This article was published in Drying Technology, 32 (13), 1575-1586, 2014
http://dx.doi.org/10.1080/07373937.2014.909843

The influence of microencapsulation with a modified chitosan (water soluble) on β-galactosidase activity

Running Title: β-galactosidase microencapsulation with a modified chitosan

Berta N. Estevinho[1,] *, Ana M. Damas[2], Pedro Martins[1,2], Fernando Rocha[1]

1 – LEPAE, Departamento de Engenharia Química, Faculdade de Engenharia da Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

2 – ICBAS - Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto, Rua de Jorge Viterbo Ferreira n.º 228, 4050-313 Porto, Portugal

Corresponding author: Tel: +351225081678 Fax: +351225081449; e-mail: berta@fe.up.pt

Abstract

The aim of this work was to investigate the possibility of producing microparticles containing β-galactosidase, using a modified chitosan (water soluble) as encapsulating agent. β-galactosidase microparticles were prepared by a spray-drying method and were characterized in terms of particle size, surface morphology, zeta potential and stability over a storage period of 6 months. Microparticles were also analysed by FTIR spectroscopy and thermogravimetry techniques . Structural analysis of the surface of the particles was performed by Scanning Electron Microscopy (SEM). SEM results show that the obtained microparticles have an average diameter smaller than 3.5 μm and a regular shape.

The β-galactosidase activity decreases when microencapsulated. The parameters *Km* and *Vmax* of the Michaelis-Menten equation were calculated for the different experimental conditions. The optimal pH ranges from 6.4 to 7.2, approximately, depending on the enzyme concentration in the microcapsule. After 6 months storage the enzyme activity presents a small decrease, although no significant differences in the appearance, colour and particle size distribution were identified.

Keywords: Immobilization, Michaelis-Menten, Spray-drying, Water soluble chitosan, β-galactosidase.

Introduction

Entrapment of bioactive molecules, such as enzymes, drugs, vitamins, flavours and peptides, in stabilizing matrices has been practiced by the pharmaceutical, food, biomedical, chemical, and waste-treatment industries for many years. [1–4]

In the present work, we propose the use of a modified chitosan (water soluble) to encapsulate the enzyme β-galactosidase. This study focuses on this enzyme due to its importance in health and industry. There are several well know problems where the enzyme β-galactosidase has a primordial role in their solution. For example, a large percentage of the world population suffers from lactose intolerance caused by the lack of β-galactosidase activity. This problem is avoided if lactose is hydrolysed by β-galactosidase to the readily utilisable sugars, glucose and galactose. [5] Other example is the pollution problem caused by the cheese whey that is disposed in wastewater causing serious environmental problems on account of high organic matter content. Whey disposal is a serious problem for dairy industry. In order to reduce pollution load, whey should be treated to obtain commercial products. β-galactosidase hydrolyses lactose found in the whey, allowing the recovery of glucose and galactose. [6–8] Further¸ the functional galacto-oligosaccharides (GOS), which promote the growth of bifido-bacteria in vivo can be produced by enzymatic reaction with β-galactosidase. [9,10]

β-galactosidase has been used in large-scale processes in both free and immobilized forms. [5,11,12] Enzyme immobilization has attracted a wide range of interest to many different industrial applications. [13] The enzyme immobilization can be obtained using a variety of techniques [5,14,15] that should be selected according to the physicochemical properties of the enzyme (such as molecular weight, protein chain length, and position of the active site), immobilization matrix, reaction conditions, type

of reactor, among others. [5,16] An important concern in the production of commercial proteins and enzymes is the preservation of their properties, namely stability and activity, during storage. The stability of immobilized enzymes is subject of continuous researches. [17–21] The results of the immobilized enzymes are compared with those of the free enzymes. In some cases, immobilized enzymes are considerably more stable against different effects (temperature, pH) in comparison with the free one. [17] It is assumed that enzyme immobilization on a carrier saves enzyme activity by increasing its conformational stability. [22] Several authors proposed mechanisms to explain the enzymes immobilization and how the immobilization can affect the activity of the enzyme. [18] For example, Bernal et al [18] studied the immobilization of β-galactosidase from *Kluyveromyces lactis*. The enzyme was covalently immobilised on a glyoxyl sepharose support by multi-point attachment. In the multi-point mechanism, the enzymes are immobilised through the region of the protein surface richest in lysines and generally have to be immobilised at alkaline pH. Bortone et al. [19] also reported, that a way to have multi-point interaction between the enzyme and support seems to consist in favouring the reactivity of the enzyme nucleophiles (i.e., Lys) with proper active groups of the support of choice by incubation at alkaline pH values. Verma et al. [20] immobilized β-galactosidase (from *Kluyveromyces lactis*) by covalent binding to glutaraldehyde-activated silica nanoparticles. The kinetics of the free and immobilized enzyme suggested that the enzyme undergoes a conformational change during immobilization process, resulting in a change in the optimum pH and temperature, as well as kinetic constants.

Regarding the immobilization agent, there are several possible substances even though biopolymers like chitosan have attracted special interest as a matrix for

immobilization and controlled release of cells, protein drugs, DNA and bioactive compounds like enzymes.[21,23]

Chitosan beads have been widely used as enzyme immobilization carriers in food processing, owing to their low cost, lack of toxicity and high protein affinity. [2,10,14,24] Chitosan is obtained by partial alkaline deacetylation of chitin, which is the second most abundant natural polymer in nature after cellulose and it is found in the structure of a wide number of invertebrates (crustaceans, exoskeleton insects, cuticles) among others [25]. The chitosan molecule is a copolymer of N-acetyl-D-glucosamine and D-glucosamine and differs in the degree of N-acetylation (40–98%) and molecular weight (50–2000 kDa). [25,26] Other properties with special interest for food, medical and pharmaceutical industry are related to the anticholesterolemic properties and bacteriostatic effects of the chitosan. [27,28] Although chitosan is an attractive biomacromolecule, it is a water-insoluble material, only soluble in acidic solutions because of its rigid crystalline structure and deacetylation, thus limiting its application to bioactive agents such as drug carriers. [29,30] It is possible however to modify the chitosan structure in order to produce easily soluble chitosan in neutral aqueous solutions. The chitosan solubility can be improved by chemical modifications involving the introduction of hydrophilic functional groups, and also by a depolymerisation process which leads to low molecular weight chitosans or oligomers. [29–31] The advantage of using a water soluble chitosan is that it is useful for drug carriers and for food industrial applications. [32]

Other advantage of the immobilization (microencapsulation) is the protection against humidity. Water facilitates or mediates a variety of physical and chemical degradations. Consequently, dry solid formulations of immobilized enzymes are often developed to provide an acceptable protein shelf life. [33] A way to obtain this dry

solid formulations can involve a spray-drying methodology, which has been used extensively with heat-sensitive materials including enzymes, blood products, microorganisms, and foods. [34–37] Spray-dried products exhibit more attractive properties than their pure forms and broaden their application range. [38] By definition, spray-drying is the conversion of a material from a fluid state into a dried particulate form by spraying the material into a hot drying gas medium. It is a continuous and single-cycle process that has the following main steps: the feeding of a solution or suspension to the atomizer; atomization; mixing of the spray with drying air; solvent evaporation; dried product separation and collection. [2] Efficiency of spray-drying can be easily improved by modifying design of the spray-dryer. The particle size of the microspheres prepared by spray-drying ranged from microns to several tens of microns and had a relatively narrow distribution. So, spray-drying techniques have been widely used in the food and pharmaceutical industries. [38]

In the present work, β-Galactosidase microparticles were formed by a spray-drying technique using a modified, water-soluble, chitosan. The main objective was to microencapsulate the enzyme in a healthy form, for example, to be added to food products at the consumption time. A series of β-galactosidase-chitosan particles were prepared, and their physicochemical structures were analysed by laser granulometry analysis, zeta potential analysis, and SEM. FTIR spectroscopy and thermogravimetry technique were also to characterize the microparticles. In addition, the activity of β-galactosidase from the particles was also tested at different pH levels. A new β-galactosidase microparticles system was created, and water soluble chitosan potential evaluated as an enzyme encapsulating material.

Material and Methods

Reagents

High purity reagents were used. 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 = 955 U/mg 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 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) solution was prepared after 2 hours agitation at 1200 rpm. Solutions with different concentrations of enzyme (0.1 mg/mL and 0.01 mg/mL) were prepared from stock solution in phosphate buffer 0.08 M at pH 7.7. To the enzyme stock solution was added BSA in order to obtain a final concentration of 1 mg BSA/mL. BSA is used to stabilize some enzymes and to prevent adhesion of the enzyme to reaction tubes, pipet tips, and other vessels. The solution containing the enzyme was added and mixed with the chitosan aqueous solution at constant agitation speed of 1200 rpm, during 10 min at room temperature.

The prepared chitosan-enzyme solution was 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 (15%), 32 m^3/h (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 54 ºC. The microparticles prepared had the following enzyme composition: assay A, no enzyme; assay B, 0.02% (w/w) enzyme; assay C, 0.1% (w/w) enzyme; and assay D, 0.2% (w/w) enzyme.

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).

Particle size distribution

The size distribution of the microparticles was assessed by laser granulometry using a Coulter Counter-LS 230 Particle Size Analyser (Miami, USA). The particles were characterized by number and volume average. The results were obtained as an average of three 60-seconds runs. To avoid the particle agglomeration, ethanol was used as dispersant and the samples were previously ultrasound-irradiated.

Zeta potential

The zeta potential is representative of the particle charge. The zeta potential of the particles was measured by zeta Sizer - Nano ZS (MALVERN Instruments – United

Kingdom). The zeta potential for all the samples was evaluated in deionized water, and every measured value is an average of 12 runs.

Fourier transform infrared spectroscopy (FTIR) analysis

The FTIR analysis of the microparticles with and without β-galactosidase was performed in order to confirm the encapsulation of β-galactosidase with chitosan, in a Bomem–MB Series, Arid-ZoneTM (Québec, Canada).

The spectra were obtained with KBr at 99%, at 21 scans/min, with a resolution of 4 cm^{-1} and expressed in transmittance in the 4000−650 cm^{-1} range.

Thermogravimetric analysis

Thermogravimetric analysis is a method of thermal analysis in which changes in physical and chemical properties of materials are measured as a function of increasing temperature (with constant heating rate). Thermogravimetric analysis can provide information about physical and chemical phenomena. Thermogravimetric analysis of the microparticles were made in a Setaram 92 16.18. The initial mass of the samples was generally in the range of 7-9 mg. The sample pan was placed in the balance system equipment and the temperature was raised from 25 to 550 °C at a heating rate of 10 °C per min. The mass of the sample pan was continuously recorded as a function of temperature.

β-galactosidase activity

The activity of the β-galactosidase enzyme was measured according to the methodology described in Switzer and Garrity [39]. 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 mM. After, the enzyme was exposed to different ONPG concentrations (0.225 mM, 0.198 mM, 0.180 mM, 0.135 mM, 0.090 mM, 0.068 mM, 0.045 mM and 0.018 mM). Different solutions of phosphate buffer (0.08 M) with different pH´s (6.4, 6.8, 7.2, 7.7 and 8.0) were prepared.

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 (phosphate buffer 0.08 M at different pH (6.4, 6.8, 7.2, 7.7 and 8.0)) 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 allows 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, is estimated by mass balance and corresponds to the same value used in the free enzyme assays.

Determination of optimum pH for β-galactosidase

The activity of the enzyme (free and microencapsulated form) has been tested at diferent pH´s. For each β-galactosidase reaction curve, at different pH, the corresponding enzymatic activity was determined dividing the initial velocity by the maximum value of the absorbance of the respective set of assays. [39] The relative activity, calculated as the ratio between the activity of every sample and the maximum activity of samples, was determined for the pH 6.4, 6.8, 7.2, 7.7 and 8.0 and for an enzyme concentration of 0.001 mg/mL and ONPG concentration of 0.225 mM. [39]

Determination of β-galactosidase kinetics parameters

For an enzyme concentration of 0.001 mg/mL, several concentrations of ONPG have been tested between 0.018 mM and 0.225 mM. For each β-galactosidase reaction curve the initial velocity was calculated, according to the methodology described in Switzer and Garrity [39] and a nonlinear regression method was performed to determine the Michaelis-Menten parameters [40]. Non-linear curve fit was performed using Microcal Origin 6.0 software package based on the Levenberg-Marquardt algorithm of x^2 minimization.

Results and Discussion

It was necessary to select and optimize the experimental conditions to maximize the activity of the enzyme β-galactosidase. These tests were done with the free enzyme, at different substrate and enzyme concentrations. Then, experiments were made to microencapsulate β-galactosidase with a modified chitosan (water soluble), characterize the microparticles formed and evaluate the kinetic mechanism and the pH effect of the microencapsulated enzyme.

Optimization of the experimental conditions

The optimization of the experimental conditions was performed with regard to the enzyme activity. The first step of this study was to select the enzyme concentration best adapted to the intended assays with microencapsulate enzyme. Several enzyme concentrations have been tested between 0.00001 mg/mL and 0.002 mg/mL (Figure 1). These experiments have been done at pH 7.7, with a substrate concentration of 0.225 mM.

In order to choose the enzyme concentration in solution for the experiments with microcapsules, the time required to achieve the end of the enzymatic reaction, i.e. when all the substrate is converted to ONP, was considered. The chosen concentration of 0.001 mg/mL warrants a sufficiently fast solution to accurately estimate reaction rates. This choice is a compromise between the time of reaction and the time to have reliable results. If we have long reactions we will need more time to do the experiments and we will risk having material changes (The same stock solution was used for all the experiments). So, we tried to perform all the experiments in the smallest possible period of time.

Enzyme microencapsulation and characterization of the microparticles

Different β-galactosidase microparticles systems with a modified chitosan (water soluble) were created. The enzyme microencapsulation was made by a spray-drying process. The operation conditions are optimized based on preliminary studies (section 3.1 and Estevinho *et al.* (2012) [41]) and considering the studies of other authors, namely Namaldi *et al.* (2006) [33] and Samborska et al. (2005) [42] . This technique was successfully used by other authors for thermosensitive compounds, for example, Goula and Adamopoulos (2012) [3] applied a spray-drying technique to microencapsulate lycopene, a carotenoid and Jiang *et al.* (2013) [43] to microencapsulate α-amylase. Enzymes can be spray-dried without significant activity losses, as reported by some authors. [33] The spray-drying process is very rapid, so the enzyme will be exposed only for few milliseconds at elevated temperatures (115-54 ºC). The highest temperature will be 115 ºC in the atomizer of the spray dryer; after this the temperature decreases very fast and at the entrance of the cyclone was 54 ºC, where continues decreasing till the ambient temperature. The thermal denaturation data for β-galactosidase presented by Branchu et al (1999) [44], reported that the calorimetric profile for commercial β-galactosidase displayed a single endotherm with a transition temperature, Tm, of 69.7 °C, and all samples exhibited a similar profile and Tm values in the range 68.4-70.0 °C. Other authors [45,46] concluded that the enzymatic activity of the β-galactosidases was found to be unperturbed at temperatures near 50 ºC. The enzyme loses 68% residual enzyme activity within 5 min at 60 ºC, whereas complete loss in enzyme activity was seen around 65º C.

The microencapsulation process has been performed in order to obtain microparticles with different formulations (Table 1). The product yield (quantity of powder recovered reported to the amount of raw materials) ranged between 30 and 40%.

The as-prepared microparticles were analyzed by laser granulometry, zeta potential and scanning electron microscopy (SEM). Also FTIR and TG analyzes have been made. Spherical microparticles, with regular shape, were produced in all the cases with an average size in differential volume less than 3.5 μm and an average size in differential number around 0.1 μm.

The size of the microparticles that contained enzyme (Table 1: B, C, D) was similar to the size of the microparticles obtained without enzyme (Table 1 1: A). It can be concluded that β-galactosidase does not influence the size of the particles formed. It was observed that, for higher concentrations of enzyme (Table 1: D), a slight roughness appeared at the surface of the microparticles.

The stability of many colloidal systems is directly related to the magnitude of their zeta potential. In general, if the value of the particle zeta potential is large, the colloidal system will be stable. Conversely, if the particle zeta potential is relatively small, the colloid system will tend to agglomerate. Table 1 shows zeta potential values measured in water for the microparticles formed with different formulations. It was observed that the microparticles formed with enzyme had a smaller value of zeta potential than the particles formed only with chitosan, but in all the cases the value obtained is associated to a stable colloidal system. The lower potential value was associated to the incorporation of the enzyme.

FTIR studies (Figure 2) give information about the molecular structure of chemical compounds and are useful for the characterization of biopolymers and to evaluate, in this case, the success of the microencapsulation and the presence of the enzyme in the microparticles. FTIR was performed in order to confirm binding of β-galactosidase with chitosan. Analysing FTIR spectra of all the samples (with and

without enzyme) it was possible to verify the presence of the enzyme associated to new bonds in the spectra.

For chitosan microparticles (sample A – without enzyme), the more important absorption bands at frequency values that justify the existence of the corresponding functional groups (bonds), were observed at approximately 3400 cm^{-1} corresponding to the vibrational stretching of the hydroxyl groups, at 1656 cm^{-1} corresponding to the amide I stretching of C=O and at 1410 cm^{-1} corresponding to CH_2 bending. The band at 1560 cm^{-1} has a larger intensity than at 1655 cm^{-1}, which suggests effective deacetylation of chitosan.

The spectra for the samples containing enzyme (B, C and D) have new bands at 950 cm^{-1} C-H bend (disubstituted - E) and 860 cm^{-1} C-H bend (disubstituted - 1,1). Probably other bands should appear in samples, but the overlapping of the bands of chitosan, made difficult to distinguish other bands. However, these results confirm that new bonds appear in the spectra of those samples so confirming the presence of enzyme in the microparticles.

Thermogravimetric analysis is presented in Figure 3 and was performed in order to confirm the influence of β-galactosidase in the chitosan sample. Analysing the thermogram of all the samples (with and without enzyme) it was possible to verify differences associated to the presence of the enzyme. The sample A with chitosan and without enzyme has a strong weight loss at 371 ºC. The samples containing enzyme (B, C and D) did not have this pronounced decrease at this temperature, however presented a more intense loss of weight for temperatures 50-85 ºC, associated to the enzyme decomposition.

Optimum pH determination

In this work, the influence of pH on β-galactosidase activity is analysed to determine the optimal pH range. The effect of pH on enzymatic activity is usually explained by a kinetic model in which the enzyme undergoes deprotonation. [47] Different pH values have been tested between 6.4 and 8 (Figure 4) for free and microencapsulated enzyme. The reaction product ONP, based on which the enzymatic reaction is followed, is a stronger chromophore in alkaline solutions, which justifies the higher values of absorbance. The pH effect was studied for substrate and enzyme concentration of 0.225 mM and 0.001 mg/mL, respectively. The activity of the enzyme decreases with the microencapsulation process for all the pH values investigated.

The highest value of the free enzyme activity was obtained for pH 6.8 (Figure 5), which is in agreement with the models and results obtained by other authors. [47] The enzyme relative activity decreases to 92%, 78% and 67% of the activity at pH 6.8 as pH increases to 7.2, 7.7 and 8.0, respectively. At pH 6.4 the enzyme relative activity was 97% of the activity at pH 6.8. Comparing the values obtained with free and encapsulated enzyme (Figure 5) one concludes that the loss of activity is more pronounced for pH 8.0. The optimal pH ranges from 6.4 to 7.2, approximately, depending on the enzyme concentration in the microcapsule. The microencapsulated formulations with enzyme concentration of 0.2 and 0.02% w/w present a decrease of the activity for pH higher than 7.2. Until pH 7.7 this decrease is smaller than that for the free enzyme, only presenting an equal or bigger loss of activity at pH 8. The enzyme microencapsulated with concentration of 0.1% w/w presented a decrease of the activity similar to the free enzyme for pH values between 6.4 and 7.2 and higher losses for pH 7.8 and 8.

β-galactosidase has two active-site carboxyl groups that can exist as $–COO^-$ (as nucleophile) and –COOH (as proton donor) simultaneously at neutral pH. [48] The shift

of the optimum pH to a basic pH may depend on the charge of the enzyme and of the immobilizing agent surface. Similar results, with an alteration of the pH range after enzyme immobilization have been reported by Zhou and Chen (2001). [48]

However, the chosen pH was 7.7 since it minimizes the experimental errors during the measurements of ONP concentration. The enzyme activity decreases but the magnitude of the variation range of absorbance is higher for pH 7.7. So the errors involved in the determination of the activity are smaller.

The ratio of the activity of immobilized enzyme to the activity of the free enzyme at pH 7.7 and with a substrate concentration of 0.225 mM, was 0.138, 0.115, 0.160, for enzyme microencapsulated 0.02, 0.1 and 0.2% w/w, respectively. This confirms the observations of other authors. [48,49] Carrara and Rubiolo (1994) [50] obtained activity values of the immobilized enzyme in chitosan of 10.7% of the free enzyme values. Branchu et al. (1999) [44] also report a loss of catalytic activity of the β-galactosidase enzyme spray-dried when compared with the commercial one. It should be noted that the microencapsulated enzyme activity is also influenced by mechanisms of enzyme release through the microparticle and, eventually, by conformational changes [51,52].

Kinetic parameters determination

The evolution of the enzymatic reaction (free enzyme) for different substrate concentrations is presented in Figure 6, for an enzyme concentration of 0.001 mg/mL. For each β-galactosidase reaction curve the initial velocity was calculated, and a nonlinear regression method was performed to determine the Michaelis-Menten parameters (Figure 7). The Michaelis-Menten parameters obtained present the values $Km = 0.28 \pm 0.06$ mM and $Vmax = 0.38 \pm 0.5$ μmol of hydrolysed ONPG/min.

The Michaelis-Menten kinetic constants were also determined for microencapsulated β-galactosidase as described above for the free enzyme. The fitting of the experimental data to the nonlinear regression (Figure 8) shows high correlation coefficients. The parameter *Vmax*, representing the maximum reaction rate at a given enzyme concentration, decreased its value after immobilization thus confirming what has been observed by Haider and Husain (2009). [53] Some active centres are likely to be blocked after immobilization, which reduces the reaction rate, causing the decrease of the maximum reaction velocity. The parameter *Km* is associated to the affinity between the enzyme and the substrate. A smaller value of *Km* indicates a greater affinity between the enzyme and substrate, and it means that the reaction rate reaches *Vmax* faster. The parameter *Km* decreases in the tests with immobilized enzyme. This result shows that interaction of enzyme and substrate had been strengthened after immobilization.

Evaluation of the stability of microparticles with β-galactosidase

The applicability of immobilized enzyme systems is conditioned by their storage stability. Bayramoglu et al. [54] studied the storage stability of the β-galactosidase preparations. They found that, in their experimental conditions, β-galactosidase exhibits higher storage stability than that of the free form. Also, the free enzyme lost all its activity, within five weeks.

In our case, after 6 months storage at controlled ambient conditions (4 ºC) no significant differences in the appearance, colour and particle size distribution of all the prepared formulations were observed. The formulation of microencapsulated β-galactosidase 0.1% (w/w) was selected to reassess the stability after 6 months (Figure

9). It was observed a small decrease in the enzyme activity. The parameter *Km* presents a small increase and *Vmax* decreased from 0.07 to 0.05 μmol of hydrolysed ONPG/min.

Dwevedi and Kayastha [55] microencapsulated β- galactosidase (from *Pisum sativum*) (PsBGAL) with chitosan and concluded that chitosan-PsBGAL has desirable properties like good stability, reusability, broad temperature and pH optima. These authors also reported a loss of about 50% in activity of chitosan-PsBGAL, observed after 46 days at 4 ºC.

Conclusion

Water soluble chitosan microparticles were prepared as a novel biocompatible matrix system for β-galactosidase microencapsulation using a spray-drying technique. While these microparticles are released in solution, they do not have a permanent immobilization. Spherical microparticles were produced with an average size less than 3.5 μm.

The Michaelis-Menten parameters were estimated for the different conditions. The β-galactosidase activity was observed to decrease after encapsulation; however, the enzyme can be conserved and protected in a dried and biocompatible chitosan shell, for a limited period of time.

The optimum pH ranges from 6.4 to 7.2, approximately, depending on the enzyme concentration in the microcapsule. After 6 months storage at controlled ambient conditions (4 ºC) a small decrease in enzyme activity was observed and also, no significant differences in the appearance, colour and particle size distribution were identified.

Acknowledgments

The authors thank Fundação para a Ciência e a Tecnologia (FCT) for the grant SFRH/BPD/73865/2010.

References

1. Ghanem, A.; Skonberg, D. Effect of Preparation Method on the Capture and Release of Biologically Active Molecules in Chitosan Gel Beads. Journal of Applied Polymer Science 2002;84:405–13.
2. Braga, G.K.; Oliveira, W.P.; Manufacturing Drug Loaded Chitosan Microspheres by Spray Drying: Development, Characterization, and Potential Use in Dentistry. Drying Technology 2007;25(2):303–10.
3. Goula, A.M.; Adamopoulos, K.G.; A New Technique for Spray-Dried Encapsulation of Lycopene. Drying Technology 2012;30(6):641–52.
4. Desai, K.G.H.; Park, H.J.; Recent Developments in Microencapsulation of Food Ingredients. Drying Technology 2005;23:1361–94.
5. Grosová, Z.; Rosenberg, M.; Rebroš, M. Perspectives and Applications of Immobilised β-Galactosidase in Food Industry - a Review. Czech Journal of Food Science 2008;26:1–14.
6. Makowski, K.; Białkowska, A.; Szczesna-Antczak, M.; Kalinowska, H.; Kur, J.; Cieśliński, H; Turkiewicz, M. Immobilized preparation of cold-adapted and halotolerant Antarctic β-galactosidase as a highly stable catalyst in lactose hydrolysis. FEMS microbiology ecology 2007;59:535–42.
7. Ansari, S.A.; Husain, Q. Lactose hydrolysis from milk/whey in batch and continuous processes by concanavalin A-Celite 545 immobilized Aspergillus oryzae β galactosidase. Food and Bioproducts Processing 2012;90:351–9. 8.

Singh AK, Singh K. Study on Hydrolysis of Lactose in Whey by use of Immobilized Enzyme Technology for Production of Instant Energy Drink. Advance Journal of Food Science and Technology 4(2): 2012;4(2):84–90.

9. Shin, H.; Park, J.; Yang, J. Continuous production of galacto-oligosaccharides from lactose by Bullera singularis β-galactosidase immobilized in chitosan beads. Process Biochemistry 1998;33:787–92.

10. Cheng, T.-C.; Duan, K.-J.; Sheu, D.-C. Application of tris(hydroxymethyl)phosphine as a coupling agent for β-galactosidase immobilized on chitosan to produce galactooligosaccharides. Journal of Chemical Technology & Biotechnology 2006;81:233–6.

11. Wentworth, D.S.; Skonberg, D.; Darrel, W.D.; Ghanem, A.; Application of chitosan-entrapped β-galactosidase in a packed-bed reactor system. Journal of Applied Polymer Science 2004;91:1294–9.

12. Carrara, C.R.; Rubiolo, A.C. A method for evaluating lactose hydrolysis in a fixed bed reactor with β-galactosidase immobilized on chitosan. Chemical Engineering Journal 1997;65:93–8.

13. Taqieddin, E.; Amiji, M. Enzyme immobilization in novel alginate–chitosan core-shell microcapsules. Biomaterials 2004;25:1937–45.

14. Biró, E.; Németh, A.S.; Sisak, C.; Feczkó, T.; Gyenis, J, Preparation of chitosan particles suitable for enzyme immobilization. Journal of Biochemical and Biophysical Methods 2008;70:1240–6.

15. Zhang, S.; Gao, S.; Gao, G. Immobilization of β-galactosidase onto magnetic beads. Applied biochemistry and biotechnology 2010;160:1386–93.

16. Gaur, R.; Pant, H.; Jain, R.; Khare, S. Galacto-oligosaccharide synthesis by immobilized Aspergillus oryzae β-galactosidase. Food Chemistry 2006;97:426–30.
17. Vasileva, N.; Iotov, V.; Ivanov, Y.; Godjevargova, T.; Kotia, N. Immobilization of β-galactosidase on modified polypropilene membranes. International Journal of Biological Macromolecules 2012;51:710–9.
18. Bernal, C.; Marciello, M.; Mesa, M.; Sierra, L.; Fernandez-Lorente, G.; Mateo, C.; Guisan, J. M. Immobilisation and stabilisation of β-galactosidase from Kluyveromyces lactis using a glyoxyl support. International Dairy Journal 2013;28:76–82.
19. Bortone, N.; Fidaleo, M.; Moresi, M. Immobilization / Stabilization of Acid Urease on Eupergit V Supports. Biotechnology Progress 2012;28:1232 –1244.
20. Verma, M.L.; Barrow, C.J.; Kennedy, J.F.; Puri, M. Immobilization of β-d-galactosidase from Kluyveromyces lactis on functionalized silicon dioxide nanoparticles: characterization and lactose hydrolysis. International journal of biological macromolecules 2012;50:432–7.
21. Singh, N.; Kayastha, A.M.; Cicer α-galactosidase immobilization onto chitosan and Amberlite MB-150: optimization, characterization, and its applications. Carbohydrate research 2012;358:61–6.
22. Tavares, A.P.M.; Rodríguez, O.; Fernández-fernández, M.; Domínguez, A.; Moldes, D.; Sanromán, M.A.; Macedo, E. A. Immobilization of laccase on modified silica: Stabilization , thermal inactivation and kinetic behaviour in 1-ethyl-3-methylimidazolium ethylsulfate ionic liquid. Bioresource Technology2013;131:405–12.

23. Oliveira, B.F.; Santana, M.H.A, Ré, M.I.; Spray-Dried Chitosan Microspheres as a pDNA Carrier. Drying Technology 2006;24(3):373–82.
24. Liu ,Y., Sun, Y., Li, Y., Xu, S., Tang, J., Ding, J.; Xu, Y. Preparation and characterization of α-galactosidase-loaded chitosan nanoparticles for use in foods. Carbohydrate Polymers 2011;83:1162–8.
25. Kumar, M.N.V.R. A review of chitin and chitosan applications. Reactive and Functional Polymers 2000;46:1–27.
26. Guliyeva, U.; Oner, F.; Ozsoy, S.; Haziroğlu, R.; Chitosan microparticles containing plasmid DNA as potential oral gene delivery system. European Journal of Pharmaceutics and Biopharmaceutics 2006;62:17–25.
27. Aranaz, I.; Mengíbar, M.; Harris R; Paños I; Miralles B; Acosta N; Galed, G.; Heras, Á.; Functional characterization of chitin and chitosan. Current Chemical Biology 2009;3:203–30.
28. Fernández-Pan, I.; Ziani, K.; Pedroza-Islas, R.; Maté, J.I.; Effect of Drying Conditions on the Mechanical and Barrier Properties of Films Based on Chitosan. Drying Technology 2010;28(12):1350–8.
29. Sashiwa, H.; Kawasaki, N.; Nakayama, A. Chemical modification of chitosan. 14:1 Synthesis of water-soluble chitosan derivatives by simple acetylation. Biomacromolecules 2002;3:1126–8.
30. Zhang, H.; Wu, S.; Tao, Y.; Zang, L.; Su, Z. Preparation and Characterization of Water-Soluble Chitosan Nanoparticles as Protein Delivery System. Journal of Nanomaterials 2010;2010:1–5.
31. Choi, W.; Ahn, K.; Lee, D.; Byun, M.; Park, H. Preparation of chitosanoligomers by irradiation. Polymer Degradation and Stability 2002;78:533–8.

32. Estevinho, B.N.; Rocha, F.; Santos, L.; Alves, A.Using Water Soluble Chitosan for Flavour Microencapsulation in Food Industry. Journal of Microencapsulation 2013;30:571–9.
33. Namaldi, A.; Çalik, P; Uludag, Y. Effects of Spray Drying Temperature and Additives on the Stability of Serine Alkaline Protease Powders. Drying Technology 2006;24:1495–500.
34. Kurozawa, L.E.; Park, K.J.; Hubinger, M.D. Effect of carrier agents on the physicochemical properties of a spray dried chicken meat protein hydrolysate. Journal of Food Engineering 2009;94:326–33.
35. Çakaloz, T.; Akbaba, H.; Yesügey, E.T.; Periz, A. Drying Model for α-Amylase in a Horizontal Spray Dryer. Journal of Food Engineering 1997;31:499–510.
36. Sloth, J.; Bach, P.; Jensen, A.D.; Kiil, S. Evaluation method for the drying performance of enzyme containing formulations. Biochemical Engineering Journal 2008;40:121–9.
37. Tonon, R. V.; Pedro, R.B.; Grosso, C.R.F.; Hubinger MD. Microencapsulation of Flaxseed Oil by Spray Drying: Effect of Oil Load and Type of Wall Material. Drying Technology 2012;30(13):1491–501.
38. Liu, C.; Desai, K.G.; Tang, X.; Chen, X.; Drug Release Kinetics of Spray-Dried Chitosan Microspheres. Drying Technology 2006;24(6):769–76.
39. Switzer, R.; Garrity, L.; Experimental Biochemistry. third edit. Freeman; 1999.
40. Johnson, K.A.; Goody, R.S.; The original Michaelis constant: translation of the 1913 Michaelis-Menten paper. Biochemistry 2011;50:8264–9.
41. Estevinho, B.N.; Damas, A.M.; Martins, P.; Rocha, F.; Study of the Inhibition Effect on the Microencapsulated Enzyme β-galactosidase. Environmental Engineering and Management Journal 2012;11:1923–30.

42. Samborska, K.; Witrowa-Rajchert, D.; Gonçalves, A. Spray-Drying of α-Amylase — The Effect of Process Variables on the Enzyme Inactivation. Drying Technology 2005;23:941–53.
43. Jiang, H.; Zhang, M.; McKnight, S.; Adhikari, B.; Microencapsulation of α-Amylase by Carrying Out Complex Coacervation and Drying in a Single Step Using a Novel Three-Fluid Nozzle Spray Drying. Drying Technology 2013;31(16):1901–10.
44. Branchu, S.; Forbes, R.; York, P.; Petrén, S., Nyqvist, H., Camber, O.; Hydroxypropyl-β-cyclodextrin inhibits spray-drying-induced inactivation of β-galactosidase. Journal of Pharmaceutical Sciences 1999;88:907–11.
45. Kishore, D., Kayastha, A.M., Optimisation of immobilisation conditions for chick pea β-galactosidase (CpGAL) to alkylamine glass using response surface methodology and its applications in lactose hydrolysis. Food Chemistry 2012;134:1650–7.
46. Kishore, D.; Kundu, S.; Kayastha, A.M. Thermal, chemical and pH induced denaturation of a multimeric β-galactosidase reveals multiple unfolding pathways. PloS one 2012;7:e50380.
47. Jurado, E.; Camacho, F.; Luzón, G.; Vicaria, J.M. Kinetic models of activity for β-galactosidases: influence of pH, ionic concentration and temperature. Enzyme and Microbial Technology 2004;34:33–40.
48. Zhou, Q.Z.K.; Chen, X.D. Effects of temperature and pH on the catalytic activity of the immobilized β-galactosidase from Kluyveromyces lactis. Biochemical Engineering Journal 2001;9:33–40.
49. Tanriseven, A.; Doğan, Ş. A novel method for the immobilization of β-galactosidase. Process Biochemistry 2002;38:27–30.

50. Carrara, C.R.; Rubiolo, A.C. Immobilization of β-Galactosidase on Chitosan. Biotechnology Progress1994;10:220–4.
51. González Siso, M.I.; Lang, E.; Carrenõ-Gómez, B.; Becerra, M.; Otero Espinar, F.; Blanco Méndez, J. Enzyme encapsulation on chitosan microbeads. Process Biochemistry 1997;32:211–6.
52. Haider, T.; Husain, Q. Concanavalin A layered calcium alginate-starch beads immobilized β-galactosidase as a therapeutic agent for lactose intolerant patients. International Journal of Pharmaceutics 2008;359:1–6.
53. Haider, T.; Husain Q. Hydrolysis of milk/whey lactose by β galactosidase: A comparative study of stirred batch process and packed bed reactor prepared with calcium alginate entrapped enzyme. Chemical Engineering and Processing 2009;48:576–80.
54. Bayramoglu, G.; Tunali, Y.; Arica, M.Y. Immobilization of β-galactosidase onto magnetic poly(GMA–MMA) beads for hydrolysis of lactose in bed reactor. Catalysis Communications 2007;8:1094–101.
55. Dwevedi, A.; Kayastha, A.M. Optimal immobilization of beta-galactosidase from Pea (PsBGAL) onto Sephadex and chitosan beads using response surface methodology and its applications. Bioresource technology 2009;100:2667–75.

Figure Captions

Figure 1: Evolution of the enzymatic reaction (pH 7.7, with a substrate concentration of 0.225 mM) with time for different concentrations of free enzyme (0.00001 - 0.002 mg/mL). The enzymatic reaction was evaluated, based on absorbance values, by UV-visible spectrophotometry at 420 nm and at room temperature.

Figure 2: FTIR spectra of the microparticles prepared with the composition: assay A, no enzyme; assay B, 0.02% (w/w) enzyme; assay C, 0.1% (w/w) enzyme; and assay D, 0.2% (w/w) enzyme. Spectra obtained with KBr at 99%, at 21 scans/min, with a resolution of 4 cm^{-1} and expressed in transmittance in the 4000−650 cm^{-1} range.

Figure 3: Thermogram of the microparticles prepared with the composition: assay A, no enzyme; assay B, 0.02% (w/w) enzyme; assay C, 0.1% (w/w) enzyme; and assay D, 0.2% (w/w) enzyme. Temperature raised from 25 to 550 °C at a heating rate of 10 °C per min.

Figure 4: Evolution of the enzymatic reaction with time for free and microencapsulated enzyme at different pH´s (6.4, 7.2, 7.7, 8.0). The enzymatic reaction was studied for substrate and enzyme concentration of 0.225 mM and 0.001 mg/mL, respectively, based on absorbance values, by UV-visible spectrophotometry at 420 nm and at room temperature.

Figure 5: Effect of the pH on the relative activity of free and microencapsulated β-galactosidase (with a substrate concentration of 0.225 mM and an enzyme concentration in solution of 0.001 mg/mL).

Figure 6: Evolution of the enzymatic reaction (at pH 7.7 and with an enzyme concentration of 0.001 mg/mL) for different ONPG concentrations. The enzymatic reaction was evaluated based on absorbance values, by UV-visible spectrophotometry at 420 nm and at room temperature.

Figure 7: Michaelis-Menten kinetics for free enzyme. The curve represents the fitting of experimental data by a nonlinear regression method. The correspondent *Km* and *Vmax* are presented (with a 95% confidence interval).

Figure 8: Michaelis-Menten kinetics for microencapsulated β-galactosidase. The microparticles were prepared with different compositions: 0.02% (w/w) enzyme; 0.1% (w/w) enzyme; and 0.2% (w/w) enzyme. The curves represent the fitting of experimental data by a nonlinear regression method. The correspondent *Km* and *Vmax* are presented (with a 95% confidence interval).

Figure 9: Comparison of Michaelis-Menten kinetics for fresh and 6 months storage for microencapsulated β-galactosidase 0.1% (w/w).

Table Captions

Table 1: Results of the characterization of the microparticles prepared with different concentrations of enzyme (A – Average size, CV – Variation coefficient of the distribution).

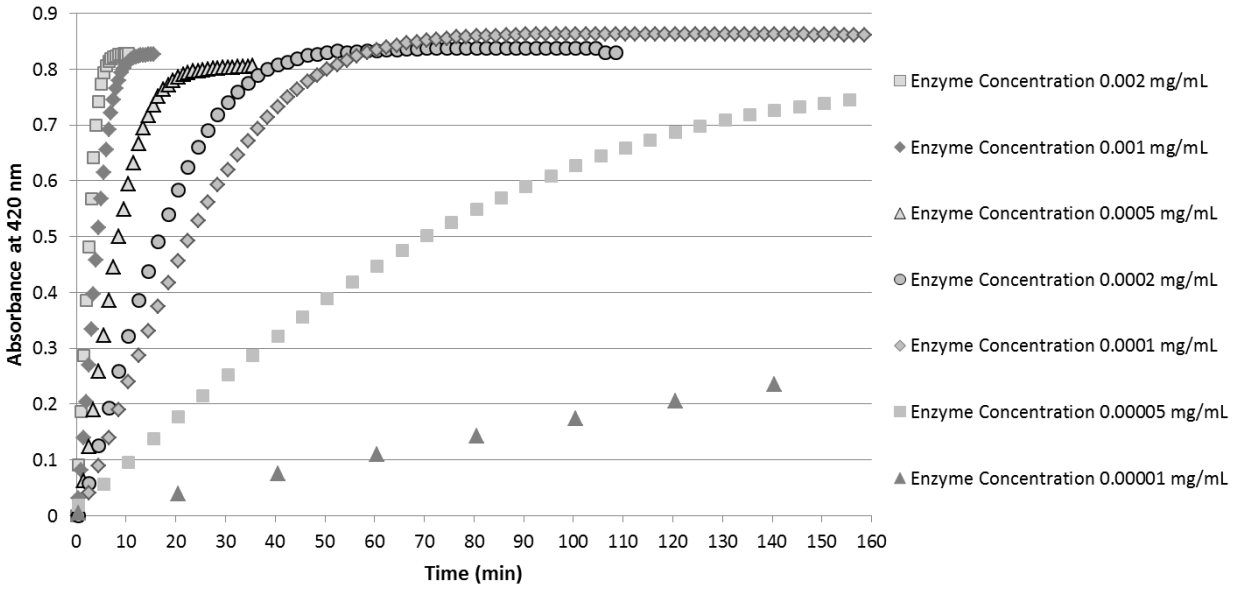


Figure 1: Evolution of the enzymatic reaction (pH 7.7, with a substrate concentration of 0.225 mM) with time for different concentrations of free enzyme (0.00001 - 0.002 mg/mL). The enzymatic reaction was evaluated, based on absorbance values, by UV-visible spectrophotometry at 420 nm and at room temperature.

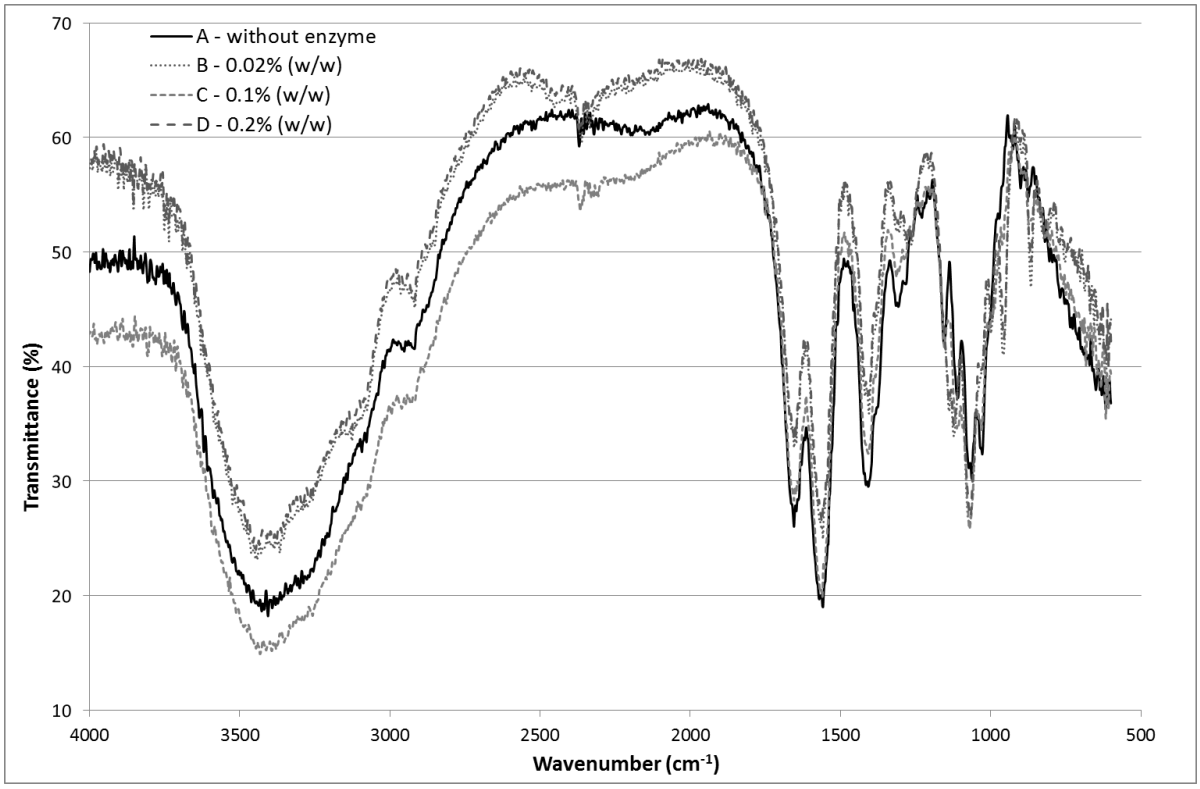


Figure 2: FTIR spectra of the microparticles prepared with the composition: assay A, no enzyme; assay B, 0.02% (w/w) enzyme; assay C, 0.1% (w/w) enzyme; and assay D, 0.2% (w/w) enzyme. Spectra obtained with KBr at 99%, at 21 scans/min, with a resolution of 4 cm^{-1} and expressed in transmittance in the 4000−650 cm^{-1} range.

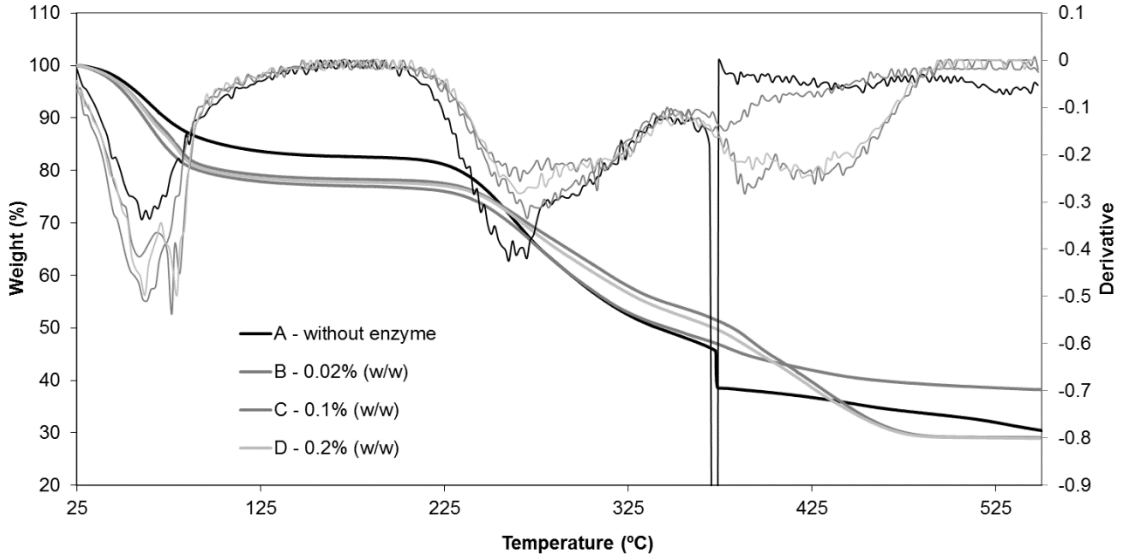


Figure 3: Thermogram of the microparticles prepared with the composition: assay A, no enzyme; assay B, 0.02% (w/w) enzyme; assay C, 0.1% (w/w) enzyme; and assay D, 0.2% (w/w) enzyme. Temperature raised from 25 to 550 °C at a heating rate of 10 °C per min.

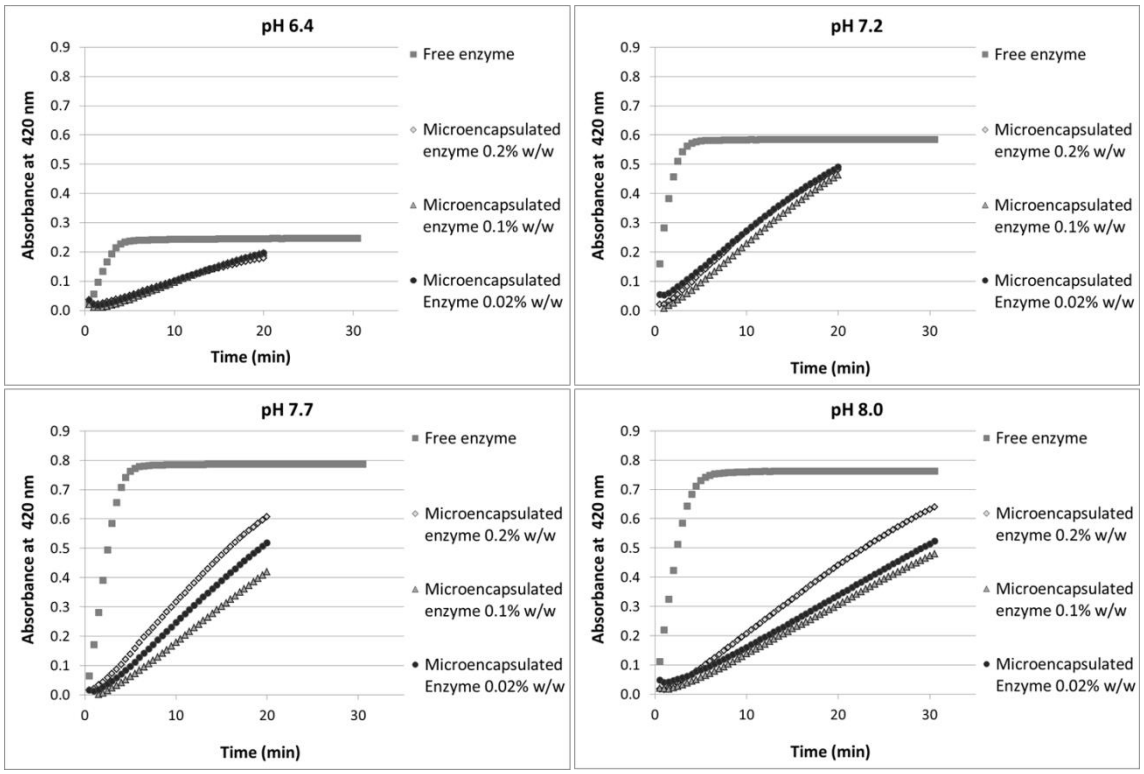


Figure 4: Evolution of the enzymatic reaction with time for free and microencapsulated enzyme at different pH´s (6.4, 7.2, 7.7, 8.0). The enzymatic reaction was studied for substrate and enzyme concentration of 0.225 mM and 0.001 mg/mL, respectively, based on absorbance values, by UV-visible spectrophotometry at 420 nm and at room temperature.

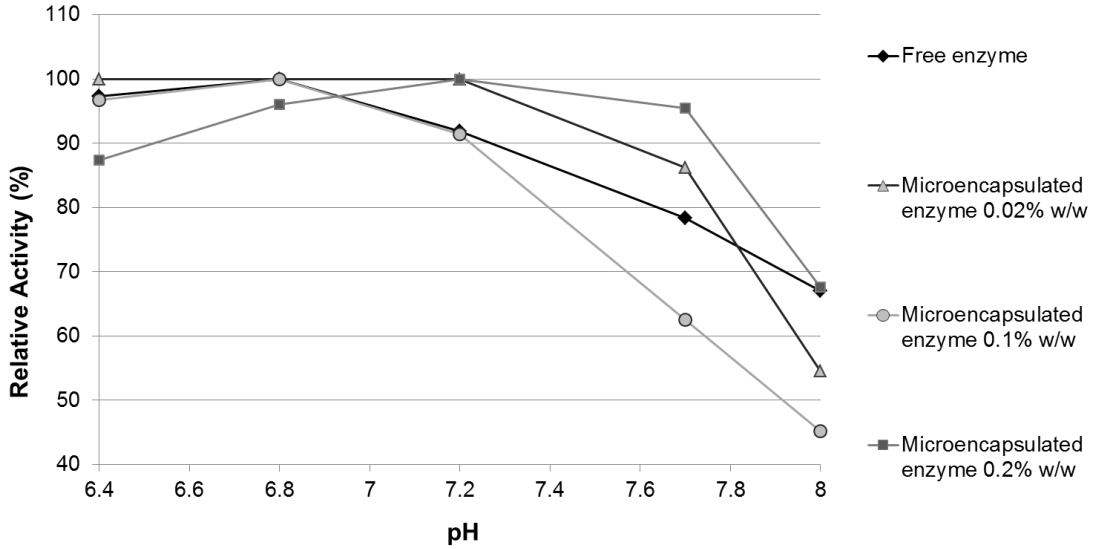


Figure 5: Effect of the pH on the relative activity of free and microencapsulated β-galactosidase (with a substrate concentration of 0.225 mM and an enzyme concentration in solution of 0.001 mg/mL).

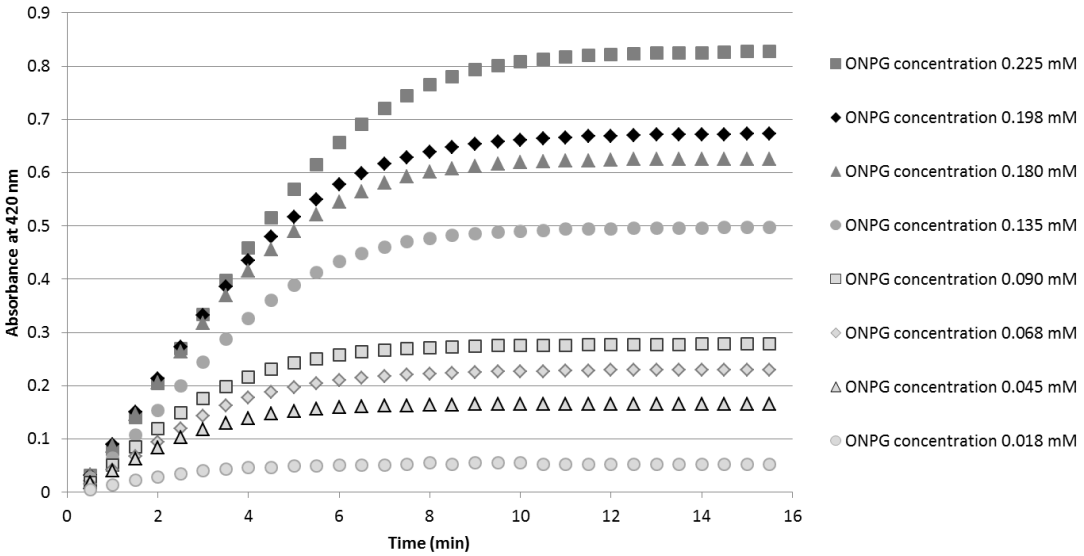


Figure 6: Evolution of the enzymatic reaction (at pH 7.7 and with an enzyme concentration of 0.001 mg/mL) for different ONPG concentrations. The enzymatic reaction was evaluated based on absorbance values, by UV-visible spectrophotometry at 420 nm and at room temperature.

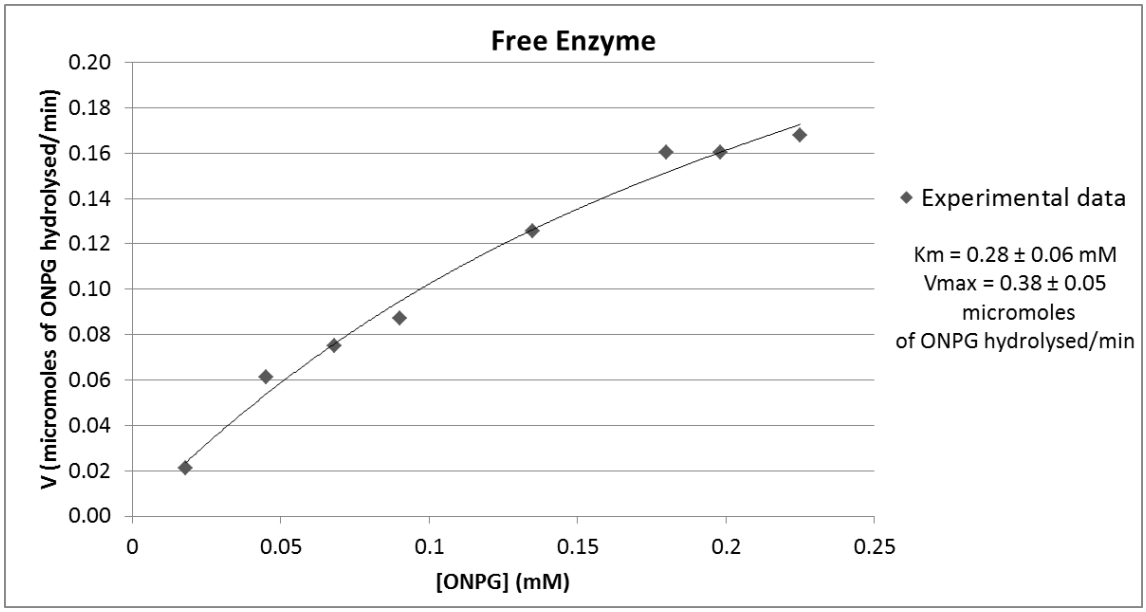
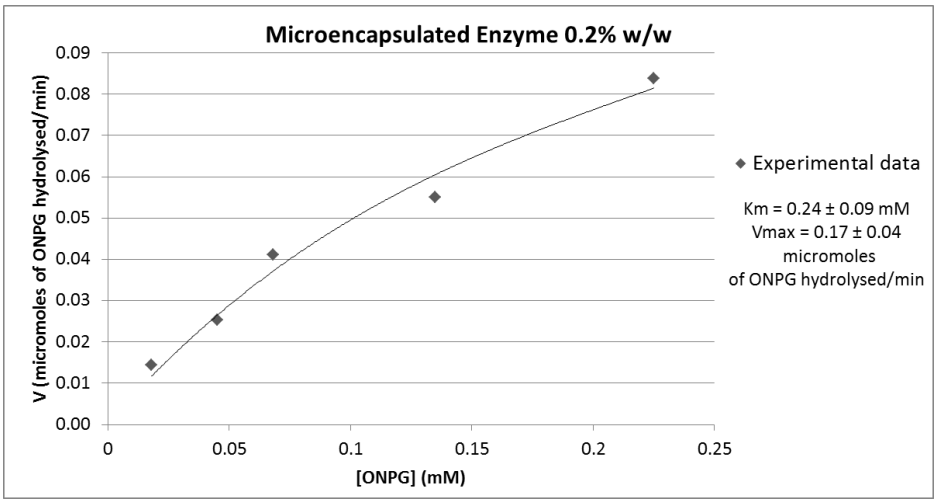
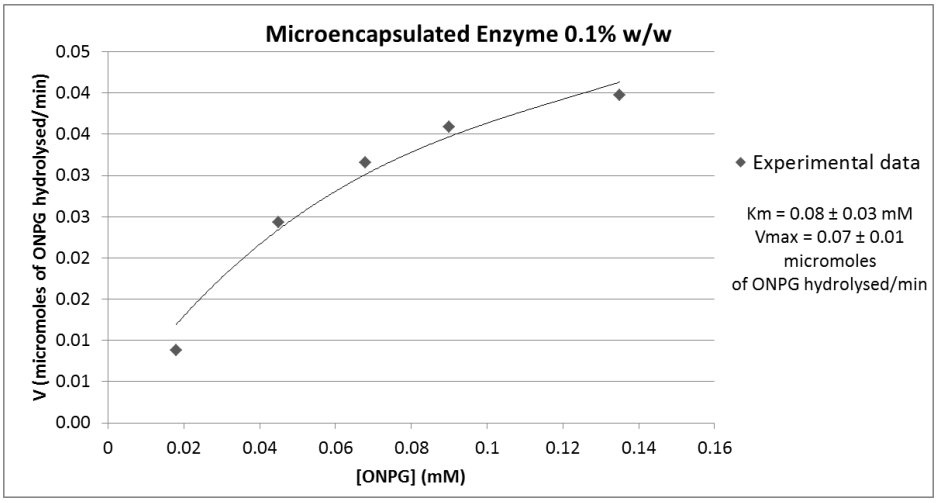


Figure 7: Michaelis-Menten kinetics for free enzyme. The curve represents the fitting of experimental data by a nonlinear regression method. The correspondent *Km* and *Vmax* are presented (with a 95% confidence interval).





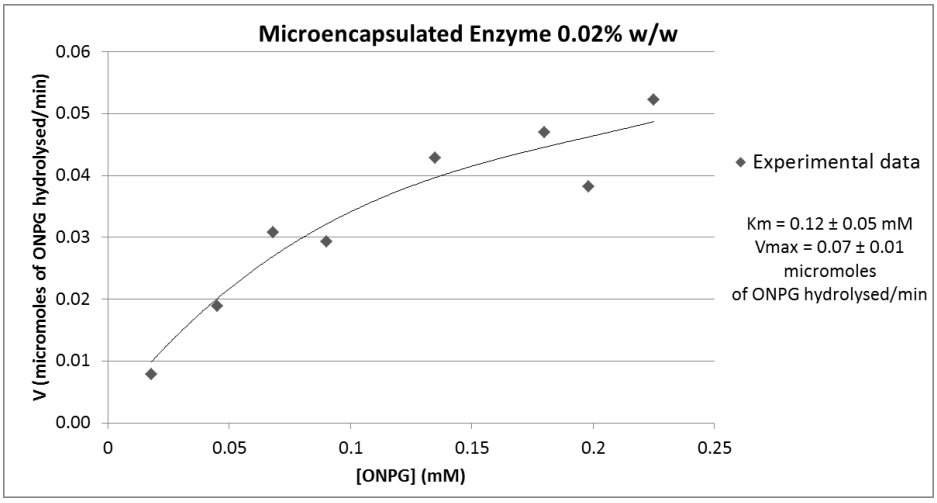


Figure 8: Michaelis-Menten kinetics for microencapsulated β-galactosidase. The microparticles were prepared with different compositions: 0.02% (w/w) enzyme; 0.1% (w/w) enzyme; and 0.2% (w/w) enzyme. The curves represent the fitting of experimental data by a nonlinear regression method. The correspondent *Km* and *Vmax* are presented (with a 95% confidence interval).

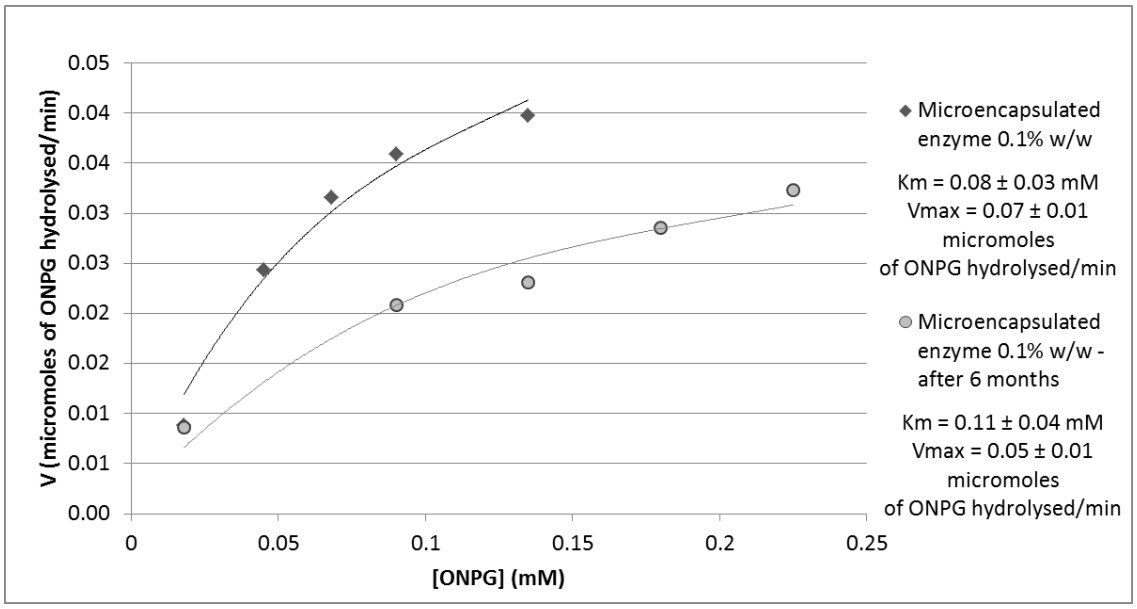
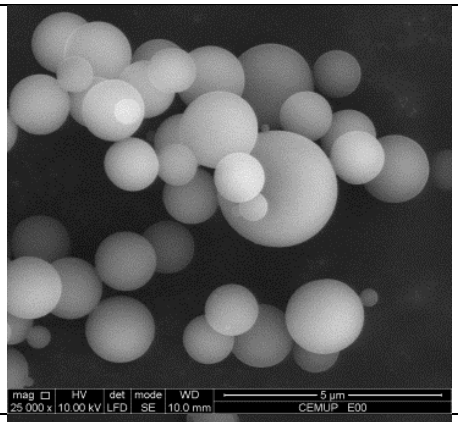
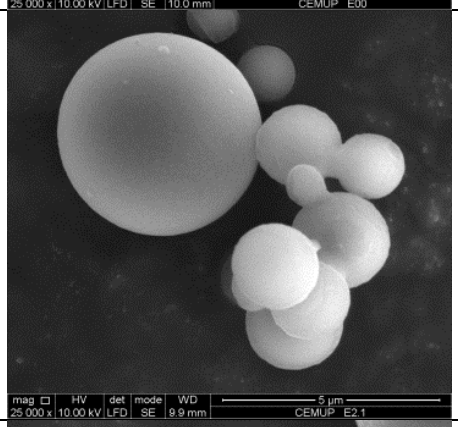
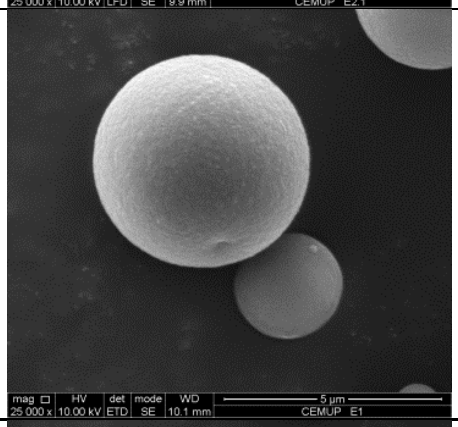
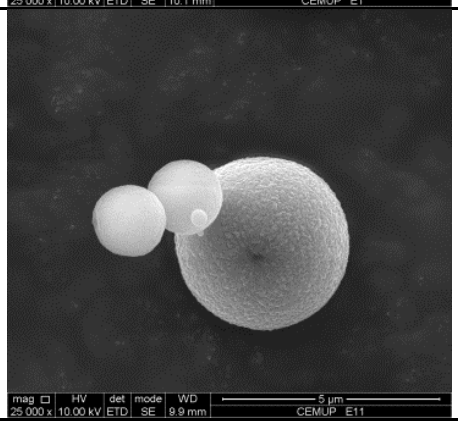


Figure 9: Comparison of Michaelis-Menten kinetics for fresh and 6 months storage for microencapsulated β-galactosidase 0.1% (w/w).

Table 1: Results of the characterization of the microparticles prepared with different concentrations of enzyme (A – Average size, CV – Variation coefficient of the distribution).

Sample	Particle size		Zeta Potential	SEM Images (25000x)
	Differential Number	Differential Volume		
A - without enzyme	A = 0.112 µm CV = 115%	A = 3.366 µm CV = 55.4%	-53.0 ± 6.1 mV	
B - 0.02% (w/w)	A = 0.096 µm CV = 88.8%	A = 3.449 µm CV = 58.8%	-50.3 ± 5.1 mV	
C - 0.1% (w/w)	A = 0.100 µm CV = 109%	A = 3.300 µm CV = 58.0%	-50.9 ± 4.5 mV	
D - 0.2% (w/w)	A = 0.100 µm CV = 111%	A = 3.326 µm CV = 54.5%	-47.4 ± 7.0 mV	

1