

Article

Development, Characterization, and Evaluation of Chitosan Nano/Microcapsules with *Bacillus subtilis* Extract and Their Effect on Tomato (*Solanum lycopersicum*) Seed Germination

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Abstract

The use of chemical fertilizers has led to significant environmental pollution. An alternative to these fertilizers is the use of natural compounds, such as phytohormones, which promote germination and crop development. However, environmental factors can affect natural compounds, reducing their effectiveness. Therefore, increasing their stability without decreasing their activity to improve crop quality is essential. This study produced and characterized chitosan and sodium tripolyphosphate (TPP) nano-microparticles (NMP) loaded with *Bacillus subtilis* extract and evaluated their impact on tomato seed germination. We employed two experimental designs (Box–Behnken and Box–Hunter–Hunter) to determine the optimal production conditions and characterized the NMP using DLS, SEM, and FTIR. The optimal treatment consisted of 8 min of homogenization, followed by 8 min of ultrasound at a 70% amplitude, resulting in a particle size of 330.7 nm, a polydispersity index of 0.25, a zeta potential of 34.3 mV, and an encapsulation efficiency of 68.8%. The NMP loaded with bacterial extract was applied to tomato seeds as a 50% dilution pretreatment. NMP achieved the best results, with a 72% germination rate (1.6 seeds per day) and an average germination time of 3.8 days. It is concluded that the experimental designs helped improve particle properties and that the chitosan and TPP coating enhances the stability and activity of the bacterial extract, potentially benefiting agronomic applications.

Keywords: *Bacillus subtilis*; chitosan; nanoparticles; seed germination; tomato seeds



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1. Introduction

The world's population is increasing, and with it, demand for food is rising. Hence, the agricultural sector aims to increase yields to meet the population's needs, with a focus on products that enhance field production [1]. Despite global recommendations, current agricultural practices still rely heavily on pesticides and synthetic fertilizers, which harm the environment. The consequences include reduced soil fertility, reduced microbial diversity, the development of resistant phytopathogens, and contamination of air, soil, and water resources [2,3].

Tomato (*Solanum lycopersicum* L.) represents a staple horticultural species whose biochemical composition provides essential nutrients and bioactive compounds, thereby conferring it strategic significance within global agri-food systems at both national and international scales [4]. Mexico is the seventh-largest producer globally and the leading exporter [5,6], and in 2024, Mexico's national production reached 3,723,803 tons, representing 19.4% of total national vegetable production [7].

Nevertheless, tomato production in Mexico is constrained by multiple factors, including phytosanitary pressures, imbalances in nutrient management, limitations in postharvest conservation, and market-related constraints [8]. Among the most critical phytopathogens affecting this crop are *Clavibacter michiganensis*, the etiological agent of bacterial canker, and *Fusarium oxysporum*, the causal organism of Fusarium wilt. Both pathogens are associated with substantial yield reductions in tomato production, exerting a considerable economic impact [9,10].

Modern systems aim to ensure sustainable crop production, driving the search for environmentally friendly solutions to enhance crop protection and yield [11]. Numerous studies demonstrate the potential of plant-associated microorganisms to enhance plant efficiency and yield in cropping systems [12]. This approach establishes sustainable agricultural practices that can serve as alternatives to chemical treatments for plant development and pest and disease control.

These microorganisms can be applied to seeds, plant surfaces, or the rhizosphere to promote plant growth, improve plant and soil health, and enhance productivity [2]. However, it is essential to comprehend the role of these microorganisms and their mechanisms in promoting growth and controlling disease and to apply them as biofertilizers, biostimulants, and biopesticides [13,14].

Many bacterial species interact with a wide range of plant species through different mechanisms of action [15]. Bacteria of the species *Bacillus subtilis* can produce antimicrobial compounds, including antibiotics, volatile organic compounds, and cyclic lipopeptides, as well as phytohormones such as auxins, in the presence of tryptophan as a precursor [16]. Auxins are present throughout the plant's life cycle and regulate cell and tissue growth [17]. The interaction between bacterial auxins and the plant root can increase root surface area and improve nutrient and water uptake, positively affecting plant growth [18,19].

One technique for delivering microorganisms and their compounds is encapsulation, a technology that involves enclosing them in small, sealed capsules. These capsules can be nano (<1.0 µm) or micrometric (1.0–1000 µm) in size and can be made from various materials to form the capsule membrane [20]. These capsules can prolong the release of nutrients from the core. The encapsulated nutrient is released once it dissolves or breaks down [21].

The current use of bio-based materials, including gums, proteins, lipids, and polysaccharides such as alginate and chitosan, for the formulation of nano- and microparticles (NMP) offers an option to improve the efficacy of phytochemical compounds [22]. Chitosan is the derivative of chitin obtained by N-deacetylation. This compound is widely used in the food and biotechnology industries to encapsulate active ingredients, immobilize enzymes, act as a carrier for controlled drug delivery, and, in agriculture, as a plant growth promoter due to its antimicrobial properties [23].

The literature describes various methods for synthesizing chitosan NMP, but ionic gelation is widely preferred. This simple technique relies on interactions between chitosan cationic chains and negatively charged polyanions, with sodium tripolyphosphate (TPP) being the most used polyanion [24].

The use of chitosan-based NMP has the potential to prevent the degradation of the compounds and protect the active agents during field applications. In addition, due to

the properties of biopolymers, the release of active ingredients can be regulated, allowing sustained levels of the active agent over an extended period, lower doses, and reduced evaporative loss [22]. Moreover, they may regulate the availability of agrochemicals for sustenance and crop production [25].

This study presents an innovative approach to enhancing the stability and efficacy of natural bioactive compounds by developing chitosan–TPP nano/microparticles encapsulating a *Bacillus subtilis* extract. This delivery system provides controlled release and preserves microbial metabolite activity more effectively than conventional agricultural inputs. This work aimed to develop and characterize the encapsulated formulation and to evaluate its capacity to improve tomato seed germination. This work contributes to sustainable agriculture by demonstrating that nano-encapsulation significantly enhances the functional performance of microbial extracts, underscoring their potential as a sustainable and eco-friendly alternative to synthetic agrochemicals.

2. Results

2.1. Bacterial Extract Production and Auxin Quantification

The *Bacillus subtilis* strain is known to produce bioactive compounds like auxins under submerged fermentation. These molecules participate in the plant life cycle, are involved in plant growth and development, and have the main functions of cell elongation, regulation of cell membrane permeability, and crop growth [16]. The effects of this type of hormone depend on concentration: at low auxin concentrations, the elongation of hypocotyls, stems, and roots is induced, leading to growth; at high concentrations, cell elongation is inhibited [26].

After the fermentation process for auxins production with *B. subtilis*, extracts were centrifuged and filtered to obtain a cell-free solution for auxin quantification, yielding a concentration of 29.4 ± 4.2 mg/L. This filtered extract was later used in the NMP formulation.

2.2. Production and Characterization of Nano-Microparticles (NMP)

The particle size of the NMP was evaluated using a Box–Hunter–Hunter experimental design to observe the effect of the factors (homogenization, ultrasound, and amplitude) on the size of the NMP. The results of the four treatments are presented in Table 1, where particle sizes ranging from 384 to 462 nm were achieved under this design.

Table 1. Characterization of NMP under the Box–Hunter–Hunter experimental design.

Treatment	Homogenization (min)	Ultrasound (min)	Amplitude (%)	Results		
				Particle Size (nm)	Polydispersion Index	Zeta Potential (mV)
1	3	3	70	462.9 ± 8.8^a	0.46 ± 0^{ab}	27.2 ± 1.9^a
2	5	3	60	450.9 ± 8.1^a	0.55 ± 0^b	29 ± 0.3^a
3	3	5	60	389.9 ± 7.2^b	0.44 ± 0^{ab}	31.1 ± 2.6^a
4	5	5	70	384.5 ± 5.5^b	0.39 ± 0^a	28.8 ± 0.4^a

Means followed by the same letter show no significant difference according to Tukey's test at 0.05.

A Pareto diagram (Figure 1) shows that both ultrasound and homogenization times have a significant effect on particle size at high values. Therefore, a Box–Behnken design was performed to reduce particle size by adjusting the levels of the significant factors. Nine treatments were obtained, as shown in Table 2, along with the corresponding results. The particle size of all treatments was reduced compared to the results obtained from the first experimental design, yielding values between 319 and 365 nm due to the increased

homogenization and ultrasound times, which enabled the mixture's ingredients to interact more efficiently.

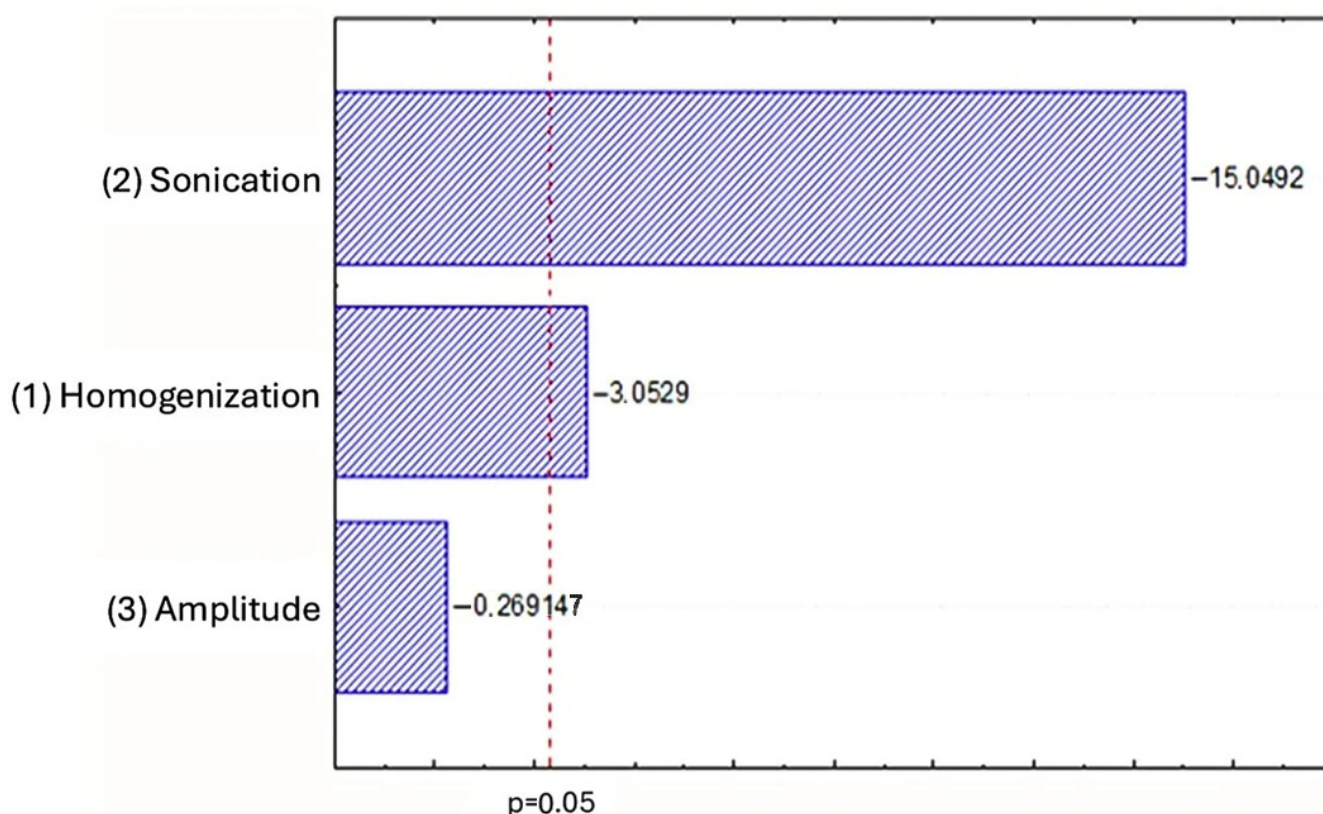


Figure 1. Pareto chart of the effect of factors on different parameters evaluated under a Box–Hunter–Hunter experimental design.

Table 2. Characterization of NMP under a Box–Behnken experimental design.

Treatment	Homogenization (min)	Ultrasound (min)	Results			
			Particle Size (nm)	Polydispersion Index	Zeta Potential (mV)	Encapsulation Efficiency (%)
1	7	8	319.8 ± 17.3 ^a	0.25 ± 0.0 ^a	34.5 ± 0.4 ^{cd}	50 ± 0.0 ^d
2	8	7	326 ± 10 ^a	0.25 ± 0.0 ^{ab}	33.5 ± 0.2 ^{ab}	28.1 ± 0.0 ^b
3	6	6	326.1 ± 28.3 ^a	0.27 ± 0.0 ^d	34.9 ± 0.4 ^d	14.1 ± 0.0 ^a
4	6	7	326.5 ± 4.1 ^a	0.27 ± 0.0 ^{cd}	34.5 ± 0.7 ^{cd}	46.9 ± 0.0 ^d
5	8	8	330.7 ± 8.3 ^{ab}	0.25 ± 0.0 ^{abc}	34.3 ± 0.7 ^{bcd}	68.8 ± 0.0 ^e
6	7	6	365.5 ± 7.1 ^d	0.27 ± 0.0 ^{cd}	33.3 ± 1.4 ^a	43.8 ± 0.0 ^{cd}
7	8	6	349.4 ± 13.3 ^{cd}	0.26 ± 0.0 ^{bcd}	33.8 ± 0.4 ^{abc}	39.1 ± 0.0 ^{bcd}
8	6	8	322.1 ± 17.4 ^a	0.25 ± 0.0 ^a	34 ± 0.4 ^{abc}	34.4 ± 0.0 ^{bc}
9	7	7	347.9 ± 7.3 ^{bc}	0.27 ± 0.0 ^{cd}	33.8 ± 0.8 ^{abc}	46.9 ± 0.0 ^d

Means following the same letter do not show a significant difference according to the Tukey test at 0.05.

With the increase in homogenization and ultrasound times, an improvement in the polydispersion index (PI) and zeta potential of the nine treatments was also observed; these variables are important since PI is a parameter used to define the degree of heterogeneity of a particle size distribution for a colloidal system, where a perfectly uniform sample tends to have a PI of zero [27]. The use of zeta potential predicts suspension stability, which describes the surface charge of nanoparticles; high values of positive or negative

zeta potential indicate adequate electrical repulsion between suspended nanoparticles, preventing their agglomeration [28].

This property can be observed by Scanning Electron Microscopy (SEM), where an agglomeration of larger particles attached to the matrix material (highlighted with red arrows) is visible in the NMP obtained from treatment 1 of the Box–Hunter–Hunter experimental design (Figure 2a). Figure 2b shows smaller, better-dispersed NMP enclosed in red circles, resulting from treatment 5 of the second experimental design (Box–Behnken). These results could be the result of the increase in ultrasound and homogenization times, since in the case of PI, the values decreased in all treatments from 0.55 of the first experimental design to 0.25 with the Box–Behnken design, which means a more homogeneous particle size in the system, and for the zeta potential, there was an increase from 27.7 to 34.9 mV, which prevents NMP from agglomerating due to this increase in surface charge.

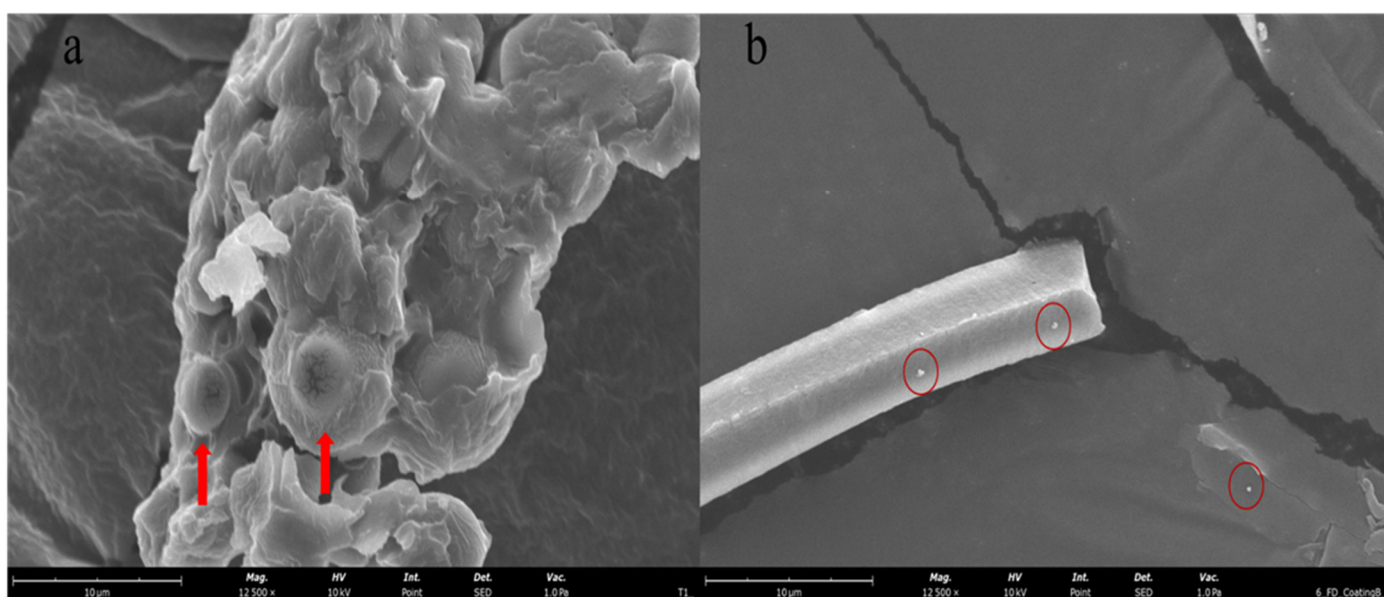


Figure 2. Images obtained by SEM of (a) treatment one under the Box–Hunter–Hunter experimental design and (b) treatment 5 under the Box–Behnken experimental design under the same magnification.

The encapsulation efficiency of NMP was also measured in the second experimental design, yielding a minimum value of 14.1% with treatment 3, which has the lowest values of homogenization and ultrasound times, and a maximum value of 68.8% in treatment 5, which, unlike treatment 3, has the highest values of the factors.

To observe the interaction between the components of the NMP, Fourier transform infrared spectroscopy (FTIR) spectra were obtained from chitosan, chitosan NMP unloaded, and loaded with indole-3-acetic acid (IAA) standard, as shown in treatment 5 (Figure 3). This treatment showed the highest encapsulation efficiency. In the spectra corresponding to chitosan, characteristic bands of this compound, according to the literature, are observed in the region of 3436 cm^{-1} , as well as the overlapped stretching bands of the groups NH_2 and OH , and the stretching of the CH from the methylene group in the region of 2876 cm^{-1} . In the regions between 1660 cm^{-1} and 1598 cm^{-1} , the absorption and stretching of the amide $\text{C}=\text{O}$ and $\text{C}=\text{N}$ groups, the formation of the CH_3 group at 1372 cm^{-1} , and the vibration of $\text{C}=\text{O}$ at 1075 cm^{-1} are observed [29].

In spectra b corresponding to uncharged NMP, there is a shift in chitosan's amino group band from 3286 cm^{-1} to 3250 cm^{-1} , which, in the literature, is mentioned to correspond to the interaction between the NH_3 group of chitosan and TPP. A change is also observed in the regions at 1542 cm^{-1} and 1404 cm^{-1} , which correspond to the interaction of the

anionic phosphate groups of the TPP with the cationic NH_3 group of the chitosan and to the stretching of the phosphate groups in the 1018 cm^{-1} region [30]. Finally, spectrum c, corresponding to the NMP loaded with IAA, shows a shift towards 3215 cm^{-1} , which, according to the literature, could correspond to the C-H stretching vibration group of IAA and the increased intensity of the band at 1650 cm^{-1} due to stretching of the C-O union from the IAA carboxyl group [29], which, according to the literature, demonstrates the interaction between nano-microparticle ingredients and the correct encapsulation of IAA.

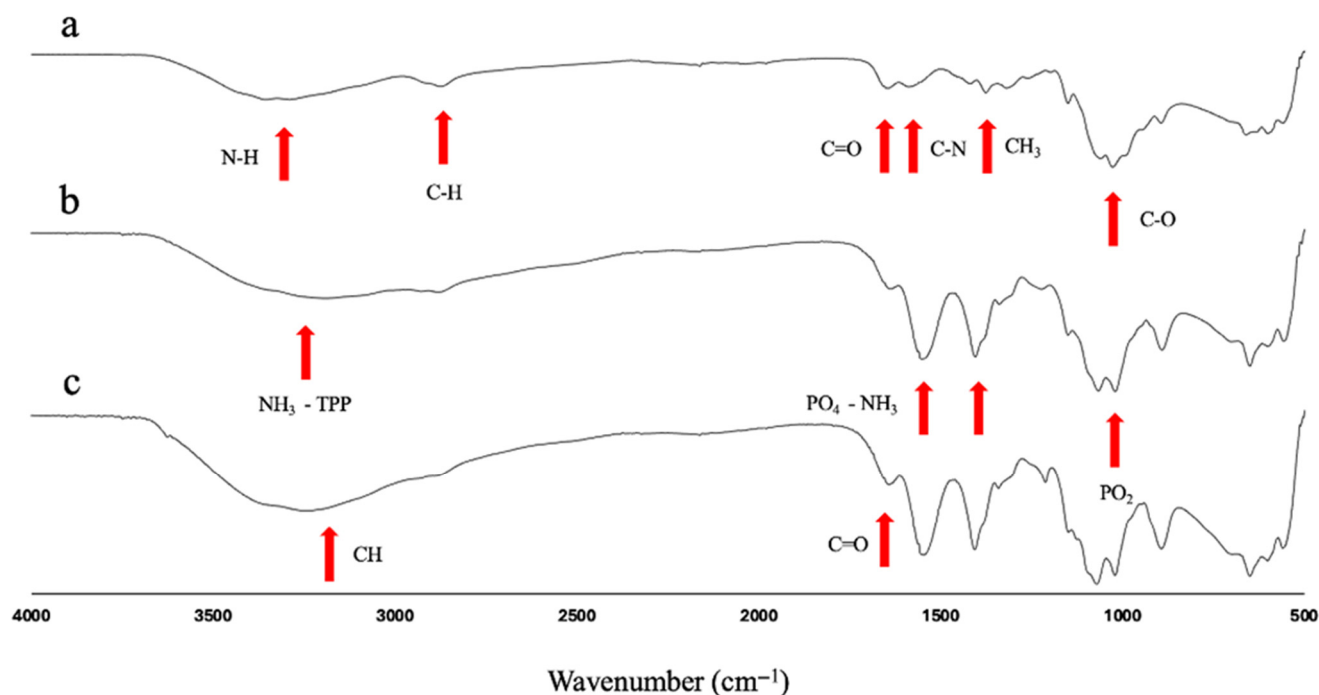


Figure 3. FTIR spectra and characteristic bands highlighted with red arrows of (a) chitosan, (b) treatment 5 (chitosan/TPP), and (c) treatment 5 (chitosan/TPP/IAA).

2.3. Germination Assays on Tomato Seeds

Once NMP treatment 5 was selected, it was used to evaluate its functionality in trials with tomato seeds, involving a total of seven treatments (Table 3). Various germination parameters were measured, and daily data were collected during the 11-day trial.

Table 3. Germination parameters of different treatments in tomato seeds.

Treatment	Germination Percentage (%)	Germination Rate (Seeds/Day)	Mean Germination Time (Days)
Control	60 ± 1.6^a	1.4 ± 0.1^{ab}	4.4 ± 0.4^c
IAA	69 ± 0.5^{bc}	1.6 ± 0^{bc}	3.8 ± 0.8^{ab}
Bacterial extract	58 ± 0.6^a	1.3 ± 0.1^a	4.3 ± 0.9^{bc}
NMP	61 ± 1^{ab}	1.4 ± 0.1^{ab}	3.6 ± 0.2^a
NMPE 25%	66 ± 3^{abc}	1.5 ± 0.3^{abc}	3.6 ± 0.9^a
NMPE 50%	72 ± 2.6^c	1.6 ± 0.2^c	3.8 ± 0.1^{ab}
NMPE 100%	66 ± 2.3^{abc}	1.5 ± 0.1^{abc}	3.8 ± 0.4^{ab}

Mean values followed by the same letter do not show a significant difference according to the LSD Fisher test at 0.1. Indole-3-acetic acid (IAA), nano-microparticles (NMP), nano-microparticles with extract (NMPE).

One of the parameters measured was germination percentage, in which the 50% NMPE treatment showed the highest (72%), with significant differences from the control (60%) and the bacterial extract (58%). The IAA treatment achieved the second-best result,

with a germination percentage of 69%, higher than the bacterial extract (58%) and the control (60%).

The IAA treatment yielded a GR value of 1.6 seeds/day and an MGT of 3.8 days, indicating better results compared to the control treatment (1.4 seeds/day and 4.4 days, respectively). These results are statistically equal to those obtained with NMP treatments with and without loading (Table 4).

Table 4. Experimental matrix of the Box–Hunter–Hunter and Box–Behnken designs to produce nano/microparticles with free cell *B. subtilis* extracts.

Factors	Levels	
	Box–Hunter–Hunter	Box–Behnken
Homogenization (min)	3, 5	6, 7, 8
Ultrasound (min)	3, 5	6, 7, 8
Amplitude (%)	60, 70	70

3. Discussion

During fermentation, temperature and pH can affect enzymatic activity in the auxin biosynthetic pathway. Suliashi and Widawati [31] mention that with a strain of *Bacillus siamensis*, when the temperature increased, they observed an increase in the concentration of total auxins, reaching a concentration of 9 µg/mL at a temperature of 35 °C. Kumari et al. [32], working with a strain of *B. subtilis*, obtained an auxin concentration of 141.92 µg/mL, also using a temperature of 35 °C, highlighting the importance of temperature in the production of indole molecules. Yousef [33], working with a strain of the genus *Bacillus*, obtained the best result at pH 8 (41.19 mg/L). do Prado et al. [34] report that strains of this genus produce higher levels of metabolites at neutral and alkaline pH.

In the case of tryptophan, the literature reports that auxin production increases linearly with tryptophan concentration [35]. Ham et al. [36] observed this behavior with a strain of the genus *Pseudarthrobacter*: as the concentration of tryptophan increased, auxin production also increased.

The literature indicates that nanoparticles must have dimensions between 1 and 100 nm [23]. However, particles with a size less than 1000 nm can still be classified as nanoparticles based on their physicochemical properties. Smaller sizes are sought, as studies have reported that smaller particles are more easily assimilated into plant tissues [37]. Therefore, it is important to decrease the size of the NMP as a function of the independent variables and to determine which factors have a more significant influence on the response variable.

Methods such as homogenization and ultrasound are widely used for nanoparticle synthesis [23]. Ultrasound causes the breakdown and dispersion of smaller particles. Prolonged times allow for greater interaction between the solution and cavitation forces, thereby decreasing particle size [38]. Regarding homogenization, Bhattacharya [39] reports that longer homogenization times result in a greater balance between vortex and shear forces, leading to a smaller particle size. Therefore, it was decided to conduct another experimental design, increasing the homogenization and ultrasound times while keeping the amplitude constant, as this factor does not significantly affect the particle size, and using the highest value (70%).

Although the effect of both factors was significant, ultrasound time was the most important factor; this could be due to the cavitation process, which includes the formation, growth, and implosive collapse of microbubbles in the medium, which creates hot spots that cause a reduction in the size of the droplets in the emulsion. Ruiz et al. [38] mention that the increase in sonication time allows a longer duration of the interaction of the emulsi-

fied medium with the cavitation force. Yanat and Schroën [24] demonstrated the effects of ultrasonication on chitosan nanoparticles as a function of duration and amplitude. They reported that particle size was reduced with an increase in duration and amplitude of applied ultrasound, and Garrido-Maestu et al. [27], with chitosan nanoparticles, obtained smaller particle sizes (189.4 nm), increasing ultrasound time to 40 min compared to treatments at 20 min (241.8 nm) and without ultrasound (838.6 nm). Additionally, Auwal et al. [40], using chitosan/TPP nanoparticles and a longer homogenization time (30 min), reported a particle size of 162 nm. In a study on lignin, and Matsakas et al. [28] reported that the homogenization process had a positive effect, reducing nanoparticle size.

These results align with the findings reported in the literature. Auwal et al. [40] reported that, with an increase in homogenization time, an increase in encapsulation efficiency is achieved, accompanied by a decrease in particle size. However, the authors note that prolonged homogenization time is associated with leakage of the bioactive compound and a decrease in encapsulation efficiency, as they used homogenization times of 20, 30, and 40 min. Using peptide-loaded chitosan and TPP nanoparticles, the authors report an encapsulation efficiency of 75% with a homogenization time of 30 min. Moreover, Bhattacharya [39] reported an encapsulation efficiency of 68.6% using the ionic gelation technique with drug-loaded chitosan nanoparticles. The authors note that various factors, such as increased speed and homogenization time, surfactant concentration, and polymer concentration, can increase encapsulation efficiency by enabling optimal interactions among the ingredients. Pereira et al. [41] mention that in chitosan and TPP nanoparticles, the encapsulation of the active ingredient can occur due to interaction with the free amino groups of chitosan. Considering the results obtained, treatment 5 was selected for subsequent trials, as it presented the best particle size values (330.7 nm), polydispersity index (PI) (0.25), zeta potential (34.3 mV), and the highest encapsulation efficiency (68.8%) among the nine treatments.

The results of the germination assay could be explained by the protective effect of the coating on the extract in the treatments with charged NMP, improving the physical and chemical stability of the auxins present in the extract [42], thereby protecting them from external factors that could cause degradation, such as photolysis or sorption. Therefore, the active agent increases its bioavailability and efficacy for the plant, allowing lower applied concentrations to achieve the same effect as free active agents. In addition, microparticle and nanoparticle systems can provide extended-release profiles, resulting in a longer duration of biological action [41,43].

Priming with IAA has already been reported to promote germination in cottonseed by regulating endogenous phytohormone metabolism, increasing the endogenous levels of IAA and gibberellic acid (GA), and inhibiting abscisic acid (ABA) synthesis during the germination period [44]. In the same work, the authors report that priming with IAA significantly enhanced seed germination, as evidenced by higher germination rates and faster germination. This process can accelerate seed germination by regulating endogenous hormones and sucrose metabolism.

In the literature, Li et al. [45] mention that chitosan can accelerate the germination rate of corn seeds. In addition, chitosan has been reported to be widely used in the agricultural sector; it is recognized for improving crop growth and yield by stimulating plant growth through antibacterial and antifungal activity, enhancing nutrient uptake, and promoting seed germination [46,47]. Li et al. [45] reported that chitosan nanoparticles positively affect seed germination and wheat seedling growth. However, the effect or mechanism of chitosan nanoparticles on seed germination has not been reported in the literature.

4. Materials and Methods

4.1. Reagents

The following reagents were used in the assays performed in this study: glacial acetic acid (J.T. Baker, Madrid, Spain), NaOH, low-molecular-weight chitosan, sodium tripolyphosphate (TPP), and Tween 40 (Sigma-Aldrich, Darmstadt, Germany). Standard solutions were prepared using 98% pure, reactive-grade indole-3-acetic acid (IAA) (Sigma-Aldrich).

4.2. Plant Material

The seeds used were DELGARDEN commercial tomato seeds of the Roma/Saladette variety (Sonae MC, Porto, Portugal), purchased at a local store in Porto, Portugal.

4.3. Bacterial Strain

The local isolated strain of *Bacillus subtilis* belongs to the microorganism collection of the Nanobioscience group at the Universidad Autónoma de Coahuila, in Saltillo, México.

4.4. Bacterial Extract Production

Fermentation with the bacterium *Bacillus subtilis* was carried out in a modified LB culture medium supplemented with tryptophan to produce auxins. The LB medium was prepared as follows: dextrose 10 g/L, yeast extract 7.5 g/L, NaCl 10 g/L, and tryptophan 3 g/L. A pre-inoculum was performed in 50 mL of modified LB medium lacking tryptophan, incubated for 18 h at 28 °C. From the pre-inoculum, fermentation was carried out at a concentration of 1×10^7 cel/mL in the LB medium mentioned above in flasks with a volume of 20% of culture medium, leaving the remaining volume of the flask for oxygen supplementation, and stirred for 8 h, at 120 revolutions per minute (rpm) in a rotary agitator at 38 °C. The fermentation broths were centrifuged at 15,000 rpm for 15 min, filtered with 0.1 µm syringe filters to remove cells, and a fraction of the filtered extract was diluted 1:10 to obtain the supernatant for auxin quantification.

4.5. Auxin Quantification

Auxin quantification was performed according to the methodology described by Anguiano-Cabello et al. [48]. The Salkowski reagent was prepared as follows: 15 mL of H₂SO₄, 25 mL of distilled water, and 0.75 mL of FeCl₃·6H₂O (0.5 M). An IAA calibration curve was prepared from a solution at 2000 parts per million (ppm) in ethanol. To measure total auxin concentration in the samples, Salkowski reagent was added, incubated for 30 min in the dark, and read at 520 nm in a Cintra 202 spectrophotometer (GBC Scientific Equipment, Knox, Australia). The curve was performed in triplicate. This method quantifies the total indolic compounds (auxins) present in the sample rather than a specific individual compound. The concentration of total auxins in the samples was calculated using the calibration curve with the highest R² value.

4.6. Production and Characterization of NMP Using the Iontropic Gelation Method

The production of NMP was carried out through the ionotropic gelation method proposed by Machado et al. [49]: 2 mL of a 2% low molecular weight chitosan solution, which was prepared with a 1% glacial acetic acid solution in water; the 2% chitosan solution was adjusted to a pH of 5 with 2 M NaOH. This solution was mixed with 3 mL of ultrapure water for 5 min. Subsequently, 1 mL of cell-free bacterial extract of *Bacillus subtilis* was added to the mixture at a concentration of 11.6 mg auxins/L to obtain a final extract concentration of 1.1 mg/L, and the mixture was stirred for an additional 10 min. After 10 min, 1 mL of a 1% (*w/v*) sodium tripolyphosphate (TPP) solution was added to water, and the mixture was mixed using an ULTRA-TURRAX homogenizer (IKA, Staufen,

Germany) and then finished using a Vibra-cell ultrasound equipment (Artisan, Champaign, IL, USA).

Different experimental designs were employed to establish optimal conditions: first, a Box–Hunter–Hunter design was used for preliminary screening of influential factors, and subsequently, a Box–Behnken design was applied to optimize homogenization and ultrasound parameters, as shown in Table 4.

4.7. Characterization of Nano and Microparticles

4.7.1. Size, Zeta Potential, and Polydispersity Index

The nano-microparticle suspensions were analyzed concerning their physical properties by dynamic light scattering (DLS) using a Nano Zetasizer Pro (Malvern Instruments, Malvern, UK). The measured parameters were particle size, polydispersity index (PI), and zeta potential. All assays were performed using a disposable folded capillary cell (Malvern Instruments, Malvern, UK), with a 90° laser angle and at room temperature (25 °C). All assays were performed in quadruplicate.

4.7.2. pH Determination

pH levels were measured with an Ion Meter 450 potentiometer (Corning Incorporated, Corning, NY, USA) to assess the degradation of the formulation components. The analysis was performed in triplicate at 25 °C.

4.7.3. Nano-Microparticle Morphology

The morphology of NMP was observed by SEM. Samples were placed on aluminum pins with double-sided adhesive carbon tape (NEM tape; Nisshin, Tokyo, Japan) and analyzed using a Phenom XL G2 equipment (Thermo Fischer Scientific–FEI, Eindhoven, Netherlands). Before SEM observation, the samples were freeze-dried and mounted on conductive adhesive tape, and a thin layer of Pt was spray-coated on the surface. SEM images were taken at an acceleration voltage of 3.0 kV, and a lens magnification between 500x and 12,500x, and observations were performed using the backscattered electron detector (BSD).

4.7.4. FTIR

A Fourier transform infrared spectrometer, Spectrum 100 (PerkinElmer, Waltham, MA, USA) was used to characterize the nano chitosan and microparticles. Among them, chitosan was compressed into slices after mixing it with KBr. The TDI was tested immediately after being brushed onto KBr tablets. The microparticles mixed with KBr were ground and compressed into slices, which were then immediately analyzed by FTIR. With a resolution of 4 cm^{−1}, the FTIR spectra were recorded within the 400–4000 cm^{−1} range in absorption mode.

4.7.5. Encapsulation Efficiency

The encapsulation efficiency was determined using a Cintra 202 spectrophotometer, as described by Taban et al. [50]. The absorbance of a standard IAA solution at 25 mg/L was measured, serving as the active ingredient in the NMP. Then, the charged NMP were centrifuged, and the supernatant absorbance was measured. The encapsulation efficiency (EE) was calculated using the following formula (1):

$$EE = \left(\frac{T_0 - S_0}{T_0} \right) \times 100. \quad (1)$$

where T₀ is the absorbance of the IAA solution, and S₀ represents the supernatant absorbance of the charged NMCs.

4.8. Germination Assays

Before use, tomato seeds were washed with distilled water under agitation for 5 min, then suspended in a 0.1% Tween 40 solution (*w/v*) for 40 min to remove fungicides. Afterward, seeds were strained, rinsed with distilled water, and placed in a 1% chlorine solution for 30 s. They were then washed again with distilled water and left to dry at room temperature for 1 day. For the germination assays, different treatments were evaluated in seeds: a control treatment (water only) and treatments with IAA standard (1.1 mg/L), bacterial extract (1.1 mg/L), NMP without extract, and NMP loaded with bacterial extract (NMPE) at different percentages (25, 50, and 100%). Treatments were applied by immersing the seeds in each solution (2:100 *v/v*) for three hours under constant agitation.

The germination trial was carried out according to Tucuch-Pérez et al. [22], with some modifications; 25 seeds were placed in germination pots, with four replicate pots per treatment for a total of 100 seeds. The pots were filled with a planting substrate (COMPO SANA, Münster, Germany) in a Fitoclima 5000 EH climate chamber (Aralab, Rio de Mouro, Portugal) at the Universidade Católica Portuguesa in the city of Porto. The pots were watered daily for 11 days. The trial was conducted in June 2024 at 22 °C, with a relative humidity of 65% and a 16:8 h light:dark cycle, with a light intensity of 200 µmol/s/m². Different parameters were measured according to Mzibra et al. [51], including germination percentage (%), which was measured following Formula (2):

$$\text{Germination percentage} = (\text{number of germinated seeds} / \text{total number of seeds}) * 100 \quad (2)$$

The germination rate (seeds/day) was also calculated according to formula (3):

$$\text{GR} = \Sigma(\text{Gt}/\text{Dt}) \quad (3)$$

where Gt is the number of newly germinated seeds on day t, and Dt is the number of days of assay

Moreover, finally, the mean germination time (days) was calculated following formula (4):

$$\text{MGT} = \Sigma(\text{D} * \text{n}) / \Sigma \text{n} \quad (4)$$

where n is the number of newly germinated seeds on day D, and D is the number of days counted from the beginning to the final day of trial.

4.9. Data Analysis

Data analysis was performed using STATISTICA 7.0, and Tukey's method was used to determine significant differences between treatments at the 95% confidence level.

5. Conclusions

In this study, the NMP system containing the *Bacillus subtilis* extract showed superior performance across all evaluated parameters compared with the control and the free extract treatments. Although the 50% NMPE treatment produced the highest values, it did not differ significantly from the 25% and 100% treatments in most parameters. However, both germination percentage and germination rate were significantly higher than those of the control, free extract, and unloaded NMP treatments. These results indicate that the extract promotes germination at low concentrations and that the observed activity is not attributable solely to chitosan, consistent with the known physiological role of indolic phytohormones such as auxins during early plant development. Overall, this work demonstrates that nano/microparticle encapsulation enhances the functional effectiveness of microbial bioactive compounds, suggesting that this technology could

be applied in agriculture to improve seed vigor, increase germination uniformity, and support the development of sustainable seed-treatment formulations as alternatives to conventional agrochemicals.

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Abbreviations

The following abbreviations are used in this manuscript:

TPP	Sodium tripolyphosphate
NMP	Nano-microparticles
DLS	Dynamic light scattering
SEM	Scanning Electron Microscopy
FTIR	Fourier transform infrared spectroscopy
rpm	Revolutions per minute
EE	Encapsulation efficiency
IAA	Indole-3-acetic acid
GR	Germination rate
MGT	Mean germination time
PI	Polydispersion index
NMPE	Nano-microparticles with extract

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