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ORIGINAL ARTICLE

Preparation, Activity and operational Stability of mexicain Entrapped in Alginate Beads

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ABSTRACT

Mexicain, cysteine protease contained in unripe fruit latex known as cuaguayote (Jacaratia mexicana), presents similar properties than papain, enzyme widely used in food industry and the pharmaceutical industry. Enzymatic preparations of mexicain were used in previous studies for colloidal stabilization of beer and in fish protein solubilization, obtaining in both cases a great performance. Now, the aim of this work is to enhance the operational stability of mexicain entrapped in sodium alginate beads, as an alternative for its optimal reuse in those processes where it is used. The influence sodium alginate concentration, calcium chloride concentration, and the hardening time on the entrapment of mexicain, are evaluated. The enzyme immobilization was prepared according to the response surface methodology with a composite central design with five repetitions in the central point with 13 runs. The selected response variables were: capsule size, strength, entrapment, and proteolytic activity; which were evaluated during three months storage at 5°C. The surface morphology of the beads was made also by optical microscopy and by scanning electronic microscopy. The entrapment system rendered spherical capsules with diameters between 1.41-2.38 mm. The strongest capsules were obtained with 1% sodium alginate and 0.1 M calcium chloride. The immobilized enzymes were more stable than free enzymes, the stability limits of the immobilized enzymes were within a pH range from 5.0 to 10 and temperatures of 25°C to 65°C. The immobilized mexicain retained greater activity (60%) than the free enzyme (8%), with 43% of residual activity after being used 5 times.

Keywords: *mexicain, Jacaratia mexicana, immobilization, operational stability*

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INTRODUCTION

The interest by the proteases enzymes has been derived from its primary function within the cell, which has allowed them to find several biotechnological applications [1]. In the food industry, proteases are often used in different processes, the largest amounts of these enzymes are derived from microbial sources, but some cysteine proteinases from plant origin are preferred for food purposes. In particular, proteases such as papain, ficin and bromelain, are widely used because they belong to the list of enzyme preparations considered by the Food and Drug Administration of United States as GRAS substances (substances generally recognized as safe), a condition shared by similar proteases isolated from the edible fruits of another Caricaceae species (www.fda.gov/default.htm).

An important factor to be considered when a protease is chosen for an industrial process is the stability of the preparation. It is well known that enzymes are very sensitive biomolecules when exposed to processing conditions that may affect there stability. Within this context, a technological alternative like the enzyme immobilization on different media types or matrices, can maintain and enhance the stability

of enzymes when operating under extreme conditions of temperature and pH, and organic solvent media; a strategy that encourages repeated use, and for the particular case of proteases enzymes, reduce its self-digestion.

Thus, in recent years has increased the interest in the preparation of immobilized enzymes through various media and immobilization techniques [2, 3]. The improved stability of enzymes after immobilization has been attributed to: the multi-bonds between enzyme and the support favoring the tertiary structure of the enzyme to become more rigid and more resistant to thermal or chemical deactivation; to the induced protection against autolysis; also to the fact that intermolecular aggregation is avoided, keeping the enzyme molecules trapped in a region of space, and to the alteration of the microenvironment that promotes a positive interaction between the enzyme and the support through a dampening effect that keeps the optimum pH for the enzyme, although the operational medium may suffer significant changes in pH

Enzymes can be immobilized by a great variety of methods, which can be classified as physical methods and chemical methods; the firsts are characterized through weak interactions between the support and the enzyme, while the latters are characterized by the formation of covalent bonds between the enzyme and the support [4]. In every case it has to be assayed the best-suited method for a particular enzyme preparation depending on chemical characteristics and composition, thus as the intended application for the immobilized enzyme. As a consequence, the optimal conditions for the immobilization and its applications are established through a series of tentative tests directed to the fulfillment of criteria such as the retention of enzymatic activity and the highest possible maximum operational stability. Among these methods, ionic gelation (ionotropic) of aqueous solutions of polysaccharides like alginate or pectin, added drop by drop to solutions with counter ions like calcium, produces a complex between oppositely charged species, with the precipitation of the polysaccharides as spherical particles causing the ionic gelation as it is known properly [5].

Among the predominant polymers used in the different techniques of entrapment or encapsulation of enzymes we have the sodium alginate, whose industrial applications have increased considerably in the recent years [6]. In a process of immobilization, the enzyme activity may be diminished or even be lost for several reasons: the type of binding of the enzyme to the support that could prevent the passage of the substrate; the effect of the reactive groups on the support with any amino acid essential for the catalytic activity of the enzyme; any conformational change that may arise in restraint, giving rise to an inactive form; or by denaturation or inactivation of the enzyme during the process [7].

The majority of the known plant proteases (papain, ficin, bromelain, and mexicain) are cysteine endopeptidases [8, 9]. Some proteases from mexican plants as papain, mexicain and hemisphaericin, obtained from the latex of fruits like the papaya (*Carica papaya*), cuaguayote (*Jacaratia mexicana*) and timbirichi (*Bromelia hemisphaerica*), respectively, all of them have been extensively studied [10]. Mexicain is a cysteine protease with molecular weight of 23.8 kDa, was described initially as a monomorphic type enzyme with high stability to pH and temperature [11]. In recent studies it has been reported that the latex from *J. mexicana* contains not only mexicain but also five proteases; including fraction IV previously identified as mexicain [12]. In previous studies on some technical applications, a refined mexicain preparation (industrial grade) for high volume applications was developed by Briones et al., (1994)[13]. It has been found that this refined preparation from J. mexicana shows better stability and specific activity than papain, when used in several processes like the colloidal stabilization of beer, in meat tenderization, in the hydrolysis of fish and vegetable proteins, in the modification of functional properties of some proteins, inferring its industrial potential for processes where papain has been widely used [14].

In previous reports, sodium alginate has been used in the immobilization of proteases from laticiferous plants, especially in the entrapment of cysteine proteases like papain [15], and araujiaína from the latex of Araujia (*Fourna hortorum*) [16]. The aim of this paper is to enhance the operational stability of mexicain, a cysteine protease entrapped in sodium alginate as an alternative to enable the optimal reuse in the processes of transformation like the preparation of protein hydrolysates.

MATERIALS AND METHODS

Chemicals

Sodium alginate was purchased from Química Meyer®, México; calcium chloride (J. T. Baker® USA), casein, tricholoacetic acid, bovine serum albumin all of them were purchased from Sigma, USA.

Plant material

Refined mexicain was obtained from unripe fruits of cuaguaote (*Jacaratia Mexicana*), the latex was partially refined (homogenized, clarified by centrifugation and stabilized) at the Plant Enzymes

Laboratory of the Center of Development Biotic Products (CEPROBI-IPN).

Protein content determination

Protein determination was carried out according to the method described by Bradford [17], using bovine serum albumin (BSA) as a standard.

Protease assay

Analyses of proteolytic activity were carried out to evaluate the stability of mexicain after the entrapment process and along the storage [18]. 60 µL of 0.8 M cysteine (pH 7.6) were added to 180 µL of the sample (free or immobilized mexicain), 25 µL of this mixture, were added to 475 µL of 1% casein in 0.05 M phosphate buffer, pH 7.6. After 10 minutes of hydrolysis at 35°C ± 2, the digestion of casein was stopped by the addition of 750 µL of 5% trichloroacetic acid (TCA). Each test tube was centrifuged at 13000×*g* for 10 min and the absorbance of the supernatant was measured at 280 nm. The proteolytic activity was reported as Tyrosine Units (TU)/mL (micrograms of tyrosine released in one hour, per mL).

Experimental design

The proteolytic activity changes during mexicain immobilization process was analyzed by a central composite experimental design (CCD) of response surface methodology (RSM), the selected factors were sodium alginate concentration and calcium chloride concentration, the analyzed variables consisted in area size, compression force, the rate of immobilization and proteolytic activity. The experimental design was built through the program Design Expert 7 (Stat-Ease Inc., Minneapolis, MN) with 13 experimental runs completely random and 5 replicates at the central point. The experimental conditions are shown in Table 1.

Table 1. Central composite design for the mexicain immobilization.

Run	Sodium alginate concentration (%)	Calcium chloride concentration (M)
1	1.00	0.10
2	2.00	0.10
3	1.00	0.30
4	2.00	0.30
5	0.79	0.20
6	2.21	0.20
7	1.50	0.06
8	1.50	0.34
9	1.50	0.20
10	1.50	0.20
11	1.50	0.20
12	1.50	0.20
13	1.50	0.20

Entrapment of mexicain on alginate beads

Alginate solutions at different concentrations (1.0, 1.5 and 2.0% w/v in water) were mixed with a mexicain solution of 1 mg/mL. The beads were obtained by dropping the alginate-enzyme mix, using a syringe with 0.80 mm inner diameter, into 50 mL of $CaCl_2$ solutions at 0.1, 0.2 and 0.3 M and under continuous stirring at 125 rpm for 6 h of hardening. The beads were separated from the calcium chloride solution by filtration.

The Entrapment Efficiency (EE) was determined by preparing the beads under the methodology previously proposed; where a sample was taken at the beginning of the enzyme preparation, after 6 h of hardening the sample was taken of the solution of $CaCl_2$ to determine the percentage of immobilized enzyme [15]:

$$\text{Entrapment efficiency} = \frac{\text{Entrapped protein (loaded)}}{\text{Initial protein loading}} \times 100 \quad \ldots(1)$$

Production yield

The yield was determined using the method described by Calero et al., [3], according with the equation 2.

$$\text{Production Yield (\%)} = \frac{\text{Enzyme alginate beads obtained}}{\text{Theoretical enzyme alginate beads}} \times 100 \quad \ldots(2)$$

Effect of pH and temperature

The effect of pH on proteolytic activity of free and immobilized mexicain was performed with 25 µL of enzyme preparation previously activated with 0.8 M cysteine; the samples were incubated at 35 °C with

475 µL of 1% of casein in different regulators prepared as follows: 50 mM acetate buffer (pH 5.0–5.5), 50 mM potassium phosphate buffer (pH 6.0–8.0), and 50 mM borate buffer (pH 8.5–10.0). The proteolytic activity assay was performed in triplicate, for 10 minutes, stopping the reaction with 750 µL of 5% TCA.
The effect of temperature on the proteolytic activity of free and immobilized mexicain was determined using casein as substrate and cysteine 0.8 M as an activator, it was performed in triplicate at different temperatures (25, 30, 35, 40, 45, 50, 55, 60 and 65°C), after 10 minutes of reaction. All the results are the average values of three replicates for each experimental condition.

Storage stability of entrapped mexicain

The stability of the mexicain was determined by evaluating the proteolytic activity in its free and trapped form for a period of 11 weeks. The beads were stored in phosphate buffer pH 7.6 at refrigeration temperature (6°C). The experiments were performed in triplicate and data were expressed as mean values.

Reusability of entrapped mexicain

To test the reusability for entrapped mexicain, the enzymatic activity was determined as previously described. After first use, the beads were removed from the reaction mixture and washing thrice with double distilled water for subsequent reuse. The decrease in activity for each cycle was determined assuming the first cycle activity as 100%. The experiments were performed in triplicate and data were expressed as mean values.

Optical microscopy

The spheres were cut transversely and placed on a slide, then observed under an optical microscope model CX31 (Olympus, Japan) with a 40X magnification and the images were captured digitally.

Stereomicroscopy

The morphology of the spheres was determined by stereomicroscopy, the spheres were placed on a slide and observed at different magnifications (1.2X, 2.5X and 3.2X). A Carl Zeiss stereo microscope (Germany) was used and images were captured digitally.

Scanning electron microscopy

The spheres were mounted on a slide with ionized carbon ribbon double-sided and covered with a silver film for 2 min in a shader gold / silver Desk IV (Denton Vacuum, USA). They were then observed in a scanning electron microscope JSM-6390LV model (JOEL, Germany) at a voltage of 5, 10 and 20 KV and 200X and 1000X increases. Images were captured digitally.

Compression test

Texture tests were performed on a TA-XT2 texture analyzer (Stable Micro Systems, England). The 15 spheres were placed in calcium alginate on a flat cylindrical platform, aluminum, and 3 inches in diameter. The compression test used a cylindrical aluminum tube 2 inches in diameter (P2), located at a constant distance between the platform and probe 3 mm, and a spindle speed of 2 mm/s. This test was performed in triplicate. The results were analyzed using the Texture Expert for Windows based on the following relationship: Gel Strength (g) = Force required to compress the sample and produce the highest peak during compression.

Particle size analysis

The size of the sodium alginate beads was determined by two methods: using a vernier (Mitutoyo, Japón) (average of a sample of 100 spheras) and by the evaluation of the spheres pass-through a battery of sieves with stainless steel mesh No. 8, 10 and 12, which correspond to 2.38, 1.68 and 1.41 mm, respectively.

Statistical analysis

The statistical experimental designs and data analysis were carried out using Design Expert 7 (Stat-Ease Inc., Minneapolis, MN) software. All experiments were performed in triplicate and data were analyzed according to a comparison of means analysis (STATGRAPHICS Centurion XV, software package). An analysis of response surface was used to evaluate the effect of the concentration of sodium alginate and calcium chloride ($CaCl_2$) on both index of immobilization and proteolytic activity.

RESULTS AND DISCUSSION

Optical microscopy and stereomicroscopy

The spheres size is an important parameter because it can influence the release profile and other parameters, such as encapsulation efficiency [19]. Alonso et al. [20] inform on several experimental conditions that affect the size of the beads, among which are the type of polymer and its molecular mass, the ratio between the polymer and the substance to be encapsulated [21], and the speed of agitation at the time of particles forming [22]. Calero et al. reported that the ionic gelation produces beads with sizes ranging from 1 to 5 mm [3]. The sizes of sodium alginate beads obtained in this study are within this

range. The analysis in the light microscope with 40x magnification showed that in the interior of the sphere there is a reticular structure (Figure 1-A). The stereoscopic microscope beads obtained by ionic gelation showed the formation of entrapment matrices with well-defined spherical structure with a hollow interior (Figure 1-B).

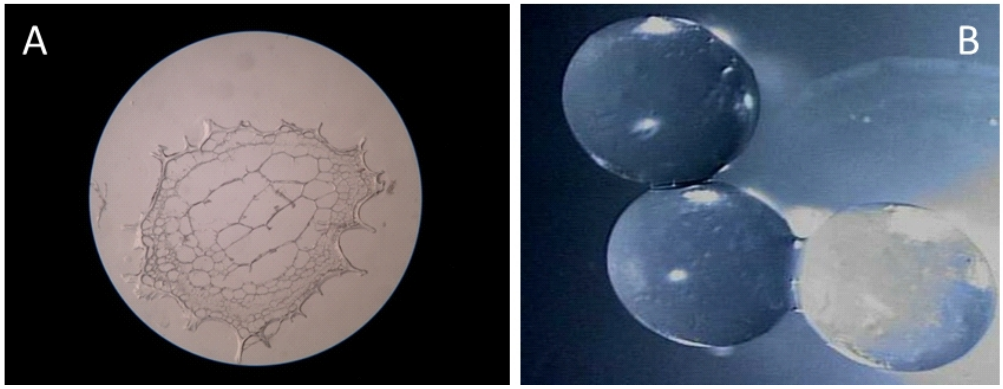
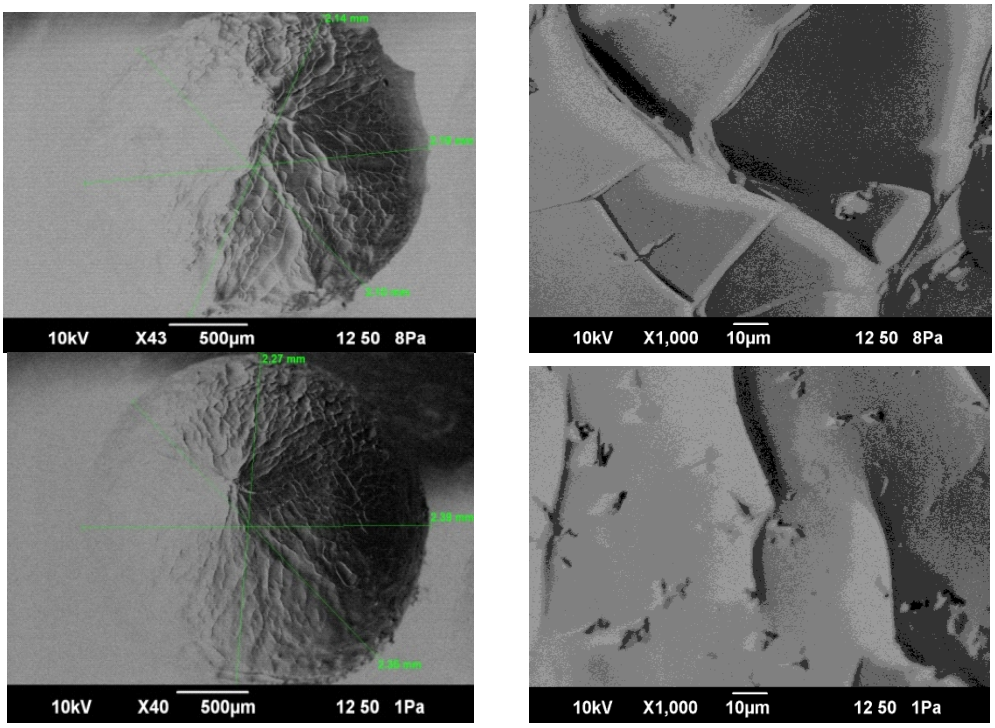


Figure1. Optical micrograph of 1% calcium alginate beads at 40X magnification (A), and by stereomicroscopy at 32X magnification (B).

Scanning electron microscopy (SEM)

The SEM analysis of the alginate spheres showed a surface with less wrinkles as the alginate concentration increased during the spheres formation. Sankalia et al. (2005) reported that the augment in sodium alginate concentration from 1.0 to 2.0% caused an increase in viscosity and slowed the calcium penetration into the area, with a lower crosslinking, and a reduction in porosity and roughness in the bead surface [15]. Similar results were obtained in this study (Figure 2 A, B, C) with 1.0, 1.5 and 2.0% sodium alginate, the surface roughness of the sphere was lower compared to the 1.0% alginate developed areas.



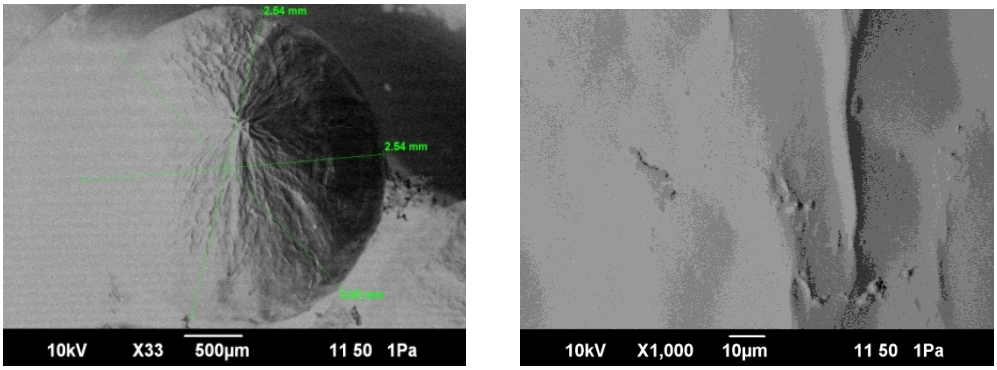


Figure 2. SEM micrographs of the surfaces of spheres with different concentrations of alginate: (A) 1.0% (B) 1.5% and (C) 2.0%.

Effect of pH on mexicain proteolytic activity

Figure 3 shows the effect of pH on proteolytic activity of free and immobilized mexicain. Proteolytic activity for the free enzyme increases gradually to a maximum value of 20,364 TU/mL at pH 7.5-8.0 and then gradually decreases as the pH is increased from 8.5 to10 units; the free mexicain retains 59.5% of proteolytic activity at pH 10 and 14.14% at pH 5.0. By the other hand, the maximum proteolytic activity for the immobilized enzymes was obtained in the pH interval of 8.5 to 9.5 units, with a maximum proteolytic activity of 22,433 TU/mL. Throughout the pH range tested, the proteolytic activity of the immobilized enzymes was always greater than the proteolytic activity of the free enzymes; except at pH 7.5, where the proteolytic activity of both enzymes was practically the same; phenomenon that has been observed by other investigators [23], in others enzymatic systems. At pH 5.0 the immobilized enzyme retains the 17.73% of the proteolytic activity that present at pH 7.5, and they were 8.89% more activity at pH 10 than at pH 7.5.

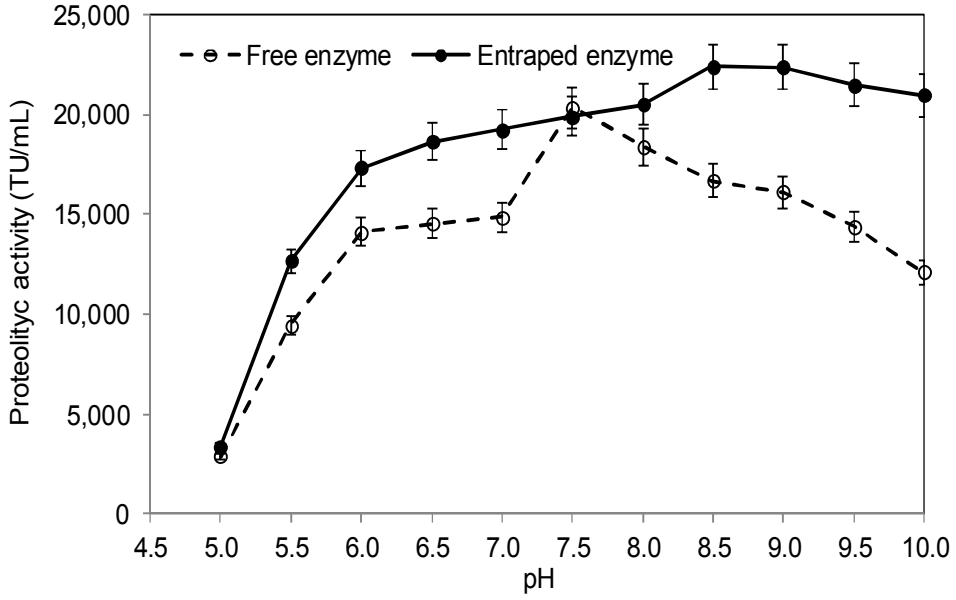


Figure 3. Effect of pH on the proteolytic activity of mexicain beads (1% casein in 0.05 M phosphate buffer at 35°C).

The difference between the behavior of free and immobilized enzymes is a consequence of the greater stability conferred on them by sodium alginate capsules. The optimum pH for free enzyme is within the range of optimal pH values (pH 7-8) for cysteine proteases like papain and for serine enzymes like trypsin [24]. In studies by Lei et al. (2004) [25], the proteolytic activity was determined at several pH values from 5.0 to 10.0, at 40 °C, for free and immobilized papain on magnetic microspheres, and it was found to be optimal for the free soluble enzyme at pH 6.5, and for the immobilized enzyme the pH optimum was at

pH 8.0. These obtained results are similar to those from Lei et al.,[24], mexicain a cysteine protease comes from another plant from the same Caricaceae family and both immobilized enzymes, papain and mexicain, exhibit good adaptability to alkaline media, in a similar way.

Effect of temperature on mexicain proteolytic activity

The effect of temperature on proteolytic activity for the free and immobilized mexicain is shown in Figure 4. It is observed that the optimum temperature for free mexicain is around 40°C, while the immobilized enzyme showed greater activity at 65°C. The immobilized form of the enzyme exhibits proteolytic activity at a higher temperature range than that exhibited by the free enzyme. Studies of Lei et al. (2004) [24] showed that the optimum temperatures for the best proteolytic activity of free and immobilized papain on magnetic microspheres were 65°C and 80°C, respectively; showing that the immobilized enzymes exhibit better resistance to high temperatures comparatively with the free proteases.

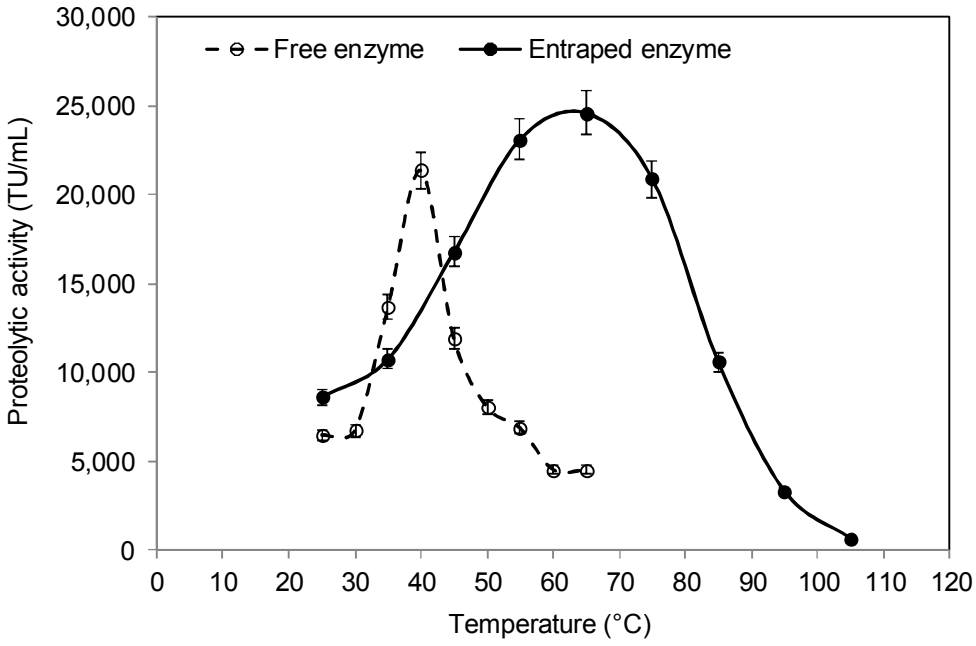


Figure 4. Effect of temperature on the proteolytic activity of mexicain (1% casein in 0.05 M phosphate buffer).

Several reports indicate an increase in stability to temperature of immobilized enzymes, particularly in the case of proteolytic enzymes, which in the free state present a self-digestion process. Wang et al. (1979) [26], mention that the immobilization of the enzymes can diminish the self-digestion phenomen in a high grade. The optimum temperature of an enzyme is an operational parameter rather than a real feature [27]. The optimum temperature for an immobilized enzyme is a characteristic parameter of the immobilized enzyme. Chang and Huang (2005) [23] mentions that the immobilization of the enzyme causes an increase in the rigidity of the enzyme, which is commonly reflected by an increase in the activity of some enzymes.

Entrapment efficiency and production yield

Table 2 shows that the entrapment efficiency was directly proportional to the amount of used polymer, with entrapment efficiencies of 90.7% for 2% sodium alginate; 88.4% for 1.5% alginate, and 87.6% efficiency with 1% sodium alginate. These results can be attributed to an increase in polymer concentration, which leads to a more complete gel with a stronger matrix structure, as a consequence of the higher concentration of glucuronic acid units that react with calcium ions.

Table 2. Effect of sodium alginate concentration on entrapment efficiency and production performance

Sodium alginate (%)	Entrapment efficiency (%)	Production yield (%)
1.0	87.61 ± 0.2	94.29 ± 2.71
1.5	88.45 ± 0.5	90.71 ± 2.06
2.0	90.79 ± 0.3	85.83 ± 1.13

Roy and Gupta (2004) [28] have reported an entrapment efficiency of 89% -92% for glucoamylases enzymes. We found it similar results in this study for the effect of high concentrations of alginate in the improvement of the encapsulation efficiency, i.e. with 2% sodium alginate, the enzyme entrapment was >90%. With 1% sodium alginate the production yield was increased. This dispersion was easier to handle because it has lower viscosity (387.6 cp) compared with alginate concentrations of 1.5% and 2.0%, which have viscosity levels of 1346 and 3240 cp, respectively. Our results agrees with those obtained by other authors [29, 30, 31], who indicate that the most important factors that affect the entrapment efficiency and production yield are the concentration of polymer, the polymer type and the timing of maturation.
Figure 5 shows the response surface plot for the immobilization parameter depending on the concentration of sodium alginate and calcium chloride. The response surface plot shows that the increase in the calcium chloride concentration corresponds to a decrease in the rate of immobilization, while concentrations of 2% sodium alginate show an increase.

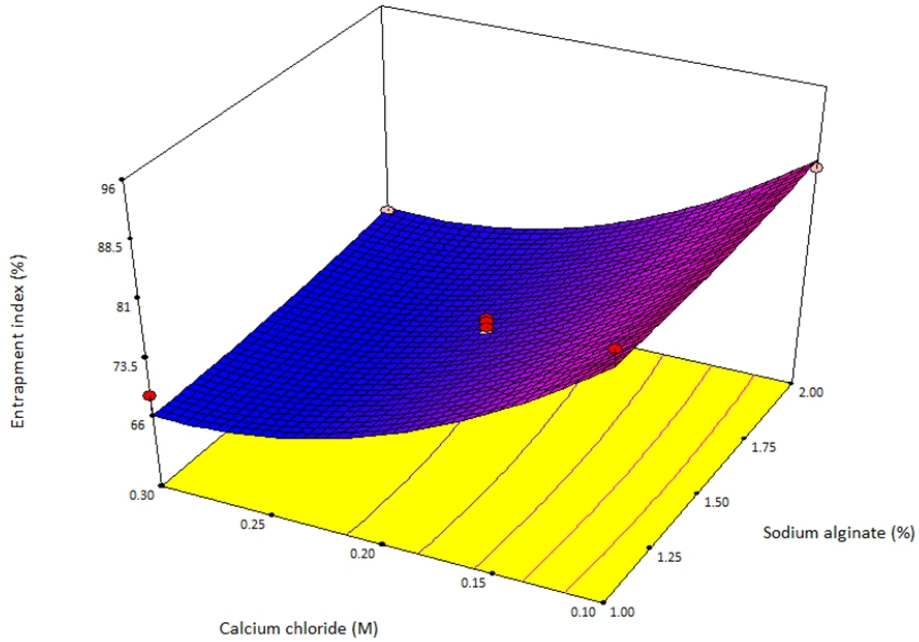


Figure 5. Response surface for the mexicain entrapment index with respect to the concentration of calcium chloride and sodium alginate.

Equation 3 represents the behavior of the immobilization process with respect to the entrapment index (EI) as the response variable. A represent sodium alginate concentration (%) and B: $CaCl_2$ concentration (M).

$$EI = 70.85 + 0.68A - 9.72B + 0.67AB + 0.63A^2 + 5.70B^2 \ldots (3)$$

The analysis variance of the entrapment index model indicates that the linear and quadratic parameter of the concentration of calcium chloride (B) had a significant effect on this response, whereas the interactions between alginate and the calcium chloride did not significantly affect the entrapment index. The highest entrapment index was obtained by using an intermediate concentration of sodium alginate (1.5%) and a very low concentration of $CaCl_2$ (0.06 M).

Proteolytic activity

Figure 6 shows the results of proteolytic activity at time zero. The evaluation of the immobilized mexicain proteolytic activity at time zero (AP_0) in the response surface analysis is represented by Equation 4.

$$APo=20271 - 1819A - 2232B - 1470AB - 1579A^2 + 1069B^2 \ldots (4)$$

The variance analysis of the AP_0 model indicates that the linear and quadratic parameter of the concentration of sodium alginate (A) and calcium chloride (B) has no significant effect ($p<0.05$) compared to the response. Likewise, interactions between alginate and the calcium chloride did not significantly affect the response of AP_0.

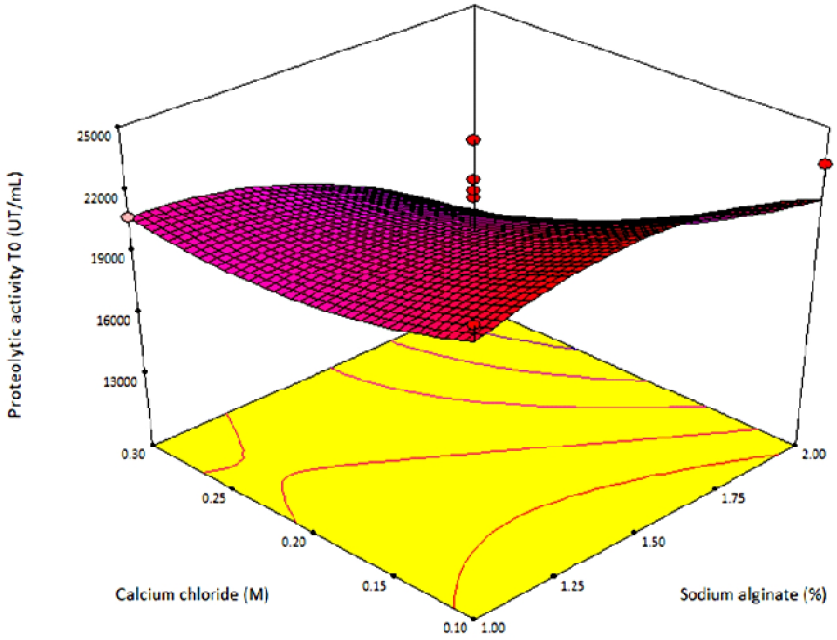


Figure 6. Response surface for the proteolytic activity of immobilized mexicain evaluated at time zero.

For assessment of proteolytic activity at three months of storage using the response surface methodology was performed analysis of variance, where the linear term in the quadratic model, which has to do with the concentration of calcium chloride (term B Equation 5) showed a significant effect on proteolytic activity in the values of "Prob> F". The other linear and quadratic terms, and their interaction, had no significant effect ($p<0.05$).

$$AP = 4129 - 1037A - 21481B + 2321AB + 735A^2 + 1989B^2 \quad ...(5)$$

It was observed that the beads prepared with low concentrations of sodium alginate (1.0%) and low concentrations of $CaCl_2$. (0.1 M) showed the highest proteolytic activity (13694.3 UT/mL), while the lowest activity value (3280 UT/mL) was obtained by using an intermediate concentration of alginate (1.5%) and a concentration of 0.34M $CaCl_2$ (Table 3).

Table 3. Matrix of experiments and results for the response surface central composite design

Run	Size (mm)	Compression (g)	Entrapment efficiency (%)	Proteolytic[a] activity (TU/mL)	Proteolytic[b] activity (TU/mL)
1	1.63	35,515	88.91	23,107	13,694
2	2.00	24,256	85.83	23,277	5,495
3	1.93	23,716	68.78	20,798	5,333
4	2.03	22,438	68.37	15,089	6,415
5	1.50	25,252	68.18	19,494	4,967
6	2.20	24,158	74.47	13,121	4,132
7	1.90	14,950	95.66	24,204	10,173
8	1.80	18,924	67.28	19,000	3,279
Central point	1.98	24,619	70.86	23,033	4,128

a: Initial proteolytic activity; b: Proteolytic activity after three months storage.

The response surface shows the increase in proteolytic activity as the concentration of calcium chloride and alginate decreases (Figure 7).

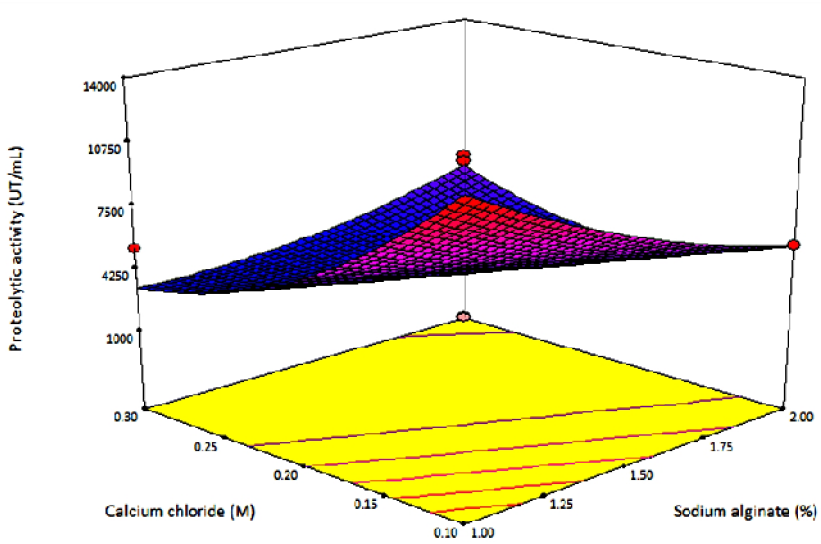


Figure 7. Response surface for the proteolytic activity of immobilized mexicain evaluated after 3 months of storage.

The analysis of the proteolytic activity indicates that occurs an oxidation during the immobilization process, which can be attributed to the cysteine enzymes strong tendency to oxidation [32], besides an incorporation of oxygen is expected from the mixing up of the alginate and the enzyme, nevertheless proteinases with a cysteine residue in their active center recover the activity with the addition of thiol compounds [9].

The immobilized mexicain can be stored at 6 ° C for 11 weeks with minimal loss of enzyme activity compared to its soluble counterpart. Lei et. al. (2004) [25] observed a similar stability pattern in immobilized papain, concluding that immobilization provides better stability during storage than the obtained with free enzyme and attributed these result to the prevention of autolysis as a result of fixing the molecules of the enzyme to the polymeric support.

Reusability of immobilized mexicain

To determine the reusability of the immobilized mexicain, we determined the proteolytic activity of several batches of beads under the same conditions. The residual activity of different batches of beads is shown in Figure 8. The operational stability study for immobilized mexicain shows a residual activity of 44% after being reused five times. It was assumed the activity of beads in the first cycle (time zero) as 100%.

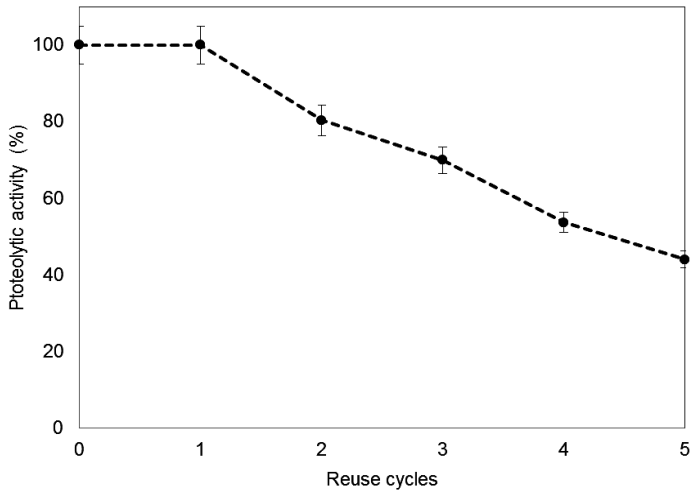


Figure 8. The operational stability of mexicain as measured by the residual proteoytic activity at different reuse cycles

The number of reuses here presented is below those reported by other authors. Lei et. al. (2004) [25] showed that papain on magnetic beads retained 70% of the initial activity after having been used ten times. The differences in the number of reuses and the residual activity level, between mexicain and papain, may be due to the different susceptibility to physical damage of the alginate beads compared to that of the magnetic support.

Similarly Mansour and Dawoud (2003) [33] have reported values of residual activity of 98% for proteases of fungal origin (invertase) after 18 cycles, using celite and polyacrylamide as a support for immobilization. Also Won et al. (2005) [34] obtained a relative activity of 72% after three cycles of action of lipase immobilized in alginate spheres coated with chitosan and silica. All these authors agree that repeated use of entrapped enzymes, results in loss of activity due to possible release of the enzyme from the alginate spheres, in addition to the potential mechanical damage when used repeatedly. From the foregoing it is concluded that the residual enzyme activity levels obtained in immobilization systems will depend on the type and properties of the enzyme and its interaction with the support used.

CONCLUSIONS

Immobilized mexicain was comparatively characterized with the free soluble enzyme in terms of the pH and temperature of optimal activity. The immobilized mexicain were more stable than the free enzymes. The stability limits of the immobilized enzymes were within a pH range from 5.0 to 10 and temperatures between 25°C to 65°C. The highest encapsulation efficiency (90.8%) was obtained by using 2% sodium alginate, while the higher production yield (94.3%) was found at a concentration of 1% sodium alginate. It was observed that the beads prepared with low concentrations of sodium alginate (1.0%) and low concentrations of $CaCl_2$. (0.1 M) showed the highest proteolytic activity after 3 months of storage. After 5 reused cycles, the immobilized mexicain retained 44% of its initial proteolytic activity.

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