Chitosan beads with neem and encapsulated entomopathogenic fungal spores for pest control

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ABSTRACT

Objective: To synthesize chitosan beads with neem and encapsulated entomopathogenic fungal spores for pest control through their controlled release.

Methodology: Beads were synthetized by means of chitosan suspensions with each of the spores of the entomopathogenic fungi (*B. bassiana*, *M. robertsii*, and *I. javanica*), and the neem extract (Azadirachtin extract in water and in oil), which were crosslinked with sodium tripolyphosphate. The synthesized beads were characterized through the determination of their average diameter, moisture percentage, and morphology, using scanning electron microscopy. Subsequently, the swelling kinetics of each bead were carried out and the first and second order kinetic models were adjusted.

Results: The synthesized beads had stable structures and homogeneous diameters, with a >90% moisture content in all cases. The morphological analysis revealed that the internal structure of the beads has cavernous networks with homogeneously distributed pores. Finally, the swelling kinetics of the beads showed a better adjustment to the second order model.

Study Limitations: The study was limited to three entomopathogenic fungi and two neem extracts.

Conclusions: The neem and the entomopathogenic fungal spores were encapsulated in the chitosan beads, which will allow the controlled release of both the neem and the spores due to their porous structure and swelling capacity.

Keywords: chitosan, neem, *B. bassiana*, *M. robertsii*, *I. javanica*.

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INTRODUCTION

Currently, more than 600 agrochemicals are used to control various organisms that damage crops all over the world. These pesticides benefit agricultural production, but small amounts of this substances can be accumulated in food, water, air, and soil, causing significant exposure in humans and therefore a health risk (Rodríguez Aguilar *et al.*, 2019; Galindo-Reyes & Alegria, 2018; Gyawali, 2018; Ghorab & Khalil, 2016). Health and environmental

risks are not easy to explain, because they not only depend on the type of pesticide, but also involve factors such as exposure period, toxicity, persistence, and the environmental characteristics of affected areas; furthermore, not all the population is fully exposed to the pesticide (Kim *et al.*, 2017; Aktar *et al.*, 2009).

Studies on the consumption of pesticides worldwide, carried out from 1990 to 2018, revealed that the amount consumed increased from 2.3 to 4.1 million metric tons during this period (76%). In addition, the demand for pesticides in 2018 was concentrated in 11 countries (78% of total consumption): China is the largest consumer (43%), while Mexico participated with only 1.3% (Centro de Estudios para el Desarrollo Rural Sustentable y la Soberanía Alimentaria [CEDRSSA], 2020). The increase in the use of pesticides in Mexico is linked to the problems that pests cause in crops and their control. The agricultural sector uses 85% of these products, which increases the exposure of farmers and the probability of poisoning (Martínez Valenzuela *et al.*, 2019). The excessive use of highly dangerous pesticides is linked to negative effects on the health of farmers and their families, particularly the children. Pesticides can cause hematological, behavioral, and hormonal changes, as well as genetic, cellular, reproductive, and neuronal damages; additionally, they impact agricultural areas and ecosystems (Quijada & Gómez, 2019; García Hernández *et al.*, 2018).

Given the negative effects of pesticides on the population, it has been currently proposed to replace them with new eco-friendly alternatives —for example, biopesticides derived from materials produced by animals, plants, microorganisms, or minerals (Kumar *et al.*, 2021; Acuña Jiménez *et al.*, 2015; Nava-Pérez *et al.*, 2012). These biopesticides have allowed the rational management and use of natural resources, preserved the biodiversity of ecosystems, lowered costs, and reduced health risk among the population. They can be extracted from plants (azadirachtin extract) or pathogenic fungi (*Beauveria*, *Metarrhizium* and *Isaria* genera) with relevance for the biological pest control (Zelaya-Molina *et al.*, 2022; Figueroa Gualteros *et al.*, 2019).

Azadirachtin extract is obtained from the neem tree (*Azadirachta indica* A. Juss.). This extract is a tetranortriterpenoid used as a natural insecticide, because it impacts more than 400 pest insects (by reducing their feeding, survival, viability of the nymphs, and progeny). In addition, azadirachtin extract is compatible with natural enemies and beneficial microorganisms, without serious impact on the environment (Kilani-Morakchi *et al.*, 2021; Aguilar-Acosta *et al.*, 2020; Esparza-Díaz *et al.*, 2010; Zada *et al.*, 2018; Muñiz-Reyes *et al.*, 2016). Entomopathogenic fungi are also considered major biopesticides. Their spores come into contact and germinate on the cuticle of the insect. Subsequently, they grow inside the host, producing toxins and feeding on the host, which loses mobility and appetite and dies after 7 to 10 days (Litwin *et al.*, 2020; Mantzoukas & Eliopoulos, 2020; Pacheco Hernández *et al.*, 2019; Mora *et al.*, 2017). Entomopathogenic fungi are grouped into 115 genera, among which *Beauveria*, *Metarrhizium*, *Isaria*, *Nomuraea*, and *Hirsutela* are considered parasites capable of infecting pest insects in all the development stages of their biological cycle (Estrada Martínez, 2019; Kovač *et al.*, 2021).

Agricultural producers have sought new alternatives to reduce the excessive spraying of pesticides on crops and, consequently, any type of health or environmental risk.

Therefore, some studies are aimed at the use of natural polymeric matrices (*e.g.*, corn starch, cellulose, alginate, wheat bran, and chitosan), in order to microencapsulate pesticides for their controlled release in agricultural crops (Campos *et al.*, 2015; Korbecka-Glinka *et al.*, 2022; Rosas-García & Luna-Santillana, 2006). Chitin and its by-products are obtained from the exoskeleton of crustaceans and they are used in various areas of the food, medicine, agriculture, cosmetics, and pharmacy industries (Pellis *et al.*, 2022; Triunfo *et al.*, 2021; Triunfo *et al.*, 2022; Hernández Cocoletzi *et al.*, 2009). Chitosan (Ch) is a biodegradable, biocompatible, polycationic, and non-toxic biopolymer derived from chitin, which does not produce a response from the immune system. This molecule has several applications in the food, pharmaceutical, chemical, and agricultural industries. In the last one, chitosan has been used for the dosed release of various agrochemicals or pesticides, through the production of films, beads, microencapsulates, or hydrogels (Rossainz-Castro *et al.*, 2018; Iber *et al.*, 2022; Valderrama Negrón *et al.*, 2017).

This work is focused on the synthesis and characterization of chitosan beads with neem (azadirachtin extract in water and in oil) and encapsulated spores of entomopathogenic fungal strains (*Beauveria*, *Metarrhizium* and *Isaria*) as an alternative pest control. The synthesis of the Ch-neem-spore beads was carried out by adding spores (produced through solid fermentation of the strains) and commercial neem (Azadirachtin extract in water (Aw) and Azadirachtin extract in oil (Ao)) into the chitosan matrix. Once the beads were synthesized, their diameter, moisture percentage, morphology, and swelling kinetics were determined.

MATERIALS AND METHODS

High-density industrial grade, America Alimentos® chitosan (degree of acetylation 84.54% and molecular weight of 82.683 g/mol) was used. The reagent grade substances were sodium tripolyphosphate (Sigma), acetic acid (J.T. Baker), Potato Dextrose Agar (PDA) culture medium (MCDLAB), and Tween 80 sterile solution (Sigma). The neem, azadirachtin extract in water (PHC NEEEM), and azadirachtin extract in oil (Azanim, Biokrone) were commercial products. The solutions were prepared with deionized water. The entomopathogenic fungal strains used were *Beauveria bassiana* 885.2, *Metarrhizium robertsii* Xoch 8.1, and *Isaria javanica* CHE-CNRCB303. The glassware was washed with running water and rinsed with deionized water before its use.

Solid fermentation of entomopathogenic fungi

The entomopathogenic fungi strains, *B. bassiana* (Bb), *M. robertsii* (Mr), and *I. javanica* (Ij), were reactivated and propagated in Petri dishes with PDA medium. Then, they were incubated for 16 days in the laboratory at 22±2 °C, 75±5% relative humidity, and photoperiods of 12 h light/12 h darkness (12:12, L:D). After the propagation of the strains, a suspension was made by cutting 1 cm^2 of PDA medium with each strain, in order to count the spores. Nine mL of 0.1% (v/v) Tween 80 were added in a test tube, which was stirred for 2 minutes in a Vortex until the elements were homogenized and the spores were detached from the substrate. Afterwards, serial dilutions (1:10, 1:100,

and 1:1000) were made (Rodríguez-Gámez *et al.*, 2017). During the spore counting tests, 0.05 mL aliquots were taken from each sample. The samples were placed in the Neubauer chamber for direct counting, where 13 random squares of the 25 chambers (integrated by the 16 squares) were counted (Marín Cervantes, 2006). The following formula was used to express the number of spores per milliliter of suspension:

$$N = nFD \quad (1)$$

Where: N=number of spores/mL of initial suspension; n=average number of spores counted in the Neubauer chamber; F=factor of the chamber used (25×10^4); D=dilution used for counting.

Rice (*Oriza sativa* L.) was used as a substrate for solid fermentation. First it was washed with running water for two minutes to remove foreign particles; subsequently, it was soaked for 15 minutes and was drained for 10 minutes. The rice was weighed (100 g wet weight) and placed in 500 g high-density polyethylene (HDPE) bags for sterilization in an autoclave at 121 °C (15 psi) for 15 minutes. Finally, the sterile material was cooled for 24 hours for its inoculation.

Prior to inoculation, 1 g of solid fermentation from each entomopathogenic fungal strain was diluted in 5 mL 0.1% (v/v) Tween 80 in a test tube and stirred vigorously. The subsequent inoculation was carried out using 5 mL sterile syringes to inject a spore suspension at a concentration greater than 1×10^8 spores/mL, striving to homogeneously spread the suspension in the substrate bag. Afterwards, the spores were incubated at 22±2 °C, exposing them to photoperiods of 12 h light/12 h darkness (12:12, L:D) during 16 days. The bags were shaken every third day to achieve a homogenous dispersion of the spores in the substrate. At the end of the inoculation, the spores were counted again using the Neubauer chamber (Rodríguez-Gámez *et al.*, 2017).

Inhibition test

The inhibition entomopathogenic fungi by neem extract was tested in a sterile PDA medium, to which different neem (azadirachtin extract in water or in oil) concentrations were added (0, 0.5, 1.0, 1.5, and 2.0 % (v /v)), with a standard volume of 20 mL for the culture medium. Subsequently, the medium was poured into Petri dishes and allowed to solidify. The culture media with different extract concentrations were inoculated in triplicate with the spores of each entomopathogenic fungi, using a platinum loop at three points in the Petri dish. They were incubated at 23 °C, during 20 days, with photoperiods of 12 h light/12 h darkness (12:12, L:D). Finally, the growth of each strain on the dishes was recorded (Castiglioni *et al.*, 2003; Hirose *et al.*, 2001).

Synthesis of Ch-neem-spores beads

The different chitosan-based beads were obtained by dripping a chitosan suspension through a nozzle onto a 2% (w/w) aqueous solution of sodium tripolyphosphate (TTP) under continuous stirring. The control bead (Ch-Control) was prepared with a 4% (w/v) Ch

solution in 1.5% (v/v) acetic acid in water (Sánchez-Duarte *et al.*, 2017). Two different neem extracts were encapsulated in chitosan: azadirachtin extract in water (Aw) and azadirachtin extract in oil (Ao). The Aw or Ao beads were prepared with a 1.5% (v/v) acetic acid in water solution and 1.5% (v/v) neem in water; subsequently, chitosan was added to generate a 4% (w/v) suspension. To obtain chitosan beads with spores of each strain (Bb, Mr, and Ij), spore suspensions with a concentration greater than 1×10^8 spores/mL in 0.1% (v/v) Tween 80 were prepared. Next, a 1.5% (v/v) acetic acid in water solution was prepared, to which the previously prepared spore suspension was added, along with the Ch needed to obtain a 4% (w/v) suspension. To obtain chitosan beads with spores of each strain (Bb, Mr, and Ij) and neem extract (Aw and Ao), a 1.5% (v/v) acetic acid in water solution and a 1.5% (v/v) neem in water solution were prepared. The spore suspension (Bb, Mr, and Ij) was added, followed by the chitosan, to obtain a 4% (w/v) suspension.

Bead characterization

Each of the different characterizations performed is described below.

Viscosity, diameter, and moisture percentage of the beads

Before each suspension was dripped to obtain the beads, the viscosity of 250 mL of each suspension was determined using a calibrated digital rotational viscometer (Cole-Parmer VCPL 150192). The viscosity was determined in triplicate at 22±2 °C (Costa *et al.*, 2015; Singh & Kumari, 2013).

In order to determine the diameter, excess water was removed from the wet beads with absorbent paper; they were then placed in a stereoscopic microscope and the diameter of each bead was measured with a digital vernier. The average diameter was obtained from the measurement of 50 beads (Sánchez-Duarte *et al.*, 2017).

The moisture percentage was determined from 2 g of wet beads, using an oven at 60 °C until constant weight was obtained (Rodríguez Hamamura *et al.*, 2010). The moisture percentage was determined with the following formula:

$$Moisture\ (\%) = \left(\frac{Wet\ bead\ weight - Dry\ bead\ weight}{Wet\ bead\ weight}\right)100 \qquad (2)$$

Morphology

The morphology of the different beads was determined in order to verify their structural, superficial, and internal characteristics (texture, roughness, and porosity). First, the beads were lyophilized and some of them were sectioned and covered with a gold bath at 30 mA for 45 s in a Denton Vacuum Desk V system. Finally, each material was observed in a JSM-6390LV scanning electron microscope (JEOL). The operating conditions were: high resolution, accelerating voltage of 20kV, and a high vacuum with bombardment of backscattered electrons on the solid structure of the surface of the beads (Nuñez-Reyes *et al.*, 2019).

Swelling kinetics

In order to determine their swelling kinetics, the lyophilized beads were weighed and placed in double distilled water at 22 °C. Each kinetic was carried out in triplicate. First, the beads were weighed at different times (0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, and 39 min), drying their surface with absorbent paper to remove excess water (Ortega, 2013; Kipcak *et al.*, 2014; Kowalski *et al.*, 2019; Ostrowska-Czubenko *et al.*, 2015). Then, the swelling at each of the different times was quantified using the following equation:

$$W = \left(\frac{Wet\ weight - Dry\ weight}{Dry\ weight} \right) \tag{3}$$

Each kinetic was adjusted to first and second order swelling kinetic models (Katime *et al.*, 2005). These kinetic models had the following variables:

W is the swelling (g of hydrogel/g of xerogel), t is the time (min), $W\infty$ is the swelling when equilibrium is reached at t equal to ∞, and k is the maximum swelling rate (min^{-1}). The first order model is based on the swelling kinetics of hydrogels. Assuming a prolonged time or greater shear stress, equilibrium can be reached and consequently a complete swelling can be achieved. The maximum swelling rate k is inverse to shear stress. Likewise, the relationship between the shear stress and the longitudinal osmotic modulus is close to 1. Therefore, the difference equation is integrated as follows:

First order:

$$\ln = \left(\frac{W\infty}{W\infty - W} \right) = kt \tag{4}$$

The second order model represents the interval from t equal to zero to an infinite time where the hydrogel reaches equilibrium and is completely swollen. $W\infty$ is when where a maximum swelling rate (k) is reached.

Second order:

$$W = \left(\frac{ktW\infty^2}{1 + ktW\infty} \right) \tag{5}$$

RESULTS AND DISCUSSION

Measurement of production of spores in the substrate

After the solid fermentation of rice, each strain produced spores. The coloration of the rice matches the spores produced by each entomopathogenic fungus. Figure 1 shows that *I. javanica* produced beige spores, *B. bassiana* white spores, and *M. robertsii* green spores.

Figure 1. Production of spores by solid fermentation: a) *I. javanica*, b) *B. bassiana*, and c) *M. robertsii*.

During the spore counting process in the mixture with Tween 80 solution, 1.76×10^8, 1.96×10^8, and 8.58×10^8 spores/mL were obtained for *B. bassiana*, *I. javanica*, and *M. robertsii*, respectively. This result show that the three strains met the desired number of spores ($>1\times10^6$ spores/mL), which matches the number of fungal spores required for germination and infection of pests and the subsequent effect on the insect growth (Acuña Jiménez *et al.*, 2015).

Inhibition test

Regarding the Aw extract, the test showed that the *B. bassiana* and *I. javanica* strains were inhibited by the 1.5 and 2% Aw extracts; meanwhile, the *M. robertsii* strain only showed inhibition with the 2% Aw extract. Regarding the Ao extract, the tests showed that none of the entomopathogenic fungal strains recorded an inhibition effect at the different concentrations (0, 0.5, 1.0, 1.5, and 2.0%).

Viscosity

Table 1 shows the viscosity of each precursor suspension of the different synthesized beads obtained with a 1 rpm constant speed. On the one hand, the viscosities used to

Table 1. Viscosities of the precursor suspensions.

Sample	μ(cP)	Torque (%)
Ch-Control	1780±0.020	29.7
Ch-Aw	1787±0.035	29.8
Ch-Ao	1793±0.027	28.5
Ch-Bb	1494±0.029	24.8
Ch-Mr	1465±0.024	26.3
Ch-Ij	1423±0.019	28.3
Ch-Aw-Bb	1491±0.018	21.4
Ch-Aw-Mr	1405±0.014	26.5
Ch-Aw-Ij	1388±0.017	23.1
Ch-Ao-Bb	1456±0.019	28.2
Ch-Ao-Mr	1416±0.017	24.8
Ch-Ao-Ij	1314±0.020	22.5

obtain the beads with neem (Aw and Ao) increased less than 1% with respect to the Ch-Control. On the other hand, the viscosities used to synthesize the beads with spores recorded an average decrease of 18% with respect to the Ch-Control. This effect is related to the addition of spore suspension in Tween 80, which modifies the pH causing the alteration of the rheology (viscosity) of chitosan (Giraldo, 2015; Labrada-Hechavarría *et al.*, 2018; Prudkin & Perez, 2016). The viscosities of the precursor suspensions used to obtain the beads with neem and spores are lower than those of Ch-Control and beads with neem (Aw and Ao); however, they are similar to those used to obtain the beads that only contain spores.

Diameter and moisture percentage of the beads

According to the data reported in Table 2, the variability of the diameter of the beads is not very dispersed compared to Ch-Control, because these data fall within the range of 2.49 to 2.89 mm (a 0.19 average deviation standard).

The addition of neem (azadirachtin extract) to chitosan decreases the bead size, as can be observed in the diameters of Ch-Aw and Ch-Ao with respect to the Ch-Control. Conversely, the addition of the spore suspension to the chitosan increases its size with respect to the Ch-Control. This phenomenon seems to be related to the decrease in the viscosity of their precursor suspensions, with regard to the viscosity of the suspension that generates the Ch-Control. Although the precursor suspensions have a lower viscosity than the control suspension, the six beads containing neem and some spores are smaller than the Ch-Control.

The moisture percentage in each bead was >90% (Ch-Ao and Ch-Mr recorded >98%). Likewise, in all cases, the data did not express a >0.31 standard deviation: the difference between the Ch-Aw-Bb, Ch-Aw-Mr, and Ch-Aw-Ij beads does not show changes regarding the Ch-Control; meanwhile Ch-Ao-Bb, Ch-Ao-Mr, and Ch-Ao-Ij have lower moisture percentages than the Ch-Control.

Table 2. Diameter and moisture percentage of the beads.

Sample	Diameter (mm)	Moisture (%)
Ch-Control	2.69±0.22	91.91±0.24
Ch-Aw	2.50±0.20	92.81±0.31
Ch-Ao	2.57±0.23	98.10±0.19
Ch-Bb	2.88±0.24	91.30±0.31
Ch-Mr	2.89±0.24	98.76±0.28
Ch-Ij	2.87±0.19	90.93±0.30
Ch-Aw-Bb	2.61±0.18	92.37±0.29
Ch-Aw-Mr	2.58±0.14	92.28±0.17
Ch-Aw-Ij	2.54±0.15	91.94±0.27
Ch-Ao-Bb	2.49±0.18	90.50±0.27
Ch-Ao-Mr	2.61±0.17	90.44±0.25
Ch-Ao-Ij	2.52±0.20	90.24±0.29

Morphological study

Figures 2, 3, and 4 show the micrographs of the external and internal surfaces of each bead. As can be seen in Figure 2, the beads were spherical; however, in some cases, there was minimal deformation (Ch-Ao, Ch-Mr, Ch-Aw-Mr, and Ch-Ao-Ij) or an ovoid appearance (Ch-Aw-Ij, Ch-Ao-Bb, and Ch-Ao-Mr). This phenomenon is not necessarily related to the content of the different beads, perhaps as a result of the lyophilization process. Regarding the morphology of the external surface, the beads showed cracks. In some cases, they were isolated (Ch-Control, Ch-Aw, Ch-Ao, Ch-Bb, Ch-Mr, Ch-Aw-Bb, and Ch-Aw-Ij), and in others they were numerous (Ch-Ij, Ch-Aw-Mr, Ch-Ao-Bb, Ch-Ao-Mr, and CH-Ao-Ij). This phenomenon is not correlated with the content of the synthesized materials and it could be caused by the sample preparation process.

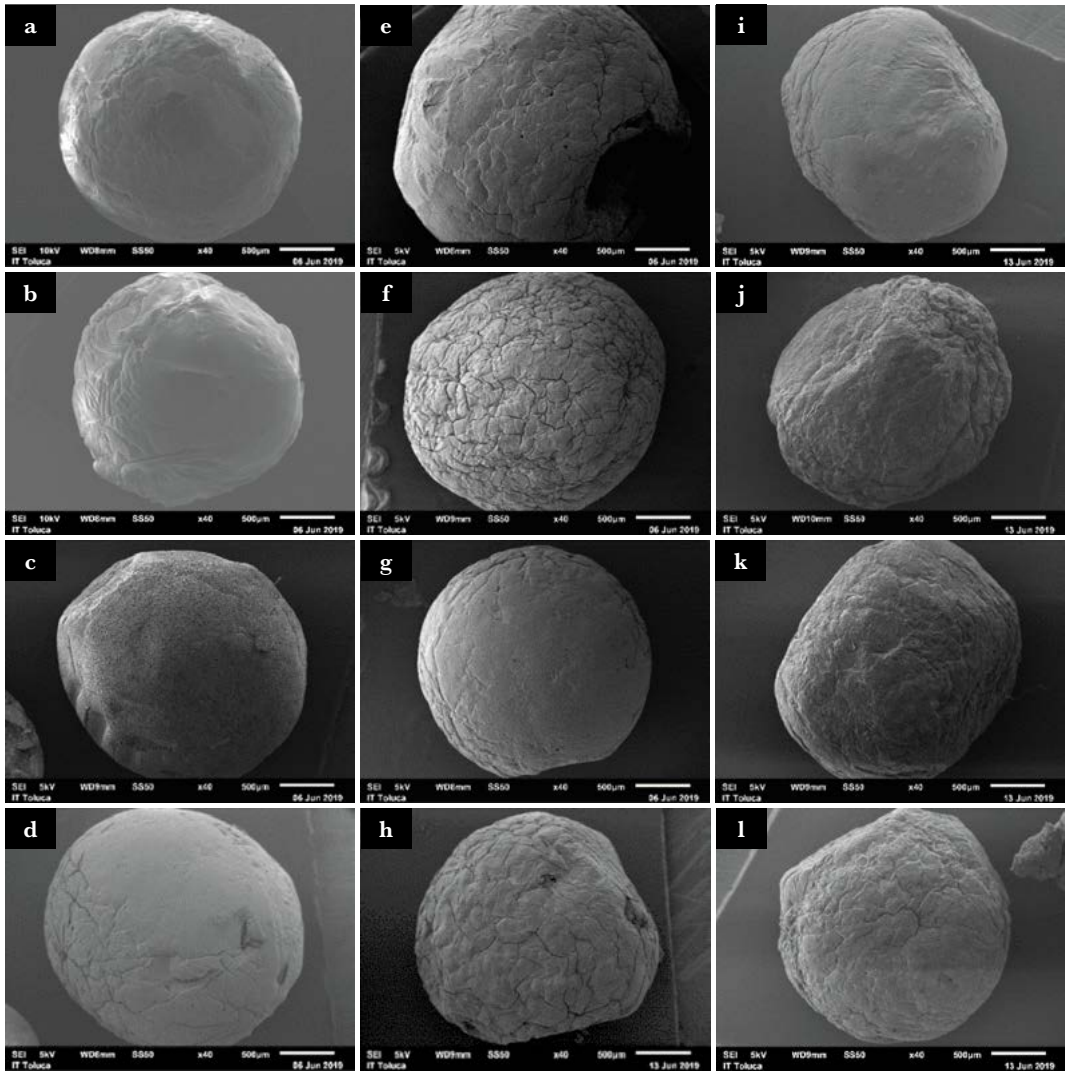

Figure 2. Micrographs at 40x of the external surface of Ch-neem-spore beads: a) Ch-Control; b) Ch-Aw; c) Ch-Ao; d) Ch-Bb; e) Ch-Mr; f) Ch-Ij; g) Ch-Aw-Bb; h) Ch-Aw-Mr; i) Ch-Aw-Ij; j) Ch-Ao-Bb; k) Ch-Ao-Mr; l) Ch-Ao-Ij.

Figure 3 shows the morphology of the external surface of each of the beads. The beads with greater porosity are the Ch-Bb and Ch-Aw-Bb; however, the Ch-Ao-Bb bead does not have a similar surface. The Ch-Aw, Ch-Mr, Ch-Ij, Ch-Aw-Mr, Ch-Aw-Ij, Ch-Ao-Bb, Ch-Ao-Mr, and Ch-Ao-Ij beads have a surface with a fine roughness. When the micrographs are enlarged, fine pores are revealed; they are similar to those observed in Ch-Control. Spherical shapes were adhered to the surface of the Ch-Aw-Bb, Ch-Aw-Mr, and Ch-Ao-Mr beads, which could be indicative of *B. bassiana* (Ch-Aw-Bb bead) and *M. robertsii* (Ch-Aw-Mr and Ch-Ao-Mr beads) spores. Particularly in the Ch-Aw-Mr bead, small spherical protuberances were detected under the surface.

Small cavities can be seen in the images corresponding to the Ch-Mr, Ch-Aw-Ij, and Ch-Ao-Ij beads; they could have been generated by the detachment of the spores that were previously attached to the surface of the beads at the time of the synthesis. A special case

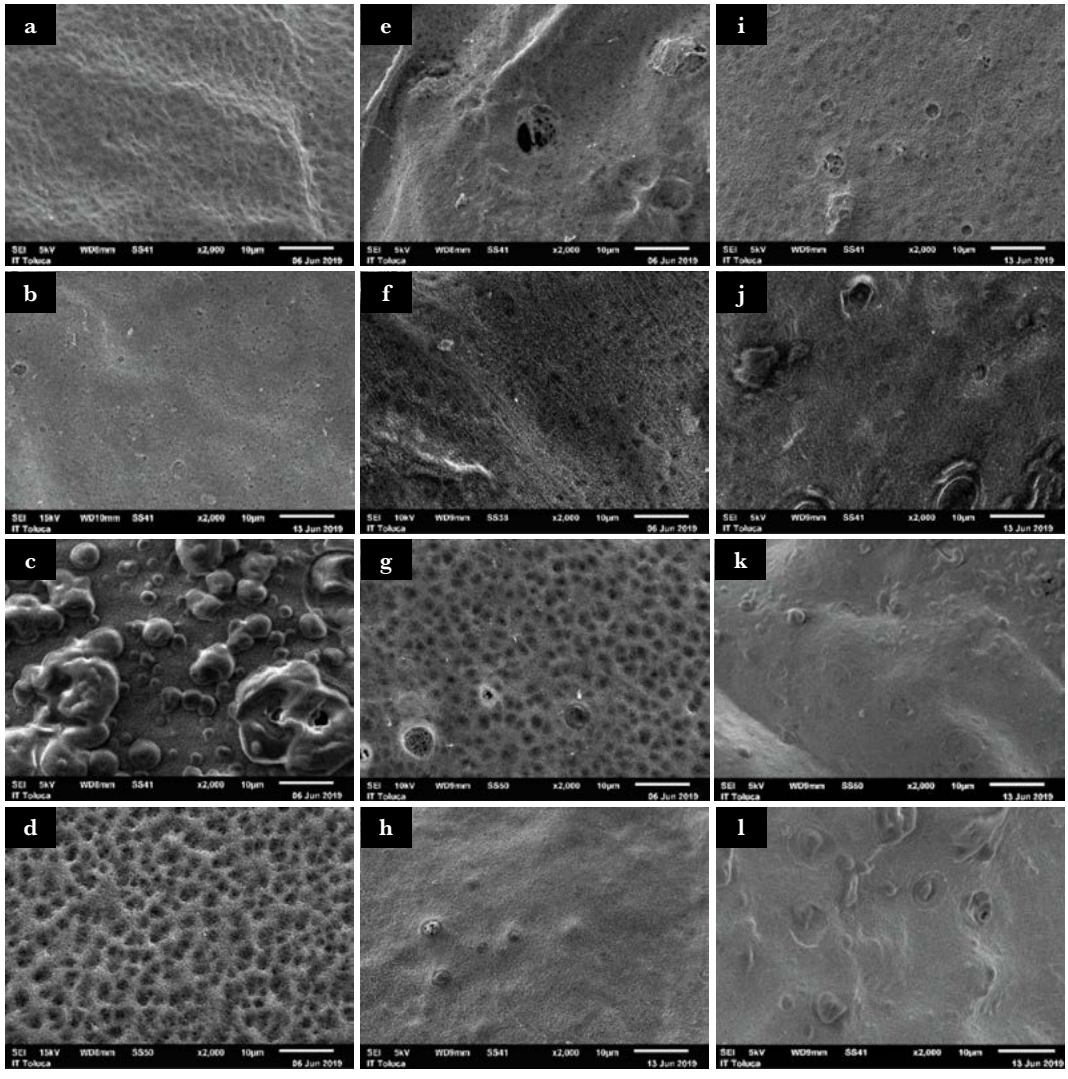

Figure 3. Micrographs at 2000x of the external surface of Ch-neem-spore beads: a) Ch-Control; b) Ch-Aw; c) Ch-Ao; d) Ch-Bb; e) Ch-Mr; f) Ch-Ij; g) Ch-Aw-Bb; h) Ch-Aw-Mr; i) Ch-Aw-Ij; j) Ch-Ao-Bb; k) Ch-Ao-Mr; l) Ch-Ao-Ij.

is the morphology observed on the surface of the Ch-Ao bead: the adhered particles could be part of the oil that was not homogenized with the matrix during the synthesis.

Finally, Figure 4 shows the 3500x magnification of the internal surface of the fractionated beads. The pores of all the beads have different characteristics, such as networks or bone-like meshes with caverns and tubules.

Adjustments to swelling models

Tables 3 and 4 show how each swelling kinetic is adjusted to the first and second order swelling models. According to these tables, the second order model of the swelling kinetics had a slightly higher coefficient of determination (R^2) than the first order model, but there was no significant change in the adjustment of the swelling kinetics. Therefore, the chi-square test was carried out in order to determine if there is a significant difference between

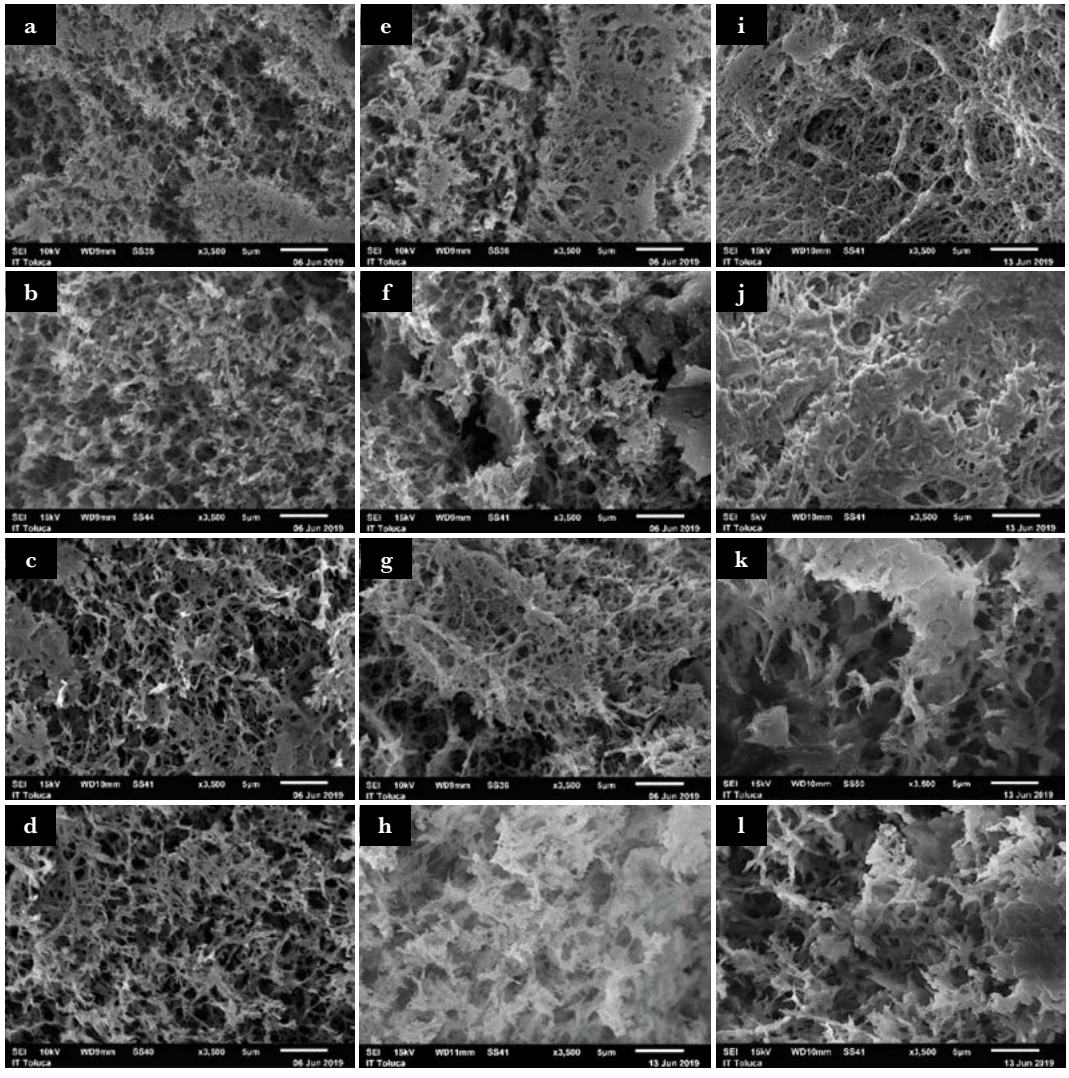

Figure 4. Micrographs at 3500x of the internal surface of Ch-neem-spore beads: a) Ch-Control; b) Ch-Aw; c) Ch-Ao; d) Ch-Bb; e) Ch-Mr; f) Ch-Ij; g) Ch-Aw-Bb; h) Ch-Aw-Mr; i) Ch-Aw-Ij; j) Ch-Ao-Bb; k) Ch-Ao-Mr; l) Ch-Ao-Ij.

Table 3. Adjustments to the first order swelling model.

Sample	$W\infty$	k	R^2	Chi-Sqr
Ch-Control	0.632620	0.73651	0.97683	7.28E-04
Ch-Aw	0.631160	0.89146	0.99419	1.79E-04
Ch-Ao	0.635650	0.93930	0.99698	9.41E-05
Ch-Bb	0.684730	1.03038	0.99875	4.52E-05
Ch-Mr	0.667360	0.90848	0.99804	6.73E-05
Ch-Ij	0.647990	0.92563	0.99311	2.24E-04
Ch-Aw-Bb	0.626830	0.93736	0.99603	1.20E-04
Ch-Aw-Mr	0.623960	1.00813	0.99420	1.75E-04
Ch-Aw-Ij	0.614520	0.92019	0.99462	1.57E-04
Ch-Ao-Bb	0.641050	0.95537	0.99280	2.29E-04
Ch-Ao-Mr	0.649130	0.94973	0.99198	2.62E-04
Ch-Ao-Ij	0.623720	0.80736	0.99134	2.61E-04

Table 4. Adjustments to the second order swelling model.

Sample	$W\infty$	k	R^2	Chi-Sqr
Ch-Control	0.654770	2.65987	0.98913	3.42E-04
Ch-Aw	0.643900	4.74278	0.99835	5.07E-05
Ch-Ao	0.645740	5.93387	0.99925	2.35E-05
Ch-Bb	0.692550	7.65708	0.99985	5.47E-06
Ch-Mr	0.677740	5.60120	0.99975	8.51E-06
Ch-Ij	0.660900	4.78650	0.99750	8.13E-05
Ch-Aw-Bb	0.637390	5.72565	0.99880	3.65E-05
Ch-Aw-Mr	0.634190	6.17635	0.99732	8.07E-05
Ch-Aw-Ij	0.625990	5.30507	0.99821	5.22E-05
Ch-Ao-Bb	0.653170	5.15825	0.99685	1.00E-04
Ch-Ao-Mr	0.662040	4.86027	0.99656	1.12E-04
Ch-Ao-Ij	0.639440	3.75259	0.99739	7.85E-05

the actual values of the swelling kinetics and the adjustment with the theoretical first and second order models. The results showed a better adjustment of the swelling kinetics to the second order model, since there is a greater error (Chi-Sqr) with the first order model. Consequently, there is a better adjustment to the model of second order swelling.

The swelling equilibrium ($W\infty$) in the second order model showed that Ch-Bb recorded the highest value with a lower degree of chitosan crosslinking than the Ch-Control and other beads (Ostrowska-Czubenko *et al.*, 2015). Meanwhile, the maximum swelling rate k in the Ch-Bb bead had a value of 7.65708 min^{-1}; this result means that it reaches a maximum swelling in a shorter time. This behavior could be caused by a structural change in the chitosan molecule, since some authors have reported that the coupling of some functional groups in the main chain of the biopolymer can improve its physicochemical properties (*e.g.*, solubility) and therefore generate some interesting synergistic characteristics (Carrero Gallardo *et al.*, 2019).

CONCLUSIONS

It is feasible to obtain chitosan beads crosslinked with sodium tripolyphosphate containing in its matrix entomopathogenic fungal spores and neem. Pests in crops could be eliminated by the potential controlled release of neem and fungal spores. This alternative for the biological control of pest insects avoids the use of agrochemicals. The morphological study showed that all the beads have similar porosity characteristics that would allow the neem extract to be released in a controlled manner. Likewise, the swelling kinetics results showed that the maximum swelling of the beads can be achieved in a relatively short time, according to the results obtained in the adjustment of the second order model.

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