prepare the systematic mapping.

1.4. Results and Discussion

1.4.1. Publication trends over the years, main fields of application, and *Bacillus* species

The graphical representations in Figure 1.2 facilitate a nuanced understanding of *Bacillus* research, illustrating the distribution of studies across various application domains and elucidating prevailing research priorities and trends based on the analysis of 150 articles. An analysis of publications between 2000 and 2024 aimed to elucidate trends concerning “factors influencing sporulation and resistance in *Bacillus*”. Figure 1.2A illustrates a significant increase in the number of studies starting from 2005, persisting through 2024. This trend underscores sustained interest within the scientific and industrial communities in the study of *Bacillus*.

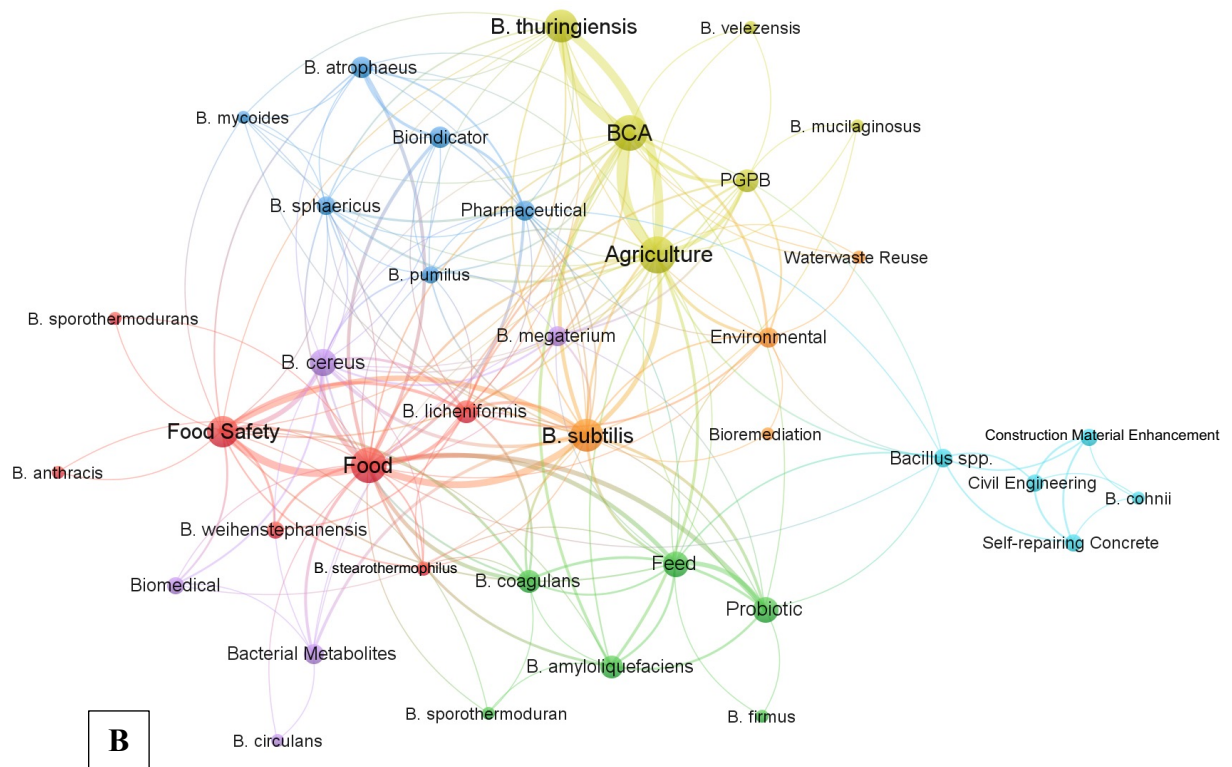
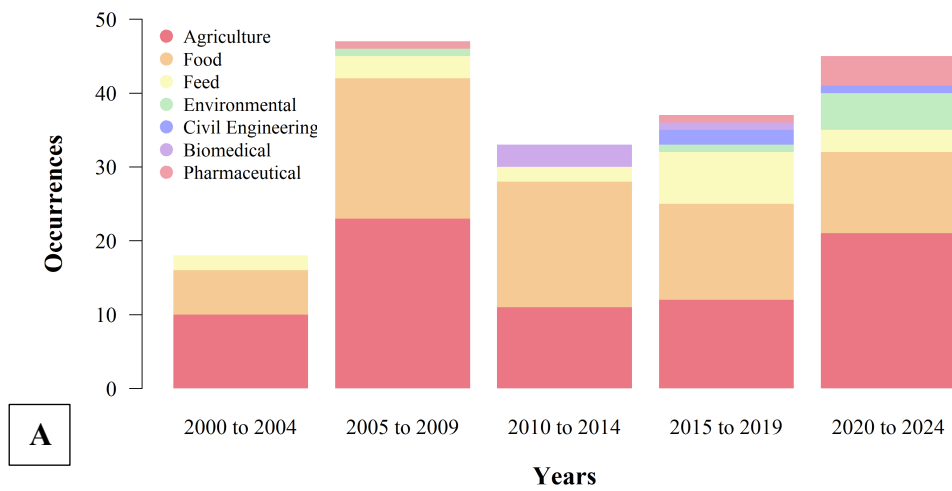
Investigations pertaining to *Bacillus* span a diverse array of interdisciplinary domains (Fig. 1.2B and 1.2C). Notably, the agriculture and food industries emerge as predominant areas of focus, collectively comprising 79.4% of the reviewed literature. Within the agricultural context, *Bacillus* is dichotomized into two primary classifications: Plant Growth-Promoting Bacteria (PGPB, representing 15%) and Biological Control Agents (BCA, constituting 85%), including species such as *B. thuringiensis*, *B. amyloliquefaciens* and *B. subtilis* (DOS SANTOS et al., 2024; FU et al., 2023; MA et al., 2022). These bacteria enhance plant growth and development, directly and indirectly, through a symbiotic relationship, benefiting from the protection and nutrients provided by the plants. Direct mechanisms include the production of phytohormones and enzymes, and nutrient availability, while indirect mechanisms involve biological control of phytopathogens and systemic resistance

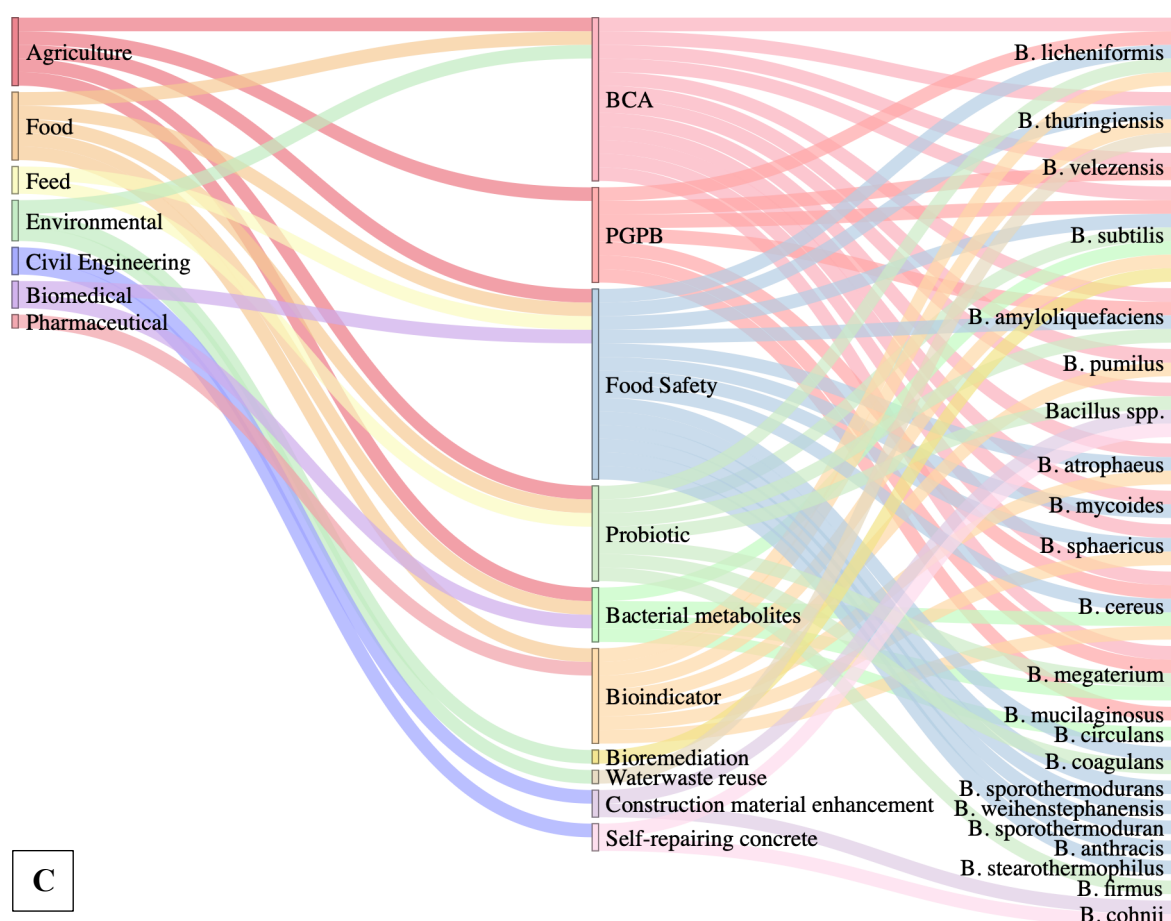
induction (BHARDWAJ et al., 2014; GLICK, 2015; SANTOS; NOGUEIRA; HUNGRIA, 2019; TAKEI et al., 2018).

In the food sector, food safety (54.5%) and the role of *Bacillus* strains as probiotics (22.7%) stand out (Fig. 1.2.B) (DAS et al., 2010; VITTORIA et al., 2023). Regarding food safety, concerns regarding the potential for toxicity and resistance, notably from strains such as *B. cereus* and *B. anthracis*, have encouraged investigations into their suitability as food additives (BHUNIA, 2018; TODOROV et al., 2022). Additionally, the propensity of some *Bacillus* strains to harbor antibiotic resistance genes has raised alarms regarding the possible horizontal gene transfer to pathogenic bacteria (ZHAI et al., 2023). Challenges also abound in devising effective sterilization and pasteurization techniques capable of neutralizing the resilient spores produced by *Bacillus* (CHO; CHUNG, 2020). From a probiotic perspective, *Bacillus* is instrumental in fostering gut health and modulating the immune system, with strains such as *B. coagulans*, *B. clausii*, and *B. licheniformis* notable for their gastrointestinal viability and efficacy in promoting digestive wellness and mitigating symptoms of disorders like irritable bowel syndrome (BIERMANN et al., 2023; DAS et al., 2010; ELISASHVILI; KACHLISHVILI; CHIKINDAS, 2019).

Furthermore, *Bacillus*'s role extends into waste treatment, bioremediation (DING et al., 2024b, 2024c; NING et al., 2024), biomedicine (VOORT; ABEE, 2012), civil engineering (HONG; KIM; PARK, 2019; JIANG et al., 2020), feed (MAHARIAWAN et al., 2021; SETIARTO et al., 2020), and the pharmaceutical industry (ESCHLBECK; BAUER; KULOZIK, 2017; LI et al., 2022), attesting to its broad applicability and potential for innovation. *B. thuringiensis* stands out as the most researched species (Fig. 1.2C), given its applicability as a biocontrol agent in agriculture. Other relevant species include *B. subtilis*, *B. licheniformis*, and *B. cereus* (Fig. 1.2C). The ability of this genus of bacteria to form resistant spores offers significant advantages in terms of stability and viability under adverse conditions, making them ideal candidates for biotechnological innovations and large-scale use.

In summary, ongoing research and the development of knowledge about *Bacillus* demonstrate their role in addressing contemporary challenges in health, environment, agriculture, and industry. The strategic selection of culture media and a deep understanding of the factors influencing sporulation are imperative to optimizing the sterilization process and yield of resistant spores, a critical consideration throughout the lifecycle of bio-input production, from inception to application. The impact of cultivation and operating conditions on *Bacillus* sporulation and resistance will be discussed in the next section.





C

Figure 1.2. Assessment of publication trends over time demonstrating the focus of *Bacillus* research in different areas through the interaction network and Sankey plot. A. Articles published each year that focus on the production and resistance of *Bacillus* spores, highlighting their relevance in different areas of application. B. Network analysis of *Bacillus* species researched in various application areas. Node size reflects study frequency in each species, while edge thickness indicates the strength of co-occurrence between *Bacillus* species and application areas. The colors signify the level of association between these variables, determined by the analysis software (Method: Strength of Association; Attraction: 2; Repulsion: -2). C. Sankey plot revealing the most investigated *Bacillus* species in different application areas, providing a clear visual representation of research priorities and trends. Data are derived from 150 articles deemed significant to our review question in the full-text analysis phase of this systematic mapping.

1.4.2. Influence of Cultivation and Operational Conditions on *Bacillus* Sporulation and Spore Resistance

Distinct characteristics of each *Bacillus* species crucially influence their sporulation behaviors. This section delineates how various cultivation and operational parameters, namely, culture medium, pH, temperature, water activity, agitation, and aeration, affect the sporulation efficacy and spore resistance in *Bacillus*.

1.4.2.1. Culture Medium

Most investigations (75.3%) have delved into the role of culture medium composition on sporulation and/or spore resilience. For optimal *Bacillus* growth and sporulation, the culture medium must be economical, widely available, and contain the necessary nutrients for the development of the bioprocess. The nutrient types and concentrations, notably the carbon to nitrogen (C:N) ratio, alongside additional additives and ions, markedly influence sporulation dynamics and spore robustness, highlighting the species-specific nutrient requirements.

- *Carbon and Nitrogen Sources*

Understanding the optimal carbon and nitrogen sources is paramount for the effective sporulation of different *Bacillus* species, as highlighted in numerous studies (ANGELO et al., 2015; CHEN et al., 2010; DING et al., 2024a, 2024b, 2024c; KHardZIANI et al., 2017a, 2017b; UONO et al., 2019). For example, sucrose (3.5 g/L) and yeast extract (4 g/L) favor growth and sporulation in *B. cohnii* (JIANG et al., 2020), while lactate and nitrate are preferable for aerobic incubation of *Bacillus* spp (ZHANG et al., 2017). Additionally, L-arabinose was found to double the concentration and sporulation efficiency of *B. subtilis* compared to glucose, positively modulating the expression of crucial sporulation genes (FU et al., 2023). A mixture of 1.8 g/L (NH₄)₂SO₄ and 8.0 g/L peptone was the ideal nitrogen source combination for *B. amyloliquefaciens* spore production (BERIKASHVILI et al., 2018).

The balance between glucose concentration and sporulation highlights a nuanced relationship; optimal levels (between 2.0 and 5.0 g/L) favor sporogenesis, whereas excessive amounts can inhibit the process through mechanisms such as catabolic repression and spo0A regulator inhibition (MONTEIRO et al., 2005, 2014; POSADA et al., 2015; SONENSHEIN, 2000). Furthermore, glucose abundance favors secondary metabolite formation that hampers spore recovery (CRISTIANO-FAJARDO et al., 2019). Remarkably, carbon depletion stands out as the main trigger for sporulation (ELISASHVILI; KACHLISHVILI; CHIKINDAS, 2019). Sporulation can also be induced by high carbon availability and nitrogen limitation (ELSAYED et al., 2014). While glucose exhaustion leads to a decline in vegetative cell and spore populations, attributed to metabolic activity cessation and

subsequent cell death, nitrogen source deficiency does not result in death (ABD GHANI et al., 2017). Moreover, spore germination, stimulated by nutrients released during cell lysis, and the subsequent death of emerging vegetative cells due to carbon source deficiency, underscore the critical role of nutrient management (FAN et al., 2023).

Strategies such as co-feeding, which involves the careful adjustment of glucose and amino nitrogen at different stages of growth and sporulation, have been shown to effectively maintain cell viability and enhance spore production (DING et al., 2024c). Similarly, innovative approaches like a nitrogen feeding system tailored to CO₂ concentration feedback have yielded improvements in sporulation efficiency and economic viability by reducing nitrogen source costs in *B. licheniformis* cultures, increasing cell and spore counts by 31.25% and 97.7%, respectively. Replacing peptone with NH₄Cl resulted in an 8.46% and 9.25% decrease in spore quantity for 5 and 50 L cultures, respectively and a substantial reduction in the costs of the nitrogen source by 87.08% and 91.39%, respectively, offering a viable approach for the economical production of spores on a large scale (DING et al., 2024b).

- *C:N Ratio*

The carbon:nitrogen (C:N) ratio is widely recognized as a fundamental regulator of vegetative growth and cell differentiation across various bacterial species, playing a pivotal role in the sporulation process. Some research underscores a direct correlation between lower C:N ratios and enhanced spore concentration, suggesting a precise mechanism through which this ratio influences sporulation efficiency (FARRERA; PÉREZ-GUEVARA; DE LA TORRE, 1998; MAHARIAWAN et al., 2020; MONTEIRO et al., 2014; VELLOSO et al., 2023a; YUNIARTI et al., 2019a).

Studies investigating the impact of total solids concentration (TSC) alongside the C:N ratio on *B. thuringiensis* HD-73 have shown that optimal spore concentrations are achieved at higher TSC levels (150 g/L) with a C:N ratio around 4:1 (FARRERA; PÉREZ-GUEVARA; DE LA TORRE, 1998). However, an increase in the C:N ratio resulted in a rapid decrease in sporulation. This optimal range indicates the process's sensitivity to nutritional balance, where deviations can significantly affect sporulation outcomes. For instance, while an increased C:N ratio promotes rapid vegetative growth in *Bacillus* spp. SB4, adversely affects sporulation efficiency, underscoring the nuanced balance required for optimal bacterial development and differentiation (YUNIARTI et al., 2019a).

Further evidence from cultivating *B. megaterium* with wheat flour, maintaining a C:N ratio of 5:1, demonstrates the highest vegetative cell concentration ($17 \cdot 10^8$ CFC/mL), and spore production ($14.7 \cdot 10^8$ spores/mL), illustrating the beneficial impact of carefully managed nutrient ratios in solid-state fermentations (MAHARIAWAN et al., 2021). Additionally, adjusting the C:N ratio and salt

content within agro-industrial waste has proven effective in enhancing sporulation efficiency, highlighting the potential of resource recycling and sustainable bioproduction practices (DOS SANTOS et al., 2024).

Conversely, specific instances, such as achieving maximal spore production in *B. firmus* SB4 with a C:N ratio of 10:1, illuminate the diverse nutritional requirements across different *Bacillus* strains and species (KUSUMANINGRUM; ANDAYANI; YUNIARTI, 2020). This diversity necessitates a tailored approach to nutrient management, emphasizing the importance of individualized investigations into the optimal carbon and nitrogen sources, their respective feeding rates, and control strategies to maximize sporulation efficiency.

- *Agro-Industrial Residues*

The adoption of agro-industrial byproducts as alternative carbon and nitrogen sources in *Bacillus* spore cultivation represents a significant leap toward sustainable biotechnological practices. This approach embodies the principles of the circular economy, focusing on resource efficiency, waste reduction, and lowering production costs, thus reinforcing the sector's commitment to environmental sustainability and economic viability.

An interesting fact is that approximately 37.3% of studies focusing on cultivation media for *Bacillus* spore production have utilized agro-industrial residues. This substantial percentage underscores the growing recognition within the scientific community of the potential these residues hold for bioproduction, emphasizing a broad-based shift towards more sustainable cultivation practices. Moreover, spores cultivated on these agro-industrial substrates exhibit enhanced heat resistance, a critical property for applications subjected to high-temperature conditions, such as agricultural biocontrol agents and sterilization processes in the food industry (SANTO et al., 2022; SELLA et al., 2008, 2012).

Figure 1.3 shows the variety of agro-industrial residues explored for *Bacillus* spore production, highlighting the adaptability of these microorganisms to a range of organic substrates. The strategic utilization of agro-industrial residues not only capitalizes on otherwise underutilized or discarded resources but also demonstrates the potential for innovation in waste valorization. By converting agricultural waste into valuable bioproducts, such as thermally resilient *Bacillus* spores, this approach fosters a more sustainable and cost-effective paradigm in biomanufacturing. This shift towards the use of agro-industrial residues in spore production not only aligns with global sustainability goals but also opens new avenues for research and development in the field of microbial biotechnology. It reflects a growing acknowledgment of the importance of sustainable practices in the industry, paving the way for more environmentally friendly and economically viable bioproduction methods.

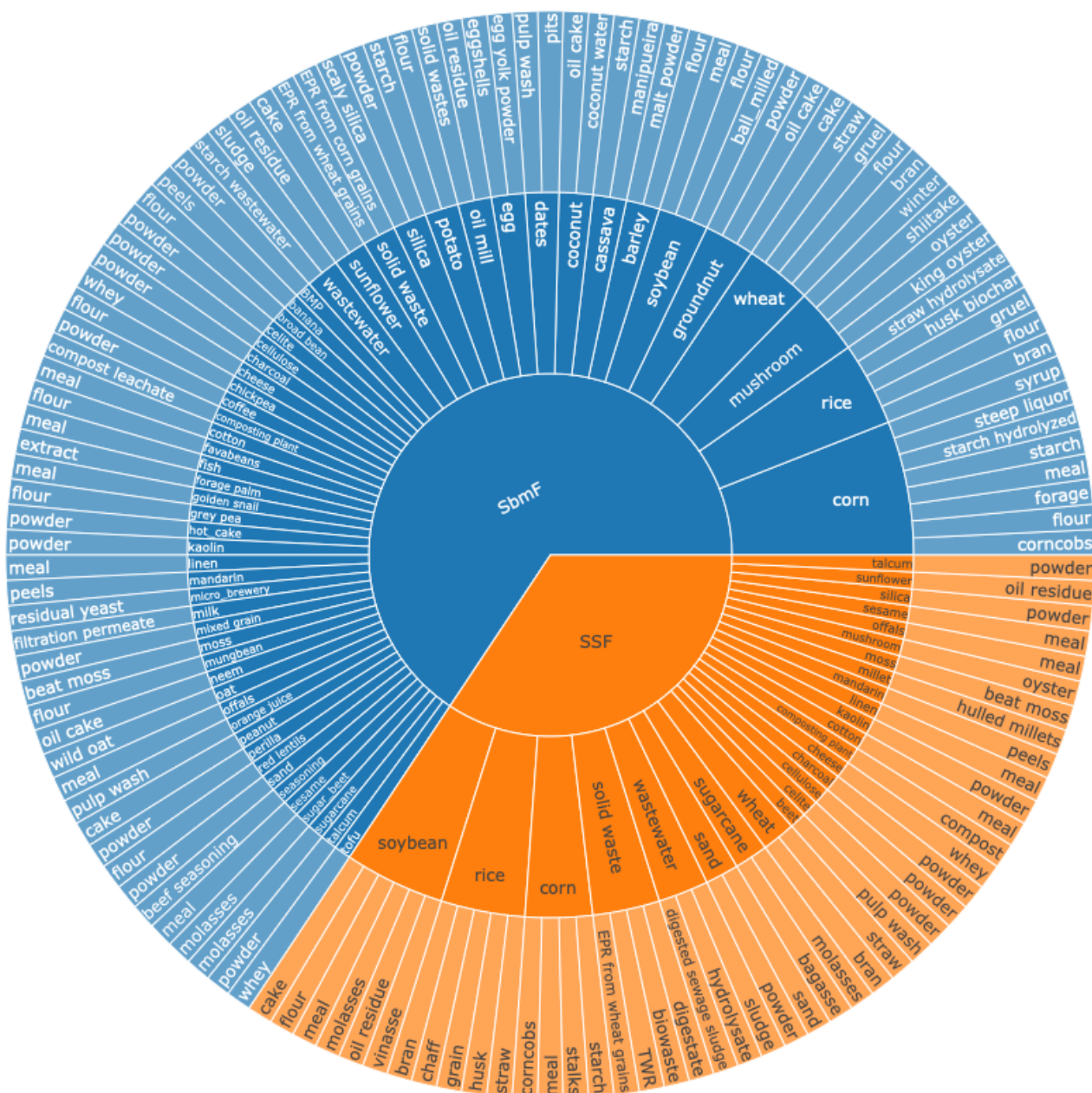


Figure 1.3. Predominantly researched agro-industrial residues for *Bacillus* spore production. SSF: Solid-State Fermentation, SbmF: Submerged Fermentation, EPR: ethanol production residue, TWR: tobacco waste residue.

- *Metallic Ions and Other Additives*

The sporulation process in *Bacillus* species is intricately linked to the metabolic balance within the cell, a balance that is heavily influenced by the presence of specific metallic ions and other additives (FREESE et al., 1974). Metal ions such as manganese (Mn^{2+}), iron (Fe^{2+}), zinc (Zn^{2+}), copper (Cu^{2+}), and calcium (Ca^{2+}) are essential cofactors that catalyze enzymatic activities crucial for sporulation and contribute to the structural stability of the resulting spores (ATRIH; FOSTER, 2001; KOLODZIEJ; SLEPECKY, 1964; MUKHOPADHYAY et al., 2004; OH; FREESE, 1976; SERIO; PECHTER; SONENSHEIN, 2006).

Mn²⁺ is particularly noteworthy for creating an environment conducive to the catalytic activity of enzymes crucial to the onset of sporulation. The effectiveness of this process is directly influenced by the ion's concentration and source (KOŁODZIEJ; SLEPECKY, 1964; MUKHOPADHYAY et al., 2004; OH; FREESE, 1976; SINGH; SETLOW, 1979; VASANTHA; FREESE, 1979). Optimal levels of Mn²⁺ facilitate the synthesis of secondary structures and metabolites essential for sporulation, whereas deviations from this optimum can impair the process (EBE et al., 2019; GRANGER et al., 2011; IÇGEN; IÇGEN; ÖZCENGİZ, 2002; IGURA et al., 2003; RYU; KIM; BEUCHAT, 2005; VERMA et al., 2013; WEINBERG, 1964). The source of manganese salt also impacts, with MnCl₂ promoting greater cellular growth and sporulation in *B. subtilis* compared to MnSO₄, possibly due to sulfate inhibition (YUNIARTI et al., 2019b). Furthermore, adding manganese to the culture medium can accelerate the sporulation process, achieving high efficiency in a short period (STÖCKEL et al., 2009).

Similarly, the supplementation of Fe²⁺ accelerates sporulation across various *Bacillus* species, including *B. anthracis*, *B. subtilis*, *B. thuringiensis*, *B. megaterium*, and *B. cereus*, suggesting a more rapid differentiation of these bacteria in the presence of iron. This may be due to the expression of late sporulation genes dependent on aconitase, an iron-requiring enzyme (PUROHIT; SASSI-GAHA; REST, 2010; SERIO; PECHTER; SONENSHEIN, 2006; VELLOSO et al., 2023a). However, excessive concentrations of Fe²⁺, Zn²⁺, and Cu²⁺ can be detrimental, inhibiting sporulation by interfering with the biosynthesis of spore components or damaging vegetative cells (KOŁODZIEJ; SLEPECKY, 1964; LEE et al., 2011). Determining the optimal concentration of these ions is crucial, as low concentrations can inhibit sporulation and high concentrations are toxic to the bacteria, preventing their growth.

Calcium addition has also shown a significant impact on sporulation and spore heat resistance, suggesting an important role for Ca²⁺ in producing more robust and resilient spores, possibly through complex formation with dipicolinic acid (DPA) and consequent reduction of water content in the spore core (CAZEMIER; WAGENAARS; TER STEEG, 2001; SETLOW, 2006; SINNELÄ et al., 2021). However, this effect is species-dependent, with *B. licheniformis* and *B. cereus* showing lower sporulation efficiency in media with minimal calcium supplementation, whereas *B. subtilis* and *B. coagulans* exhibited the opposite effect (SINNELÄ et al., 2019). Moreover, calcium ion restriction in the culture medium can inhibit sporulation (O'HARA; HAGEMAN, 1990), and its omission results in a large fraction of non-refractory spores (KOŁODZIEJ; SLEPECKY, 1964).

Manipulating inorganic salts during cultivation has revealed the importance of the timing of these components' addition to the medium. Magnesium, manganese, iron, zinc, and copper salts should be added in the early phases of *Bacillus* growth, negatively influencing when added at the end

of cellular growth; zinc salts are unnecessary after vegetative growth; adding manganese salts at the end of vegetative growth inhibits sporulation; and adding copper after autoclaving reduces sporulation, indicating copper binding with medium constituents during sterilization (KOŁODZIEJ; SLEPECKY, 1964). Moreover, depletion of Ca^{2+} or Mg^{2+} led to the release of more hydrophobic and adhesive spores (HAMIOT et al., 2023), while a five-fold increase in salt concentration altered spore morphology, producing elongated *B. megaterium* spores with an average volume increase of nearly 30% (XU ZHOU et al., 2017).

Interestingly, studies have also highlighted the impact of less commonly examined elements, such as Tb^{3+} , Sm^{3+} , and Dy^{3+} (rare earth elements), which can increase sporulation and heat tolerance of spores at low concentrations, while higher concentrations may inhibit sporulation (NING et al., 2024; RUMING et al., 2004; ZHAO et al., 2004). Furthermore, the addition of tricalcium phosphate has been shown to not only support cell survival at room temperature but also to elevate the rate of sporulation (SHAHROKH ESFAHANI et al., 2016). Introducing phosphate into the sporulation medium has significantly boosted spore heat resistance, whereas sodium and potassium chlorides did not exhibit a notable effect (MAZAS et al., 2009). Additionally, the presence of free fatty acids in the cultivation medium markedly diminishes the heat resistance of *Bacillus* spores, particularly in the case of unsaturated fatty acids (DAS et al., 2010).

These insights into the role of metallic ions and additives in *Bacillus* sporulation not only deepen our understanding of the physiological requirements for spore formation but also highlight potential strategies for industrial applications. By manipulating the concentration, source, and timing of these critical elements, it is possible to significantly improve the efficiency and outcomes of sporulation, paving the way for advancements in biotechnological applications involving *Bacillus* spores.

1.4.2.2. Temperature, pH, and Water Activity/Moisture Content

Variations in temperature, pH, and water activity/moisture content directly influence the sporulation rate, viability, and resistance of spores. Precise adjustments in these environmental conditions can significantly enhance the production of resistant spores, underscoring the importance of these variables in the sporogenesis process (VELLOSO et al., 2023a).

- *Temperature*

Bacillus spp. (mesophilic microorganisms) typically thrive at temperatures between 20 and 40 °C (STAMENKOVIĆ-STOJANOVIĆ et al., 2019). Literature suggests that temperatures below the optimum tend to decrease or delay the growth of vegetative cells, while temperatures above 85 °C are lethal to these cells (MONTEIRO et al., 2014; STAMENKOVIĆ-STOJANOVIĆ et al., 2019).

The cultivation temperature also significantly affects the sporulation process. Low temperatures reduce the efficiency of *Bacillus* sporulation, whereas high temperatures promote greater spore heat resistance. This resistance is attributed to an increased concentration of dipicolinic acid (DPA), greater mineralization in the spore core, and a reduction in the water content within the spore (BARIL et al., 2012a; BRESSUIRE-ISOARD; BROUSSOLLE; CARLIN, 2018; GARCIA; VAN DER VOORT; ABEE, 2010; ISTICATO et al., 2020; MINH et al., 2011; PALOP; SALA; CONDÓN, 1999). Studies show that DPA concentrations are higher in spores of *B. cereus* and *B. anthracis* cultivated at high temperatures (30 and 45 °C) compared to those grown at lower temperatures (10 and 25 °C) (BAWEJA et al., 2008; PLANCHON et al., 2011), and that the water content is lower in spores of *B. subtilis* formed at elevated temperatures (37 and 48 °C) (MELLY et al., 2002). Interestingly, studies report that sporulation under suboptimal temperatures of 25°C, although slower and less efficient, results in a high number of fully functional spores. Conversely, the optimal temperature of 42°C accelerates the sporulation process, but the spores produced tend to exhibit structural and morphological defects (VITTORIA et al., 2023). This contrast highlights the complexity of *Bacillus* adaptive responses to different temperature regimes, emphasizing the importance of carefully selecting cultivation conditions to maximize both the quantity and quality of spores.

Table 1.1 illustrates the specific temperature and pH conditions that various *Bacillus* strains prefer for optimal sporulation, highlighting the diversity in their environmental requirements.

Table 1.1. Temperature and pH for sporulation in selected *Bacillus* species.

<i>Bacillus</i> species	Temperature and pH for spore production	Ref.
<i>B. megaterium</i> B119	37°C and pH 5.6	(VELLOSO et al., 2023a)
<i>B. thuringiensis</i> KH4	30°C and pH 6.5	(SALEHI JOUZANI et al., 2015)
<i>B. thuringiensis</i> var. <i>tenebrionis</i> -BN2	28°C and pH 8.0	(SABERI et al., 2023)
<i>B. thuringiensis</i> var <i>israelensis</i> ATCC 700872	30°C and pH 7.0	(ELSAYED et al., 2014)
<i>B. thuringiensis</i> VCRC B-17	30°C and pH 7.2	(PRABAKARAN; HOTI, 2008)
<i>B. velezensis</i> 83	30°C and pH 6.8	(CRISTIANO-FAJARDO et al., 2024)
<i>B. cohnii</i>	30°C and pH 10.0	(JIANG et al., 2020)
<i>B. coagulans</i> BL32	30°C and pH 8.5	(SOUZA; RODRIGUES; AYUB, 2009)
<i>B. coagulans</i> RK-02	41.4°C and pH 6.3	(SEN; BABU, 2005)
<i>B. amyloliquefaciens</i> BS-20	37°C and pH 7.0	(SU et al., 2019)

<i>B. weihenstephanensis</i> KBAB4	30°C and pH 7.2	(BARIL et al., 2012a)
<i>B. licheniformis</i> AD978	45°C and pH 7.2	(BARIL et al., 2012a)
<i>B. subtilis</i> BSB1	46.9°C and pH 6.8	(GAUVRY et al., 2021)
<i>B. subtilis</i> CCM1 355	34°C and pH 8.0	(ANWAR; SABRI; HASNAIN, 2007)
<i>B. subtilis</i> MB24	37°C and pH 7.5	(MONTEIRO et al., 2005)

- *pH*

The interplay between pH and the growth and sporulation dynamics of *Bacillus* reveals notable complexity with significant variations across different species. Generally, most bacteria prefer a pH range of 6.0 to 7.0 for optimal vegetative cell growth (STAMENKOVIĆ-STOJANOVIĆ et al., 2019). However, sporulation presents a more diverse scenario regarding the ideal pH, highlighting variation in the adaptive responses of the microorganisms. Studies indicate that an alkaline medium favors sporogenesis, while extremely acidic conditions can delay or even inhibit the growth of *Bacillus*, negatively affecting spore production (ANWAR; SABRI; HASNAIN, 2007; BARIL et al., 2012a; MONTEIRO et al., 2005; POSADA et al., 2015; SU et al., 2019). In contrast, evidence suggests higher sporulation efficiency and spore production under slightly acidic to neutral conditions, with the pH ranging from 5.6 to 7.0 (CRISTIANO-FAJARDO et al., 2024; SEN; BABU, 2005; VELLOSO et al., 2023a). The presence of divalent metal ions in the medium has been shown to increase the tolerance of *B. coagulans* ATCC 10545 to acidic pH environments (CAZEMIER; WAGENAARS; TER STEEG, 2001). However, the mineralization of *Bacillus* spores is intensified in alkaline pH, contributing to their thermal resistance, a relevant aspect for industrial applications and preservation (ABBAS et al., 2014a, 2014b; BRESSUIRE-ISOARD; BROUSSOLLE; CARLIN, 2018; MINH et al., 2008; XU ZHOU et al., 2017). It has also been reported that sporulation can be accelerated in alkaline pH environments (BAWEJA et al., 2008; VELLOSO et al., 2023a), and that pH control can significantly influence sporulation efficiency, as observed in a sevenfold increase when scaling from shaker to reactor (BIERMANN et al., 2023).

The relationship between cultivation pH and the characteristics of *Bacillus* spores also exhibits significant interspecies variability. Some *Bacillus* species, when cultivated at high pH, form more hydrophobic and adhesive spores (ESCHLBECK; BAUER; KULOZIK, 2017). In contrast, other species develop spores with similar properties when cultivated under acidic conditions (HAMIoT et al., 2023). Interestingly, in some cases, high pH is associated with lower resistance to hydrogen peroxide (ESCHLBECK; BAUER; KULOZIK, 2017), while greater heat resistance was observed under alkaline conditions, especially when combined with additional treatments like nisin (AOUADHI et al., 2016).

These observations underscore the complex interaction between cultivation conditions and spore biology, indicating that the effect of pH on sporulation and the surface properties of spores varies among different *Bacillus* species. Therefore, it is crucial to carefully adjust the pH during *Bacillus* cultivation to optimize both growth and sporulation, considering the specificities of each species. Initiatives such as the two-stage process proposed by CRISTIANO-FAJARDO et al., (2024) for *B. velezensis* 83 spores production (growth at pH 5 and sporulation at pH 6.8), and the development of a cardinal model by GAUVRY et al., (2021) to predict sporulation of *B. subtilis* BSB1 based on environmental factors, are significant steps in exploring and leveraging the potential of these microorganisms.

- *Water Activity and Moisture Content*

Water activity (a_w) directly affects the availability of water for biochemical reactions and cellular growth, thereby influencing sporulation. Suboptimal water activities can lead to a delay in the initiation of sporulation and a significant reduction in the rate of spore formation and final spore yield. This is attributed to the direct impact of a_w on cellular physiology and metabolic pathways that regulate sporulation (BARIL et al., 2012b). Furthermore, the presence of abundant water on the medium's surface allows for flagellar motility, resulting in a heterogeneous cell and spore population, including small and damaged spores. High-viscosity media have led to the emergence of filamentous cells and spores more sensitive to heat (STECCHINI et al., 2009).

Water activity is also a determinant factor for the thermal resistance of *Bacillus* spores. Studies have shown that spores cultivated on solid media, such as agar plates and agro-industrial residues, possess greater resistance to heat and chemicals compared to those cultivated in liquid media. This difference is attributed to variations in the composition of spore coat proteins (ABHYANKAR et al., 2016; ROSE et al., 2007; SELLA et al., 2012). Moreover, the investigation of the effect of osmotic agents, such as NaCl and glycerol, on water activity revealed that the addition of NaCl to the culture medium increases spore resistance, while glycerol does not have a significant impact on this (AOUADHI et al., 2016).

In terms of solid-state cultivation, substrate moisture is a determining factor for maximizing spore production in species like *B. amyloliquefaciens*, *B. pumilus*, and *B. atrophaeus*. It was observed that optimal substrate moisture around 50% is ideal for maximum spore production of *B. amyloliquefaciens* and *B. pumilus*, while an increase to 60% results in a notable decrease in sporulation (LEE et al., 2020; SU et al., 2019). On the other hand, for *B. atrophaeus*, the best condition for sporulation was an initial moisture of 80% without aeration (SELLA et al., 2009b). This effect can be explained by the significant impact of moisture on the physical properties of different

substrates used, such as elasticity, hardness, adhesiveness, and morphology.

These findings highlight the importance of controlling water activity and underscore the distinct influence that different osmotic agents can have on spore resistance. It points out the complexity of environmental factors affecting sporulation and spore resilience.

- *Combined Effect*

The interplay between temperature, pH, and water activity (including substrate moisture content) significantly influences the resistance and quality of *Bacillus* spores (Jagannath et al., 2003). Strategies that optimize these environmental conditions can lead to spores with enhanced thermal resistance and other desirable characteristics, suitable for a wide range of applications. For example, high temperatures increase the resistance of *B. atrophaeus* spores to hydrogen peroxide (H₂O₂) and *B. megaterium* spores to heat, but this enhancement is pH dependent. The effect of temperature on spore resistance exhibited a quadratic character concerning pH, with optimal resistance to H₂O₂ at neutral pH and to heat in more acidic environments (STIER; KULOZIK, 2020; VELLOSO et al., 2023a). Furthermore, the pH-temperature interaction influences spore size, resulting in smaller spores under alkaline conditions and high temperatures (XU ZHOU et al., 2017). Understanding and manipulating these factors offer pathways to improve both the efficiency of sporulation and the functional properties of the produced spores, highlighting the complexity and adaptability of *Bacillus* to varying cultivation conditions.

1.4.2.3. Agitation and Aeration

Optimizing oxygenation through careful management of agitation and aeration conditions is key for efficient sporulation in *Bacillus* spp. The availability of oxygen is crucial for cellular growth, but its scarcity or excess due to improper agitation and aeration can adversely affect the production dynamics (STAMENKOVIĆ et al., 2018; STAMENKOVIĆ-STOJANOVIĆ et al., 2019), potentially leading to increased operational costs and diminished sporulation efficiency (SAHOO et al., 2003, 2004). Interestingly, only 20% of the articles evaluated have specifically focused on optimizing agitation and aeration conditions. This statistic suggests that while the importance of oxygenation is well-recognized, there remains significant potential for further research and optimization in this area.

Agitation affects the distribution of oxygen and nutrients, influencing the overall sporulation efficiency. Aeration is vital for ensuring sufficient oxygen levels within the culture and optimal aeration conditions significantly enhance *Bacillus* spore production. TZENG et al., (2008) found that agitation at 300 rpm in a conventional 5 L bioreactor can suppress the sporulation of *B. amyloliquefaciens*, with the optimal spore concentration achieved at 200 rpm. This example underscores the negative impact of excessive agitation, including increased shear stresses that can

adversely affect cells. POSADA et al., (2015) recorded the highest spore yield of *B. subtilis* in a 14 L bioreactor, operating at 400 rpm and an air flow rate of 12 L/min. CHEN et al., (2010) scaled up *B. subtilis* spore production by controlling agitation (400-800 rpm) and maintaining dissolved oxygen tension at 30-40%, resulting in high spore density. The concentration of dissolved oxygen proved essential for spore production in *B. amyloliquefaciens* and *B. thuringiensis*, with significant increases under optimized conditions in 20 L and 7 L bioreactors, respectively. Oxygen transfer, encompassing oxygen uptake rate (OUR), oxygen transfer rate (OTR), and volumetric mass transfer coefficient (k_La), has been identified as critical factors in maintaining effective sporulation at larger scales. For example, NDAO et al., (2017) demonstrated improvements in growth and spore production in 2000 L bioreactors through the careful management of the oxygen transfer rate.

Oxygen limitation has distinct effects on sporulation. ELSAYED et al., (2014) and DE CARVALHO et al., (2010) found that limited mass transfer prevented *B. thuringiensis* and *B. subtilis* sporulation, emphasizing the need for adequate air flow rates. Low k_La limits growth and sporulation, while increases up to a certain threshold promote spore and endotoxin production (DA SILVA et al., 2021; HA et al., 2018; MOUNSEF et al., 2015). The absence of oxygen reduced the formation of spores of *B. cereus* and *B. thuringiensis* var. *israelensis* (ABBAS et al., 2014a; BONIOLO et al., 2012), and led to 100% sporulation of *B. thuringiensis* H14 (SARRAFZADEH; NAVARRO, 2006). On the other hand, *B. cereus* spores formed under such conditions show greater resistance to heat and chemical agents (ABBAS et al., 2014b). Interestingly, the formation of spores under low oxygen transfer or anaerobic conditions often results in spores with increased heat resistance due to a higher accumulation of dipicolinic acid (ABBAS et al., 2014a, 2014b; PLANCHON et al., 2011; SETLOW, 2006), in addition to exhibiting greater hydrophobicity and adhesiveness (HAMMOT et al., 2023). The complex of water, Ca^{2+} ions, and dipicolinic acid is vital for the thermal resistance of spores, protecting proteins and lipids (COWAN et al., 2003, 2004). Oxygen also influences the pH of the culture broth and the composition of the sporangia, with high oxygen transfer rates elevating the pH during sporulation. Interruption in oxygen supply results in a more acidic pH and accelerates sporangia lysis (SARRAFZADEH et al., 2014).

Biphasic approaches and two-stage aeration and agitation strategies have been shown to optimize spore production in submerged and solid-state fermentation (BONIOLO et al., 2012; SOUZA; RODRIGUES; AYUB, 2009; STIER; KULOZIK, 2022; ZHAO et al., 2019). SEN; BABU, (2005) alternated agitation and aeration conditions in a 20 L bioreactor to increase biomass during the exponential phase and induce sporulation during the stationary phase (247 rpm and 1.05 vvm; 115 rpm and 0.33 vvm). Similarly, MOHD MASRI; MAIDIN; BIN ARIFF, (2021) implemented a control of dissolved oxygen tension, adjusting it to 80% during growth and reducing it during the mid-

exponential phase to enhance sporulation efficiency. Conversely, MEJIAS et al., (2020) used digested and biological waste in solid fermentation, applying a two-stage aeration strategy that starts with microaeration and follows with intensive aeration to optimize temperature and biodegradability, aiming to increase spore production.

The examples provided illustrate the nuanced impact of agitation and aeration on *Bacillus* sporulation, highlighting the necessity of optimizing these parameters for improved spore production. Tailored strategies, acknowledging the specific oxygenation needs of different *Bacillus* strains, are essential for maximizing the efficiency and quality of spore production, with significant implications for their industrial application.

Overall, studies indicate that reducing the agitation speed favors sporulation, suggesting that gentler agitations favor spore formation, possibly due to less turbulence and mechanical stress. Similarly, lowering the aeration rate during sporulation in relation to the growth phase increases sporulation, suggesting that conditions of lower oxygen concentration are beneficial, whether by reducing the oxygen demand of cells in the stationary phase or affecting sporulation signaling. High levels of dissolved oxygen can promote high spore production but only up to a certain threshold, after which, specific to each microorganism, sporulation inhibition can occur. Anaerobiosis, while increasing spore resistance, has a strain-specific effect on sporulation.

The interplay between agitation and aeration conditions in *Bacillus* cultivation underscores the complexity of optimizing sporulation conditions. Achieving the right balance of oxygen availability through careful manipulation of agitation and aeration conditions is paramount for producing high-quality and resilient spores. Tailored strategies that consider the specific oxygen needs of *Bacillus* strains can lead to more efficient and cost-effective bioprocesses, with significant implications for industrial applications of *Bacillus* spores.

1.4.2.4. Additional Insights into *Bacillus* Sporulation

- *Cultivation Scale, Reactor Type, and Fermentation process*

⇒ Most studies (64.4%) in this area have been conducted on a small scale, primarily using Erlenmeyer flasks, focusing on the preliminary research and optimization of growth, spore production, and secondary metabolite expression parameters. This preference is attributed to the ease of manipulation, cost-effectiveness, and experimental flexibility offered by Erlenmeyer flasks. However, bioreactors have been utilized in 17% of the papers, highlighting a significant portion of research dedicated to scaling up and optimizing processes for industrial applications. The transition from small-scale experiments to bioreactor trials in 10% of the studies underscores the strategy of validating initial findings and assessing the feasibility of larger-scale production.

⇒ Submerged fermentation is favored in most articles, chosen 5.5 times more frequently than solid-state fermentation. This preference likely stems from the scalability, control over process parameters, and continuous monitoring capabilities inherent to submerged fermentation, which facilitate process optimization.

⇒ The choice of reactor type significantly influences spore production outcomes. For instance, bubble column reactors have shown superior performance in producing heat-resistant *Bacillus* spores compared to stirred tank reactors (STR), achieving higher sporulation efficiency and energy efficiency in spore production (VELLOSO et al., 2023a). Similarly, the 20L airlift reactor (ALR) outperformed the 5L STR in the production of *B. amyloliquefaciens* B128 spores, achieving a spore productivity five times greater than that of shaker flasks and eight times more than STR. Moreover, the cultivation time in ALR was 19 hours shorter than in STR significantly affecting the process productivity (TZENG et al., 2008).

- *Advanced Cultivation Strategies*

⇒ Incorporating inert supports like polyurethane foam in solid cultures has emerged as an effective strategy for enhancing metabolite production, including spore yield and productivity. This approach has shown to increase spore production and confer greater thermal resistance to spores, compared to liquid cultures (FLORES-TUFIÑO et al., 2021; LIMA-PÉREZ et al., 2019).

⇒ While batch cultivations are common, studies have demonstrated the benefits of semicontinuous and fed-batch processes in achieving higher spore concentrations and productivity, underlining the importance of innovative cultivation strategies (MONTEIRO et al., 2005; PRADELLA; TACIRO, 2000; SILVA et al., 2011; YEZZA et al., 2005).

⇒ Co-cultivation experiments reveal that the interaction between different microbial strains can significantly impact cellular growth and spore production, with outcomes varying based on the microorganism ratio (DAI et al., 2020).

- *Modeling and Optimization*

⇒ The development of kinetic models and hybrid models combining traditional dynamic modeling with neural networks has provided new avenues for optimizing the sporulation process, allowing for precise adjustments of key cultivation parameters to enhance spore yield (DING et al., 2024a; GAUVRY et al., 2019).

This comprehensive overview of *Bacillus* spp. cultivation underscores the complexity and diversity of approaches in optimizing spore production. From the choice of cultivation scale and method to the strategic use of reactor types and advanced modeling techniques, each aspect plays a critical role in maximizing sporulation efficiency and spore quality. As the field continues to evolve,

leveraging these insights will be crucial for advancing the industrial and agro-industrial applications of *Bacillus* spores, promoting more efficient, scalable, and sustainable bioproduction processes.

1.5. Conclusion

This systematic mapping of the sporulation of *Bacillus* species underscores the intricate nature of this biological process and highlights the critical need for customized cultivation and operational strategies to optimize spore efficiency and enhance resistance. Our analysis reveals that pinpointing the ideal conditions for sporulation—tailored to the specific needs of each *Bacillus* species and application context—can substantially boost spore yield. This optimization holds promising implications for a wide range of applications, from agriculture and food safety to environmental sustainability and bioremediation.

A significant insight from our study is the pivotal role of foundational research and the meticulous optimization of conditions at a small scale, which are indispensable for understanding the fundamental aspects of *Bacillus* sporulation. There is a growing momentum toward scaling these findings for industrial application, highlighting a keen interest in leveraging *Bacillus*' biotechnological potential. The exploration of diverse approaches, including the use of inert supports in solid-state fermentation and the development of kinetic models for process optimization, showcases *Bacillus*' remarkable adaptability.

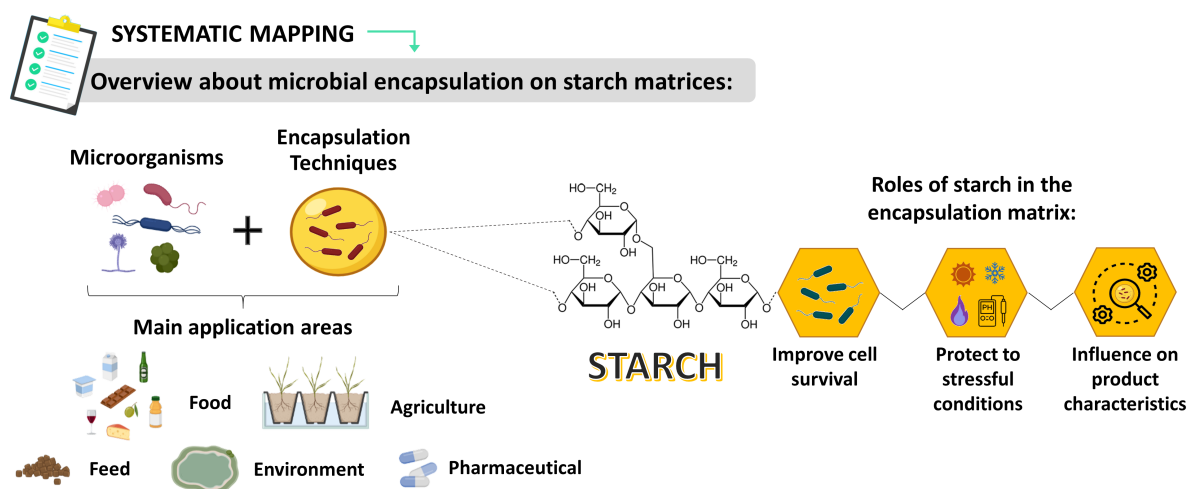
The transition from benchtop studies to large-scale production emphasizes the necessity for interdisciplinary collaboration and innovation. Bridging the gap between fundamental research and industrial application unlocks new opportunities for leveraging *Bacillus* spores in developing efficient, sustainable biotechnological solutions. Future investigations should focus on addressing research gaps such as the environmental factors affecting sporulation processes, integrating advanced modeling techniques like machine learning, and exploring the co-cultivation of *Bacillus* with other microorganisms.

Moreover, the potential of spore functionalization for applications in medical and healthcare fields, such as targeted drug delivery, is in its early stages but holds promising potential. Addressing regulatory and safety considerations for the use of *Bacillus*, especially genetically modified strains, in various applications is crucial for advancing the field responsibly. As we move forward, integrating advanced modeling, novel fermentation strategies, and cross-sectoral applications will be crucial in harnessing the full potential of *Bacillus* spores, offering significant advancements in science and technology. Focusing research efforts on these areas could significantly advance our understanding of *Bacillus* sporulation and its practical applications, leading to scientific breakthroughs and biotechnological innovations.

2. Exploring the roles of starch for microbial encapsulation through a systemic mapping review

* The content of this chapter is an adaptation of the scientific article entitled: “Exploring the roles of starch for microbial encapsulation through a systematic mapping review” by Velloso, C. C. V.; Lopes, M. M.; Badino, A. C.; Farinas, C. S., published in Carbohydrate Polymers.

Reference: Carbohydr. Polym., 2023, 306, 120574.



2.1. Abstract

Microorganism encapsulation protects them from stressful conditions and assists in maintaining their viability, being especially beneficial when the carrier material is a renewable and biodegradable biopolymer, such as starch. Here, a systematic mapping was performed to provide a current overview on the use of starch-based systems for microbial encapsulation. Following well-established guidelines, a systematic mapping was conducted and the following could be drawn: 1) there was a significant increase in publications on microbial encapsulation using starch over the past decade, showing interest from the scientific community, 2) ionotropic gelation, emulsification and spray drying are the most commonly used techniques for starch-based microbial encapsulation, and 3) starch play important functions in the encapsulation matrix such as assisting in the survival of the microorganisms. The information gathered in this systematic mapping can be useful to guide researchers and industrial sectors on the development of innovative starch-based systems for microbial encapsulation.

Keywords: Starch, encapsulation, microorganisms, microbial carrier, polysaccharide, systematic mapping.

2.2. Introduction

Microbial cells have been used in several fields, such as food processing, pharmaceutical products, environmental protection, fermentation products, agricultural biotechnology, wastewater treatment, and other industries (RATHORE et al., 2013; SABERI-RISEH et al., 2021; SCHOEBITZ et al., 2021; SCHOEBITZ; LÓPEZ; ROLDÁN, 2013; VASSILEV et al., 2020). However, microorganisms can have their efficiency and survival reduced due to external stressing factors when applied in the free form. A good strategy to overcome these limitations is encapsulation, a technology in which the entire encapsulated material is fully retained, trapped, and/or covered by a matrix (OLIVEIRA et al., 2021; VOS et al., 2010). This methodology has been used to deliver active ingredients from inks to highly innovative systems for pharmaceutical and food applications (SHISHIR et al., 2018; VEMMER; PATEL, 2013). Encapsulation has also been used to deliver many products other than microorganisms, such as flavors, volatile oils, plant extracts, and enzymes (HOYOS-LEYVA et al., 2018b; RATHORE et al., 2013; SHISHIR et al., 2018; ZHU, 2017). When a microorganism is encapsulated, the matrix should protect it against biotic or abiotic factors, such as mechanical stresses, the presence of toxic substances, other microorganisms in the environment, and stressful physicochemical conditions. This protection should allow for increased cell survival in these adverse situations that prevail in production, transport, and during the application, increased shelf life of commercial products, besides the controlled and targeted release of microbial cells (JOHN et al., 2011; OLIVEIRA et al., 2021; SABERI-RISEH et al., 2021; WESTMAN et al., 2012).

Thus, when effective, microorganism encapsulation can bring several advantages regarding their application in the most diverse fields and can be done by using different techniques, such as ionotropic gelation, emulsification, casting, extrusion, and spray drying. It is important to point out that there is no consensus on the best technique because the type and characteristics of the microorganism, the carrier material, and the application of the product interfere with this choice (OLIVEIRA et al., 2021). For example, microspheres containing probiotic cells need to be smaller than 100 µm to avoid a gritty sensation when consumed. On the other hand, microspheres containing fermenting microorganisms in bioreactors must be mechanically strong to withstand harsh mechanical and physical conditions. Therefore, the selected method should produce microspheres with the necessary attributes while causing minimal damage to cell integrity and viability, and should be easy to scale up with acceptable processing costs (RATHORE et al., 2013). Therefore, among the issues that should be evaluated for the encapsulation of microorganisms, an important one is the choice of carrier material.

The selected carrier should remain stable in the environment, have mechanical strength to withstand the adverse conditions throughout production, transport, storage, and application, have

good physicochemical characteristics, as well as adequate properties regarding their degradation or dissolution for controlled release into the environment (JOHN et al., 2011; VASSILEV et al., 2020). Among the natural polysaccharides used to encapsulate microorganisms, the following can be mentioned: alginate, carrageenan, agar/agarose, gum, starch, cellulose, pectin, and chitosan (VASSILEV et al., 2020; VEMMER; PATEL, 2013). Starch, a polymer made up of amylose and amylopectin chains (AI; JANE, 2015), has been showing promising results for microbial encapsulation given that it is a natural, abundant, biodegradable, renewable, non-toxic, and inexpensive biopolymer. This material reduces the water content and increases the amount of dry matter in the beads, reducing the drying rate, increasing mechanical resistance and microbial survival, allowing the progressive release of cells, and it can be used as a carbon source for the microorganism (GUO et al., 2021; HOYOS-LEYVA et al., 2018a).

Due to the advantages of encapsulation and the use of starch as a carrier, excellent reviews on this topic can be found in the literature. The synthesis, processing, application methods, potential opportunities for commercial products (GARCIA; GARCIA; FARACO, 2020; GOPINATH et al., 2018; GUO et al., 2021; HOYOS-LEYVA et al., 2018a; QI; TESTER, 2019; RODRIGUES; EMEJE, 2012; ROSTAMABADI; FALSAFI; JAFARI, 2019; VERSINO et al., 2016), as well as evaluation of the mechanical, thermal and barrier properties of starch-based materials (HOYOS-LEYVA et al., 2018a; NIRANJANA PRABHU; PRASHANTHA, 2018; SHAH et al., 2016) have been thoroughly studied. Recently, the antibacterial and antioxidant properties of starch films, their ability to serve as an ultraviolet (UV), water vapor, and oxygen barrier (CUI et al., 2021; LAUER; SMITH, 2020), as well as the fundamental concepts of release control and the role of starch in the encapsulation process (BOOSTANI; JAFARI, 2021; ZHU, 2017) have also been reviewed. However, these reviews mostly summarized advances in starch-based encapsulation for specific areas such as food, biomedical or pharmaceutical. Therefore, a systematic mapping gathering an overview of starch-based microbial encapsulation covering the different requirements of distinctive fields (food, agriculture, pharmaceutical, and environmental) would be highly useful for the scientific community as well as the industrial sector.

Evidence reviews, which are commonly published, can be defined as articles that discuss and elucidate a specific question or subject based on research articles that use scientific methods to answer a question or hypothesis through data collection (GONÇALVES et al., 2021). On the other hand, systematic mappings are designed to give an overview of a specific question or topic of interest (JAMES; RANDALL; HADDAWAY, 2016; PETERSEN; VAKKALANKA; KUZNIARZ, 2015), and its output is a complete narrative report of the literature that identifies areas with sufficient or saturated studies, as well as gaps in the literature (BERGER-TAL et al., 2019). The latter is very

important for the possibility of developing innovative works with fewer biases commonly found in traditional literature reviews (such as selection, publication, and detection biases). They are considered efficient, reliable, transparent, unbiased, objective, and have all the procedures planned and documented, allowing for their reproducibility (MULROW, 1994; TRANFIELD; DENYER; SMART, 2003). These advantages are due to the fact that these systematic mappings have a series of well-established, rigorous, and standardized guidelines and methods to be followed (BERGER-TAL et al., 2019; CEE, 2013; HIGGINS et al., 2021).

Considering the importance of expanding the use of beneficial microorganisms in different sectors, the need, and advantages of encapsulating them, the good characteristics of starch and their potential to act as a carrier or part of the matrix in encapsulation procedures, a systematic mapping on these topics was proposed here. The following was addressed: 1) trends in publications over the years (1990–2022), main fields of application and encapsulated microorganisms, 2) the techniques used for the encapsulation of microorganisms involving starch in the matrix, the materials used with starch, and the functions they can have in the encapsulation system, and 3) the roles and properties of starch, such as its capacity to help microorganisms survival during and after the encapsulation procedure, the specific roles by which starch can assist this survival, the better performance of microorganisms encapsulated in this kind of matrix towards adverse conditions, and the influence of starch in the final encapsulated products.

2.3. Methodology

The first step of this systematic mapping was to develop a protocol in which were defined the topics and questions of interest of the study, keywords, strings, and databases used to search for the primary studies in the available literature, the inclusion and exclusion criteria to select these studies, and how to extract and analyze the information from the selected articles. Overall, it was a step-by-step review of the systematic mapping to be followed. Throughout the steps, all the work was documented in spreadsheets. The methodology followed was based on guidelines (CEE, 2013; HIGGINS et al., 2021; PULLIN; STEWART, 2006) already described, and shown in Figure 2.1.

For the construction of the string (combination of keywords using Booleans), an exploratory analysis was performed, in which 32 articles were selected based on previous knowledge and research about the topic of interest in this systematic mapping (Table 2.1S). The string was submitted to the database machines (Web of Science, Scopus, and Compendex- Engineering Village), and returned the results shown in Figure 2.2. After excluding the duplicates, 2,496 articles were organized in a spreadsheet and their abstracts were analyzed according to the inclusion and exclusion criteria previously defined in the protocol. The excluded and included articles were organized in spreadsheets (Table 2.2S and Table 2.3S). Finally, 204 articles were selected that met the inclusion criteria to carry

out the data extraction stage, which was also organized in a spreadsheet (Table 2.4S). In addition to the basic information (title, authors, publication year, and abstract), information was extracted such as the encapsulation technique, materials used in the procedure, type and role of the starch, the microorganisms that were encapsulated, and the field for application of the product, as also defined previously in the protocol. The data analysis was made by constructing graphics and figures using statistical software R and network maps drawn by VOSviewer software.

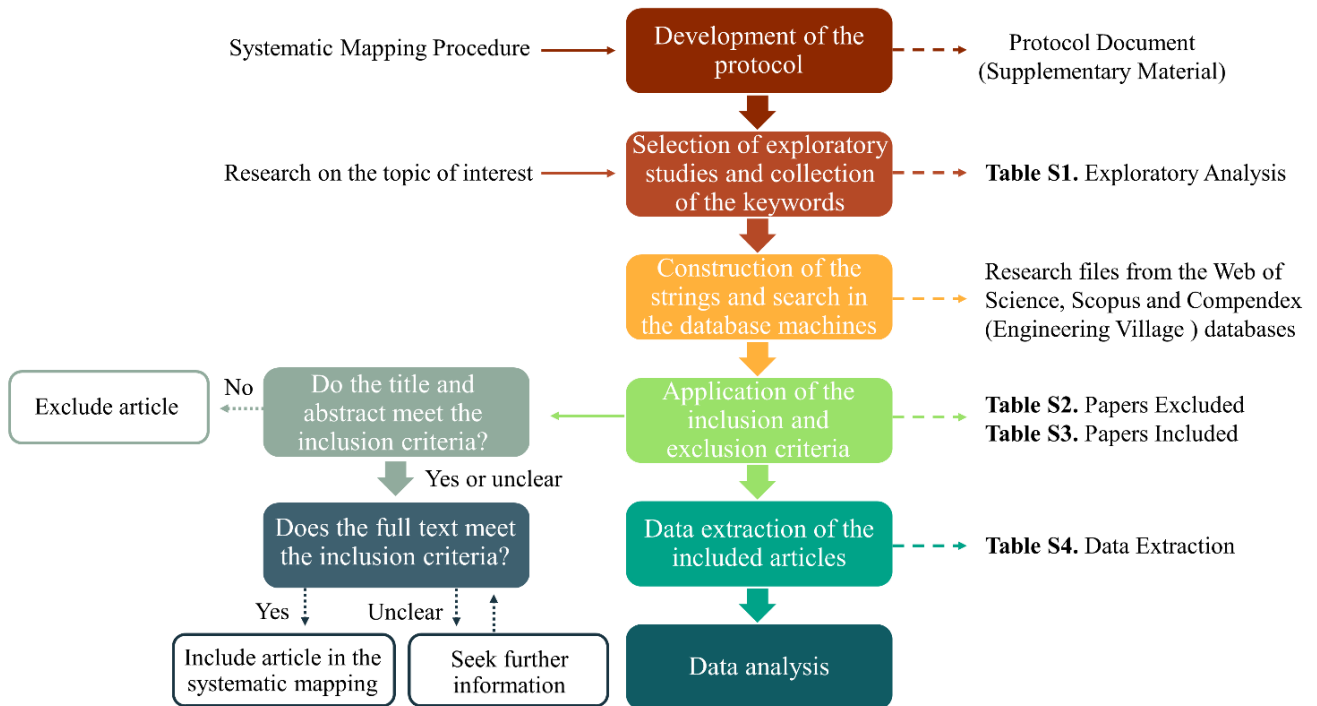


Figure 2.1. Flow diagram of the methodology used to prepare the systematic mapping.

STRING:
 (("formul*" OR "bioformul*" OR "entrapment*" OR "immobil*" OR "bioimmobil*" OR "matrix" OR "film*" OR "coat*" OR "composit*" OR "nanocomposit*" OR "dispers*" OR "prill" OR "emulsion*" OR "coacervat*" OR "hydrogel*" OR "encapsul*" OR "macroencapsul*" OR "microencapsul*" OR "nanoencapsul*" OR "capsul*" OR "microcapsul*" OR "nanocapsul*" OR "macrocapsul*" OR "bead*" OR "microbead*" OR "macrobead*" OR "sphere*" OR "microsphere*" OR "nanosphere*" OR "macrosphere*" OR "particle*" OR "nanoparticle*" OR "microparticle*" OR "macroparticle*" OR "granul*" OR "pellet*" OR "spray")) AND TOPIC: (("release" OR "plant growth" OR "carrier*" OR "delivery" OR "fixation" OR "solubiliz*" OR "agricult*" OR "inocul*" OR "bioinocul*" OR "biofertil*" OR "biostimul*" OR "fertili*" OR "plant nutrition" OR "nutrient uptake" OR "biocontrol" OR "bioinsecticide" OR "bioherbicide" OR "biofungicide" OR "biobactericide" OR "viability")) AND TOPIC: (("microb*" OR "microorganism*" OR "bacteri*" OR "rhizobacteri*" OR "PGPR" OR "PGPB" OR "plant growth-promoting bacteria" OR "plant growth-promoting rhizobacteria" OR "fung*" OR "probiotic*")) AND TOPIC: (("starch*"))

DATABASES:

- Web of Science (1,378 results)
- Scopus (1,602 results)
- Compendex - Engineering Village (353 results)

837 duplicates

2,496 articles to analyze
the inclusion and
exclusion criteria

**FINAL: 204 ARTICLES
FOR DATA EXTRACTION**

Inclusion criteria:

- (a) Studies will be included in which the microorganism is inside a matrix.
- (b) Studies will be included that use the starch in the procedure of encapsulation of microorganisms.
- (c) Studies will be included that describe the methodology used for the encapsulation of microorganisms.

Exclusion criteria:

- (a) Studies will be excluded that do not address the encapsulation of microorganisms in a matrix.
- (b) Studies will be excluded that do not use starch in the procedure of encapsulation of microorganisms.
- (c) Studies will be excluded that do not describe the methodology used for the encapsulation of microorganisms.
- (d) Studies will be excluded that are not in the form of research articles (such as reviews, posters, dissertations and thesis).
- (e) Papers and articles will be excluded that are not found in a language other than English.

Figure 2.2. Flow diagram of the search and selection of studies.

2.4. Results and Discussion

2.4.1. Publication trends over the years, main fields of application, and encapsulated microorganisms

To assess the publication trends on the topic of ‘encapsulation of microorganisms in a starch matrix for applications in different areas’, the number of studies over the years for each field is shown in Figure 2.3. This figure shows the publication trend on this topic from 1990 to 2022. It indicates that the number of articles began to increase in the last eight years and has shown an acceleration trend, which is a good indicator of the field's relevance. It can also be deduced that this is a subject of great interest to the scientific community.

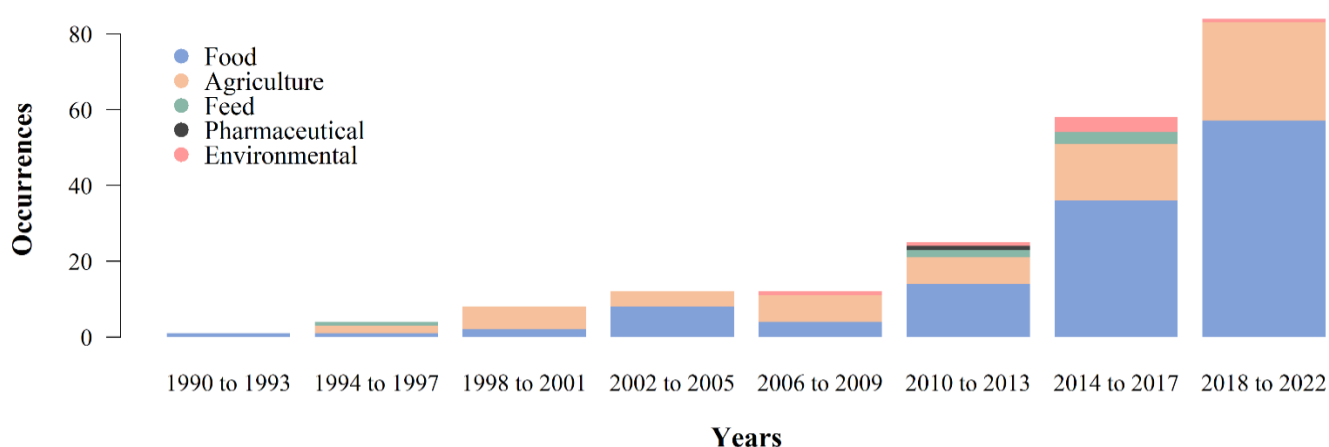


Figure 2.3. Articles published over the years presenting data on the encapsulation of microorganisms in a matrix containing starch. The 204 articles presented are limited to those included in this systematic mapping, considered relevant to our full-text-level review question. The bars indicate the number of articles published over the years in the (●) food, (●) agriculture, (●) feed, (●) pharmaceutical and (●) environmental fields.

The main areas of interest are food and agriculture, which together represent 93% of articles related to this study. In the food sector, encapsulation using starch matrix has been explored mainly for the delivery of probiotics and in fermentation food, and the most present microorganism genera were *Lactobacillus* and *Bifidobacterium* as probiotics and *Saccharomyces* in fermented food (Figure 2.4), which is compatible with previous literature (FAO; WORLD HEALTH ORGANIZATION: FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS; FAO, 2006; PAPIZADEH et al., 2017). The term probiotic is a word that means “for life” and it is currently used to name bacteria capable of bringing health benefits to the host (FAO; WORLD HEALTH ORGANIZATION: FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS; FAO, 2006). For this field, the performances of microorganisms encapsulated in starch matrices were evaluated when used in olive paste (ALVES et al., 2015), chocolate milk

(GHASEMNEZHAD et al., 2017), cream-filled cake (ALI et al., 2012), ice cream (ABDOLHOSSEINZADEH et al., 2018; KATARIA; ACHI; HALAMI, 2018; ZAEIM et al., 2020; ZANJANI et al., 2018a), fruit juice (RENGADU; GERRANO; MELLEM, 2021; YING et al., 2012), chocolate (HOSSAIN et al., 2021), and yogurt (DONTHIDI; TESTER; AIDOO, 2010; KAILASAPATHY, 2006; MURÚA-PAGOLA et al., 2021; SAMEDI; CHARLES, 2019; WATTANANAPAKASEM; COSTABILE; SUWANNAPORN, 2018; ZIAR; GÉRARD; RIAZI, 2012).

On the other hand, the agricultural sector is subdivided into two large groups: Plant Growth-Promoting Microorganisms (PGPM) and Biological Control Agents (BCA). The main representatives of the PGPM group are *Pseudomonas* sp., *Azospirillum* sp., and *Bacillus* sp. The BCA group includes bacteria of the *Bacillus* genus and yeasts of *Saccharomyces* genus (Figure 2.4). PGPM can stimulate the growth and development of plants at different stages of their life cycle, directly or indirectly, through a symbiotic association, in which the plant provides protection and nutrients for these microorganisms. Among the direct mechanisms used by PGPM is the production of phytohormones and enzymes, as well as the availability of nutrients, while the indirect ones used by BCA comprise the biological control of phytopathogens and induced systemic resistance (BHARDWAJ et al., 2014; GLICK, 2015; SANTOS; NOGUEIRA; HUNGRIA, 2019; TAKEI et al., 2018). In this area of application, the release profiles of the encapsulated microorganism in the soil were evaluated to develop formulations for biofertilizers (WU et al., 2012). In addition, cultivation studies were carried out in a greenhouse and in the field to obtain a bioproduct for pest management (VIMALA DEVI et al., 2021).

Other areas also explored for the application of encapsulated microorganisms using starch matrix are pharmaceuticals (BEHERA et al., 2014; HEINEMANN; CARVALHO; FAVARO-TRINDADE, 2013; LAVARI et al., 2017), environmental solutions such as effluent treatment and bioremediation (HAMEED; ISMAIL, 2021; KARABIKA et al., 2009; KARAMALIDIS et al., 2010; LAKSHMI et al., 2017; NUSSINOVITCH et al., 2017; SAFITRI; PRIADIE; INDAH PERMATASARI PERMATASARI, 2015), and animal feed (CIANNAMEA et al., 2013; MA et al., 2015) (Figure 2.4).

The technology for encapsulating live microorganisms has evolved from the immobilized cell technology used in the biotechnology industry. Unlike the encapsulation of vitamins, essential oils, medicines and pigments, in the encapsulation of microorganisms, the process conditions that can affect the viability of the microorganism are highly important (BURGAIN et al., 2011). In addition to protecting cells against adverse environments and increasing the shelf life, the encapsulation of microorganisms using starch may allow the release of the microorganism in a viable and

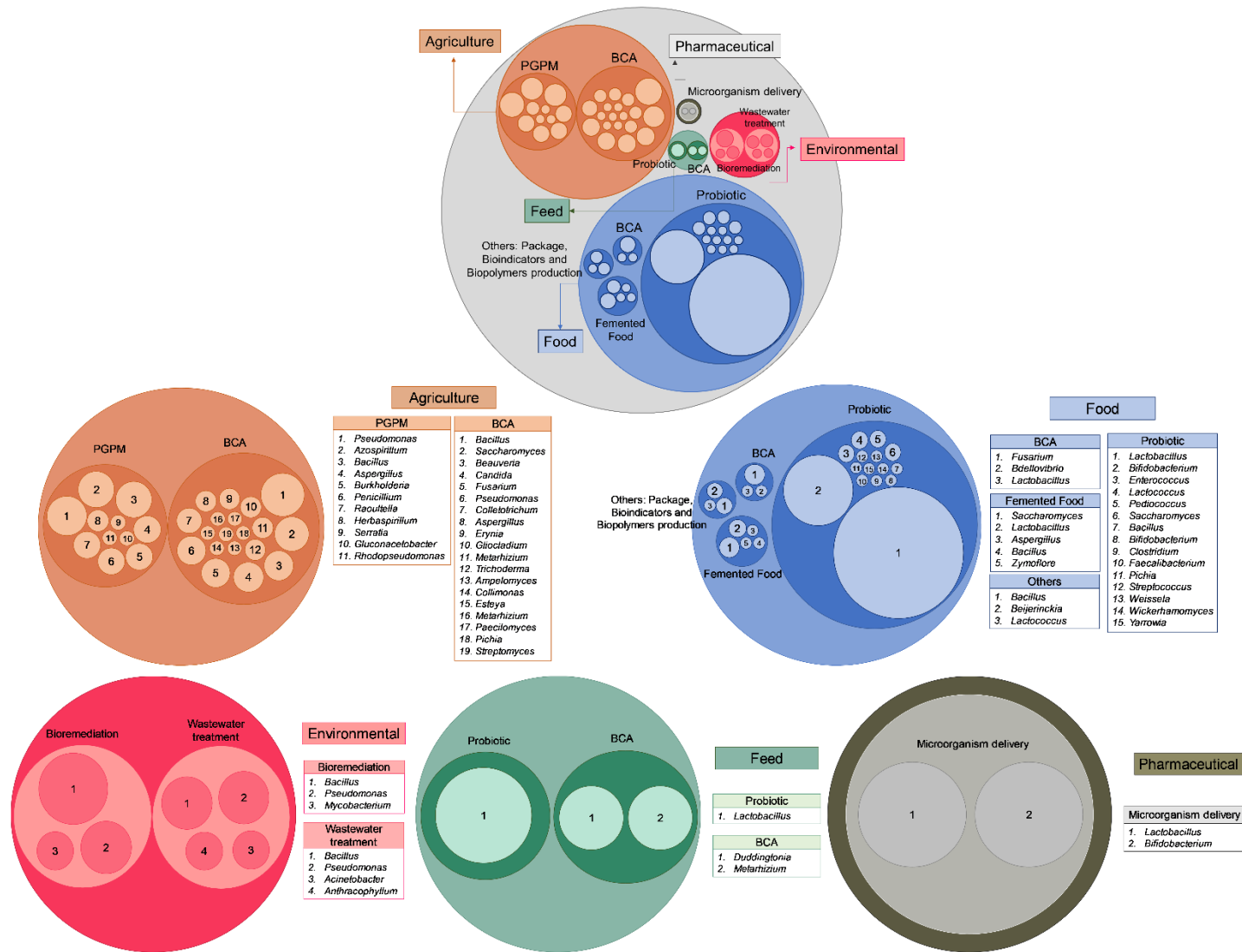


Figure 2.4. Groups of microorganisms encapsulated in starch matrix according to each field of application: (●) food, (●) agriculture, (●) feed, (●) pharmaceutical and (●) environment, and the respective microorganism's genus found in each sub-area. The larger the size of the circle, the greater the number of articles published on that subject. PGPM: Plant Growth-Promoting Microorganisms; BCA: Biological Control Agents.

metabolically active state at the site of interest. An important issue to be taken into consideration to achieve these matters is the encapsulation technique that will be used, as discussed in the next topic.

2.4.2. Techniques and use of materials with starch for microbial encapsulation

Figure 2.5. A shows the network of studies that describe microorganisms encapsulated using starch through some of these techniques: ionotropic gelation, emulsification, spray drying, freeze-drying, fluidized bed drying, casting, extrusion, dispersion, or a combination of them. Figure 2.5. B shows the materials used with starch in the encapsulation procedures. In this topic, the theory involving the encapsulation techniques and the use of these other materials will be addressed. Table 2.5S clusters the encapsulating matrices used for each encapsulation technique.

2.4.2.1. Ionotropic gelation

The ionotropic gelation technique can be described as a physicochemical process of making beads with hydrocolloids (JOHN et al., 2011). In this technique, a polymeric solution is first mixed with the microbial cells and then dripped through an orifice as droplets into a hardening solution. When these droplets reach the hardening solution, the beads are formed by the contact with a cross-linking agent (usually Ca^{2+} ions). The size and shape of the beads can vary depending on the concentration, temperature, viscosity and flow rate of the polymeric solution containing the cells, the distance of free-fall into the hardening solution, and the size of the orifice of dripping (JOHN et al., 2011; RATHORE et al., 2013).

The advantages of this technique are its relative simplicity and low cost, the mild conditions used in the process without toxic solvents, enabling high viability of the organism during the encapsulation procedure. Some disadvantages are the difficulty of producing beads smaller than 500 μm , the necessity of using low to moderate viscosity hydrocolloid solutions, the slow formation of beads, and scaling-up difficulties (OLIVEIRA et al., 2021; SABERI-RISEH et al., 2021). Furthermore, the products usually need to undergo a drying step (OLIVEIRA et al., 2021; SABERI-RISEH et al., 2021).

Figure 2.5. B. shows a vast number of studies involving the combination of alginate and starch for microbial encapsulation. Especially for the ionotropic gelation technique, this matrix was the most used among the articles found in this systematic mapping. To improve mechanical properties, for example, such a matrix can be used by adding clays such as bentonite and kaolin, and zeolite. Sugars and other protective agents such as maltodextrin, whey, and sucrose (Table 2.5S) can be added to

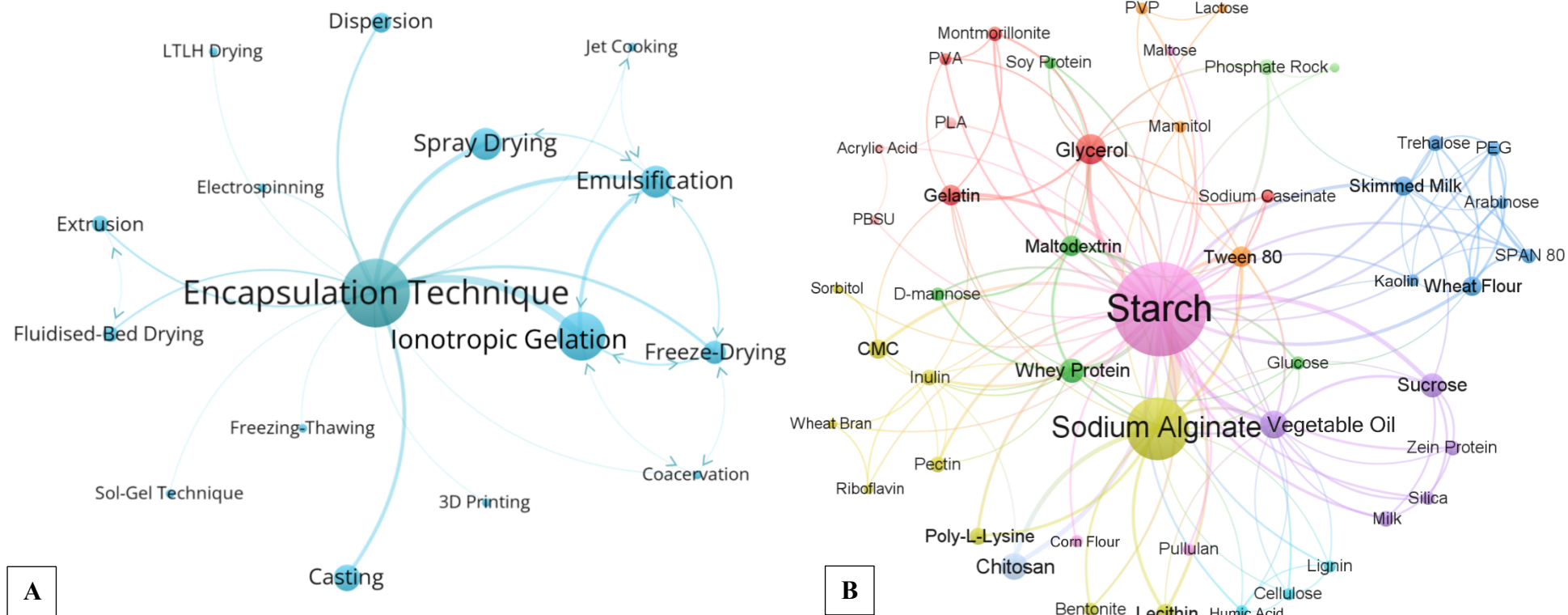


Figure 2.5. Network analysis of A) microorganism encapsulation techniques using starch in the matrix composition addressed by our dataset, and B) different materials used in the composition of the encapsulation matrix that appears in at least 2 articles covered by our dataset. The node size is proportional to the number of occurrences, arrows and edge thickness represents co-occurrences between encapsulation techniques (A) or materials (B). Color groups represent the association strength between variables calculated by the software itself (method: association strength; attraction: 6; repulsion: 0). LTLH drying: Low-temperature, low-relative humidity drying, and edge thickness represents co-occurrences between the materials; PVP: Polyvinylpyrrolidone; PEG: Polyethylene glycol; PLA: Polylactic acid; CMC: Carboxymethyl Cellulose; PBSU: Polybutylene succinate; PVA: Polyvinyl alcohol.

protect the cells as they are known as drying protectants (COGHETTO; BRINQUES; AYUB, 2016; MORGAN et al., 2006; VEMMER; PATEL, 2013). Therefore, they can help microorganisms survive at this stage of the procedure, which can be performed in laboratory ovens or through more sophisticated techniques such as freeze-drying (ARAÚJO et al., 2016; ARAÚJO ETCHEPARE et al., 2016; DONTIDI; TESTER; AIDOO, 2010; EDWARDS et al., 2020; NUSSINOVITCH et al., 2017). Furthermore, some materials can be used as a source of nutrients by encapsulated microorganisms, helping their survival (COGHETTO; BRINQUES; AYUB, 2016; MORGAN et al., 2006; VEMMER; PATEL, 2013).

Similar to the alginate, pectin can form beads via crosslinking with the Ca^{2+} ion (DAFE et al., 2017), and chitosan can form beads by the dripping method via crosslinking both with tripolyphosphate (PEREZ et al., 2018) and with phytic acid (KIM et al., 2017). In these studies, starch was used to improve the properties of the beads for microbial encapsulation. Therefore, starch can play an important role in the matrices and encapsulation procedures involving ionotropic gelation despite the lack of a direct interaction with the ions Ca^{2+} .

2.4.2.2. Emulsification

The emulsification technique consists of dispersing the microbial cells into a polymeric aqueous suspension (dispersed phase) and adding them to an oil or organic solvent (continuous phase). These two phases are homogenized to form a water-in-oil emulsion using a surfactant and/or stirring. If the dispersed phase is the oil or an organic solvent, it is classified as an oil-in-water emulsion, and if the dispersed and continuous phases are aqueous solutions, the emulsion can be classified as a water-in-water emulsion (COGHETTO; BRINQUES; AYUB, 2016; LI et al., 2018; RATHORE et al., 2013). After the homogenization of the phases, the beads or microspheres can already be formed, or, as most often, it is necessary to break the emulsion by cooling or by adding a crosslinking agent such as the CaCl_2 , which solidifies the droplets via ionotropic gelation with Ca^{2+} ions (RATHORE et al., 2013; VEMMER; PATEL, 2013).

The size of the microspheres in this method is determined by the stirring speed, the phases ratio, and the addition of the crosslinking solution. The concentration of the surfactant used also affects the droplet size of the dispersed phase, which eventually affects the final size of the product. This technique usually results in microspheres with a wide size distribution that are harvested from the emulsion by filtration or centrifugation (RATHORE et al., 2013; SABERI-RISEH et al., 2021). Some advantages of this method are its versatility, and the capacity of producing macro, micro, and even nanoparticles size, besides keeping cells viability on a larger scale. A disadvantage is the potential toxicity of the use of organic solvents, surfactants, and other adjuvants that may remain in

the polymer or be difficult to remove (OLIVEIRA et al., 2021; RATHORE et al., 2013; SABERI-RISEH et al., 2021).

Similar to the ionotropic gelation technique, the matrix of alginate and starch is commonly used for emulsification (Table 2.5S). THANGRONGTHONG et al., (2020) used the calcium lactate as a crosslinking agent instead of the CaCl_2 , whereas MARTIN et al., (2013) used acetic acid in the emulsification. The alginate and starch matrix can be used by adding glucose, mannitol, glycerol inulin, and gelatin (Table 2.5S). RADDATZ et al., (2020) and CARINE et al., (2020) used pectin and starch as the matrix for the emulsification, followed by the break of the emulsion with CaCl_2 , and HEIDEBACH; FÖRST; KULOZIK, (2010) studied the emulsification with starch as matrix followed by enzymatic gelation, with casein as a crosslinking agent.

As a final step, the beads from the emulsification procedure can be dried by spray drying and freeze-drying techniques (BULL; KUMAR; ANN, 2016; CRITTENDEN et al., 2006; YING et al., 2011, 2010). Therefore, as discussed, both ionotropic gelation and emulsification procedures are usually followed by a drying step (GUO et al., 2021) which will be addressed in the following topics.

2.4.2.3. Spray drying

In the spray drying method, living cells are dispersed in a carrier material (usually a polymer) forming a dispersion or emulsion, which is atomized into a hot drying air followed by rapid evaporation of the water or solvent. Then, the formed product is separated as a dry powder from the conveying air in a cyclone (RATHORE et al., 2013; SCHOEBITZ; LÓPEZ; ROLDÁN, 2013; VEMMER; PATEL, 2013). The main advantage of this technique is the combination of particle formation and drying in one step (VEMMER; PATEL, 2013). Other advantages are the low cost, fast processing, high productivity, easy scale-up, and simple continuous processing operation. Some disadvantages are the high temperatures that may harm the survival of microorganisms and the fast-drying rates that normally do not allow for the encapsulation of drying-sensitive living cells. Furthermore, the range of polymers that can be used as carriers is limited (OLIVEIRA et al., 2021; RATHORE et al., 2013; VEMMER; PATEL, 2013).

The appropriate carriers should be carbohydrates that undergo a glass transition when the dispersion or emulsion is rapidly evaporated in the process (VOS et al., 2010). Starch, for example, is one polymer that is extensively used for encapsulation by spray drying as it tends to form spherical particles when dried (SABERI-RISEH et al., 2021; VOS et al., 2010). However, the production of these starch spherical aggregates may require the addition of binding agents to enhance the encapsulation efficiency and microcapsule stability, such as other polysaccharides and proteins (BEIRÃO-DA-COSTA et al., 2011; HOYOS-LEYVA et al., 2018a). These materials may improve

the formation of the product and the performance of the encapsulation via physical and chemical mechanisms, such as adhesion and hydrogen bonds, respectively (HOYOS-LEYVA et al., 2018b).

Furthermore, studies show that other additives may be required for the protection of microorganisms upon drying since in these processes they undergo conditions of stress that decrease their survival. A good solution for this issue is the use of protective agents, which comprise chemically diverse substances such as sugars, polymers, and amino acids. Some examples are sucrose, skim milk, maltodextrin, and cysteine (BERNINGER et al., 2018). Regarding sugars, it was reported that trehalose and sucrose, for example, can increase desiccation tolerance in several microorganisms. These materials are capable of stabilizing membranes and proteins by replacing water around polar residues with the formation of hydrogen bonds. This preserves the structure and function of some important proteins during water-free conditions such as drying (MORGAN et al., 2006). Given that starch is considered a good encapsulating agent to be applied in the spray drying technique, several works used it as a single carrier material or combination of materials (Table 2.5S).

2.4.2.4. Freeze-drying

In the freeze-drying (or lyophilization) technique, cells are frozen with a carrier and then dried by sublimation of the solvent, normally water, under a high vacuum (COGHETTO; BRINQUES; AYUB, 2016). In the freezing stage, porous dried product structures are formed as a consequence of the creation of ice crystals from the present residual water, which subsequently sublime during the drying stage under reduced pressure (DOLLY et al., 2011).

The freeze-drying technique forms a particle and dries it in one step, such as spray drying. However, freeze-drying is relatively more effective than spray drying in overcoming the limitations associated with the high temperature, enabling better survival of microorganisms (RATHORE et al., 2013). Some disadvantages of the procedure involve high capital and operating costs due to the low temperatures, high vacuum, and long residence times required (DOLLY et al., 2011). In this process, the cells may be damaged because of the crystal formation and the stress condition of high osmolarity (COGHETTO; BRINQUES; AYUB, 2016).

Therefore, as in the spray drying technique, several cell protectants (such as sugars, polyols, and macromolecules) can be used to assist in the viability of the cells during the dehydration step of the freeze-drying procedure (Table 2.5S). Cryoprotectants can act by forming hydrogen bonds with the polar groups of the phospholipid membrane, reducing the amount of water bound on the surface and maintaining the stability of the membrane. Likewise, they can absorb part of the residual water, increasing viscosity and reducing water efflux and osmotic stress. Also, cryoprotectants can accumulate in cells and decrease the osmotic difference between the internal and external

environments, thus enabling the better survival of the microorganisms (COGHETTO; BRINQUES; AYUB, 2016; ROCKINGER; FUNK; WINTER, 2021). Starch was used as a single encapsulating agent, and once more the matrix of starch and alginate was used, alone or with the addition of other materials (Table 2.5S).

2.4.2.5. Fluidized-bed drying

The fluidized-bed drying is a technique to dry granulated solids (MORGAN et al., 2006). It consists of a bed of solid particles with a stream of air or gas blowing through mechanical shaking so high that puts the particles upward, creating a fluidized effect in the solid products. Thus, they are freely suspended in the air stream and dehydrated by the rapid exchange of heat and mass with air (MORGAN et al., 2006; SANTIVARANGKNA; KULOZIK; FOERST, 2007). To this method, a spray can be incorporated, and it can coat the products (MORGAN et al., 2006).

The fluidized-bed process is generally considered less stressful for the cells than the spray-drying (SCHOEBITZ; LÓPEZ; ROLDÁN, 2013). Furthermore, it is a fast process, of low cost, high production, viable for large-scale continuous production, and allows the use of several encapsulating materials. Some disadvantages are the difficulty to operate it, relatively long duration of the process, and formation of irregular particle sizes (OLIVEIRA et al., 2021; SANTIVARANGKNA; KULOZIK; FOERST, 2007). Starch was used as single carrier material and with sucrose and skimmed milk (Table 2.5S).

In general, the drying processes are of industrial interest, because of the possibility of scale-up (SANTIVARANGKNA; KULOZIK; FOERST, 2007). However, as discussed, some adjustments in the parameters and the use of additives may be necessary for better encapsulation and survival of the microorganisms.

2.4.2.6. Casting

The casting technique is used to produce films and involves the preparation of a solution of polymer and cells, followed by heating with excess water. The solution is then spilled out into a casting surface (such as plates) using a film caster with a uniform gap (such as a draw-down bar for plane surfaces) to obtain a film of uniform and controlled thickness. After the spreading in the casting surface, it is taken to be dried under specific conditions of temperature and relative humidity, making the solvent (usually water) evaporates, and enabling the formation of a film through the rearrangement of its macromolecules into a cohesive, free-standing layer (FITCH-VARGAS et al., 2016; OTONI et al., 2017; PARIKH et al., 2015). In this method, both the mixing speed and time are significant parameters to ensure a homogeneous dispersion of the polymeric solution before it is spread on the casting surface. The major problems of the casting technique are the high processing cost and the

long drying times, making it difficult to scale up (MADHUMITHA et al., 2018; OCHOA-YEPES et al., 2019; OTONI et al., 2017).

Starch is a material with great potential to be used to produce films and encapsulate microorganisms using the casting technique. However, some properties need to be adjusted according to the intended application, such as the poor mechanical and thermal characteristics, and barrier properties (related to the hydrophilic nature of the material) (NIRANJANA PRABHU; PRASHANTHA, 2018; WU; ZHANG, 2001). Thus, to overcome such unsatisfactory properties, starch can be blended with other polymers. Some examples are the use of starch in combination with nonbiodegradable polymers such as low-density polyethylene (LDPE), and with biodegradable polymers, such as poly (lactic acid) (PLA), poly (vinyl alcohol) (PVA), and carboxymethylcellulose (CMC) (KALAMBUR; RIZVI, 2006; NAFCHI et al., 2013; WU; ZHANG, 2001). In addition, various proteins used along with starch blends have resulted in films that have resistance to water vapor permeability and improved tensile strength (NIRANJANA PRABHU; PRASHANTHA, 2018). Generally, to be considered a blend, the mixture of starch with another polymer should form a single phase, whereas the mixture of starch with non-reactive polymers or other materials (fillers) consisting of two or more different phases, separated by a distinct interface, is usually referred to as composites (SABU et al., 2012). Among the different fillers that can be used, clays (such as kaolin and montmorillonite) are considered the most abundant, environmentally friendly, and low-cost ones. They can strongly improve the mechanical and thermal properties of starch films, for example (MADHUMITHA et al., 2018). Furthermore, it was observed that these fillers can reduce the water uptake by improving barrier properties and decrease the biodegradability of starch materials according to their addition (EINOLLAHI et al., 2021; NIRANJANA PRABHU; PRASHANTHA, 2018).

In addition to polymers and fillers, there is a variety of plasticizers, such as water and glycerol (the most used with starch), as well as urea, maltodextrin, and phosphate sorbates that have been used to reduce water absorption and increase the flexibility and applicability of starch materials. Furthermore, the plasticizers are important to be applied in starch systems to reduce their brittleness during storage caused by the retrogradation phenomena, in which the mechanical properties of the starch structure change due the recrystallization process (NAFCHI et al., 2013; NIRANJANA PRABHU; PRASHANTHA, 2018). Unmodified starch was used as a single material in the casting method and with other materials (Table 2.5S).

Another strategy to improve the properties of starch is the modification of starch (NIRANJANA PRABHU; PRASHANTHA, 2018). Several modification methods (physical, chemical, and enzymatic) have been used to improve the functional characteristics of native starches,

and consequently their film-forming ability and the characteristics of the final product (GUO et al., 2021; HAQ et al., 2019). For example, DONTIDI; TESTER; AIDOO, (2010) related different sources of gelatinized starch with the viability patterns of *L. casei* in the encapsulation; IVANOVA; TEUNOU; PONCELET, (2006) used octenyl succinate starch as an emulsifier to produce beads; ALTERIIS; PARASCANDOLA; SCARDI, (1990) used oxidized starch as a cross-linker to form a gelatin film; LI et al., (2016) showed that formulations using hydrolyzed starch showed significantly larger initial viable cells compared to native starch after freeze-drying, and CIANNAMEA et al., (2013) produced dialdehyde starch as a cross-linking agent.

2.4.2.7. Extrusion

Extrusion is a process used to mix and disperse the cells and the wall materials forming highly viscous matrices. Under thermal and mechanical input, energy pressure, moisture, and shear, this dispersion undergoes many order-disorder transitions passing inside the extruder forming a mass, and when leaves, it is shaped and solidified into the shape undercooling and evaporation of water (EMIN et al., 2012; LAI; KOKINI, 1991; SCHOEBITZ; LÓPEZ; ROLDÁN, 2013). An advantage of this process is the good shelf life and stability it can provide to the encapsulated cells since the atmosphere gasses diffuse very slowly through the matrix making it an impermeable barrier against oxygen. However, this barrier may be ineffective due to structural defects such as cracks, thin walls, or pores formed in the product during or after the procedure (EMIN et al., 2012; GOUIN, 2004; SCHOEBITZ; CEBALLOS; CIAMPI, 2013). Other drawbacks of this technology are the formation of large particles (typically 500 – 1000 µm), the high processing temperature often used that makes necessary an adjustment of parameters to enable the survival of microorganisms, and the materials used for the extrusion that are limited to carbohydrates and mixture of additives (GOUIN, 2004).

Among the materials that can be used, carbohydrates of various dextrose equivalents are important encapsulating agents for applications in the extrusion process. Starch is one of them, especially for its well-known gelatinization phenomena in the formation of thermoplastic starch (GOUIN, 2004; LAI; KOKINI, 1991; WONGPHAN; HARNKARNSUJARIT, 2021). However, pure starch materials can be unsuitable for the extrusion process because of limitations, such as the poor mechanical properties (essential during the handling of the materials) and barrier properties due to the water sensitivity of hydrophilic starch (KALAMBUR; RIZVI, 2006; YU et al., 2013). The strategies to improve such properties are similar to those already discussed for the casting technique and involve the use of polymeric blends, composites, plasticizers (forming thermoplastic starch), and modifications of starch (NIRANJANA PRABHU; PRASHANTHA, 2018). Among the properties that these strategies can improve, an important one for the extrusion technique is that they can

facilitate the flow behavior of starch during the process in the extruder (YU et al., 2013). Starch was used alone in extrusion procedures and with other materials as well (Table 2.5S).

2.4.2.8. Dispersion

The reports that applied the transitions of the starch (its gelatinization) as a principle to encapsulate the microorganisms but did not use extruder equipment were classified here as dispersions. Starch gelatinization is normally achieved by heating the starch with water (or other plasticizers) as a first step, and by cooling and shaping it as a final step, forming a gel. The formation of this gel is a result of interactions between the amylose and amylopectin molecules upon the cooling, which forms networks to hold water and other compounds such as the microorganisms, opening possibilities for their encapsulation (AI; JANE, 2015; LAI; KOKINI, 1991). The dispersions can be shaped with sieves (JACKSON; SHASHA; SCHISLER, 1996; ROSAS-GARCÍA, 2006) and in mill (KLAIC et al., 2018) for example, to obtain the most suitable shape, such as in the form of granules and powders (RIDDECH; PHIBUNWATTHANAWONG; SARIN, 2021).

Starch networks can physically interact with microbial cells, retaining and encapsulating them. Chemically, these networks can interact with the cells through hydrogen bonds, van der Waals forces, and electrostatic forces (HOYOS-LEYVA et al., 2018b). In the case of *Bifidobacterium* strains, a study showed that its adhesion to starch could be attributed to interactions with their cellular or extracellular proteins (CRITTENDEN et al., 2001). Dispersion has been applied using starch as a single material and combined with other materials (Table 2.5S).

2.4.2.9. Other encapsulation techniques

Electrospinning, freeze-thawing, coacervation, sol-gel and 3D printing techniques were also used to encapsulate microorganisms into starch matrices (Table 2.5S).

2.4.3. Roles of starch in the encapsulation matrix

Commercially available starches (for example, corn, potato, wheat, rice, and cassava) are the most used as wall material for the encapsulation of microorganisms, with corn starch used in 48.3% of the studies evaluated here. Other unconventional starch sources (tapioca, taro, chestnut, sago, durum, banana, amaranth, etc.) have also been evaluated for microorganism encapsulation (Fig. 2.6). Starches from different sources can have different encapsulation efficiencies and cell viability (KANMANI; LIM, 2013). In the group of articles analyzed in this systematic mapping, it was observed that in 19%, the sources of starch used in the encapsulation matrices were not defined.

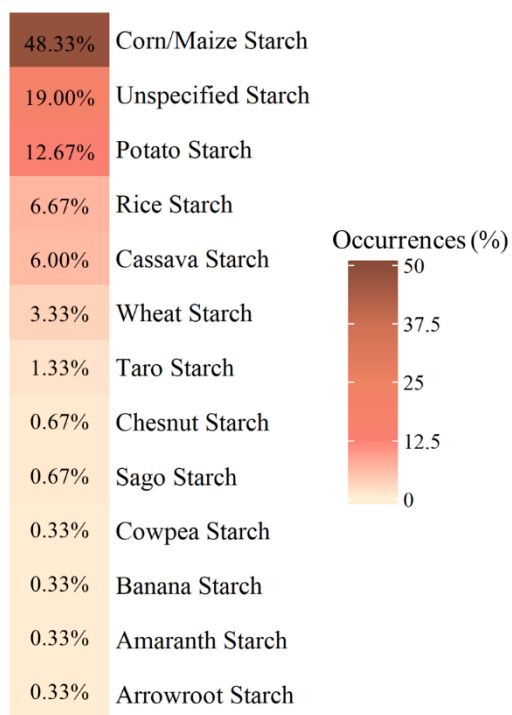


Figure 2.6. Heatmap representing the frequency in which starch sources were used in the articles covered in this systematic mapping.

Table 2.1 summarizes the roles that the starch can play when present in the matrices for the encapsulation of microorganisms. Each role will be approached fully and in more detail in the next topics.

Table 2.1. Roles of starch when present in the matrix for the encapsulation of microorganisms.

Cell survival <i>Cell survival at the encapsulation procedure</i> <i>Cell survival at storage</i>	According to the results of the articles found in this systematic mapping, the presence of starch in the encapsulating matrix has the capacity of assisting the cells to survive in the encapsulation procedure and during the time and conditions of storage of the product.
Specific roles of starch in the cell survival <i>Starch as filler</i> <i>Starch as drying protectant</i> <i>Starch as coating</i> <i>Suitable microenvironment: starch as physical barrier and carbon supplier</i>	Starch can act as filler increasing stability, and decreasing the water content in the product, besides leading to a higher cell adhesion. As a drying protectant, it can help the viability of the microorganisms through these kinds of processes. Furthermore, starch can offer a suitable microenvironment for cells to survive and grow, due to the physical barrier that it can provide, as well as due to the carbon source that is available for microorganisms' consumption.
Performance of microorganisms encapsulated in starch <i>Resistance to gastric conditions</i> <i>Resistance to stressful conditions</i>	The microorganisms encapsulated in starch matrix can show better performance towards some situations, such as the resistance to gastric conditions aiming the application especially in food industry, and resistance to stressful conditions in general such as conditions of low pH, salinity, and high temperatures.
Influence in the characteristics of the product	The presence of starch in the matrix for the encapsulation of microorganisms can influence some characteristics of the product. For example, in its shape, size, mechanical properties, biodegradability and properties of controlled release.

2.4.3.1. Cell survival

Maintaining cell survival during and after the encapsulation procedure is a very important factor regardless of the type of encapsulation technique and intended application, as will be further discussed as follows.

- *Cell survival during the encapsulation procedure*

The cell survival (or viability) during the encapsulation procedure can be expressed in values of colony forming unit (CFU) or encapsulation efficiency percentage. The latter is mostly used and is a term used for calculating the relation between the number of cells offered at the beginning of the encapsulation procedure and the number of encapsulated cells that remains viable in the matrix. In any case, it is usually done by disrupting the structure in which the microorganism was encapsulated and measuring the number of viable cells soon after this disruption, in most cases by the plate counting method. With this systematic mapping, we showed that the presence of the starch usually leads to high and considerable values of cell survival in the encapsulation procedure when compared to formulations without starch. In general, its presence leads to better results due to the capacity of the microorganisms to adhere to their structure and network and be protected.

KATARIA; ACHI; HALAMI, (2018) showed that the addition of 2% of starch to alginate beads produced by emulsification led to a better recovery of the viable *Bifidobacterium* encapsulated. The beads of alginate alone showed values of $14 \cdot 10^3$ CFU g⁻¹, while the beads of alginate and starch were $2 \cdot 10^9$ CFU g⁻¹. DAM et al., (2018) studied formulations involving glycerol and starch (at concentrations of 5 or 10%) produced by ionotropic gelation and coacervation followed by freeze-drying. Among the microcapsules obtained, the one with 10% of starch showed the higher viable bacteria count after the encapsulation, with a value of $1.04 \cdot 10^{10}$ CFU g⁻¹, which was the best result among all formulations, indicating the benefits of the incorporation of the starch. LUCA; OROIAN, (2021), RADDATZ et al., (2020), THANGRONGTHONG et al., (2020), and RENGADU; GERRANO; MELLEM, (2021) showed encapsulation efficiencies among 81.55 and 88.78% for their encapsulating matrices containing starch, obtained through different techniques.

MARTIN et al., (2013) produced microparticles by emulsification and gelation followed by freeze-drying and observed that the encapsulation efficiency was significantly higher for microparticles with starch, with a value of $97.26 \pm 0.33\%$ compared to $74.41 \pm 1.76\%$ for microparticles without starch. ROHMAN et al., (2020) used cassava starch and sodium alginate to produce beads by ionotropic gelation and obtained an encapsulation efficiency of 70.83% for 4% (w/v) starch, while the control beads (without starch) showed a value of 50.56%. Moreover,

BOMMASAMUDRAM; MUTHU; DEVAPPA, (2022) obtained 95.6% encapsulation efficiency using starch by spray drying, which was the best result among the formulations studied.

DAFE et al., (2017) encapsulated a probiotic by the ionotropic gelation technique using pectin alone, and pectin with different concentrations of starch. The percentage of encapsulation efficiency with pectin was $72.2 \pm 1.2\%$, and this value was increased according to the increase of starch concentrations, reaching a value of $94.8 \pm 0.82\%$ using 1.5% of the material. WANG et al., (2020) produced alginate beads with corn starch and noticed that as the proportion of starch increased, the encapsulation efficiency initially increased. The maximum encapsulation efficiency of beads was $93.39 \pm 2.75\%$ in 4% starch concentration, but when the starch concentration reached 8%, the encapsulation efficiency decreased significantly. This may be because, in the CaCl_2 solution, there was an excess of starch that was not encapsulated, resulting in many cells adsorbed on it.

- *Cell survival at the storage*

In addition to maintaining cell survival during the encapsulation procedure, it is essential to ensure that the encapsulated microorganism remains viable under different conditions and times of storage, until its application. As already discussed, the two main fields in which encapsulated microorganisms can be applied are the food industry and agriculture. For these two areas, there are regulations concerning this matter, which are guidelines to be followed to develop and commercialize new products.

For the food industry, it is usually of interest to encapsulate probiotics, which should be viable at the time of consumption at a minimum recommended level of 10^6 CFU ml^{-1} required by the food industry in general, including the US FDA (ETCHEPARE et al., 2015; TRIPATHI; GIRI, 2014). Moreover, it is known that probiotic food products should preferably be stored at a temperature of 4–5 °C (MORTAZAVIAN et al., 2007).

LUCA; OROIAN, (2021) produced microcapsules of sodium alginate and soluble potato starch by emulsification and ionotropic gelation, and obtained very good viability (between $8.30 \pm 0.00 - 9.00 \pm 0.02 \log_{10}$ CFU g^{-1}) during the 30 days of storage at 2–8 °C. NUNES et al., (2018) showed that a formulation using hi-maize starch, maltodextrin, tween, glycerol, and Arabic gum to encapsulate *L. acidophilus* by spray drying, kept the microorganism count above the recommended level for the food industry field ($> 6 \log_{10}$ CFU g^{-1}) at the end of 120 days of storage at room temperature (25 °C). REYES et al., (2018) encapsulated *L. plantarum* in high maize starch and other formulations through the spray drying technique. The cell viability was measured after storage under 97 and 10% vacuum at refrigerated (4°C) and room (23°C) temperatures, for 60 days. The results showed that the *L. plantarum* encapsulated with high maize starch, packed under 97% vacuum, and

stored at refrigerated temperature maintained the highest cell viability during the 60 days of storage, implying that the starch material can act as a protective agent, and leads to higher viabilities at storage.

In the agriculture field, it is desirable to develop biofertilizers formulations of encapsulated microorganisms following the specifics of the guidelines for each country. For example, in Brazil, the MAPA (Ministry of Agriculture, Livestock and Food Supply) requires a minimum of 10^9 viable cells per gram or ml of the product until the validity, which should be at least six months. In China, the regulation depends on the kind of bacteria utilized to produce the biofertilizer, however, the required amounts are also around 10^9 CFU ml or g^{-1} , with at least six months of validity. In India, the Ministry of Agriculture prescribes production and marketing standards about the different kinds of microorganisms utilized to produce the biofertilizer. For bacteria, the minimum count of viable cells is $5 \cdot 10^7$ cells per gram of solid carrier, or 10^8 cells per ml of the liquid carrier. For products containing mycorrhizal fungi, at least 100 viable propagules must be present per gram of the product (MALUSÁ; VASSILEV, 2014).

Therefore, in the agriculture field, it is also important to develop formulations with a good shelf life as studied by MARCELINO et al., (2016). They produced composites by extrusion with cassava starch, sugarcane bagasse, glycerol, rock phosphate, and skim milk as a bacterial carrier, and showed a viability of *A. brasilense* Ab-V5 cells (a PGPM) for up to 4 months with values around 10^6 CFU g^{-1} , at room temperature. GUIMARÃES et al., (2018) encapsulated *A. niger* through extrusion in a matrix of corn starch and rock phosphate or elemental sulfur, aiming to apply its phosphorus solubilization capacity in the agriculture field. It was observed that the starch matrix was effective in keeping the spores viable after 30 days of storage at room temperature. SHAH; AEBI; TUOR, (2000) produced beads by ionotropic gelation with sodium alginate, potato starch, and sucrose. The storage studies showed better viability of the cells in the granules containing starch than the control beads (without starch). PEREZ et al., (2018) produced beads by ionotropic gelation using a matrix of chitosan and starch blend and sodium tripolyphosphate as a cross-linking agent, aiming to encapsulate and co-encapsulate PGPB bacteria. After one year of storage, the viability of *Azospirillum brasilense* Az39 encapsulated separately or together with *Pseudomonas fluorescens* ZME4 in dried beads remained constant (10^9 CFU g^{-1}). These studies show that starch can assist in the survival of the microorganisms, both in the encapsulation procedure and in long-term storage. In the next topics, we further elaborate on the mechanisms by which the starch can improve cell survival.

2.4.3.2. Specific roles of starch in cell survival

- *Starch as a filler*

The use of starch as a matrix filler for microbial encapsulation can slow down the water inflow and water content into the product structure by increasing the dry matter and acting as a bulking agent or blocking pores. This strategy can lead to high cell retention and survival in the matrix, protection to drying, and stability at long-term storage and stressful environmental conditions. This function is mostly explored in beads produced by the technique of ionotropic gelation. Studies show that corn and sago starch can act as fillers by blocking pores in the Ca-alginate network (WIDANINGRUM et al., 2019; ZAEIM et al., 2019). TA et al., (2021) noticed the same mechanism in their work, leading to the reduction of the pores size of the alginate network with the incorporation of starch. It was discussed that it provides better bacteria retention in the stirring step due to the presence of the granules of this carbohydrate within the alginate gel cavities. SUVARNA; DSOUZA; LAKSHMI, (2018), ZANJANI et al., (2018a) and ZANJANI et al., (2018b) also observed the good adhering capacity of the microorganisms in the gelatinized starch used as filler in alginate capsules leading to the improvement of viability of the encapsulated cells.

ROHMAN et al., (2020) reported that the cassava starch as filler in the sodium alginate beads lowered the water content and alleviated the effects of osmotic stress on cell physiology, so that cell viability was stabilized in long-term storage. SCHOEBITZ; CEBALLOS; CIAMPI, (2013b) used potato starch, corn and modified corn starch with the sodium alginate beads to increase the dry matter, resulting in good cell survival during and after the encapsulation procedure. Furthermore, the presence of the starch resulted in better mechanical stability of the beads, and in the longer-term, the survival and establishment of cells in soil conditions was improved. POP et al., (2015) used starch as filler in alginate-based granules, and higher encapsulation efficiency and an enhancement in cells stability in the food product were also noticed remaining with good viability until the consumption. KRELL et al., (2017) and HUMBERT; VEMMER; PATEL, (2018) incorporated corn starch in alginate beads as filler and observed better protection of cells during the drying, helping in the survival of the encapsulated microorganisms.

Given that starch can act as a filler protecting the microorganisms during and after the encapsulation procedures, improving the mechanical properties of the product, and also protecting from drying processes, this material was used in encapsulation procedures such as the spray and freeze-drying as bulking agents (GARCÍA; BARRERA-CORTE; MARTI, 2014; KHAN; DIJL; HARMSSEN, 2014). Furthermore, KANMANI; LIM, (2013) used different types of starches in their films produced by casting, and the water vapor permeability and the water absorption rates decreased

considerably with increasing starch contents. Extrusion with cassava starch was also an encapsulation technique in which the starch was used as a filler (MARCELINO et al., 2016). Finally, more recently, the starch was used as filler in nanofibers for the encapsulation of microorganisms obtained through the electrospinning technique (GHORBANI; MARYAM, 2021). In overall, these studies demonstrate that the use of starch as a filler can improve the properties of the encapsulating matrices, such as survival during processing and storage, protection upon drying, and improvement of mechanical resistance.

- *Starch as a drying protectant*

The presence of starch can protect microorganisms from dehydration during the encapsulation procedure, thus enabling their survival. Such osmotic protection could be related to the action of starch as a structural support when spaces filled with water became voids after drying, added to the property of cells adhesion to starch (SCHOEBITZ; SIMONIN; PONCELET, 2012; VASSILEV et al., 2020). These dehydration or drying processes can occur in the drying step of beads, such as after the ionotropic gelation in laminar air flow (PRZYKLENK et al., 2017) or in the freeze-drying process (DAM et al., 2018; DONTIDI; TESTER; AIDOO, 2010). These studies approached the role of starch as a drying protectant, helping in cell survival throughout these processes. HONGPATTARAKERE; URAIPAN, (2015) also noticed this phenomenon when producing starch films by casting and drying the material in a vacuum oven. Furthermore, encapsulation techniques involving drying methods such as spray drying, freeze-drying, and fluidized-bed drying (APONTE et al., 2016; CRITTENDEN et al., 2006; GOTOR-VILA et al., 2017; LAVARI et al., 2014; MARÍN et al., 2017; REYES et al., 2018; TANG et al., 2020; UMASHANKAR et al., 2018) show the protective effects of starch enabling better cell survival.

- *Starch as a coating*

The starch can be present in the formulation materials also as a coating of the product. ANSARI et al., (2021) produced beads by ionotropic gelation using sodium alginate, resistant corn starch as coating, and a double coating with nanoparticles. It was found that the coatings offered higher protection for the encapsulated cells, leading to an increase in its viability in the whey drink and in the human gastrointestinal tract. LUO et al., (2019) used modified cassava starch as a coating for alginate beads and observed a lower permeability than in the uncoating beads. Furthermore, the coating made the microcapsules more compact and formed a more confined environment which hindered exchanges between bacterial cells and the environment. KHORASANI; SHOJAOSADATI, (2017) produced beads by ionotropic gelation using pectin and bacterial nanocellulose and starch as a coating. It was observed that the coating enhanced the thermal protection, in addition to assisting in

the survivability after drying and simulated gastrointestinal digestion. GHASEMNEZHAD et al., (2017) produced beads also by ionotropic gelation with sodium alginate, and resistant starch as a coating. The effects of the coating were observed mostly regarding the protection of cells during the storage of the product.

- *Suitable microenvironment: starch as a physical barrier and carbon supplier*

Other than the capacity of protecting cells from the drying processes, the presence of the starch offers a good microenvironment for the microorganisms to survive and grow. The starch can protect cells from external stresses by acting as a physical barrier, and acting as a carbon supplier for microorganisms, and is a metabolizable material that can help with their survival.

OYEAGU et al., (2018) noticed an increase over time in the cell density of encapsulated *Bacillus subtilis* and *Saccharomyces cerevisiae* in alginate and starch beads produced by ionotropic gelation. The values were 36.5% and 74% higher, for these two microorganisms, respectively, for beads with starch than the obtained for the control beads. SOTO et al., (2011) produced beads of alginate, and alginate with corn starch by ionotropic gelation. After 9 h and 18 h of incubation, capsules made of alginate with starch had higher counts than the alginate capsules. These works indicated the suitable micro-climate offered by the starch for cells to remain viable, protected, and multiply.

VOBLIKOVA et al., (2019) and ALVAREZ; LEAL, (2018) observed that the starch in combination with alginate has a synergistic effect on the ionotropic gelation process that provides additional protection for the probiotic cells in its network. In addition, OLUSEYI et al., (2020) observed that in the ionotropic gelation procedures, starch forms a strong bond with alginate through electrostatic interactions, which leads to the formation of a membrane on the surface of the granules and forms a good protective barrier for cells. MA et al., (2015) used porous starch in combination with alginate for encapsulation of microorganisms through the technique of emulsification and ionic gelation followed by freeze-drying and noticed that the external surface of the microcapsules showed many micropores, which is fundamental for guaranteeing higher protection, since it can retain and protect the probiotic cells, offering a suitable microenvironment.

During the spray drying, it was also noticed that the hi-maize resistant starch is capable of protecting the encapsulated substances during the procedure (NUNES et al., 2018), as it can isolate the cells from the stresses of the external environment (YING et al., 2012). The same occurs for spray drying with taro and rice starch (ALFARO-GALARZA et al., 2020). Carboxymethyl cellulose and corn starch films produced by casting can act by protecting the microorganisms in their network, as shown by LAN et al., (2021).

This protection and physical barrier that the starch offer for the microorganisms is important to maintain the viability in the encapsulation procedures, to ensure the survival in long term, and towards unfavorable conditions in the environment that the encapsulated microorganism intends to be applied, such as the survival of probiotics in gastric conditions and applications in agriculture for controlled release, for example. These applications will be further discussed in the next topic. Other than the physical barrier, the microorganisms need a source of energy to remain viable, which can be achieved using a carbon supplier material such as starch.

LAN et al., (2021) produced films of carboxymethyl cellulose and corn starch, and discussed that beyond offering physical protection, the encapsulated bacteria can use the active ingredients of the film for obtaining energy. YING et al., (2011) encapsulated *Lactobacillus rhamnosus* CG through the spray drying technique with tapioca starch as matrix and discussed the role of starch as a metabolizable material. On the other hand, KRELL et al., (2017) encapsulated *Metarhizium* through ionotropic gelation using alginate and corn starch, and discussed that the latter served as a carbon source, assisting in the fungal growth. GUIMARÃES et al., (2018) performed an extrusion with corn starch and rock phosphate and elemental sulfur to encapsulate *A. niger* and observed the same role of the starch in providing a carbon source to support microorganism growth. Finally, SILVA et al., (2012) also approached this role of starch when encapsulating bacteria in a dispersion of carboxymethyl cellulose and starch. HUMBERT; VEMMER; PATEL, (2018) reported that it is of great advantage if the matrix materials fulfill several tasks simultaneously. In their work, they produced beads for encapsulation of *Saccharomyces cerevisiae*, and observed that the starch acts as filler and carbon supplier. NUSSINOVITCH et al., (2017) commented that the starch acts as an internal carbon source and a matrix enhancer filler. Likewise, TANTRATIAN; PRADEAMCHAI, (2020) showed that the soluble starch used as a matrix acts as a protective agent and carbon supplier.

2.4.3.3. Performance of microorganisms encapsulated in starch matrices

- *Resistance to gastrointestinal conditions*

Starch is an excellent encapsulating agent to help survival and growth, in addition to its role as a prebiotic, which is generally defined as ‘nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/ or activity of one or a limited number of bacterial species already established in the colon, and thus in effect improve host health’ through the modulation of the intestinal flora (FAO; WORLD HEALTH ORGANIZATION: FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS; FAO, 2006; GIBSON; ROBERFROID, 1995).

In this systematic mapping, we found some studies that discuss and evaluate encapsulated probiotics in starch matrix regarding their resistance and performance in surviving towards certain gastric conditions. ARAÚJO ETCHEPARE et al., (2016) produced microparticles of sodium alginate and resistant corn starch by ionotropic gelation, and the product was exposed to simulated gastrointestinal juice. At the end of the experiment, log reductions of 3.67 and 3.01 were obtained for the control (only alginate) and alginate and starch microparticles, respectively, with significant differences between the treatments. Therefore, it was shown that the addition of starch increased the bacteria resistance to low pH and bile salts in the simulated digestive system, resulting in a higher number of viable cells. TA et al., (2021) produced alginate and resistant starch beads, and other six formulations also by the ionotropic gelation technique. Overall, the most effective protection could be noted for the resistant starch-reinforced beads, for which viable cells could be still detected even after 135 min of gastric and 150 min of intestinal treatment, occasioned due to the better protection offered by the starch against low pH and bile salt conditions. It is worth noting that, the number of these survivor cells was above the recommended minimum level necessary for the achievement of the probiotic effects. Also by ionotropic gelation, DAFE et al., (2017) encapsulated a probiotic in pectin and corn starch beads and evaluated its performance after sequential exposure to simulated gastric and intestinal fluids. After 2 h, almost complete death of free cells was observed, while the numbers of surviving cells were 5.15 and 6.67 Log CFU/g for control beads (without starch) and pectin and corn starch, respectively. HONGPATTARAKERE; URAIPAN, (2015) evaluated the encapsulated *L. plantarum* CIF17AN2 in a matrix of resistant starch from unripe saba banana. The starch film produced by casting was first exposed to simulated gastric juice and gastrointestinal transit, containing pepsin, bile and pancreatin at acid pHs. Without adding starch, loss of viability (72.46% reduction) was much higher than the product with starch, in which 40.83% reduction was observed. Therefore, it can be observed that starch effectively protects the probiotic from gastric acid and bile exposures.

- *Resistance to other stressful conditions*

Assays evaluating the resistance to high temperatures are interesting when aiming for applications in the food area. The control of tolerance of probiotic bacteria to temperature stress holds potential practical benefits especially in industrial fermentation processes when strains are usually exposed to thermal processing and thus require enhanced thermo-tolerance (ROSS et al., 2005). MANDAL et al., (2014) encapsulated *L. casei* in alginate and alginate with a starch matrix. The formulations were tested regarding their heat treatment resistance (65 °C for 20 min). The results showed that the number of the free cells drastically reduced on the heat treatment. Survival of the *Lactobacilli* increased upon encapsulation in the alginate matrix, and it was further improved by the

incorporation of resistant starch. The starch might have increased the total solid content of the capsules and interacted with alginate polymers that led to a decrease in the diffusion of hot water molecules through the matrix of the capsules, leading to better results. SETIARTO et al., (2020) encapsulated probiotics in taro starch and maltodextrin formulations by spray drying. The product was incubated in the water bath at 50, 60, 70, and 80 °C, for 30 min. The results showed that the encapsulated *L. plantarum* SU-LS36 with taro starch had the highest heat resistance survivability among the others. AHMAD et al., (2019) encapsulated probiotics in native starch and nano-sized starch, and evaluated their thermal resistance at 50, 60, 70, and 80 °C, for 5 and 15 min. The results showed that the nano-sized starch may not be able to retain the cells inside its core due to its small size since more cells appear to be attached at the surface of the wall material, whereas the native starch provided better protection to cells against all time-temperature combinations. These findings show that the thermal resistance offered by encapsulation using starch depends on the properties and characteristics of each starch.

Concerning resistance to salinity conditions, it is important to evaluate it, since many foods contain salts that act as a flavor enhancer or as a preservative, and their content may jeopardize the cell viability of probiotic cultures (ALBARRACÍN et al., 2011). TEOH et al., (2011) evaluated this matter for two strains of probiotic bacteria encapsulated by the ionotropic gelation technique in a matrix of alginate and starch, and SABIKHI et al., (2010) evaluated *L. acidophilus* LA1 encapsulated by emulsification and ionotropic gelation also in matrix of alginate and starch. Finally, KHORRAMVATAN et al., (2014) evaluated the performance of microencapsulated *Bacillus thuringiensis* in the sodium alginate and starch matrix under UV radiation stressful conditions aiming application in agriculture. In all these studies, the starch used in the matrix increased microorganism survival under stressful conditions.

2.4.3.4. Influence in the characteristics of the product

The presence of starch can interfere in the shape, size, and mechanical properties of the final product. Furthermore, as starch is a hydrophilic and biodegradable polymer, its presence can enhance the swelling capacity and biodegradability of the formulations, facilitating the release of entrapped cells into the environment, which is a desired property especially for the agriculture field, that will be discussed throughout this topic.

ZANJANI et al., (2018b) microencapsulated a probiotic by emulsification and ionotropic gelation with sodium alginate and Hylon starch, and discussed that starch creates an integrated and uniform mixture with alginate because of their good binding capacity and adequate solubility, which facilitates capsule formation. WU et al., (2014) produced beads by ionotropic gelation using different

proportions of sodium alginate and corn starch. Regarding their size, the diameter of the beads prepared with starch was larger than that of the beads prepared with alginate alone, which was probably related to the shrinkage of beads that significantly reduced with increasing concentrations of starch and alginate with hydrophilic nature.

SCHOEBITZ; SIMONIN; PONCELET, (2012) produced beads by ionotropic gelation with sodium alginate and corn starch. The SEM images showed that the alginate-starch beads were spherical with a diameter of 2.3 ± 0.16 mm, while alginate presented very small wrinkled beads and 1.05 ± 0.11 mm in diameter. MARTIN et al., (2013) produced microparticles by emulsification, ionotropic gelation, and freeze-drying with sodium alginate and corn starch. It was also observed that the addition of starch to the alginate formulation increases the size of microparticles from $49 \mu\text{m} \pm 17$ to $70 \mu\text{m} \pm 17$ ($p < 0.05$), with a significantly higher percentage in the range 60-90 μm . Furthermore, the addition of starch leads to a spherical shape and smooth surface without pores, which confers higher protection to the encapsulated bacteria compared to the porous surface of the alginate microcapsule. ZAEIM et al., (2019) produced beads by ionotropic gelation with sodium alginate, corn starch, and chitosan as a coating. It could be observed that the starch granules led to an excessive size increment up to 1 mm and a much wider size distribution curve, which indicates the loss of size uniformity. In addition to the increase in particle size, the addition of starch decreased surface wrinkles of the capsules when compared to non-starch capsules, which is a result likely due to the increased solids content.

ALFARO-GALARZA et al., (2020) produced microparticles by spray drying with taro and rice starch, and it was shown that the taro starch led to a greater tendency in forming spherical aggregates. This tendency may be related to the presence of residual protein on the periphery of the starch granules, which acts as an adherent material facilitating aggregation (GUO et al., 2021; HOYOS-LEYVA et al., 2018a). MUHAMMAD et al., (2021) also produced microparticles by spray drying, using different kinds of resistant starch (rice, maize, and potato). It was observed the same tendency of forming round-shaped microcapsules, with small differences in shape regarding the different sources of starch. produced beads by ionotropic gelation with sodium alginate and cassava starch, and it was observed that the starch content helped beads to retain their spherical shape after drying. The neat alginate beads were smaller and more irregular in shape with wrinkled and cracked surfaces, whereas the beads that contained starch were larger, more spherical, showing a more homogenous and smoother surface, with less cavitation. In conclusion, the effects of starch were attributed to filling the voids inside the beads. DAM et al., (2018) and SUVARNA; DSOUZA; LAKSHMI, (2018) also observed similar characteristics for the starch products in their procedures.

SÁENZ-SANTOS et al., (2021) produced films with tapioca starch, sodium alginate, and glycerol by casting. This formulation was compared with other films in the study, and a smoother surface was observed through micrography, indicating greater homogeneity. Furthermore, the starch films showed the highest percentage of elongation, lower thickness, and higher tensile stress (MPa). RODRIGO et al., (1998) encapsulated bacteria by ionotropic gelation and freeze-drying in the sodium alginate and different concentrations of modified corn starch (2, 4, 6, 8, 10, and 12%). An increase in mechanical strength was observed, in which the 12% starch particle was significantly stronger than the others. WU, (2008b) produced films by casting using polybutylene succinate (PBSU) with starch, and acrylic acid grafted PBSU with starch. It was observed that the increase of the starch content led to a lower final torque, a decrease in melting temperature and melting enthalpy, in addition to a clear decrease in crystallinity. OYEAGU et al., (2018) produced beads by ionotropic gelation using alginate and different concentrations of starch. It was obtained that the compressive strength of the starch beads was higher than that of the control beads.

For agricultural applications, WANG; TANG; WANG, (2020) produced capsules through ionotropic gelation with sodium alginate and corn starch in different concentrations. The swelling ratio significantly varied from $163.33 \pm 7.57\%$ to $240.33 \pm 5.51\%$, with the increase of the amount of starch. This was related to the OH groups of starch, which have excellent hydrophilicity. Furthermore, it was observed an improvement in the biodegradability with the addition of starch, due to the use of the carbon by the microorganisms in the environment, leading to an accelerated degradation of the entire capsules. The release properties were tested showing that the characteristics of microcapsules were improved with the increase of starch dosage, releasing a higher number of bacteria than the formulation without starch. WU et al., (2014), WU, (2010), WU; WU; CHANG, (2007) and WU, (2008a) also showed that by increasing the starch concentration, the swelling, permeability, and average pore diameter was higher, as well as the biodegradability of the material, leading to a higher cell release compared to materials without starch. Therefore, the rate and number of cells released can be adjusted by altering the composition of the matrix materials.

2.5. Conclusion

In this systematic mapping, we applied methodological rigor standards to develop a reliable and complete overview of the literature of the last 30 years addressing the encapsulation of microorganisms using starch as a matrix or part of the matrix, leading to the following conclusions:

- The encapsulation of microorganisms in the starch matrix has been studied to be applied mainly in the food and agricultural fields, being the probiotics and the PGPM microorganisms the

most investigated so far. The number of publications on the subject showed an increase in the last 10 years, highlighting a crescent interest of the scientific community about this topic.

- Encapsulation techniques such as ionotropic gelation, emulsification, spray drying, freeze-drying, casting, and extrusion techniques can be employed in the encapsulation of microorganisms using starch in the matrix. Furthermore, it was possible to understand the roles of other materials used along with starch in some matrices to improve cell survival and properties of the final encapsulated product.

- There are several important roles that the starch plays when it is present in the encapsulating matrix, regarding the microorganism survival at the encapsulation procedure and storage, performance towards adverse conditions, and characteristics of the product (such as size, morphology, roughness, and mechanical properties).

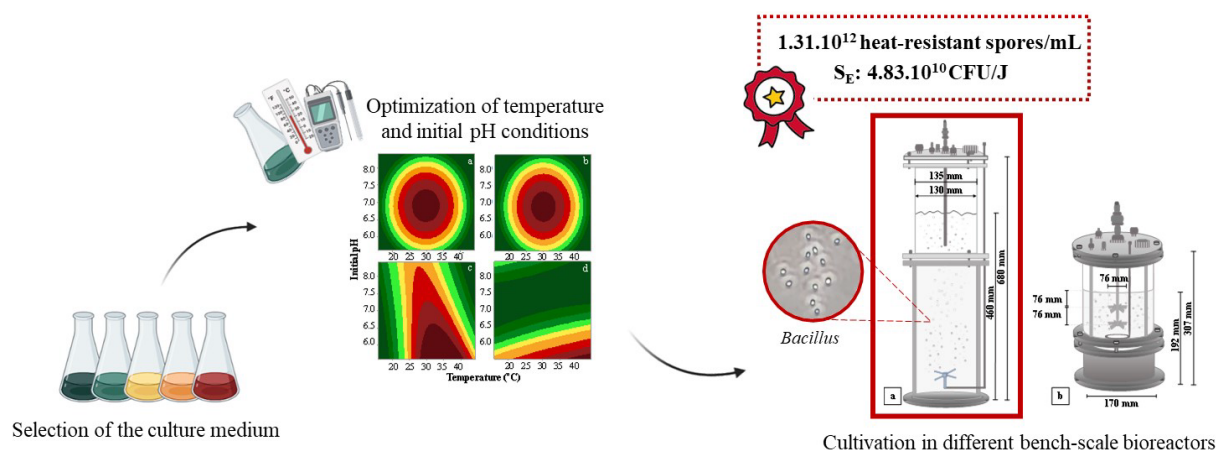
- An important issue we could observe among the articles of this systematic mapping is that only few studies characterized the encapsulation matrix. The focus of most articles is on carrying out experiments about the function and application of the final product. Through the characterization of the matrix, knowledge about the characteristics of the final product could be obtained, facilitating the achievement of desired modifications and their continuous improvement. Therefore, we suggest that further research be carried out to improve the understanding of the structure and formation of encapsulation matrices, as well as to explore the mechanisms of retention and release of microorganisms, and how changes in the encapsulating matrix influence the controlled and targeted release.

- In addition to the lack of characterization, the articles usually lack in addressing the specific functions of the materials used in the matrices. Therefore, the present systematic mapping was important to collect these data and elucidate the functions of starch. Thus, it was possible to obtain valuable information that can help the scientific community to develop innovative starch-based systems to encapsulate microorganisms and apply them in different sectors.

3. High yield of heat-resistant spores of *Bacillus megaterium* in bioreactors

* The content of this chapter is an adaptation of the scientific article entitled: “High yield of heat-resistant spores of *Bacillus megaterium* in bioreactors” by Velloso, C. C. V.; Camargo, B. C. P.; Sousa, M. D. B.; Buffo, M. M.; de Oliveira Paiva, C. A.; Farinas, C. S.; Badino, A. C., published in Biochemical Engineering Journal.

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3.1. Abstract

Microbial inoculants have been proposed as a sustainable and cost-effective alternative to conventional agricultural practices. However, the effectiveness of these bio-based products can be negatively impacted by reduced cell viability during product development, storage, and commercialization. To address these limitations, it is critical to identify and optimize the cultivation conditions that can lead to the production of highly resistant spores for use as inoculants. In this study, optimal cultivation conditions for obtaining *Bacillus megaterium* B119 spores in bioreactors were reached, with increases in the sporulation efficiency and heat resistance. The best nutrient medium and optimal temperature and initial pH conditions were obtained on shake flask experiments and subsequently validated in two bench-scale bioreactor models (stirred and aerated tank and the bubble column). The optimized temperature and initial pH were found to be 37°C and 5.6. The highest concentration of heat-resistant *Bacillus* spores of 1.31·10¹² CFU/mL was obtained in the bubble column bioreactor with a sporulation efficiency of 100.0%, a heat resistance of 78.4%, and an energetic efficiency on spores production of 4.83·10¹⁰ CFU/J. The production of highly resistant spores can significantly improve the survival rate of microorganisms during inoculant production and application, thereby enhancing their effectiveness in the field.

Keywords: microbial inoculant, bioprocess engineering, *Bacillus megaterium*, cultivation conditions, heat-resistant spores, sporulation efficiency.

3.2. Introduction

Microbial inoculants are increasingly recognized as an economically important agricultural product with the potential to replace traditional fertilization practices. These inoculants can enhance yields, protect crops, and restore soil fertility (TIWARI; PRASAD; LATA, 2019; VELLOSO et al., 2020). However, the survival of microorganisms during production, formulation, storage, transportation, and field application under varying environmental conditions, remains a challenge (KAMINSKY et al., 2019; VASSILEV et al., 2015). Efforts have been made to extend the shelf-life of microbial inoculants, particularly at the downstream stage. Several formulations have been developed to provide a favorable environment for microbial survival and physical protection, thereby improving cell viability over an extended duration (BERNINGER et al., 2018; STAMENKOVIĆ-STOJANOVIĆ et al., 2019). Technological advances, including microbial delivery systems (e.g., seed coating) and genetic modification of microorganisms, have also been employed to augment the effectiveness of inoculants (QIU et al., 2019; YADAV et al., 2018). Additionally, bioprocess engineering also can be used at the upstream stage to cultivate microorganisms with resistance to different environmental conditions (VASSILEV et al., 2015).

Submerged (SmF) and solid-state fermentations (SSF) are the most commonly employed cultivation methods for the production of a variety of bioproducts, including microbial inoculants. Both present advantages and disadvantages and the choice of the cultivation method are usually based on the characteristics of the microorganism, bioproduct, and the production process, where the cultivation mode, downstream steps, and production scale must be considered. Solid-state fermentation involves the utilization of a solid substrate with low moisture content and offers some advantages, including the use of economically and environmentally sustainable substrates (e.g., agro-industrial residues), as well as reduced water and energy consumption. Furthermore, by providing a more natural environment for microorganisms, solid-state fermentation can yield benefits in terms of specific metabolite production and improved biomass quality (VASSILEVA et al., 2021). The primary challenges associated with this cultivation method are related to downstream and scale-up processes and the control of variables at a large scale. In contrast, submerged fermentation in liquid media is the prevailing approach adopted in industrial settings, primarily due to its well-established advantages in terms of bioprocess instrumentation, control, and scale-up. Additionally, submerged fermentation exhibits remarkable versatility by allowing the use of various bioreactors, operating

conditions, and cultivation modes (batch, fed-batch, and continuous), thereby creating diverse growth environments for microorganisms (BUFFO et al., 2020; FAZENDA et al., 2008). The main objective of submerged cultivation is to provide optimal conditions for microorganisms to achieve high biomass and/or spores. This fact, in combination with the aforementioned information, renders submerged cultivation an attractive choice for bacterial production, particularly for species belonging to the *Bacillus* genus.

Bacillus is a highly promising inoculant due to its properties that promote plant growth, biocontrol performance, and ability to form endospores (DE SOUSA et al., 2020; TIWARI; PRASAD; LATA, 2019). For instance, three *Bacillus* strains (CNPMS B116, CNPMS B119, and CNPMS B2084) that were isolated from efficient maize genotypes have demonstrated potential for enhancing seedling growth of maize under water stress in hydroponics (VELLOSO et al., 2020), millet in the greenhouse (RIBEIRO et al., 2018), and maize and soybean in the field (DE SOUSA et al., 2020; OLIVEIRA et al., 2020). However, *Bacillus* spore production can be affected by various cultivation parameters, including temperature and pH, with different *Bacillus* species and strains responding differently (POSADA et al., 2015; VEHAPE et al., 2022). Temperature and pH are crucial factors that affect the growth, sporulation, and resistance of *Bacillus* spores. Acidic pH inhibits growth and lowers sporulation efficiency, while slightly alkaline pH enhances sporogenesis and spore mineralization, thereby improving heat resistance (BRESSUIRE-ISOARD; BROUSSOLLE; CARLIN, 2018; POSADA et al., 2015; XU ZHOU et al., 2017). Similarly, low temperatures reduce *Bacillus* growth and sporulation, while high temperatures generate more heat-resistant spores (BRESSUIRE-ISOARD; BROUSSOLLE; CARLIN, 2018; ISTICATO et al., 2020; PALOP; SALA; CONDÓN, 1999). However, the optimal temperature and pH for *Bacillus* growth and sporulation depend on the strain and species of the microorganism, and the combined effect of multiple factors can further alter *Bacillus* metabolism and morphology (JAGANNATH; NAKAMURA; TSUCHIDO, 2003). Therefore, to enhance the sporulation efficiency and heat resistance of *Bacillus* spores, it is necessary to evaluate the combined effect of different cultivation conditions, such as pH and temperature, on the morphology, density, and resistance of the spores.

In this study, it was investigated how cultivation conditions could be optimized to enhance the sporulation efficiency and heat resistance of *Bacillus megaterium* B119 spores in two different bioreactor models. The work goal was to identify the best culture medium for sporulating *B. megaterium* B119 and to optimize pH and temperature for a higher concentration of heat-resistant spores. A central composite rotatable design (CCRD) was applied in shake flasks cultivations to optimize the culture parameters. Subsequently, optimized conditions for obtaining a high yield of heat-resistant *Bacillus* spores were validated in two different models of bioreactors, conventional

stirred and aerated tank and pneumatic bubble column bioreactors. The results provide important insights into bioprocess engineering for sustainable agriculture by enabling efficient and consistent production of microbial inoculants.

3.3. Materials and Methods

3.3.1. Microorganism

B. megaterium CNPMS B119 was utilized in this work. The strain was isolated from maize genotypes efficient in phosphorus utilization (OLIVEIRA et al., 2009) and deposited in the Collection of Multifunctional and Phytopathogenic Microorganisms of Embrapa Maize and Sorghum (CNPMS) (Minas Gerais, Brazil).

3.3.2. Working stock and inoculum

The working stock of *B. megaterium* B119 was created for inoculum standardization. A sample of the B119 strain backup stock obtained from the CNPMS collection was streaked to ensure its purity. Then, an isolated colony was inoculated into 50 mL of tryptone soy broth (TSB) in a 500 mL Erlenmeyer and incubated at 30 °C and 250 rpm for 16 h. The cell suspension was centrifuged and the pellet was resuspended in a 25% (v/v) glycerol solution. A volume of 2.5 mL of this suspension was transferred to 5 mL cryotubes and stored in ultra-freezer (-80 °C) forming the working stock ($\sim 2.10^8$ CFU/mL). For the inoculum preparation, a cryotube was removed from the ultra-freezer, thawed at room temperature, and inoculated into a 500 mL Erlenmeyer flask containing 50 mL of TSB culture medium under the same conditions as the working stock. At the beginning of all cultivations in shake flasks and conventional and pneumatic bioreactors, a proportion of 10% (v/v) of the inoculum was used.

3.3.3. Selection of culture medium for sporulation of *B. megaterium* B119

Five different culture media (Table 3.1) were tested for growth and sporulation of *B. megaterium* B119 in shake flasks at 30°C and 250 rpm for 72 h. An inoculum solution (10% v/v) of reactivated B119 strain was used to inoculate each medium. Carbon and nitrogen sources, as well as salts, were autoclaved separately to avoid any interference with the nutrient composition of the media. The initial pH of all culture media was adjusted to 7.0 using 5 M NaOH solution before autoclaving. The sporulation process was evaluated using a phase contrast microscope (Olympus, model BX51, Japan). The total nitrogen and carbon contents of the culture media were determined by elemental analysis using the CHNS/O elemental analyzer (model 2400, PerkinElmer, Massachusetts, USA). The medium that produced the highest *Bacillus* sporulation was selected for subsequent experiments.

Table 3.1. Composition of culture media tested for *Bacillus* growth and sporulation.

Culture medium	Composition (g.L ⁻¹)	Reference
Sporulation <i>Bacillus</i> Medium (SBM)	Glucose (1.04), MgSO ₄ ·7H ₂ O (0.59), KH ₂ PO ₄ (6.0), yeast extract (5.0); peptone (3.0), NaCl (0.01), and salt stock solution (1.136 mL.L ⁻¹ of FeSO ₄ ·7H ₂ O 0.1 M; 300 µL.L ⁻¹ of ZnSO ₄ ·7H ₂ O 0.1 M; 9.9 mL.L ⁻¹ of CaCl ₂ ·2H ₂ O 0.1 M; 30 mL.L ⁻¹ of MnSO ₄ ·H ₂ O 0.1 M).	(POSADA et al., 2015)
Difco Sporulation Medium (DSM)	Difco nutrient broth (8.0); KCl (1.0); MgSO ₄ ·7H ₂ O (0.25); glucose (3.5). In 1 L of this solution. add 1 mL of each of the solutions: CaCl ₂ ·2H ₂ O 1 M; MnSO ₄ ·H ₂ O 10 mM and FeSO ₄ ·7H ₂ O 1 mM.	(MONTEIRO et al., 2005)
Minimum Medium (MM)	Glucose (5.0); (NH ₄) ₂ SO ₄ (2.0); Na ₃ C ₆ H ₅ O ₇ ·2H ₂ O (1.0); MgSO ₄ ·7H ₂ O (0.2); 0.1 mM of MnSO ₄ ·H ₂ O; 0.1 mM of CaCl ₂ ·2H ₂ O; and 3.6 µM of FeSO ₄ ·7H ₂ O.	(ENGLISH; VARY, 1986)
Winogradsky's mineral salt solution (WSG)	NaCl (2.5); K ₂ HPO ₄ (2.5); KH ₂ PO ₄ (2.5); MgSO ₄ ·7H ₂ O (2.5); MnSO ₄ ·H ₂ O (0.05); FeSO ₄ ·7H ₂ O (0.5); 1 mL of the solution of trace elements (ZnSO ₄ ·7H ₂ O (2.25); CuSO ₄ ·5H ₂ O (1.0); CaCl ₂ ·2H ₂ O (2.0); H ₃ BO ₄ (0.2)). The salt solution was supplemented with glycerol (30); urea (0.8); and yeast extract (0.05).	(PORRAS et al., 2017)
Luria Bertani + salts (LB)	Peptone (10.0); yeast extract (5.0); NaCl (5.0); salt stock solution (FeSO ₄ ·7H ₂ O (0.002); ZnSO ₄ ·7H ₂ O (0.02); MnSO ₄ ·H ₂ O (0.02); MgSO ₄ ·7H ₂ O (0.3)); and glucose (2.0).	(VALICENTE; MOURÃO, 2008)

3.3.4. Central composite rotatable design (CCRD) in shake flasks

A Central Composite Rotatable Design (2² factorial design with 4 trials under the axial conditions and 3 repetitions at the central point) totaling 11 trials was carried out in independent duplicates (RODRIGUES; IEMMA, 2015). Effects of the independent variables temperature (x₁) and pH (x₂) on the production of resistant spores of *B. megaterium* B119 were evaluated using SBM culture medium (Table 3.1). The coded and true values of the independent variables were as follows. Temperature (x₁): -1,41 (15.9 °C), -1 (20 °C), 0 (30 °C), +1 (40 °C), and +1,41 (44.1 °C). pH (x₂): -1.41 (5.6), -1 (6.0), 0 (7.0), +1 (8.0), and +1.41 (8.4). The dependent variables evaluated were viable cells concentration (y₁ in CFU/mL), spore concentration (y₂ in CFU/mL), sporulation efficiency (y₃ in %), and heat resistance (y₄ in %). The results were analyzed using samples from 48 h of cultivation and for differences among means via Tukey's test (p < 0.05).

Data were evaluated using the statistical software Minitab 17. Second-order models were obtained and analyzed statistically by analysis of variance (ANOVA). From the resulting models, the

Derringer and Suich desirability function (DERRINGER; SUICH, 1980) was used to determine in the evaluated sample space the temperature and pH values that would maximize the sporulation efficiency, the heat resistance and the spore production from *B. megaterium* B119, selecting a target of 10^8 spores/mL. Finally, an experiment was conducted in duplicate under the conditions indicated by the desirability test for model validation in shake flasks. Experimental and predicted responses were compared.

3.3.5. Comparison of resistant *B. megaterium* B119 spore production in shake flasks and bioreactors

The production of resistant spores of *B. megaterium* B119 was evaluated in 500 mL shake flasks with 50 mL of cultivation broth, in a conventional stirred and aerated tank (SAT) bioreactor of 4-L working volume (Bioflo IIC, New Brunswick Scientific), equipped with two 6-blade Rushton turbines (RT-RT), and in a pneumatic bubble column (BC) bioreactor of 5-L working volume (Fig. 3.1). The temperature was maintained at 37°C and the initial pH was adjusted to 5.6 with 1 M NaOH solution based on the optimization conditions from previous CCRD. All components of the SBM medium were sterilized together to ensure the sterilization of the process. The shake flasks were maintained at 250 rpm, like previous experiments. The operating conditions of the SAT (400 rpm and 0.5vvm) and BC (1.5 vvm) bioreactors were chosen based on the oxygen transfer quantified by initial volumetric oxygen transfer coefficient (k_{La}), similar in both devices. The k_{La} for SAT was obtained from Bustamante et al. (BUSTAMANTE; CERRI; BADINO, 2013) with a value of 146 h^{-1} , while for the BC bioreactor the k_{La} of 134 h^{-1} was obtained according to Mendes and Badino (MENDES; BADINO, 2015).

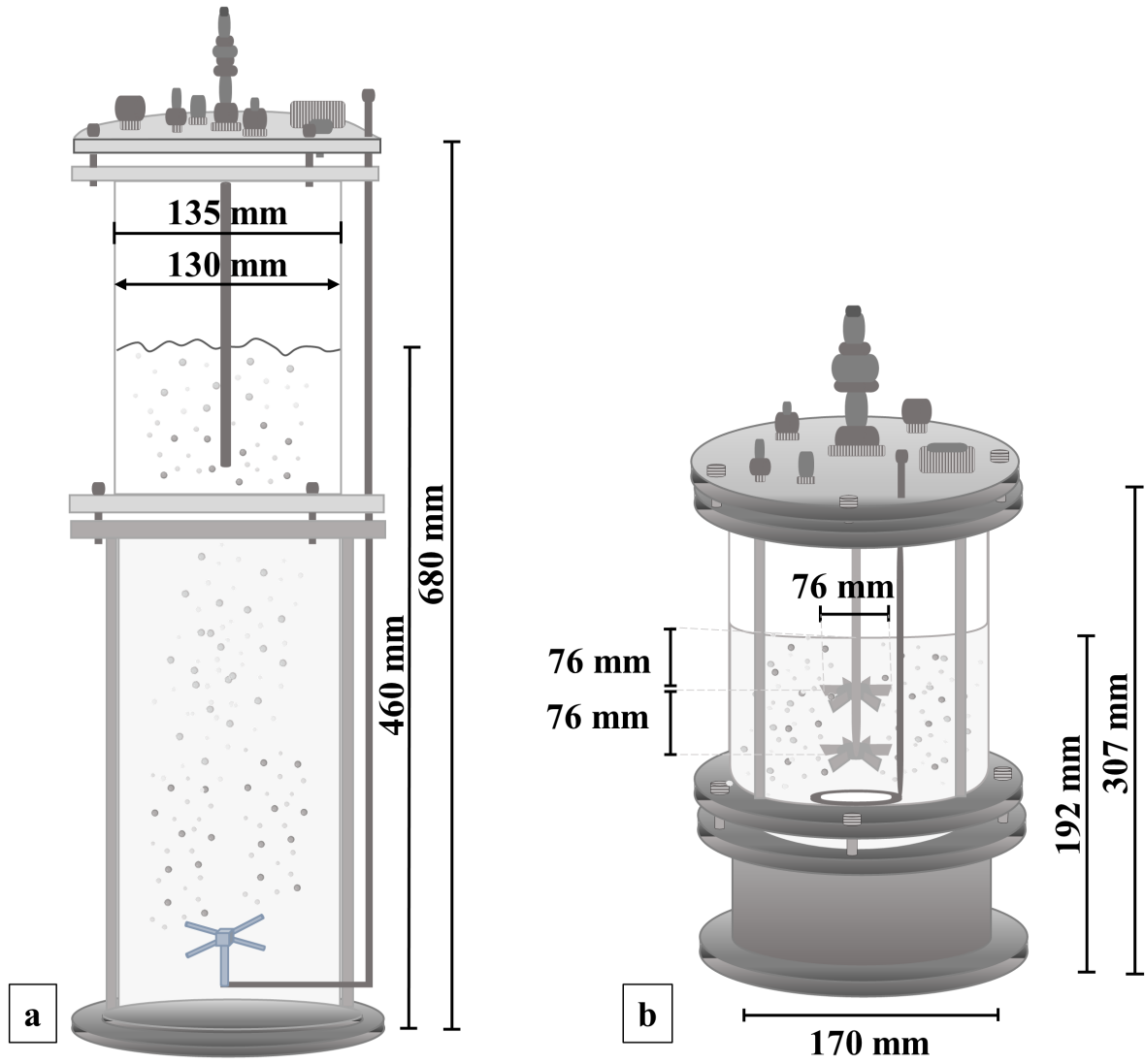


Figure 3.1. Geometric characteristics and main dimensions of (a) 5-L bubble column (BC) bioreactor, (b) 4-L stirred and aerated tank (SAT) bioreactor.

3.3.6. Analytical methods

Samples were intermittently withdrawn along the cultivations and subjected to analysis. The quantification of the biomass concentration (C_X) was performed by gravimetry. Initially, a predetermined volume of 2 mL of the fermentation broth was introduced into pre-dried and weighed Eppendorf tubes ($m_{initial}$ in g). Subsequently, the contents were centrifuged at 4 °C and 9,000 rpm for 10 min and the supernatant was discarded. The cells were then washed twice with an equal volume of distilled water and centrifuged again. Afterwards, the Eppendorf tubes were dried at 60 °C for 24 h and weighed (m_{final} in g). Cell concentration (C_X) was calculated at each time interval (0.0, 1.5, 3.0, 4.5, 6.0, 10, 15, 24, 32, and 48 h) by Equation 1.

$$C_X \left(\frac{g}{L} \right) = \frac{m_{final} - m_{initial}}{2 \cdot 10^{-3}} \quad (1)$$

Total viable cells (C_{VC}) and spore (C_S) concentrations were determined by the serial dilution technique at 15, 24, 32, and 48 h. Colony forming unit (CFU) counts were performed after 12 h of incubation at 30 °C for dilutions containing 15–60 colonies by drop plate method (20 µL). Total viable cells are obtained without any heat treatment. For spore concentration (C_S) analysis, the samples were subjected to thermal shock (50 °C for 30 min) before serial dilution to render vegetative cells inactive while preserving all spores. These conditions were chosen based on the results obtained in preliminary tests.

Sporulation efficiency (SE) was determined as the ratio between spore (C_S) and total viable cell (C_{VC}) numbers. The response variables C_{VC} , C_S , and SE were calculated from Equations 2, 3, and 4.

$$C_{VC} \left(\frac{CFU}{mL} \right) = \frac{n_{CFU} \cdot D}{V_S} \quad (2)$$

$$C_S \left(\frac{CFU}{mL} \right) = \frac{n_{CFU_{50^\circ C}} \cdot D}{V_S} \quad (3)$$

$$SE(\%) = \frac{C_S}{C_{VC}} \cdot 100 \quad (4)$$

where n_{CFU} is the number of CFU, D is the dilution factor and V_S is the sample volume (mL) for plating.

To verify the resistance of the spores under high thermal conditions, spores were incubated at 90 °C for 10 min (conditions commonly employed in literature studies (JOSE-LUIS et al., 2011; MELLY et al., 2002)) and quantified using the serial dilution technique. The spore heat resistance (HR) was determined as the ratio between the numbers of surviving spores and total spores (C_S) at 15, 24, 32, and 48 h (Equation 5).

$$HR(\%) = \frac{n_{CFU_{90^\circ C}} \cdot D}{V_S \cdot C_S} * 100 \quad (5)$$

Finally, Equation 6 was used to compare the bioreactors in terms of energy efficiency (S_E) in spore production.

$$S_E \left(\frac{CFU}{J} \right) = \frac{C_S * 10^3}{\frac{P}{V} * t} \quad (6)$$

where P is the power consumption of the bioreactor (J/s), V is the working volume (L), and t is the cultivation time (s).

3.4. Results and Discussion

3.4.1. Qualitative selection of the culture medium for *B. megaterium* B119 sporulation

Figure 3.2 illustrates the morphologies of *B. megaterium* B119 cultivated in various media for 72 h or until complete sporulation. The images obtained with a phase contrast microscope indicate that the SBM medium exhibited the highest sporulation efficiency for *B. megaterium* B119 in the shortest time (34-48 h), while the DSM showed good efficiency but late (58 h). The MM demonstrated delayed spore maturation (72 h). However, under the given conditions and periods analyzed, no evidence of sporulation was observed in LB and WSG media.

The sporulation of *Bacillus* endospores relies on the maintenance of a balanced concentration of intracellular metabolites. Disruption of this balance, whether through enzyme inhibition or mutation, can lead to suppressed sporulation (FREESE et al., 1974). Enzyme activation, critical for sporulation, can be achieved through metallic ion supplementation, which can enhance the efficiency and stability of spore formation (KOŁODZIEJ; SLEPECKY, 1964; MUKHOPADHYAY et al., 2004). Additionally, the carbon-to-nitrogen ratio (C:N) significantly affects bacterial growth and cell differentiation (FARRERA; PÉREZ-GUEVARA; DE LA TORRE, 1998; MONTEIRO et al., 2014). Table 3.2 presents the C:N ratio and the trace element concentrations of the media used in the study.

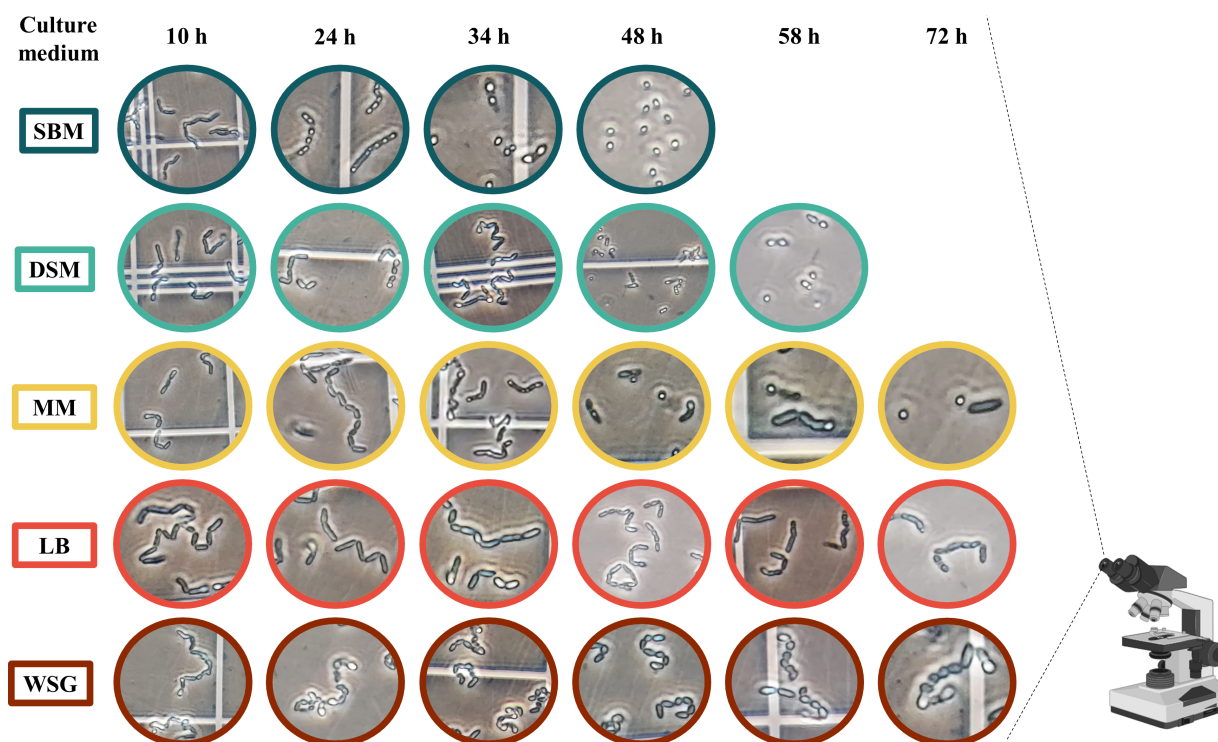


Figure 3.2. Phase contrast photomicrographs were captured to illustrate the cell differentiation of *B. megaterium* B119 cultured in various media. The experiments were conducted at 30°C and 250 rpm

in shake flasks containing different culture media, with an initial pH of 7.0, and sampled over a 72h cultivation period. Difco Sporulation Medium (DSM), Sporulation *Bacillus* Medium (SBM), Minimum Medium (MM), Winogradsky's mineral salt solution (WSG), and Luria Bertani + salts (LB).

Table 3.2. Chemical composition (C:N ratio and trace elements concentrations) of the culture media evaluated to produce *Bacillus megaterium* B119 resistant spores.

Culture Media	C:N ratio	Concentration (mg. L ⁻¹)					
		Fe	Mn	Ca	Zn	Cu	Mg
DSM	4.4:1	0.06	0.55	40.00	-	-	24.39
SBM	4.3:1	0.64	16.50	3.96	0.20	-	51.80
MM	5.3:1	0.20	5.50	4.00	-	-	19.51
LB	4.5:1	0.40	6.51	-	4.53	-	29.27
WSG	37:1	101.00	16.30	0.54	0.51	0.26	243.90

Difco Sporulation Medium (DSM), Sporulation *Bacillus* Medium (SBM), Minimum Medium (MM), Winogradsky's mineral salt solution (WSG), and Luria Bertani + salts (LB).

The chemical composition data presented in Table 3.2 demonstrate a correlation between a lower C:N ratio and a higher level of sporulation in both SBM and DSM media. Conversely, the WSG medium, which showed no spore formation, had the highest C:N ratio. These results support existing literature, which reports that the highest spore concentrations and sporulation efficiency were obtained in cultures of *Bacillus thuringiensis* HD-73 and *Bacillus* sp. SB4 with lower C:N ratios (FARRERA; PÉREZ-GUEVARA; DE LA TORRE, 1998; YUNIARTI et al., 2019a).

Regarding trace element concentration, the SBM medium had the highest levels of iron (Fe²⁺), magnesium (Mg²⁺), and manganese (Mn²⁺) among the media where the sporulation of the B119 strain occurred (Table 3.2). The adequate concentration of these ions can accelerate the differentiation process and lead to high sporulation efficiency in different *Bacillus* species (PUROHIT; SASSI-GAHA; REST, 2010; STÖCKEL et al., 2009). Fe²⁺ contributes to the expression of the delayed sporulation gene of *Bacillus* sp., which is dependent on aconitase, an iron-dependent enzyme (SERIO; PECHTER; SONENSHEIN, 2006). Mg²⁺ is essential for the growth of *Bacillus* and plays a crucial role in stabilizing the polysomes of sporulated organisms, which can potentially enhance heat resistance of the spores of this bacteria genus (AL-ADHAM, 1989; IGURA et al., 2003). Al-Adham (1989) reported slower sporulation in *Bacillus stearothermophilus* at lower Mg²⁺ concentrations (AL-ADHAM, 1989). Similarly, Mn²⁺ is essential for creating a favorable microenvironment for key regulatory elements during the onset of sporulation, ensuring that Mg²⁺ binds and catalyzes the phosphotransfer necessary for sporulation to occur (KOŁODZIEJ; SLEPECKY, 1964; MUKHOPADHYAY et al., 2004). Several studies have reported that in adequate concentrations, Mn²⁺ stimulates the growth and sporulation of several species of *Bacillus*, including *B. megaterium*

(GHOSH et al., 2011; KOLODZIEJ; SLEPECKY, 1964), *B. subtilis* (GRANGER et al., 2011; STÖCKEL et al., 2009), *B. mycoides* (STÖCKEL et al., 2009), *B. brevis* (CURRAN; EVANS, 1954), *B. atrophaeus* (STÖCKEL et al., 2009), *B. sphaericus* (STÖCKEL et al., 2009), *B. cereus* (RYU; KIM; BEUCHAT, 2005), and *B. thuringiensis* (IÇGEN; IÇGEN; ÖZCENGİZ, 2002; STÖCKEL et al., 2009). However, concentrations higher than 3.10^{-4} mol/L can inhibit or delay sporulation (GRANGER et al., 2011; IÇGEN; IÇGEN; ÖZCENGİZ, 2002). Ghosh et al. (GHOSH et al., 2011) found that spores of *B. megaterium* exhibited greater resistance to UVC radiation when exposed to high levels of Mn^{2+} (up to 1 mM). Additionally, spores of *B. subtilis* showed greater resistance to wet heat, dry heat, and H_2O_2 . This makes high levels of Mn^{2+} potentially interesting for application in the production of microbial inoculants.

High concentrations of Fe^{2+} (WSG), Mg^{2+} (WSG), and Zn^{2+} (LB) and low or no Ca^{2+} concentration (WSG and LB, respectively) were observed in media where sporulation did not occur. While iron and magnesium are crucial for many metabolic processes, an excess of free iron can be toxic to the cell, impairing its development, and high magnesium concentrations can reduce sporulation efficiency in *Bacillus* (BROWN; HODGES, 1974). Similarly, high levels of zinc can reduce sporulation in various bacteria, including *Bacillus* species, by damaging vegetative cells and interfering with spore component biosynthesis (IÇGEN; IÇGEN; ÖZCENGİZ, 2002; LEE et al., 2011). However, the effect of calcium ion concentration on sporulation efficiency varies among different *Bacillus* strains. Some studies have shown that a low concentration of calcium can reduce sporulation efficiency, while others have observed the opposite effect (MONTEIRO et al., 2014; SINNELÄ et al., 2019). Nevertheless, high Ca^{2+} concentration in the spore nucleus has been found to increase heat resistance in *Bacillus* species, including *B. megaterium* (LEVINSON; HYATT, 1964). Calcium can chelate to pyridine-2,6-dicarboxylic acid (DPA) to form Ca^{2+} -DPA, which efficiently reduces the water content in the spore nucleus, strengthening the spore structure and aiding sporulation (SETLOW et al., 2006). Based on these findings, SBM medium was chosen for subsequent cultivation experiments.

3.4.2. Optimization of temperature and initial pH conditions in shake flasks

To assess the potential of *B. megaterium* B119 to produce high concentrations of heat-resistant spores, a central composite rotatable design (CCRD) was employed to investigate the impact of the independent variables temperature and initial pH in shake flask cultivations. Table 3.1S presents the coefficients and their statistical significance ($p < 0.05$) for the dependent variables, including viable cell concentration (CFU/mL), spore concentration (CFU/mL), sporulation efficiency (%), and heat resistance (%). Regression coefficients were calculated based on significant and linear terms, and mathematical models were generated for each of the dependent variables (Table 3.3). All significant

and linear terms were considered to obtain the models. The adequacy of the models was assessed by ANOVA, which revealed that all independent variables had a statistically significant impact on the responses (Table 3.4). The R^2 and calculated F values (Table 3.4) were suitable for developing second-order models for all dependent variables under the studied experimental conditions (Equations y_{1-4}). The equations presented in Table 3.4 were employed to produce contour curves for each of the dependent variables (Figure 3.3).

Figures 3.3a and 3.3b show similar profiles for the concentration of viable cells and spores. Optimal conditions for cell growth and spore production were found to occur at a temperature of 30 ± 5 °C and a pH of around 7.0 ± 0.5 . Outside of this range, extreme temperature and pH values reduced cell growth and inhibited sporulation (Figures 3.3a and 3.3b). The highest sporulation efficiencies (SE) were achieved in the temperature range of 30-40 °C and $pH \leq 6.5$ (Figure 3.3c). At pH 7.0, minor temperature fluctuations resulted in significant changes in the sporulation efficiency (SE), particularly outside the optimal temperature range (30-37 °C). However, within the optimal temperature range, pH variation did not exert a substantial influence on sporulation efficiency. Therefore, temperature variation was found to be more influential than pH in the studied ranges. In Figure 3.3d, it is evident that the highest heat resistance (HR) of *B. megaterium* B119 spores was attained at higher temperatures and lower initial pH. However, at temperatures above 40 °C and pHs below 3.5, and temperatures above 45 °C for any pH, cells died, and sporulation did not occur (Figures 3.3a and 3.3b). In contrast to sporulation efficiency (SE), pH variation had a greater impact on heat resistance (HR) than temperature variation.

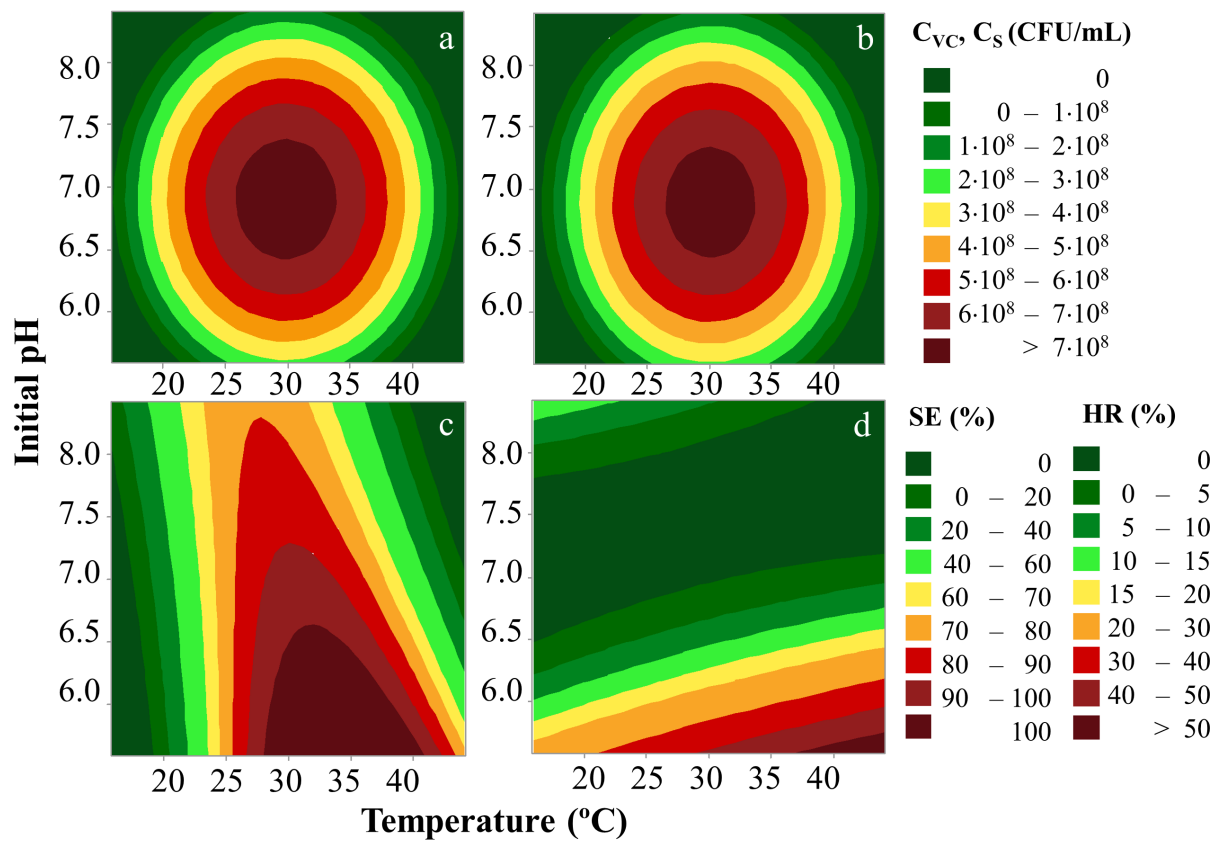


Figure 3.3. Contour curves for the dependent variables (a) viable cell concentration (C_{VC}), (b) spore concentration (C_S), (c) sporulation efficiency (SE), and (d) heat resistance of formed spores (HR). The submerged cultivation of *B. megaterium* B119 was carried out in the SBM medium at 250 rpm for 48 h.

Table 3.3. Design matrix of the Central Composite Rotatable Design (CCRD) with independent variables temperature (°C) and pH, and the results for responses: viable cells concentration (CFU/mL), spore concentration (CFU/mL), sporulation efficiency (%), and heat resistance (%), for the cultivation of *B. megaterium* B119 for 48 h.

Trial	Temperature (°C)	Initial pH	Viable cells concentration (CFU/mL)	Spore concentration (CFU/mL)	Sporulation efficiency (%)	Heat resistance (%)
	x ₁	x ₂	y ₁	y ₂	y ₃	y ₄
1	-1(20)	-1(6)	$1.28 \pm 0.41 \cdot 10^7$ ^d	$8.63 \pm 5.01 \cdot 10^5$ ^d	6.43 ± 1.84 ^{bc}	0.04 ± 0.00 ^c
2	1(40)	-1(6)	$4.23 \pm 0.27 \cdot 10^7$ ^c	$4.23 \pm 0.27 \cdot 10^7$ ^b	100 ± 0.00 ^a	25.9 ± 0.88 ^b
3	-1(20)	1(8)	$5.98 \pm 0.80 \cdot 10^7$ ^{bc}	$7.06 \pm 1.09 \cdot 10^6$ ^c	11.8 ± 0.25 ^b	0.88 ± 0.15 ^c
4	1(40)	1(8)	$1.42 \pm 0.00 \cdot 10^5$ ^e	$1.11 \pm 0.29 \cdot 10^3$ ^e	0.78 ± 0.21 ^c	0.00 ± 0.00 ^c
5	-1,41(15,9)	0(7)	$4.23 \pm 0.14 \cdot 10^7$ ^c	$3.21 \pm 0.18 \cdot 10^5$ ^d	0.76 ± 0.07 ^c	0.14 ± 0.03 ^c
6	1,41(44,1)	0(7)	$2.84 \pm 0.93 \cdot 10^5$ ^e	$0.00 \pm 0.00 \cdot 10^0$ ^f	0.00 ± 0.00 ^c	0.00 ± 0.00 ^c
7	0(30)	-1,41(5,6)	$4.20 \pm 0.03 \cdot 10^8$ ^a	$4.18 \pm 0.05 \cdot 10^8$ ^a	99.7 ± 0.42 ^a	55.5 ± 6.38 ^a
8	0(30)	1,41(8,4)	$1.09 \pm 0.02 \cdot 10^8$ ^b	$1.03 \pm 0.06 \cdot 10^8$ ^b	95.1 ± 6.96 ^a	1.16 ± 0.11 ^c
9	0(30)	0(7)	$8.29 \pm 1.36 \cdot 10^8$ ^a	$8.21 \pm 1.24 \cdot 10^8$ ^a	99.1 ± 1.27 ^a	1.83 ± 0.15 ^c
10	0(30)	0(7)	$6.53 \pm 1.11 \cdot 10^8$ ^a	$6.53 \pm 1.11 \cdot 10^8$ ^a	100 ± 0.00 ^a	2.28 ± 0.02 ^c
11	0(30)	0(7)	$8.05 \pm 1.58 \cdot 10^8$ ^a	$7.79 \pm 1.47 \cdot 10^8$ ^a	98.8 ± 0.65 ^a	2.32 ± 0.21 ^c

Coded and () true values of the independent variables. Values are mean $\pm$ SD of duplicates of the dependent variables. For the same response, means with different small letters in the same column differ significantly ($p < 0.05$) by Tukey's test. The logarithmic transformation was used in the Tukey test for the variables viable cell concentration and spore concentration.

Table 3.4. Percentage of variance explained (R^2), calculated F ($F_{\text{calc.}}$) value, tabulated F (F_{Table}), and desirability value for the responses viable cells concentration, spore concentration, sporulation efficiency, and heat resistance, by analysis of variance (ANOVA).

	Response	Unit	Goal	Regression Equation in Coded Units	R^2 (%)	$F_{\text{calc.}}$	F_{Table}	d
y_1	Viable cells concentration (C_{VC})	10^7 CFU/mL	-	$y_1 = 76.26 - 1.12 x_1 - 39.91 x_1^2 - 5.43 x_2 - 27.77 x_2^2$	91.65	46.62	2.96	-
y_2	Spore concentration (C_{Sp})	10^7 CFU/mL	target 1.10^8	$y_2 = 75.11 + 0.42 x_1 - 4.05 x_1^2 - 6.02 x_2 - 27.46 x_2^2$	92.51	52.52	2.96	0.99
y_3	Sporulation efficiency (SE)	%	maximize	$y_3 = 93.55 + 10.19 x_1 - 52.32 x_1^2 - 12.55 x_2 - 26.15 x_1 x_2$	88.75	33.52	2.96	1.00
y_4	Heat resistance (HR)	%	maximize	$y_4 = -0.59 + 3.10 x_1 - 12.74 x_2 + 12.07 x_2^2 - 6.69 x_1 x_2$	80.48	17.53	2.96	0.83

x_1, x_2 : coded independent variables for temperature and initial pH, respectively.

Bacillus species are mesophilic microorganisms that grow optimally in a temperature range of 30-37 °C and a pH range 6.0-7.0 (STAMENKOVIĆ-STOJANOVIĆ et al., 2019). Low temperatures have been reported to reduce the efficiency of *Bacillus* sporulation, whereas high temperatures increase the heat resistance of the spores (BARIL et al., 2012b; PALOP; SALA; CONDÓN, 1999), which is consistent with our findings. The increase in spore resistance with high temperatures is attributed to the reduction of water content in the spore nucleus (BRESSUIRE-ISOARD; BROUSSOLLE; CARLIN, 2018; ISTICATO et al., 2020). Contrarily to our findings, some studies have reported that an alkaline pH favors sporulation while a very acidic pH can delay and/or suppress the growth and spores production of *Bacillus* (POSADA et al., 2015; STAMENKOVIĆ-STOJANOVIĆ et al., 2019; VEHAPI et al., 2022). This is because an alkaline pH can increase the mineralization of spores and, therefore, enhance heat resistance (BRESSUIRE-ISOARD; BROUSSOLLE; CARLIN, 2018; XU ZHOU et al., 2017). CAZEMIER; WAGENAARS; TER STEEG, (2001) observed that the presence of divalent metal ions in the sporulation medium increased the ability of *B. coagulans* ATCC 10545 to grow at low pH values, which may be related to the results obtained in this work. Sporulation efficiency (*SE*) is generally maximum at the optimal growth temperature and pH and decreases as these values vary, prolonging the sporulation process (BARIL et al., 2012b; BAWEJA et al., 2008; MINH et al., 2011).

The models presented in Table 3.4 were used to determine the temperature and initial pH that maximize sporulation efficiency and heat resistance, achieving a target of 10⁸ spores/mL, according to Derringer and Suich's desirability function (Fig. 3.4, Table 3.4). The composite desirability obtained was 0.9382, indicating a high probability of obtaining results close to the predicted values provided by the software. *B. megaterium* B119 was cultivated in shake flasks at an optimized temperature of 37 °C and initial pH of 5.6. The experimental results were compared with the responses predicted by the models for all response variables (Table 3.5). It was observed that the differences between experimental and predicted values for *SE* and *HR* were insignificant. However, the concentrations of viable cells and spores obtained experimentally were 10 times higher with relative errors of 95.39 and 97.03%, respectively. This discrepancy may be due to the higher growth of the B119 strain at 37 °C, which was not predicted by the model. Overall, these results suggest that the developed mathematical model has a good predictive capacity for *SE* and *HR*.

POSADA et al., (2015) utilized an initial pH of 5.5 and a temperature of 30 °C to sporulate *B. subtilis* EA-CB0575 in a 14 L bioreactor employing the same culture medium (SBM). The

authors obtained a sporulation efficiency (SE) of $92.9 \pm 5.1\%$ and a spore concentration (HR) of $2.27 \pm 0.56 \cdot 10^9$ spores/mL, which is comparable to the results obtained in this study. Therefore, the temperature and initial pH of 37°C and 5.6, respectively, determined by the desirability function, were selected for the subsequent experimental procedures.

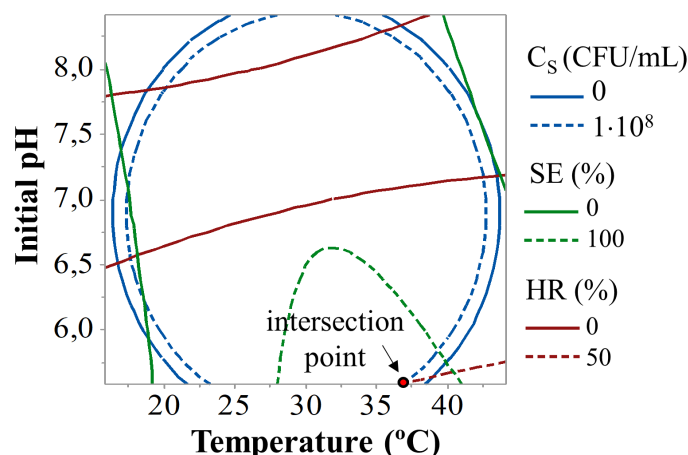


Figure 3.4. Overlaid contour plot with the limits defined in Table 3.4 for optimizing the production of heat-resistant spores of *B. megaterium* B119. The intersection point is Temperature $\cong 37^\circ\text{C}$ and Initial pH $\cong 5.6$.

Table 3.5. Predicted values (Pred.), experimental values (Exp.), and relative error ($RE = (\text{Exp} - \text{Pred})/\text{Exp} \cdot 100$) for the responses viable cells concentration (C_{VC}), spore concentration (C_S), sporulation efficiency (SE), and heat resistance (HR).

Response variable	Predicted value	Experimental value	Relative Error (%)
C_{VC} (CFU/mL)	$1.13 \pm 0.73 \cdot 10^8$	$2.45 \pm 0.58 \cdot 10^9$	95.39
C_S (CFU/mL)	$1.14 \pm 0.70 \cdot 10^8$	$3.84 \pm 1.65 \cdot 10^9$	97.03
SE (%)	111.9 ± 12.0	100.0 ± 0.0	-11.9
HR (%)	49.3 ± 5.5	53.6 ± 7.4	8.10

The predicted values were obtained using the model equations for each response variable and they are presented as fitted values $\pm$ standard error. The experimental values are presented as mean $\pm$ standard deviation for triplicates.

3.4.3. Cultivation of *B. megaterium* B119 in different bench-scale bioreactors

The present study investigated the impact of bioreactor type (stirred and aerated tank: SAT and bubble column: BC) on the production of resistant *Bacillus* spores, as shown in Figure 3.5 and Table 3.6. Figure 3.5 revealed a clear and expressive difference in the heat resistance exhibited by the spores generated. Specifically, spores obtained in shake flasks and the BC bioreactor exhibited heat resistance of approximately 50 and 80%, respectively, at the end of

the cultivation period. In contrast, spores synthesized in the SAT bioreactor showed negligible heat resistance, below 1%. This phenomenon may be attributable to a possible scenario of oxygen limitation. Dissolved oxygen (*DO*) profiles of the SAT and BC bioreactor cultivations (Figures 3.5b and 3.5c) indicated a transient period in terms of *DO* during the first 18 h. Specifically, the SAT cultivation reached a minimum *DO* tension near 20%, while the BC bioreactor showed critical *DO* tension close to 0%, an indication of oxygen limitation.

According to ABBAS et al., (2014b), anaerobic conditions tend to favor the production of spores with higher heat resistance, resulting in lower spore concentration compared to aerobic conditions. In this study, both cultures were carried out under aerobic conditions to avoid the reduction in cell growth caused by anaerobic growth. It was observed that under conditions without oxygen limitation (SAT), the spores formed showed less heat resistance. In contrast, the condition under oxygen limitation (BC) may have stimulated the increased production of heat-resistant spores. Furthermore, the spore concentration (C_S) at the end of the bioprocess was 9.52% higher in the BC bioreactor compared to the SAT bioreactor (Figure 3.5 and Table 3.6).

Pneumatic bioreactors are commonly known to promote a less shearing and more homogeneous environment for cells (BADINO et al., 2016). Based on data from ESPERANÇA et al., (2022) under the same operating conditions as those in the present study, it was found that the energy dissipation and circulation rate (EDCF), which is associated with the maximum shear rate, was 3.3 times greater in the SAT than in the BC. In addition, the comparison between the two bioreactors in terms of average shear rate under the operating conditions of this study revealed an average shear rate approximately twice as high in the SAT (BUFFO et al., 2020). It has been reported in the literature that shear stress strongly affected the sporulation of *B. amyloliquefaciens* (TZENG et al., 2008) and *B. subtilis* (SAHOO et al., 2004). TZENG et al., (2008) observed that a higher stirrer speed in a conventional 5 L bioreactor resulted in lower sporulation of *B. amyloliquefaciens*, possibly due to the generation of high shear stresses. Similarly, SAHOO et al., (2004) reported that a higher shear rate inhibited *B. subtilis* sporulation due to an increase in Reactive Oxygen Species (ROS) inside the cell, which in turn influenced the regulation of gene expression (σ_B). In their previous work, SAHOO et al., (2003) demonstrated the effect of shear stress on the genome of the same microorganism, with increased shear resulting in lower expression of the gene (σ_B), which plays an essential role in protecting against various stresses, such as oxidative stress, heat, and osmotic shocks. These reports provide a rationale for the lower sporulation observed in the SAT compared to the BC

and shake flasks in the present study.

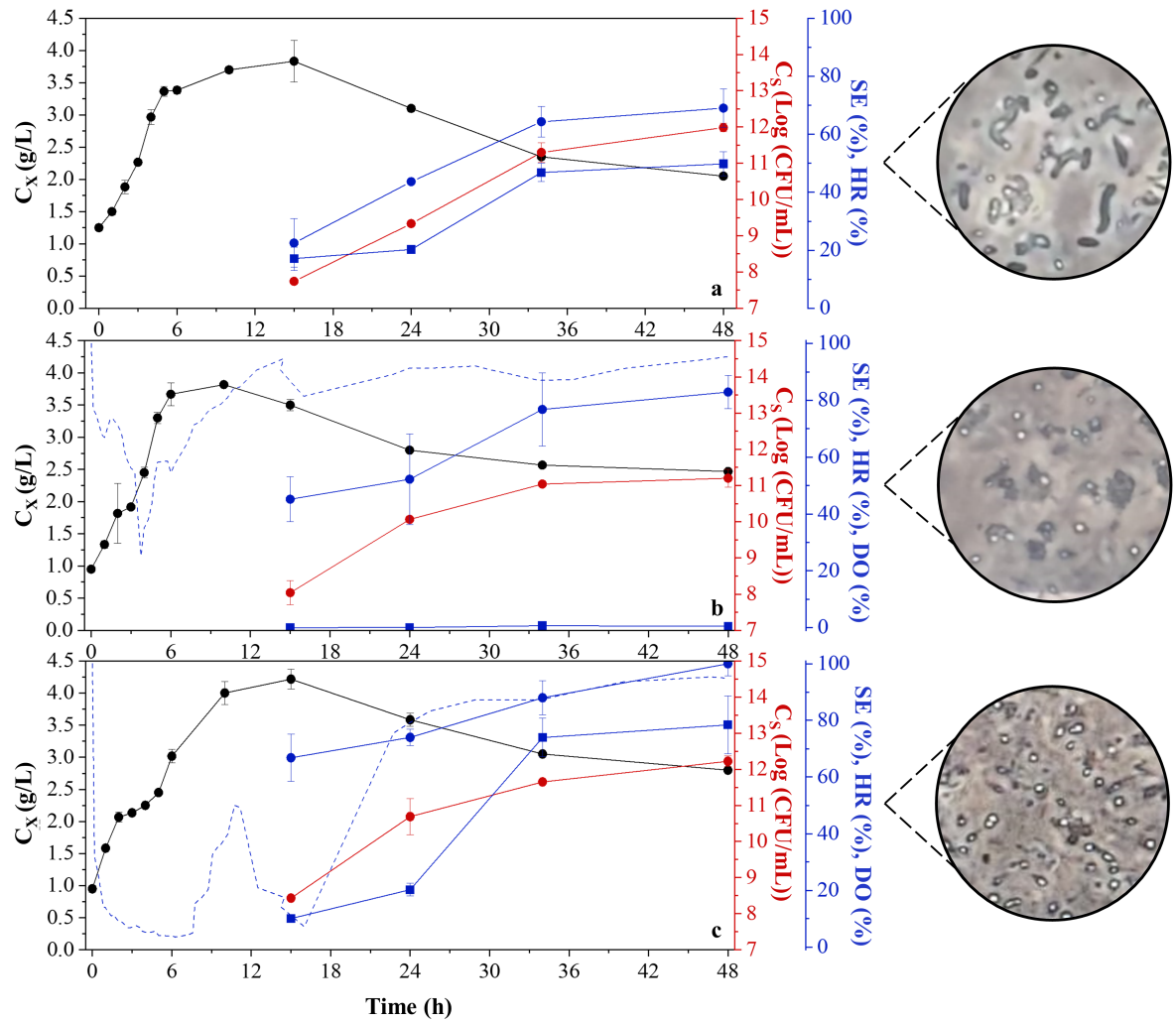


Figure 3.5. Time courses of cell concentration (C_X in g/L) (—●—), spore concentration (C_S in CFU/mL) (—●—), sporulation efficiency (SE in %) (---●---), resistance to heat (HR in %) (—■—), and dissolved oxygen (DO in %) (---) in submerged cultivation of *B. megaterium* B119 under optimized growth conditions over 48 h in: (a) shake flasks, (b) stirred and aerated tank (SAT) bioreactor, and (c) bubble column (BC) bioreactor. Besides the graphs, there are photomicrographs of the culture broth after 48 h of submerged cultivation at 37 °C and initial pH of 5.6. Spores are represented by the shape of bright circles and vegetative cells by the shape of gray rods in the photomicrographs.

Table 3.6. Spore concentration (C_S), sporulation efficiency (SE), heat resistance (HR) of *B. megaterium* B119 spores, and energetic efficiency on spores' production (S_E) obtained in shake flasks, stirred and aerated tank (SAT) bioreactor, and bubble column (BC) bioreactor, cultivated at 37 °C and initial pH 5.6.

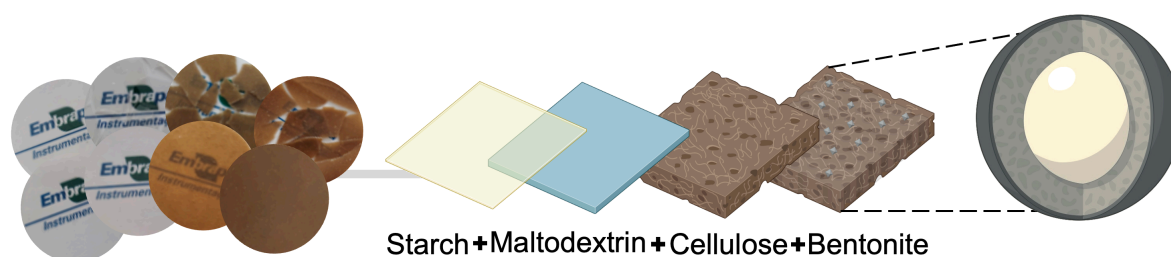
Reactor model	C_S (CFU/mL)	SE (%)	HR (%)	S_E (CFU/J)
Shaker flasks	$9.64 \pm 0.27 \cdot 10^{11}$	$69.0 \pm 6.6 \%$	$49.78 \pm 4.18 \%$	-
SAT	$1.59 \pm 0.66 \cdot 10^{11}$	$82.9 \pm 5.8 \%$	$0.45 \pm 0.01 \%$	$7.08 \cdot 10^8$
BC	$1.67 \pm 0.12 \cdot 10^{12}$	$100.0 \pm 14.3 \%$	$78.41 \pm 10.23 \%$	$4.83 \cdot 10^{10}$

Although there were differences in spore production between the three trials, all of them achieved significant amounts of *B. megaterium* spores, particularly when compared to other reports in the literature (MAHARIAWAN et al., 2020; SANTIAGO BADILLO et al., 2021). However, the BC bioreactor was found to be the most favorable to produce resistant spores of the B119 strain. This finding can be attributed to the oxygen limitation and/or lower shear stress present in the BC, both provided by the hydrodynamic characteristics of the equipment. Furthermore, this device presents several operational advantages compared to the SAT, such as the absence of moving parts, much lower power consumption, and consequently lower energy expenditure. For the operating conditions of the present study, the specific power consumptions in the SAT and BC bioreactors were 1.3 and 0.2 kW/m³, respectively (BUFFO et al., 2020; CHISTI; MOO-YOUNG, 1987). In terms of energetic efficiency for spores' production or spores produced per energy unit (S_E in CFU/J) (Table 3.6), it would represent a 98.5% higher production in the BC bioreactor compared to the SAT bioreactor.

3.5. Conclusion

This study developed a bioprocess engineering strategy for obtaining high yields of heat-resistant spores of *B. megaterium* that can enhance cell viability in the production of microbial inoculants. Our findings demonstrate that cultivating *B. megaterium* in SBM culture medium under specific conditions (37°C and pH 5.6) in a bubble column bioreactor yielded a high heat-resistant spores' concentration, while also being more cost-effective in terms of energy expenditure compared to a stirred tank bioreactor. These results provide valuable insights for bioprocess engineering and could contribute to the development of more efficient and sustainable methods for microbial inoculant production, ultimately benefiting agriculture and the environment.

4. Modulation of starch-based film properties for potential application as coating systems



4.1. Abstract

Starch-based coating systems find application in different industrial sectors as eco-friendly carriers of bioactive compounds such as in fertilizers and seeds. However, starch cannot be used as the only matrix component because of its high hygroscopicity and poor mechanical qualities. Here, we investigated the effects of the sequential addition of maltodextrin, cellulose, glycerol, and bentonite on the physicochemical characteristics of starch-based films potentially applied in coating systems. It observed that the addition of bentonite tends to increase hydrophobicity, but association with glycerol contributed to its reduction. The sequential reduction of water vapor permeability was observed, achieving lower values with the addition of bentonite. Viscosity variation was also verified, still in gel form. The matrices with or without glycerol showed a significant increase in viscosity with the addition of bentonite. The adjustment of this parameter is essential in the coating system. This trend is also observed for mechanical properties. In this case, cellulose addition provided a remarkable variation. Additionally, the water content between the starch chains is considerably reduced, as well as the brittleness and uniformity are improved with the simultaneous addition of fillers and glycerol-containing matrices.

Keywords: starch-based films, biocomposite, casting, coating system.

4.2. Introduction

Coating systems carrying bioactive compounds have been applied in several industrial sectors. Applications include edible films with probiotics and prebiotics (PAULO et al., 2021; ZOGHI; KHOSRAVI-DARANI; MOHAMMADI, 2020), seed coating with plant growth-promoting microorganisms (AFZAL et al., 2020), fertilizer coating containing nutrient-solubilizing microorganisms (MAJARON et al., 2023), and nanocomposites containing bioactive compounds for packaging application (SHARABY et al., 2022). The development of

eco-friendly carriers should seek the preservation of both the coated system (for example, the shelf life of the seeds) and the bioactive compounds (for example, microorganisms). It is also desirable that, when required, the matrix acts as a nutritional source for bioactive cells, such as microorganisms, playing an essential role in cell release and multiplication. Thus, gelatin (GIRARDI et al., 2018), gums (HUANG et al., 2019), cellulose and its derivatives (AMIRKHANI et al., 2016), alginates (BERNINGER; MITTER; PREININGER, 2016), and starch (VERCELHEZE; MARIM; OLIVEIRA, 2019) are some of the biopolymers used alone or in combination with other materials.

Starch has many benefits as a raw material for coating. However, this material is limited due to its high hygroscopicity and low mechanical properties, leading to water-sensitive brittle materials (AMIRKHANI et al., 2016; VERSINO et al., 2016). The modification of starch to thermoplastic starch (TPS) improves the desired properties for coating application due to the reduction of the strong interaction between amylose and amylopectin molecules, providing greater flexibility and improving its mechanical strength (MONTILLA-BUITRAGO et al., 2021). In terms of degradation during use and storage, it is highly fragile due to its organic nature, having reduced mechanical properties when exposed to hydrolytic, oxidative, and extreme temperature conditions, negatively impacting the products' quality and shelf life (XIE et al., 2014). The incorporation of fillers can improve TPS physicochemical properties to a desired level. Cellulose and bentonite fillers significantly influence the mechanical properties of starch films (FREITAS; GONZÁLEZ-MARTÍNEZ; CHIRALT, 2023). Furthermore, the ultraviolet radiation protection conferred by bentonite (MOVAHEDI et al., 2014) and cellulose (FREITAS; GONZÁLEZ-MARTÍNEZ; CHIRALT, 2023) is desirable as it could provide better protection, for example, for biologically active compounds such as encapsulated bacteria.

Any formulation should consider that an important economic factor is the water content in the matrix before and after drying and the bioactive compounds' preservation during and after drying. Water-soluble sugar-based preservatives, such as maltodextrin, can increase the total solid content of the mixture and increase bioactive cells survival in the drying process (CHURIO; VALENZUELA, 2018; MAHDI et al., 2020). Furthermore, the moisture contents of the final coating systems are critical to ensure product stability during storage. For instance, higher moisture values correlate with poor microorganisms' survival and reduced vigor of coated seeds (JACOB et al., 2016; KANG et al., 2019).

This work aims to develop a starch-based carrier material for coating systems using biodegradable, renewable, and low-cost polymers. We hypothesize that, in addition to

increasing the solids content in the precursor starch gels, the sequential addition of fillers (maltodextrin, cellulose, and bentonite) can modulate the physicochemical properties of the starch-based films, such as the morphology, thermal behavior, mechanical and barrier properties.

4.3. Materials and methods

4.3.1. Materials

Maize starch (Amidex 3001) and maltodextrin (MOR-REX 1920) were purchased from Ingredion (Brazil). Calcium bentonite clay was donated from Buntech (Brazil). Micro and nanocellulose were obtained from Tetra Pak® carton packs. The extraction methodology was adapted from BORGES et al., (2023).

4.3.2. Film preparation

All films were prepared under the same experimental conditions. Briefly, the initial components of the film (starch, maltodextrin, cellulose, bentonite, and glycerol, in different compositions according to Table 4.1) were cold homogenized and then heated in a water bath at ~ 96 °C for 25 min for the matrix gelation. Then, 0.85% NaCl solution was added to the previously prepared gel in a 1:1 (v/v) proportion. The bentonite was initially homogenized in NaCl solution and later added to the gel.

The purpose of this preparation protocol is to allow the possibility of inserting thermosensitive bioactive components into the saline solution, such as biomolecules or microorganisms (e.g., bacterial spores), because the process enables prior gelation and subsequent lowering of the temperature to approximately 40°C before adding saline solution. After the formulations, the gels were placed in silicone molds with 16.5 x 19 cm dimensions and kept at 25 °C for 48 h. An illustrative scheme of the techniques used to produce the composites, a detailed description of each formulation, and an illustrative scheme of the composite containing all these materials are described in Figure 4.1.

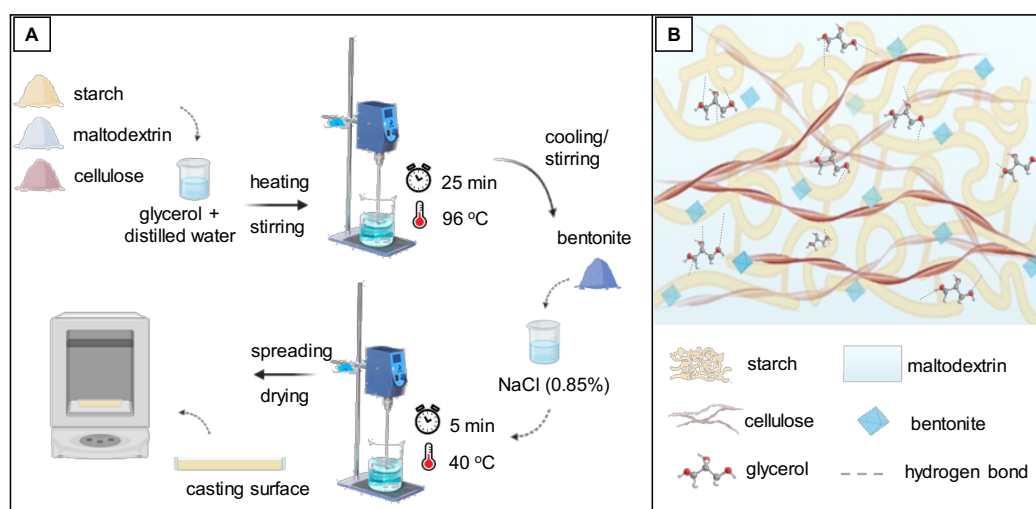


Figure 4.1. Protocol to obtain matrices based on starch, maltodextrin, cellulose, and bentonite. Schematic illustration of (A) the casting technique used to produce polymer films and (B) the complete composite of thermoplastic starch, maltodextrin, cellulose, and bentonite.

Table 4.1. Detailed mass composition of formulations based on starch (S), maltodextrin (M), cellulose (C), bentonite (B), and glycerol (Gly), with possible application as coating systems.

Sample code	Gel composition (% w/v)	Saline solution composition (% w/v)
S	starch (10%)	NaCl (0.85%)
SM	starch (10%), maltodextrin (25%)	NaCl (0.85%)
SMC	starch (10%), maltodextrin (25%), cellulose (5%)	NaCl (0.85%)
SMCB	starch (10%), maltodextrin (25%), cellulose (5%)	NaCl (0.85%), bentonite (5%)
S-Gly	starch (10%), glycerol (7.5%).	NaCl (0.85%)
SM-Gly	starch (10%), maltodextrin (25%), glycerol (7.5%)	NaCl (0.85%)
SMC-Gly	starch (10%), maltodextrin (25%), cellulose (5%), glycerol (7.5%)	NaCl (0.85%)
SMCB-Gly	starch (10%), maltodextrin (25%), cellulose (5%), glycerol (7.5%)	NaCl (0.85%), bentonite (5%)

Using a JEOL microscope type JSM 6510, scanning electron microscopy (SEM) was used to examine the films' morphology. The carbon tape that was applied to the surface of a metallic disc (stub) was covered in samples. After being coated with carbon, the disc was scanned in the secondary electron mode using a Leica EM SCD050 chamber.

To determine the phases present in the material, all films were characterized using X-ray diffraction (XRD) (Shimadzu) in a ShimadzuXRD-6000 diffractometer using $\text{CuK}\alpha$ radiation

($\lambda = 1.54178 \text{ \AA}$). The relative intensity was measured within the 5–50° diffraction range (2 θ). The voltage was 30 kV, the current was 30 mA, and the scanning speed was 1° min⁻¹. Using a Thermogravimetric Analyzer TGA-Q500 (TA Instruments, USA) in an air atmosphere at a flow rate of 60 mL/min, Thermal Gravimetric Analysis (TGA) was used to assess the thermal behavior and stability of the films. At 10 °C per minute, the materials were heated from about 25 °C to 700 °C.

Using the attenuated total reflection (ATR) method and a spectrophotometer called VERTEX 70 (Bruker Corporation), Fourier-transform infrared spectroscopy (FT-IR) was used to assess the molecular structure of the composites both before and after UV degradation behavior. At room temperature, 64 spectra scans with wavenumbers between 400 and 4000 cm⁻¹ and a nominal resolution of 2 cm⁻¹ were recorded.

4.3.3. Hydrophilic Properties

The composites' hydrophilicity was studied by the moisture content, contact angle, and water vapor permeability (WVP) of the matrices. Moisture was gravimetrically determined by heating them to 105 °C using an ID50 infrared balance (Mars Scientific, Brazil) until constant weight. Water contact angle measurements were performed with five replicates using the BiolinScientific Optical Tensiometer (Theta Lite Model). Water contact angles were measured from 0 to 60 seconds after the droplet landed on the film surface, and images were recorded every 1 second. The WVP was determined gravimetrically at 25 °C by the ASTM E96-10106 method. The glycerol films were applied in permeation cells of 2.4 cm diameter with 2 mL of distilled water inside. These cells were then placed in an Ethik Technology climate chamber (Model 420-2TS 295 L, Brazil) with 50% RH at 25°C and monitored by weighing for 36 hours at least 1-hour intervals. The WVP was calculated from Equation (1). This experiment was carried out with eight replicates of each material.

$$\text{WVP} \left(\frac{g \cdot mm}{kPa \cdot m^2 \cdot h} \right) = \frac{\Delta m * e}{\Delta p \times A \times \Delta t} \quad (1)$$

where:

$$\frac{\Delta m}{\Delta t} = \text{lost mass difference over time} \left(\frac{g}{h} \right)$$

A = exposed area for moisture transfer (m²)

e = film thickness (mm)

Δp = water vapor pressure gradient (kPa)

4.3.4. Viscosity and Mechanical properties

The viscosity measurements were performed using an Anton Paar–Physica MCR 301 Rheometer (Anton Paar, GmbH, Germany). The measuring system had a 27 mm inner diameter concentric cylinder geometry (CC27). The rheometer utilized a temperature-controlled water bath and a Peltier heating system for accurate control. The shear rate ($\dot{\gamma}$) was set from 1 to 1000 1/s, totaling 50 points. The test temperatures (25 °C and 40 °C) were set before adding the material, and the run started when the test material achieved equilibrium temperature. The measurements were performed twice to obtain the average values.

The TA.XTplus Texture Analyser (Stable Micro Systems, Godalming, Surrey, UK) was used to assess the films' mechanical properties using tension tests per ASTM D882-10 (2010) by the American Society for Testing and Materials. The film samples utilized in the testing had dimensions of 10 mm × 50 mm and were cut using sharp scissors. Samples were clamped between grips, and at an initial distance of 20 mm between the grips, force/deformation was recorded during extension at 0.5 mm/sec. In this way, tensile strength (calculated by dividing the maximum force by film cross-section (thickness x width), MPa), Young's module (slope of the force-deformation curve, MPa), and elongation at break (deformation divided by initial probe length and multiplied by 100, %) were determined from ten replicates for each film formulation. Films were conditioned at 25°C and 50% RH for three days before testing.

4.4. Results and Discussion

4.4.1. Physiochemical characterization

Figure 4.2 shows the macroscopic and microscopic morphological characteristics of the starch-based matrices containing the different fillers (maltodextrin, cellulose, bentonite – with and without glycerol). The macroscopic (Fig. 4.2A) and microscopic (Fig. 4.2B) characteristics of the films without glycerol were significantly altered after maltodextrin addition. All SM-based samples are fragile and brittle, a characteristic observed by the cracks. Adding cellulose and bentonite makes the film less brittle, but minor cracks remain. However, this characteristic practically disappears for the films with glycerol. Small cracks remain only in the 10% starch, 25% maltodextrin, and 7.5% glycerol matrix. These findings suggest the importance of the plasticizer in starch films, especially when additional fillers tend to impair their characteristic plasticity (BEHERA; MOHANTA; THIRUGNANAM, 2022; TARIQUE; SAPUAN; KHALINA, 2021).

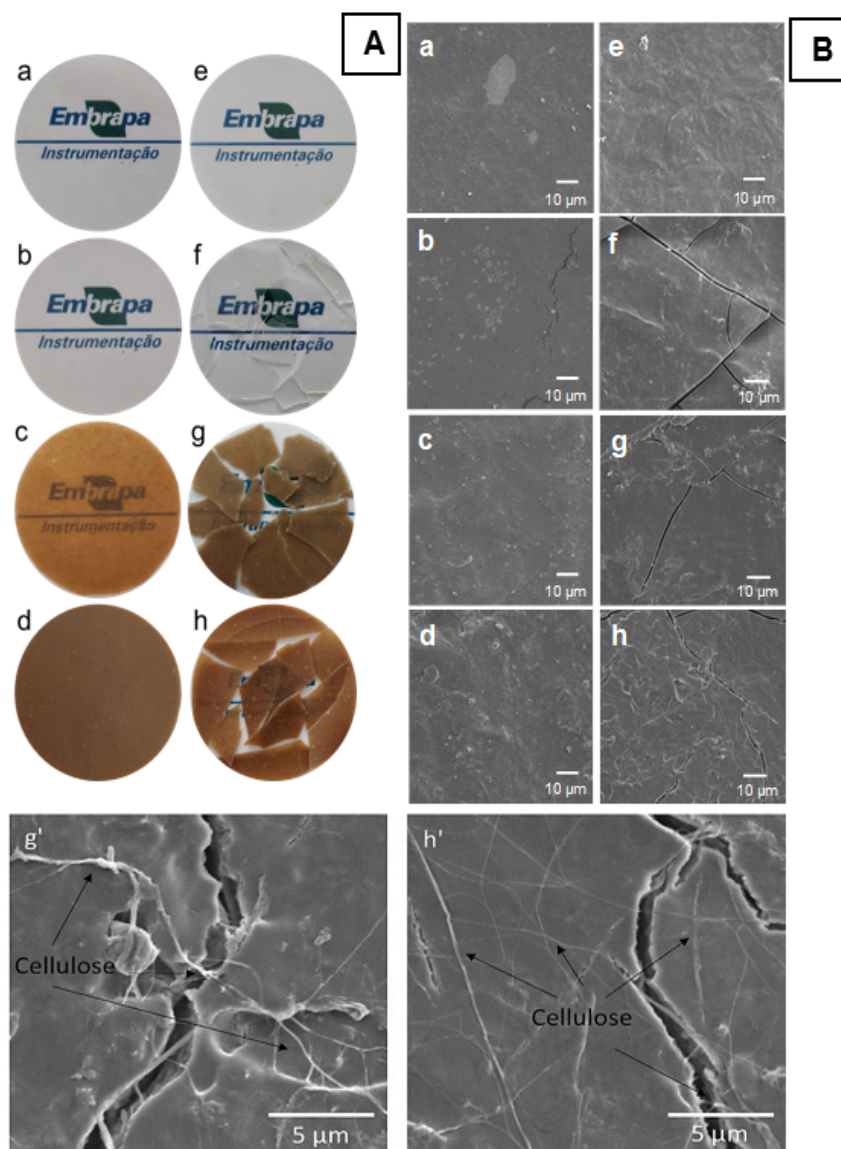


Figure 4.2. Morphological characteristics of matrices. A) Pictures and B) SEM images of starch film 10% with (a) and without glycerol (e); starch film 10% containing maltodextrin 25% with (b) and without glycerol (f); starch film 10% containing maltodextrin 25%, cellulose 5% with (c) and without glycerol (g); starch film 10% containing maltodextrin 25%, cellulose 5%, and bentonite 5% with (d) and without glycerol (h).

The plasticizer role in the matrix improves the component's compatibility since the cellulose fibers in films without adding glycerol are observed (Fig. 4.2 g', 4.2 h'). On the other hand, for the analogous films containing glycerol, the cellulose fibers are not easily observed, indicating that they are finely dispersed in the matrices. The good interfacial adhesion between the cellulose fiber and the matrix indicates better chemical compatibility, potentially leading to better stress transfer and increased mechanical properties.

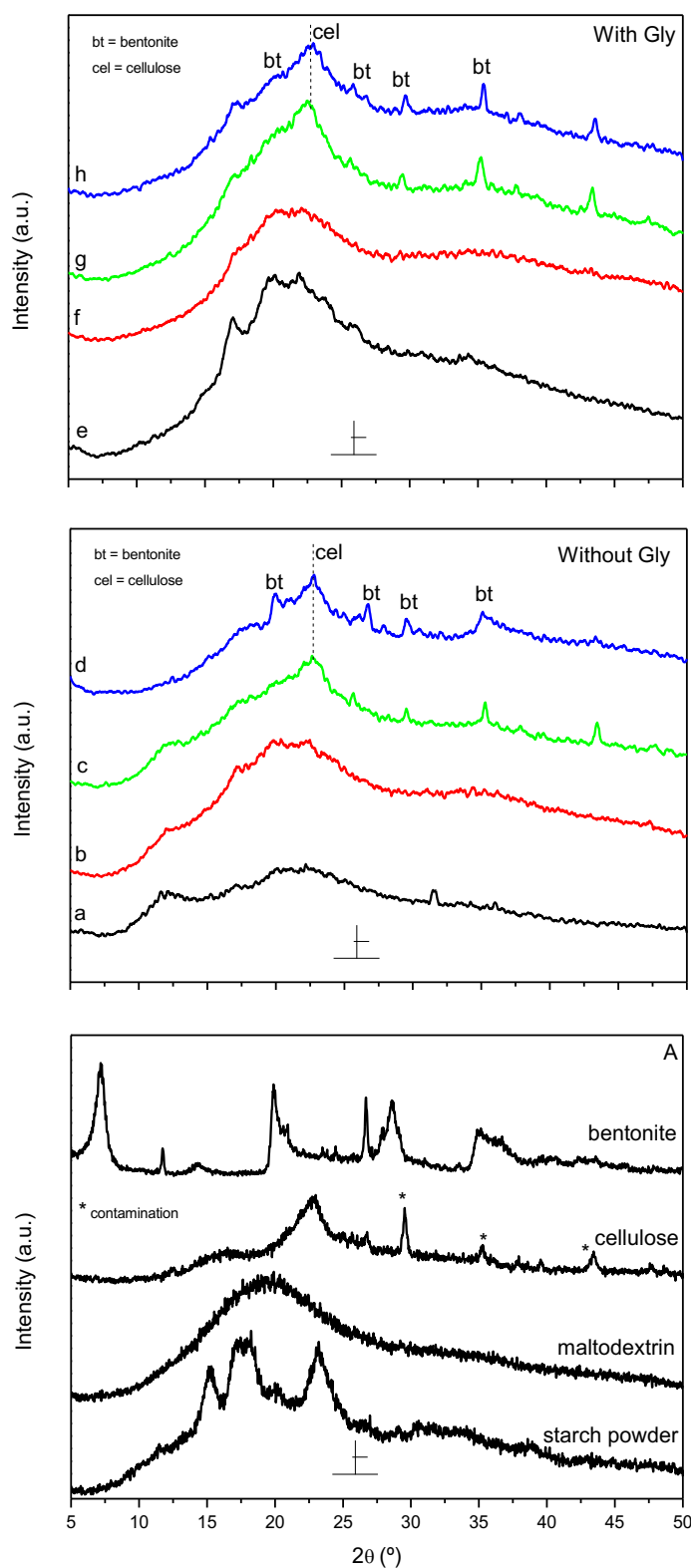


Figure 4.3. XRD pattern of the precursor materials (A) and starch film 10% without (a) and with glycerin (e); starch film 10% containing dextrin 25% without (b) and with glycerin (f); starch film 10% containing dextrin 25%, cellulose 5% without (c) and with glycerin (g); starch film 10% containing dextrin 25%, cellulose 5%, and bentonite 5% without (d) and with glycerin (h).

After the gelation process in the starch back-hydration, the polymeric chains are disorderly rearranged since the characteristic peaks of the crystalline phases of the starch are practically absent (WANG et al., 2019). This effect is less pronounced in the film with glycerol. Maltodextrin is an initially amorphous material. Therefore, its addition in large amounts in the starch matrix increases the amorphous profile of the films obtained. With the addition of the two crystalline fillers, cellulose, and bentonite, the XRD profile of the samples now presents some of the prominent peaks related to these materials (GONG et al., 2017). The suppression of the bentonite basal peak at 7.2° (001) indicates the montmorillonite delamination (CORTES-ROJAS; SOUZA; OLIVEIRA, 2017), which can significantly alter the barrier and mechanical properties of the starch matrix.

Thermogravimetric Analysis (TGA) analyzed the films' thermal behavior. The thermal decomposition of the starch films under the N_2 atmosphere shows two main decomposition regions (Fig. 4.2S). The first step up to $200^\circ C$ is attributed to water-releasing content. The second step at $250^\circ C$ is related to the overlapping of the polysaccharide decomposition components: starch, maltodextrin, and cellulose. The profile above $300^\circ C$ suggests the formation of stable carbonaceous materials up to $700^\circ C$ (GAO et al., 2021). There is a clear difference between the films with and without glycerol by DTG curves, specifically in the second region of thermal decomposition (Fig. 4.2S B). Films without glycerol have lower thermal decomposition peaks than their respective glycerol-containing counterparts, an effect particularly evident for pure starch films. It suggests increased interactions between starch molecules and/or the other matrix components when glycerol is present (NORDIN et al., 2020). Without glycerol, the incorporation of insoluble components (cellulose and bentonite) tends to decrease the flexibility of the amylopectin chains (SANTANA et al., 2017), probably acting as impurities in the starch polymeric network, which would explain the reduction in thermal stability.

Figure 4.1S shows the infrared spectroscopy (FTIR) spectra of the starch-based composites. The bands at 3200 to 3600 cm^{-1} are related to the axial deformation of OH, the band at 2935 cm^{-1} is related to the CH stretch, and the band at 1157 cm^{-1} is related to the C-O-C glycosidic bond. The bands observed at 1157 , 1108 , 1082 , and 928 cm^{-1} are related to C-O and C-C elongation; at 1644 cm^{-1} , it can be attributed to water bounded to starch (BODIRLAU; TEACA; SPIRIDON, 2013; REN et al., 2017; WONGPHAN; HARNKARNSUJARIT, 2021). The bands at 1460 , 1248 , and 857 cm^{-1} are related to the vibrations of the CH_2 group (BODIRLAU; TEACA; SPIRIDON, 2013; PAVLOVIC; BRANDÃO, 2003). Although all

formulations have very similar spectra, significant differences are observed in formulations according to glycerol addition due to starch and glycerol bands overlapping (MARIANO-TORRES; LÓPEZ-MARURE; DOMIGUEZ-SÁNCHEZ, 2015).

4.4.2. Hydrophilic Properties

Moisture for the composites (Fig. 4.4C) shows a good correlation with the data obtained by TGA (Fig. 4.4A, 4.4B and 4.2S), which demonstrated that by comparing all samples, pure starch films (S and S-Gly) present higher water content, 7.00% and 10.40% at 105 °C, respectively, associated with the existence of hydrogen bonds between the chains of amylose and amylopectin formed by water molecules (BASIAK; LENART; DEBEAUFORT, 2018). In general, the added fillers tend to reduce the samples' water content, and this is particularly evident for films with glycerol.

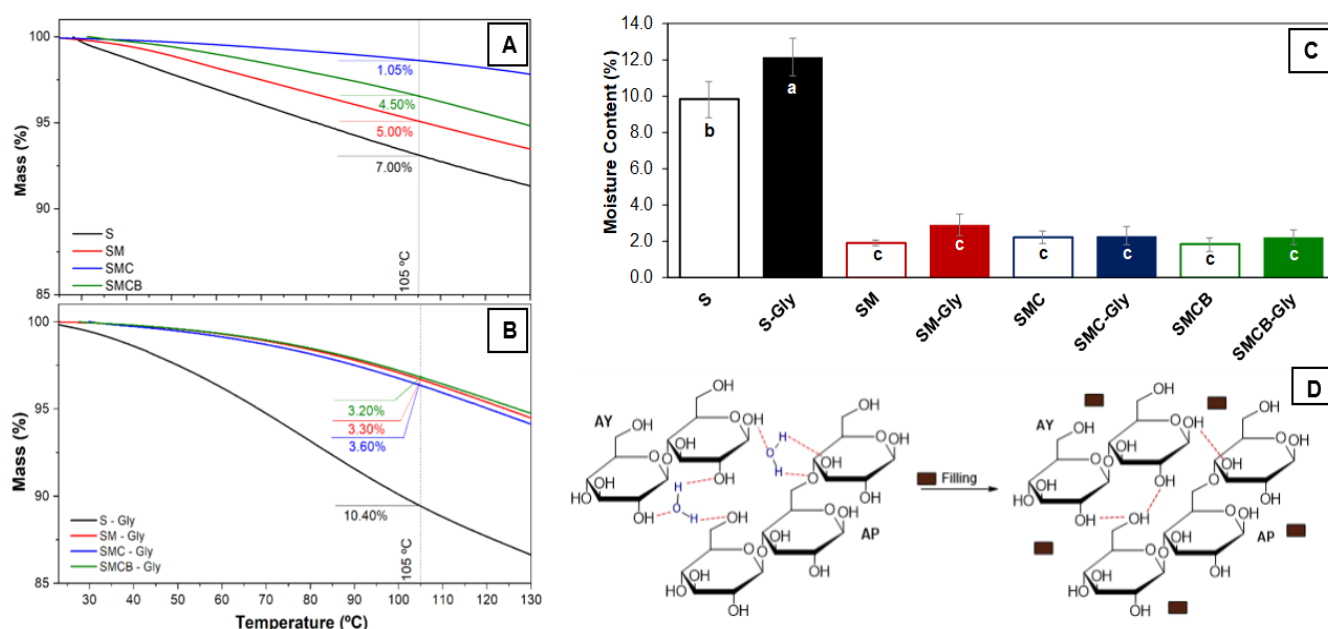


Figure 4.4. Residual moisture in starch matrices promises to carry biologically active components in the coating. A) TGA curves of starch-based films with glycerol and B) without glycerol. C) Moisture content of starch matrices; D) Schematic representation of the modification of water content between amylose (AY) and amylopectin (AP) molecules. Values sharing the same letter are not significantly different by Tukey's test ($p < 0.05$). S: starch; M: maltodextrin; C: cellulose; B: bentonite; Gly: glycerol.

The matrices with glycerol showed a slightly higher moisture content than the material without glycerol. Still, it was only statistically significant for the matrix containing only starch (Fig. 4.4C). The moisture content of all starch films with the addition of fillers studied was less

than 3%, with no statistical difference at 95% significance between treatments, being suitable for long-term storage (CHEW; TAN; NYAM, 2018; PREMI; SHARMA, 2017). The lower water/moisture content of films with added fillers can be explained by a breakage of amylose (AY) and amylopectin (AP) chains so that intertwining with other starch chains occurs by intermolecular hydrogen bonds (as opposed to extra molecular hydrogen bonding with water), leading to a lower water loading capacity (Fig. 4.4D). Moisture content is a critical aspect correlated with water activity, fluidity, viscosity, drying efficiency, oxidation of bioactive agents, and microbial growth (MAHDI et al., 2020). High moisture content can affect storage stability, leading to degradation and release of encapsulated material earlier than desired (KANG et al., 2019), as well as affecting, for example, seed longevity by inducing deterioration and negatively impacting seed vigor (JACOB et al., 2016).

The contact angle and WVP are shown in Figures 4.5 and 4.6, respectively. The contact angle reflects the surface hydrophobicity and wetting properties. Contact angle lower than 90° indicates a hydrophilic surface with low surface hydrophobicity and high wettability of the films (WONGPHAN; HARNKARNSUJARIT, 2021). On the other hand, WVP is attributed to the absorption behaviors, diffusion, and desorption of water vapor. Thus, surface hydrophobicity, solubility, and molecular diffusion significantly impact this characteristic (GAUDIN et al., 2000). WVP was analyzed only for materials containing glycerol since materials without glycerol were fragile and brittle.

In general, the glycerol addition has more influence on materials whose contact angle is higher than 90° . That is, S and SMCB films, reducing this value and making them hydrophilic (from $111.6 \pm 3.1^\circ$ to $61.5 \pm 2.0^\circ$ and from $99.7 \pm 6.9^\circ$ to $49.8 \pm 1.6^\circ$, respectively). Maltodextrin reduced the contact angle in films probably due to its influence on reducing water content after drying, though not statistically significantly different for WVP, considering 95% significance. This behavior can be related to the higher wettability of the maltodextrin molecules in the composites.

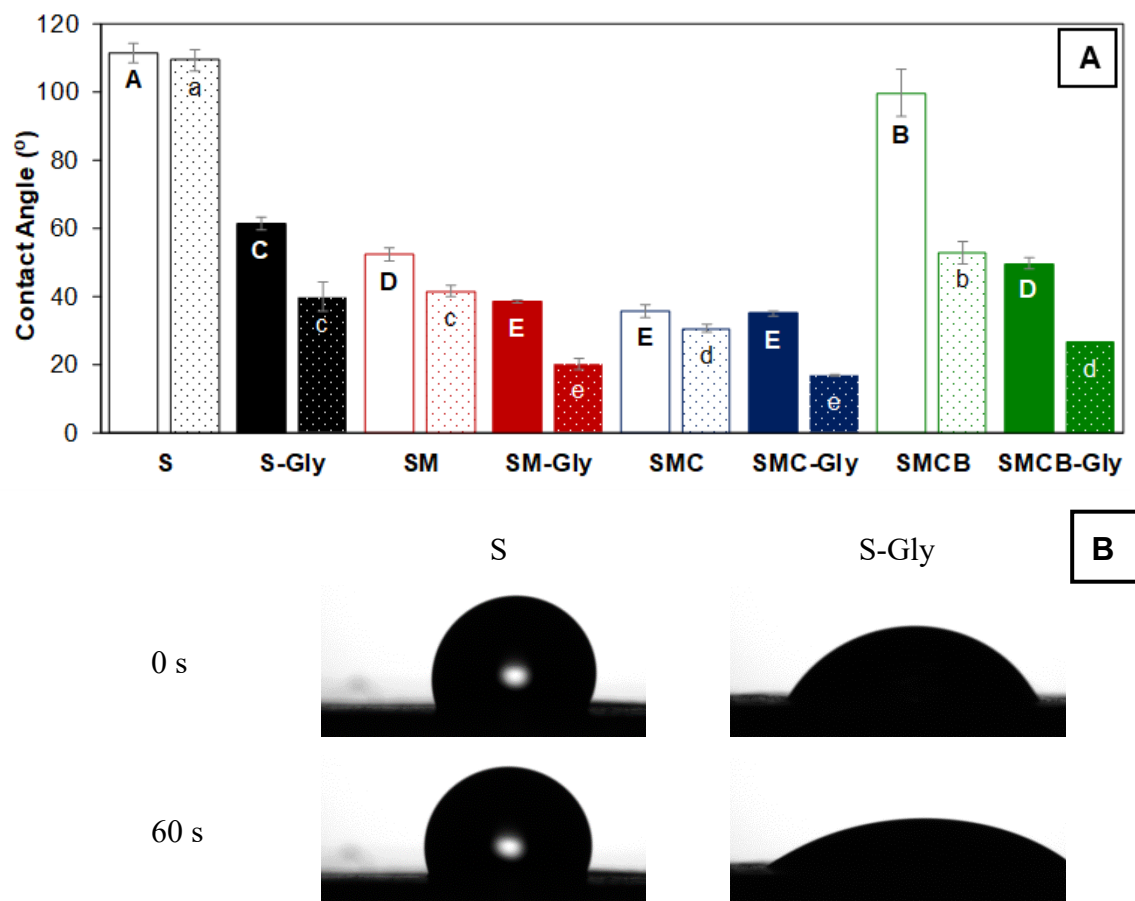


Figure 4.5. Barrier properties of starch matrices with the addition of fillers. A) Contact angle as the drop comes into contact with the material (smooth surface) and after 60 seconds (cross-hatched surface) of the starch matrices. B) Photo of the water droplet in contact with the starch matrix without and with glycerol, representing a hydrophobic and hydrophilic material, respectively. According to Tukey's test, values that do not have the same letter are statistically different ($p < 0.05$). The capital letter was used for the angle values when the drop of water came into contact with the material, and the lower-case letter for the angle after 60 minutes. S: starch; M: maltodextrin; C: cellulose; B: bentonite; Gly: glycerol.

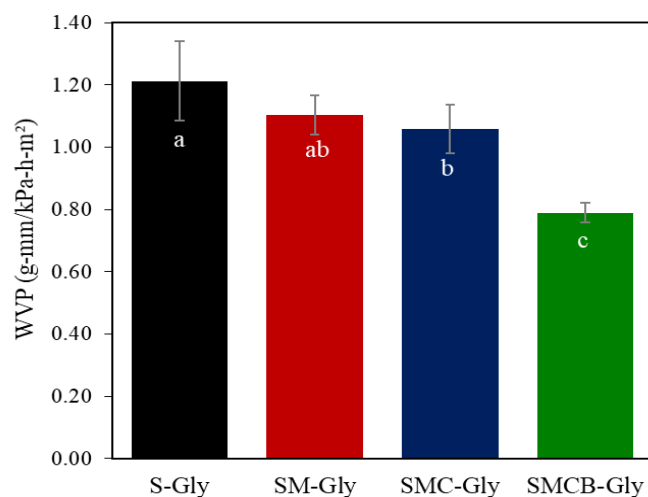


Figure 4.6. Water vapor permeability (WVP) of starch matrices with glycerol. Tukey's test shows values with different letter combinations are statistically different ($p < 0.05$). S: starch; M: maltodextrin; C: cellulose; B: bentonite; Gly: glycerol.

Incorporating cellulose in the SM and SM-Gly matrices did not present statistical differences in the contact angle or for the WVP, indicating no significant change in the hydrophilicity. However, a significant reduction is observed for WVP concerning the S-Gly film. This result can be explained by the crystalline domains, which arise when cellulose nanofibers are uniformly distributed throughout the polymeric matrix and exhibit a high degree of orientation (SVAGAN; HEDENQVIST; BERGLUND, 2009). These domains function as a labyrinth by preventing water vapor from diffusing through the matrix, lengthening the diffusion path, and causing tortuosity (GARCIA et al., 2009; SANTANA et al., 2017; YU; DEAN; LI, 2006).

Including bentonite in the SMC and SMC-Gly matrices significantly increased the contact angle and reduced the WVP, indicating increased hydrophobicity on the film's surface. MÜLLER; LAURINDO; YAMASHITA, (2011) and SHANMATHY; MOHANTA; THIRUGNANAM, (2021) also found a reduction in WVP and an increase in the contact angle with increasing bentonite concentration in the starch film. The permeation rate in the polymer depends on the number of permeant molecules, presence of voids in the matrix, density, and degree of crosslinking of the chains—the more compact the matrix structure, the lower the permeability. Bentonite also presents the maze effect, which increases the barrier properties of the films due to the delay in gas exchange or even the entrapment within the bentonite structure, which may explain the results found with the addition of this filler (KONGKHLANG et al., 2008).

The hydrophilic properties of starch-based films can be adjusted on demand using the proposal fillers. For instance, moisture content can be decreased by 70-80% to promote bioactive species protection in the matrix, such as microorganism cells. Moreover, if high hygroscopic matrices are required, maltodextrin would be a potential alternative. If a starch-maltodextrin matrix is essential but does not increase the hygroscopic character, bentonite could be added to the matrix. However, glycerol in the films generally tends to increase hydrophilic behavior, probably due to its high affinity for water molecules. The results showed that the simultaneous addition of cellulose and bentonite to the starch matrix is an effective way to adjust the barrier properties (permeability to water vapor), a critical aspect, for example, in seed coating application (BABAEE et al., 2015; GHANBARI et al., 2018a, 2018b).

4.4.3. Viscosity and Mechanical properties

The viscosity of gels/films is a property that must often be adjusted according to the application of interest. For example, in seed coating, the gel viscosity must be adequate to the point that the film adheres to the seed and forms a smooth and homogeneous layer in the coating and drying process. Gels with very low or very high viscosity are typically unsuitable for seed coatings (AFZAL et al., 2020; CISNEROS-ZEVALLOS; KROCHTA, 2003).

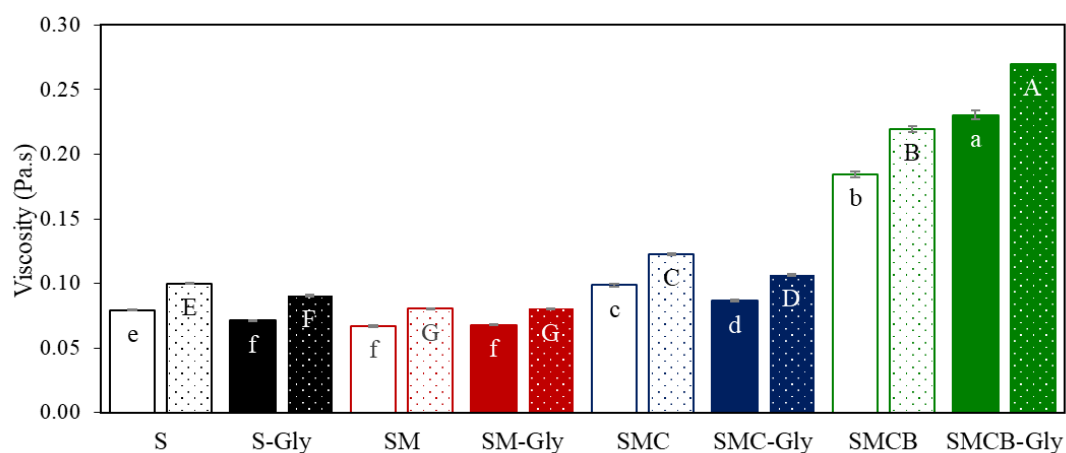


Figure 4.7. The viscosity of starch matrices with adding fillers at 25°C (cross-hatched surface) and 40°C (smooth surface). According to Tukey's test, values that do not have the same letter are statistically different ($p < 0.05$). The capital letter was used for 25 °C, and the lower-case letter for 40 °C.

In Figure 4.7, the viscosity data of the composite gels obtained before the drying step are presented for temperatures of 40 °C (approximate temperature for Industrial Seed Treatment) and 25 °C (ambient temperature). In general, the following behavior was observed regarding

the viscosity of the gels: i) a slight reduction in viscosity after the incorporation of maltodextrin; ii) then, with the sequential addition of cellulose, the viscosity tends to increase; iii) the most significant increase comes after the addition of bentonite, representing a threefold increase.

The initial viscosity reduction behavior with the addition of maltodextrin can be explained by the breaking of the entanglement of amylose and amylopectin chains, thus increasing their mobilities (HUANG et al., 2020). Cellulose and bentonite as swellable materials (BRACHMAN et al., 2021; NGENE; ROUX; LACHENAL, 2022) tend to reduce water availability between the starch polymer chains, reducing their mobility. In addition, as they are crystalline and water-insoluble materials, their dispersion in the matrix of starch and maltodextrin tends to restrict the mobility of the starch chains, changes the starch self-association mechanism, which causes an increase in the viscosity of the gels (DÍAZ-CALDERÓN et al., 2022; ODEH; EDEGBE; OYEDOH, 2021).

For all films, there was an increase in viscosity between 0.0126 Pa.s (SM-Gly) and 0.0395 Pa.s (SMC-Gly-B), decreasing the temperature from 40 °C to 25 °C. It is due to the greater intermolecular cohesion force formed when the temperature is reduced and the water evaporates, increasing the viscosity (ZHOU et al., 2018). However, when comparing gels with and without glycerol, there is no clear relationship to explain an increase or decrease in viscosity between different materials. Therefore, it can be inferred that the viscosity of starch gels can be adjusted according to the demand and application of interest. For example, to increase viscosity, bentonite can be added.

The mechanical properties of matrices composed of starch, maltodextrin, cellulose, bentonite, and glycerol are shown in Table 4.2. Regarding the tensile strength, it is observed that there was no statistical difference when adding maltodextrin to the starch matrix. However, the highest elongation was obtained among all formulations, and a significant increase in Young's modulus was probably due to the plasticization effects. WONGPHAN; HARNKARNSUJARIT, (2021) observed that when maltodextrin was added to a starch matrix, this material did not form joint networks and acted as a filler (filler material) in the formulations, resulting in a reduction in tension and an increase in elasticity, corroborating what was observed.

Table 4.2. Mechanical properties (tensile strength, percent elongation at break, and Young's modulus) of formulations based on starch and glycerol.

Sample code	Tensile Strength (MPa)		Percent elongation at break (%)		Young's Modulus (MPa)	
S-Gly	1.721 ± 0.075	c	65.99 ± 4.704	b	8.706 ± 0.566	d
SM-Gly	1.754 ± 0.088	c	101.8 ± 9.457	a	34.97 ± 2.428	c
SMC-Gly	6.542 ± 0.114	b	8.725 ± 0.599	c	317.0 ± 9.453	b
SCMB-Gly	9.989 ± 0.173	a	4.865 ± 0.269	c	413.8 ± 9.730	a

Values that do not share the same letter are significantly different by Tukey's test ($p < 0.05$).

The cellulose addition to the SM-Gly matrix significantly increased the tensile strength (280%) and Young's modulus (36 times). It decreased the elongation (7.5 times), indicating a stiffness increase and reduction of the plasticity of the material, corroborating previous data from previous data (CHENG et al., 2019; JOHAR; AHMAD, 2012; KALIA et al., 2011). The increase in Young's modulus may be related to typical cellulose stiffness and strength. The reduction in elongation may be due to the strong interaction of the cellulose with the matrix (SANTANA et al., 2017). According to CHANG et al., (2012), Strong interactions between fillers and starch macromolecules can increase the film's tensile strength at the expense of the polymer chains' flexibility and extensibility. Generally speaking, cellulose fibers can be added to polymer matrices to create three-dimensional networks that enhance the composite's qualities (ALLOUI et al., 2016).

By including bentonite in the SMC-Gly formulation, there was a significant increase in tensile strength and Young's modulus but no significant difference in elongation, despite having reduced it to approximately half of its value, as also previously observed in the literature (CURVELO; DE CARVALHO; AGNELLI, 2001; OSMAN et al., 2020). DE MORAES; MÜLLER; LAURINDO, (2011) discovered that including nanoclay and cellulose fibers affected the values of Young's modulus. Still, the cellulose fibers' activity can be mainly attributed to the rise in the film's tensile strength. Although the effect of the cellulose fibers appeared to be predominant, the nanoclay concentration significantly impacted the films' ability to elongate.

The increase in tensile strength of TPS using bentonite as a filler may be associated with polar interactions, mainly hydrogen bonds between hydroxyl groups of starch molecules and silicate/clay (MÜLLER; LAURINDO; YAMASHITA, 2012). In this case, the alkaline cations in the bentonite structure provide interfacial interactions with the starch matrix, improving the layered exfoliation of the clay mineral and the mechanical properties of the composite.

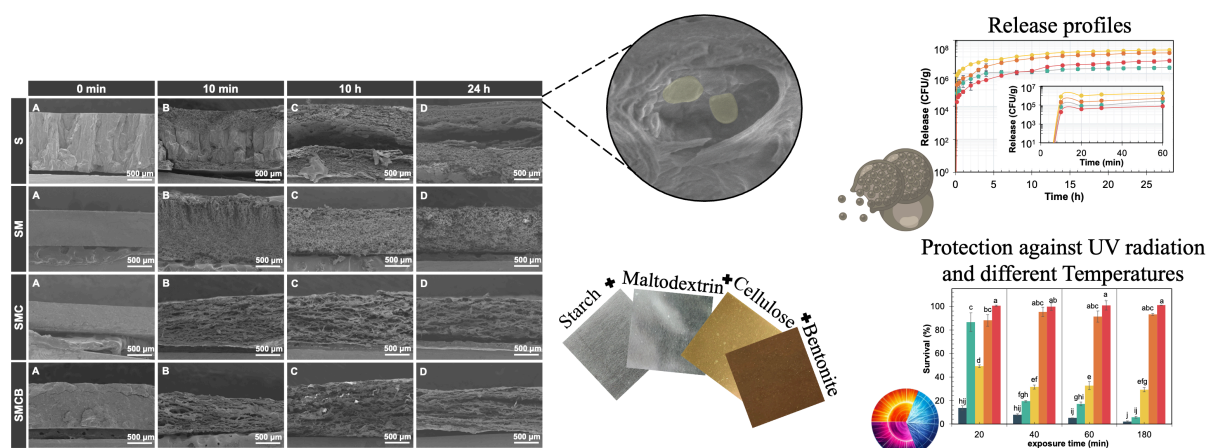
Furthermore, incorporating up to 6% of the clay content reduces elongation at break, corroborating the data. It can be explained by the fact that layered silicates can provide some new nucleation sites and contribute to the growth of crystallites (CYRAS et al., 2008).

Viscosity and mechanical properties adjustments are more related to the matrices' processing part and shelf life. For instance, among the proposed fillers, bentonite represents the most effective filler to adjust viscosity on demand depending on the coating system used. From the viscosity and mechanical properties is also possible to highlight the potential modulation of the matrices. The results indicate that hybrid fillers (cellulose and bentonite) significantly increased the tensile tenacity of the starch. In contrast, the addition of maltodextrin improved the elongation of the material. High material strength depends on how starch-based a matrix is applied, such as when covering seeds to prevent germination and possible damage during handling, transportation, and planting (QIU et al., 2020), i.e., highly rigid materials are avoided.

4.5. Conclusion

This investigation aimed to develop a carrier material with high solids content from the gelled starch matrix produced by casting, with favorable or adjustable characteristics as a carrier matrix for bioactive compounds, such as microorganisms' cells potentially applied, for example, in seed coatings. Adding fillers in the starch matrix results in a considerable increase in solids content, an exciting feature to minimize drying steps. Furthermore, the different fillers reduce the residual water content of the films, as well as improve their mechanical and morphological characteristics, mainly due to the addition of bentonite and glycerol, respectively. In our approach, it is possible to modulate the properties of starch-based films according to the demand for coating films. Therefore, the proposed filler components are promising to adjust properties to apply the starch-based matrix as a coating system better.

5. Modulation of starch-based film properties for encapsulation of microbial inoculant



5.1. Abstract

Controlled release of beneficial microorganisms in agriculture using polymeric matrices can improve biofertilizer efficacy, but it requires the modulation of properties to ensure more efficient and predictable release patterns. This study investigated the effect of a starch-based system to protect and release *Priestia megaterium* (former *Bacillus megaterium*), processed as films modified with potential cell-protective additives (maltodextrin, cellulose, and bentonite). The release kinetics, physicochemical and morphological film characteristics, and their protection against UV radiation and temperature were evaluated. The microorganism release was dependent of the film microstructure and composition, both in initial and extended-release rates. Maltodextrin incorporation increased cell release, while cellulose and bentonite delayed due to influences in the water uptake (swelling) and diffusion across polymer structure. Modified films protected the microorganisms against UV radiation and extreme temperatures, being the film with all additives (SMCB) the best protective formulation (100% UV_C survival) compared to starch matrix (< 20% UV_C survival after 40 min). These insights pave the way for targeted, efficient, and sustainable agricultural practices, aligning with evolving needs in modern agriculture.

Keywords: carrier, controlled release, kinetic behavior, abiotic stresses protection, plant growth promoting bacteria, sustainable agriculture.

Highlights

- Maltodextrin aids early microbial release and microbial growth
- Cellulose ensures stable, prolonged microbial release post-burst
- Bentonite enhances microbial retention and protects against environmental stressors
- SMCB film: promising formulation with controlled release & UV/temperature protect

- Advancements for controlled and extended-release biofertilizers

5.2. Introduction

The challenges of sustainable agriculture demand innovative approaches to address climate change and the environmental risks of intensive agriculture. The increase in demand for food has led to excessive utilization of fertilizers, resulting in environmental problems such as eutrophication, acidification, salinization, and soil contamination (KOPITTKKE et al., 2019). In this context, Plant Growth-Promoting Bacteria (PGPB) emerged as a promising alternative to reduce fertilizer application by improving nutrient absorption, as well as promoting phytohormone production and resistance to environmental stresses (SHAH et al., 2021; VELLOSO et al., 2020).

PGPB effectiveness depends on the ability of these microorganisms to survive, multiply, and disperse within the agricultural environment. PGPB encapsulation in biopolymeric matrices, making controlled-release systems, can protect them against biotic and abiotic stresses, enable gradual release, and facilitate prolonged storage without compromising microorganism viability (BRONDI et al., 2022; LOPES; OLIVEIRA-PAIVA; FARINAS, 2023; VEJAN et al., 2019). The challenges to obtain a functional product include a mild route to process the encapsulating polymer, adequate for the microorganism medium conditions (pH, temperature); an application route adequate to the desired delivery route; and a material microstructure that allows the water transference and the microorganism protection when dry. Finally, specific properties of the encapsulating material (e.g., water uptake of superabsorbent polymers, beneficial under water deficit conditions (SABERI RISEH et al., 2023), or UV-absorption capacity of mineral loads (KIM et al., 2020; MOVAHEDI et al., 2014)) should be taken in account.

A typical application is in seed coating, where the goal frequently is the increase of seed germination and vigor. Thus, fast microorganism release is necessary (MA, 2019). On the other hand, if the target is an action at different stages of the plant's life cycle, the gradual release tends to be more advantageous (VELLOSO et al., 2020). A detailed analysis of the interplay among carriers, additives, microorganisms, and plant systems is essential for understanding the attributes of immobilized PGPB and devising applicable strategies for agriculture.

Here we investigate the release mechanisms and protection of *Priestia megaterium* in starch-based formulations for coating, using three modifiers – maltodextrin, a starch-miscible carbohydrate used in microorganism cultivation media; cellulose, a fibrous modifier used to

reinforce starch matrices, but also a carbon source for microorganism growth after reactivation; and bentonite, a mineral phase used to reinforce starch but not used as nutrient source by microorganism. Our findings contribute to understanding the release mechanisms of microorganisms within modified starch-based films with additives, highlighting the influence of these modifications on the physicochemical and morphological characteristics of the films, as well as on protection against adverse environmental factors. These insights are pivotal for developing effective controlled-release systems tailored to specific applications in sustainable agriculture.

5.3. Materials and methods

5.3.1. Materials

Maize starch (Amidex 3001) and maltodextrin (MOR-REX 1920) were purchased from Ingredion (Brazil). Calcium bentonite clay was donated from Buntech (Brazil). Micro and nanocellulose were obtained from Tetra Pak® carton packs using the extraction methodology proposed by BORGES et al., (2024). AMG 300L from *Aspergillus niger* (EC 3.2.1.3; glucoamylase; amyloglucosidase) and α -amylase BAN 480L were donated from Novozymes A/S (Bagsværd, Denmark).

5.3.2. Microorganism cultivation

Priestia megaterium CNPMS B119 was utilized in this work. The strain was isolated from maize genotypes efficient in phosphorus utilization (OLIVEIRA et al., 2009) and deposited in the Collection of Multifunctional and Phytopathogenic Microorganisms of Embrapa Maize and Sorghum (CNPMS) (Minas Gerais, Brazil).

P. megaterium B119 was cultured in Sporulation *Bacillus* Medium - SBM (pH adjusted to 5.6) at 37°C, 250 rpm for 48 hours to obtain the highest concentration of spores (VELLOSO et al., 2023a). Following cultivation, the microbial suspension was centrifuged at 8,000 rpm and 4°C for 20 minutes and then resuspended in sterile saline solution (NaCl 0.85% w/v). OD_{600nm} was determined using a spectrophotometer to establish the microorganism concentration in the suspension (OD_{600nm} = 1.0, equivalent to 10⁸ CFU/mL).

5.3.3. Film preparation by casting

The formulations assessed in this study consist of starch (S: starch 10% w/v and glycerol 7.5% w/v), starch and maltodextrin (SM: starch 10% w/v, maltodextrin 25% w/v, and glycerol 7.5% w/v), starch, maltodextrin, and cellulose (SMC: starch 10% w/v, maltodextrin 25% w/v, cellulose 5% w/v, and glycerol 7.5% w/v), and starch, maltodextrin, cellulose, and bentonite

(SMCB: starch 10% w/v, maltodextrin 25% w/v, cellulose 5% w/v, glycerol 7.5% w/v, bentonite 5% w/v). All films were prepared under similar experimental conditions.

Briefly, the initial film components (starch, maltodextrin, cellulose, and glycerol) were cold homogenized and subsequently heated in a water bath at approximately 96°C for 25 minutes to form a gel matrix. The gel matrix was then cooled to around 40°C, and a 0.85% NaCl solution containing *P. megaterium* B119 spores (10⁸ CFU/g of material) was added to the gel at a 3:2 (v/v) ratio. Bentonite was homogenized initially in the bacterial suspension and then added to the gel. Following formulation, the gels were poured into silicone molds measuring 16.5 x 19 cm and subsequently incubated at 25°C for 48 hours.

5.3.4. Cumulative release

The cumulative release of *P. megaterium* B119 from the films was recovered at various time intervals using the following procedure: film samples sized 1x1cm were weighed and immersed in 5 mL of sterile saline solution (0.85% NaCl w/v) in tubes maintained at 25°C and 150 rpm. At each subsequent time interval, the film was aseptically transferred to a new tube containing 5 mL of sterile saline solution. CFU (Colony Forming Units) from the previous tube were determined using serial dilution and plating techniques. All experiments were conducted with four replicates. The release rate (CFU/h) was defined by the angular coefficient of each point.

5.3.5. Release kinetics and mechanisms

To simulate the release mechanism and kinetics, we employed the classical Korsmeyer-Peppas model as described by KORSMEYER et al., (1983) (equation (1), $\frac{M_t}{M_\infty} \leq 0.6$).

$$\frac{M_t}{M_\infty} = kt^n \quad (1)$$

Here, M_t (CFU/g) represents the concentration of bacteria released at time t (h), M_∞ (CFU/g) denotes the amount released at infinite time, k is the release rate constant associated with the characteristics of the macromolecular network, and n represents the release exponent linked to the release mechanism. Additionally, the Higuchi model ($\frac{M_t}{M_\infty} = kt^{\frac{1}{2}}$), zero-order model ($\frac{M_t}{M_\infty} = kt$), and first-order model ($1 - \frac{M_t}{M_\infty} = e^{-kt}$) were used to analyze release profiles (LAKSHANI et al., 2023).

5.3.6. Cell viability after exposure to stressful conditions

Cell viability following exposure to UV radiation and different temperatures was evaluated for both free bacteria and encapsulated formulations. Essays were conducted in triplicate, and results were expressed as mean survival percentage $\pm$ standard deviation. In the UV exposure test, both free bacteria and encapsulated formulations were subjected to UV radiation within a chamber equipped with six UV-C lamps (Philips 15 W G15T8) for durations of 20, 40, 60, and 180 minutes. For the heat exposure test, both free bacteria and encapsulated formulations were incubated at temperatures of 15 °C and 45 °C, relative humidity 75%, for periods of 7, 14, and 21 days. Following exposure to each stress condition, sterile saline solution (NaCl 0.85% w/v) was added for free bacteria. In contrast, for encapsulated formulations, 0.1% (v/v) of the enzymes α -amylase and AMG were added to the saline solution (NaCl 0.85% m/v). The formulations were then stirred for 1 hour at 50°C and 150 rpm to facilitate matrix degradation. Subsequently, serial dilution and plating were performed under all conditions.

5.3.7. Film physicochemical and morphological characterization

Samples (1 x 1 cm) in triplicate were dried in the oven for 24 hours at 100°C, weighed (m_0), and subsequently immersed in 5 mL of sterile saline solution (NaCl 0.85% w/v). The experimental setup involved placing the samples in a 360° rotational shaker at 25°C and 150 rpm. After immersion, samples were carefully removed from the saline solution at 10 minutes, 10 hours, and 24 hours. Their surfaces were lightly dried with absorbent paper to remove excess liquid, and then they were weighed again (m_1) to evaluate swelling over different immersion times (equation (2)).

$$Swelling = \frac{m_1 - m_0}{m_0} \quad (2)$$

Following swelling assessment, the samples were dried again in an oven for 24 hours at 100°C and reweighed (m_2), allowing for the calculation of material solubility (equation (3)).

$$Solubility (\%) = \frac{m_0 - m_2}{m_0} \cdot 100 \quad (3)$$

The fracture morphology of the films was analyzed using scanning electron microscopy (SEM) with a JEOL microscope model JSM 6510. X-ray microtomography (Model 1172, SkyScan) was employed to evaluate the internal structures of the films.

The open porosity (ϕ , corresponding to the fraction of pores connected to the surface) of the materials was estimated using Archimedes' Principle (equation (4)). Briefly, film samples (1x1 cm) were dried in an oven at 100°C for 24 hours, then placed in a desiccator until they

cooled to room temperature and immediately weighed (m_{dry}). Subsequently, the films were submerged in 5 mL of mineral oil for 24 hours, followed by the application of vacuum for 30 minutes to ensure thorough pore filling. The samples were then suspended in mineral oil and weighed while fully immersed (m_{sub}). After removal from the liquid, the excess surface liquid was carefully removed, and the sample was immediately reweighed (m_{sat}). Five samples of each material were used in the experiment.

$$\phi (\%) = \frac{m_{sat} - m_{dry}}{m_{sat} - m_{sub}} \cdot 100 \quad (4)$$

The microporosity of the material was measured by evaluating images of cross sections in the software CT Analyzer v.1.14.4.1 (CTAn, Bruker, Kontich, Belgium) from an X-ray microtomography image (Model 1172, SkyScan).

UV protection percentages (S_{UV}) in three distinct wavelength regions (S_{UVA} for the range between 320 and 400 nm, S_{UVB} for wavelengths between 280 and 320 nm, and S_{UVC} for the range between 200 and 280 nm) were measured in a UV/Visible Spectrophotometer (Model UV-1600PC, VWR®) in the transmission, and calculated using equations (5-7) (LYU; GU; MAO, 2020).

$$S_{UVA} = 1 - \frac{\sum_{320}^{400} T_{\lambda} \cdot \Delta\lambda}{\sum_{320}^{400} \Delta\lambda} \cdot 100 \quad (5)$$

$$S_{UVB} = 1 - \frac{\sum_{280}^{320} T_{\lambda} \cdot \Delta\lambda}{\sum_{280}^{320} \Delta\lambda} \cdot 100 \quad (6)$$

$$S_{UVC} = 1 - \frac{\sum_{200}^{280} T_{\lambda} \cdot \Delta\lambda}{\sum_{200}^{280} \Delta\lambda} \cdot 100 \quad (7)$$

5.3.8. Statistical Analysis

All experimental results were submitted through analysis of variance (ANOVA) followed by the Tukey mean comparison test ($p < 0.05$). The Pearson correlation analysis and the subsequent heatmap visualization were conducted using the R software environment, utilizing the ComplexHeatmap library for generating the heatmap.

5.4. Results and Discussion

5.4.1. Release behavior

The bacterial release profile from all films exhibited an initial burst followed by a gradual and controlled increase in released quantity (Fig. 5.1A). A high release rate of bacteria was observed for all formulations within the initial minutes (Fig. 5.1B), which is beneficial for

competing with the existing soil microbiota and for effective colonization of the plant. The inclusion of maltodextrin in the starch matrix led to a tenfold increase in the release rate of the microorganism. Conversely, the addition of cellulose in SM film and bentonite in SMC film reduced the average release rate by approximately 40% and 70%, respectively (Fig. 5.1B). Notably, SM and SMC films showed the highest release rates and continued microorganism release throughout the experiment (Fig. 5.1A-B). On the other hand, the starch (S) film exhibited a noticeable reduction in microorganism release rate after 2 hours, while the release rate for the SMCB film gradually increased up to 10 hours. Beyond this point, the release rate for all materials declined, although the cumulative release amount continued to increase (Fig. 5.1A-B). The rapid initial cell release can be attributed to the bacteria's release from the outer layer and surface of the films, triggered by the solubilization and swelling of the materials upon contact with the liquid. Subsequently, a slower diffusion of bacteria located deeper within the films likely occurred.

The kinetics and extent of bacteria release from polymer matrices involve a combination of diffusion and dissolution processes, with the dynamics being regulated by the interactions among water, polymer, and strain properties (WU et al., 2012). The release kinetics of the microorganism in starch-based films were evaluated using four different models: the Korsmeyer-Peppas model (Fig. 5.1C), zero-order model (Fig. 5.1D), first-order model (Fig. 5.1E), and Higuchi model (Fig. 5.1F). As observed in Table 5.1, for film S with only starch, the Higuchi model provides a better fit ($R^2 = 0.9943$), indicating that the primary release mechanism is predominantly modulated by diffusion. Conversely, for all the other films, both the zero-order model and the Korsmeyer-Peppas model exhibit high correlation coefficients (SM: $R^2_Z = 0.9847$ and $R^2_{KP} = 0.9912$, SMC: $R^2_Z = 0.9930$ and $R^2_{KP} = 0.9953$, SMCB: $R^2_Z = 0.9918$ and $R^2_{KP} = 0.9914$). Zero-order kinetics defines a constant release process, which is desirable for microbial inoculants.

Employing the Korsmeyer-Peppas model (Fig. 5.1B), an assessment of the diffusion type can be conducted. The obtained 'n' value between 0.5 and 1.0 for the films S and SM indicates Anomalous (non-Fickian) transport, wherein the release is modulated by both diffusion and polymer matrix behavior, including swelling and erosion. Such mechanisms imply that the substance's migration is not purely diffusive but also involves significant structural rearrangement within the polymer, leading to a non-linear, time-dependent release profile (PARMAR; SHARMA, 2018). Moreover, it was observed that the release kinetic constant, 'k', which encompasses the overall diffusion coefficient of the solute and the geometric

characteristics of the system, was approximately thirtyfold greater for the film SM than for S. This suggests that bacteria are released from the matrix more rapidly in SM than in S, likely due to the swelling of the matrix and removal of the maltodextrin through solubilization. Furthermore, the SMC matrix presents $n = 1$ (zero-order release), while the SMCB matrix shows $n > 1$ (Non-Fickian Model – Super Case II), indicating complex release kinetics beyond traditional diffusion processes (RITGER; PEPPAS, 1987). Super Case II model suggest that polymer relaxation properties controlled the cell release and determined the release rate. Similar microorganism release mechanism model (Super Case II) has been observed in other formulations containing biopolymers and clay (HE et al., 2015; WU et al., 2012). This insight into the polymer matrix's physical and chemical nuances underscores the intricate interplay dictating the controlled release of active agents.

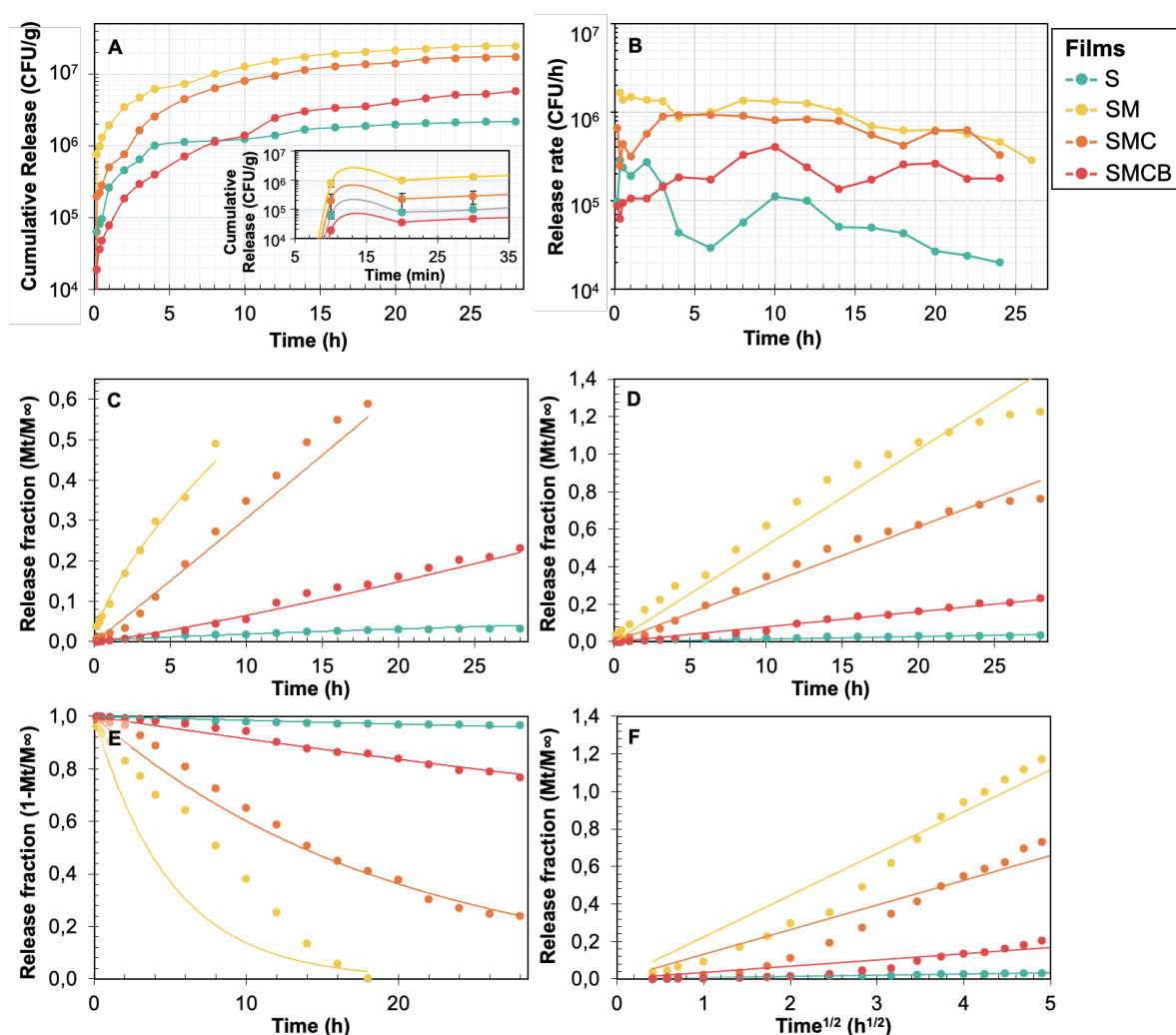


Figure 5.1. Investigation into the release mechanism and kinetics of *P. megaterium* B119 from starch-based films (A-B), employing four distinct models: (C) Korsmeyer-Peppas model, (D) zero-order model, (E) first-order model, and (F) Higuchi model. Films composition: starch (S),

starch and maltodextrin (SM), starch, maltodextrin, and cellulose (SMC), starch, maltodextrin, cellulose, and bentonite (SMCB).

Table 5.1. Release kinetic constant, n value and correlation coefficients (R^2) obtained in the release of *P. megaterium* B119 from starch-based films using the Korsmeyer-Peppas model, zero-order model, first-order model and Higuchi model.

Films	Korsmeyer-Peppas model			Zero-order model		First-order model		Higuchi model	
	k_{KP}	n	R^2_{KP}	k_Z	R^2_Z	k_F	R^2_F	k_H	R^2_H
S	0.0035	0.73	0.9627	0.0014	0.9531	0.0015	0.9197	0.0064	0.9943
SM	0.1082	0.68	0.9912	0.0512	0.9847	0.1986	0.8864	0.2227	0.9819
SMC	0.0291	1.02	0.9953	0.0307	0.9930	0.0508	0.9905	0.1313	0.9595
SMCB	0.0042	1.19	0.9914	0.0081	0.9918	0.0089	0.9858	0.0337	0.9126

Films composition: starch (S), starch and maltodextrin (SM), starch, maltodextrin, and cellulose (SMC), starch, maltodextrin, cellulose, and bentonite (SMCB).

These findings indicate that modifying the structure and properties of films using different fillers is essential for regulating the release patterns of PGPB. A polymeric matrix with rapid initial release followed by sustained release is crucial for the effective distribution of biofertilizers throughout the plant's life cycle.

5.4.2. Correlation between the controlled release, physicochemical and morphological characteristics of starch-based films

Figure 5.2 provides a visual representation of the dynamic changes in matrix characteristics during bacterial release, offering insights into determining the correlation between these features and bacterial release rate. Figure 5.3 and Figure 5.4 showcase the evolving morphology of the films over a 24-hour release period, as captured by SEM and microtomography images, respectively.

Starch film (S) exhibits a significant decline in bacterial release rate over time, followed by the lowest microorganism release after 24 hours (Fig. 5.2A-B). This behavior indicates a preferential release of bacteria from the material's surface and outer regions during the initial stages, with a tendency to retain deeper-located microorganisms. The starch film demonstrated the highest swelling rate and the lowest solubility (Fig. 5.2C-D). While overall porosity increased with time, open pore count remained relatively low (Fig. 5.2E-F). Morphological analyses (Fig. 5.3, Fig. 5.4) revealed an initially compact central region surrounded by a porous zone in starch films, highlighting a structural opening from exterior to interior. The gradual increase in pore size and concentration over time led to the emergence of a central void zone

after 10 hours, followed by a coalescence of pores and voids after 24 hours. Figure 5.4 highlights a sizable void within the structure disconnected from the external environment. The presence of such voids potentially contributed to the observed lower microorganism release, as indicated by the recovery rate of only 3.3% after 24 hours (Fig. 5.1D).

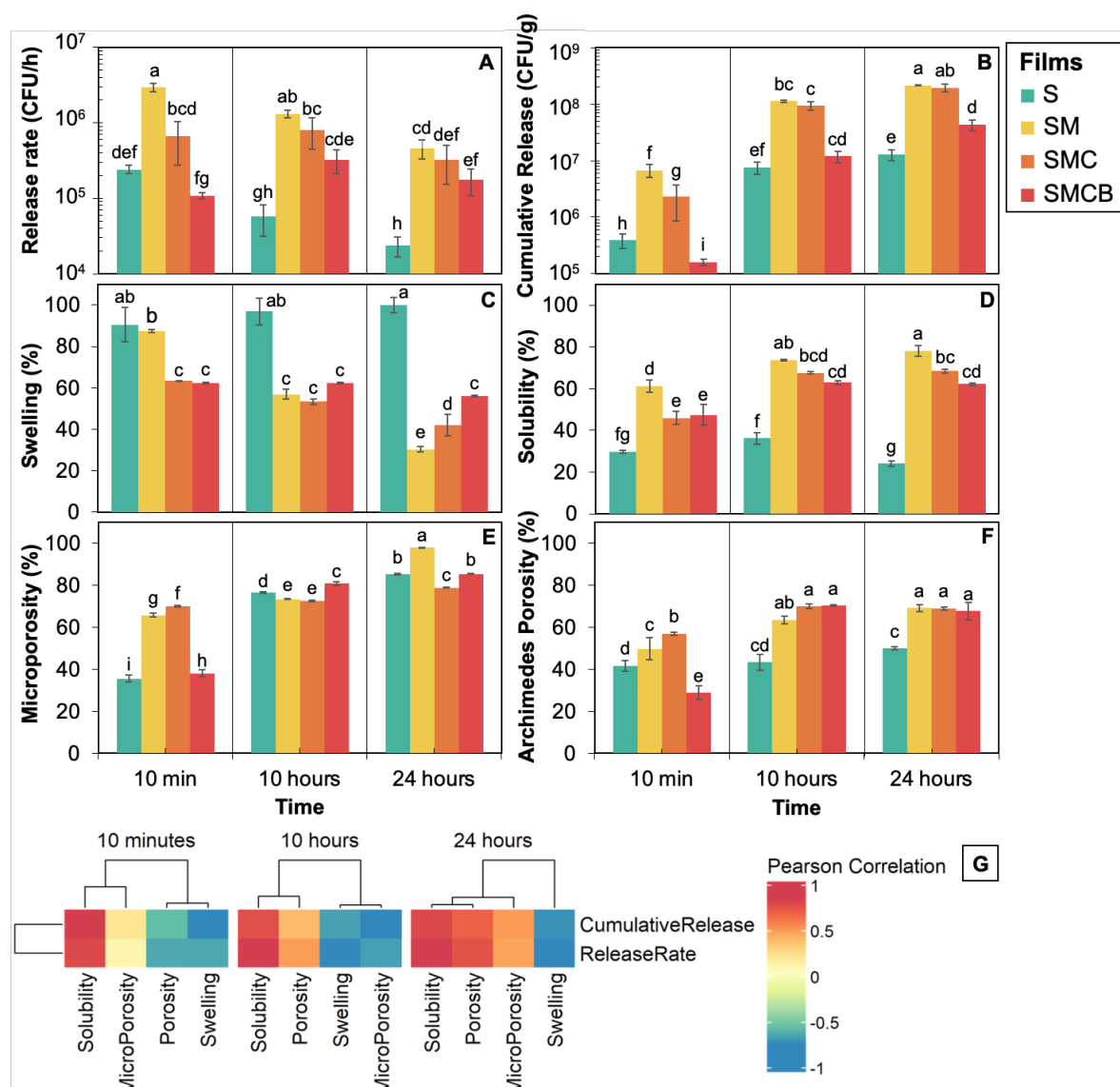


Figure 5.2. Modification in the physical and morphological attributes of starch-based films during the release of *P. megaterium* B119. (A) B119 release rate in saline solution, (B) B119 concentration, (C) swelling, (D) solubility, (E) microporosity, and (F) Archimedes porosity in starch (S), starch and maltodextrin (SM), starch, maltodextrin, and cellulose (SMC), and starch, maltodextrin, cellulose, and bentonite (SMCB) films at 10 minutes, 10 hours, and 24 hours of release. (G) Correlation between the films' physical and morphological properties and the release of *P. megaterium*. Different letters indicate significant differences between values ($p < 0.05$).

Conversely, Starch and Maltodextrin film (SM) exhibited the most remarkable release rate, releasing the highest quantity of microorganisms compared to the other matrices (Fig. 5.2A-B). This matrix also displayed the highest total porosity by the end of the process (Fig. 5.2E). However, the swelling of the matrix decreased over time (Fig. 5.2C). The addition of maltodextrin accelerated structural opening, driven by increased matrix solubility (Fig. 5.2D), resulting in a uniform increase in pore size and quantity over time (attributed to molecular intermixing) and an earlier and quicker release rate of microorganisms (Fig. 5.3, Fig. 5.4). This property is significant for the application of PGPB, especially in assisting with seed germination and the early stages of plant development. Maltodextrin, acting as a carbon source, likely supported microbial growth within the matrix, leading to a post-24-hour recovery of microorganisms surpassing the initially added quantity (Fig. 5.1D). This behavior holds promise for sustaining microbial activity and fostering competition with the natural microbiota in the soil environment.

Incorporating cellulose into the formulation (SMC) reduced the initial microorganism release rate and initial microorganism concentration compared to the SM film (Fig. 5.2A-B). Furthermore, the SMC film exhibited lower solubility compared to SM, yet effectively retained higher swelling, thereby preserving the material's porous architecture after 24 hours of immersion (Fig. 5.2C-D). There were no significant differences in the porosity of SMC compared to SM (Fig. 5.2E-F). However, Figure 5.3 and Figure 5.4 clearly illustrate morphological changes introduced by cellulose resulted in larger and irregular pores that formed wide channels, favoring a stable release pattern (zero-order release kinetics) over time without significant structural collapse.

The inclusion of bentonite (SMCB) did not affect matrix solubility and swelling (Fig. 5.2C-D) but influenced the release kinetics by retaining microorganisms within the matrix. After a 10-minute immersion, this material exhibited the lowest concentration of microorganisms among all films (Fig. 5.2B). This observation may be associated with the smaller number of open pores (Fig. 5.2F) and the matrix displaying a dense core surrounded by a porous region (Fig. 5.4) at this time – a pattern similar to the pure starch film (S). Another notable difference is the statistically significant increase in the release rate of this material (SMCB) at 10 hours (Fig. 5.2A), potentially linked to the corresponding increase in solubility and, consequently, in open porosity. While no significant morphological differences are observed when comparing matrices with and without bentonite (Fig. 5.3), the addition of bentonite significantly reduced the microorganism release (Fig. 5.2B). This characteristic may

be correlated with lower water vapor permeability and higher hydrophobicity of the SMCB film compared to SM and SMC films (BORGES et al., 2024). Similarly, ZHU et al., (2023) observed that composite polymers with higher viscosity and hydrophobicity reduced the mass transfer of *Pseudomonas putida* Rs-198. This controlled release behavior aligns with previous studies on bentonite-incorporated matrices (HE et al., 2015; SINGH et al., 2009). For example, the presence of bentonite in the alginate formulation was found to delay the release of *Raoultella planticola* Rs-2, as evidenced by the longer time taken to release 50% of the bacteria, despite this formulation also exhibiting a higher swelling rate (HE et al., 2015). This gradual and controlled release characteristic is desirable for the application of microbial inoculants, as they can aid in development and growth throughout the plant's life cycle.

Pearson correlation analysis indicated that the main factors influencing the microorganism's release and release rate are initially the solubility and subsequently also the porosity of the material (Fig. 5.2G). Additionally, a negative correlation was observed between swelling and microorganism release throughout the analysis period (Fig. 5.2G). It corroborates the greater influence of maltodextrin in increasing the rate of cell release. However, to maintain the advantageous characteristics of maltodextrin while achieving intermediate release rates between starch film (S) and maltodextrin-containing starch film (SM), insoluble components or those with low solubility in aqueous solutions, such as cellulose and bentonite, can be integrated.

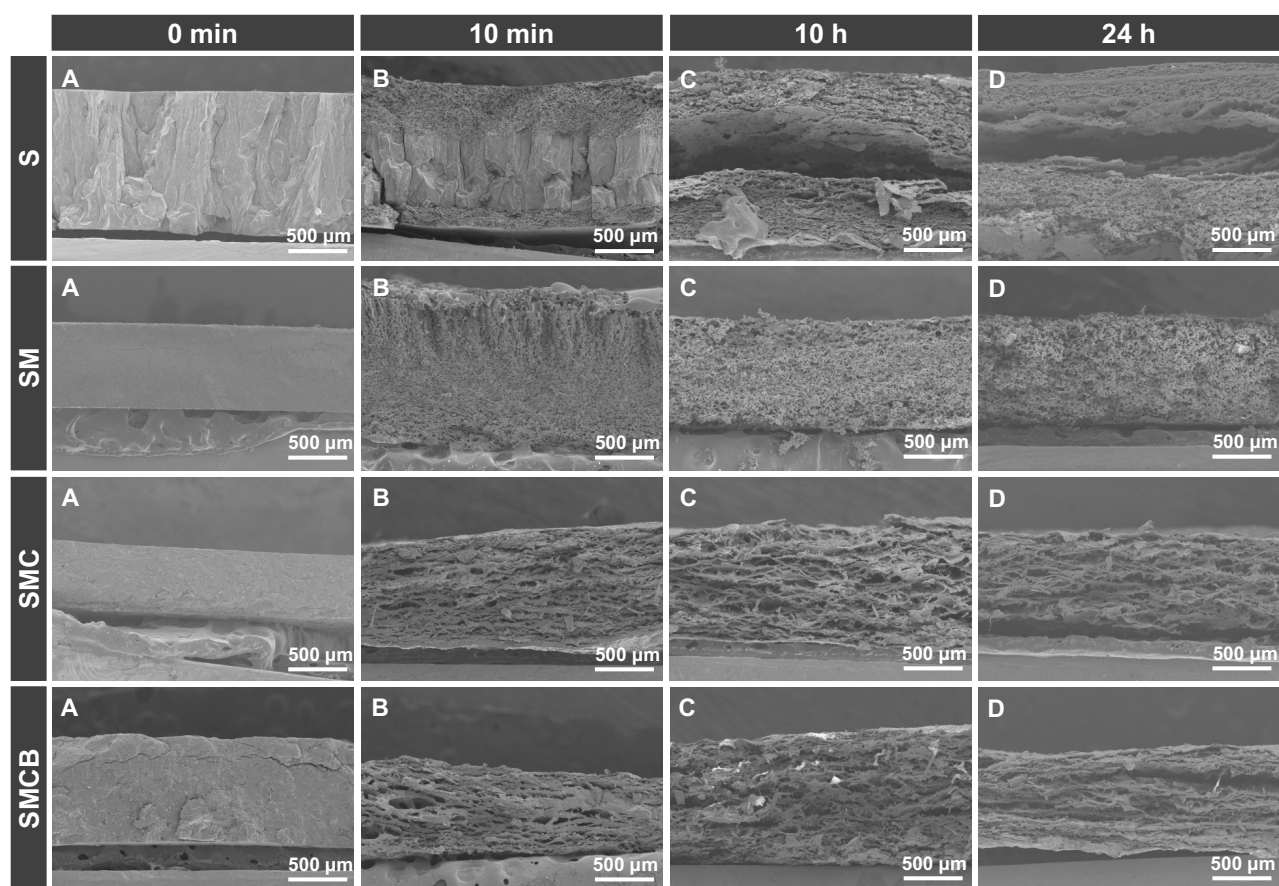


Figure 5.3. SEM images of starch (S); starch and maltodextrin (SM); starch, maltodextrin, and cellulose (SMC); and starch, maltodextrin, cellulose, and bentonite (SMCB) films along the releasing process.

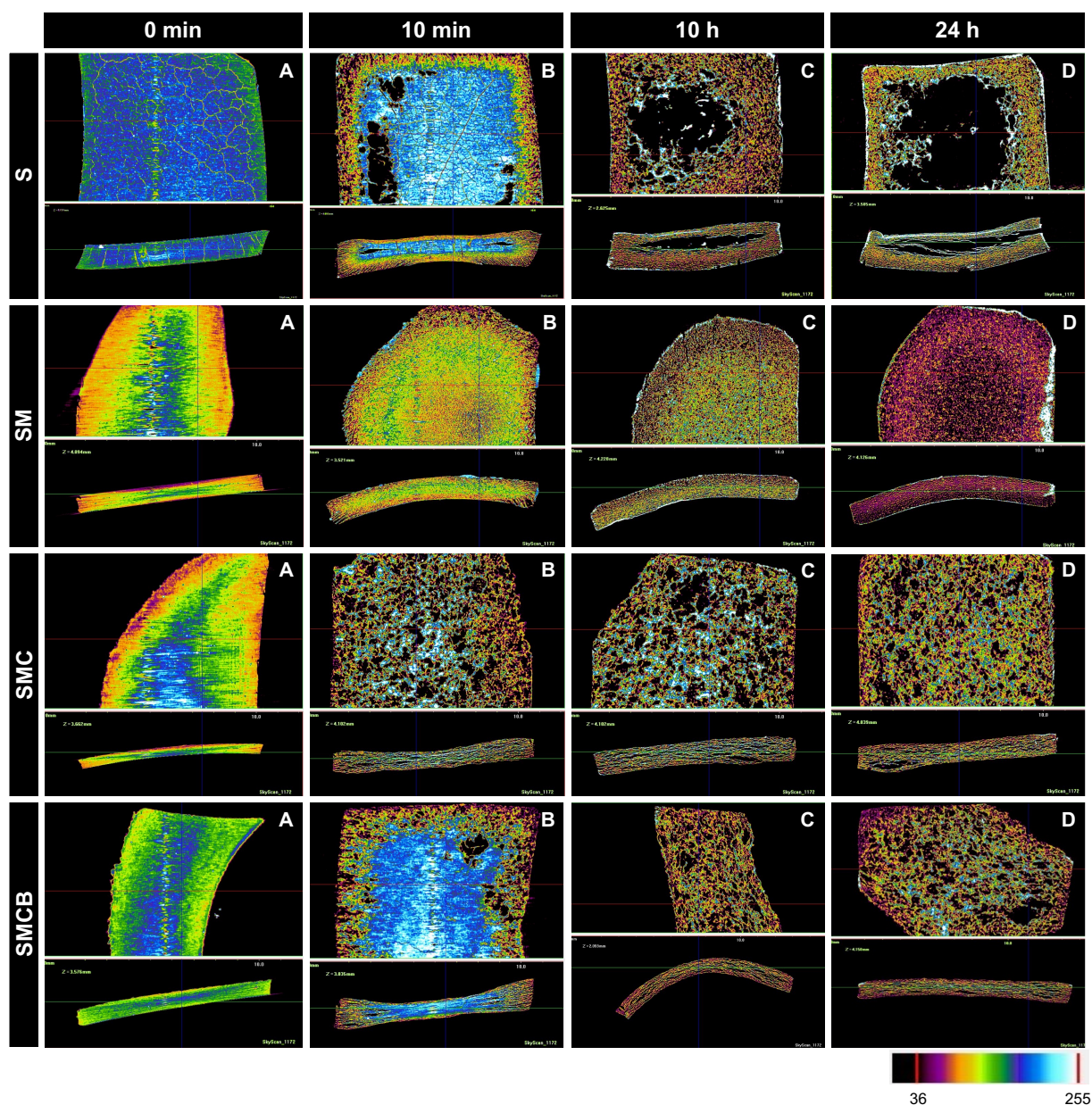


Figure 5.4. Microtomography images of starch (S); starch and maltodextrin (SM); starch, maltodextrin, and cellulose (SMC); and starch, maltodextrin, cellulose, and bentonite (SMCB) films along releasing process.

The results highlight the crucial role of adjusting the physicochemical and morphological properties of starch-based films to regulate the release kinetics of microorganisms like *Priestia megaterium*. This adaptability is valuable for developing tailored materials, especially in controlled release systems for agriculture, allowing precise adjustment of PGPB release rates and concentrations to meet specific plant growth requirements.

5.4.3. Protection against UV radiation and temperature

Incorporation of maltodextrin, cellulose, and bentonite into the starch matrix improved its protection against UV radiation, especially in the UVA and UVB regions, where SMC and SMCB films exhibited S_{UV} values of 100% (Fig. 5.5A). Only the starch film (S) showed lower UV protection in the S_{UVC} region, likely due to the darker color of the materials after cellulose and bentonite addition (BORGES et al., 2024).

Evaluating the microorganism's survival after UV exposure revealed that the film S provided more efficient protection during short exposures (20 min) compared to the film SM, but SM offered higher protection after that (Fig. 5.5B). Films with cellulose (SMC) and bentonite (SMCB) consistently showed superior UV protection regardless of exposure duration, as reported in previous studies (BORGES et al., 2024; FREITAS; GONZÁLEZ-MARTÍNEZ; CHIRALT, 2023; MOVAHEDI et al., 2014). Additionally, maltodextrin is also known to provide visible and ultraviolet light stability to encapsulated materials (XIAO et al., 2022).

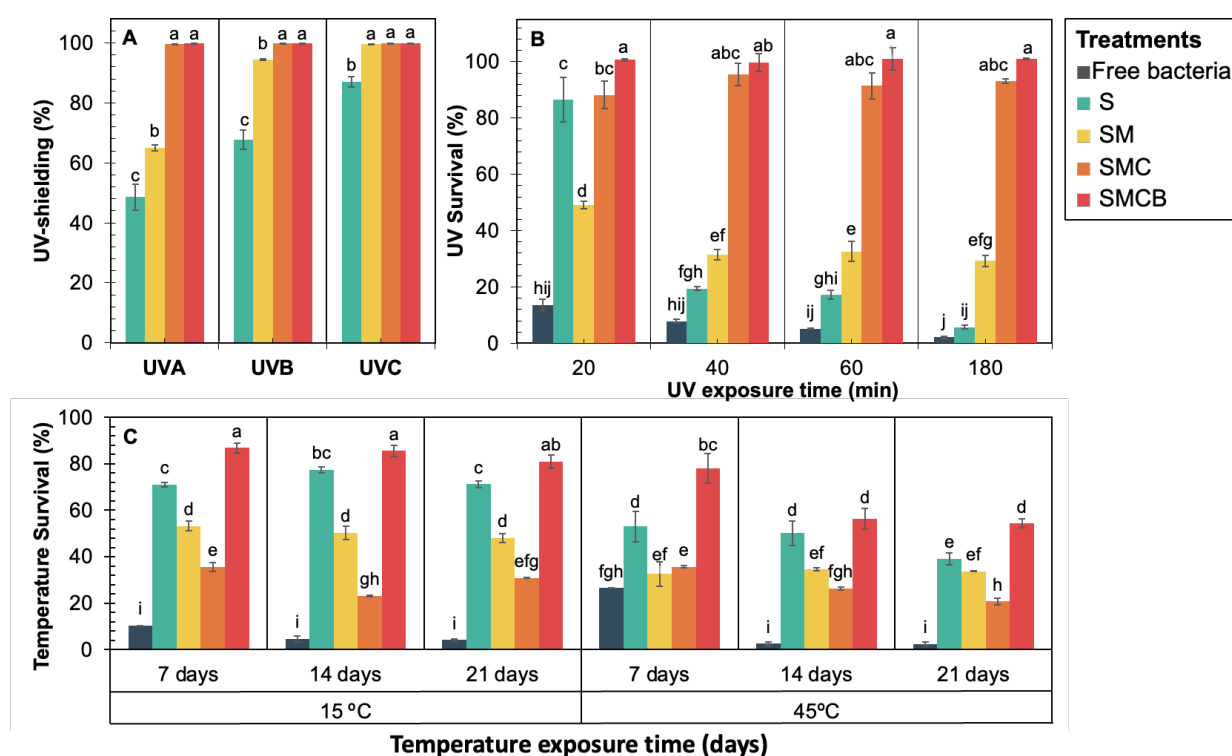


Figure 5.5. Protective effects of starch-based films against UV radiation, low, and high temperatures on *P. megaterium* B119 survival. (A) UV-shielding capability against various types of ultraviolet light rays, and bacterial survival upon exposure to UV-C light (B) and different temperatures (C) for free cells (dark blue) and bacteria encapsulated in starch (light

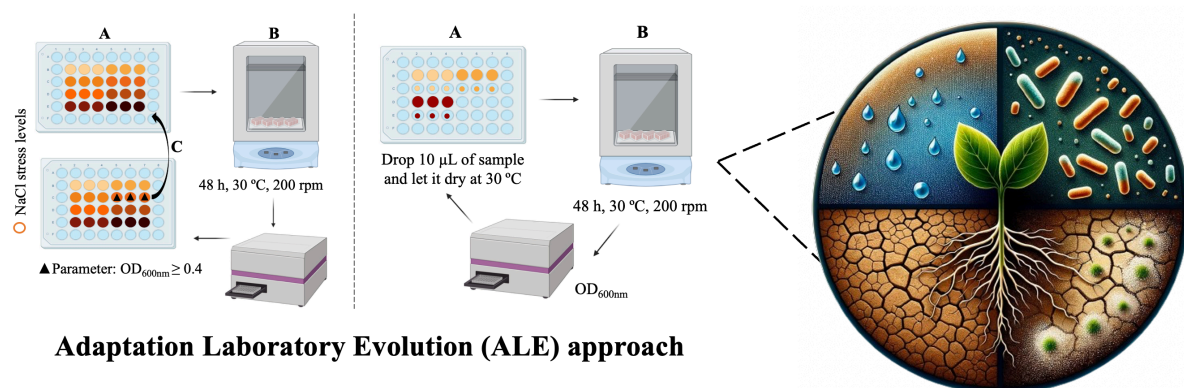
blue), starch and maltodextrin (yellow), starch, maltodextrin, and cellulose (orange), and starch, maltodextrin, cellulose, and bentonite (pink) films.

All tested materials also provided protective effects against temperatures of 15°C and 45°C compared to free cells (Fig. 5.5C). At lower temperatures, initial decreases in microorganism viability were observed in the films, stabilizing over time. Notably, the S and SMCB films demonstrated superior performance in maintaining bacterial survival, with SMCB showing the highest viability at higher (54%) and lower (80%) temperatures. Conversely, the SMC film exhibited the highest decline in microorganism viability compared to the other films. Maltodextrin and cellulose additions may increase thermal conductivity, leading to reduced survival at high temperatures (POURMOHAMMADI et al., 2018; YILDIRIM et al., 2014), while bentonite, acting as an insulator and mass transport barrier (DHIBAR; KAR; KHATUA, 2012), contributes to the greater thermal protection observed in SMCB films.

5.5. Conclusion

Understanding how material characteristics influence microorganism release is crucial for optimizing product applications. Our findings demonstrated that integrating maltodextrin, cellulose, and bentonite into the starch matrix enhanced protection against UV radiation and extreme temperatures while also modifying the cells release profile. While the addition of maltodextrin accelerated the release of microorganisms, cellulose and bentonite increased retention and allowed a gradual release. Among the studied films, the SMCB stands out as a promising option for formulating controlled-release biofertilizers due to two key advantages. Firstly, it protects beneficial bacteria from environmental stressors in the rhizosphere, where they compete with native microflora. Secondly, its rapid initial release enables prompt root colonization, followed by gradual and prolonged release, prolonging the microbial inoculant's effects. This understanding enables to formulate materials for precise control over cell release, leading to targeted and effective applications of microbial inoculants under adverse field conditions.

6. Adaptive Laboratory Evolution of *Bacillus* strains to drought and saline stress



6.1. Abstract

The adaptation of plant growth-promoting microorganisms to environmental stresses, such as drought and salinity, is crucial for sustainable agriculture in the face of climate change. In this study, we investigated the ability of *Bacillus subtilis* and *Bacillus megaterium* strains to adapt to such conditions using the adaptive laboratory evolution (ALE) technique. The results demonstrated that the adapted *B. subtilis* strain (BSdry-40) exhibited significant adaptation to drought conditions, while *B. megaterium* (BMdry-40) did not show such adaptation. Both strains were subjected to saline stresses, with adaptation observed only in *B. subtilis* (BSdry-40). Evaluations of biofilm formation and extracellular polysaccharides (EPS) production did not reveal significant differences between the tested strains and their respective wild-type strains. However, the BMdry-40 strain exhibited superior phosphorus solubilization capacity compared to the wild-type strain. Additionally, *Bacillus* strains adapted to saline stress conditions demonstrated resistance to drought and salinity conditions, with the resistance more evident in the strains adapted to salinity (BSNaCl-18, BNaCl-29, and BMNaCl-37). EPS production was higher in the BMNaCl-37 strain compared to the wild-type strain, while differences between the *B. subtilis* strains were not significant. The BMNaCl-37 and BNaCl-29 strains exhibited significantly greater phosphorus solubilization capacity compared to their respective wild-type strains. These results highlight the successful adaptation of the tested strains to adverse conditions and suggest potential for the development of more resilient and effective microbial inoculants for plant growth promotion in stressful environments. To elucidate the genetic changes underlying these adaptations, the selected strains were subjected to whole-genome sequencing and analysis (work in progress).

6.2. Introduction

In recent decades, global climate change has emerged as a significant global concern, particularly regarding its implications for food security and environmental sustainability. Notably, the acceleration of soil salinity and the occurrence of more severe droughts have become prominent issues (BOGATI; WALCZAK, 2022; MUKHOPADHYAY et al., 2021). These environmental shifts can impact various aspects of plant physiology and soil microbial community structure, leading to reduced crop productivity and yield (BOGATI; WALCZAK, 2022; FORNI; DUCA; GLICK, 2017; ULLAH; BANO; KHAN, 2021).

In response to these challenges, the utilization of plant growth-promoting bacteria (PGPB) has emerged as a promising strategy to mitigate the adverse effects of environmental stresses (BITTENCOURT et al., 2023; JANSSON; HOFMOCKEL, 2020). PGPB employ various mechanisms to enhance plant tolerance to abiotic stresses, including phosphate solubilization, nitrogen fixation, 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase activity, modulation of plant homeostasis through biofilm formation, and the production of metabolites such as volatiles, siderophores, indole-3-acetic acid (IAA), and exopolysaccharides (BITTENCOURT et al., 2023; KUMAR et al., 2020; QIN et al., 2016).

However, the efficacy of PGPB is contingent upon their ability to survive under adverse environmental conditions. In this regard, genetic engineering techniques and adaptive laboratory evolution (ALE) have been widely employed to enhance the growth-promoting characteristics and stress resistance of PGPB (ALI; KHAN, 2021; GAN et al., 2022; SARAPAT et al., 2020). For instance, WEN et al., (2021) enhanced nitrogen fixation in *Klebsiella varicola* 137 by 122-fold using computational and synthetic biology tools, while RODRÍGUEZ; GONZALEZ; SELMAN, (2000) improved phosphorus solubilization in *Pseudomonas* and *Burkholderia* strains through recombinant DNA techniques. DA-SILVA et al., (2017) demonstrated 1.5 times greater symbiotic efficacy in chickpea plants inoculated with a *Mesorhizobium* strain modified with extra groEL gene copies (stress response gene). SARAPAT et al., (2020) showed that *Bradyrhizobium* sp. SUTN9-2, adapted via ALE, improved nodulation, maintained nitrogen fixation under water deficit, and increased plant biomass. Similarly, GAN et al., (2022) enhanced macrolactin production in *Bacillus siamensis* A72 under salt stress through ALE.

In this context, this project aims to enhance the resistance of the P-solubilizing strains *Bacillus subtilis* NRRL B-354 and *Bacillus megaterium* NRRL B-14308 (DO CARMO et al., 2019) to drought and salinity stresses while maintaining or improving their growth promotion

capabilities. We employed the ALE technique to adapt these strains to drought (desiccation) and saline stress conditions. Furthermore, we evaluated in vitro biofilm formation, EPS production, and phosphorus solubilization of both wild-type and adapted strains. Subsequently, strains exhibiting the desired characteristics underwent whole-genome sequencing (WGS). The results of genomic analysis will elucidate molecular mechanisms underlying the observed phenotypic changes.

6.3. Materials and Methods

6.3.1. Adaptive Laboratory Evolution (ALE)

Salt stress was induced using NaCl in the adaptive laboratory evolution (ALE) process of strains *B. subtilis* 354 (BS) and *B. megaterium* 14308 (BM). Briefly, NaCl was added to tryptone soy broth (TSB) to achieve the osmolarity conditions specified in Table 6.1. Culture experiments were conducted in 48-well plates, with each well containing 500 μ L of medium. Each condition (medium osmolarity) was replicated three times. After each cultivation, a serial transfer of 10 μ L to fresh medium was performed every 48 hours. Transfer to the next round of higher osmolarity conditions occurred when an optical density at 600 nm ≥ 0.4 was observed, indicating potential adaptation.

Table 6.1. Media osmolarity employed for the adaptive laboratory evolution of *B. subtilis* (BS) and *B. megaterium* (BM) strains.

	Osmolarity (Mpa)						
BS	0.0	-1.0	-5.0	-10.0	-12.5	-15.0	-17.5
BM	0.0	-1.0	-3.0	-5.0	-7.0	-9.0	-11.0

The protocol by (SLININGER et al., 2021; SLININGER; SCHISLER, 2013) was adopted with adaptations for both strains to induce drought stress. Briefly, every 48 hours, 10 μ L of each bacterium from the previous cycle was dispensed in triplicate into a new 48-well plate, allowed to dry at 35°C for 30 minutes, and then resuspended using 500 μ L of fresh TSB medium. This process was repeated for forty iterations.

An overview schematic of the entire procedure is shown in Figure 6.1. All experiments were conducted on a shaker at 200 rpm and 30°C. Growth in TSB medium was used as a control. The adapted bacteria are denoted by acronyms that include the initials of the wild strain (BS or BM) from which they originated, the stress applied during the adaptation process (dry for desiccation, NaCl for salinity), and the number of cycles completed during ALE to produce these strains.

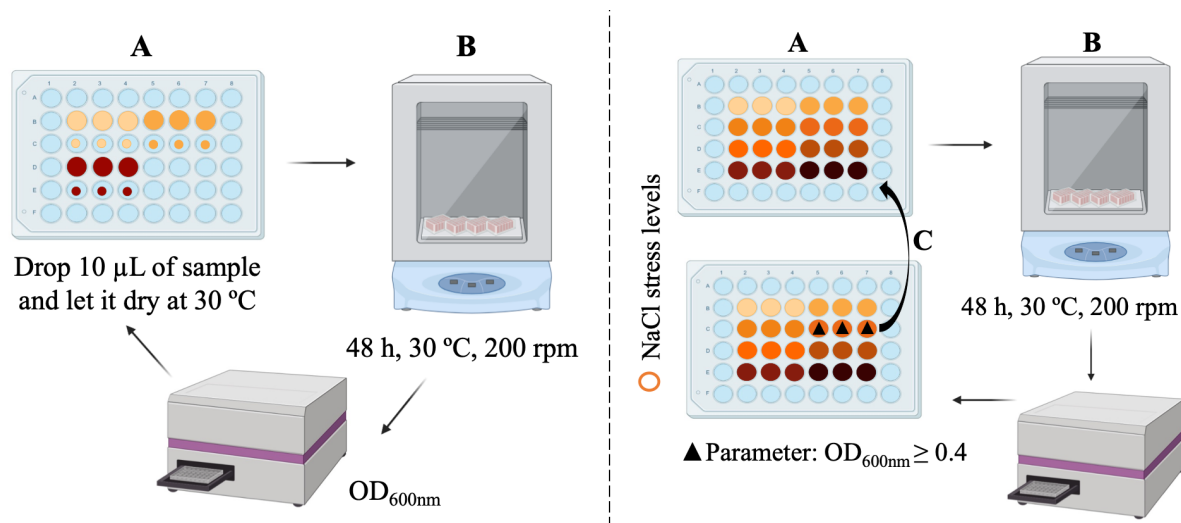


Figure 6.1. Schematic view of procedures for Adaptation Laboratory Evolution (ALE) approach. Microplate evolution involves submission to stress (osmotic or desiccation) (A), growth and reconstitution after prolonged drying stress exposure (B), and repeat (A-C) by transferring to the next round of survivors of most challenging stress conditions.

6.3.2. Evaluation of plant growth-promoting characteristics and stress resistance of wild-type and ALE strains

6.3.2.1. Calcium phosphate solubilization

The microorganism was initially cultured in TSB at 30°C, 200 rpm for 16 hours (pre-inoculum), and then centrifuged and resuspended in NBRIP medium (g/L ingredients: glucose, 10; $\text{Ca}_3(\text{PO}_4)_2$, 5; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25; KCl, 0.2; and $(\text{NH}_4)_2\text{SO}_4$, 0.15) to achieve a $\text{OD}_{600\text{nm}}$ of 1.0 (inoculum). Subsequently, 500 μL of the inoculum was added to 20 mL of NBRIP in 125 mL Erlenmeyer flasks, and the cultivation was carried out at 30°C, 200 rpm for 9 days. At the end of the cultivation, serial dilution, and plating are performed to assess microbial growth. Each sample was also centrifuged and filtered, and the final pH was measured.

The soluble P content of the samples was determined using a colorimetric method adapted from (MURPHY; RILEY, 1986). In summary, a 1 mL aliquot of the clear supernatant reacts with 0.4 mL of ascorbic acid solution (0.4 mol/L), 0.04 mL of citric acid (0.03 mol/L), 2 mL of a reagent consisting of 25 mL of sulfuric acid solution (4.7 mol/L), 5.5 mL of ammonium molybdate solution (0.08 mol/L), and 0.6 mL of antimony potassium tartrate solution (0.05 mol/L). This mixture was incubated at 50°C for 15 minutes, forming then a phosphoantimonymolybdenum blue complex. The concentration of the complex is determined

by UV-Vis spectrometry at a wavelength of 880 nm. The calibration curve is constructed using a soluble phosphorus source (KH_2PO_4) at concentrations ranging from 0.1 to 6 mg/L of P.

6.3.2.2. Biofilm formation

Biofilm production was assessed using a spectrophotometric method described by (STEPANOVIĆ et al., 2007) with modifications. Briefly, the microorganism was initially cultured in TSB at 30°C, 200 rpm for 16 hours (pre-inoculum), and then centrifuged and resuspended in TSB medium supplemented with 1% glucose to achieve an $\text{OD}_{600\text{nm}}$ of 1.0 (inoculum). 10 μL of the inoculum solution was added to 200 μL of TSB supplemented with 1% glucose and incubated at 30°C for 48 hours in a static incubator. After incubation, the contents of the wells were decanted into a discard container, and each well was washed three times with 300 mL of distilled water. Following washing, the remaining attached bacteria were fixed by adding 200 μL of methanol at 37°C for 15 minutes. Subsequently, the remaining methanol was removed, and the plate was allowed to air-dry at room temperature. The adherent biofilm layer formed in each microtiter-plate well was stained with 200 μL of Crystal violet used for Gram staining (2% Hucker crystal violet) for 15 minutes at room temperature. After staining, the stain was aspirated with a pipette, and the excess stain was rinsed off by placing the microtiter plate under running tap water until the washings were free of the stain. The plate was then air-dried at room temperature, and the dye bound to the cells was resolubilized and eluted from attached cells with 200 μL of 95% ethanol per well. Ethanol was gently added, and the microtiter plate was covered with a lid to minimize evaporation and left at room temperature for at least 30 minutes without shaking.

The optical density (OD) of each well stained with crystal violet was measured at 570 nm using a microtiter-plate reader. Average OD values were calculated for all tested strains and negative controls. Tests were performed in triplicate and repeated two times. The cut-off value (OD_c) was established as three standard deviations (SD) above the mean OD of the negative control: $\text{OD}_c = \text{average OD of negative control} + (3 \times \text{SD of negative control})$. Strains were classified as follows for easier interpretation of the results based upon the previously calculated OD values: $\text{OD} \leq \text{OD}_c$ = no biofilm producer; $\text{OD}_c < \text{OD} \leq 2 \times \text{OD}_c$ = weak biofilm producer; $2 \times \text{OD}_c < \text{OD} \leq 4 \times \text{OD}_c$ = moderate biofilm producer; $4 \times \text{OD}_c < \text{OD}$ = strong biofilm producer.

6.3.2.3. Exopolysaccharide (EPS) production

The microorganism was initially cultured in TSB at 30°C, 200 rpm for 16 hours (pre-inoculum), and then centrifuged and resuspended in TSB medium supplemented with 5 g/L sucrose to achieve an $\text{OD}_{600\text{nm}}$ of 1.0 (inoculum). 500 μL of the inoculum solution was added

to 20 mL of TSB supplemented with 5 g/L sucrose in a 125 mL Erlenmeyer flask and incubated at 30°C, 200 rpm for 5 days. Falcon tubes were labeled, dried in a 60°C incubator for 48 hours, and weighed (w_0). After 5 days, 3 mL of each sample was added to a 15 mL Falcon tube containing 9 mL of chilled 95% ethanol and left in the refrigerator overnight. Subsequently, the Falcon tube was centrifuged, the supernatant was removed, and the Falcon tube was placed back in a 60°C incubator for 48 hours and weighed (w_1). The EPS produced was calculated using the formula:

$$EPS \left(\frac{g}{L} \right) = \frac{w_1 - w_0}{3 \cdot 10^{-3}}$$

6.3.2.4. Evaluation of wild-type and ALE strains resistance to simulate drought and saline stress

The microorganism was initially cultured in TSB at 30°C, 200 rpm for 16 hours (pre-inoculum), and then centrifuged and resuspended in TSB medium to achieve an OD_{600nm} of 1.0 (inoculum). 10 µL of the inoculum solution was added to 500 µL of culture media simulating the different stress conditions. For drought stress, 450 g/L of sorbitol or 200 g/L of PEG 8000 was added to TSB, while for salt stress, NaCl was added to TSB at concentrations of 80, 100, 120, and 150 g/L. Cultivation was carried out in triplicate under the same conditions as the pre-inoculum. After 48 hours of cultivation, the optical density at 600 nm was measured using a microplate reader.

6.3.3. Whole Genome Sequence Analyses and Assessment of Epigenetic Contributions

The software RAST, Prokka, and Panzer were utilized for genome annotation, and QUAST was used to assess sequencing quality and examine genome characteristics. Additionally, sequence alignment of the adapted and wild-type strains was performed using the Bowtie2 software. Post-processing mapping and duplicate removal were carried out using the Samtools and Picard tools. Alignment results with mapping quality scores above 30 were retained.

Single nucleotide polymorphisms (SNPs) and insertion and deletion variants (InDels) in the genome will be evaluated using GATK and VarScan. Sequences containing identified SNPs and other mutations relative to the wild-type will be cross-referenced with genes previously associated with resistance to abiotic stresses and plant growth-promoting characteristics. Mutations in shared genes or pathways will also be assessed to find a source of circumstantial evidence that may suggest a common genetic solution for the stress conditions used.

Additionally, the sequences will be analyzed for epigenetic mechanisms including methylation, histone modifications, chromatin structure, and selected noncoding RNA (ncRNA) (Skinner 2015), which may have significantly contributed to observed phenotypic changes in the ALE strains.

6.4. Results and Discussion

Results demonstrated adaptation to drought stress conditions using the dry-method approach for the adapted *B. subtilis* strain (BSdry-40), whereas no adaptation was observed for *B. megaterium* (BMdry-40). This finding was further supported by testing the selected adapted strains under drought stress conditions induced by 200 g/L PEG or 450 g/L sorbitol (Fig. 6.2A). Similar results were obtained when subjecting both *Bacillus* strains to saline stress conditions using different NaCl concentrations (Fig. 6.2B), i.e., adaptation was observed only in the adapted *B. subtilis* strain (BSdry-40). Biofilm formation and extracellular polysaccharide (EPS) production did not exhibit significant differences among the tested bacterial strains compared to their respective wild-type strains. However, when assessing P solubilization, strain BMdry-40 showed remarkably higher solubilization capacity compared to the wild-type strain (Figs. 6.2C-E). This result may be attributed to the increased production of organic acids, utilized in P solubilization, as evidenced by the lower pH at the end of the BMdry-40 experiment compared to BM (data not shown).

Desiccation-tolerant bacteria are highly desirable as they can persist in soils and inoculant formulations for extended periods, thereby enhancing plant colonization under conditions of low water availability (MOLINA-ROMERO et al., 2017). Furthermore, it is noteworthy that some studies have reported increased phosphorus release after desiccation in soils inoculated with various microorganisms (BÜNEMANN et al., 2013; DINH et al., 2017), suggesting a possible correlation in the mechanisms of action between desiccation-resistant microorganisms and P solubilization.

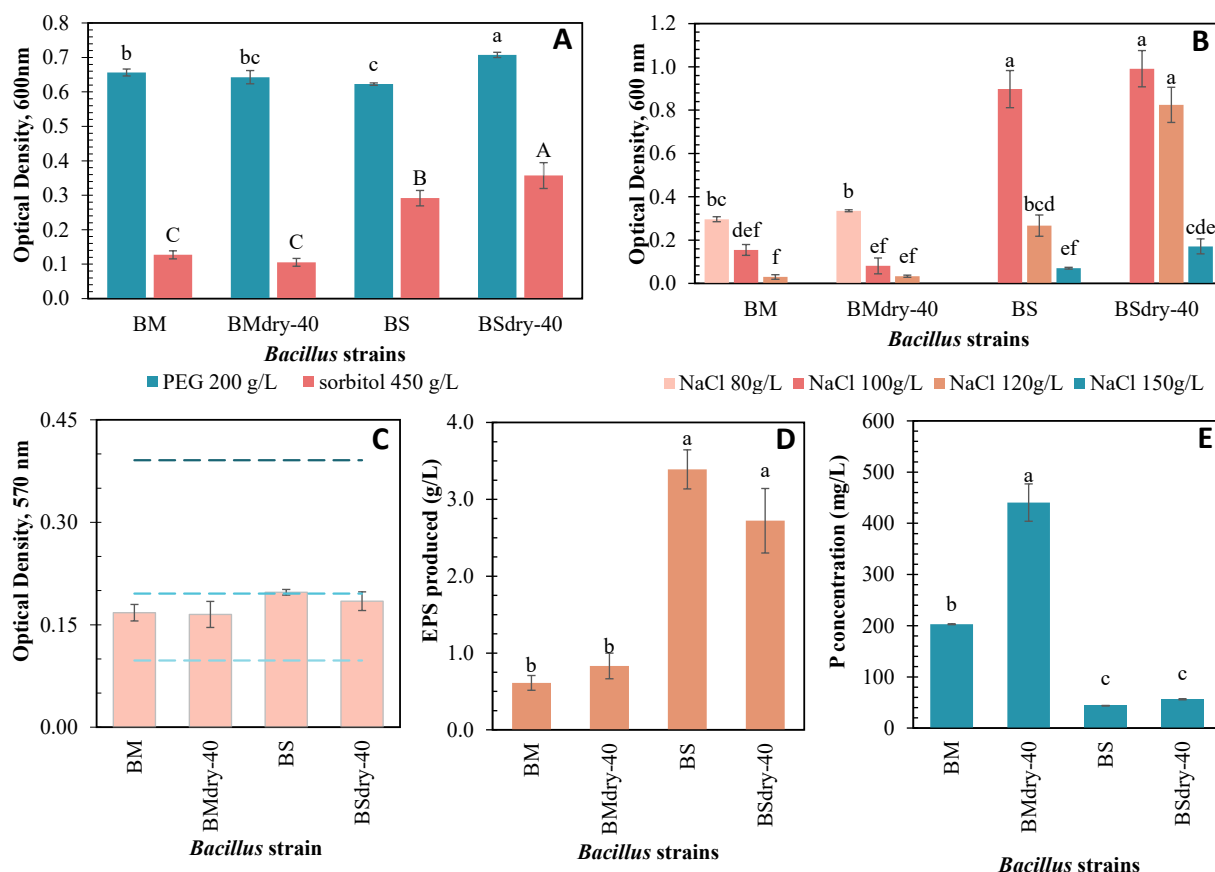


Figure 6.2. Resistance of bacteria adapted by ALE to simulated drought stress (A) and saline stress (B) conditions. Measurement of biofilm formation (C), EPS production (D), and phosphorus solubilization (E) was performed. Strains. BS: *B. subtilis* 354, BM: *B. megaterium* 14308, dry: adapted through dry drop stress (40 cycles). (C) Samples between the light blue and the middle blue dashed line are categorized as poorly biofilm-forming. Samples between the dark blue and middle blue dashed lines are considered moderately biofilm-forming. Samples above the dark blue line are classified as strongly biofilm forming. Means followed by the same letter indicate that the treatments do not differ significantly by the Tukey test ($p < 0.05$).

Bacillus strains were also adapted to saline stress conditions and then tested for resistance to drought conditions (Fig. 6.3A) and saline stress with different NaCl concentrations (Fig. 6.3B). Notably, adaptation to drought conditions was observed for *B. subtilis* (BSNaCl-18; BNaCl-29) and *B. megaterium* (BMNaCl-37) adapted strains, with differences more evident when the test was performed using sorbitol (Fig 6.3A). This result may be related to the mechanisms used by microorganisms to survive drought and salinity conditions being similar (BITTENCOURT et al., 2023; KUMAR et al., 2020; QIN et al., 2016). Similarly, drought and salinity induce similar global stresses (water deficit conditions) and trigger similar initial plant

responses. Thus, microorganisms that assist plants resist salinity can also make plants tolerant to drought stress (FORNI; DUCA; GLICK, 2017).

When testing the bacterial strains under different salt concentrations for saline stress conditions, BNaCl-29 and BSNACL-18 exhibited significantly higher resistance compared to their respective wild types (Fig. 6.3B). BMNaCl-37 grew better than wild-type BM under simulated salt stress in solid media (data not shown), although this growth was not statistically different in liquid media (Fig. 6.3B). No major differences in biofilm formation were observed among the tested strains (Fig. 6.3C). However, BMNaCL-37 showed significantly higher EPS production than wild-type BM, while no differences were observed between the *Bacillus subtilis* strains (Fig. 6.3D). BMNaCl-37 and BNaCl-29 showed significantly higher capacity for P solubilization compared to their respective wild-type strains, while no other major differences were observed for the additionally tested strains (Fig. 6.3E). EPS production is one of the mechanisms adopted by microorganisms in response to stress. In soil, EPS forms complexes with metal ions ($\text{Al}^{3+} > \text{Cu}^{2+} > \text{Zn}^{2+} > \text{Fe}^{3+} > \text{Mg}^{2+} > \text{K}^{+}$), and this mechanism can be extrapolated as a means of phosphorus solubilization (NASEEM et al., 2018).

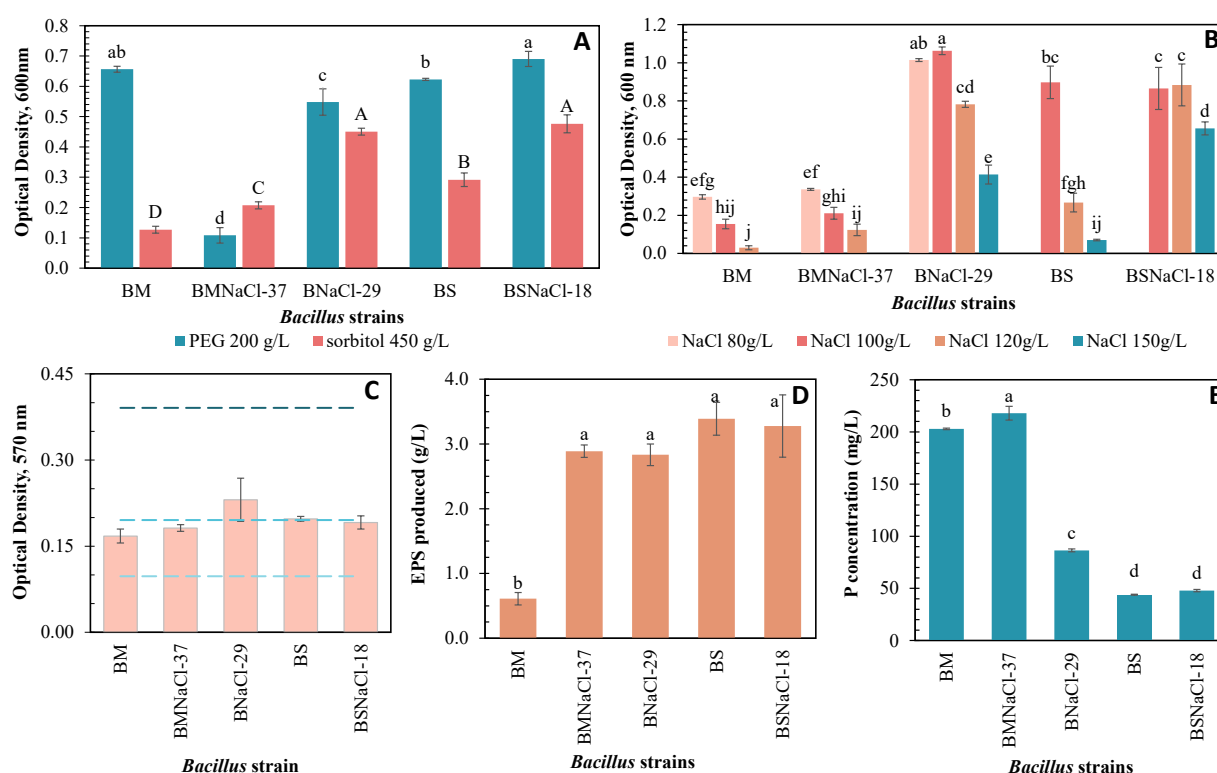


Figure 6.3. Resistance of bacteria adapted by ALE (saline stress conditions) and wild-type strain to simulated drought stress (A) and saline stress (B) conditions. Measurement of biofilm formation (C), EPS production (D), and phosphorus solubilization (E) was performed. Strains:

BS: *B. subtilis* 354, BM: *B. megaterium* 14308, NaCl: adapted through salinity stress. (C) Samples between the light blue and the middle blue dashed line are categorized as poorly biofilm-forming. Samples between the dark blue and middle blue dashed lines are considered moderately biofilm-forming. Samples above the dark blue line are classified as strongly biofilm-forming. Means followed by the same letter indicate that the treatments do not differ significantly by the Tukey test ($p < 0.05$).

The findings highlight the successful adaptation of the tested *Bacillus* wild-type strains to diverse conditions, showcasing a spectrum of newly acquired traits. To elucidate the genetic alterations underlying these adaptations and to gain insights into the mechanisms employed by the microorganisms, we selected and submitted the following strains for whole-genome sequencing and analysis (work in progress): *Bacillus* wild-type strains, BSdry-40, BMdry-40; BSNaCl-18; BNaCl-29; BMNaCl-37. Table 6.2 provide an overview of the general characteristics of the genomes of the sequenced *Bacillus* strains.

Table 6.2. General features of *Bacillus* strains genomes.

General features	BM	BMNaCl-37	BS	BSdry-40	BSNaCl-18	BNaCl-29
Contigs	103 (5,584,748 bp)	83 (5,476,081 bp)	53 (3,986,938 bp)	55 (3,986,894 bp)	54 (3,986,921 bp)	52 (4,238,025 bp)
Contigs N50 (bp)	283,351	514,901	294,318	263,165	294,317	1,070,065
GC Content (%)	37.69	37.75	43.84	43.84	43.84	43.6
Number of Subsystems	327	325	333	333	333	335
Protein-coding genes	6,039	5,594	4,179	4,168	4,170	4,556
Genes with function predicted	4,020	3,669	3,520	3,523	3,525	3,678
Hypothetical protein	2,019	1,925	659	645	645	878
RNA genes	100	102	80	79	80	77

BS: *B. subtilis* 354, BM: *B. megaterium* 14308, NaCl: adapted through salinity stress, dry: adapted through dry drop stress.

6.5. Conclusion

This study demonstrated the adaptability of *B. subtilis* and *B. megaterium* strains to drought and salinity stress conditions. The adaptive laboratory evolution (ALE) technique using the desiccation method allowed the *B. subtilis* strain (BSdry-40) to significantly adapt to drought conditions, while the *B. megaterium* strain (BMdry-40) did not show adaptation. The strains adapted to salinity showed resistance to both drought and salinity stresses (BSNaCl-18, BNaCl-29, and BMNaCl-37). The phosphorus solubilization capacity was notably higher in the BMdry-40 strain compared to the wild-type strain, suggesting an improvement in the efficacy of plant growth promotion under stress conditions. Additionally, the production of extracellular polysaccharides (EPS) was higher in the BMNaCl-37 strain, indicating a possible adaptive response to saline stress. These findings emphasize the potential use of adapted *Bacillus* strains as microbial inoculants to promote plant growth in stressful environments, contributing to agricultural sustainability in the face of climate change. Whole-genome sequencing and analysis of the selected strains are underway to elucidate the genetic changes underlying the observed adaptations, which may provide additional insights for the development of strategies to mitigate abiotic stresses in plants.

7. General Conclusions and future perspectives

This research has made significant progress in increasing the amount of heat-resistant *Bacillus* spores in bioreactors, in creating a versatile composite material for the controlled release of the microorganism, and in adapting *Bacillus* strains to resist stress from drought and salinity through the Adaptive Laboratory Evolution (ALE) technique. Additionally, a few remarks deserve to be highlighted for each Chapter:

Chapter 1: The systematic analysis of *Bacillus* sporulation underscores the necessity for tailored cultivation strategies to enhance spore production, catering to diverse applications in agriculture, food safety, and environmental sustainability. Identifying species-specific and application-oriented optimal conditions is paramount. Moreover, the significance of foundational research and small-scale process optimization to comprehend sporulation mechanisms cannot be overstated. There lies a substantial scope for advancing research, particularly in employing advanced modeling, co-cultivation techniques, and exploring the potential of spores in bioremediation and medical applications. Integrating these insights into scalable practices while addressing regulatory concerns is crucial for unlocking the full biotechnological potential of *Bacillus*, paving the way for significant scientific and biotechnological breakthroughs.

Chapter 2: The systematic mapping noted gap is the scant characterization of the encapsulation matrix in studies, which mainly focus on the product's application rather than its structural properties. The mapping emphasizes the need for further research into matrix characterization, microorganism retention and release mechanisms, and the impact of matrix modifications on targeted release, aiming to advance the development of innovative systems for diverse applications.

Chapter 3: The cultivation of *B. megaterium* in SBM culture medium under specific conditions (37°C and pH 5.6) within a bubble column bioreactor resulted in an efficient and cost-effective production of high concentrations of heat-resistant spores, particularly when compared to traditional stirred tank bioreactors regarding energy consumption. Considering the impact of cultivation conditions on spore revival in response to nutrients, it is advisable to conduct assays to assess the obtained strains' germination capacity and growth promotion characteristics. Furthermore, achieving genetic stability in microbial strains is essential to ensure the preservation of their desired traits over time. Future research could explore the influence of using consortia versus single strains on inoculant survival and functionality. The development of diverse consortia with strains carrying redundant or complementary functions holds promise, yet the dynamics of their establishment in various environments and the potential limitations imposed by certain members on others' functions in specific scenarios remain unexplored. Additionally, the direct interactions between added co-innoculums in soils, such as biofilm formation, merit investigation. Lastly, the two-step strategy for

inoculant production and the utilization of low-cost materials warrants further exploration. Despite recent advancements in microbial inoculant production and formulation, additional research is needed to refine upstream operational productivity through alternative fermentation strategies, such as continuous fermentation or various bioreactor configurations.

Chapter 4: The study on modulating the properties of starch-based films for coatings has demonstrated the ability to customize the release and protection of beneficial microorganisms. The incorporation of diverse additives has facilitated the development of coating systems with optimized physicochemical characteristics tailored for specific agricultural applications. Building on this work, future endeavors could involve applying these formulations with microorganisms to seeds and evaluating their impact on germination, colonization, and growth promotion in plants.

Chapter 5: The controlled release analysis of beneficial microorganisms from modified starch-based films revealed distinct release profiles and enhanced protection against UV radiation and extreme temperatures. This underscores the effectiveness of film modifications in improving microbial survival and targeted delivery in the agricultural environment. A thorough understanding of how morphophysiological alterations of materials are affected by additives is crucial for precisely controlling additive proportions to maximize efficacy. Future directions for this formulation could include conducting a cost analysis of the bioformulation production process. Additionally, investigating the impact of polymer inoculation on plant microbiomes, focusing on microbial function and structure, would be insightful. Expanding the production and application of encapsulated bioformulations and extending the target species beyond cereals, legumes, and certain vegetables is also one option.

Chapter 6: The application of laboratory adaptive evolution (ALE) to enhance the resistance of *Bacillus* strains to drought and salinity stress has proven to be a promising strategy. The adapted strains exhibited not only greater resilience to these stresses but also maintained or enhanced their plant growth-promoting capabilities, suggesting a viable path for developing robust microbial inoculants for agriculture in the face of climate change. The next step involves evaluating the sequenced genomes to identify potential mutations or SNPs related to stress response, thereby gaining deeper insights into the genes associated to the resistance of stressful environmental conditions. Furthermore, efforts should be directed towards understanding the genetic and physiological mechanisms underlying adaptations and the transferability of ALE results to field conditions. A long-term assessment of the ecological effects of adapted strains is imperative to ensure the safety and sustainability of their agricultural application.

This research has significantly advanced the field of microbial biotechnology, particularly in the context of agriculture and environmental sustainability. By optimizing *Bacillus* spore production and characterizing starch-based encapsulation matrices, we have laid the groundwork for the development of robust microbial inoculants. The efficient production of heat-resistant spores and the tailored release of beneficial microorganisms from modified starch-based films demonstrate the potential for enhancing plant growth and resilience to environmental stressors. The application of laboratory adaptive evolution further highlights the promise of genetically adapted strains in facing the challenges of climate change. Collectively, these findings offer innovative solutions for sustainable agriculture, with implications for food security and environmental preservation.

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SUPPLEMENTARY MATERIAL

Table 3.1S. Coefficient values for the responses: viable cells concentration, spore concentration, sporulation efficiency, and heat resistance, by analysis of variance (ANOVA).

Term	Viable cells (CFU/mL)		Spore (CFU/mL)		Sporulation efficiency (%)		Heat resistance (%)	
	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
Mean	7.63E+08*	0.000	7.51E+08*	0.000	98.640*	0.000	2.150	0.534
Temperature	-1.12E+07	0.689	4.24E+06	0.874	10.190*	0.038	3.100	0.153
Initial pH	-5.43E+07	0.066	-6.02E+07*	0.036	-12.550*	0.013	-12.740*	0.000
Temperature ²	-3.99E+08*	0.000	-4.05E+08*	0.000	-53.910*	0.000	-2.910	0.254
Initial pH ²	-2.78E+08*	0.000	-2.75E+08*	0.000	-5.410	0.328	11.220*	0.000
Temperature*Initial pH	-2.23E+07	0.575	-1.21E+07	0.749	-26.150*	0.001	-6.690*	0.036

Significant parameters ($p \leq 0.05$) are indicated (*).

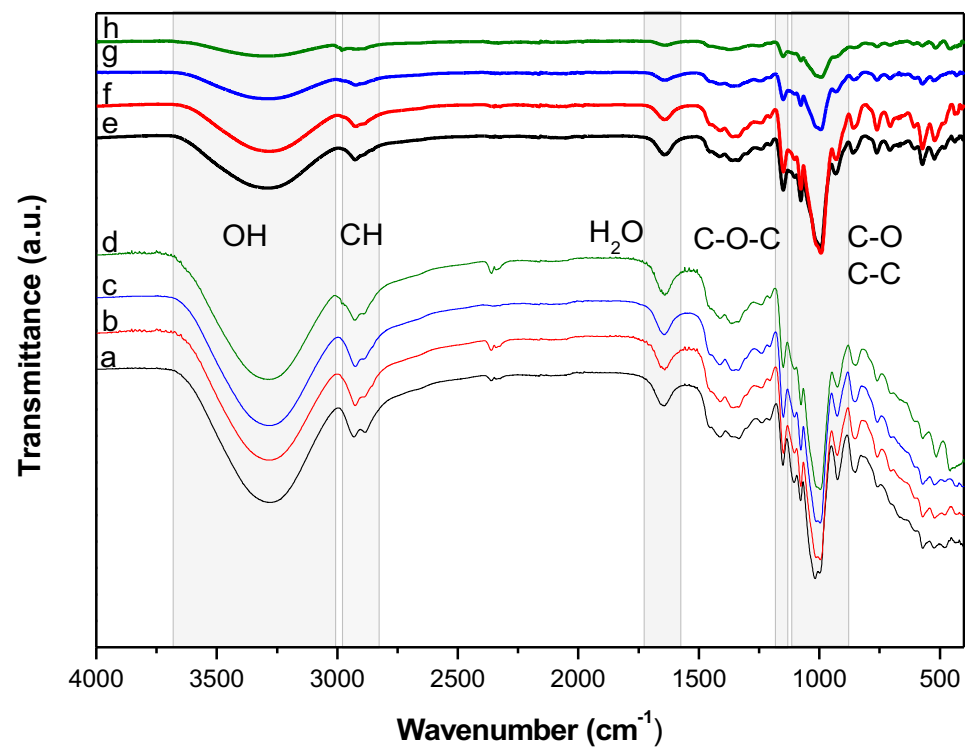


Figure 4.1S. FTIR spectra of starch film 10% with (a) and without glycerin (e); starch film 10% containing dextrin 25% with (b) and without glycerina (f); starch film 10% containing dextrin 25%, cellulose 5% with (c) and without glycerina (g); starch film 10% containing dextrin 25%, cellulose 5%, and bentonite 5% with (d) and without glycerina (h).

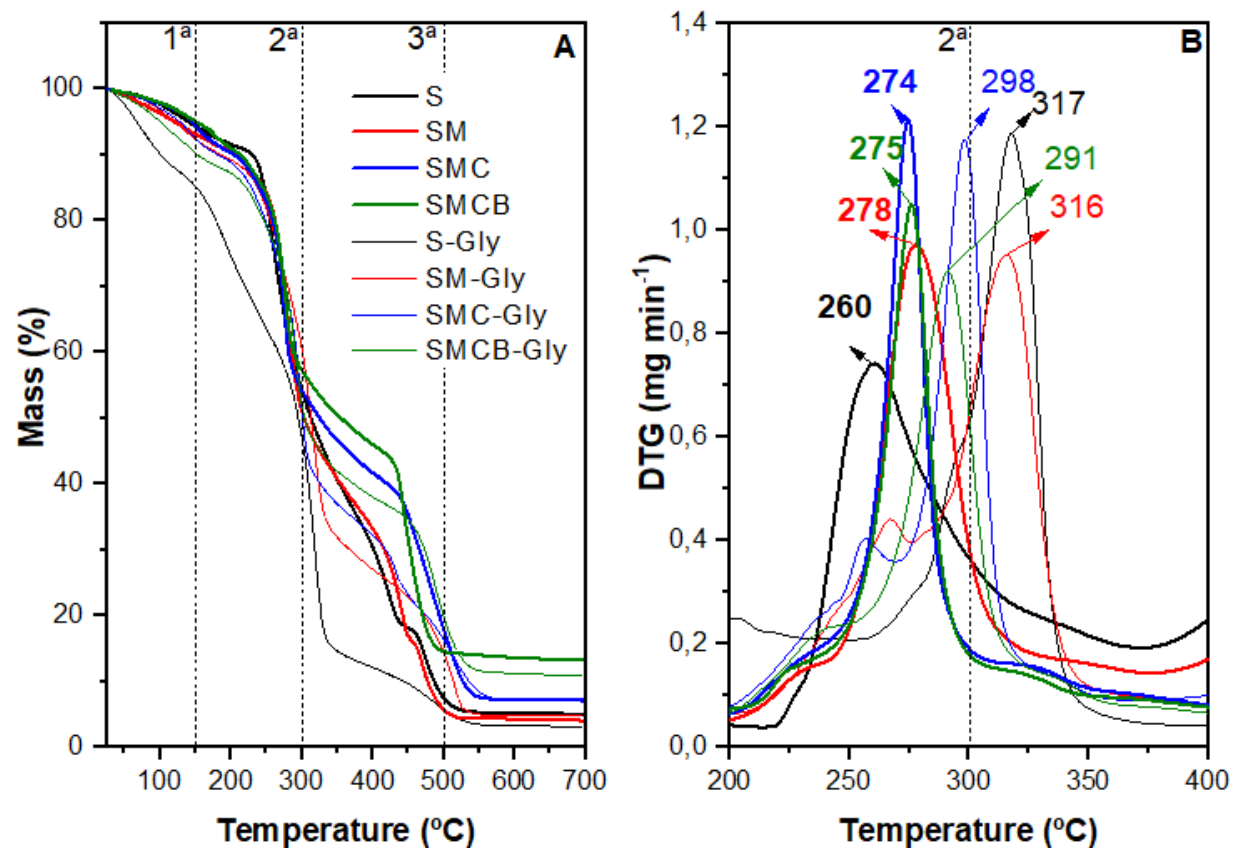


Figure 4.2S. TGA curves (A) and DTG curves (B) of composites containing starch (S), maltodextrin (M), cellulose (C), bentonite (B) and glycerol (Gly).

APPENDIX A

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