UNIVERSIDADE FEDERAL DE SÃO CARLOS

CENTRO DE CIÊNCIAS EXATAS E DE TECNOLOGIA

DEPARTAMENTO DE ENGENHARIA QUÍMICA

PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA QUÍMICA

"Encapsulamento de *Trichoderma harzianum* em matrizes à base de nanocelulose e carboximetilcelulose para aplicação na agricultura"

Mariana Govoni Brondi Wolf

Tese de doutorado apresentada ao Programa de Pós-Graduação em Engenharia Química da Universidade Federal de São Carlos como parte dos requisitos necessários para a obtenção do título de Doutor em Engenharia Química, área de concentração em Pesquisa e Desenvolvimento de Processos Químicos.

Orientadora: Dra. Cristiane Sanchez Farinas

São Carlos, SP, Brasil

Junho, 2023

FEDERAL UNIVERSITY OF SÃO CARLOS

CENTER OF EXACT SCIENCES AND TECHNOLOGY

CHEMICAL ENGINEERING DEPARTMENT

GRADUATE PROGRAM IN CHEMICAL ENGINEERING

"Encapsulation of *Trichoderma harzianum* in nanocellulose and carboxymethyl cellulose based matrices for agricultural applications"

Mariana Govoni Brondi Wolf

Doctoral thesis presented to the Graduate Program of Chemical Engineering at the Federal University of São Carlos as part of the requirements to obtain the title of Doctor in Chemical Engineering, area of concentration: Research and Development of Chemical Processes

Supervisor: Cristiane Sanchez Farinas, PhD

São Carlos, SP, Brazil

June, 2023

UNIVERSIDADE FEDERAL DE SÃO CARLOS

Centro de Ciências Exatas e de Tecnologia
Programa de Pós-Graduação em Engenharia Química

Folha de Aprovação

Defesa de Tese de Doutorado da candidata Mariana Govoni Brondi Wolf, realizada em 21/06/2023.

Comissão Julgadora:

Profa. Dra. Cristiane Sanchez Farinas (EMBRAPA)

Prof. Dr. Cauê Ribeiro de Oliveira (EMBRAPA)

Prof. Dr. Eduardo de Aquino Ximenes (IU)

Prof. Dr. Francys Kley Vieira Moreira (UFSCar)

Prof. Dr. Alberto Colli Badino Junior (UFSCar)

O Relatório de Defesa assinado pelos membros da Comissão Julgadora encontra-se arquivado junto ao Programa de Pós-Graduação em Engenharia Química.

AGRADECIMENTOS

A Deus pela vida maravilhosa, repleta de amor, aprendizados, oportunidades e conquistas.

Ao meu marido, meus pais, minha irmã e meus avós por todo a amor, apoio, carinho, ensinamentos e conversas. Vocês são meus exemplos de vida e inspiração e minha jornada acadêmica não seria possível sem o encorajamento de vocês. Obrigada por estarem sempre ao meu lado, me incentivando quando eu mais precisava e celebrando cada conquista ao longo do caminho. O apoio de vocês foi a força motriz por trás da minha determinação.

À minha orientadora, Dra. Cristiane, que me acompanha desde o final da minha graduação e que foi fundamental para o meu crescimento pessoal e profissional. Agradeço imensamente pela sua orientação, paciência, ensinamentos, apoio, oportunidades e confiança que sempre depositou em mim. Seu profissionalismo é um exemplo para mim.

Ao Dr. Caue, pela orientação, apoio, ensinamentos e pelas oportunidades, que com certeza me possibilitaram um grande crescimento pessoal e profissional.

Aos meus amigos Vanessa, Ariane, Camila, Thalita e Thalyne, por todas as conversas, paciência, ajuda e pelos momentos de descontração.

Aos grandes amigos da Lehigh University (Sahanaz, Diego, Emmanuel e Mohamed). A amizade, conversas e o apoio incondicional deles foram fundamentais para a minha adaptação e para o meu crescimento pessoal e profissional.

Aos colegas da Embrapa Instrumentação pelas colaborações e disposição em ajudar no desenvolvimento deste doutorado.

Aos professores Valter e Alberto, que me apresentaram à Engenharia Química e contribuíram para que eu trilhasse esse caminho profissional.

Aos analistas e técnicos da Embrapa Instrumentação, que estavam sempre dispostos a ajudar e foram fundamentais no desenvolvimento deste projeto.

À Embrapa Instrumentação pela excelente infraestrutura e pelo apoio institucional no desenvolvimento deste projeto.

À UFSCar e ao Departamento de Engenharia Química, pela oportunidade, apoio institucional e pela infraestrutura.

Aos professores que eu tive a oportunidade de ter durante a minha graduação e na pós-graduação. Seus ensinamentos foram essenciais para o meu crescimento pessoal e profissional.

Às agências de fomento, CNPq (processo n° 141303/2019-0), CAPES (financiamento 001) e FAPESP, pelo apoio financeiro para o desenvolvimento deste projeto.

E a todos que de alguma forma contribuíram para o desenvolvimento deste projeto de doutorado.

A todos vocês, o meu muito obrigada!

LIST OF FIGURES

LIST OF TABLES

RESUMO

Inoculantes microbianos são uma alternativa sustentável para se aumentar a produtividade agrícola e reduzir os problemas ambientais e de saúde causados pelo uso exacerbado de produtos agroquímicos. Estes bioprodutos são formulações com microrganismos vivos que são capazes de aumentar o crescimento e o desenvolvimento das plantas. No entanto, para que os inoculantes tenham sucesso comercial, alguns desafios precisam ser enfrentados, tais como o aumento da vida útil desses bioprodutos e a proteção dos microrganismos após a aplicação no solo. Nesse sentido, uma formulação adequada desempenha um papel crucial na sobrevivência e eficácia dos inoculantes. O encapsulamento de microrganismos em matrizes de polímeros naturais tem se mostrado uma solução promissora em atender esses requisitos. Ademais, esses materiais apresentam vantagens como biocompatibilidade, biodegradabilidade, são renováveis e podem ser utilizados como fonte de nutrientes pelos microrganismos. Embora ainda pouco explorados para esse tipo de aplicação, a avaliação de materiais obtidos a partir da celulose, tais como a carboximetilcelulose ou a nanocelulose, podem apresentar resultados interessantes no aumento da proteção de inoculantes. Desse modo, esta tese teve como objetivo avaliar o uso de nanocristais de celulose (CNC) e do compósito CNC:carboximetilcellulose (CNC:CMC) no aumento do tempo de prateleira e da proteção do fungo *Trichoderma harzianum*, um inoculante microbiano muito reportado na literatura, principalmente devido a sua ação como agente de controle biológico. Para tanto, dispersões de 5% (m/v) CNC e a mistura de 5% (m/v) CNC com 1,5% (m/v) CMC na razão volumétrica de 3:1 (CNC:CMC), contendo uma concentração de esporos de 10^9 esporos/g polímeros foram gotejadas em uma solução reticulante de $CaCl_2$ (1 M). Após a coagulação, a viabilidade do microrganismo foi avaliada e as esferas produzidas foram armazenadas úmidas e sob refrigeração (4 °C). Caracterizações como microtomografia de raio-X e microscopia eletrônica de varredura (MEV) mostraram a boa dispersão dos polímeros nas matrizes de encapsulamento, além da presença do microrganismo na superfície do material. Experimentos de tempo de prateleira ressaltaram que ambas as matrizes foram capazes de reduzir a mortalidade dos esporos encapsulados em comparação com o microrganismo livre (armazenado nas mesmas condições) após até 12 meses de armazenamento. O encapsulamento também foi capaz de aumentar a proteção do microrganismo contra a exposição prolongada a temperatura de 40 °C, a incidência direta de radiação UV-C e ao contato com um fungicida químico comercial. Além disso, após 1 ano de armazenamento, a ação de biocontrole do *T. harzianum* contra o fitopatógeno *Fusarium solani* manteve-se preservada. Sendo assim, os materiais aqui avaliados se mostraram eficientes no aumento da proteção de inoculantes microbianos, mostrando-se uma alternativa interessante para a formulação desses materiais e para fazer com que a agricultura caminhe no sentido de práticas mais sustentáveis, alinhadas aos conceitos de bioeconomia.

Palavras chave: nanocelulose, carboximetilcelulose, inoculantes microbianos, encapsulamento, tempo de prateleira, *T. harzianum*.

ABSTRACT

Microbial inoculants are a sustainable alternative to increase agricultural productivity and reduce environmental and health problems caused by the exacerbated use of agrochemicals. These bio-based products are formulations with living microorganisms that are able to increase the productivity of vegetal crops. Nevertheless, for the commercial success of these bioproducts, some challenges still need to be overcome, particularly those related to increasing the inoculant shelf life, and its protection during storage and after its application. In this sense, a suitable formulation plays a crucial role in the survival and efficacy of inoculants. For instance, encapsulating microorganisms in natural polymeric matrices has shown promising results in meeting the requirements for a good formulation. These materials have advantages such as biocompatibility, biodegradability, renewability and can be used as a nutrient source by microorganisms. In this context, although still underexplored for this type of application, the evaluation of cellulose-based materials, such as carboxymethyl cellulose or nanocellulose, may result in positive outcomes on increasing microbial inoculants protection. Therefore, the objective of this thesis was to assess the use of cellulose nanocrystals (CNC) and the CNC:carboxymethyl cellulose (CNC:CMC) composite for increasing the shelf-life and protection of the fungus *Trichoderma harzianum*, a well-known microbial inoculant mainly due to its effect as a biocontrol agent. To this end, dispersions of 5% (w/v) CNC and a 5% (w/v) CNC mixed with a 1.5% (w/v) CMC at a volumetric ratio of 3:1 (CNC:CMC), containing a spore concentration of 10^9 spores/g of polymers, were dripped to a crosslinking solution of $CaCl_2$ (1 M). After coagulation, the viability of the microorganism was evaluated, and the produced beads were stored wet under refrigeration (4 °C). Characterizations such as X-ray microtomography and scanning electron microscopy (SEM) showed a good dispersion of the polymers in the encapsulating matrices, as well as the presence of the microorganism on the capsules' surface. Shelf-life experiments highlighted that both matrices were able to reduce the mortality of encapsulated spores compared to the free microorganism (stored under the same conditions) after 9 and 12 months of storage. Additionally, the encapsulation increased the fungus protection when exposed to a temperature of 40 °C, a direct UV-C radiation, and during the contact with a commercial fungicide. Furthermore, after 1 year of storage, the biocontrol action of *T. harzianum* against the phytopathogen *Fusarium solani* remained preserved. Thus, the evaluated materials proved to be efficient in enhancing the protection of microbial inoculants, offering an interesting alternative for the formulation of these materials and for moving agriculture towards more sustainable practices aligned with the concepts of bioeconomy and climate change concerns.

Keywords: nanocellulose, carboxymethylcellulose, microbial inoculant, encapsulation, shelf-life, *T. hazianum*.

SUMMARY

CHAPTER 1 – Structure of the Thesis, Literature Review and Objectives

1.1. STRUCTURE OF THE THESIS

This thesis was organized in four chapters. Chapter 1 provides a brief introduction about microbial inoculants, the importance of their formulations and cellulose derivatives as interesting materials for inoculants encapsulation. In the end of this chapter, the general and specific goals of this thesis are presented.

Chapter 2 reports the production and characterization of environmentally friendly beads made of cellulose nanocrystals (CNC) and a composite of CNC and carboxymethyl cellulose (CNC:CMC). We also evaluated if these materials are promising candidates for the encapsulation of the biocontrol fungus *Trichoderma harzianum*.

Chapter 3 describes the encapsulation effect on the microorganism shelf-life and protection against some stressful conditions such as heat exposure, UV-radiation incidence, and the contact with a chemical fungicide. Additionally, the ability of the encapsulated spores stored for more than one year in keeping their antagonistic effect was also assessed.

Both chapters 2 and 3 were written in the form of scientific research papers, presenting theoretical basis, methodology, results, and discussion of the obtained data. Some parts of these chapters are somewhat similar since the scientific research of each one has many aspects in common. It is noteworthy that Chapter 2 is an adapted version of the scientific article published in the journal "Carbohydrate Polymers"

(https://doi.org/10.1016/j.carbpol.2022.119876). Chapter 3 is still being prepared for submission.

Chapter 4 presents an overview of the developed research, briefly describing what was done, the general conclusions of the Thesis and the future perspectives on the developed research.

1.2. LITERATURE REVIEW

1.2.1. Microbial Inoculants

Increasing crop productivity is of paramount importance to meet the ever-growing food demand of the global population. With the global population projected to reach 9.7 billion by 2050 (United Nations Department of Economic and Social Affairs, 2022), it is crucial to optimize agricultural practices to ensure sufficient food supply (FAO, 2021). Chemical fertilizers and pesticides have played a significant role in enhancing crop productivity by providing essential nutrients and controlling pests and diseases. These inputs have revolutionized agriculture, allowing for increased yields and improved crop quality (Devi et al., 2022). However, the uncontrolled and excessive use of chemical fertilizers and pesticides can lead to detrimental environmental and health consequences (Devi et al., 2022; Gill et al., 2022).

The unregulated application of chemical fertilizers contributes to soil degradation, water pollution, and greenhouse gas emissions (Devi et al., 2022; Gill et al., 2022). Nitrogen-based fertilizers, for instance, can leach into water bodies, causing eutrophication and damaging aquatic ecosystems (Kremser and Schnug, 2002; Sharpley et al., 1987). Moreover, pesticide misuse can harm beneficial insects, birds, and other wildlife, disrupting ecological balance (Narayanan et al., 2022). To address these environmental challenges and achieve sustainable agricultural productivity, it is imperative to adopt more eco-friendly and low-carbon practices within a bioeconomy framework (Trigo et al., 2023).

In this context, microbial inoculants have emerged as a promising and sustainable alternative to agrochemicals. Microbial inoculants consist of beneficial microorganisms such as bacteria and fungi, which are applied to seeds, roots, soil or leaves to enhance

plant growth and protection against pests and diseases (Sammauria et al., 2020; Santos et al., 2019). These microorganisms can live free or can establish symbiotic relationships with plants, promoting nutrient uptake, improving soil structure, and stimulating plant defense mechanisms. Moreover, microbial inoculants offer the potential for reducing chemical inputs, thus minimizing environmental pollution, and preserving biodiversity (Sammauria et al., 2020; Santos et al., 2019). Integrating microbial inoculants into agricultural systems aligns with the principles of a low carbon and bioeconomy concept, fostering sustainable food production while minimizing negative ecological impacts (Trigo et al., 2023).

Figure 1 illustrates a few ways of how microbial inoculants can increase plants growth and mitigate a phytopathogen infection. Some microorganisms can carry out biological nitrogen fixation, reducing atmospheric nitrogen to forms that plants can more easily assimilate (NH_4^+), through the action of nitrogenase enzymes (Franche et al., 2009). Most of the phosphorus (P) presented in soil are in insoluble forms (immobilized), which cannot be uptaken by plants (Krishnaraj and Dahale, 2014; Ribeiro and Carmo, 2019). Some microorganisms such as bacteria from the genus *Bacillus, Pseudomonas and Rhizobium*, and the fungi from genus *Aspergillus, Penicillium* and *Trichoderma* are well-known as phosphorus solubilizers (Babalola and Glick, 2012; Sharma et al., 2013; Verma et al., 2014). Inorganic P can be solubilized by organic acids produced by the microorganisms (Klaic et al., 2018; Sharma et al., 2013). Organic P can be released by the action of enzymes (produced by the inoculants), such as phytase and phosphatase (Sharma et al., 2013).

Phytohormones such as gibberellins, cytokinins, ethylene and indole-3-acetic acid can be produced and released by some microorganisms, which can end up stimulating plants growth, attenuate stressful conditions and induce the plants defense mechanism

(Bulegon et al., 2019; Vessey, 2003). Additionally, some inoculants can act as a biocontrol agent, inhibiting the harmful effect of phytopathogens (Mona et al., 2017; Zhang et al., 2016).

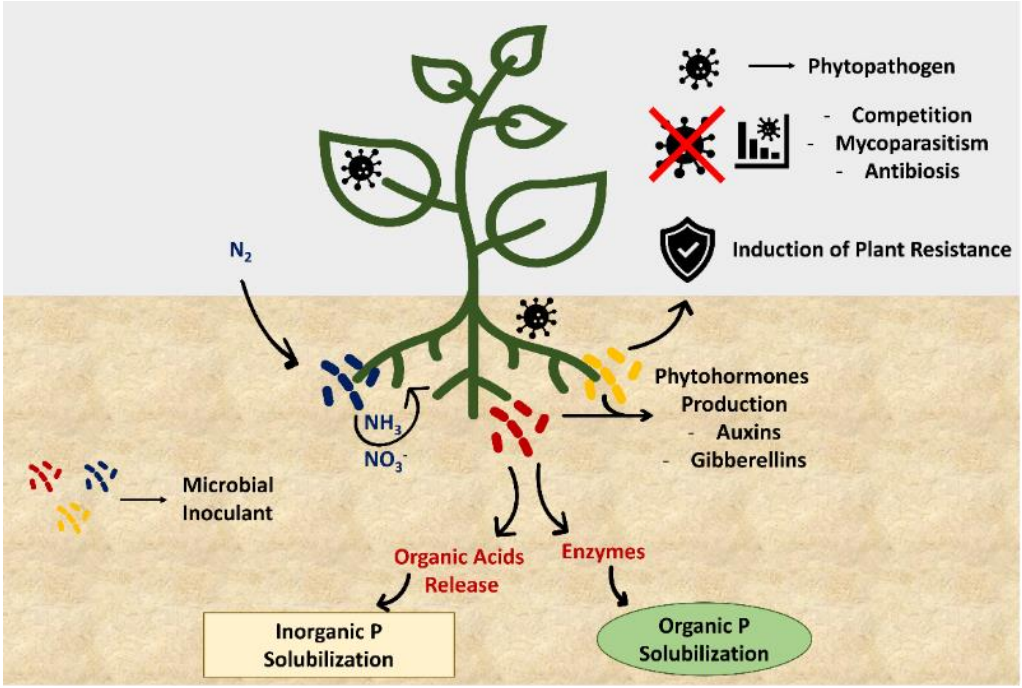


Figure 1. Illustration of how a microbial inoculant can act to increase plant growth and to mitigate a phytopathogen infection.

Trichoderma harzianum is a fungus known for its remarkable efficacy as a biocontrol agent in agriculture (Saravanakumar et al., 2017; Stummer et al., 2020; Zhang et al., 2016). This fungus exhibits antagonistic properties against various plant pathogens, including fungi, bacteria, and nematodes. *T. harzianum* can reduce the phytopathogen incidence through multiple mechanisms, such as mycoparasitism, and the production of antifungal compounds. By colonizing the rhizosphere and plant roots, it actively competes for resources with pathogenic organisms suppressing their growth and preventing disease establishment. Its ability to degrade fungal cell walls and release lytic

enzymes further contributes to its biocontrol potential (Mancera-López et al., 2019; Stummer et al., 2020; Zhang et al., 2016).

Additionally, *Trichoderma harzianum* offers several other benefits to plants. It can enhance nutrient availability in the soil by solubilizing phosphate and mobilizing micronutrients, making them more accessible for plant uptake (Ali et al., 2022). This feature is particularly beneficial in nutrient-deficient soils, leading to improved plant nutrition and growth. Moreover, it has been found to mitigate stressful conditions such as drought and salinity by inducing stress tolerance mechanisms in plants (Mona et al., 2017). It can produce phytohormones, such as auxins and cytokinins, which regulate plant growth and development, including root and shoot growth, flowering, and fruiting (Illescas et al., 2021; Zhang et al., 2013). These characteristics make *Trichoderma harzianum* a valuable microbial inoculant for enhancing plant health, productivity, and resilience in agricultural systems.

Besides the positive effects reported previously, the establishment of microbial inoculants as commercial products faces several challenges that need to be overcome (Kaminsky et al., 2019; Qiu et al., 2019; Vassilev et al., 2020). Since these products contain live microorganisms, maintaining the viability and efficacy of these organisms throughout the production, packaging, storage, and application processes is crucial. Another challenge lies in their compatibility with existing agricultural practices and inputs (Kaminsky et al., 2019; Qiu et al., 2019; Vassilev et al., 2020). Integrating microbial inoculants into conventional farming systems requires addressing issues such as compatibility with chemical fertilizers and pesticides, as well as compatibility with different crop varieties and soil types. Furthermore, protecting the microorganism's post-application is crucial for their survival and long-term efficacy in the field. Factors like UV radiation, temperature fluctuations, soil conditions, and competition with native

microorganisms can pose risks to the introduced microbial populations (Kaminsky et al., 2019; Qiu et al., 2019; Vassilev et al., 2020). Additionally, scalability and cost-effectiveness are significant factors in the commercialization of microbial inoculants. In this sense, developing cost-efficient production methods, optimizing formulation and application techniques, and demonstrating long-term economic benefits for farmers are essential steps in establishing microbial inoculants as viable and widely adopted commercial products (Kaminsky et al., 2019; Qiu et al., 2019; Vassilev et al., 2020). Robust research and development efforts are required to optimize these aspects and ensure the successful commercialization of microbial inoculants.

1.2.2. Microbial Inoculant Formulations

As mentioned before, the formulation in which microbial inoculant is delivered plays a crucial role in the successful application and efficacy of this bioproduct in agriculture. Proper formulation helps protect the microorganisms, enhance their viability, and ensure targeted delivery to the intended plant or soil environment (Qiu et al., 2019). One key aspect of formulation is the choice of carrier materials, which provide a protective matrix for the microorganisms. Common carrier materials include peat, vermiculite, clay, and various organic materials (Bashan et al., 2014). These carriers offer physical support, protect against environmental stressors, and promote microbial survival during storage and application. Additionally, formulation techniques such as granulation, pelletization, and encapsulation can further enhance the stability and protection of microbial inoculants (Bashan et al., 2014). The formulation process also allows for the addition of additives such as nutrients, growth-promoting substances, and protectants, which can improve the performance and efficacy of microbial inoculants. Different types of formulations are available, providing options that suit various application methods and

environmental conditions. A summary of the available formulations is illustrated by Figure 2.

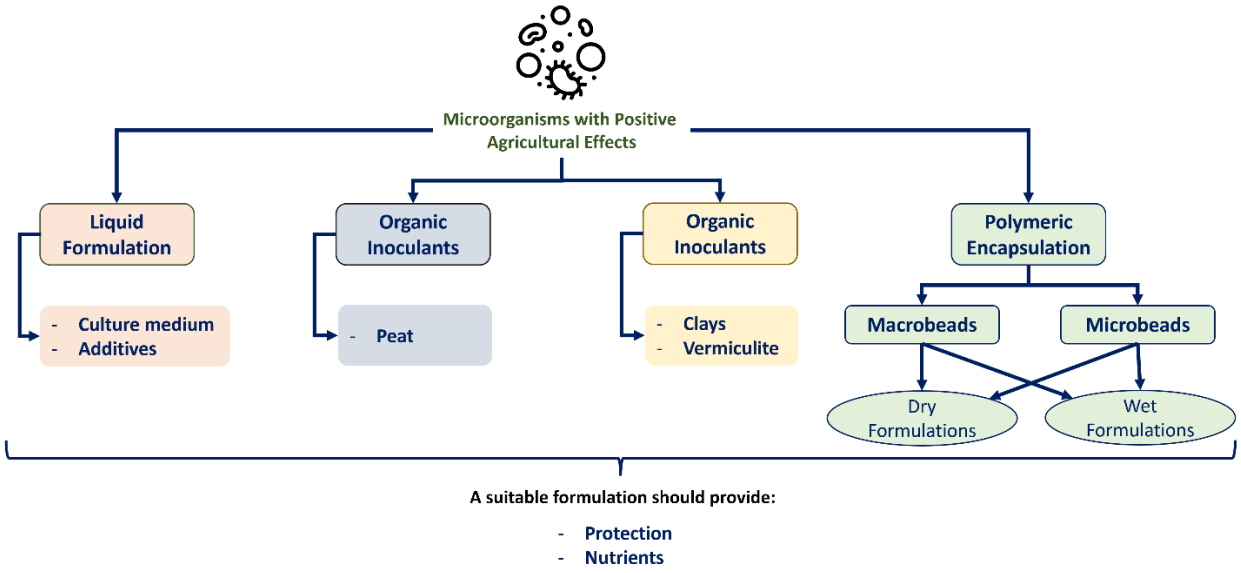


Figure 2. Schematic representation of the possible formulation types for microbial inoculant and how it can be applied in agriculture (adapted from Bashan et al., 2014).

Liquid formulations

Liquid formulations for microbial inoculants offer several advantages such as an easier application, uniform distribution, and rapid colonization on plant surfaces or in the rhizosphere (Bashan et al., 2014). These formulations typically consist of suspensions or solutions containing the desired microorganisms. One advantage of liquid formulations is their convenience and flexibility in application. They can be easily applied as seed treatments, foliar sprays, or through irrigation systems, allowing for efficient and targeted delivery of the microorganisms. Their production is generally simpler than other carrier methods, with low cost, and can work with a high concentration of cells in the product (Bashan et al., 2014; Goljanian-Tabrizi et al., 2016; Lobo et al., 2019).

To further improve the performance of liquid formulations, various additives can be incorporated. These additives may include nutrients, surfactants, adhesives, and protectants (Anjali et al., 2019; Gopal and Baby, 2016). Nutrients can provide essential elements that support microbial growth and activity. Surfactants help improve the spread and adherence of the formulation on plant surfaces, ensuring better contact between microorganisms and the target area. Adhesives can enhance the attachment of the formulation to seeds or plant tissues, improving its longevity and effectiveness. Protectants, such as stabilizers or preservatives, can be added to extend the shelf life and maintain the viability of the microorganisms during storage and application. In this regard, Anjali et al. (2019) reported that liquid formulations with additives (Carboxymethylcellulose (CMC), Polyvinylpyrrolidone (PVP), and Trehalose) were able to maintain high viability of *Mesorhizobium* and *Rhizobium* bacteria compared to free cells and solid formulations on charcoal for a period of 300 days. However, it is important to note that these additives may impact the final product price (Anjali et al., 2019; Gopal and Baby, 2016).

Several commercial microbial inoculants are available in liquid formulations (Berger et al., 2018; Lobo et al., 2019). It is worth highlighting Biomaphos here in Brazil, the first Brazilian liquid inoculant for phosphorus solubilization developed in a partnership between EMBRAPA and the company Bioma. This biofertilizer was developed with the bacteria species *Bacillus subtilis* and *B. megaterium* and, when applied in corn plantations, resulted in a 10% increase in grain yield, besides enhancing plant growth (EMBRAPA, 2019).

As disadvantages of these formulations, the liquid nature of the product often results in a large volume, affecting transportation and storage (Bashan et al., 2014; Zayed, 2016). Additionally, in order to ensure higher cell viability, storage usually needs to occur

under refrigerated conditions (4°C). Moreover, cells in these formulations are more sensitive to stress conditions (although some additives can mitigate these effects), which ended up decreasing the product shelf-life. However, the main disadvantage lies in the reduced cell protection after soil application, which may impact viability and require larger quantities of the product to mitigate this issue (Bashan et al., 2014; Zayed, 2016).

Solid formulations

Microbial inoculants in solid formulations offer several advantages in agricultural and environmental applications. First, solid formulations provide enhanced shelf life and stability, allowing for long-term storage without compromising the viability and efficacy of the microbial inoculants (Bashan et al., 2014; Schoebitz et al., 2013a). This ensures that the desired microbial populations remain intact until the time of application. Secondly, solid formulations are easy to handle, transport, and apply, making them convenient for large-scale production and distribution. They can be formulated into granules, pellets, powders, or tablets, depending on the specific needs and requirements (Florencio et al., 2022; Santos et al., 2019; Zayed, 2016). Additionally, solid formulations offer protection to the microorganisms against harsh environmental conditions, such as extreme temperatures and UV-radiation and chemical stressors, thereby increasing their survival and effectiveness upon application.

However, one potential drawback of solid formulations is the slower release of microorganisms compared to liquid formulations, which may affect their immediate impact on target systems (Bashan et al., 2014; O'Callaghan et al., 2022; Santos et al., 2019). Nevertheless, this characteristic can be desired when an immediate effect is not required. Additionally, this controlled release property combined with the environmental

stressors' protection can be particularly interesting when a severe condition (such as an extreme drought) happens, which can significantly affect the microorganism viability. In this case, the microorganisms entrapped in a solid matrix, by presenting a lower release rate, will definitely have a higher protection than the free ones in the soil, increasing their survivability. An additional issue related to these formulations is the production cost, which is usually higher than liquid products (Bashan et al., 2014; Santos et al., 2019; Schoebitz et al., 2013a; Vemmer and Patel, 2013). Nonetheless, it is important to highlight that usually, a solid formulation requires fewer applications during crops' development, which may end up compensating the higher production cost.

Encapsulation of microbial inoculants is gaining attention in recent years. This technique involves the coating or entrapment of microorganisms within a protective matrix, providing numerous benefits (Pereira et al., 2023; Schoebitz et al., 2013a; Vassilev et al., 2020). The use of natural polysaccharides as encapsulation matrices for microbial inoculants provides several advantages in terms of biocompatibility and sustainability. Natural polysaccharides, such as alginate, chitosan, and starch, are abundant, renewable, and biodegradable materials, making them environmentally friendly alternatives to synthetic encapsulation matrices (Campos et al., 2014; Rojas-Sánchez et al., 2022; Vassilev et al., 2020). These polysaccharides offer excellent encapsulation properties, allowing for the efficient entrapment and protection of microorganisms. Moreover, natural polysaccharides can be easily modified to improve their encapsulation efficiency, stability, and release characteristics, further enhancing their utility as encapsulation matrices for microbial inoculants (Campos et al., 2014; Rojas-Sánchez et al., 2022; Vassilev et al., 2020).

Microbial inoculants can be encapsulated using various production processes, each offering unique advantages and considerations. One commonly used method is

extrusion, where the microbial cells are mixed with a matrix material and extruded through a nozzle to form droplets or beads. The droplets are then solidified through processes such as coagulation or drying to create the encapsulated microbial inoculants (Schoebitz et al., 2013a). Another technique is spray drying, which involves atomizing a suspension or solution of the microorganisms and matrix material into a drying chamber. The microorganisms and matrix material are rapidly dried, forming encapsulated particles with improved stability (Felizatti et al., 2021; Schoebitz et al., 2013a). Encapsulation can also be achieved through emulsion or coacervation methods, where the microbial cells are dispersed within a liquid phase and then surrounded by a polymer or polysaccharide matrix, forming microcapsules (Saberi Riseh et al., 2021; Schoebitz et al., 2013a). Freeze drying is another approach where the microbial cells and matrix material are frozen, followed by sublimation of the ice to remove moisture, resulting in a dry powder with encapsulated microorganisms (Lopes et al., 2020). These different production processes offer flexibility in terms of encapsulation efficiency, particle size control, and release characteristics, allowing for the development of tailored microbial inoculant formulations to meet specific application requirements. Figure 3 illustrate some encapsulation methods.

One of the most commonly reported polysaccharides for encapsulating microbial inoculants is alginate (Locatelli et al., 2018; Pereira et al., 2023; Riseh et al., 2021). Sujarit et al. (2020) encapsulated the bacterium *Streptomyces palmae* in alginate for use in biocontrol against the fungus *Ganoderma boninense*. The encapsulation spheres were produced by dripping the polymeric solution into a calcium chloride coagulation bath. The authors observed that, in addition to increasing the shelf life of the bacterium compared to free spores, the use of encapsulated microorganisms reduced the pathogen's effect on palm trees, demonstrating even a superior effect to that of commercial

fungicides. Furthermore, there was a slow and controlled release of the bacterium in the soil, allowing for improved colonization compared to the use of free microorganisms (Sujarit et al., 2020).

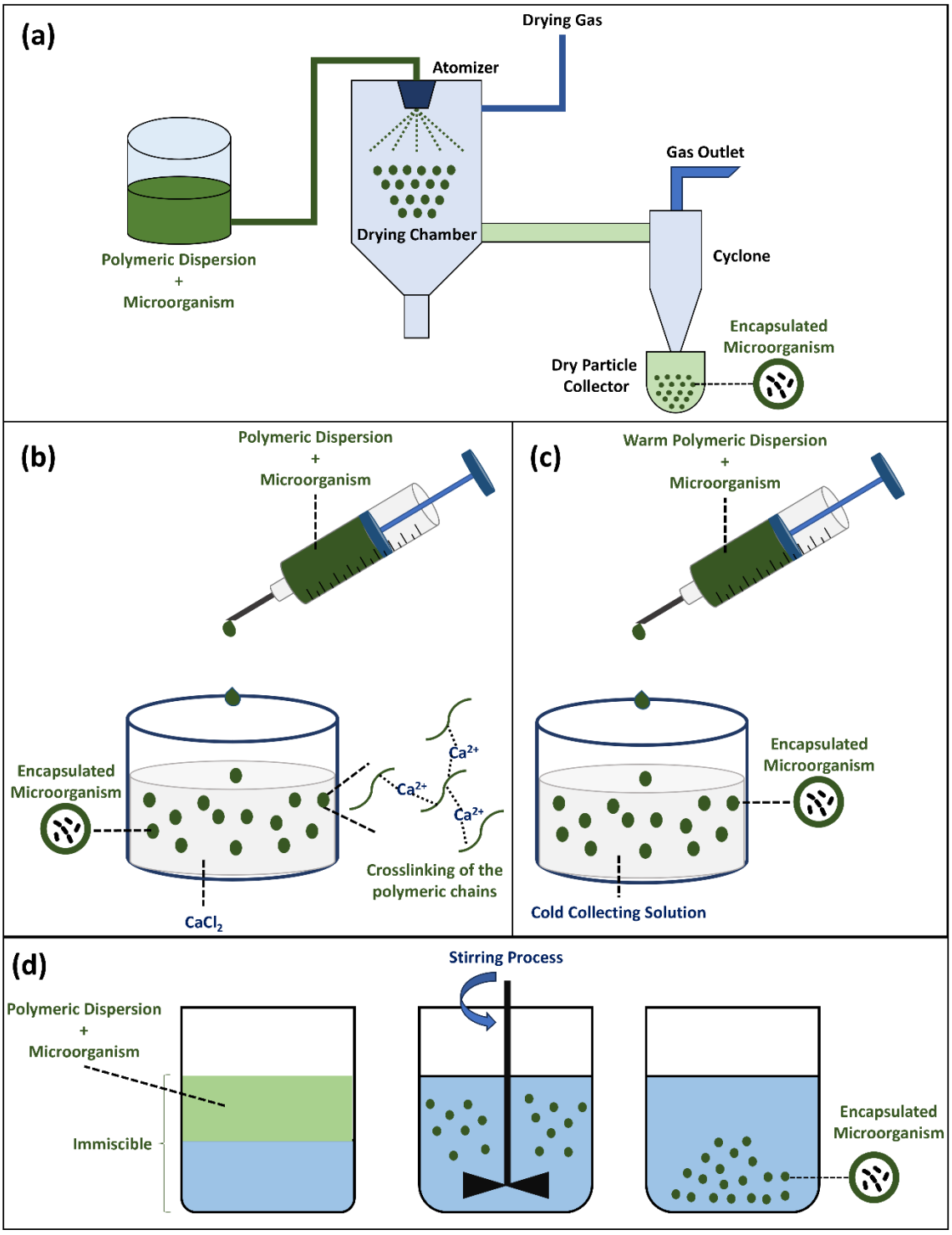


Figure 3. Illustration of some methods for microbial inoculant encapsulation. (a) spray dryer, (c) ionic crosslinking, (d) thermal gelation, and (e) emulsion.

Often, the use of polysaccharides alone is insufficient to ensure good mechanical properties of the encapsulation matrix, controlled release of the microorganism in the soil, and source of nutrients for its development when applied in the field. Therefore, additives such as clay minerals, humic acids, skim milk, nanomaterials, among others, are employed to enhance these properties (He et al., 2015; Marinković et al., 2018; Minaxi and Saxena, 2011). Clay minerals such as bentonite have been reported to increase the mechanical strength of the material, enable slow and controlled release of cells, improve shelf life, and protect microorganisms from the effects of ultraviolet (UV) radiation (He et al., 2015; Wu et al., 2012).

1.2.3. Cellulose nanocrystals (CNC) and carboxymethyl cellulose (CMC)

Cellulose is a complex carbohydrate that serves as the main structural component of plant cell walls (40 – 50%), being the most abundant natural polymer on earth. It is composed of long chains of glucose monomers linked together through β-1,4-glycosidic bonds (Nimz, 1984). Cellulose can be extracted from lignocellulosic biomass through mechanical or chemical processes (Pinto et al., 2022). The resulting cellulose is characterized by a hierarchical structure, consisting of microfibrils that are formed by the packaging of cellulose chains due to hydrogen and Van de Waals bonds (Ballesteros, 2010; Horn et al., 2012). These microfibrils have crystalline regions, which exhibit a high ordering degree, and amorphous regions, which can be more easily depolymerized by the synergistic action of cellulolytic enzymes produced by microorganisms. The relative crystalline and amorphous content can vary depending on the plant source and processing methods (Horn et al., 2012; Pinto et al., 2022). Cellulose unique physical and chemical properties, such as high tensile strength and biodegradability, make it a versatile material

with a wide range of applications, including in the fields of textiles, paper manufacturing, biofuels, and biomedical engineering.

Cellulose derivatives are modified forms of cellulose that have been chemically altered to introduce new properties and functionalities. These modifications involve substituting hydroxyl groups on the cellulose chain with different chemical functional groups (Heinze et al., 2018; Shaghaleh et al., 2018). Common cellulose derivatives include cellulose acetate, cellulose ethers (such as methylcellulose and hydroxyethylcellulose), and carboxymethylcellulose. The introduction of these functional groups imparts various desirable properties to cellulose, such as improved solubility, increased thermal stability, enhanced film-forming abilities, and controlled rheological behavior (Heinze et al., 2018; Shaghaleh et al., 2018).

Carboxymethylcellulose (CMC) is a cellulose derivative produced through introduction of carboxymethyl groups onto the cellulose structure (Figure 4). This material possesses several notable properties that make it a valuable material in various applications (Rahman et al., 2021). Firstly, CMC is highly water-soluble, forming clear and viscous solutions when dispersed in water. This property allows for easy incorporation of CMC into formulations and facilitates its use as a thickening, stabilizing, and binding agent. Additionally, CMC exhibits excellent film-forming capabilities, forming flexible and transparent films when cast. The film-forming property of CMC enables its utilization in coatings, packaging materials, and controlled-release systems (Rahman et al., 2021). CMC is also known for its ability to absorb and retain water, which contributes to its application as a hydrogel or moisture-retaining agent in agriculture and personal care products (Bauli et al., 2021). Furthermore, CMC is biodegradable, biocompatible, and non-toxic, making it environmentally friendly and safe for various applications. These properties collectively make CMC a versatile material with a wide

range of uses in industries such as food, pharmaceuticals, textiles, and agriculture (Rahman et al., 2021).

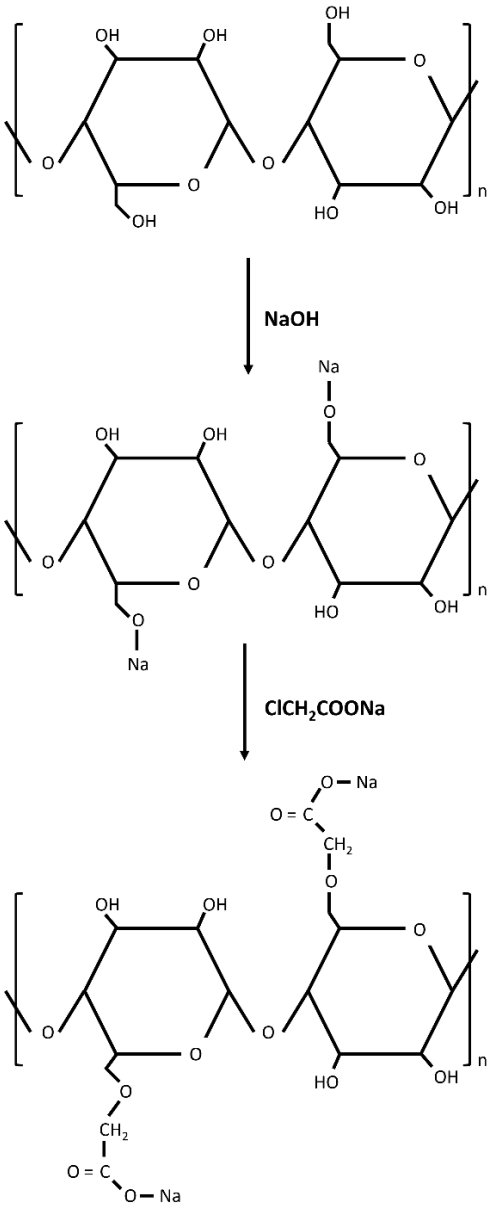


Figure 4. Sodium CMC production from cellulose.

Carboxymethyl cellulose has found significant agricultural applications, particularly in the form of hydrogels and encapsulation matrices for bioactive compounds (pesticides) and fertilizers (Davidson et al., 2013; Rahman et al., 2021; Saberi Riseh et al., 2023). CMC hydrogels, with their water-absorbing properties, are utilized to improve

soil water retention, enhance nutrient availability, and promote plant growth. These hydrogels act as reservoirs, releasing water and nutrients gradually to the plant roots, allowing controlled nutrient release and minimizing nutrient leaching, thereby increasing water-use efficiency and nutrient uptake (Bauli et al., 2021; Rahman et al., 2021; Saberi Riseh et al., 2023). Moreover, CMC is employed as an encapsulation matrix for bioactive compounds, such pesticides and plant growth regulators. The encapsulation process protects these bioactives from environmental degradation, ensuring their controlled release and prolonged activity in the soil (Rahman et al., 2021; Saberi Riseh et al., 2023).

For instance, a CMC hydrogel used as a root targeted delivery vehicle to encapsulated and slowly release a liquid fertilizer was able to reduce in 78% the amount of fertilizer needed to achieve similar levels of plant yield (in comparison with the incapsulated agrochemical). Additionally, the CMC matrix presented a similar lifetime in soil than wheat crops, which allowed a fertilizer delivery over the entire plant's growth and development (Davidson et al., 2013).

The addition of fillers to CMC matrices can significantly improve certain material properties. Fillers, such as nanomaterials, can enhance the mechanical strength and thermal stability of CMC matrices. The incorporation of fillers can reinforce the structure, increase the tensile strength, and improve the resistance to deformation or fracture (Bauli et al., 2021; Jannatyha et al., 2020; Singh et al., 2017). Additionally, fillers can modify the rheological properties of CMC matrices, improving their viscosity, flow behavior, and handling characteristics. Fillers can also impact the barrier properties of CMC matrices, enhancing their resistance to moisture, gases, or UV radiation. Furthermore, the addition of fillers can provide additional functionalities to CMC matrices, such as antimicrobial properties, or enhanced release kinetics of encapsulated substances (Bauli et al., 2021; Jannatyha et al., 2020; Saberi Riseh et al., 2023). For instance, cellulose nanocrystals

(CNC) additions to a CMC matrix were able to increase the material performance as a nutrient delivery system, providing a controlled release of NPK fertilizer (Bauli et al., 2021). Additionally, CNC was able to increase in 60% the water absorption capacity of the hydrogel. The authors highlighted that both CNC and CMC are highly compatible materials and can have a strong interaction due to the formation of hydrogen bonds between them (Bauli et al., 2021).

Cellulose nanocrystals (CNCs) are a type of nanomaterial produced from cellulose fibers through a controlled hydrolysis process. The production of CNCs involves the extraction of cellulose fibers from biomass sources, such as wood or agricultural waste, followed by acid or enzymatic hydrolysis (Figure 5) to break down the cellulose chains into nanoscale crystalline structures (Aziz et al., 2020; Squinca et al., 2020; Thomas et al., 2018). The resulting CNCs possess unique properties such as high aspect ratio (length to diameter, L/D), large surface area, and excellent mechanical strength. CNCs exhibit remarkable thermal stability, biocompatibility, and biodegradability, making them attractive for various applications (Dufresne, 2013; Thomas et al., 2018). In the field of materials science, CNCs are used as reinforcing agents in composites. Their exceptional strength and stiffness enable the development of lightweight and high-performance materials. Due to its high crystallinity, CNC can have a stiffness between 100 – 130 GPa, which is superior to or in the same order of magnitude as materials such as bronze, aluminum alloys and Kevlar (Dufresne, 2013). Additionally, CNCs find applications in the biomedical field, such as drug delivery systems, tissue engineering scaffolds, and wound dressings, due to their biocompatibility and ability to interact with biological systems. The versatile nature of CNCs make them promising candidates for a wide range of applications (Figure 6) in areas including electronics, textiles, energy storage, environmental remediation, and agriculture (do Nascimento et al., 2022; Thomas et al.,

2018; Ventura et al., 2020). As reported before for CMC, nanocellulose hydrogels has also draw attention as promising materials in fields such as medicine, food, and agriculture (Nascimento et al., 2018). In the later field, these materials can be a source of water, nutrients, and pesticides for crops, acting as a controlled release system (Nascimento et al., 2018).

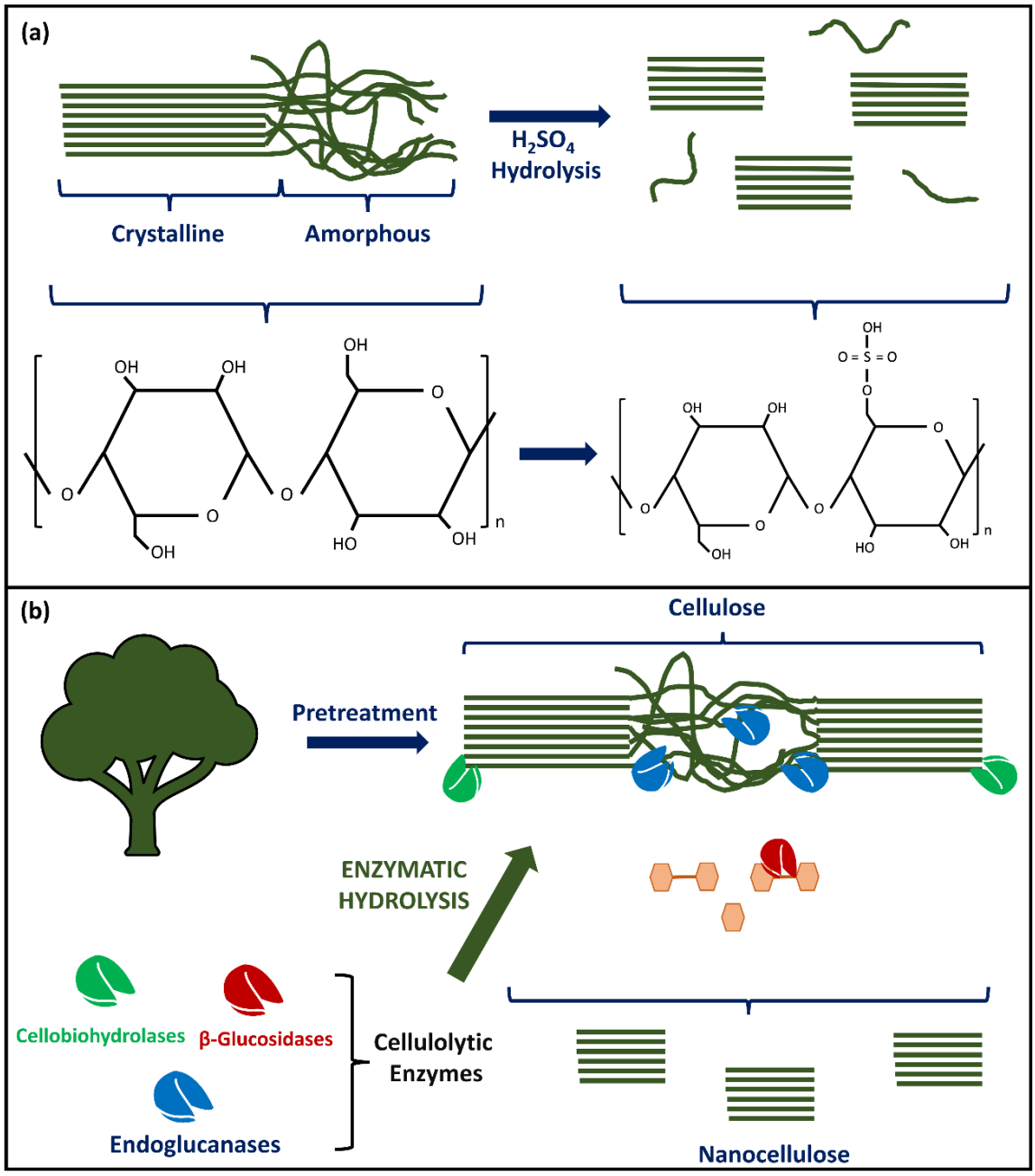


Figure 5. Illustration of (a) acid (sulfuric acid) and (b) enzymatic hydrolysis for nanocellulose production.

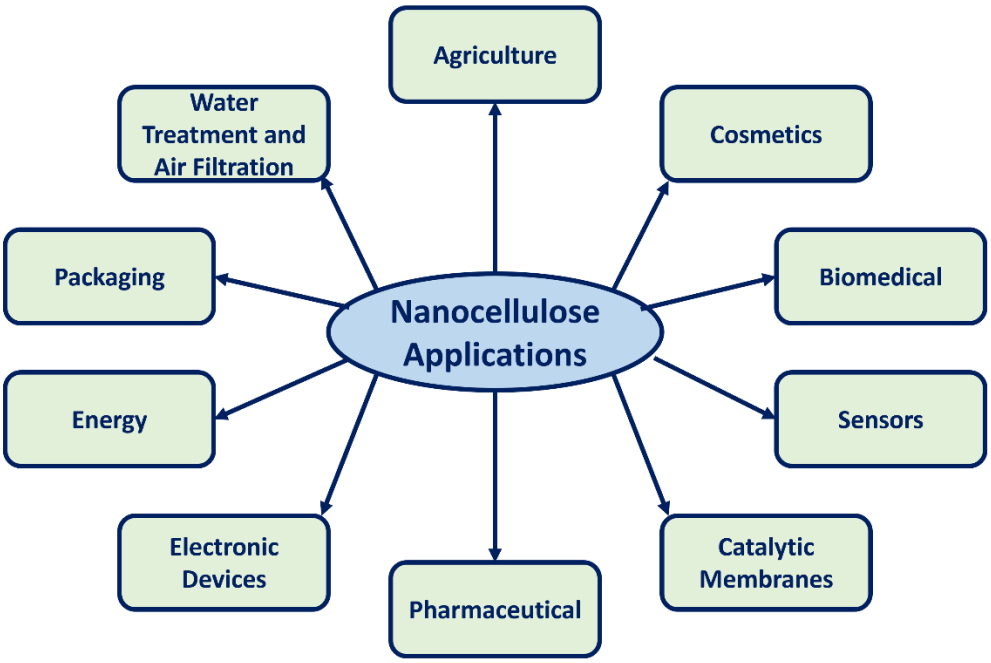


Figure 6. Possible applications of cellulose nanocrystals.

Several research articles report CNC applications as encapsulation matrices for probiotics, drug delivery systems and enzyme immobilization (de Carvalho et al., 2016; Fiorati et al., 2020; Lima et al., 2020). As highlighted in the previous section, these beads can be produced by several methods, such as ionic or thermal gelation, spray-drier, emulsion, etc., with the final diameter of these materials ranging from nanometers to a few millimeters, depending on the technique used for their production (de Carvalho et al., 2016; Gericke et al., 2013; Levin et al., 2018; Lima et al., 2020). The ionic crosslinking with $CaCl_2$ is possible when CNC has functional groups that allow the chains reticulation by the calcium ions (de Carvalho et al., 2016). A particular advantage of this process is that it can be carried out at room temperature, which is a condition that do not harm microorganisms (de Carvalho et al., 2016; Ferrari et al., 2021). Figure 7 illustrates the crosslinking process of TEMPO-oxidized (a) and sulfated (b) nanocellulose.

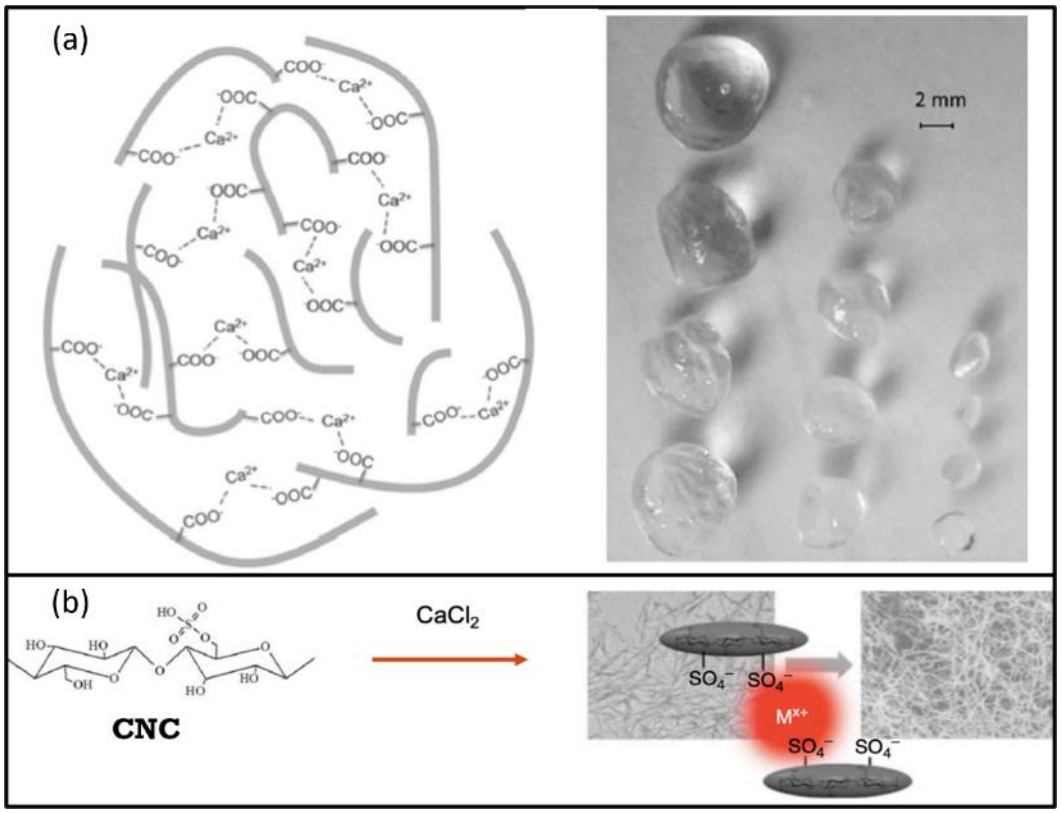


Figure 7. Illustration of the crosslinking mechanism of TEMPO-oxidized CNC and the produced beads (a) and sulfated CNC (b) in the presence of calcium ions (adapted from Chau et al., 2015; de Carvalho et al., 2016).

As can be noticed, CNC and CMC based materials have a wide range of applications, including those related to agriculture. However, up to now, there is no work on literature that evaluates CNC and the CNC:CMC composites as encapsulation matrices for microbials inoculants. Nonetheless, the particular properties and applications reported so far for these materials show that they might be very promising candidates for excellent inoculant carriers. Additionally, while the most reported material for microbial inoculant (alginate) encapsulation has a market price of approximately US$ 10/kg (Strobel et al., 2020), some techno-economic analysis reports a minimum selling price for nanocellulose obtained from sulfuric acid hydrolysis of US$ 4.89/kg – US$ 5.47/kg (Bondancia et al.,

2022; Rajendran et al., 2023), which indicates that CNC may also be a more economic approach.

1.3. OBJECTIVES

The goal of this thesis was to evaluate the effectiveness of the encapsulation of microbial inoculants in a matrix of cellulose nanocrystals (CNC) and in the nanocellulose:carboxymethylcellulose (CNC:CMC) composite in increasing the microorganism shelf-life and its protection against stressful conditions. Furthermore, it was assessed whether, after encapsulation, the microorganism kept its ability to work as a biological control agent, towards suppressing the growth of a phytopathogen. For these experiments, the well-known microbial inoculant *Trichoderma harzianum* was used as a model microorganism.

Specific goals

(i) Evaluate the effectiveness of beads production from dispersions of CNC and CNC:CMC composites by using a simple crosslinking method in $CaCl_2$ solution that can be carried out at room temperature;

(ii) Perform a thermo-chemical and morphological characterization of the beads produced;

(iii) Add *T. harzianum* spores to the polymeric matrices and evaluate the encapsulation effect on the microorganisms viability;

(iv) Perform a thermo-chemical and morphological characterization of the beads containing the microorganism;

(v) Evaluate the encapsulation effect on the microorganism shelf-life;

(vi) Evaluate the effect of encapsulation on the microorganism protection when subjected to the following conditions:

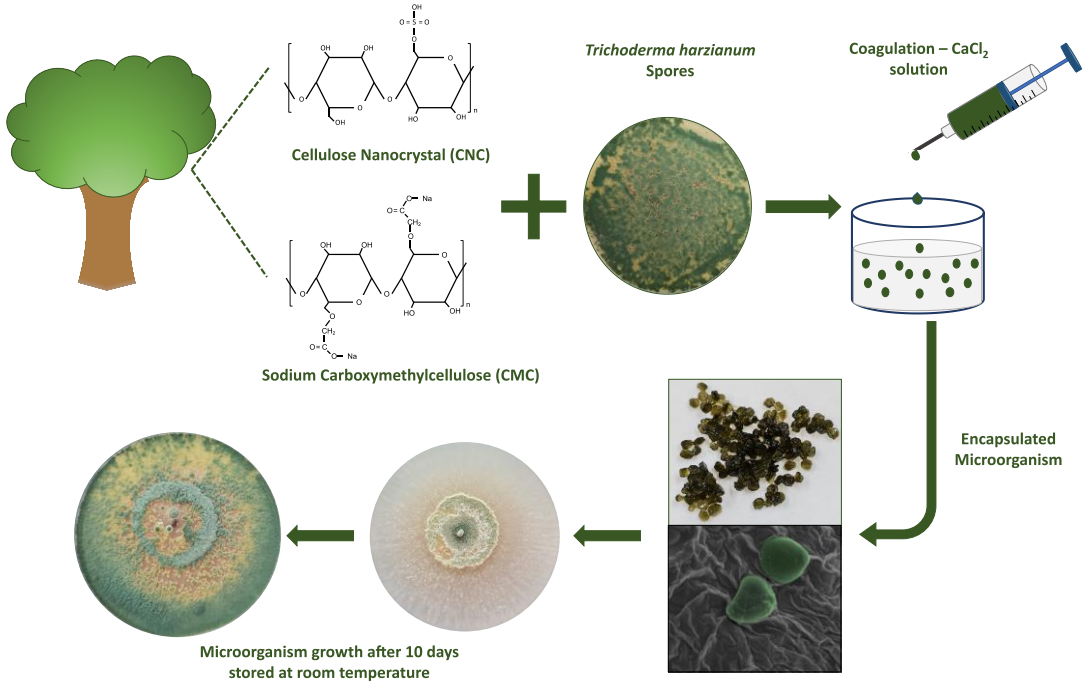
- Heat exposure for different periods;
- UV-light radiation exposure;
- Direct contact with a commercial fungicide;

(vii) Evaluate if the encapsulated microorganisms kept its antagonistic ability of inhibiting a phytopathogen growth in an *in vitro* test after being stored for one year.

CHAPTER 2 - Beads Production, Characterization, and the Evaluation of their Effect on *Trichoderma harzianum* Encapsulation

*The content of this chapter is an adaptation of the scientific article entitle: "Encapsulation of Trichoderma harzianum with nanocellulose/carboxymethyl cellulose nanocomposite", published in 2022 in the journal Carbohydrate Polymers, volume 295, ISSN 0144-8617, doi: 10.1016/j.carbpol.2022.119876 by Brondi, M.; Florencio, C.; Mattoso, L.; Ribeiro, C.; Farinas, C.

GRAPHICAL ABSTRACT



ABSTRACT

This study proposes the use of green matrices of cellulose nanocrystals (CNC) and a nanocomposite of CNC with carboxymethyl cellulose (CMC) for efficiently encapsulating the plant biocontrol agent *Trichoderma harzianum*. Beads containing spores of the microorganism were produced by dripping dispersions of the polymers into a $CaCl_2$ coagulation bath, resulting in the crosslinking of CNC chains by Ca^{2+} ions. SEM micrographs evidenced the *T. harzianum* spores in the encapsulation matrices. X-ray microtomography confirmed the random distribution of the microorganism within the polymeric matrix and the presence of internal pores in the CNC:CMC:spores beads. Encapsulation in the CNC:CMC nanocomposite favored growth of the fungus after 10 days of storage at room temperature, which could be attributed to the presence of internal pores and to the extra carbon source provided by the CMC. The results indicated that CNC:CMC nanocomposites are promising materials for protecting and delivering microbial inoculants for agricultural applications.

Keywords: cellulose nanocrystals, carboxymethyl cellulose, microbial inoculant, encapsulation

2.1. INTRODUCTION

Increased crop productivity is crucial for meeting the growing global demand for food. Chemical fertilizers and pesticides are essential tools for enhancing agricultural production, but the uncontrolled use of these products can negatively affect the environment. Therefore, the challenge is to identify and develop more sustainable practices, such as biofertilization employing materials inoculated with beneficial

microorganisms (Sammauria et al., 2020; Santos et al., 2019). These bio-based products contain living microorganisms that can increase nutrient availability and plant growth, act as biocontrol agents against phytopathogens, mitigate the effects of stressful conditions (such as salt, drought, pH, and temperature), and/or release biostimulants and phytohormones (Egamberdieva et al., 2017; Kour et al., 2020; Seenivasagan and Babalola, 2021). Among the microbial inoculants, the fungus *Trichoderma harzianum* has been widely reported as a biocontrol agent for important crops including soybean, wheat, and maize (Mona et al., 2017; Saravanakumar et al., 2017; Zhang et al., 2016). For instance, its inoculation on soybean plants decreased the effect of the phytopathogen *Sclerotinia sclerotiorum* by 56% (Zhang et al., 2016). *T. harzianum* can also act to increase nutrient solubilization and uptake by plants, as well as the release of phytohormones, enhancing the resistance of plants to stressful conditions (Ghorbanpour et al., 2018; Mona et al., 2017; Ye et al., 2020).

The shelf-life of these bio-based products remains a challenge that can hinder their applications, with the inoculant formulation being of paramount importance. A suitable carrier should maintain the microorganism viable from the time of manufacture of the product until its targeted use (Kaminsky et al., 2019; Qiu et al., 2019). The encapsulation of microbial inoculants can provide a suitable medium for microorganism delivery, protecting against weathering and other stresses, and ideally ensuring conditions for adaptation of the organism after application on crops (Schoebitz et al., 2013b; Vassilev et al., 2020). Natural polysaccharides such as alginate, chitosan, and starch have the advantage of biodegradability (Vassilev et al., 2020), although they may suffer from unstable capsule structure or limited loading capacity (Vassilev et al., 2020). Several studies using the encapsulation method with the alginate matrix have been carried out, the results show that the alginate in the presence of other polymers improves the

microorganism protection against some abiotic stresses, increasing the shelf-life (Locatelli et al., 2018; Maruyama et al., 2020). However, characterized by a low mechanical strength due to its structure, alginate as a carrier presents an unsettled and uncontrolled release of its content (Vassilev et al., 2020). One way to address these problems is to use compositions with nanoparticles, such as nanocomposites (Kumar et al., 2021). For example, the use of nanocellulose-reinforced polymers can maintain complete biodegradability and improve the mechanical properties of the formulation, enabling a wide range of applications (Nascimento et al., 2018).

Carboxymethyl cellulose (CMC) has emerged as an ideal matrix for these nanocomposites, given its natural compatibility with nanocellulose structures (Oun and Rhim, 2017). Both cellulose nanocrystals (CNC) and CMC can be easily used to prepare capsules by different techniques (Calegari et al., 2022; Fitri et al., 2022; Levin et al., 2018; Nie et al., 2021). Therefore, the present work proposes the use of nanocellulose:CMC nanocomposite (CNC:CMC) as a system suitable for protecting *T. harzianum*, employed as a model microorganism. The results showed that the nanocomposite capsules could be prepared by a simple coagulation route, according to a straightforward dripping method. Following the encapsulation process, the protected *T. harzianum* was capable of growth after 10 days of dry storage at room temperature. The procedure could be extended to other microorganisms, making CNC:CMC a versatile and sustainable platform for the delivery of agriculturally relevant microorganisms.

2.2. MATERIALS AND METHODS

2.2.1. Materials

Cellulose nanocrystals (CNCs) (Celluforce, Canada) and sodium carboxymethyl cellulose (CMC - medium viscosity and with a degree of substitution of 0.7) (Synth, Brazil) were used as the encapsulation matrices. The CNCs were obtained by hydrolysis with sulfuric acid (64 wt%), leading to the addition of sulfate half-ester groups at the surface (Reid et al., 2017). According to the manufacturer, it has an average nominal diameter of 7.5 nm and a length of 150 nm, with an aspect ratio of 20. Calcium chloride ($CaCl_2$) (Synth, Brazil) was used as the crosslinking agent. Tween 80 (Dinâmica, Brazil) was employed to extract the spores of the filamentous fungus. All the materials were used as received. The *Trichoderma harzianum* LQC-99 strain was provided by Embrapa Environment (Brazil).

2.2.2. *Trichoderma harzianum* cultivation and spore extraction

Trichoderma harzianum LQC-99 was used as a model microorganism to evaluate the effects of encapsulation in CNC and CNC:CMC on a well-known microbial inoculant (Fraceto et al., 2018; Maruyama et al., 2020). The spores were germinated at 28 ˚C in Petri dishes containing potato dextrose agar (PDA). After 7 days, a 1% (w/v) Tween 80 solution was used to collect the spores, which were then concentrated by centrifugation for 10 min at 8000 rpm. The spore concentration was determined using a Neubauer chamber.

2.2.3. Beads preparation

A preliminary evaluation (reported in the Supplementary Material, Table 1) was used to define the CNC, CMC, and $CaCl_2$ concentrations employed. Nanocomposite beads were produced by coagulation. For the reference samples without spores (Figure 8a), CNC and CMC were dispersed in distilled water and stirred overnight by a magnetic stirrer at room temperature, resulting in dispersions with final concentrations of 5% (w/v) and 1.5% (w/v), respectively. Dispersions of pure CNC and a CNC:CMC mixture (3:1 by volume) were evaluated as the primary materials of the encapsulation matrices. The process was carried out by dripping the polymeric dispersions into a 1 M $CaCl_2$ coagulation bath (kept under mild stirring), at room temperature, using a 3 mL syringe and a needle. $CaCl_2$ is widely used as a crosslinking agent in the production of alginate beads (Lan et al., 2018). The Ca^{2+} ions can form ionic interactions between the negatively charged groups of cellulose (and its derivatives) and nanocellulose molecules (after previous functionalization of the polymers to induce charges), resulting in the formation of beads when the polymeric dispersion is dripped into $CaCl_2$ solution (de Carvalho et al., 2016; Ferrari et al., 2021). The beads formed were kept in the salt solution for 30 min, washed with distilled water to remove the excess salt, and then dried in an oven at 28 ˚C for 48 h. Additionally, some of the beads were lyophilized to remove residual water, prior to characterization using FTIR, TGA, and DSC techniques.

For the encapsulation of the microorganism (Figure 8b), all materials were previously autoclaved at 121 ˚C for 15 min, and the procedure was performed under sterile conditions in a laminar flow of air. A concentration of 10^9 spores/g (dry polymer) was added to the CNC and CNC:CMC solutions, with stirring for 15 min and then dripping into 1 M $CaCl_2$ solution. The beads were kept in the bath for 30 min, followed by washing with distilled water and storing in water in a refrigerator (at 4 ˚C). Some of

the beads were also dried in an oven at 28 ˚C for 48 h and subsequently stored at room temperature. The encapsulation efficiency was determined using the Neubauer chamber to quantify the spores remaining in the $CaCl_2$ solution after the coagulation process.

2.2.4. Films preparation

The thermal and chemical characteristics of the CNC, CMC, and CNC:CMC materials were investigated by preparing films after dispersion of the samples in water at concentrations of 5% (w/v) CNC, 1.5% (w/v) CMC, and CNC:CMC composite at a volume ratio of 3:1 (CNC dispersion:CMC solution). These solutions were stirred overnight until complete dispersion. For the contact angle measurements, *T. harzianum* spores were added to the solutions at a concentration of 10^9 spores/g of material, under stirring for 15 min. The solutions were then spread on Teflon plates and oven-dried for 48 h at 28 ˚C.

2.2.5. Characterizations

Morphological evaluation of the CNC and CNC:CMC beads with and without the microorganism spores was performed using an optical stereomicroscope (Stemi 2000-C, Zeiss) and a scanning electron microscope (SEM) (JSM-6510, JEOL). X-ray microtomography (Model 1172, SkyScan) was used to evaluate the internal structures of the bead matrices and the dispersions of CNC, CMC, and spores. Static water contact angle measurements (CAM 101, KVS Instruments) were performed to evaluate the interactions of the CNC and CNC:CMC films with water, and to determine the effect of spore addition on the hydrophilic properties of the films.

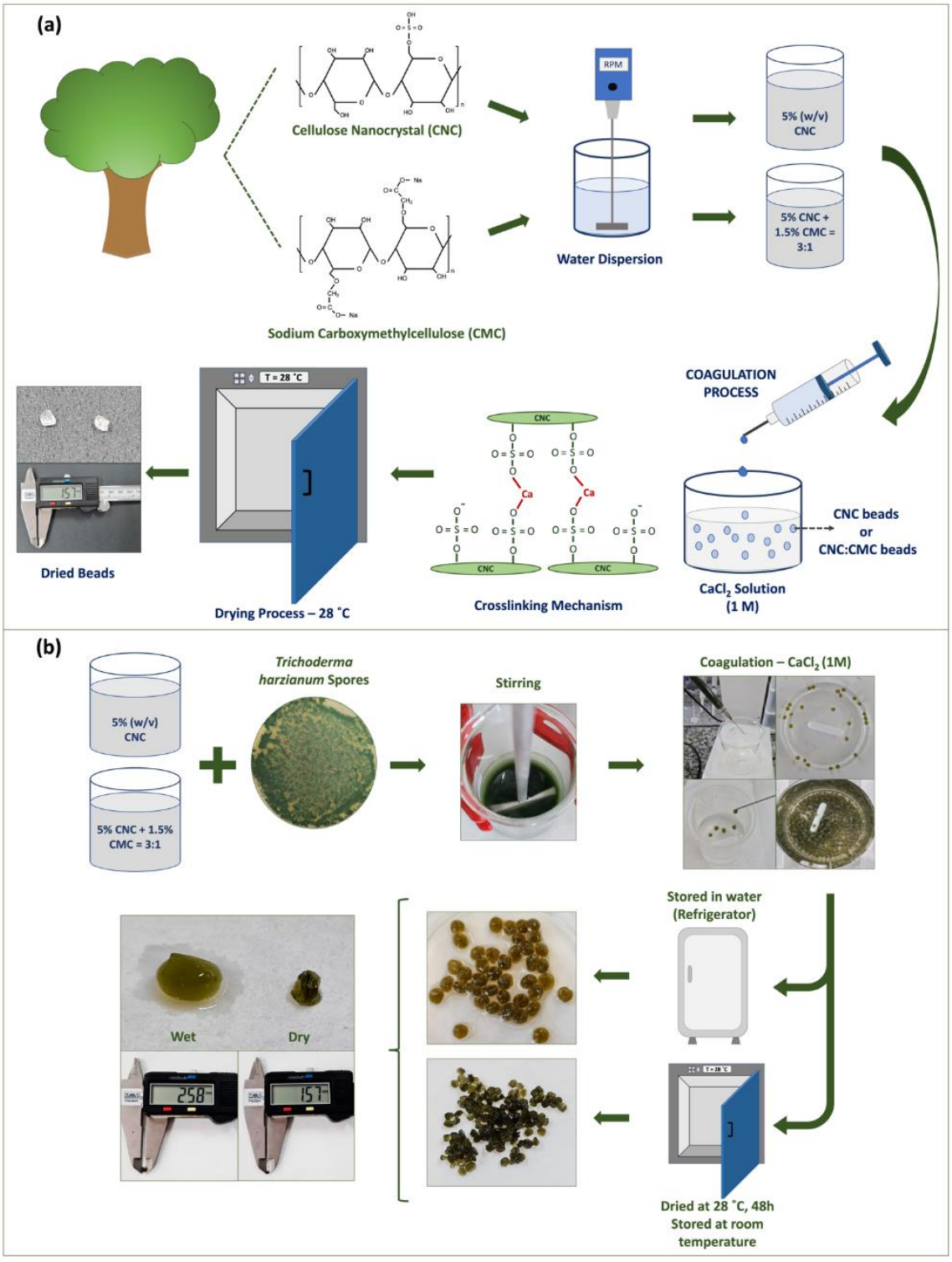


Figure 8. Schematic representation of production of the beads: (a) without the microorganism and (b) with the *Trichoderma harzianum* spores (10^9 spores/g).

The thermal and chemical characterization of the beads and films (without the spores) was performed by thermogravimetric analyses (TGA – Q500 analyzer, TA Instruments), differential scanning calorimetry (DSC - Q100 analyzer, TA Instruments) and Fourier Transform Infrared Spectroscopy (FTIR - Vertex 70 instrument (Bruker) equipped with an attenuated total reflectance (ATR) accessory). A detailed description of the characterization techniques is presented in Supplementary Material.

2.2.6. Effect of encapsulation on microorganism growth

The effect of encapsulation on the growth of the microorganism was assessed 10 days after the coagulation process. The beads (CNC and CNC:CMC matrices) containing the encapsulated microorganism, which had been kept in a refrigerator (wet) with temperature between 2 and 6 ˚C or at room temperature (dry) with temperature around 25 ˚C, were placed in the centers of Petri dishes containing potato dextrose agar (PDA). The PDA solution was prepared according to the manufacturer's data (Acumedia, USA). After microorganisms' inoculation, the plates were kept in a Bio-Oxygen demand (BOD) incubator at 28 ˚C for 10 days. The development of the fungus was then followed by imaging analysis, using ImageJ software. For comparison, the solution of free spores that had been stored under refrigeration was also evaluated, using the same spore concentration as for the encapsulated spores.

2.3. RESULTS AND DISCUSSION

2.3.1. Preparation and morphological characterization of the beads

Figure 8a shows the coagulation of the beads without *T. harzianum*, as well as a schematic representation of the crosslinking mechanism, while Figure 8b shows the process with encapsulation of the fungus. The spore encapsulation efficiency was 99.98 ± 0.01%. It can be seen from Figure 8a and 8b that the presence of *T. harzianum* in the matrix did not influence the size of the spheres. The average diameter of the wet beads was 2.58 ± 0.12 mm, while the dried beads presented a lower average value of 1.57 ± 0.19 mm (Figure 8b), due to moisture loss. The drying process carried out at mild temperatures was considered essential for this type of product, since it could mitigate the effect of high temperature on cell viability (Fraceto et al., 2018; Su et al., 2008). It is believed that performing the encapsulation process at lower temperatures results in less inhibition of the growth of microorganisms, including fungi of the genus *Trichoderma*, for which the best growth occurs at temperatures close to 30 ˚C. Therefore, in addition to the thermal and chemical characterizations of the beads, experiments were carried out to observe the growth of the microorganism in the presence of the different polymers.

Morphological characterizations of the beads of pure CNC and the CNC:CMC nanocomposite, in the presence and absence of *T. harzianum* spores, were performed by stereomicroscopy (Figure 9a and 9b) and SEM (Figure 9c-9e). The pure CNC beads presented a rougher surface, which could have resulted from the oven-drying process (at 28 ˚C for 48 h) and stronger ionic crosslinking interactions, while the surfaces of the CNC:CMC beads were less irregular. The CNC and CNC:CMC beads both exhibited a dense and compact microstructure, with some cracks on their surfaces. There was no evidence of agglomeration of the polymers (within the detection limits of the equipment),

indicating good miscibility of CMC in the CNC matrix. Neither matrices (without the spores) showed a porous structure, which could be a result of the ionic interactions (crosslinking) between the Ca^{2+} ions and the CNC, resulting in a denser structure (Huq et al., 2017; Nie et al., 2021).

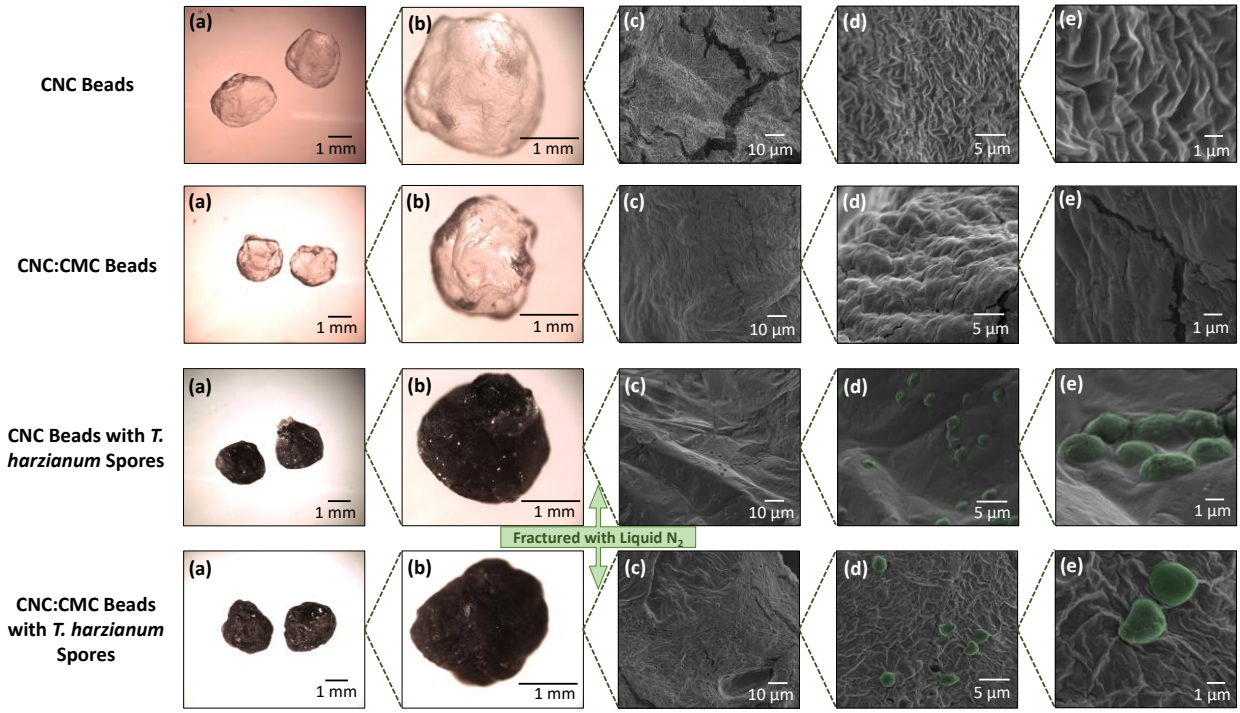


Figure 9. Stereomicroscopy photomicrographs (a, b) and SEM micrographs (c-e), obtained at different magnifications, of the CNC and CNC:CMC nanocomposite beads with and without *Trichoderma harzianum*. The spores were colored green by the GIMP 2.10 software.

The spheres were fractured by immersion in liquid nitrogen, in order to be able to visualize the microorganisms inside the matrix structure by SEM analysis. The *T. harzianum* spores present within the capsules can be seen (green color) in Figure 9d and 9e. The average spore diameter calculated using ImageJ software was 2.14 ± 0.22 µm, which was in agreement with the average size reported by Muñoz-Celaya et al. (2012).

The green color of the beads with the encapsulated microorganism resulted from addition of the *T. harzianum* spores (Figure 8b).

2.3.2. X-ray microtomography and contact angle analyses

The X-ray microtomography results (Figure 10) showed no evidence of agglomeration, indicating good dispersion of the components in the encapsulation matrix. Considering pores and cracks, all the beads presented a dense structure (within the detection limits of the equipment), with the exception of the CNC:CMC:*T. harzianum* beads, for which the structure was probably influenced by air bubbles trapped inside the matrix after the coagulation process. The addition of the concentrated spores solution, followed by the mixing process, resulted in the entrapment of some air bubbles within the encapsulation matrix, due to the high viscosity of the CNC:CMC dispersion.

The contact angle analysis indicated the interactions between the materials and water. Sodium CMC is water-soluble (Guo et al., 2019), while cellulose nanocrystals usually have strong affinity for water, although they do not dissolve in water (Vanderfleet and Cranston, 2021). As shown in Figure 10, the CNC and CNC:CMC films both presented a contact angle smaller than 90˚ (66.8˚ and 57.7˚, respectively), reflecting hydrophilic behavior. The smaller angles for the nanocomposite (CNC:CMC) could be explained by the addition of CMC, which has higher water affinity, compared to CNC (Mandal and Chakrabarty, 2019). The presence of spores did not change the wettability of the CNC:CMC film. However, for the CNC film, the addition of the spores caused the contact angle to decrease from 66.8˚ to 45.1˚. In all cases, the contact angles decreased after 60 s of interaction, especially for the nanocomposite film (with and without spores) and the CNC film with spores. Although the CNC and CNC:CMC films were hydrophilic, it is important to highlight that they presented higher water contact angles than those

obtained for alginate, the most widely used material for the encapsulation of microbial inoculants (Holler et al., 2018; Lavrič et al., 2021). This property could be attractive for agricultural applications, enabling slower and controlled release of the microorganism after application to crops (Campos et al., 2014; Vassilev et al., 2020).

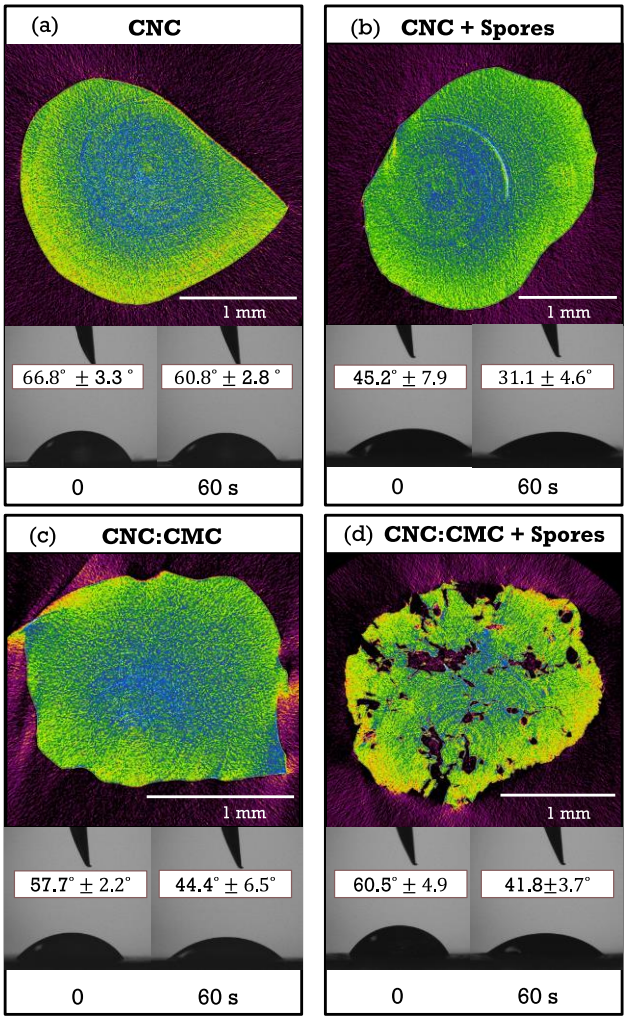


Figure 10. X-ray microtomography images of the beads and water contact angles of the films, in the presence and absence of the microorganism spores: (a) CNC, (b) CNC:*T. harzianum*, (c) CNC:CMC, and (d) CNC:CMC:*T. harzianum*.

2.3.3. Thermal and chemical characterizations

The results of TGA analyses and the thermo-gravimetric derivative (DTG) analyses of the samples are shown in Figure 11a and 11b, respectively. All the films showed slight water evaporation at around 50-120 ˚C. The onset of thermal degradation of the CNC and CNC:CMC films occurred at ~215 ˚C. The TGA curves showed mass losses in the first thermal degradation event of approximately 30% for the CNC film (at 215-275 ˚C) and 5% for the CNC:CMC film (at 215-235 ˚C). These results reflected slower degradation of the composite, compared to the pure CNC, which could be explained by the existence of hydrogen bonds between CNC and CMC (pseudo-crosslinks between the polymers) (Mandal and Chakrabarty, 2019). In the temperature range 275-450 ˚C, the CNC film showed 25% mass loss, compared to a value of approximately 50% for the CNC:CMC film. Thermal degradation of the CMC film started at ~250 ˚C, with mass loss of around 20% up to the maximum temperature of 500 ˚C used in the TGA measurements. The lower degradation temperature of CNC obtained by sulfuric acid hydrolysis, compared to CMC, has been reported previously (Choi and Simonsen, 2006; H. Li et al., 2020). The higher surface area of CNC could act to increase the exposure to heat, consequently reducing the thermal stability (Hu et al., 2014). It has also been reported that the concentration of the sulfuric acid used in the production of CNC influences the thermal degradation behavior of the resulting material (Gong et al., 2017).

The thermal degradation profiles of the CNC and CNC:CMC beads were similar. In both cases, a water evaporation event occurred between 50 and 120 ˚C, with mass losses of ~28% and ~21% for CNC and CNC:CMC, respectively. These higher water contents of the beads obtained using the crosslinking process with $CaCl_2$ could have been due to the entrapment of water within the bead structures. Furthermore, since $CaCl_2$ is a

hygroscopic salt, its residues remaining in the beads could have acted to increase the water content. The onset temperature for thermal degradation of the beads (~175 ˚C) was lower than for the films. From 175 to 300 ˚C, the CNC beads presented a mass loss of 17%, while the CNC:CMC composite beads lost ~22%. Previous studies have also reported decreases in the thermal degradation onset temperatures of composites, compared to pure polymers, due to the effect of Ca^{2+} ionic crosslinking (Abitbol et al., 2021; Kumar et al., 2017; Uyanga and Daoud, 2021).

Figure 11c shows the DSC results for the films and beads. The endothermic peaks in DSC curves, in the temperature range 50-150 ˚C, are usually related to heat adsorption as water evaporates from the materials (Asma et al., 2014; Lima et al., 2020). The DSC curve for the CNC film showed a shallow endothermic event. Although the hydroxyl groups on CNC attract water molecules, the presence of sulfate groups reduces the hygroscopicity of this material, leading to a smaller energy demand to evaporate water (H. Li et al., 2020). In the case of CMC, the presence of hydroxyl and carboxylate groups increases the affinity for moisture absorption, leading to an increase in the width of the endothermic peak corresponding to water evaporation (Mandal and Chakrabarty, 2019). The curve for the CNC:CMC composite (Figure 11c) showed an intermediate behavior of the endothermic peak.

The behaviors of the DSC curves for the CNC and CNC:CMC beads were similar, with the ionic crosslinking process resulting in increased endothermic peak widths and temperatures for water evaporation. The CNC:CMC composite beads required higher energy absorption for water evaporation, compared to the pure CNC beads, due to the higher water affinity of CMC. Another endothermic event was observed at 180-210 ˚C, for both types of beads. As found in the TGA analysis, these peaks were related to the

thermal degradation of the materials. A Table highlighting the main events observed by the TGA and DSC analyses is provided in the Supplementary material (Table 2).

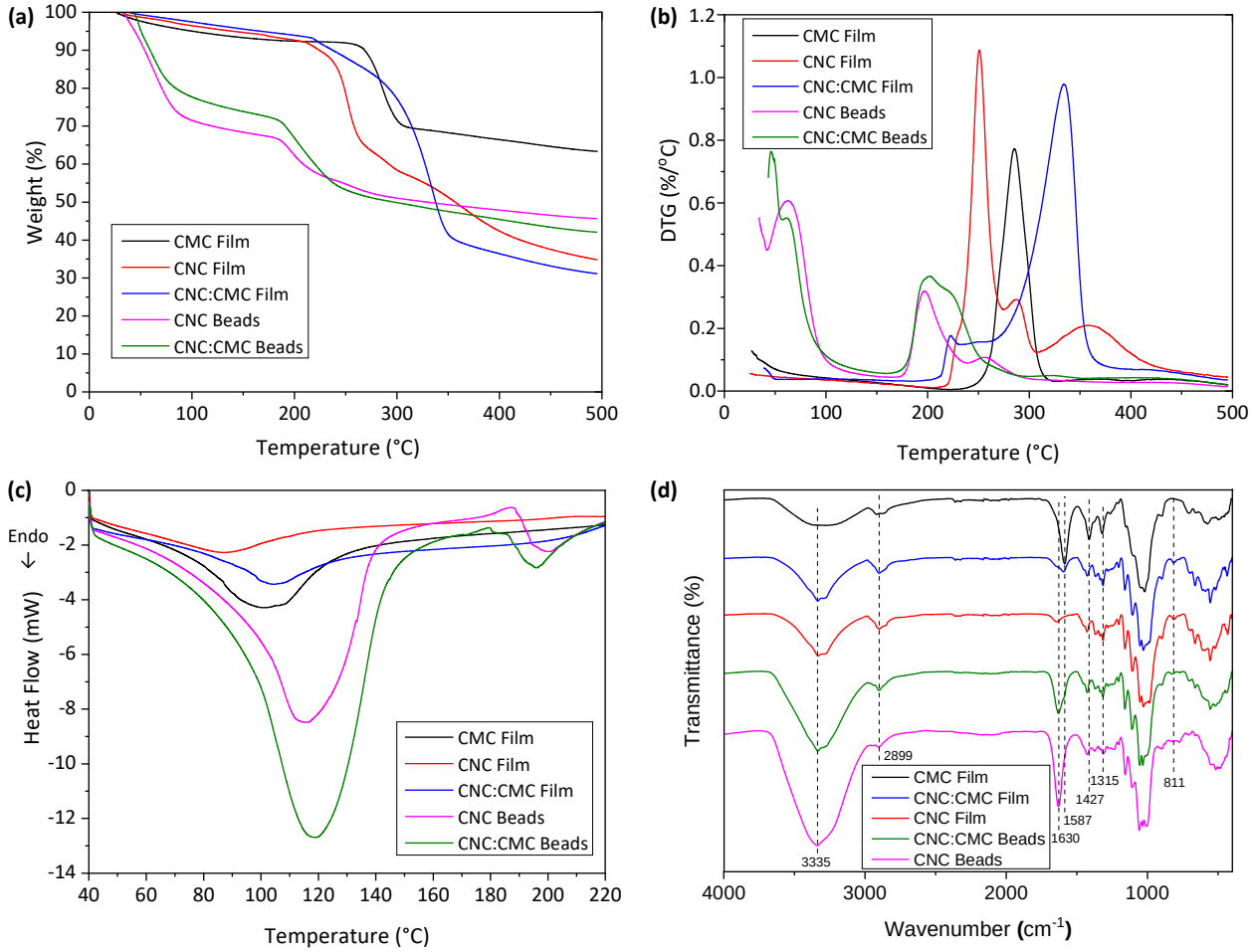


Figure 11. Thermal and chemical characterizations of CNC, CMC, a mixture of CNC and CMC (3:1 ratio), and the lyophilized CNC and CNC:CMC beads: (a) thermogravimetric analysis (TGA), (b) derivative thermogravimetry (DTG), (c) differential scanning calorimetry (DSC), and (d) Fourier transform infrared spectroscopy (FTIR).

Fig. 11d shows the FTIR spectra of the films formed by dispersing the materials (CNC, CNC:CMC, and CMC) in water and then oven-drying, together with the spectra for the lyophilized CNC and CNC:CMC beads. The spectra reflected the similar chemical structures of CNC and CMC. The spectra for the beads obtained after the crosslinking

process showed an increase of the band at 3335 cm^{-1} (ascribed to stretching of the -OH groups present in both CNC and CMC), compared to the spectra for the films. This could be explained by the Ca^{2+} crosslinking process, with formation of stronger hydrogen bonds between the CNC and CNC:CMC chains (Mandal and Chakrabarty, 2019; Nie et al., 2021). The intensity of the -OH band was lower for CMC, because some of the -OH groups had been substituted by $-CH_2COONa$ during the production process (Alizadeh Asl et al., 2017). Bands at 2900 and 1630 cm^{-1} were related to C-H stretching and -OH bending vibrations, respectively. The CMC spectrum presented a peak at 1587 cm^{-1}, attributed to the $-COO^-$ group, which was not observed in the spectra for the other matrices (or was very small for CNC:CMC, due to the low CMC content). This peak was related to the carboxymethyl groups present at the CMC surface. Bands at around 1427 cm^{-1} (1412 cm^{-1} for CMC), 1371 cm^{-1}, and 1315 cm^{-1} were assigned to $-CH_2-$ symmetric bending and the bending vibrations of C-H and C-O groups at the ring, respectively (Kusmono et al., 2020; Mandal and Chakrabarty, 2019). Peaks in the range 1000-1200 cm^{-1} were attributed to ether group -O- stretching (Alizadeh Asl et al., 2017). A small peak at 811 cm^{-1}, observed in the spectra for all the samples with CNC, was related to the C-O-S group of sulfate ester, which was present on the surface due to the use of sulfuric acid hydrolysis in CNC production (Jasmani and Adnan, 2017; Kusmono et al., 2020).

2.3.4. Effect of encapsulation on microorganism growth

Figure 12a shows the effect of encapsulation on growth of the fungus in the Petri dishes containing PDA, observed 10 days after the encapsulation process. The CNC and CNC:CMC beads containing the microorganism spores were previously stored wet (under refrigeration) and dried (at room temperature). The growth of the encapsulated microorganism was compared to that of the free spores in solution, stored under

refrigeration. For most of the conditions, with the exception of the dried CNC beads, the fungus was able to grow on the PDA, but at different growth rates. The fastest rate was observed for the refrigerated (wet) CNC:CMC beads (Figure 12b), where the CMC present in the matrix provided an additional source of carbon for the microorganism (Bashan et al., 2014; Gelain et al., 2021). *T. harzianum* has been found to produce cellulases capable of degrading cellulosic substrates after 24 h of cultivation (da Silva Delabona et al., 2016; Li et al., 2020). Therefore, the release of these enzymes could assist the microorganism in degrading the CNC:CMC matrix, enhancing its growth in the PDA medium. In addition, as shown by the X-ray microtomography results (Figure 10), the internal pores in the beads could act to increase diffusion of the spores from the beads to the PDA.

The growth rate observed for the refrigerated (wet) beads was similar to, or better than, that for the free spores. Possible explanations were the extra protection that encapsulation provided against stress occurring during the storage period, as well as the additional carbon source present in the encapsulation matrix (Chanratana et al., 2018; He et al., 2015; Vassilev et al., 2020). It is also important to point out that the viability of the microorganism was affected by the storage temperature and the drying process (Chanratana et al., 2018; He et al., 2015), since the fungus present in the refrigerated (wet) CNC beads was able to occupy the entire dish after 4 days, while the fungus in the dried CNC beads was unable to grow. It is also possible that the lower growth rate observed for the pure CNC beads, compared to the CNC:CMC composite, could have been influenced by damage to the cell membranes of the microorganism by the sharp edges of the CNC, causing cell inactivation (Noronha et al., 2021). The addition of CMC to the encapsulation matrix may have provided protection against this effect.

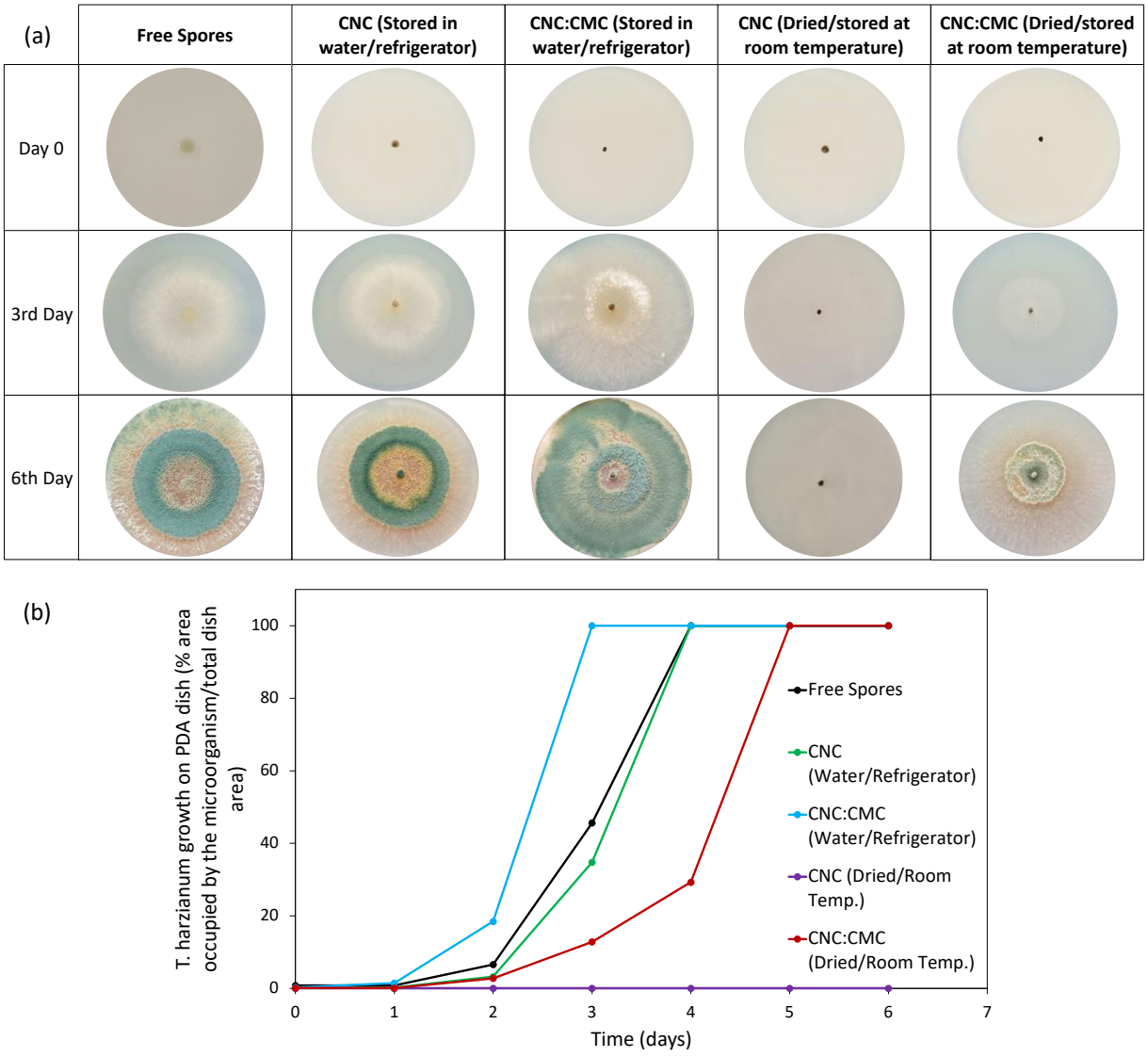


Figure 12. (a) Growth in PDA medium of the free spores and the spores encapsulated in the CNC and CNC:CMC (3:1) beads previously stored for 10 days under wet/refrigerated or dry/room temperature conditions, evaluated by photographs obtained during 6 days. (b) Percentages of the Petri dish areas occupied by *Trichoderma harzianum*, determined using ImageJ software.

Comparison of the dried/room temperature CNC:CMC with the refrigerated/wet CNC:CMC nanocomposite showed that the latter presented faster growth. The drying process and the storage at room temperature could have decreased the viability of the

fungus, because the spores had greater exposure to environmental factors such as temperature and humidity changes. However, it was expected that encapsulation would provide the cells with protection against these conditions. The lower water content within the matrix of the dried beads would lead to slower metabolic activity of the microorganisms, consequently decreasing the growth rate (which could assist in increasing the shelf-life of the product) (Santos et al., 2019), as well as reduce diffusion of the cells from the bead matrix to the PDA medium. The compact structure of the dried beads, shown in the SEM micrographs (Figure 9), could also have contributed to reducing diffusion of the cells. These attractive characteristics would ensure the desired slow release of the microorganism, following application of the product to crops.

2.4. CONCLUSIONS

Beads composed of CNC and CNC:CMC nanocomposite were produced by ionic crosslinking using Ca^{2+} ions and were effectively employed for fungus encapsulation. Determination of the growth rates of the free and encapsulated microorganisms in PDA medium showed that the growth was influenced by the drying process and the storage temperature. The addition of CMC to the CNC matrix was essential for the growth of *T. harzianum* after storage under dry conditions for 10 days following the encapsulation process. These results indicated that CNC:CMC beads are an attractive green matrix for the encapsulation of microorganisms used in agriculture and open possibilities for field application in different cultures, which, if generate positive outcomes, can offer a way to increase productivity and reduce dependence on chemical fertilizers and pesticides in field.

2.5. SUPPLEMENTARY MATERIAL

2.5.1. Effect of CNC, CMC and $CaCl_2$ concentrations in beads production

The effect of the concentrations of CNC, CMC, $CaCl_2$ and the composite CNC:CMC ratio on the beads production and characteristics were presented in Table S1. After these results, the conditions that resulted in uniform spherical particles after the drying process were chosen to be evaluated in the following steps of this study (fungus encapsulation and beads characterization). These conditions were: CNC dispersion of 5% (w/v); a composite of CNC (5%) and CMC (1.5%) on a volume mixing rate of 3:1; and a $CaCl_2$ coagulation bath of 1M.

Table 1. Effect of the concentrations of CNC, CMC and $CaCl_2$ on beads production.

Effect	CNC (% w/v)	CMC (% w/v)	Volume ratio (CNC:CMC)	$CaCl_2$ (M)	Comments
CMC Concentration	0	0.5	0:1	1	No capsule was formed by the coagulation in $CaCl_2$ bath.
		1			
		1.5			
		2			
		3			
CNC Concentration	1	0	1:0	1	Obtention of non-uniform particles.
	2.5	0	1:0	1	Obtention of spherical particles, however they collapsed after the drying process.

	5	0	1:0	1	Obtention of spherical beads that kept their shape even after the drying process.
CNC:CMC Composite	2.5	0.5	1:1	1	Obtention of spherical particles, however they collapsed after the drying process
		1	1:1	1	
		1.5	1:1	1	
	5	1.5	1:1	1	Obtention of spherical particles, however they collapsed after the drying process
			2:1	1	Obtention of spherical particles, however they collapsed after the drying process
			3:1	1	Obtention of uniform beads after the coagulation and the drying process, as illustrated in Fig. 8a.
	5	2	3:1	1	Solution with high viscosity, which hampered the dripping process.
$CaCl_2$ concentration	5	0	1:0	0.05	Obtention of fragile beads, which broke up when they were manipulated. Also, when oven dried, the beads flattened.
				0.1	
				0.5	
				1	Obtention of uniform beads after the coagulation and the drying process, as illustrated in Fig. 8a.

2.5.2. Characterization Methodology

Morphological evaluation of the CNC and CNC:CMC beads with and without the microorganism spores, after oven-drying at 28 ˚C for 48 h, was performed using an optical stereomicroscope (Stemi 2000-C, Zeiss) and a scanning electron microscope (SEM) (JSM-6510, JEOL) operated at an acceleration voltage of 5.0 kV. For the SEM analyses, the samples were sputter-coated with gold and the beads containing the microorganism were previously fractured using liquid nitrogen. The images were treated using GIMP 2.10 software, enabling the features to be highlighted in different colors. X-ray microtomography (Model 1172, SkyScan) was used to evaluate the internal structures of the bead matrices and the dispersions of CNC, CMC, and spores.

Water contact angle measurements were performed to evaluate the interactions of the CNC and CNC:CMC films with water, and to determine the effect of spore addition on the hydrophilic properties of the films. Angles higher than 90˚ are observed for materials with hydrophobic characteristics (Law, 2014). The static water contact angles of the CNC and CNC:CMC films, with and without the presence of spores, were measured at times of 0 and 60 s after dripping water onto the film surface, using an automatic optical goniometer (CAM 101, KVS Instruments). The images were recorded every 1 s, during 60 s. Each film was tested 5 times and the values were presented as means and standard deviations.

Thermogravimetric analyses (TGA) of the films and spheres were performed using a Q500 analyzer (TA Instruments). A mass of approximately 5 mg of the sample was transferred to a platinum crucible and heated from room temperature to 700 ˚C, at a rate of 10 ˚C.min^{-1}, under an inert atmosphere of nitrogen supplied at a flow rate of 40 mL.min^{-1}.

Differential scanning calorimetry (DSC) thermograms were acquired using a Q100 analyzer (TA Instruments), with heating of around 5 mg of the sample (films and spheres) from room temperature to 240 ˚C, at a rate of 10 ˚C.min^{-1}, under an atmosphere of nitrogen supplied at 5 mL.min^{-1}.

Fourier transform infrared spectroscopy (FTIR) analyses were performed using a Vertex 70 instrument (Bruker) equipped with an attenuated total reflectance (ATR) accessory, in order to obtain information about the chemical changes of the matrices after the crosslinking process. The spectra were obtained from 4000 to 400 cm^{-1}, with 32 scans per sample and resolution of 4 cm^{-1}. All the spectra were normalized using the intensity at 1032 cm^{-1}. The CNC and CNC:CMC beads obtained after the ionic crosslinking process in $CaCl_2$ solution were dried by lyophilization before the analysis. Samples of CNC, CMC, and CNC:CMC films were obtained after dissolution in distilled water and subsequent drying of the films in an oven at 30 ˚C for 48 h.

2.5.3. Thermal observations

The main thermal observations from Figure 11 a, b and c) were highlighted in Table 2.

Table 2. Main thermal events observed after the TGA and DSC analyses.

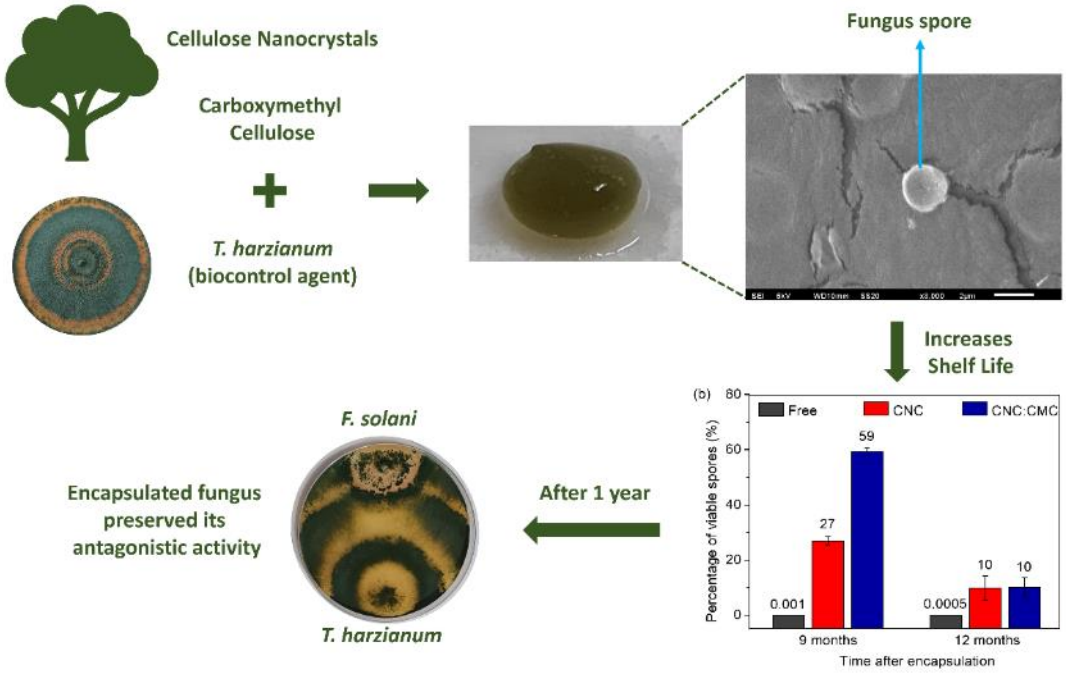
Analysis	Sample	Temperature (˚C)	Main Observations
TGA	All films	50 - 120	Slight water evaporation (~ 5% mass loss)
	CNC film	215 - 275	First thermal degradation event (~ 30% mass loss)

		275 - 310	Thermal degradation event (~ 5% mass loss)
		310 - 450	Thermal degradation event (~ 20% mass loss)
	CNC:CMC film	215 - 235	First thermal degradation event (~ 5% mass loss)
		275 - 450	Thermal degradation event (~ 50% mass loss)
	CMC film	250 - 320	Thermal degradation event (~ 20% mass loss until the final temperature of 500 ˚C)
	CNC bead	50 -120	Water evaporation (~ 28% mass loss)
		175 - 300	Thermal degradation (~17% mass loss)
	CNC:CMC bead	50 -120	Water evaporation (~ 21% mass loss)
		175 - 300	Thermal degradation (~22% mass loss)
DSC	CNC film	50 - 150	Shallow endothermic peak (water evaporation)
	CMC film		Endothermic peak (water evaporation) with higher intensity than CNC film
	CNC:CMC film		Endothermic peak (water evaporation) with an intermediate behavior
	CNC and CNC:CMC beads	50 - 150	Endothermic peak (water evaporation) with higher intensity than the films due to the crosslinking process CNC:CMC peak > CNC due to the higher water affinity of CMC
		180 - 210	Endothermic peak related to the thermal degradation of the beads

CHAPTER 3 – Encapsulation Effect on Microorganism Shelf-Life, Protection, and Antagonistic Activity

*The content of this chapter is an adaptation of the manuscript entitle: "Increasing Shelf-Life and Protection of the Biocontrol Agent *Trichoderma harzianum* by Encapsulation in Green Matrices of Nanocellulose and Carboxymethyl Cellulose", which will be submitted for publication.

GRAPHICAL ABSTRACT



ABSTRACT

Enhancing microbial inoculant shelf-life, by using biodegradable and renewable materials, is essential to make these bio-based products more competitive in comparison to chemical fertilizers and pesticides. Biofertilizer application can contribute to reduce the negative environmental impact caused by agrochemicals, while increasing crops productivity in a bioeconomy concept. Here, green matrices of cellulose nanocrystals (CNC) and a composite of CNC and carboxymethyl cellulose (CNC:CMC) were used to encapsulate the spores of the biocontrol fungus *Trichoderma harzianum*. Our results showed that, besides increasing the microorganism shelf-life (after 1 year, approximately 10% (~10^8 CFU/mL from an initial concentration of ~10^9 CFU/mL) of the encapsulated spores remained viable, while approximately all free microorganism (without encapsulation) were dead ($5x10^3$ CFU/mL)), the encapsulation process can also enhance the microorganism protection when exposed to stressful conditions, such as heat, UV-radiation and the contact with a chemical fungicide. In this latter case, while the free fungus presented a survivability of $1.4x10^7$ CFU/mL after its inoculation with the fungicide, the CNC and CNC:CMC matrices increased it to $2.7x10^8$ CFU/mL and $4.7x10^8$ CFU/mL, respectively. Additionally, encapsulation was able to preserve the antagonistic effect of *T. harzianum* against the phytopathogen *Fusarium solani* for up to one year after its production. These results highlight that these cellulose-based matrices are very promising materials for microbial inoculants formulations, which can help our agriculture to move towards more sustainable practices.

Keywords: cellulose nanocrystals, carboxymethyl cellulose, microbial inoculant, encapsulation, shelf life.

3.1. INTRODUCTION

Chemical fertilizers and pesticides are essential products to increase the agricultural productivity, but the uncontrolled and exacerbated usage of these products can cause severe problems to the environment (eutrophication, greenhouse gases emissions, etc) and for human health (Devi et al., 2022; Gill et al., 2022). It means that more sustainable agricultural practices are required to increase productivity in accordance with the concept of bioeconomy (Trigo et al., 2023). In this context, microbial inoculants are rising attention in recent years. These products consist of alive microorganisms that are applied to soil or plants to enhance nutrient availability and uptake, increase plant's resistance against environmental stressful conditions (extreme temperatures, drought, flooding, UV light exposure, etc.) and control phytopathogens diseases. Besides increasing crops productivity, microbial inoculants can also improve soil health (Li et al., 2022; O'Callaghan et al., 2022; Santos et al., 2019).

The fungus *Trichoderma harzianum* is an example of a commonly used microbial inoculant due to its beneficial effect on enhancing plant growth and disease suppression (Ali et al., 2022; Fraceto et al., 2018). *T. harzianum* effect as a biocontrol agent have been well reported and it can be explained by its good ability to compete against phytopathogens for nutrients, produce secondary metabolites that inhibits their growth and to stimulates the plant's defense mechanism, enhancing plant ability to resist against a pathogen attack (Asad, 2022; Zhang et al., 2016). As reported by Zhang et al. (2016), the use of *T. harzianum* in soybean plants can increase their growth due to the production of phytohormones, such as indole acetic acid. It can also reduce the severity of white mold disease, caused by the fungus *Sclerotinia sclerotiorum,* due to the formation of an oppressive structure against the phytopathogen hyphae and due to the production of lytic

enzymes (chitinase and β-glucanase), which can degrade the cellular membrane of *S. sclerotiorum* (Zhang et al., 2016).

While microbial inoculants have shown great results on improving plant growth in line with concepts of more sustainable practices, there are still a few challenges associated with their use (Kaminsky et al., 2019; O'Callaghan et al., 2022). One of the main issues is ensuring the viability and stability of the inoculant during its production, storage, transportation and during and after its application on crops, since these microorganisms can be very sensible to environmental stressors. The use of a suitable formulation can help to increase the microorganism protection, optimizing the efficacy of these products (O'Callaghan et al., 2022; Qiu et al., 2019; Rojas-Sánchez et al., 2022). Moreover, the formulation should use environmentally friendly materials, to avoid environmental problems, it should help to promote inoculant colonization and survival in the target plant, and it should be easy to apply and cost-effective, making it accessible and practical for farmers and researchers (O'Callaghan et al., 2022; Qiu et al., 2019; Vassilev et al., 2020).

In this sense, encapsulation techniques are presenting positive results on meeting the criteria reported previously. In addition to increasing the inoculant shelf-life, this formulation can improve the efficacy of the inoculant by providing a slow and controlled release of the microorganisms and protecting them from degradation by soil enzymes or other microorganisms (Pereira et al., 2023; Rojas-Sánchez et al., 2022; Vassilev et al., 2020). The use of polysaccharides as encapsulation matrices is an interesting approach, since these materials are abundant, biocompatible, and biodegradable, making them an attractive choice for sustainable agriculture practices (Pereira et al., 2023; Rojas-Sánchez et al., 2022; Vassilev et al., 2020). They can provide physical protection to the microorganisms, as well as nutrients and energy. Natural polysaccharides can also be

modified to optimize the release of the microorganisms, ensuring their effective delivery to the target plant. The lower releasing rate provided by the matrices can reduce the fertilization cost by the farmers, since fewer applications are necessary throughout the growth of agricultural crops (Pereira et al., 2023; Vassilev et al., 2020).

As previously reported by our research group, cellulose-based materials such as cellulose nanocrystals (CNC) and carboxymethyl cellulose (CMC) are suitable candidates for *T. harzianum* encapsulation due to their excellent properties (Brondi et al., 2022). These beads can be produced by simple coagulation routes using $CaCl_2$ as a crosslinker. Although these encapsulating beads have already been characterized, their effect on microorganism protection and shelf-life is still unclear. In this sense, here we evaluated for the first time the effect of green matrices made of only CNC and the composite CNC:CMC on the protection and shelf-life of the biocontrol fungus *T. harzianum*. Free and encapsulated spores were submitted to stressful situations such as UV light, fungicide contact and temperature and their survivability after these exposures were determined. Additionally, spores' viability was evaluated during the period of 1 year, showing that these materials are excellent candidates for microbial inoculant encapsulation.

3.2. MATERIAL AND METHODS

3.2.1. Materials

Cellulose nanocrystals (CNC, Celluforce, Canada), obtained by sulfuric acid hydrolysis and sodium carboxymethyl cellulose (CMC, Synth, Brazil), with a degree of substitution of 0.7 and medium viscosity, were used as the polymeric matrix for the beads production. The crosslinking agent was calcium chloride ($CaCl_2$, Synth, Brazil).

Trichoderma harzianum LQC-99 (donated by Embrapa Environment, Brazil) was used as a model microorganism for the encapsulation experiments. The fungus spores were germinated for 7 days at 28 °C in Petri dishes containing potato dextrose agar (PDA, Acumedia, Brazil). Spores were extracted by a 0.85% (w/v) NaCl solution and the final dispersion was centrifuged to increase the spore's concentration. A Neubauer chamber was utilized to determine the final fungus concentration.

3.2.2. Preparation of CNC and CNC:CMC beads

For beads production, first all materials (apart from the solid polymers) were autoclaved at 121 °C for 15 minutes and the encapsulation process was carried out under sterile condition in a laminar of air. Briefly CNC (5% w/v) and CMC (1.5% w/v) were dispersed in distilled water, following the methodology reported by Brondi et al. (2022). For the pure CNC dispersion and a mixture of CNC:CMC in a volume ration of 3:1 (with a final mass concentration of 3.75% (w/v) CNC and 0.375% (w/v) of CMC) a spore's solution of *T. harzianum* was added in a concentration of approximately 10^9 spores/g dry polymer. These dispersions were then dripped in a 1 M $CaCl_2$ coagulation bath. The produced beads were kept in salt solution under mild agitation for 30 min, followed by distilled water washing to remove the salt excess. The spheres were then stored in a refrigerator at 4 °C for further experiments. The reason why the beads were kept under this condition is found in the Supplementary Material (section 3.5).

3.2.3. Viability experiments

To determine the free and encapsulated spore viability after the beads production and after being stored under refrigeration (4 °C) for 9 and 12 months, viability

experiments were carried out. For that, 0.5 mL of the free spores solution, and 20 beads of CNC and CNC:CMC matrices were added in a Erlenmeyer containing 10 mL of NaCl 0.85% (w/v). Then, 20 µL of the commercial enzymatic cocktail Cellic CTec3 was added to speed up the spore's release from the beads. These Erlenmeyer's were then incubated in an orbital shaker at 30 °C, 200 rpm, for 24 hours. The resulting solution was then serially diluted with NaCl (0.85%) and inoculated in a Petri dish with PDA medium, which was incubated at 28 °C for 24 hours. After that, the colony-forming units (CFU/mL) was determined visually. Additionally, the total amount of spores released in the Erlenmeyer flask was determined in a Neubauer chamber. All experiments were carried out with biological and technical triplicates. The concentrations in CFU/ml or viability percentage were reported as mean ± standard deviation. To easier the comparison, the total spore's concentration was standardized as 10^9 spores/mL and the spores survivability was then calculated assuming this initial concentration.

3.2.4. Resistance experiments

The ability of the encapsulation matrix to protect the microorganism against some stressful situations was also evaluated. For that, free and encapsulated microorganisms were exposed to three different situations: (1) the temperature of 40 °C for 24, 48 and 192 hours; (2) Ultraviolet (UV-C) radiation for 30 and 90 minutes, at room temperature, and in a distance of 20 cm from the light source, in a dark room; (3) direct contact to a commercial fungicide solution (difenoconazole, 0.0167% (w/w), Forth, Brazil) for 24 hours. After being submitted to these conditions, free and encapsulated fungus were placed in an Erlenmeyer in a 0.85% NaCl solution with 20 µL of Cellic CTec3 (as described before on topic 3.2.3) and incubated in a shaker at 200 rpm, 30 °C for 24 hours. The resulting solution was then serially diluted and inoculated in a Petri dish with PDA

medium, followed by the colony-forming units (CFU/mL) determination. All experiments were carried out with biological and technical triplicates.

3.2.5. Antagonism experiments

Fusarium solani is a phytopathogenic fungi that can infect plants roots (e.g., tomatoes, soybean, eggplants, etc), decreasing their productivity. This phytopathogen was used to evaluate the antagonistic activity of *T. harzianum,* in its free and encapsulated form. *F. solani* strains were cultivated in potato dextrose agar (PDA) at 28 °C for 10 days. For the antagonism's experiments, a 5 mm disk containing the mycelia of *F. solani* was placed in one side of o 9 cm Petri dish with PDA medium. On the opposite side of the dish, *T. harzianum* was placed (free spores (20 μL), or one bead the encapsulated fungus in the CNC or CNC:CMC matrices). The encapsulated microorganism was evaluated right after its production and after being stored for one year. The Petri dishes containing both fungi were then kept at 28 °C and the microorganisms' growth across the PDA medium over time was evaluated by daily photos. This test was carried out in triplicate.

3.2.6. Scanning electron microscopy (SEM)

A morphological study of the lyophilized CNC and CNC:CMC beads containing the spores was performed using a Scanning Electron Microscope (SEM - JEOL JSM-6510) with an accelerating voltage of 5.0 kV. For the SEM analysis, the samples were coated by sputtering with gold.

3.2.7. Evaluation of the fungus growth over the beads

The fungus growth over the beads placed in PDA medium was evaluated by photos taken over time. For that, the Petri dishes containing the nutrient medium and the beads were incubated at 28 °C for up to 7 days.

3.3. RESULTS AND DISCUSSION

3.3.1. Characterization of lyophilized beads

Both the CNC and the CNC:CMC lyophilized beads, containing the microorganism, were characterized by SEM (Figure 13). As can be seen, the CNC (Figure 13. (a)) presented a higher amount of macropores on its surface than the composite CNC:CMC (Figure 13. (b)). Beside these pores, the CNC bead's surface seems to be smoother with some surface cracks. At higher magnifications, it was also possible to visualize the presence of surface cracks. For both matrices it was possible to see in the surface the fungus spores, as highlighted at the higher magnification micrographs. When compared with the beads previously characterized by our research group (Brondi et al., 2022), the final morphology has some differences, which could be a result of the drying process. While here the beads were lyophilized, in the previous study they were air-dried, which ended up resulting in a more dense and compact structure.

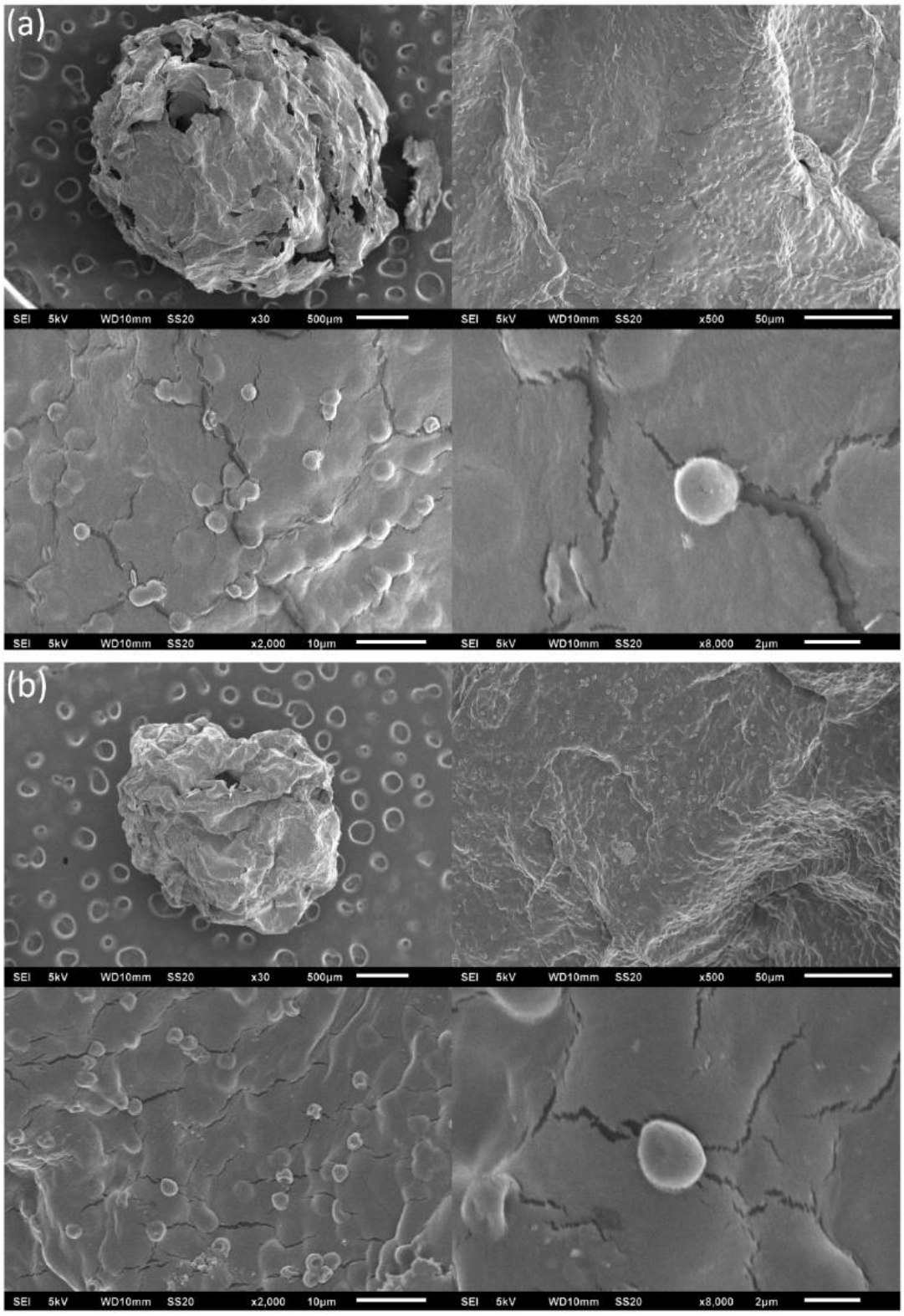


Figure 13. Morphological characterization by SEM of lyophilized CNC beads (a) and CNC:CMC beads (b), with different magnifications.

3.3.2. Indicative of substrate consumption

To verify if the microorganism was able to use the beads as substrates for its development, we evaluated the fungus growth over the beads (CNC and CNC:CMC) placed in PDA medium by photos taken in different time periods (Figure 14). After 3 days of incubation, for both matrices, it was possible to see some fungus hyphae over the beads. After 7 days, both the CNC and CNC:CMC composite were covered by the *T. harzinum* spores, which indicates that the microorganism can use these matrices as a substrate for carbon and energy source. *T. harzianum* can produce a set of enzymes (cellobiohydrolase, endo-glucanase and β-glucosidase) that work synergistically to degrade cellulosic substrates into oligomers and monomers (Lee et al., 2017; J.-X. Li et al., 2020). Although the higher crystallinity of CNC can slow down the degradation rate, some studies report that these materials can also be hydrolyzed by microorganisms' enzymes (Erdal and Hakkarainen, 2022; Leppänen et al., 2020). Also, depending on the surface group and the substitution degree, this degradation can happen with a faster rate than in CMC or other cellulose derivatives (Erdal and Hakkarainen, 2022; Singh et al., 2016).

3.3.3 Effect of the encapsulation on microorganism shelf-life

The effect of encapsulation on the fungus viability in CNC and CNC:CMC matrices was evaluated and compared with the free microorganism stored under the same conditions. Initially, the entrapped fungus was released from both beads by an enzymatic hydrolysis method. This method was chosen to accelerate the spore release from the beads, since without the enzymes the release happened very slowly in the 0.85% NaCl solution (after 48 hours of incubation, the amount of free spores in the salt solution was almost zero). *T. harzianum* viability was determined right after the encapsulation process,

and after the time periods of 9 and 12 months of storage under 4 °C. Aiming to easier the comparison, the total cells released concentration for all samples were standardized to 10^9 spores/ml.

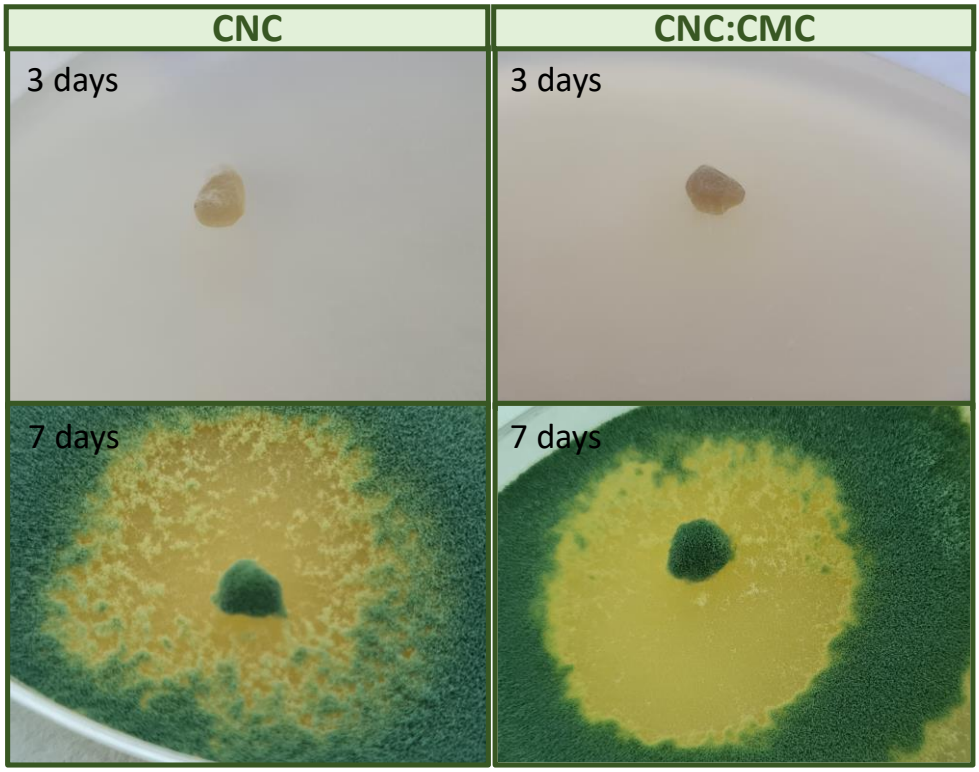


Figure 14. Evaluation of the microorganism's growth over the beads after 3 days and 7 days in a Petri dish with PDA medium.

As reported in Figure 15, after 9 and 12 months, the beads stood out by their great effect in preventing spore inactivation. While the free microorganism had a viability of ~ $1.3x10^4$ CFU/mL (0.0013%) after 9 months and $4.5x10^3$ CFU/mL after 12 months, the CNC beads resulted in a viability of $2.7x10^8$ CFU/mL (27%) and $9.9x10^7$ CFU/mL (10%), while the composite had $5.9x10^8$ CFU/mL (59%) and $1x10^8$ CFU/mL (10%), after 9 and 12 months, respectively. When compared with other encapsulation matrices, *T. harzianum*

survival was between 10 – 40% when it was encapsulated in matrices of maltodextrin or gun Arabic or in a mixture of these polymers, which were stored at 4 °C for 8 weeks (~ 2 months). After 12 weeks at the same conditions, the survivability was approximately zero (Muñoz-Celaya et al., 2012). When alginate was used as encapsulating matrix for *T. harzianum* spores (also coagulated in a $CaCl_2$ crosslinking bath and stored wet and under refrigeration) the microorganism viability reduced from 10^8 CFU/mL after encapsulation to 10^6 CFU/mL after 120 days (~ 4 months) (Maruyama et al., 2020). It means that under similar conditions, CNC and CNC:CMC beads were more efficient in keeping the fungus alive than alginate (one of the most used matrix for microbial inoculant encapsulation) and other natural polymers. It could be explained by the fact that alginate beads produced by $CaCl_2$ coagulation are usually fragile. They are easily disintegrated during handling, storage, and processing, which will leave the microorganism more exposed. In order to improve these matrix's properties, CNC can be added, increasing the mechanical properties of alginate (Huq et al., 2017). Additionally, CNC incorporation increases matrix tortuosity, which will slow down diffusion processes, increasing microorganisms protection (Huq et al., 2017). In this sense, a CNC based matrix results in less fragile beads, with more protected microorganisms. Another advantage of CNC in comparison with alginate is that its high surface area-to-volume ratio results in a higher cells loading capacity (Hasan et al., 2020; Nascimento et al., 2018).

However, it is possible to notice that right after the encapsulation process, the spores viability was 74% in CNC and 84% in the CNC:CMC composite, which were lower than the viability of the free microorganism (98%). It means that the encapsulation process can slightly decrease the fungus activity, and this reduction could be a result of the vigorous stirring process used to well disperse the spores in the polymeric matrices or it could be due to the osmotic stress faced by some microorganism at the surface of the

beads when they were in contact with the salt coagulation bath (Delgado-Jarana et al., 2006).

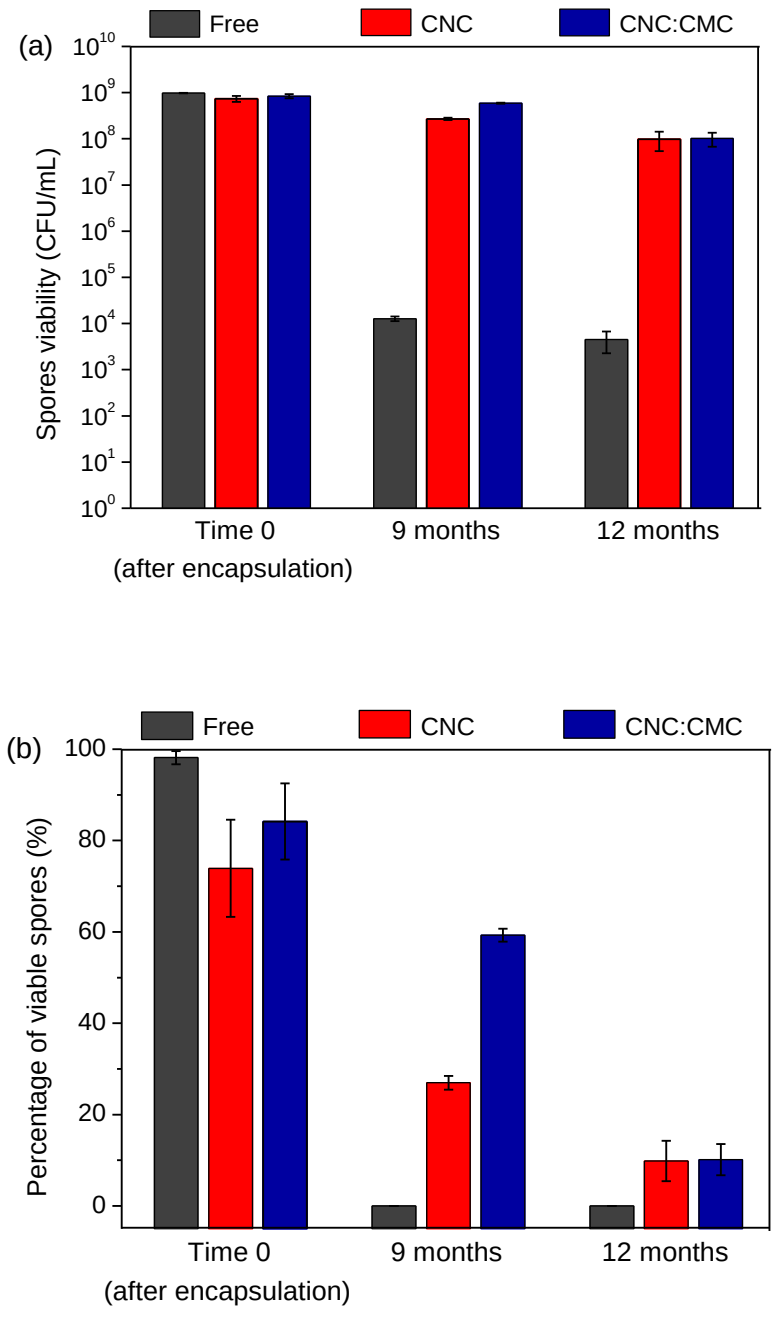


Figure 15. Shelf-life of the free and encapsulated microorganism stored under refrigeration. (a) Viable cells (UFC/mL). (b) Percentage of viable cells.

3.3.4. Effect of abiotic stress conditions on free and encapsulated microorganism

Ensuring the microbial inoculant protection during its manufacturing process, storage, transportation and after its application by farmers on crops is an essential request for the product success. Some field conditions that these products can find are: pH and temperature variations, excess or lack of water, UV light exposure, contact with other agrochemical products, different soil salinity, etc. Here, the protection provided by the CNC and the CNC:CMC matrices over the spores was evaluated during the following situations: (a) an elevated temperature of 40 °C, which was kept during 24, 48 and 192 hours; (b) the fungus was exposed to UV-C radiation for 30 and 90 minutes; (c) the microorganism was incubated with a commercial fungicide for 24 hours. The obtained results as the spores that remained viable are shown in Figure 16 and the free fungus was also exposed to the same conditions for comparison. For comparison, the total spore's concentration (dead and living cells) was standardized in ~ 10^9 spores/mL.

Thermal stress

Thermal stress can significantly impact the viability of the fungus *T. harzianum*. When exposed to high temperatures, the microorganism can face proteins denaturation, enzymes inactivation and DNA and RNA oligonucleotides rupturing, which will affect its survivability (Fernández-Sandoval et al., 2012). Our results (Figure 16. (a)) showed that when the free fungus was continuously exposed to the temperature of 40 °C, the spores' viability was greatly affected. While free spores started with a viability of $9.8x10^8$ CFU/mL, its incubation for 24, 48 and 192 hours decreased the amount of cells that remained viable to $3.4x10^8$ CFU/mL, $2.8x10^8$ CFU/mL and $3.9x10^5$ CFU/mL,

respectively. Clearly, the encapsulation acted as a protective barrier to reduce the heat propagation across the beads, decreasing the fungus inactivation. For the CNC matrix (that presented a spore's viability of $7.4x10^8$ CFU/mL right after the beads production), the spores that remained viable was $5.4x10^8$ CFU/mL, $4.8x10^8$ CFU/mL and $2.6x10^7$ CFU/mL for 24, 48 and 192 hours. For the composite (with a starting survivability of $8.4x10^8$ CFU/mL), the viability was $6.3x10^8$ CFU/mL, $6.1x10^8$ CFU/mL and $2.1x10^7$ CFU/mL for the evaluated times. In short, while for the time of 192 h the free microorganism survivability decreased four orders of magnitude, the encapsulated ones decreased only one order.

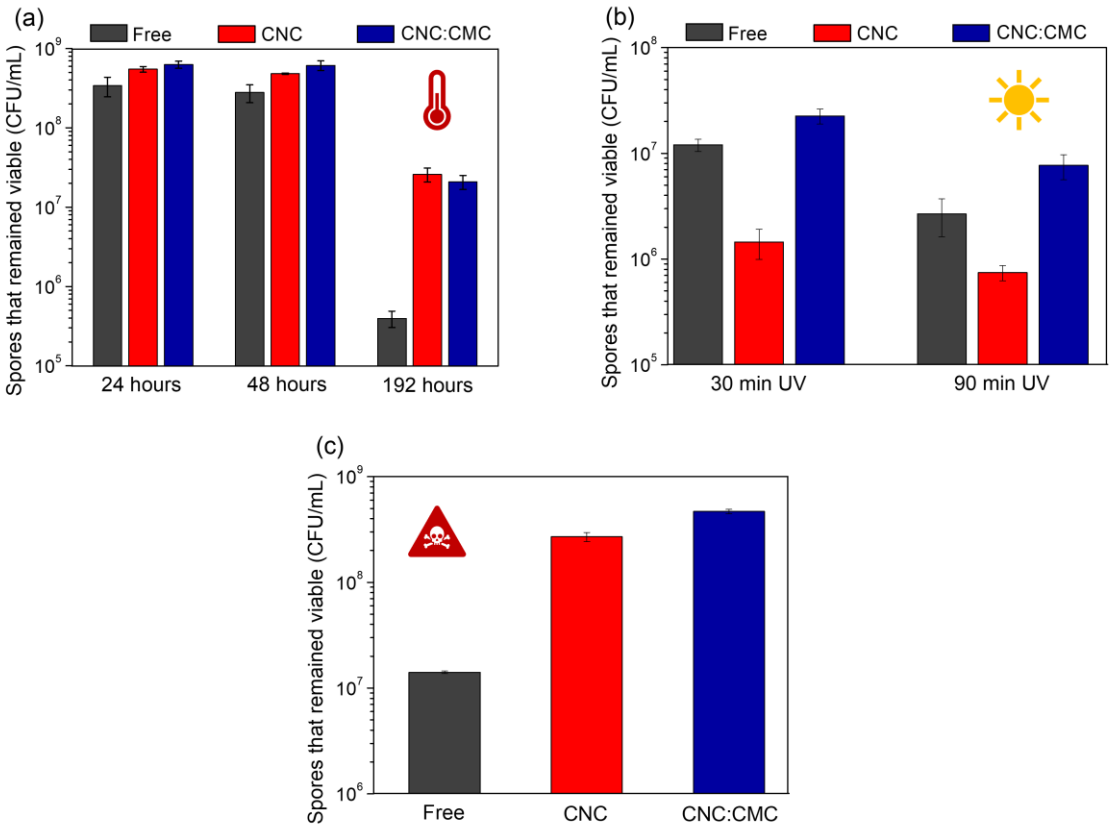


Figure 16. Effect of the free and encapsulated microorganism when submitted to stressful conditions. (a) Exposed to the temperature of 40 °C for 24, 48 and 192 hours. (b) UV light exposure for 30 and 90 minutes. (c) Commercial fungicide incubation for 24 hours.

It is important to highlight that the thermal conductivity of CNC-based materials is affected by the matrix composition, its crystallinity, the crystallite size and the degree of alignment of the nanocrystal (Apostolopoulou-Kalkavoura et al., 2021). For instance, when CNC chains are aligned, its thermal conductivity was reported as 5.7 $W.m^{-1}.K^{-1}$ (in the axial direction), and the material has anisotropic behavior. However, when CNC was self-organized (chains randomly oriented) in a film, its thermal conductivity decreased to the value of 0.25 $W.m^{-1}.K^{-1}$ and an in-plane isotropy was observed (Diaz et al., 2014). This decrease in thermal conductivity can be explained by the fact that randomly oriented chains will increase the resistance for phonons propagation across the material (Apostolopoulou-Kalkavoura et al., 2021; Diaz et al., 2014; Uetani and Hatori, 2017). This arbitrary orientation represents a condition more similar to the beads evaluated by us in this study, explaining why CNC can increase the microorganism protection.

If these beads were applied in soils, the matrix would also increase the microorganism protection. Soil thermal conductivity depends on its type/composition (sand, sandy loam, loam, clay loam, etc.), its bulk density, moisture content, salinity, among other variables (Abu-Hamdeh and Reeder, 2000). For instance, a sand soil can present a thermal conductivity in the range of 0.58 to 1.9 $W.m^{-1}.K^{-1}$, while a clay loam soil can present a conductivity from 0.36 to 0.69 $W.m^{-1}.K^{-1}$ when the bulk density varies from 1.23 – 1.59 g/cm^3 and the water content was between 1.4% to 21.2% (Abu-Hamdeh and Reeder, 2000). In this sense, if the beads were applied in a soil with the previous characteristics, they would enhance the microorganism protection by decreasing the heat propagation across the material.

UV-radiation stress

UV radiation is one of the main environmental factors that can affect fungi survivability (Hughes et al., 2003). When the spores are encapsulated in the CNC or the composite CNC:CMC matrix, the polymers will act as a UV-blocker. It happens since the CNC crystals will hindrance the light passage across the matrix, reducing UV-light transmittance (H. Li et al., 2020; Oun and Rhim, 2016). For instance, while a 2% (w/v) CMC film can have a UV-light transmittance (280 nm) of 77%, a CNC addition (10% w/w) will reduce the transmittance to 32% at the same wavelength (H. Li et al., 2020).

In this context, our results (Figure 16 (b)) show that the encapsulated *T. harzianum* in the composite matrix was relatively less sensible to UV-light exposure than its free form. Although the survivability decreased greatly for all conditions after 30 and 90 minutes under UV, the encapsulation by CNC:CMC resulted in a spore survivability of $2.3x10^7$ CFU/mL and $7.7x10^6$ CFU/mL, while the free form had $1.2x10^7$ CFU/mL and $2.6x10^6$ CFU/mL, respectively.

The CNC matrix resulted in a survival rate lower than the free spores $1.5x10^6$ CFU/mL and $7.5x10^5$ CFU/mL for 30 and 90 minutes, respectively, which is an unexpected behavior since CNC is reported as a UV-light blocker. However, the lower protection could be explained by the fact that during the hydrolysis to release the entrapped spores from the beads, the matrix made with only CNC had a lower saccharification rate than the composite (after 24 hours, small CNC beads residues were still presented in the hydrolysis medium, while the composite was completely degraded). It means that only the spores located in the most superficial layers of the beads, which were more susceptible to the UV-radiation, were released, while the ones presented in the inner parts of the matrix (more protected) were still entrapped. Since only the released spores were used for the viability determination, it can explain the lower survivability.

Incubation with a commercial fungicide

Although agrochemicals are still required to increase agricultural productivity, these products have a low compatibility with microbial inoculants, which represents a limitation for these bio-products application (Santos et al., 2021). For instance, seeds inoculated with *Bradyrhizobium* strains and treated with fungicides resulted in a bacteria mortality of up to 62% after 2 h and 95% after 24 hours (Campo et al., 2009). In this sense, a suitable microbial inoculant formulation should be able to provide protection to the microorganism against these chemicals' pesticides.

Difenoconazole is a systemic triazole fungicide that is commonly used to prevent and treat plants' diseases caused by some fungi. It can be applied to seeds, roots or leaves and it usually has a high efficiency (Liu et al., 2021; Zhang et al., 2021). However, its application was reported to decrease the soil bacteria community (Zhang et al., 2021). Additionally, when *T. hazianum* was exposed to concentrations higher than 64 ppm, this fungicide was able to inhibit in 100% the fungus growth (Khalko and Pan, 2009).

In this context, encapsulated and free spores of *T. harzianum* were inoculated for 24 hours with a commercial difenoconazole fungicide and its effect on fungus viability was evaluated (Figure 16 (c)). Free microorganism was significantly affected by the fungicide, presenting a final viability after the inoculation of $1.4x10^7$ CFU/mL. However, the encapsulation matrices were able to decrease the spore's mortality due to the polymeric barrier that reduces the direct contact between the fungus and the fungicide. While CNC beads resulted in a viability of $2.7x10^8$ CFU/mL, in the composite CNC:CMC approximately $4.8x10^8$ CFU/mL remained viable. It means that the encapsulation matrices allowed a better compatibility between a chemical and a biological product. In this sense, both matrices presented outstanding results on enhancing the fungus

protection, emphasizing that nanocellulose is an excellent material for microbial inoculant formulation.

3.4.5. Antagonistic activity of the encapsulated *T. harzianum*

Plants pathogens can significatively decrease crops production around the world (Chaloner et al., 2021). *Fusarium solani* is an example of a phytopathogen that can cause foot and root rot in several plants such as soybean, potatoes, pea, tomatoes, among others, decreasing productivity (Aoki et al., 2014). *Trichoderma harzianum* is a well-known biocontrol agent, and it has positive effects on suppressing the *F. solani* action by mechanisms such as competition, induction of plant's resistance, mycoparasitism and antibiosis (Ben Amira et al., 2017; Erazo et al., 2021). In this sense, an *in vitro* antagonism test was carried out to evaluate if the encapsulated microorganism after the beads production and after being stored for one year was still able to suppress the growth of the phytopathogen *Fusarium solani*.

As reported by Figure 17, in all evaluated samples, *T. harzianum* was able to efficiently inhibit the growth of *F. solani* in the PDA medium, completely dominating the Petri dish after 10 days. Even the beads kept under refrigeration for 1 year were able to prevent the phytopathogen from spreading across the Petri dish, showing that both the CNC and the composite were able to keep the antagonistic activity of the fungus. Additionally, the free spores presented a faster growth rate when compared with the encapsulated forms. It was expected since the spores do not have to overcome the matrices barrier to start their development at the nutrient medium. Moreover, as reported previously, the fungus at this condition presented a slightly higher viability, which could

also result in an initial faster growth rate. However, after 6 days, the growth rates seem to be very similar for all samples.

Additionally, it is important to highlight that after these beads' application on crops, besides releasing the microorganisms, they will also be a source of water, since these beads were kept wet. This water content could be important for the fungus development and also, it can improve the water retention in soil and the water supply for plants, which is an interesting resource for areas where irrigation is difficult or that suffers from drought (Ghobashy, 2020; Sahmat et al., 2022). Several studies report the positive effect of CNC and CMC as hydrogels to increase the water retention capacity in soils (do Nascimento et al., 2022; Nascimento et al., 2018; Nie et al., 2021; Saberi Riseh et al., 2023). For instance, a CMC:CNC hydrogel was reported to have a water absorption capacity of 150 times their dry weight (Araki and Yamanaka, 2014). These hydrogels can effectively enhance plants growth and development by increasing water availability for plants (do Nascimento et al., 2022; Nascimento et al., 2018; Nie et al., 2021; Saberi Riseh et al., 2023). Furthermore, the fact that the beads are not dried can be an advantage for biocontrol applications, in which the microorganisms need to have a faster release and growth rates to quickly suppress the phytopathogen development. Dried beads can provide a lower delivery and diffusion rates, which can affect the inoculant effect. Moreover, the beads application can help to increase the soil organic matter due to the polymer degradation, providing more nutrients to soil microorganisms (Sahmat et al., 2022).

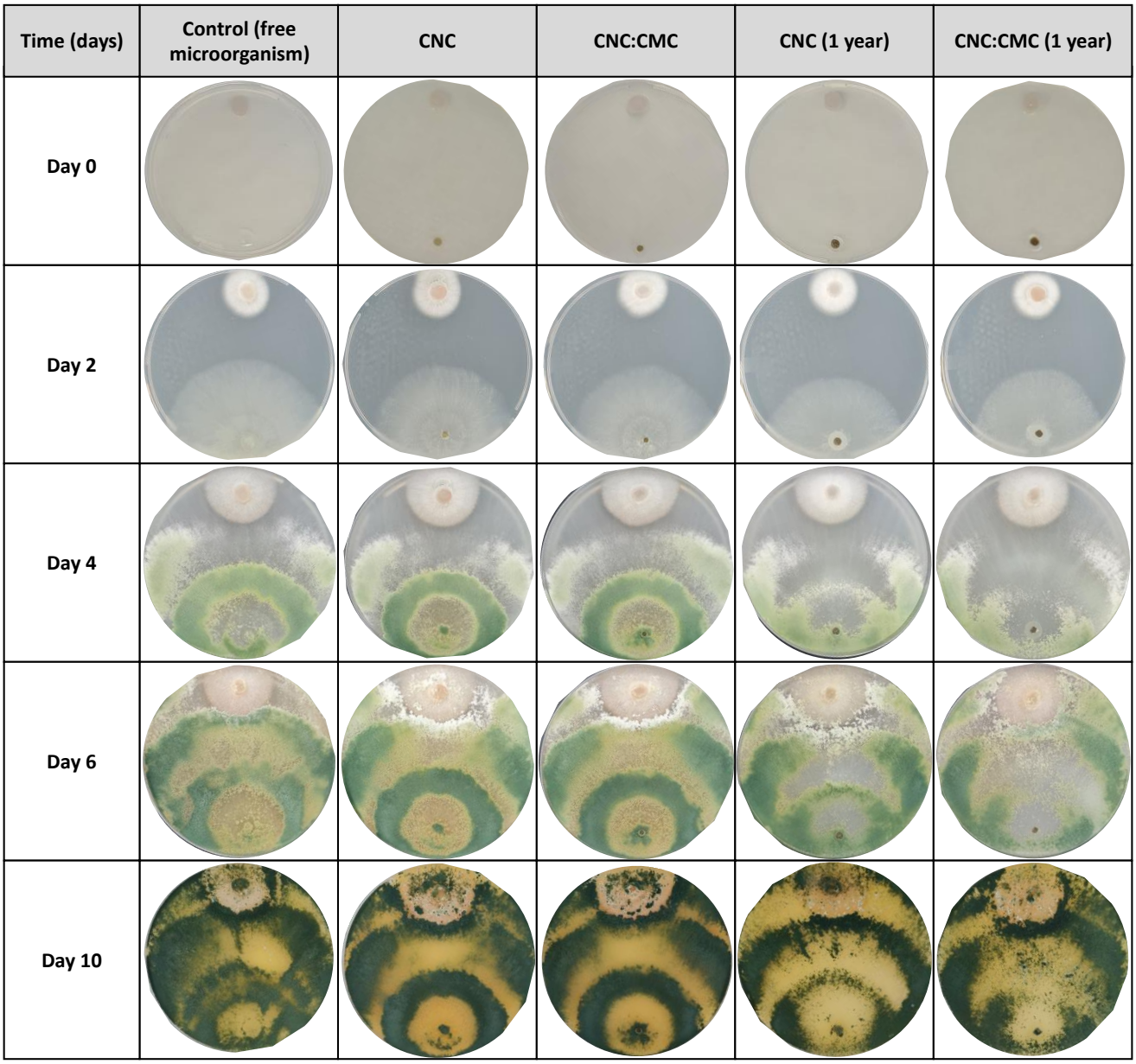


Figure 17. Antagonistic test of the biocontrol fungus (free and encapsulated) *T. harzianum* (bottom of the petri dish) against the phytopathogen *Fusarium solani* (top of the petri dish).

3.4. CONCLUSIONS

The results reported here demonstrate that the both matrices, an only CNC and the composite CNC:CMC, had excellent outcomes on enhancing the shelf-life and protection of biocontrol fungus *T. harzianum*, one of the most reported microbial inoculant. These biopolymers were able to enhance the fungus protection when it was submitted to stressful

situations commonly encountered after being applied on agricultural crops, such as heat and UV-light exposure, and the contact with a chemical fungicide. Additionally, the encapsulation using these green, renewable, and biodegradable matrices was able to keep the fungus action as a biocontrol agent even after one year of storage. These properties are very important to make these bio-based products more competitive in comparison to chemical fertilizers, which can help to reduce the carbon footprint related to agrochemical applications.

3.5. SUPPLEMENTARY MATERIAL

3.5.1. Spores' release from the beads

For the viability tests, initially the spores need to be released from the encapsulation matrices. In order to do it, we evaluated several methodologies such as the incubation of the oven dried beads (which were dried according to the methodology previously reported by Brondi et al. (2022)) in solutions with different pHs (4, 7 and 10), NaCl concentrations (0.1 M, 0.5 M, 1 M, and 5 M), and surfactant (Tween 80) concentrations (1%, 5% and 10% (w/v)). The beads were incubated for up to 96 hours at 30 °C in these mediums. The number of spores released was determined by using a Neubauer chamber. Surprisingly, the spore quantification resulted in extremely low number, showing that in all solutions, most of the microorganism remained entrapped. Figure 18 shows some pictures of the CNC:CMC beads, incubated in different conditions for 96 hours, in which it is possible to notice the spheres are still presented. In this context, in order to make the spores' release faster, the beads were incubated for 24 hours in a solution with cellulolytic enzymes (Cellic CTec3), which was able to liberate fungus. As can be seen in Figure 18, CNC:CMC capsules were completely hydrolyzed, while the

CNC matrix still presented a few visible particles, however, most of the material was also hydrolyzed.

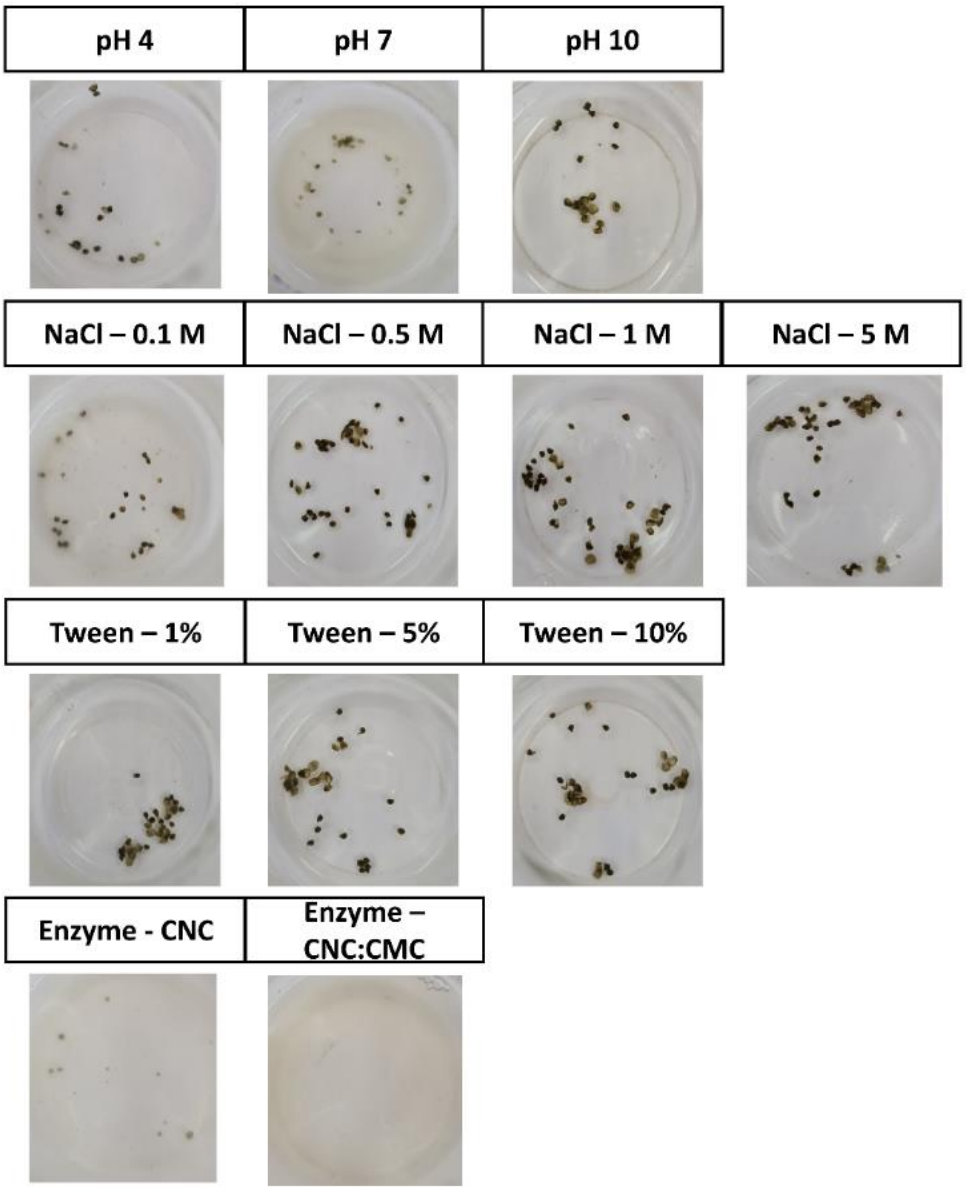


Figure 18. Matrices solubilization and spores' release in different solutions.

3.5.2. Viability of dried and wet capsules

Our previous work (Brondi et al., 2022) evaluated the wet and dried beads for spore encapsulation, however here, only the wet/refrigerated condition was evaluated. It happened due to a few difficulties in determine the spore viability. The first issue is related

to sample contamination, which happened probably during the oven drying process. This step was the only one carried out outside the laminar flow and since other microorganisms other than *T. harzianum* are studied in our laboratory, contaminations were often presented, even when the oven was previously cleaned with a 70% ethanol solution. Figure 19 shows a viability test in which the sample was contaminated with *Bacillus* strain. Since these bacteria have a much faster growth rate than *T. harzianum*, it was very difficult to determine the fungus viability. Besides that, when the viability was able to be assessed, the results showed that the percentage of viable spores was very low after the encapsulation process, especially for the CNC matrix. Table 3 summarizes the viability of the dried beads. In this sense, the experiments related to shelf-life and protection were carried out with the wet/refrigerated beads.

Figure 19. Viability test of the encapsulated fungus in which a *Bacillus* contamination was detected.

Table 3. Viability of the dried beads

Sample	Viability after encapsulation		Viability after 1 month of storage at room temperature	
	CFU/mL*	% Viable Spores	CFU/mL*	% Viable Spores
Dried CNC	$3x10^2$	0	0	0
Dried CNC:CMC	$6x10^5$	0.06	$1x10^5$	0.01

* The starting concentration was ~ 10^9 CFU/mL.

CHAPTER 4 – Overview of the Developed Research, General Conclusions and Future Perspectives

One of the most important challenges to be overcome for microbial inoculants is the increase of their shelf-life and protection (during production, storage, transportation and after its application) by using cost-effective materials. Cellulose-based materials, such as cellulose nanocrystals and carboxymethyl cellulose, stands out as interesting materials to be evaluated as encapsulation matrices due to some properties like availability, biodegradability, renewability, and biocompatibility. Additionally, these materials can be easily functionalized, in order to present different properties and a wide range of methods can be used to produce encapsulating structures. Some of these methods apply conditions that are not harmful for microorganisms, such as mild pH, room temperature, etc.

In this thesis, initially it was evaluated the production of green beads of CNC and the CNC:CMC composite by a simple coagulation method in a $CaCl_2$ solution. Different CNC, CMC and $CaCl_2$ concentrations were used and the conditions that resulted in the more consistent and stable beads were characterized and used in the subsequent experiments. The next step was the fungus addition to the formulations, followed by beads characterization and the evaluation of the spore viability. The results highlighted that the fungus addition did not influence the beads coagulation. However, drying the beads in an oven at 30 °C after their production decrease the microbial viability. One possible explanation could be the fact that during the drying process, the sharp edges of CNC can damage the fungus membrane, causing its inactivation. Due to these results, the further experiments were carried out using the wet/refrigerated capsules.

Viability tests comparing the free spores and the encapsulated fungus showed that the latter was very efficient in increasing the product shelf-life (even though a slightly decrease in viability was observed right after the coagulation process). Furthermore, excellent results were obtained for both matrices regarding the microorganism protection when submitted to stressful situations. All these conditions are essential and desired for a good microbial inoculant carrier and, when compared to other traditional encapsulation matrices reported in literature and kept under similar condition, it was possible to notice that the conditions evaluated here resulted in better results in terms of shelf-life improvement. Additionally, it was also shown that the fungus encapsulated for more than one year was able to preserve its action as a biocontrol agent, completely suppressing the growth of the phytopathogen in an *in vitro* experiment. It is also important to highlight that the fact that the beads were kept wet could be an interesting alternative to provide water for plants (specially in drought-affected regions or where irrigation is difficult) and for the microorganisms (although it can be a disadvantage for transportation). The high water retention capacity of CNC and CMC hydrogels has been extensively used for agricultural purposes and a product that provides water and a microbial inoculant can be extremely interesting. Additionally, it can provide a faster delivery and growth rate (compared to dried beads) for the fungus after its application in the field, which can be a positive characteristic for a biocontrol application. In comparison with liquid formulations, the microorganism release rate will be lower, and its protection will be higher.

In summary, our conclusions are:

- Beads were efficiently produced by a simple coagulation method using sustainable polymeric matrices.

- The drying process and the temperature of storage can significantly impact the microorganism survivability.
- The encapsulation was able to increase the microorganism shelf-life and protection, presenting better results than other natural polysaccharides well-reported in literature.
- The encapsulated fungus stored for more than 1 year was able to keep its antagonistic activity, completely suppressing the phytopathogen growth in an *in vitro* experiment.
- Although both CNC and CNC:CMC matrices (stored wet and under refrigeration) showed excellent results in keeping the cells viable under different conditions, due to the lower viability of the dried CNC beads, it can suggest that the composite CNC:CMC can be a more suitable environment for microbial inoculant encapsulation.

After the development of this research, it was possible to notice that other studies are possible and necessary in this field, to develop sustainable, and cost-effective matrices and to better understand the role of nanocellulose and other cellulose derivatives as a suitable carrier. Some research suggestions are listed below:

- Techno-economic and environmental (life-cycle analysis) evaluation of this biofertilizer production, comparing it with other encapsulation matrices such as alginate and other commercially available products.
- Evaluate the biofertilizer effect on plants experiments.
- Add additives to the formulations in order to produce oven-dried beads with better survivability rates.
- Since the beads were able to protect the microorganisms when in contact with a commercial fungicide, these matrices can be used to allow the compatibility

between the biofertilizer with a chemical fertilizer, such as urea. Usually, when urea is in contact with a microorganism, it will reduce its viability. In this sense, if CNC or CNC:CMC containing the fungus were used to coat the urea granule, it can reduce the mortality rate, allowing the compatibility between two different products. Additionally, the coating can improve the release properties of the chemical fertilizer.

- In the same context, these formulations can be used to coat seeds, allowing the compatibility between the microorganism and the fungicide that is usually presented on seeds.

REFERENCES

Abitbol, T., Mijlkovic, A., Malafronte, L., Stevanic, J.S., Larsson, P.T., Lopez-Sanchez, P., 2021. Cellulose nanocrystal/low methoxyl pectin gels produced by internal ionotropic gelation. Carbohydr. Polym. 260, 117345. https://doi.org/10.1016/j.carbpol.2020.117345

Abu-Hamdeh, N.H., Reeder, R.C., 2000. Soil Thermal Conductivity Effects of Density, Moisture, Salt Concentration, and Organic Matter. Soil Sci. Soc. Am. J. 64, 1285–1290. https://doi.org/10.2136/sssaj2000.6441285x

Ali, S., Khan, M.J., Anjum, M.M., Khan, G.R., Ali, N., 2022. Trichoderma harzianum Modulates Phosphate and Micronutrient Solubilization in the Rhizosphere. Gesunde Pflanz. 74, 853–862. https://doi.org/10.1007/s10343-022-00643-0

Alizadeh Asl, S., Mousavi, M., Labbafi, M., 2017. Synthesis and Characterization of Carboxymethyl Cellulose from Sugarcane Bagasse. J. Food Process. Technol. 08. https://doi.org/10.4172/2157-7110.1000687

Anjali, Sharma, P., Nagpal, S., 2019. Effect of Liquid and Charcoal Based Consortium Biofertilizers Amended with Additives on Growth and Yield in Chickpea (Cicer arietinum L.). Legum. Res. Int. J. https://doi.org/10.18805/LR-4131

Aoki, T., Donnell, K.O., Geiser, D.M., 2014. Systematics of key phytopathogenic Fusarium species : current status and future challenges 189–201. https://doi.org/10.1007/s10327-014-0509-3

Apostolopoulou-Kalkavoura, V., Munier, P., Bergström, L., 2021. Thermally Insulating Nanocellulose-Based Materials. Adv. Mater. 33, 2001839. https://doi.org/10.1002/adma.202001839

Araki, J., Yamanaka, Y., 2014. Anionic and cationic nanocomposite hydrogels reinforced with cellulose and chitin nanowhiskers: effect of electrolyte concentration on mechanical properties and swelling behaviors. Polym. Adv. Technol. 25, 1108–1115. https://doi.org/10.1002/pat.3361

Asad, S.A., 2022. Mechanisms of action and biocontrol potential of Trichoderma against fungal plant diseases - A review. Ecol. Complex. 49, 100978. https://doi.org/10.1016/j.ecocom.2021.100978

Asma, C., Meriem, E., Mahmoud, B., Djaafer, B., 2014. Physicochemical Characterization of Gelatin-CMC Composite Edibles Films from Polyion-Complex Hydrogels. J. Chil. Chem. Soc. 59, 2279–2283. https://doi.org/10.4067/S0717-97072014000100008

Aziz, T., Fan, H., Zhang, X., Haq, F., Ullah, A., Ullah, R., Khan, F.U., Iqbal, M., 2020. Advance Study of Cellulose Nanocrystals Properties and Applications. J. Polym. Environ. 28, 1117–1128. https://doi.org/10.1007/s10924-020-01674-2

Babalola, O.O., Glick, B.R., 2012. The use of microbial inoculants in African agriculture: Current practice and future prospects. J. Food, Agric. Environ. 10, 540–549.

Ballesteros, M., 2010. Enzymatic hydrolysis of lignocellulosic biomass, in: Bioalcohol Production. Elsevier, pp. 159–177. https://doi.org/10.1533/9781845699611.2.159

Bashan, Y., de-Bashan, L.E., Prabhu, S.R., Hernandez, J.P., 2014. Advances in plant growth-promoting bacterial inoculant technology: Formulations and practical perspectives (1998-2013). Plant Soil 378, 1–33. https://doi.org/10.1007/s11104-013-1956-x

Bauli, C.R., Lima, G.F., de Souza, A.G., Ferreira, R.R., Rosa, D.S., 2021. Eco-friendly carboxymethyl cellulose hydrogels filled with nanocellulose or nanoclays for agriculture applications as soil conditioning and nutrient carrier and their impact on cucumber growing. Colloids Surfaces A Physicochem. Eng. Asp. 623, 126771. https://doi.org/10.1016/j.colsurfa.2021.126771

Ben Amira, M., Lopez, D., Triki Mohamed, A., Khouaja, A., Chaar, H., Fumanal, B., Gousset-Dupont, A., Bonhomme, L., Label, P., Goupil, P., Ribeiro, S., Pujade-Renaud, V., Julien, J.L., Auguin, D., Venisse, J.S., 2017. Beneficial effect of Trichoderma harzianum strain Ths97 in biocontrolling Fusarium solani causal agent of root rot disease in olive trees. Biol. Control 110, 70–78. https://doi.org/10.1016/j.biocontrol.2017.04.008

Berger, B., Patz, S., Ruppel, S., Dietel, K., Faetke, S., Junge, H., Becker, M., 2018. Successful Formulation and Application of Plant Growth-Promoting Kosakonia radicincitans in Maize Cultivation. Biomed Res. Int. 2018, 1–8. https://doi.org/10.1155/2018/6439481

Bondancia, T.J., Batista, G., de Aguiar, J., Lorevice, M. V., Cruz, A.J.G., Marconcini, J.M., Mattoso, L.H.C., Farinas, C.S., 2022. Cellulose Nanocrystals from Sugar Cane Bagasse Using Organic and/or Inorganic Acids: Techno-Economic Analysis and Life Cycle Assessment. ACS Sustain. Chem. Eng. 10, 4660–4676. https://doi.org/10.1021/acssuschemeng.2c00061

Brondi, M., Florencio, C., Mattoso, L., Ribeiro, C., Farinas, C., 2022. Encapsulation of Trichoderma harzianum with nanocellulose/carboxymethyl cellulose nanocomposite. Carbohydr. Polym. 295, 119876. https://doi.org/10.1016/j.carbpol.2022.119876

Bulegon, L.G., Guimarães, V.F., Júnior, R.C., Battistus, A.G., Inagaki, A.M., Suss, A.D., 2019. Photosynthetic and Production of Urochloa ruziziensis Inoculated with Azospirillum brasilense under Drought. J. Exp. Agric. Int. 1–9. https://doi.org/10.9734/jeai/2019/v38i630315

Calegari, F., Sousa, I., Ferreira, M.G.S., Berton, M.A.C., Marino, C.E.B., Tedim, J., 2022. Influence of the Operating Conditions on the Release of Corrosion Inhibitors from Spray-Dried Carboxymethylcellulose Microspheres. Appl. Sci. 12, 1800. https://doi.org/10.3390/app12041800

Campo, R.J., Araujo, R.S., Hungria, M., 2009. Nitrogen fixation with the soybean crop in Brazil: Compatibility between seed treatment with fungicides and bradyrhizobial inoculants. Symbiosis 48, 154–163. https://doi.org/10.1007/BF03179994

Campos, E.V.R., de Oliveira, J.L., Fraceto, L.F., Singh, B., 2014. Polysaccharides as safer release systems for agrochemicals. Agron. Sustain. Dev. 35, 47–66. https://doi.org/10.1007/s13593-014-0263-0

Chaloner, T.M., Gurr, S.J., Bebber, D.P., 2021. Plant pathogen infection risk tracks global crop yields under climate change. Nat. Clim. Chang. 11.

https://doi.org/10.1038/s41558-021-01104-8

Chanratana, M., Han, G.H., Melvin Joe, M., Roy Choudhury, A., Sundaram, S., Halim, M.A., Sa, T., 2018. Evaluation of chitosan and alginate immobilized Methylobacterium oryzae CBMB20 on tomato plant growth. Arch. Agron. Soil Sci. https://doi.org/10.1080/03650340.2018.1440390

Chau, M., Sriskandha, S.E., Pichugin, D., Thérien-Aubin, H., Nykypanchuk, D., Chauve, G., Méthot, M., Bouchard, J., Gang, O., Kumacheva, E., 2015. Ion-Mediated Gelation of Aqueous Suspensions of Cellulose Nanocrystals. Biomacromolecules 16, 2455–2462. https://doi.org/10.1021/acs.biomac.5b00701

Choi, Y., Simonsen, J., 2006. Cellulose nanocrystal-filled carboxymethyl cellulose nanocomposites. J. Nanosci. Nanotechnol. 6, 633–639. https://doi.org/10.1166/jnn.2006.132

da Silva Delabona, P., Lima, D.J., Robl, D., Rabelo, S.C., Farinas, C.S., da Cruz Pradella, J.G., 2016. Enhanced cellulase production by Trichoderma harzianum by cultivation on glycerol followed by induction on cellulosic substrates. J. Ind. Microbiol. Biotechnol. 43, 617–626. https://doi.org/10.1007/s10295-016-1744-8

Davidson, D.W., Verma, M.S., Gu, F.X., 2013. Controlled root targeted delivery of fertilizer using an ionically crosslinked carboxymethyl cellulose hydrogel matrix. Springerplus 2, 1–9. https://doi.org/10.1186/2193-1801-2-318

de Carvalho, R.A., Veronese, G., Carvalho, A.J.F., Barbu, E., Amaral, A.C., Trovatti, E., 2016. The potential of TEMPO-oxidized nanofibrillar cellulose beads for cell delivery applications. Cellulose 23, 3399–3405. https://doi.org/10.1007/s10570-016-1063-2

Delgado-Jarana, J., Sousa, S., González, F., Rey, M., Llobell, A., 2006. ThHog1 controls the hyperosmotic stress response in Trichoderma harzianum. Microbiology 152, 1687–1700. https://doi.org/10.1099/mic.0.28729-0

Devi, P.I., Manjula, M., Bhavani, R.V., 2022. Agrochemicals, Environment, and Human Health. Annu. Rev. Environ. Resour. 47, 399–421. https://doi.org/10.1146/annurev-environ-120920-111015

Diaz, J.A., Ye, Z., Wu, X., Moore, A.L., Moon, R.J., Martini, A., Boday, D.J., Youngblood, J.P., 2014. Thermal conductivity in nanostructured films: From single cellulose nanocrystals to bulk films. Biomacromolecules 15, 4096–4101. https://doi.org/10.1021/bm501131a

do Nascimento, D.M., Nunes, Y.L., Feitosa, J.P.A., Dufresne, A., Rosa, M. de F., 2022. Cellulose nanocrystals-reinforced core-shell hydrogels for sustained release of fertilizer and water retention. Int. J. Biol. Macromol. 216, 24–31. https://doi.org/10.1016/j.ijbiomac.2022.06.179

Dufresne, A., 2013. Nanocellulose: A new ageless bionanomaterial. Mater. Today 16, 220–227. https://doi.org/10.1016/j.mattod.2013.06.004

Egamberdieva, D., Wirth, S.J., Alqarawi, A.A., Abd_Allah, E.F., Hashem, A., 2017. Phytohormones and Beneficial Microbes: Essential Components for Plants to Balance Stress and Fitness. Front. Microbiol. 8. https://doi.org/10.3389/fmicb.2017.02104

Erazo, J.G., Palacios, S.A., Pastor, N., Giordano, F.D., Rovera, M., Reynoso, M.M., Venisse, J.S., Torres, A.M., 2021. Biocontrol mechanisms of Trichoderma harzianum ITEM 3636 against peanut brown root rot caused by Fusarium solani RC 386. Biol. Control 164, 104774. https://doi.org/10.1016/j.biocontrol.2021.104774

Erdal, N.B., Hakkarainen, M., 2022. Degradation of Cellulose Derivatives in Laboratory, Man-Made, and Natural Environments. Biomacromolecules 23, 2713–2729. https://doi.org/10.1021/acs.biomac.2c00336

FAO, F. and A.O. of the U.N., 2021. The State of Food Security and Nutrition in the World 2021. FAO, IFAD, UNICEF, WFP and WHO. https://doi.org/10.4060/cb4474en

Felizatti, A.P., Manzano, R.M., Rodrigues, I.M.W., da Silva, M.F. das G.F., Fernandes, J.B., Forim, M.R., 2021. Encapsulation of B. bassiana in Biopolymers: Improving Microbiology of Insect Pest Control. Front. Microbiol. 12. https://doi.org/10.3389/fmicb.2021.704812

Fernández-Sandoval, M.T., Ortiz-García, M., Galindo, E., Serrano-Carreón, L., 2012. Cellular damage during drying and storage of Trichoderma harzianum spores. Process Biochem. 47, 186–194. https://doi.org/10.1016/j.procbio.2011.10.006

Ferrari, N., Maestri, C.A., Bettotti, P., Grassi, M., Abrami, M., Scarpa, M., 2021. Effect of Process Conditions and Colloidal Properties of Cellulose Nanocrystals Suspensions on the Production of Hydrogel Beads. Molecules 26, 2552. https://doi.org/10.3390/molecules26092552

Fiorati, A., Negrini, N.C., Baschenis, E., Altomare, L., Faré, S., Schieroni, A.G., Piovani, D., Mendichi, R., Ferro, M., Castiglione, F., Mele, A., Punta, C., Melone, L., 2020. TEMPO-nanocellulose/Ca2+ hydrogels: Ibuprofen drug diffusion and in vitro cytocompatibility. Materials (Basel). 13, 183. https://doi.org/10.3390/ma13010183

Fitri, I.A., Mitbumrung, W., Akanitkul, P., Rungraung, N., Kemsawasd, V., Jain, S., Winuprasith, T., 2022. Encapsulation of β-Carotene in Oil-in-Water Emulsions Containing Nanocellulose: Impact on Emulsion Properties, In Vitro Digestion, and Bioaccessibility. Polymers (Basel). 14, 1414. https://doi.org/10.3390/polym14071414

Florencio, C., Bortoletto-Santos, R., Favaro, C., Brondi, M., Velloso, C., Klaic, R., Ribeiro, C., Farinas, C., Mattoso, L., 2022. AVANÇOS NA PRODUÇÃO E FORMULAÇÃO DE INOCULANTES MICROBIANOS VISANDO UMA AGRICULTURA MAIS SUSTENTÁVEL. Quim. Nova. https://doi.org/10.21577/0100-4042.20170909

Fraceto, L.F., Maruyama, C.R., Guilger, M., Mishra, S., Keswani, C., Singh, H.B., de Lima, R., 2018. Trichoderma harzianum -based novel formulations: potential applications for management of Next-Gen agricultural challenges. J. Chem. Technol. Biotechnol. 93, 2056–2063. https://doi.org/10.1002/jctb.5613

Franche, C., Lindström, K., Elmerich, C., 2009. Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. Plant Soil 321, 35–59. https://doi.org/10.1007/s11104-008-9833-8

Gelain, L., Kingma, E., Geraldo da Cruz Pradella, J., Carvalho da Costa, A., van der Wielen, L., van Gulik, W.M., 2021. Continuous production of enzymes under

carbon-limited conditions by Trichoderma harzianum P49P11. Fungal Biol. 125, 177–183. https://doi.org/10.1016/j.funbio.2020.10.008

Gericke, M., Trygg, J., Fardim, P., 2013. Functional cellulose beads: Preparation, characterization, and applications. Chem. Rev. 113, 4812–4836. https://doi.org/10.1021/cr300242j

Ghobashy, M.M., 2020. The application of natural polymer-based hydrogels for agriculture, Hydrogels Based on Natural Polymers. Elsevier Inc. https://doi.org/10.1016/B978-0-12-816421-1.00013-6

Ghorbanpour, A., Salimi, A., Ghanbary, M.A.T., Pirdashti, H., Dehestani, A., 2018. The effect of Trichoderma harzianum in mitigating low temperature stress in tomato (Solanum lycopersicum L.) plants. Sci. Hortic. (Amsterdam). 230, 134–141. https://doi.org/10.1016/j.scienta.2017.11.028

Gill, R., Naeem, M., Ansari, A.A., Chhikara, A., Bremont, J.F.J., Tuteja, N., Gill, S.S., 2022. Agrochemicals in Soil and Environment: Conclusions and Future Perspectives, in: Agrochemicals in Soil and Environment. Springer Nature Singapore, Singapore, pp. 609–612. https://doi.org/10.1007/978-981-16-9310-6_26

Goljanian-Tabrizi, S., Amiri, S., Nikaein, D., Motesharrei, Z., 2016. The comparison of five low cost liquid formulations to preserve two phosphate solubilizing bacteria from the genera Pseudomonas and Pantoea. Iran. J. Microbiol. 8, 377–382.

Gong, J., Li, J., Xu, J., Xiang, Z., Mo, L., 2017. Research on cellulose nanocrystals produced from cellulose sources with various polymorphs. RSC Adv. 7, 33486–33493. https://doi.org/10.1039/C7RA06222B

Gopal, S., Baby, A., 2016. Enhanced Shelf-Life of Azospirillum and Psb Through. Int. J. Sci. Environ. ISSN 2278-3687 Technol. 5, 2023–2029.

Guo, T., Gu, L., Zhang, Y., Chen, H., Jiang, B., Zhao, H., Jin, Y., Xiao, H., 2019. Bioinspired self-assembled films of carboxymethyl cellulose–dopamine/montmorillonite. J. Mater. Chem. A 7, 14033–14041. https://doi.org/10.1039/C9TA00998A

Hasan, N., Rahman, L., Kim, S.H., Cao, J., Arjuna, A., Lallo, S., Jhun, B.H., Yoo, J.W., 2020. Recent advances of nanocellulose in drug delivery systems. J. Pharm. Investig. 50, 553–572. https://doi.org/10.1007/s40005-020-00499-4

He, Y., Wu, Z., Tu, L., Han, Y., Zhang, G., Li, C., 2015. Encapsulation and characterization of slow-release microbial fertilizer from the composites of bentonite and alginate. Appl. Clay Sci. 109–110, 68–75. https://doi.org/10.1016/j.clay.2015.02.001

Heinze, T., El Seoud, O.A., Koschella, A., 2018. Cellulose Derivatives, Springer Series on Polymer and Composite Materials. Springer International Publishing, Cham. https://doi.org/10.1007/978-3-319-73168-1

Holler, S., Porcelli, C., Ieropoulos, I.A., Hanczyc, M.M., 2018. Transport of Live Cells Under Sterile Conditions Using a Chemotactic Droplet. Sci. Rep. 8, 8408. https://doi.org/10.1038/s41598-018-26703-y

Horn, S.J., Vaaje-Kolstad, G., Westereng, B., Eijsink, V., 2012. Novel enzymes for the degradation of cellulose. Biotechnol. Biofuels 5, 45. https://doi.org/10.1186/1754-

6834-5-45

Hu, Y., Tang, L., Lu, Q., Wang, S., Chen, X., Huang, B., 2014. Preparation of cellulose nanocrystals and carboxylated cellulose nanocrystals from borer powder of bamboo. Cellulose 21, 1611–1618. https://doi.org/10.1007/s10570-014-0236-0

Hughes, K.A., Lawley, B., Newsham, K.K., 2003. Solar UV-B Radiation Inhibits the Growth of Antarctic Terrestrial Fungi. Appl. Environ. Microbiol. 69, 1488–1491. https://doi.org/10.1128/AEM.69.3.1488-1491.2003

Huq, T., Fraschini, C., Khan, A., Riedl, B., Bouchard, J., Lacroix, M., 2017. Alginate based nanocomposite for microencapsulation of probiotic: Effect of cellulose nanocrystal (CNC) and lecithin. Carbohydr. Polym. 168, 61–69. https://doi.org/10.1016/j.carbpol.2017.03.032

Illescas, M., Pedrero-Méndez, A., Pitorini-Bovolini, M., Hermosa, R., Monte, E., 2021. Phytohormone Production Profiles in Trichoderma Species and Their Relationship to Wheat Plant Responses to Water Stress. Pathogens 10, 991. https://doi.org/10.3390/pathogens10080991

Jannatyha, N., Shojaee-Aliabadi, S., Moslehishad, M., Moradi, E., 2020. Comparing mechanical, barrier and antimicrobial properties of nanocellulose/CMC and nanochitosan/CMC composite films. Int. J. Biol. Macromol. 164, 2323–2328. https://doi.org/10.1016/j.ijbiomac.2020.07.249

Jasmani, L., Adnan, S., 2017. Preparation and characterization of nanocrystalline cellulose from Acacia mangium and its reinforcement potential. Carbohydr. Polym. 161, 166–171. https://doi.org/10.1016/j.carbpol.2016.12.061

Kaminsky, L.M., Trexler, R. V., Malik, R.J., Hockett, K.L., Bell, T.H., 2019. The Inherent Conflicts in Developing Soil Microbial Inoculants. Trends Biotechnol. 37, 140–151. https://doi.org/10.1016/j.tibtech.2018.11.011

Khalko, S., Pan, S.K., 2009. Phytotoxicity of some fungicides and their compatibility study with a potential biocontrol agent Trichoderma harzianum. J. Crop Weed 5, 151–153.

Klaic, R., Giroto, A.S., Guimarães, G.G.F., Plotegher, F., Ribeiro, C., Zangirolami, T.C., Farinas, C.S., 2018. Nanocomposite of starch-phosphate rock bioactivated for environmentally-friendly fertilization. Miner. Eng. 128, 230–237. https://doi.org/10.1016/j.mineng.2018.09.002

Kour, D., Rana, K.L., Yadav, A.N., Yadav, N., Kumar, M., Kumar, V., Vyas, P., Dhaliwal, H.S., Saxena, A.K., 2020. Microbial biofertilizers: Bioresources and eco-friendly technologies for agricultural and environmental sustainability. Biocatal. Agric. Biotechnol. 23, 101487. https://doi.org/10.1016/j.bcab.2019.101487

Kremser, U., Schnug, E., 2002. Impact of fertilizers on aquatic ecosystems and protection of water bodies from mineral nutrients Aus dem Institut für Pflanzenernährung und Bodenkunde Ulrich Kremser Ewald Schnug Impact of fertilizers on aquatic ecosystems and protection of water bodies. Landbauforsch. Volkenrode 52, 81–90.

Krishnaraj, P.U., Dahale, S., 2014. Mineral Phosphate Solubilization: Concepts and Prospects in Sustainable Agriculture. Proc. Indian Natl. Sci. Acad. 80, 389. https://doi.org/10.16943/ptinsa/2014/v80i2/55116

Kumar, A., Choudhary, A., Kaur, H., Mehta, S., Husen, A., 2021. Smart nanomaterial and nanocomposite with advanced agrochemical activities. Nanoscale Res. Lett. 16. https://doi.org/10.1186/s11671-021-03612-0

Kumar, A., Lee, Y., Kim, D., Rao, K.M., Kim, J., Park, S., Haider, A., Lee, D.H., Han, S.S., 2017. Effect of crosslinking functionality on microstructure, mechanical properties, and in vitro cytocompatibility of cellulose nanocrystals reinforced poly (vinyl alcohol)/sodium alginate hybrid scaffolds. Int. J. Biol. Macromol. 95, 962–973. https://doi.org/10.1016/j.ijbiomac.2016.10.085

Kusmono, Listyanda, R.F., Wildan, M.W., Ilman, M.N., 2020. Preparation and characterization of cellulose nanocrystal extracted from ramie fibers by sulfuric acid hydrolysis. Heliyon 6, e05486. https://doi.org/10.1016/j.heliyon.2020.e05486

Lan, W., He, L., Liu, Y., 2018. Preparation and properties of sodium carboxymethyl cellulose/sodium alginate/chitosan composite film. Coatings 8. https://doi.org/10.3390/coatings8080291

Lavrič, G., Oberlintner, A., Filipova, I., Novak, U., Likozar, B., Vrabič-Brodnjak, U., 2021. Functional Nanocellulose, Alginate and Chitosan Nanocomposites Designed as Active Film Packaging Materials. Polymers (Basel). 13, 2523. https://doi.org/10.3390/polym13152523

Lee, H., Lee, Y.M., Heo, Y.M., Lee, J., Kim, J.S., Kang, K.Y., Kim, J.J., 2017. Utilization of agricultural residues for enhancement of cellulolytic enzyme production and enzymatic saccharification by Trichoderma harzianum KUC1716. Ind. Crops Prod. 109, 185–191. https://doi.org/10.1016/j.indcrop.2017.08.042

Leppänen, I., Vikman, M., Harlin, A., Orelma, H., 2020. Enzymatic Degradation and Pilot-Scale Composting of Cellulose-Based Films with Different Chemical Structures. J. Polym. Environ. 28, 458–470. https://doi.org/10.1007/s10924-019-01621-w

Levin, D., Saem, S., Osorio, D.A., Cerf, A., Cranston, E.D., Moran-Mirabal, J.M., 2018. Green Templating of Ultraporous Cross-Linked Cellulose Nanocrystal Microparticles. Chem. Mater. 30, 8040–8051. https://doi.org/10.1021/acs.chemmater.8b03858

Li, H., Shi, H., He, Y., Fei, X., Peng, L., 2020. Preparation and characterization of carboxymethyl cellulose-based composite films reinforced by cellulose nanocrystals derived from pea hull waste for food packaging applications. Int. J. Biol. Macromol. 164, 4104–4112. https://doi.org/10.1016/j.ijbiomac.2020.09.010

Li, J.-X., Zhang, F., Jiang, D.-D., Li, J., Wang, F.-L., Zhang, Z., Wang, W., Zhao, X.-Q., 2020. Diversity of Cellulase-Producing Filamentous Fungi From Tibet and Transcriptomic Analysis of a Superior Cellulase Producer Trichoderma harzianum LZ117. Front. Microbiol. 11. https://doi.org/10.3389/fmicb.2020.01617

Li, J., Wang, J., Liu, H., Macdonald, C.A., Singh, B.K., 2022. Application of microbial inoculants significantly enhances crop productivity: A meta-analysis of studies from 2010 to 2020. J. Sustain. Agric. Environ. 1, 216–225. https://doi.org/10.1002/sae2.12028

Li, J.X., Zhang, F., Jiang, D.D., Li, J., Wang, F. Lou, Zhang, Z., Wang, W., Zhao, X.Q., 2020. Diversity of Cellulase-Producing Filamentous Fungi From Tibet and

Transcriptomic Analysis of a Superior Cellulase Producer Trichoderma harzianum LZ117. Front. Microbiol. 11, 1–15. https://doi.org/10.3389/fmicb.2020.01617

Lima, G.F., Souza, A.G., Rosa, D. dos S., 2020. Nanocellulose as Reinforcement in Carboxymethylcellulose Superabsorbent Nanocomposite Hydrogels. Macromol. Symp. 394, 2000126. https://doi.org/10.1002/masy.202000126

Liu, R., Li, J., Zhang, L., Feng, T., Zhang, Z., Zhang, B., 2021. Fungicide difenoconazole induced biochemical and developmental toxicity in wheat (Triticum aestivum l.). Plants 10, 1–15. https://doi.org/10.3390/plants10112304

Lobo, C.B., Juárez Tomás, M.S., Viruel, E., Ferrero, M.A., Lucca, M.E., 2019. Development of low-cost formulations of plant growth-promoting bacteria to be used as inoculants in beneficial agricultural technologies. Microbiol. Res. 219, 12–25. https://doi.org/10.1016/j.micres.2018.10.012

Locatelli, G.O., dos Santos, G.F., Botelho, P.S., Finkler, C.L.L., Bueno, L.A., 2018. Development of Trichoderma sp. formulations in encapsulated granules (CG) and evaluation of conidia shelf-life. Biol. Control 117, 21–29. https://doi.org/10.1016/j.biocontrol.2017.08.020

Lopes, A.R. de O., Locatelli, G.O., Barbosa, R. de M., Lobo Junior, M., Moura Mascarin, G., Lamenha Luna Finkler, C., 2020. Preparation, characterisation and cell viability of encapsulated Trichoderma asperellum in alginate beads. J. Microencapsul. 37, 270–282. https://doi.org/10.1080/02652048.2020.1729884

Mancera-López, M.E., Izquierdo-Estévez, W.F., Escalante-Sánchez, A., Ibarra, J.E., Barrera-Cortés, J., 2019. Encapsulation of Trichoderma harzianum conidia as a method of conidia preservation at room temperature and propagation in submerged culture. Biocontrol Sci. Technol. 29, 107–130. https://doi.org/10.1080/09583157.2018.1535053

Mandal, A., Chakrabarty, D., 2019. Studies on mechanical, thermal, and barrier properties of carboxymethyl cellulose film highly filled with nanocellulose. J. Thermoplast. Compos. Mater. 32, 995–1014. https://doi.org/10.1177/0892705718772868

Marinković, J., Bjelić, D., Tintor, B., Miladinović, J., Đukić, V., Đorđević, V., 2018. Effects of soybean co-inoculation with plant growth promoting rhizobacteria in field trial. Rom. Biotechnol. Lett. 23, 13401.

Maruyama, C.R., Bilesky-José, N., de Lima, R., Fraceto, L.F., 2020. Encapsulation of Trichoderma harzianum Preserves Enzymatic Activity and Enhances the Potential for Biological Control. Front. Bioeng. Biotechnol. 8, 1–14. https://doi.org/10.3389/fbioe.2020.00225

Minaxi, Saxena, J., 2011. Efficacy of rhizobacterial strains encapsulated in nontoxic biodegradable gel matrices to promote growth and yield of wheat plants. Appl. Soil Ecol. 48, 301–308. https://doi.org/10.1016/j.apsoil.2011.04.007

Mona, S.A., Hashem, A., Abd_Allah, E.F., Alqarawi, A.A., Soliman, D.W.K., Wirth, S., Egamberdieva, D., 2017. Increased resistance of drought by Trichoderma harzianum fungal treatment correlates with increased secondary metabolites and proline content. J. Integr. Agric. 16, 1751–1757. https://doi.org/10.1016/S2095-3119(17)61695-2

Muñoz-Celaya, A.L., Ortiz-García, M., Vernon-Carter, E.J., Jauregui-Rincón, J., Galindo, E., Serrano-Carreón, L., 2012. Spray-drying microencapsulation of Trichoderma harzianum conidias in carbohydrate polymers matrices. Carbohydr. Polym. 88, 1141–1148. https://doi.org/10.1016/j.carbpol.2011.12.030

Narayanan, M., Kandasamy, S., He, Z., Kumarasamy, S., 2022. Ecological impacts of pesticides on soil and water ecosystems and its natural degradation process, in: Pesticides in the Natural Environment. Elsevier, pp. 23–49. https://doi.org/10.1016/B978-0-323-90489-6.00002-1

Nascimento, D.M., Nunes, Y.L., Figueirêdo, M.C.B., De Azeredo, H.M.C., Aouada, F.A., Feitosa, J.P.A., Rosa, M.F., Dufresne, A., 2018. Nanocellulose nanocomposite hydrogels: Technological and environmental issues. Green Chem. 20, 2428–2448. https://doi.org/10.1039/c8gc00205c

Nie, G., Zang, Y., Yue, W., Wang, M., Baride, A., Sigdel, A., Janaswamy, S., 2021. Cellulose-based hydrogel beads: Preparation and characterization. Carbohydr. Polym. Technol. Appl. 2, 100074. https://doi.org/10.1016/j.carpta.2021.100074

Nimz, H.H., 1984. Wood-chemistry, ultrastructure, reactions. Holz als Roh- und Werkst. 42, 314–314. https://doi.org/10.1007/BF02608943

Noronha, V.T., Camargos, C.H.M., Jackson, J.C., Souza Filho, A.G., Paula, A.J., Rezende, C.A., Faria, A.F., 2021. Physical Membrane-Stress-Mediated Antimicrobial Properties of Cellulose Nanocrystals. ACS Sustain. Chem. Eng. 9, 3203–3212. https://doi.org/10.1021/acssuschemeng.0c08317

O'Callaghan, M., Ballard, R.A., Wright, D., 2022. Soil microbial inoculants for sustainable agriculture: Limitations and opportunities. Soil Use Manag. 38, 1340–1369. https://doi.org/10.1111/sum.12811

Oun, A.A., Rhim, J.W., 2017. Characterization of carboxymethyl cellulose-based nanocomposite films reinforced with oxidized nanocellulose isolated using ammonium persulfate method. Carbohydr. Polym. 174, 484–492. https://doi.org/10.1016/j.carbpol.2017.06.121

Oun, A.A., Rhim, J.W., 2016. Isolation of cellulose nanocrystals from grain straws and their use for the preparation of carboxymethyl cellulose-based nanocomposite films. Carbohydr. Polym. 150, 187–200. https://doi.org/10.1016/j.carbpol.2016.05.020

Pereira, J.F., Oliveira, A.L.M., Sartori, D., Yamashita, F., Mali, S., 2023. Perspectives on the Use of Biopolymeric Matrices as Carriers for Plant-Growth Promoting Bacteria in Agricultural Systems. Microorganisms 11, 1–17. https://doi.org/10.3390/microorganisms11020467

Pinto, E., Aggrey, W.N., Boakye, P., Amenuvor, G., Sokama-Neuyam, Y.A., Fokuo, M.K., Karimaie, H., Sarkodie, K., Adenutsi, C.D., Erzuah, S., Rockson, M.A.D., 2022. Cellulose processing from biomass and its derivatization into carboxymethylcellulose: A review. Sci. African 15, e01078. https://doi.org/10.1016/j.sciaf.2021.e01078

Qiu, Z., Egidi, E., Liu, H., Kaur, S., Singh, B.K., 2019. New frontiers in agriculture productivity: Optimised microbial inoculants and in situ microbiome engineering. Biotechnol. Adv. 37, 107371. https://doi.org/10.1016/j.biotechadv.2019.03.010

Rahman, M.S., Hasan, M.S., Nitai, A.S., Nam, S., Karmakar, A.K., Ahsan, M.S., Shiddiky, M.J.A., Ahmed, M.B., 2021. Recent Developments of Carboxymethyl Cellulose. Polymers (Basel). 13, 1345. https://doi.org/10.3390/polym13081345

Rajendran, N., Runge, T., Bergman, R.D., Nepal, P., Houtman, C., 2023. Techno-economic analysis and life cycle assessment of cellulose nanocrystals production from wood pulp. Bioresour. Technol. 377, 128955. https://doi.org/10.1016/j.biortech.2023.128955

Reid, M.S., Villalobos, M., Cranston, E.D., 2017. Benchmarking Cellulose Nanocrystals: From the Laboratory to Industrial Production. Langmuir 33, 1583–1598. https://doi.org/10.1021/acs.langmuir.6b03765

Ribeiro, C., Carmo, M., 2019. Why nonconventional materials are answers for sustainable agriculture. MRS Energy Sustain. 6, 7. https://doi.org/10.1557/mre.2019.7

Riseh, R.S., Skorik, Y.A., Thakur, V.K., Pour, M.M., Tamanadar, E., Noghabi, S.S., 2021. Encapsulation of plant biocontrol bacteria with alginate as a main polymer material. Int. J. Mol. Sci. 22. https://doi.org/10.3390/ijms222011165

Rojas-Sánchez, B., Guzmán-Guzmán, P., Morales-Cedeño, L.R., Orozco-Mosqueda, M. del C., Saucedo-Martínez, B.C., Sánchez-Yáñez, J.M., Fadiji, A.E., Babalola, O.O., Glick, B.R., Santoyo, G., 2022. Bioencapsulation of Microbial Inoculants: Mechanisms, Formulation Types and Application Techniques. Appl. Biosci. 1, 198–220. https://doi.org/10.3390/applbiosci1020013

Saberi Riseh, R., Gholizadeh Vazvani, M., Hassanisaadi, M., Skorik, Y.A., 2023. Micro-/Nano-Carboxymethyl Cellulose as a Promising Biopolymer with Prospects in the Agriculture Sector: A Review. Polymers (Basel). 15. https://doi.org/10.3390/polym15020440

Saberi Riseh, R., Skorik, Y.A., Thakur, V.K., Moradi Pour, M., Tamanadar, E., Noghabi, S.S., 2021. Encapsulation of Plant Biocontrol Bacteria with Alginate as a Main Polymer Material. Int. J. Mol. Sci. 22, 11165. https://doi.org/10.3390/ijms222011165

Sahmat, S.S., Rafii, M.Y., Oladosu, Y., Jusoh, M., Hakiman, M., Mohidin, H., 2022. A Systematic Review of the Potential of a Dynamic Hydrogel as a Substrate for Sustainable Agriculture.

Sammauria, R., Kumawat, S., Kumawat, P., Singh, J., Jatwa, T.K., 2020. Microbial inoculants: potential tool for sustainability of agricultural production systems. Arch. Microbiol. 202, 677–693. https://doi.org/10.1007/s00203-019-01795-w

Santos, M.S., Nogueira, M.A., Hungria, M., 2019. Microbial inoculants: reviewing the past, discussing the present and previewing an outstanding future for the use of beneficial bacteria in agriculture. AMB Express 9, 205. https://doi.org/10.1186/s13568-019-0932-0

Santos, M.S., Rodrigues, T.F., Nogueira, M.A., Hungria, M., 2021. The challenge of combining high yields with environmentally friendly bioproducts: A review on the compatibility of pesticides with microbial inoculants. Agronomy 11. https://doi.org/10.3390/agronomy11050870

Saravanakumar, K., Li, Y., Yu, C., Wang, Q., Wang, M., Sun, J., Gao, J., Chen, J., 2017.

Effect of Trichoderma harzianum on maize rhizosphere microbiome and biocontrol of Fusarium Stalk rot. Sci. Rep. 7, 1771. https://doi.org/10.1038/s41598-017-01680-w

Schoebitz, M., López, M.D., Roldán, A., 2013a. Bioencapsulation of microbial inoculants for better soil-plant fertilization. A review. Agron. Sustain. Dev. https://doi.org/10.1007/s13593-013-0142-0

Schoebitz, M., López, M.D., Roldán, A., 2013b. Bioencapsulation of microbial inoculants for better soil-plant fertilization. A review. Agron. Sustain. Dev. 33, 751–765. https://doi.org/10.1007/s13593-013-0142-0

Seenivasagan, R., Babalola, O.O., 2021. Utilization of Microbial Consortia as Biofertilizers and Biopesticides for the Production of Feasible Agricultural Product. Biology (Basel). 10, 1111. https://doi.org/10.3390/biology10111111

Shaghaleh, H., Xu, X., Wang, S., 2018. Current progress in production of biopolymeric materials based on cellulose, cellulose nanofibers, and cellulose derivatives. RSC Adv. 8, 825–842. https://doi.org/10.1039/C7RA11157F

Sharma, S.B., Sayyed, R.Z., Trivedi, M.H., Gobi, T.A., 2013. Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. Springerplus 2, 587. https://doi.org/10.1186/2193-1801-2-587

Sharpley, A.N., Smith, S.J., Naney, J.W., 1987. Environmental impact of agricultural nitrogen and phosphorus use. J. Agric. Food Chem. 35, 812–817. https://doi.org/10.1021/jf00077a043

Singh, G., Chandoha-Lee, C., Zhang, W., Renneckar, S., Vikesland, P.J., Pruden, A., 2016. Biodegradation of nanocrystalline cellulose by two environmentally-relevant consortia. Water Res. 104, 137–146. https://doi.org/10.1016/j.watres.2016.07.073

Singh, P., Medronho, B., Alves, L., da Silva, G.J., Miguel, M.G., Lindman, B., 2017. Development of carboxymethyl cellulose-chitosan hybrid micro- and macroparticles for encapsulation of probiotic bacteria. Carbohydr. Polym. 175, 87–95. https://doi.org/10.1016/j.carbpol.2017.06.119

Squinca, P., Bilatto, S., Badino, A.C., Farinas, C.S., 2020. Nanocellulose Production in Future Biorefineries: An Integrated Approach Using Tailor-Made Enzymes. ACS Sustain. Chem. Eng. 8, 2277–2286. https://doi.org/10.1021/acssuschemeng.9b06790

Strobel, S.A., Knowles, L., Nitin, N., Scher, H.B., Jeoh, T., 2020. Comparative technoeconomic process analysis of industrial-scale microencapsulation of bioactives in cross-linked alginate. J. Food Eng. 266, 109695. https://doi.org/10.1016/j.jfoodeng.2019.109695

Stummer, B.E., Zhang, Q., Zhang, X., Warren, R.A., Harvey, P.R., 2020. Quantification of Trichoderma afroharzianum, Trichoderma harzianum and Trichoderma gamsii inoculants in soil, the wheat rhizosphere and in planta suppression of the crown rot pathogen Fusarium pseudograminearum. J. Appl. Microbiol. 129, 971–990. https://doi.org/10.1111/jam.14670

Su, Y.L., Fu, Z.Y., Zhang, J.Y., Wang, W.M., Wang, H., Wang, Y.C., Zhang, Q.J., 2008. Microencapsulation of Radix salvia miltiorrhiza nanoparticles by spray-drying.

Powder Technol. 184, 114–121. https://doi.org/10.1016/j.powtec.2007.08.014

Sujarit, K., Pathom-aree, W., Mori, M., Dobashi, K., Shiomi, K., Lumyong, S., 2020. Streptomyces palmae CMU-AB204T, an antifungal producing-actinomycete, as a potential biocontrol agent to protect palm oil producing trees from basal stem rot disease fungus, Ganoderma boninense. Biol. Control 148, 104307. https://doi.org/10.1016/j.biocontrol.2020.104307

Thomas, B., Raj, M.C., B, A.K., H, R.M., Joy, J., Moores, A., Drisko, G.L., Sanchez, C., 2018. Nanocellulose, a Versatile Green Platform: From Biosources to Materials and Their Applications. Chem. Rev. 118, 11575–11625. https://doi.org/10.1021/acs.chemrev.7b00627

Trigo, E., Chavarria, H., Pray, C., Smyth, S.J., Torroba, A., Wesseler, J., Zilberman, D., Martinez, J.F., 2023. The Bioeconomy and Food System Transformation, in: Science and Innovations for Food Systems Transformation. Springer International Publishing, Cham, pp. 849–868. https://doi.org/10.1007/978-3-031-15703-5_45

Uetani, K., Hatori, K., 2017. Thermal conductivity analysis and applications of nanocellulose materials. Sci. Technol. Adv. Mater. 18, 877–892. https://doi.org/10.1080/14686996.2017.1390692

United Nations Department of Economic and Social Affairs, P.D., 2022. World Population Prospects 2022, United Nation.

Uyanga, K.A., Daoud, W.A., 2021. Green and sustainable carboxymethyl cellulose-chitosan composite hydrogels: Effect of crosslinker on microstructure. Cellulose 28, 5493–5512. https://doi.org/10.1007/s10570-021-03870-2

Vanderfleet, O.M., Cranston, E.D., 2021. Production routes to tailor the performance of cellulose nanocrystals. Nat. Rev. Mater. 6, 124–144. https://doi.org/10.1038/s41578-020-00239-y

Vassilev, N., Vassileva, M., Martos, V., Garcia del Moral, L.F., Kowalska, J., Tylkowski, B., Malusá, E., 2020. Formulation of Microbial Inoculants by Encapsulation in Natural Polysaccharides: Focus on Beneficial Properties of Carrier Additives and Derivatives. Front. Plant Sci. 11. https://doi.org/10.3389/fpls.2020.00270

Vemmer, M., Patel, A. V., 2013. Review of encapsulation methods suitable for microbial biological control agents. Biol. Control 67, 380–389. https://doi.org/10.1016/j.biocontrol.2013.09.003

Ventura, C., Pinto, F., Lourenço, A.F., Ferreira, P.J.T., Louro, H., Silva, M.J., 2020. On the toxicity of cellulose nanocrystals and nanofibrils in animal and cellular models, Cellulose. https://doi.org/10.1007/s10570-020-03176-9

Verma, J.P., Yadav, J., Tiwari, K.N., Jaiswal, D.K., 2014. Evaluation of plant growth promoting activities of microbial strains and their effect on growth and yield of chickpea (Cicer arietinum L.) in India. Soil Biol. Biochem. 70, 33–37. https://doi.org/10.1016/j.soilbio.2013.12.001

Vessey, J.K., 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255, 571–586. https://doi.org/10.1023/A:1026037216893

Ye, L., Zhao, X., Bao, E., Li, J., Zou, Z., Cao, K., 2020. Bio-organic fertilizer with reduced rates of chemical fertilization improves soil fertility and enhances tomato

yield and quality. Sci. Rep. 10, 177. https://doi.org/10.1038/s41598-019-56954-2

Zayed, M.S., 2016. Advances in Formulation Development Technologies, in: Microbial Inoculants in Sustainable Agricultural Productivity. Springer India, New Delhi, pp. 219–237. https://doi.org/10.1007/978-81-322-2644-4_14

Zhang, F., Yuan, J., Yang, X., Cui, Y., Chen, L., Ran, W., Shen, Q., 2013. Putative Trichoderma harzianum mutant promotes cucumber growth by enhanced production of indole acetic acid and plant colonization. Plant Soil 368, 433–444. https://doi.org/10.1007/s11104-012-1519-6

Zhang, Fuli, Ge, H., Zhang, Fan, Guo, N., Wang, Y., Chen, L., Ji, X., Li, C., 2016. Biocontrol potential of Trichoderma harzianum isolate T-aloe against Sclerotinia sclerotiorum in soybean. Plant Physiol. Biochem. 100, 64–74. https://doi.org/10.1016/j.plaphy.2015.12.017

Zhang, H., Song, J., Zhang, Z., Zhang, Q., Chen, S., Mei, J., Yu, Y., Fang, H., 2021. Exposure to fungicide difenoconazole reduces the soil bacterial community diversity and the co-occurrence network complexity. J. Hazard. Mater. 405, 124208. https://doi.org/10.1016/j.jhazmat.2020.124208

APPENDIX

Publication rights and permissions – Authorization to reuse the published article on this thesis.

Chapter 2 – Brondi, M., Florencio, C., Mattoso, L., Ribeiro, C., Farinas, C., 2022. Encapsulation of Trichoderma harzianum with nanocellulose/carboxymethyl cellulose nanocomposite. Carbohydrate Polymers. 295, 119876. https://doi.org/10.1016/j.carbpol.2022.119876