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Encapsulation of lipase from *Aspergillus niger* GH1 and evaluation of their disintegration *In Vitro*

Encapsulación se lipasas de *Aspergillus niger* GH1 y evaluación de su digestión I*n Vitro*

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Abstract

Lipases are enzymes that hydrolyze triglycerides to fatty acids and glycerol. These enzymes are naturally present in the human body and are essential to digest fats. In the present study, we focused on the encapsulation of lipases produced by Aspergillus niger GH1 fungus in an alginate matrix, using three concentrations of sodium alginate (2, 2.5, and 3.0 % w/v) and two concentrations of CaCl2 were evaluated (0.1 and 0.2M) such as crosslinking agent. The results show that alginate provides a diffusion barrier which enables resist gastric conditions, so they can be released into the intestine in 28 minutes, where the enzymes hydrolysis triglycerides. The results showed that with 3.0 % alginate and 0.2 M CaCl2 concentration, the morphological characteristics and enzymatic activity are the best results, thus being effective for encapsulating and releasing lipases.

Keywords: encapsulation; enzyme; *Aspergillus niger*.

Resumen

Las lipasas son enzimas que hidrolizan los triglicéridos en ácidos grasos y glicerol. Estas enzimas están presentes de forma natural en el cuerpo humano y son esenciales para la digestión de las grasas. En el presente estudio nos centramos en la encapsulación de lipasas producidas por el hongo Aspergillus niger GH1 en matriz de alginato, utilizando tres concentraciones de alginato sódico (2, 2,5 y 3,0% p/v) y se evaluaron dos concentraciones de CaCl2 (0,1 y 0,2M) como agente gelificante. Los resultados muestran que el alginato proporciona una barrera de difusión que permite resistir las condiciones gástricas, por lo que pueden ser liberados en el intestino en 28 minutos, el lugar donde las enzimas realizan la hidrólisis de los triglicéridos. Los resultados mostraron que con un 3,0 % de alginato y una concentración de CaCl2 0,2 M, las características morfológicas y la actividad enzimática son las que mejores resultados presentan, resultandos eficaces para encapsular y liberar lipasas.

Palabras clave: Encapsulación; enzima; *Aspergillus niger*.

INTRODUCTION

Lipases (EC3.1.1.3) are a kind of enzyme with considerable physiological significance and industrial potential. (Sirisha et al., 2016). This enzyme catalyzes triacylglycerol hydrolysis at the oil-water interface to release glycerol and free fatty acids (Dheeman et al. 2010).

The interest in lipases has increased in recent years due to their diverse catalytic properties (Zuin et al., 2022). They have a big field of industrial applications, such as additives in detergent formulations, in the food industry for the manufacture of products with low dietary fat and cholesterol, in the paper industry with the objective removing the wax from the paper pulp, in the pharmaceutical industry in obtaining bioactive molecules, chemical synthesis processes for obtaining optically pure compounds (Alves et al., 2024), modification of fats and other lipids by hydrolysis and esterification(Lipases as Biocatalysts for Enzymatic Interesterification | SpringerLink, n.d.) to name a few.

With advances in immobilization techniques, the use of enzymes and immobilized cells is increasing (Di Cosimo et al., 2013), due to the fragile nature of the enzyme protein, which has limited stability in the structure and functionality (Mulinari et al., 2020). It is considered that an enzyme is suitable for a commercial application if its stability is sufficient for the application and the method of encapsulation allows it to do so. Encapsulation is a process where a continuous thin coating is formed around solid particles, liquid droplets, or gas cells that are fully contained within the capsule wall (Kok et al., 2018). This technology has been used in the food industry for more than 60 years as a way to provide liquid and solid ingredients as an effective barrier for environmental and/or chemical interactions until release is desired (Sheldon et al., 2021).

Enzyme encapsulation is an attractive method among the different immobilization strategies to improve the reusability and stability of enzymes because it can separate enzymes from a hazardous external environment (Di Cosimo et al., 2013).
The active substance is covered with a porous polymer film(Liu et al., 2022); this membrane barrier or film is usually made of components with strings to create a net with a hydrophobic and/or hydrophilic property(Basso & Serban, 2019).
The encapsulation technique allows food packaging and other materials such as oils, probiotic bacteria, enzymes, whey, vegetable pigments, minerals, vitamins, and food additives. Nearly any material that needs to be protected, isolated, slowly released over time, or released at a certain time can be encapsulated (Zou et al., 2023). The principal coating materials typically used in food industries are carbohydrates, cellulose, gums, lipids, and proteins. Encapsulation is carried out through various processes chemicals such as conservation, interfacial polymerization, ionic gelification, polymer incompatibility, entrapping in liposomes, and mechanical processes like techniques of co-crystallization, freeze drying/cooling, extrusion, and finally, there is the spray drying technique, this being the most important and used in the food industry. (Gupta et al., 2024)

In this study, we used the ionic gelification method for the lipase immobilization to fabricate calcium alginate beads. Alginate has the following advantages: a) Alginate, the major shell material, is extracted from algae, which is cheap and has been used in food and drugs for many years. It is full of human-necessary microelements and is used as a health food in many countries. Moreover, with anti-cancer activity, alginate can absorb heavy metal ions and free radicals from the human body. The capsule is very stable. The capsule's shell comprises at most 15% of the capsule weight, which can improve the loading capacity of the bioactive agent within the capsule. The purpose of this work was to encapsulate lipases obtained from the fermentation of the fungus Aspergillus niger GH1 to provide protection and stability to digestive conditions when being incorporated into a food product.

MATERIALS Y METHODS

Obtention of lipase extract by fermentation with *Aspergillus niger* GH1

The enzyme extract was obtained by fermentation in a liquid medium of Czapek-dox, this contained the following composition: 2 g/L of NaNO3; 1 g/L K2HPO4; 0.5 g/L MgSO4.7H2O; 0.5 g/L KCl; 0.01 g/L FeSO4.7H2O, also a carbon source supplemented with 1% olive oil w/w. The initial pH was adjusted to 6.0 and sterilized at 15 Lb of pressure at 121°C for 15 min.

Conditions of fermentation were inoculum; 1 X 107 spores/ml of A. niger GH1 (conidial suspension in Tween 80 0.01%), incubation time; for eight days at 130 rpm, and temperature; at 30 °C. After, the fungal cultures were vacuum filtered and then centrifuged at 12,000 rpm for 5 min (Coca and Harrison, 2001). The supernatant was collected, and enzyme activity of lipase was determined by measuring the increase in absorbance at 348 nm produced by the release of p-nitrophenol as a result of hydrolysis of 50 mM p-nitrophenyl ester propionate (p-NPP) in 25 mM phosphate buffer pH 7.0 and 37 °C (Bastida et al., 1998). The enzyme unit is required to

hydrolyze one micromole of p-NPP per minute under the described conditions.

Selection of conditions for encapsulation of lipases

The ionic gelification technique encapsulated lipases. An experimental block design was obtained, which is shown in Table 1; three repetitions were performed for each treatment. Sodium alginate was dispersed in distilled water at a temperature of 60 °C at constant agitation during 30 minutes of mixing to immobilize the enzyme subsequently 30 mL of distilled water was added to 120 mL of sodium alginate solution and mixed at room temperature at constant stirring for 10 minutes. The suspension was passed through a 20 mL syringe and was dropped into the solution of CaCl2 in continuous agitation to keep the capsules from sticking between them. It was kept for 30 minutes at room temperature in the solution (Figure 1).

Table 1. Design of the experimental matrix for each lipase encapsulation treatment

Treatment	Alginate solution (%)	$CaCl_2$ (mol/L)
A	2	0.1
B		0.2
C	2.5	0.1
D		0.2
E	3.0	0.1
F		0.2

After each treatment, the capsules were collected by filtration and washed with distilled water to remove excess Ca2+ on the surface of the capsule. The capsules were dried for 24 h at 28 ° C.

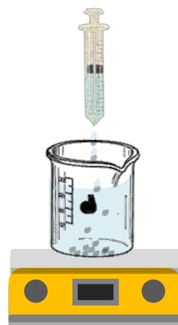

Figure 1. Schematic representation of the method used to encapsulate lipases: an alginate solution with lipases extract was dripped into a stirred calcium chloride solution.

Characterization of alginate capsules

Morphology and particle size

The particle size of the alginate beads was measured by hand using a caliper, which was determined by the average of 30 samples. Morphology was determined using an optical microscope adapted with Dino-lite AM3111 0.3MP Digital microscope.

Moisture content

The moisture contents of the capsules were determined automatically in moisture analysis equipment (Ohaus), using 0.5 g of capsules; the test was conducted at a constant temperature of 110 °C (Gangawane et al., 2024).

Colorimetry

The capsules were used to determine CIELAB color coordinates using a chromameter. CIELAB colorimetric system was interpreted as follows: L* indicates lightness read from 0 (black) to 100 (white). The positive a* value indicates the red color, while the negative a* value represents the green color. Similarly, positive and negative b* values indicate yellow and blue, respectively. Chroma values denote the saturation or purity of color. In a color wheel, values close to the center, at the same L* value, indicate dull or gray colors, whereas values near the circumference represent vivid or bright colors (Moreira et al., 2017).

Hygroscopicity

Hygroscopicity was determined according to the method proposed by Cai and Corke (2000), with some modifications. Samples of about 1g were placed in desiccators with a saturated solution of NaCl (relative humidity of 75.3%). After one week, samples were weighed, and hygroscopicity was expressed as adsorbed moisture per 100g of sample (g/100g).

Water activity

Water activity was measured using 1 g of lipase capsules in Aqualab equipment (DECAGON DEVICES INC.).

Enzymatic activity

Enzyme activity was determined to alginate beads by measuring the increase in absorbance at 348 nm produced by the release of p-nitrophenol as a result of hydrolysis of 50 mM p-nitrophenyl ester propionate (p-NPP) in 25 mM phosphate buffer pH 7.0 and 37 ° C (Bastida et al., 1998). The enzyme unit

is the enzyme required to hydrolyze 1 micromole of p-NPP per minute under the described conditions.

Disintegration in vitro of lipase encapsulated

The disintegration of capsules was determined according to disintegration tests from FEUM (FEUM, 2011) with some modifications. An ELECSA disintegrator weighing 250 mg of the capsule was used and placed in the basket tubes. A simulated gastric fluid (2.0 g dissolved sodium chloride and 3.2 g pepsin in 7.0 mL of hydrochloric acid was completed to 1000 mL of water. The solution pH 1.2) and placed in motion team for 2 h at 37 °C ±2. Elapsed capsules were changed to a simulated intestinal (dissolved 6.8 g of potassium phosphate monobasic in 250 mL of water and added 190 mL of 0.2N sodium hydroxide and was completed to 1000 mL of water, the pH of the solution was 7.5) at 37 °C ± 2, time was measured until complete disintegration of capsules.

RESULTS

To define the concentrations of the ingredients and conditions for the production of the lipase beads, three alginate concentrations (2.0 %, 2.5 % and 3.0 %) and two concentrations of the crosslinking agent (0.1 and 0.2 M of CaCl2) were tested. Capsules were obtained with all the formulations tested, as shown in Figure 2. The best morphological appearance was 3.0% of alginate, while 2.5 % and 2.0 % showed a semispherical appearance.

The size of the capsules was measured for each concentration (Table 2), and the different sizes obtained are shown below, where the capsules were in a range of 1.49 to 1.71 mm; Flores et al 2011 measured the size of the alginate capsules of three different concentrations of sodium alginate (1.0%, 1.5%, and 2.0%) the capsule diameter was from 1.86 to 2.03 mm.

The best results were obtained with the concentration of 3.0% alginate and 0.2M CaCl2; the capsules had a spherical and regular appearance, while the other concentrations showed irregularly shaped; this polysaccharide forms strong gels with Ca+2, giving spheres with good strength and flexibility (Anandharamakrishnan et al., 2010; Birchal et al., 2006). The size and morphology can be influenced by different parameters such as speed stirring, the encapsulating matrix type, the technique used, the substance to be encapsulated, and some other parameters that can be considered (Gharsallaoui et al., 2007; Jin & Chen, 2009; Langrish & Fletcher, 2001).

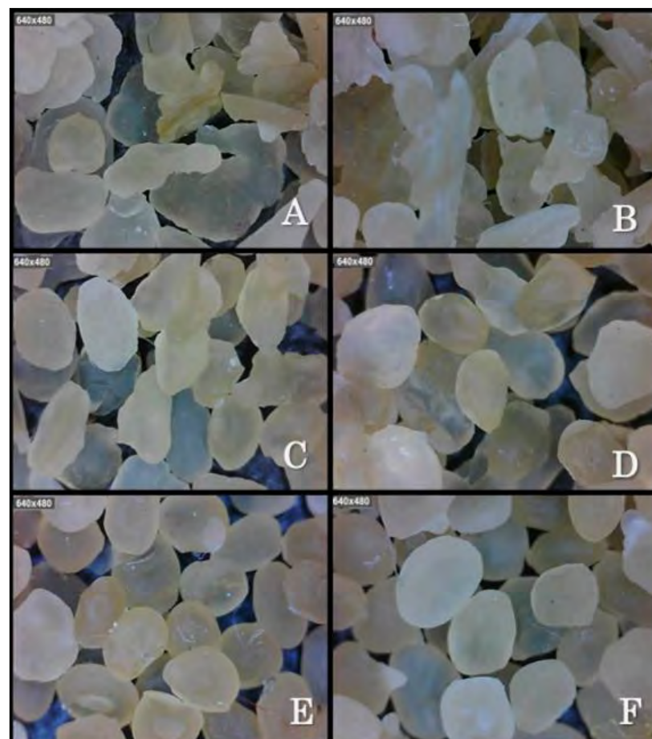

Figure 2. Morphological appearance of lipase capsules. A) 2.0%, 0.1 M, B) 2.0%, 0.2 M, C) 2.5%, 0.1 M, D)2.5%, 0.2 M, E) 3.0%, 0.1M and F) 3.0%, 0.2M

Table 2. Size of capsules with extract of lipase.

Treatment	Size (mm)
A	1.51 ± 0.15
B	1.66 ± 0.16
C	1.59 ± 0.14
D	1.52 ± 0.12
E	1.49 ± 0.13
F	1.71 ± 0.10

A) 2.0% sodium alginate and 0.1 M $CaCl_2$, B) 2.0% sodium alginate and 0.2 M $CaCl_2$, C) 2.5% sodium alginate and 0.1 M $CaCl_2$, D) 2.5% sodium alginate and 0.2 M $CaCl_2$, E) 3.0% sodium alginate and 0.1M $CaCl_2$ and F) 3.0% sodium alginate and 0.2M $CaCl_2$. Data are mean (±standard deviation) of three replicates.

The results of the moisture content determined for each of the samples can be seen in Figure 3.

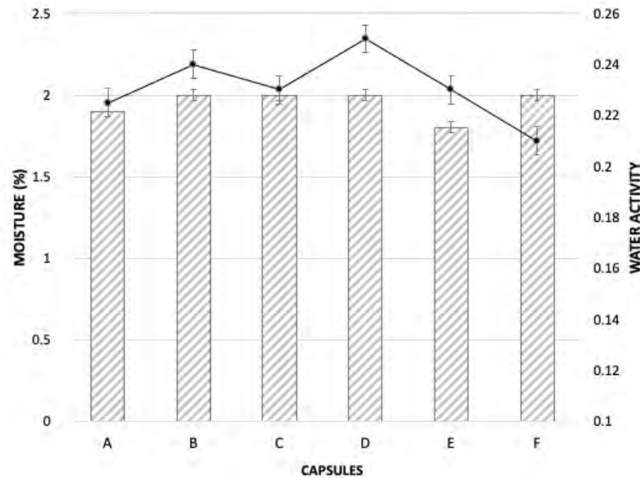

Figure 3. Moisture content (y-axis primary) and water activity (y-axis secondary) of alginate capsules at different concentrations. A) 2.0 % sodium alginate and 0.1 M $CaCl_2$, B) 2.0 % sodium alginate and 0.2 M $CaCl_2$, C) 2.5 % sodium alginate and 0.1 M $CaCl_2$, D) 2.5 % sodium alginate and 0.2 M $CaCl_2$, E) 3.0 % sodium alginate and 0.1M $CaCl_2$ and F) 3.0 % sodium alginate and 0.2M $CaCl_2$. Data are mean (±standard deviation) of three replicates.

The moisture content of the capsules was in the range of 1.8 and 2%, (Delgado et al., 2022a), showing moisture content values between 96% and 95% in alginate capsules. The difference with this work was that the capsules were pre-dried so that results were expected, and these values were not significantly different; the moisture content and the water activity are relatively low; these results indicate the stability of the capsules and prevent the growth of microorganisms that may affect its functionality.

The hygroscopicity results can be seen in Table 3; this was evaluated in a desiccator with a saturated solution of NaCl and was weighed; the capsules lost weight, indicating that they cannot absorb atmospheric moisture. (Delgado et al., 2022). Demonstrated that alginate capsules are more stable when they have a low hygroscopicity.

Figure 4 shows the color results obtained in the capsules. The results show that the color of the capsules is in the brown-yellow color range; all capsules 2.0 %, 2.5 %, and 3.0 % are in the same place, so the evaluated concentration of sodium alginate does not affect the color of the capsule.

Table 3. Hygroscopicity of alginate capsules. Initial weight and final weight of the alginate capsules after 7 days of the experiment

Capsules	Initial weight	Final weight
A	1.007	0.971 ± 0.005
B	1.003	0.970 ± 0.001
C	1.006	0.948 ± 0.005
D	1.004	0.958 ± 0.010
E	1.004	0.951 ± 0.004
F	1.003	0.968 ± 0.007

A) 2.0 % sodium alginate and 0.1 M $CaCl_2$, B) 2.0 % sodium alginate and 0.2 M $CaCl_2$, C) 2.5 % sodium alginate and 0.1 M $CaCl_2$, D) 2.5 % sodium alginate and 0.2 M $CaCl_2$, E) 3.0 % sodium alginate and 0.1M $CaCl_2$ and F) 3.0 % sodium alginate and 0.2M $CaCl_2$. Data are mean (±standard deviation) of three replicates.

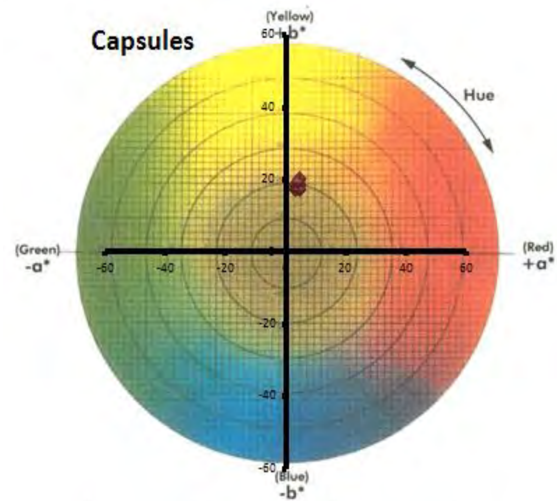

Figure 4. Polar scatter plot of color of capsules A) 2.0 % sodium alginate and 0.1 M $CaCl_2$, B) 2.0 % sodium alginate and 0.2 M $CaCl_2$, C) 2.5 % sodium alginate and 0.1 M $CaCl_2$, D) 2.5 % sodium alginate and 0.2 M $CaCl_2$, E) 3.0 % sodium alginate and 0.1M $CaCl_2$ and F) 3.0 % sodium alginate and 0.2 M $CaCl_2$. Data are mean (±standard deviation) of three replicates

The enzymatic activity was evaluated; the results are shown in Figure 5, where the enzymatic extract obtained from a previous fermentation with Aspergullus niger GH1 was 20.02

UE/mL, the highest enzyme encapsulated was with 2.0 % of alginate sodium and 0.2 M, this capsules shows an activity of 17.86 UE/mL, following by the capsules of 2.0 % 0.2 M, 2.5 % 0.1 M, 2.5 % 0.2 M, 3.0 % 0.1 M, with an activity of 15.77 UE/mL, 17.20 UE/mL, 14.44 UE/mL and 15.88 UE/mL respectively, and the and concentration which encapsulated the lowest amount of enzyme was 2.0 % and 0.1 M with and activity of 11.73 UE/mL. Flores et al. (2011) encapsulated proteolytic enzymes, and the best results were with the low alginate concentration of 70.8 % of enzymatic encapsulation, while the present work with the highest alginate concentration achieved encapsulation 87.6 %, which indicates that our method is a better combination for encapsulating enzymes.

For the disintegration of the capsules, the concentration of 3.0% sodium alginate and 0.2 M $CaCl_2$ was chosen as it showed the highest enzymatic activity. The disintegration time of the alginate capsules was determined in a tablet disintegrator (ELECSA) to see if they could resist simulated stomach and intestinal fluid conditions (Figure 6).

The results show that the capsules are resistant to acid pH since after being in contact with simulated gastric fluid at pH 1.2, they did not show any change in their morphology after the capsules were evaluated in an intestinal medium to measure the disintegration time after 5 minutes lipase capsules began to diminish in size and 28 minutes after the capsules were completely disintegrated so that they can resist gastric conditions and under intestinal conditions are completely disintegrated, alginate capsules can be designed for an active pharmaceutical ingredient with gastric solubility, enteric solubility or colon solubility easily. Moreover, it can be used in food and industry due to its perfect safety, stability, and low cost. Calcium alginate gels are widely used in food, pharmaceutical, and medical applications (Ta et al., 2021). It has been reported that calcium alginate beads can protect bioactive molecules from the stomach's harsh, acidic environment Gopal, 2022).

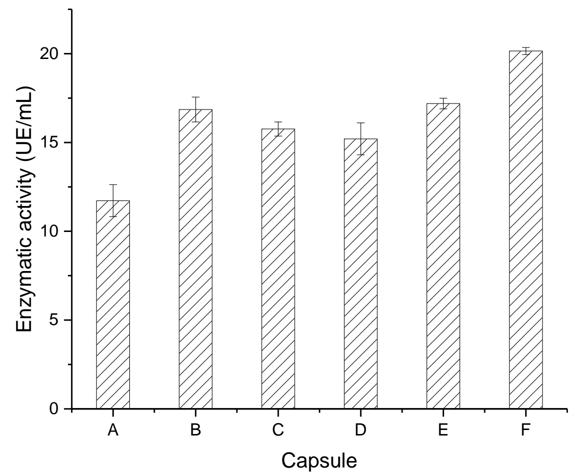

Figure 5. Lipolytic enzyme activity of capsules at different concentrations. A) 2.0% sodium alginate and 0.1 M CaCl2, B) 2.0% sodium alginate and 0.2 M CaCl2, C) 2.5% sodium alginate and 0.1 M CaCl2, D) 2.5% sodium alginate and 0.2 M CaCl2, E) 3.0% sodium alginate and 0.1M CaCl2 and F) 3.0% sodium alginate and 0.2M CaCl2. Data are mean (±standard deviation) of three replicates.

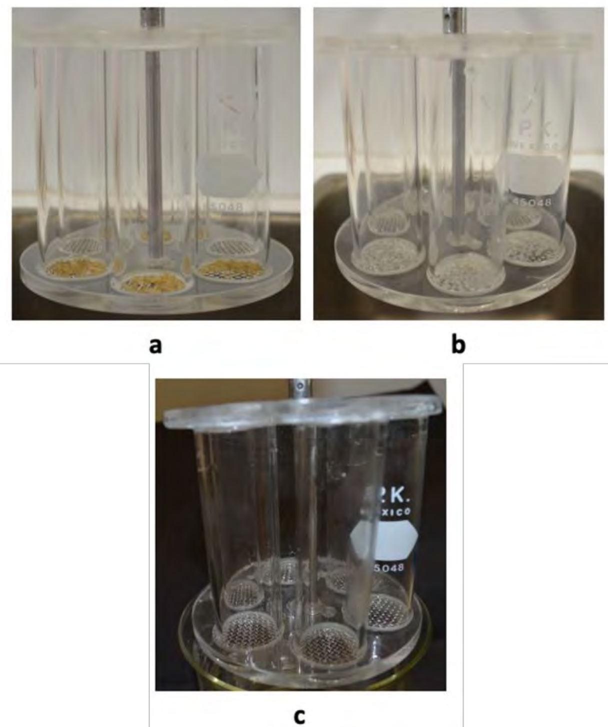

Figure 6. Disintegration of lipases capsules. a) Capsules before being in contact with the medium simulated gastric. b) Capsules after two hours in the middle gastric. c) Total Disintegration of capsules.

CONCLUSIONS

Lipase encapsulation has great potential for the food industry because it can be the vehicle for incorporating these enzymes in many food products that may benefit humans. Designing an encapsulation technique that allows for preserving the enzyme and resisting digestive conditions greatly contributes to the industry and facilitates better-quality manufacturing processes and products. In this work, the best combination to obtain capsules with desirable characteristics is 3% sodium alginate crosslinking agent CaCl2 solution 0. 2 M, which is resistant to digestive conditions. The technique used in this study for forming the capsules was an ionic gelification reaction between a polysaccharide and a counter ion, also known as the alginate drip method. The process is performed quickly and can encapsulate any food, either hydrophobic, hydrophilic thermosensitive liquid, or solid.

This research highlights that the method used to encapsulate lipases has a practical opportunity in the food industry thanks to its technological advantages since it can protect the active material from environmental degradation (heat, air, light, and humidity, among others). Encapsulated compounds are released gradually. Their physical properties can be modified according to their needs. Characteristics such as taste and odor of the material can be masked due to encapsulation.

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CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

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