Food Science and Technology
ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)
DOI: https://doi.org/10.1590/fst.31518

Study of the parameters used in the encapsulation of commercial pectinase in calcium alginate and its effect on its catalytic activity

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Abstract

Despite the high catalytic properties of pectinases, the utilization of free enzymes always presents some hindrances such as low stability, a difficulty for product recovery and the impossibility of continuous use. Enzyme encapsulation is one of the methods used to overcome these limitations; however, some kind of effect is expected to occur on its catalytic activity. The objective of this work was to study the parameters involved in the process of encapsulation of a commercial pectinase product in calcium alginate and the effect of this encapsulation on its catalytic activity. The effect of the parameters of sodium alginate, calcium chloride, enzyme concentration and reaction time on enzymatic activity and encapsulation yield were also evaluated. The effect of pH and temperature on the activity of the free and encapsulated enzyme was studied. The highest yield of immobilization was obtained with a concentration of enzyme solution of 4%. Free and encapsulated enzymes showed similar behavior regarding the catalytic activity. The encapsulated enzyme had a narrower pH range (pH 4.0) than the free enzyme (pH 3.0 to 5.0). Besides, the encapsulated enzyme showed an increase in the stability in the pH range between 7 and 8 and above 10 to 12.

Keywords: pectinase; encapsulation; parameters; pH; temperature.

Practical Application: Pectin hydrolysis in the juices, wine and paper industries, among others.

1 Introduction

The pectinolytic enzymes have many applications in different industries such as fruits and vegetable juices, wine, textile, and paper and pulp industries (Rehman et al., 2014; Gummadi & Panda, 2003; Kashyap et al., 2001). Pectinase is a generic term used for the complex group of enzymes that catalyze the hydrolysis of pectin by breaking a-1-4-glycosidic linkage of galacturonic acid to decrease the viscosity which is responsible for causing turbidity and undesirable cloudiness in fruits juices (Rehman et al., 2013; Kashyap et al., 2001).

Despite the high catalytic properties of pectinase, some problems are found in the use of enzymes in industrial processes such as poor stability under operational conditions, their susceptibility to the process conditions, difficulty for product recovery, impossibility of multiple reuses in an industrial process and the presence of inhibitory compounds, even in low concentrations (Lei et al., 2015; Rehman et al., 2013). Several methods have been proposed to overcome these limitations, and among them, the immobilization method stands out (Rehman et al., 2013; Mohamad et al., 2015; Krajewska, 2004).

Enzymes may be immobilized by various methods that can be classified as physical, with a weak interaction between enzyme and support, and chemicals whose covalent bonds are formed with the enzyme. Some examples of physical methods are: containment of an enzyme within a membrane reactor, adsorption (physical, ionic) on a water-insoluble matrix, inclusion (or gel entrapment), microencapsulation with a solid membrane (Krajewska, 2004).

When an enzyme is added to an inert material, some kind of effect is expected to occur on its catalytic activity. These effects may be steric and conformational, may interfere with the diffusional microenvironment and may affect the catalytic efficiency and the characteristics of the enzyme as optimal pH and temperature (Mohamad et al., 2015). The objective of this work was to study the parameters involved in the process of encapsulation of a commercial pectinase product in calcium alginate and the effect of this encapsulation on its catalytic activity.

2 Materials and methods

2.1 Enzyme

Commercial pectinolytic enzyme Pectinex® Ultra Clear (*Aspergillus acuteatus, Aspergillus niger*) (Novozymes Latin America Ltda) was used for the experiments. This enzyme was selected among other commercial enzymes because it presented a more significant increase of clarity when applied in cagaita (*Eugenia dysenterica* DC) pulp as preliminary work (Cardoso et al., 2014).

Received 11 Oct., 2018
Accepted 06 Nov., 2018
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2.2 Analysis of pectinase activity

The pectinase activity of the free and immobilized enzymes was determined by spectrophotometry at 540 nm using the modified dinitrosalicylic acid method (Oliveira et al., 2012). For the enzymatic reaction, citric pectin was used as the substrate for 30 min. One unit of activity was defined as the amount of sample required to release 1 μmol of galacturonic acid released per minute of reaction. The analyzes were performed in triplicate. The difference between the means was evaluated by ANOVA followed by Tukey test, being considered significant those with $p \leq 0.05$.

2.3 Effects of pH and temperature on pectinase.

The pH effect of the pectinase was studied from pH 3.0 to pH 12.0, and the buffers with different pH were mixed with the purified protease at a ratio of 1:1. The enzyme activity was determined by the assay method described previously.

The reaction mixture was incubated at different temperatures ranging from 30 to 90 °C at optimum pH determined previously.

The analyzes were performed in triplicate. The difference between the means was evaluated by ANOVA followed by Tukey test, being considered significant those with $p \leq 0.05$.

2.4 pH and thermal stability

Pectinase was pre-incubated at pH 3 to pH 12 at 10 °C for 24h to study pH stability. After this time the residual activity of each treatment was determined by the assay method described previously.

Pectinase was pre-incubated at 30 to 90 °C for 1 h to study thermal stability. After this time the residual activity of each treatment was determined by the assay method described previously.

The analyzes were performed in triplicate. The difference between the means was evaluated by ANOVA followed by Tukey test, being considered significant those with $p \leq 0.05$.

2.5 Calcium alginate capsules

The capsules were obtained by dripping sodium alginate solution and a buffer solution containing enzymes in a calcium chloride solution using Cole Parmer peristaltic pump - Model 7523-80, Masterflex YZ-06475-14 L/S 14 hose and needles 0.80 x 25 mm.

Equal volumes of sodium alginate and commercial enzymes diluted in citrate-phosphate buffer pH 4.0 were homogenized at 150 rpm for 5 min in a Fisatom 713D homogenizer. Two mL of sodium alginate solution and enzyme were dripped at a flow rate of 2 mL/min in 20 mL of calcium chloride solution. The formed capsules were submerged in calcium chloride solution at 30, 60 or 90 minutes in a refrigerator at 4 °C. The capsules were then washed in deionized water and stored in citrate-phosphate buffer solution pH 4.0. The calcium chloride solution and the deionized water used during the capsule formation process were saved for analysis of protein content and pectinase activity (Rehman et al., 2013).

2.6 Experimental design of the parameters to be used in the process of formation of calcium alginate capsules

Factorial planning was used in 3 levels of variation with 4 repetitions of the central point of use of the software STATISTICA 7.0 for the preliminary study of the parameters evaluated during the encapsulation. The parameters evaluated were: concentrations of sodium alginate, calcium chloride and commercial enzyme Pectinex® Ultra Clear, reaction time and enzyme solution concentration on the encapsulation yield and the pectinase activity of the encapsulated enzyme. The parameters and values used are shown in Table 1.

2.7 Encapsulation yield

The amount of encapsulated enzyme was determined by a difference of the initial amount of protein in the diluted commercial enzyme used for the formation of the capsules by the amount of residual protein present in the sodium chloride solution and the water used in the washing of the capsules. Proteins were quantified by the Bradford method (Li et al., 2007).

2.8 Statistical analysis

The analysis of variance was performed with the GraphPad Prism software using Tukey's test as a post-test with confidence level $p < 0.05$. The multivariate statistical methodology was also performed. A Full Factorial Design (2n) was used in 3 levels of variation with 4 repetitions of the central point using the software STATISTICA 7.0 for the exploratory tests. (Rodrigues & Iemma, 2005).

3 Results and discussion

3.1 Preliminary evaluation of the parameters used in the encapsulation process

Pectinase activity and encapsulation yield

The evaluation of the parameters: sodium alginate concentration, calcium chloride concentration, enzymes solution concentration and reaction time in the capsule formation process was performed to determine which positively and significantly influenced the pectinase activity and the yield of encapsulation. The parameters that affected positively and significantly were used in the optimization of this process.

Only the enzyme concentration parameter was significant for the response encapsulation yield at the confidence level $p \leq 0.05$.

Table 1. Parameters and values used in the complete factorial design for the pectinase encapsulation process.

Parameter	-1	0	1
Sodium alginate concentration (%)	1	2.5	4
Calcium chloride concentration (M)	0.05	0.275	0.5
Reaction time (min)	30	60	90
Enzyme solution concentration (% v/v)	0.5	2.75	5

The analysis of variance was significant for the results obtained with encapsulation yield ($p \leq 0.05$ and $p \leq 0.01$). The coefficient of determination (R^2) presented a value of 0.91.

For the pectinase activity response, the enzyme concentration parameter was the only significant at the confidence level $p \leq 0.05$. The analysis of variance was significant for pectinase activity ($p \leq 0.05$). The coefficient of determination (R^2) presented a value of 0.94. Therefore, only the enzyme concentration significantly affected the encapsulation yield and enzyme activity at the level tested ($p \leq 0.05$) and was considered for encapsulation optimization.

Other works reported the influence of the concentration of sodium alginate and calcium chloride on the activity of the enzyme and the encapsulation yield using univariate analysis. Rehman et al. (2013) evaluated the influence of sodium alginate and calcium chloride concentration on the encapsulation yield of *Bacillus lichenformis* pectinase in calcium alginate. The results revealed that both the sodium alginate concentration and the calcium chloride concentration influenced the encapsulation yield. According to the authors, this maximum yield with 3% of sodium alginate concentration is due to the formation of stable cross-links that kept the encapsulated enzymes. Ganaie et al. (2014) immobilized fructosyltransferase in calcium alginate for the production of fructooligosaccharides. The highest fructosyltransferase activity was achieved using a solution of 3% sodium alginate and 1% calcium chloride. According to the authors, increasing the concentration of alginate above 3% would cause an increase in the interaction between the active site of the enzyme and the alginate, resulting in low activity of the enzyme.

3.2 Optimization of the parameters used in the encapsulation process

All parameters were set at their minimum values for the encapsulation process, except for the calcium chloride concentration. The use of the minimum values results in the use of less reagent quantity and shorter reaction time. Sodium alginate solutions with a concentration above 2% are very viscous making it difficult to pump.

According to Lee et al. (2000), alginate hydrogels can decrease up to 60% of their initial mechanical strength when exposed for more than 15 hours in physiological buffer solutions, which was attributed to the loss of divalent ions by the exchange with monovalent ions present in the solution.

Therefore, for optimization, 1% sodium alginate solution, 0.5 M calcium chloride solution and 30 min reaction time were used. For the optimization of the capsule formation process, enzyme concentrations ranging from 1 to 10% were used.

The higher the concentration of the enzyme used in the encapsulation process, the greater the activity presented by the encapsulated enzyme (Figure 1A). The increase in activity is possibly due to the accumulation of enzyme on the surface and in the pores of the calcium alginate capsule.

Increasing the concentration of enzyme in the formation of the calcium alginate capsules results in increased encapsulation yield to the concentration of 4% (Figure 1B). From this value, a decrease in yield ($p \leq 0.05$) occurred because concentrations would lead to greater loss of enzymes to the calcium chloride solution during the drip. Busto et al. (2006) immobilized commercial pectinases in calcium alginate capsules by adsorption. The highest yields of immobilization were obtained when 1.2% of the enzyme was used. According to the authors, at higher concentrations, there is a competition of the enzymes for the same binding sites.

Comparing the two dependent variables analyzed (pectinase activity and encapsulation yield), the 4% concentration was considered optimal for the commercial enzyme in the capsule formation process.

3.3 Effect of pH and temperature on the activity of the free commercial enzyme Pectinex® Ultra Clear

The free commercial Pectinex Ultra Clear enzyme showed an optimum pH of pectinase activity between 3 and 5 (Figure 2A). The pectinase activity, among these values, was not significantly different at the confidence level $p \leq 0.05$.

The free commercial Pectinex Ultra Clear enzyme showed a range of optimum activity temperatures between 40 and 50 °C (Figure 2B). During the subsequent analyzes of activity, the temperature of 40 °C was used. The lower the temperature used,

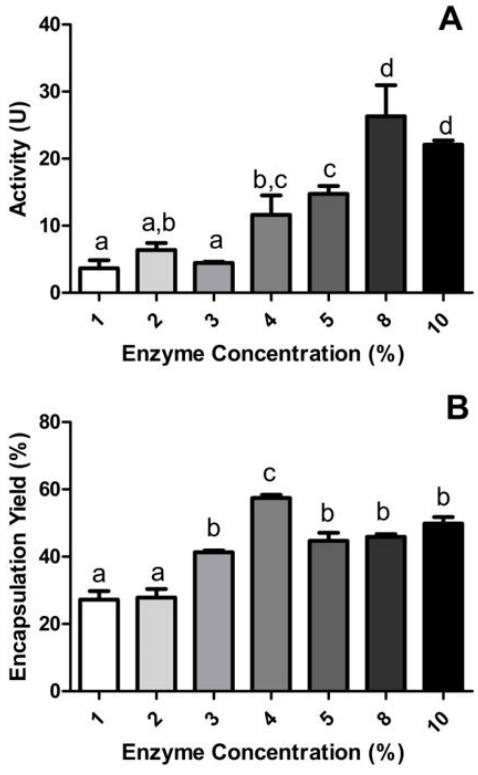

Figure 1. (A) Activity of pectinase and (B) Encapsulation Yield presented by the enzyme Pectinex® Ultra Clear encapsulated in calcium alginate using a different concentration of enzyme during its preparation. One unit of activity represents the formation of 1 µg of galacturonic acid/min of reaction Equal letters do not differ significantly from each other at the confidence level $p < 0.05$.

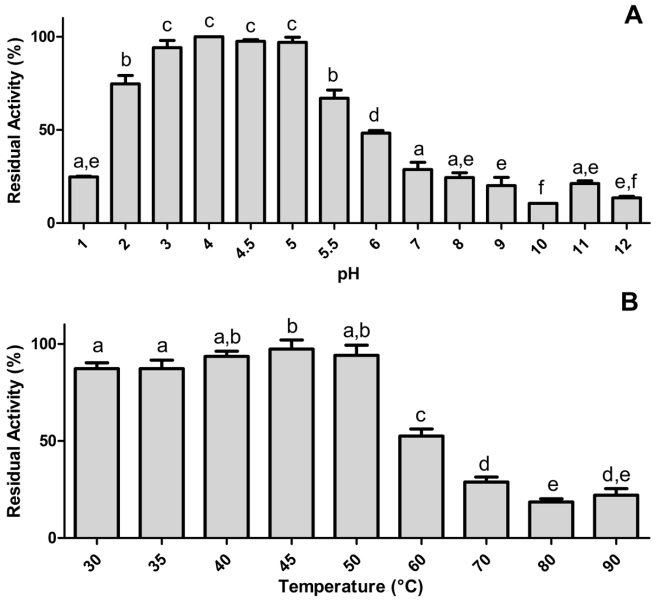

Figure 2. (A) Effect of pH on the activity of free enzyme Pectinex® Ultra Clear; (B) Effect of temperature on the activity of the free enzyme Pectinex® Ultra Clear. Equal letters do not differ significantly from each other at the confidence level $p < 0.05$.

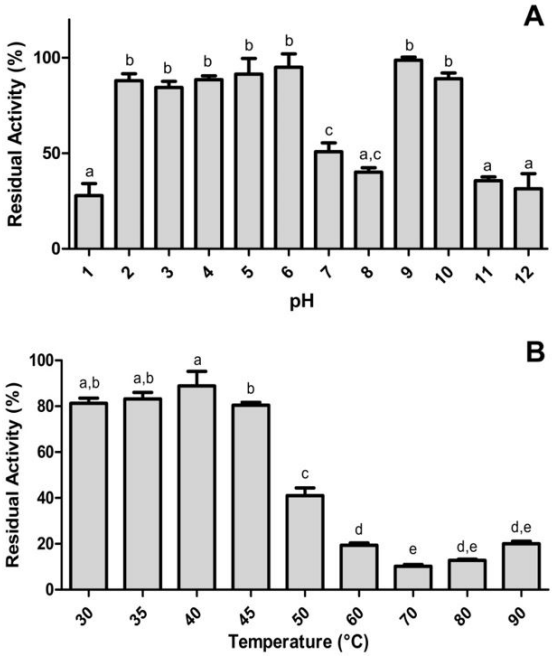

Figure 3. (A) pH stability of Pectinex® Ultra Clear commercial enzyme; (B) Pectinex® Ultra Clear commercial enzyme thermal stability. Equal letters do not differ significantly from each other at the confidence level $p < 0.05$.

the less alteration of the components of the medium, such as vitamins, pigments and aroma compounds.

Ahmed et al. (2016) purifying pectinase from Aspegillus niger obtained the optimum pH of 5.0 and an optimum temperature of 50 °C of enzyme activity.

Purified polygalacturonase from *Penicillium veridicatum* presented an optimum pH of 5.0 and an optimum temperature of 55 °C (Silva et al., 2002).

3.4 pH and thermal stability of the commercial enzyme Pectinex® Ultra Clear

The free commercial Pectinex Ultra Clear enzyme had pH stability between 2 and 6 and between 9 and 10 (Figure 3A). Encapsulated and free enzyme samples were stored in citrate-phosphate buffer pH 4.0. The two distinct pH stability ranges observed in Figure 3A may indicate the presence of isoenzymes in the commercial pectinase.

The free commercial Pectinex Ultra Clear enzyme had thermal stability between 30 and 45 after 1 h of incubation (Figure 3B).

The pectinase of *Penicillium viridicatum* RFC3 produced by solid-state fermentation by Silva et al. (2002) showed stability in the pH between 5.0 and 8.0 and stability in the temperature between 30 and 40 °C for polygalacturonase activity.

3.5 Effect of temperature and pH on the activity of the encapsulated enzyme Pectinex® Ultra Clear

After defining the parameters used in the encapsulation process of the enzyme, a characterization of the pH and temperature optimum and stability was performed to compare with the results obtained with the free enzyme.

After encapsulation, the enzyme showed narrowing in the optimum pH range of activity. The encapsulated enzyme presented higher activity at pH 4.0 (Figure 4A) while the free enzyme ranged from 3.0 to 5.0 (Figure 2A). It is possible that the enzyme's proximity to the negatively charged surface of the alginate caused a change in the ionization of the active site of the enzyme and / or in the conformation of the substrate, making enzyme-substrate fitting highly efficient only at pH 4.0 (Garg & Kumar, 2008). In general, both the free and encapsulated enzyme showed similar patterns. Both enzymes presented a marked reduction of activity from pH 6.0.

Other authors did not observe a difference between the optimal pH of the free and encapsulated enzyme. Anwar et al. (2009), immobilizing protease from *Baciilus subtilis* KOBGE-SAH on calcium alginate, found that there was no difference between the optimum pH (pH 7.5) of the free and encapsulated enzymes. According to Rehman et al. (2013), the immobilization of *Bacillus licheniformis* KIBGE-IB21 pectinase in calcium alginate did not alter the optimal pH of the enzyme, which was equal to 10.0. According to the authors, the free enzyme was more affected by the pH change in values below 7.0 due to the greater conformational stability reached by the enzyme immobilized inside the polymer alginate calcium network.

As with free Pectinex® Ultra Clear, the encapsulated enzyme had the highest activity between 40 and 50 °C (Figure 4B). From 70 °C, both the free and encapsulated enzyme presented a significant reduction ($p \leq 0.05$) of activity. Other authors also found no difference between the optimal temperature of the free enzyme and the encapsulated enzyme. Busto et al. (2006) did not

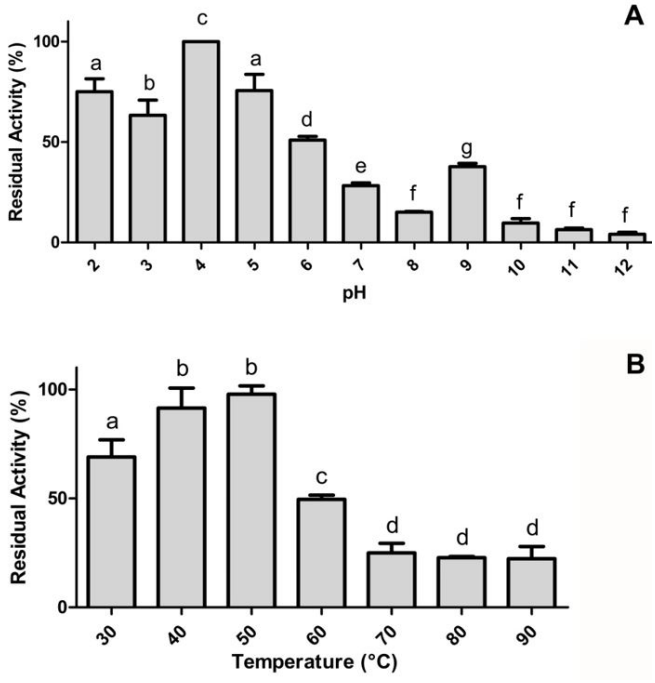

Figure 4. (A) Optimum pH of the encapsulated Pectinex® Ultra Clear commercial enzyme; (B) Optimum temperature of the encapsulated Pectinex® Ultra Clear commercial enzyme Equal letters does not differ significantly from each other at the confidence level $p < 0.05$.

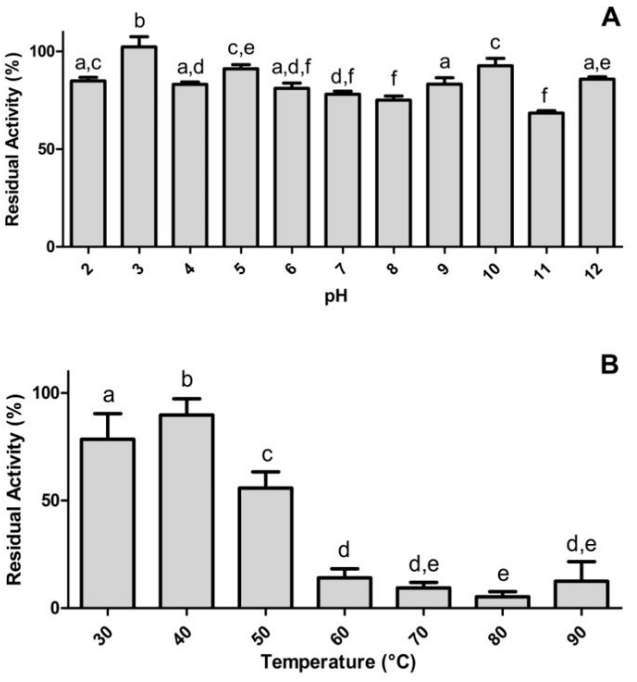

Figure 5. (A) pH Stability of the encapsulated Pectinex® Ultra Clear commercial enzyme; (B) Encapsulated Pectinex® Ultra Clear commercial enzyme thermal stability Equal letters do not differ significantly from each other at the confidence level $p < 0.05$.

notice a difference between the optimum temperature of 55 °C when using the free Rapidase C80 pectinase and encapsulated in calcium alginate. The authors report that the free enzyme maintained 80% of its maximum activity in a temperature range (37-80 °C) higher than the encapsulated (45-70 °C). Arya & Srivastava (2006) reported that after the immobilization of CGTase (cyclodextrin gliconotransferase) produced by *Bacillus macerans* ATCC 8244 in calcium alginate there was no variation of the optimum temperature of activity relative to the free enzyme that was 60 °C. The authors also reported that the enzyme pattern immobilized with temperature variation was very similar to the free enzyme as occurred in the present work. However, Rehman et al. (2013) observed a variation of the optimum temperature of activity after the immobilization of *Bacillus licheniformis* KIBGE-IB21 pectinase in calcium alginate from 45 °C, presented by the free enzyme, to 55 °C. The optimum temperature increase for encapsulated enzyme could be due to the physical limitation of the enzyme causing an increase in the activation energy required for the substrate to bind to the active site of the immobilized enzyme.

3.6 pH and thermal stability of encapsulated Pectinex® Ultra Clear

The encapsulated Pectinex Ultra Clear enzyme presented higher stability at pH 3.0 (Figure 5A). The free enzyme had a pH of stability between 2 and 6 and between 9 and 10 (Figure 3A). The encapsulated enzyme maintained 70% of its maximum activity in the pH range studied (2 to 12), differently than with the free enzyme, thus presenting higher pH stability after immobilization.

The encapsulated Pectinex Ultra Clear presented higher thermal stability at 40 °C (Figure 5B) while the free enzyme showed a temperature range between 30 and 40 °C (Figure 3B). The encapsulated enzyme maintained 55.8% of its maximal activity at 50 °C while the free enzyme 41.1%. From 60 °C, the two forms had a drastic reduction in their activity (below 20%). With the results obtained it can be affirmed that the encapsulation did not increase the stability of the enzyme against the increase of the temperature.

Ramirez et al. (2013) reported increased thermal stability of Aspergillus niger pectinase after encapsulation by adsorption on calcium alginate and chitin. The authors verified that free and encapsulated enzyme maintained their maximum activity up to 40 °C and 50 °C, respectively. The free enzyme was inactivated at 60 °C while the encapsulated enzyme still maintained 25% of its activity.

4 Conclusion

Immobilization of the enzyme Pectinex® Ultra Clear by encapsulation in calcium alginate was efficient. In the optimization of the encapsulation, the only significant variable was the enzymatic concentration. The best immobilization yield was obtained with a 4% enzymatic solution. The other conditions for immobilization of the enzyme were a concentration of 2% sodium alginate, 0.5M calcium chloride and reaction time of 30 min.

The characteristics of the encapsulated enzyme were similar to those of the free enzyme, presenting only differences in relation to the optimum pH range narrowing (4) and increased stability in the pH ranges between 7 and 8 and above 10 to 12.

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