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Effect of the pH in the formation of β-galactosidase microparticles produced by a spray-drying process

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Abstract

The objective of this work was to investigate the influence of pH in the microencapsulation process, using a modified chitosan to microencapsulate the enzyme β-galactosidase, by a spray-drying technique. Structural analysis of the surface of the particles was performed by Scanning Electron Microscopy (SEM), showing that the obtained microparticles have an average diameter smaller than 3.5 μm and in general a regular shape. The activity of the enzyme was studied by spectrophotometric methods using the substrate O-Nitrophenyl-β,D-galactopyranoside (ONPG). The parameters of Michaelis-Menten were calculated. The value of *Km* decreases with the decrease of the pH, which can be associated to an increase of the affinity between the enzyme and

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substrate to smaller pH´s. The highest value of the parameter *Vmax*, representing the maximum reaction rate at a given enzyme concentration, was obtained at pH 6.

Keywords: Microencapsulation, modified chitosan, β-galactosidase, spray-drying.

Introduction

The challenge of this work was to evaluate the effect of the pH on the β-galactosidase microencapsulation process, by a spray drying technique. We propose the use of a modified chitosan to encapsulate the enzyme β-galactosidase, which has a very important role in health and industry [1–6].

Many people experienced gastrointestinal disorders including abdominal distention, cramps, flatulence, and/or watery stools after the ingestion of milk or milk products, caused by a β-galactosidase deficient. Most people with this problem are not able to digest lactose well, they are discouraged from consuming milk, and by this way may lose a major source of calcium and high-quality proteins from their diets. For these lactose-intolerant people, hydrolyzed-lactose milk, cultured dairy products and sweet acidophilus milk that include microbial organisms producing β-galactosidase have been recommended as milk substitutes [7]. However some questions have been reported by some authors about these options. For example the hydrolyzed-lactose milk for lactase-deficient subjects has a sweeter taste than whole milk [7,8].

Encapsulation of β-galactosidase can be a solution. Microencapsulation can provide a physical barrier between the core compound and other components of the product. For example, the microencapsulation in liposomes, which can segregate β-galactosidase from lactose in milk under storage conditions. In this strategy of microencapsulation, lipid vesicles are carriers for the β-galactosidase enzymes, protecting them [7–9]. In such a lipid vesicle assisted lactose hydrolysis process, the entrapped enzyme is added to milk and is released into the stomach by the presence of bile salts, allowing an 'in situ' degradation of lactose [7,8].

An important factor in the microencapsulation process is the choice of an encapsulating agent, which is very important for the encapsulation efficiency and microcapsule stability.

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Chitosan is a widely used biopolymer [10,11]. Chitosan has interesting intrinsic properties, such as biocompatibility, biodegradability and also anticholesterolemic, hypocholesterolemic, antimicrobial, and antioxidant [12]. Modified Chitosan has been used for different microencapsulation processes considering the advantages of being soluble at neutral pH [13,14] but insoluble at acid pH. In order to develop this kind of chitosans, many attempts have been made to modify the molecular structure of chitosan, and thereby improve or control its properties [15–17].

Also the methodology and the experimental conditions will influence the type of microparticles that will be obtained. In this study, a spray-drying technique was used.

Spray drying is a relatively low cost technology, rapid, reproducible, allowing easy scale-up, when compared with other microencapsulation techniques, justifying the preference in industrial terms [18–21]. The process is flexible, offering substantial variation in microencapsulation matrix, is adaptable to commonly used processing equipment and produces particles of good quality. Spray drying production costs are lower than those associated with most other methods of encapsulation [22].

Using in the β-galactosidase microencapsulation process, a spray drying technique, we will simplify the process of obtaining β-galactosidase microencapsulated formulation, increasing the possibility of human application. Studies with β-galactosidase microencapsulated by a spray drying technique have already been developed by the authors, which optimized the spray drying methodology applicate to β-galactosidase and the selection of the encapsulating agent, in previous works [23,24] however is necessary to clarify how the pH of the immobilization can affect the activity of the enzyme. This study will focus in this question how the pH can affect the size, morphology of the β-galactosidase microparticles and activity of the enzyme.

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Experimental

Reagents

Water soluble chitosan (pharmaceutical grade water soluble chitosan) was obtained from China Eastar Group (Dong Chen) Co., Ltd ((Batch no. SH20091010). Water soluble chitosan was produced by carboxylation and had a deacetylation degree of 96.5% and a viscosity (1%, 25 ºC) of 5 mPa.s.

β-galactosidase enzyme (*Escherichia coli*) from Calbiochem (Cat 345,788 ; EC number: 3.2.1.23) with a specific activity of 955 U mg^{-1} protein and BSA (bovine serum albumin) were purchased from Sigma Aldrich (A7906-100g) . The enzyme substrate O-nitrophenyl β, D-galactopyranoside (ONPG) was purchased from Merck (ref 8.41747.0001).

Experimental conditions – Spray-drying process

The same type of procedure (methodology and operational conditions) was followed for all the types of microparticles prepared. All the solutions were prepared with deionised water at room temperature. Water soluble chitosan 1% (w/v) solutions were prepared with different pH (5.2, 6, 7. 8 and 9), after 2 hours agitation at 1200 rpm. The pH of the chitosan solution was adjusted with hydrochloric acid for different pH values.

A solution with a concentration of enzyme (0.1 mg mL^{-1}) was prepared from stock solution in phosphate buffer 0.08 M at pH 7.7. To the enzyme stock solution BSA was added to obtain a final concentration of 1 mg BSA mL^{-1}. BSA is used to stabilize some enzymes and to prevent adhesion of the enzyme to reaction tubes, pipet tips, and other vessels.

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The solution containing the enzyme (5 mL) was added and mixed with the chitosan aqueous solution (25 mL) at constant agitation speed of 1200 rpm, during 10 min at room temperature.

The five prepared chitosan-enzyme solutions (with different pH) were spray-dried using a spray-dryer BÜCHI B-290 advanced (Flawil, Switzerland) with a standard 0.5 mm nozzle. The spray-drying conditions, solution and air flow rates, air pressure and inlet temperature were set at 4 mL min^{-1} (15%), 32 m^3 h^{-1} (80%), 6.5 bar and 115 ºC, respectively. The outlet temperature, a consequence of the other experimental conditions and of the solution properties, was around 58 ºC.

Scanning electron microscopy characterization

Structural analysis of the surface of the particles was performed by Scanning Electron Microscopy (SEM) (Fei Quanta 400 FEG ESEM/EDAX Pegasus X4M). The surface structure of the particles was observed by SEM after sample preparation by pulverization of gold in a Jeol JFC 100 apparatus at Centro de Materiais da Universidade do Porto (CEMUP).

β-galactosidase activity

The activity of the β-galactosidase was measured according to the methodology described by Switzer and Garrity [25]. The enzyme activity was evaluated, based on absorbance values, by UV-visible spectrophotometry (UV-1700 - PharmaSpec - SHIMADZU) at 420 nm and at room temperature.

The enzyme activity was tested with the substrate ONPG. A stock solution of ONPG was prepared with a concentration of 2.25 mmol L^{-1}. Then, the enzyme was exposed to

different ONPG concentrations (0.225, 0.198, 0.180, 0.135, 0.090, 0.068, 0.045 and 0.018 mmol L^{-1}).

The enzymatic reaction started by adding the enzyme solution (either in the free form, or in the microencapsulated form) to the cuvette containing the buffer solution and the substrate ONPG. The reaction volume was kept constant in all the experiments and equal to 2.5 mL. The cuvette was stirred for 20 s. The formation of an orange coloured product [O-nitrophenol (ONP)] that absorbs at 420 nm allowed the monitoring of the enzymatic reaction. The value of the absorbance was recorded at time intervals of 30 s. The enzyme concentration, in the microencapsulated enzyme assays, was estimated by mass balance and corresponds to the same value used in the free enzyme assays (enzyme concentration 0.001 mg mL^{-1}).

Determination of β-galactosidase kinetic parameters

For an enzyme concentration of 0.001 mg mL^{-1}, several concentrations of ONPG have been tested between 0.018 and 0.225 mmol L^{-1}. For each β-galactosidase reaction curve the initial velocity was calculated, according to the methodology described by Switzer and Garrity [25]. A linear regression method, Lineweaver-Burk method, was performed to determine the Michaelis-Menten parameters.

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Results and Discussion

The spray drying methodology and the operation conditions were optimized based on preliminary studies [23,24,26]. The product yield (quantity of powder recovered reported to the quantity of raw materials) was in average 30%. This value is low. The authors have already reported yields ranging between 30% and 50% for the microencapsulation of β-galactosidase with different biopolymers [23,24]. Erdinc & Neufeld (2011) referred that at inlet temperatures of 150 and 175°C, higher moisture removal and product yield was observed. The final moisture content of the particles was around 6%, and the product yield was 35% [27]. In this work the inlet temperature is lower (115 ºC) increasing the probability of obtaining low product yields. At low temperatures the deposition of particles on the cylinder or/and on the cyclone wall was observed, leading to a lower product yield. On the other hand the particles formed are very small, and the efficiency of the cyclone to separate small particles decreases, some of them being aspirated with the air leaving the spray dryer. The sample volume was small (30 ml) implying also higher relative losses.

The prepared microparticles were characterized and the enzymatic activity was evaluated. Structural analysis of the surface of the particles was performed by SEM (Figure 1), showing that the obtained microparticles have an average diameter smaller than 3.5 μm, and in general a smooth surface and a regular shape. The diameter of the particles was confirmed by laser granulometry using a Coulter Counter-LS 230 Particle Size Analyser (Miami, USA). For all the assays, microparticles with an average size (differential volume distribution) around 3.4 – 3.5 μm with a variation coefficient of distribution around 55% were obtained. For a differential number distribution the average size of the microparticles is around 0.10 – 0.11 μm with a variation coefficient of the distribution around 100%. It was not observed any significant difference in the size of the

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169 microparticles obtained with different pH. On the other hand, the increase of the pH of
170 the modified chitosan solution allowed the formation of microparticles more defined, and
171 with a more regular shape (Figure 2). Further, differences in the dispersibility of the
172 microparticles in water due to pH were not observed.
173 With the present work we also intend to compare the behaviour of the enzyme β-
174 galactosidase when microencapsulated with different solutions of modified chitosan with
175 different pH. The success of the β-galactosidase microencapsulation depends on various
176 factors such as pH, ionic strength, surface and protein properties such as isoelectric point
177 of the protein and history of dependence of protein-adsorption kinetics [28]. Since the
178 activity of β-galactosidase may be significantly reduced or lost during the
179 microencapsulation process, the selection of the encapsulating agent and the pH are very
180 important. The pH is an important factor that significantly influences encapsulation
181 efficiency [29]. β-galactosidase works in a relatively broad pH range: enzymes from fungi
182 act between pH 2.5–5.4, yeast and bacterial enzymes act between pH 6.0–7.0. Depending
183 on the natural source where lactose is present, pH values range between ~ 3.5 or 5.6 of
184 acid whey to 6.5 of milk. [28]. The isoelectric point of β-galactosidase is around 4.6 [30].
185 Chitosan is a positive polymer in acidic solutions, and its positive potential decreases with
186 increasing solution pH [29]. In experimental works with α-galactosidase (isoelectric
187 point also 4.6), the positive potential of α-galactosidase decreased when the pH was
188 increased from 3.0 to 4.5, after which the repellent force between chitosan and α-
189 galactosidase weakened. [29]
190 In this work a modified chitosan was tested, which is a less positive polymer in acid
191 solutions than the normal chitosan; on the other hand β-galactosidase from a bacterial
192 source was used, these enzymes acting better between pH 6.0–7.0. So, our kinetic results
193 will be obtained for a combination of factors.

For the free enzyme, in a previous study [23], the highest value of the enzyme activity was obtained at pH 6.8, which is in agreement with results obtained by other authors [31]. In Figure 3, the evolution of the enzymatic reaction with time for the microencapsulated enzyme formed was observed. The highest velocity was reached when the enzyme was microencapsulated at pH 6. With the increase of the pH for values higher than 6, the velocity decreases and the same happened when the pH decreases for values lower than 6. So the optimal pH to do the microencapsulation of the β-galactosidase with this modified chitosan is around pH 6.

This can be explained by the fact that the enzyme β-galactosidase has two active-site carboxyl groups that can exist as $–COO^-$ (as nucleophile) and –COOH (as proton donor) simultaneously at neutral pH [32] but also it depends on the amount of carboxyl and amino group in chitosan. For example, some groups of chitosan can be charged more positively by the effect of the decrease of the pH and can establish interactions with some groups of the enzyme charged more negatively. The interactions between enzyme and chitosan can change the conformation of the enzyme and/or can make difficult the access to the active center of the enzyme, by this way the activity of the enzyme will decrease.

So, different pH will influence the structure of the enzyme and of the encapsulating agent and the type of interactions between them and as referred before, the pH of the solution will affect the strength of the interaction between chitosan and β-galactosidase.

For each β-galactosidase reaction curve, the initial velocity was calculated, and the Lineweaver-Burk linearization was performed to determine the Michaelis-Menten parameters (Figure 4).

The Michaelis-Menten parameters were determined for the microencapsulated formulations with different pH and are presented in Table 1. The values related to the free enzyme have already been determined by the authors [26].

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The parameter *Vmax*, representing the maximum reaction rate at a given enzyme concentration, decreased its value after microencapsulation process thus confirming what has been observed by other authors [26,33]. Some active centres are likely to be blocked after microencapsulation, which reduces the reaction rate, causing the decrease of the maximum reaction velocity. The highest value of the *Vmax* was obtained from the microencapsulated β-galactosidase formulation obtained at pH 6, being more than four times higher than the *Vmax* obtained from the formulations produced with different pH (Figure 5). However, this value is smaller than the *Vmax* obtained with the free enzyme [26].

The parameter *Km* was associated to the affinity between the enzyme and the substrate. A smaller value of *Km* indicated a greater affinity between the enzyme and substrate, and it means that the reaction rate reaches *Vmax* faster. The value of *Km* increased in these assays of microencapsulation assays with pH of the microencapsulation formulation, this means that the affinity between the enzyme and the substrate decreased (Figure 6). A linear correlation between the value of Km and the pH of the β-galactosidase microencapsulation solution was obtained.

The β-galactosidase immobilization on chitosan was studied by Carrara and Rubiolo [34]. These authors obtained chitosan beads of 2.2 mm diameter, bigger than the microparticles that we obtained in this work. The higher activity value of the immobilized enzyme compared with those of the free β-galactosidase is only 10.7% of the free enzyme values. In our study, for a microencapsulation process at pH 6, the enzyme keeps 55% of the activity of the free enzyme. A different pH provoked a decrease in the activity of the enzyme.

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After six months storage at controlled ambient conditions (4ºC), a small decrease in enzyme activity was observed, as described in a previous work [23], and no significant differences in the appearance, color, and particle size distribution were identified.

Comparing the results obtained in this study with the previous ones [23,24,26], we can conclude that the selection of the pH for the immobilization (microencapsulation) of the enzyme is so important as the selection of the encapsulating agent or the selection of the operational conditions of the spray dryer for the optimization of the β-galactosidase activity.

Conclusion

The main objective of this work was to study the influence of pH in the β-galactosidase microencapsulation process, with a modified chitosan through a spray-drying process.

β-galactosidase microparticles with an average diameter smaller than 3.5 μm and in general a regular shape were obtained.

The parameters of Michaelis-Menten were calculated for all the β-galactosidase formulations. The value of *Km* decreases with the decrease of the pH, which can be related to an increase of the affinity between the enzyme and substrate to smaller pH´s.

The highest value of the *Vmax* was obtained for the microencapsulated β-galactosidase formulation obtained at pH 6, being more than four times higher than the *Vmax* obtained for the formulations produced with different pH. However this value is smaller than the *Vmax* obtained with the free enzyme. For a microencapsulation process at pH 6, the enzyme keeps 55% of the activity of the free enzyme.

Acknowledgments

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TABLE CAPTIONS

Table 1: Michaelis-Menten parameters (*Km* and *Vmax*), for the different assays with free and microencapsulated β-galactosidase.

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FIGURE CAPTIONS

Figure 1: SEM images of β-galactosidase microparticles prepared at different pH´s (5.2, 6, 7, 8 and 9). Magnification = 12000 times, beam intensity (HV) = 15kV, distance between the sample and the lens (WD) = 15 mm.

Figure 2: Surface and shape of the β-galactosidase microparticles prepared at pH´s 5.2 and 9.

Figure 3: Evolution of the enzymatic reaction with time for β-galactosidase microencapsulated, formed with modified chitosan solutions at different values of pH. The enzymatic reaction was studied for substrate and enzyme concentrations of 0.135 mmol L^{-1} and 0.001 mg mL^{-1}, respectively, based on absorbance values, by UV-visible spectrophotometry at 420 nm and at room temperature.

Figure 4: Lineweaver-Burk representation for the different formulations of microencapsulated β-galactosidase.

Figure 5: Evolution of the *Vmax* with pH.

Figure 6: Evolution of *Km* with pH.

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412 Table 1: Michaelis-Menten parameters (*Km* and *Vmax*), for the different assays with free

413 and microencapsulated β-galactosidase.

	Microencapsulated Enzyme					Free Enzyme
	pH 5.2	pH 6	pH 7	pH 8	pH 9	
Km (mmol L^{-1})	0.62	0.67	0.74	0.77	0.96	0.47
Vmax (µmoles of ONPG hydrolysed min^{-1})	0.08	0.32	0.08	0.05	0.03	0.58
R^2	0.93	0.9	0.84	0.94	0.95	0.99

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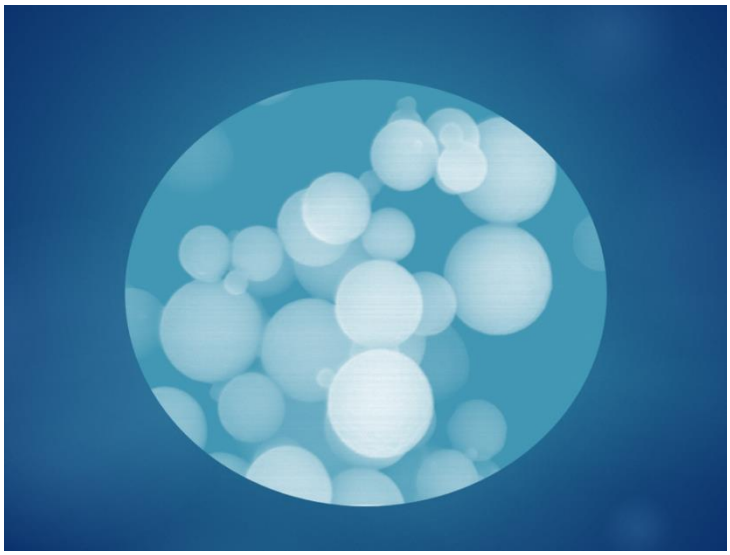

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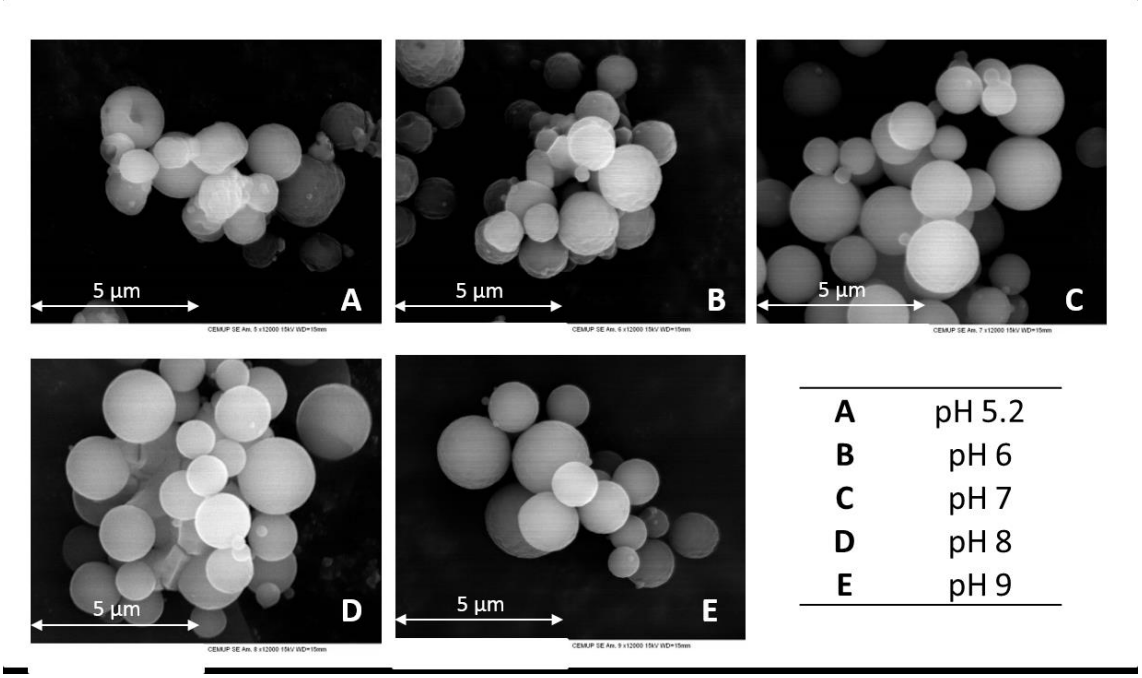

Figure 1: SEM images of β-galactosidase microparticles prepared at different pH´s (5.2, 6, 7, 8 and 9). Magnification = 12000 times, beam intensity (HV) = 15kV, distance between the sample and the lens (WD) = 15 mm.

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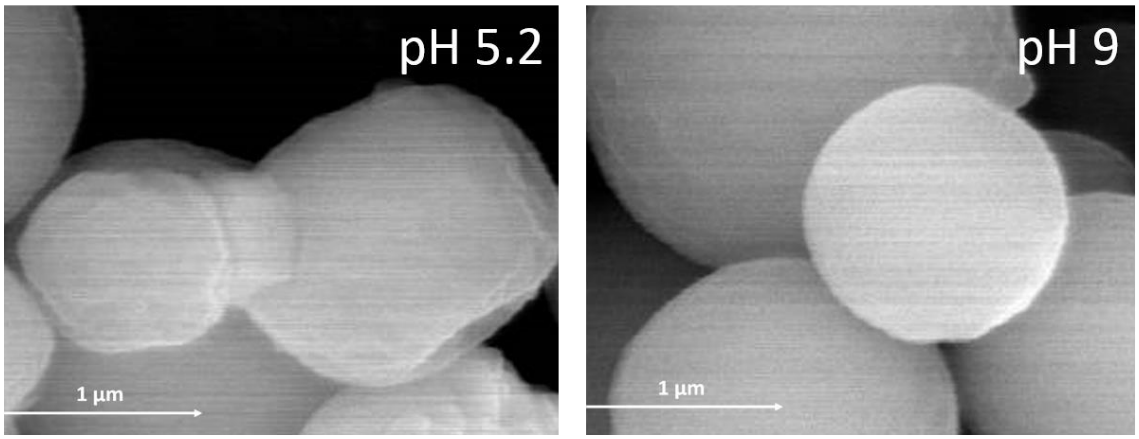

Figure 2: Surface and shape of the β-galactosidase microparticles prepared at pH´s 5.2 and 9.

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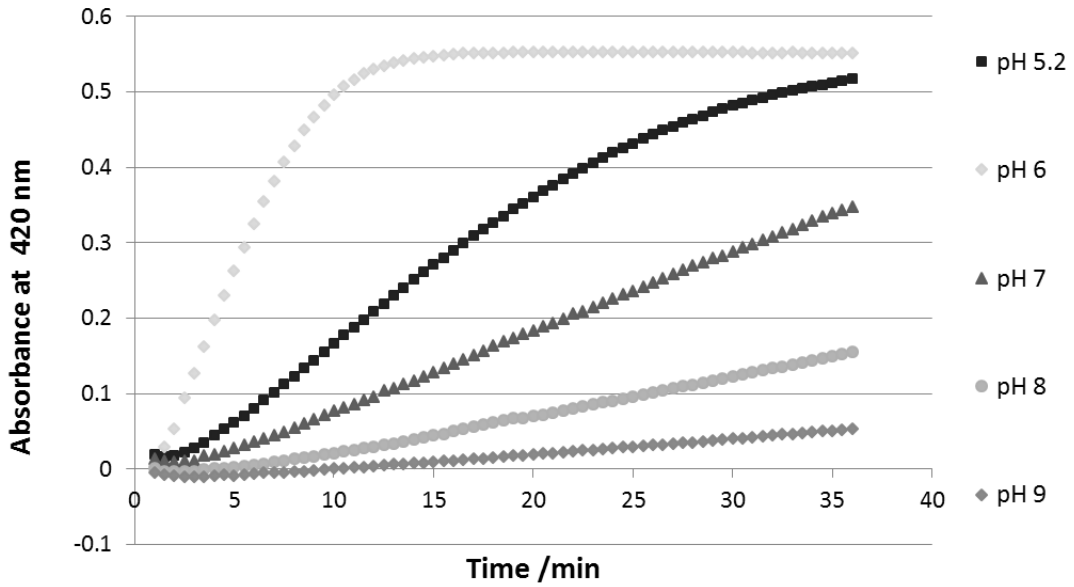

Figure 3: Evolution of the enzymatic reaction with time for β-galactosidase microencapsulated, formed with modified chitosan solutions at different values of pH. The enzymatic reaction was studied for substrate and enzyme concentrations of 0.135 mmol L^{-1} and 0.001 mg mL^{-1}, respectively, based on absorbance values, by UV-visible spectrophotometry at 420 nm and at room temperature.

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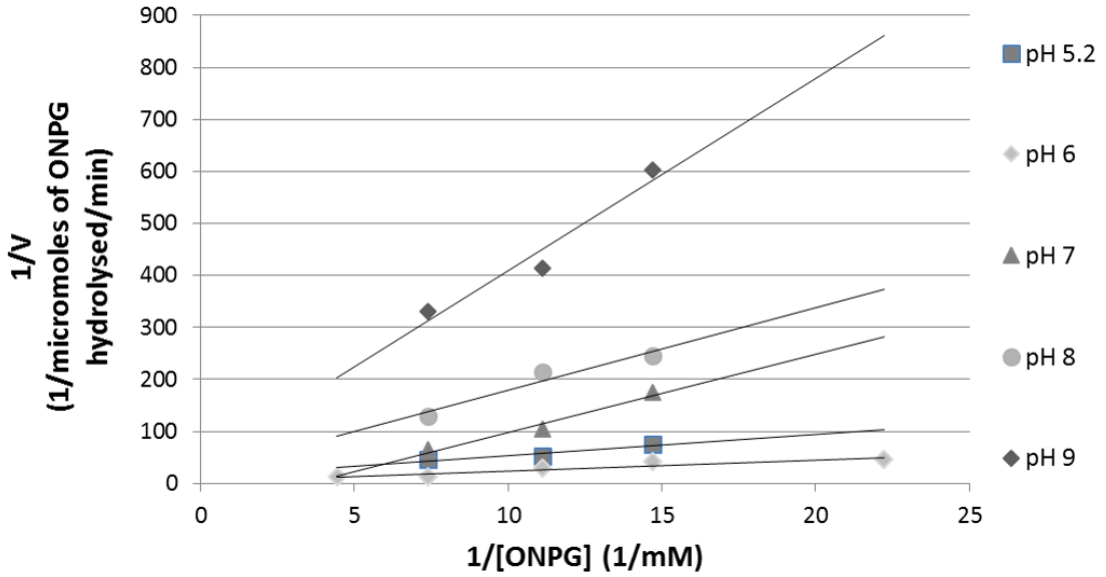

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441 Figure 4: Lineweaver-Burk representation for the different formulations of

442 microencapsulated β-galactosidase.

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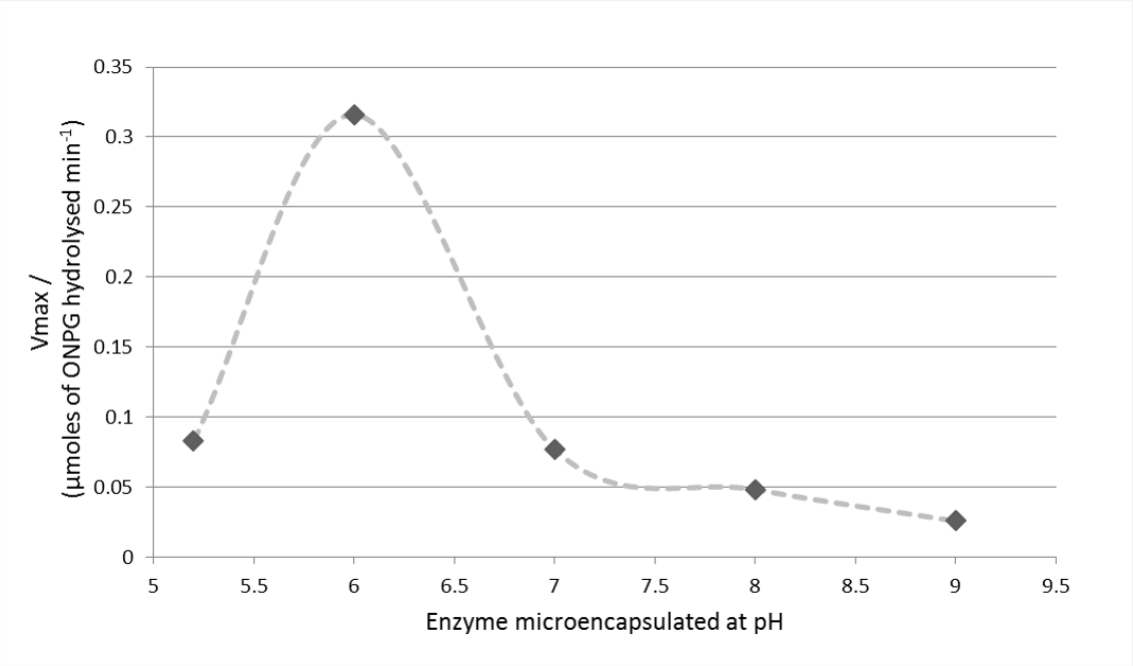

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445 Figure 5: Evolution of the *Vmax* with pH.

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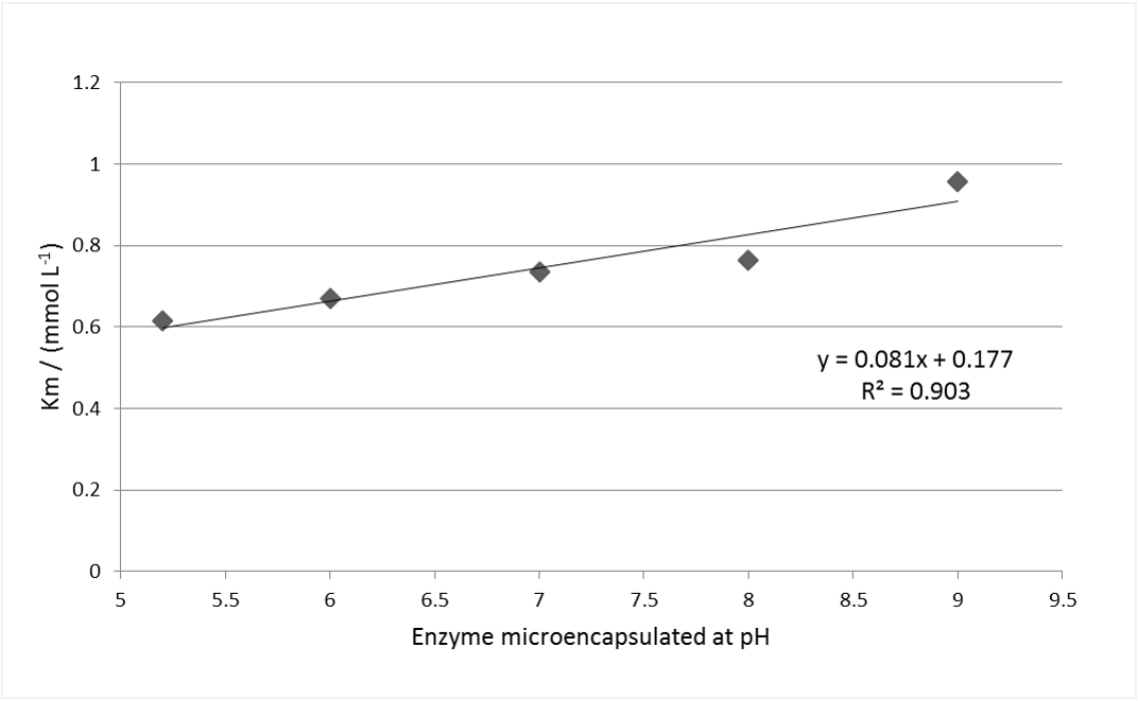

Figure 6: Evolution of *Km* with pH.

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