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Article

Stabilization of β-Galactosidase Encapsulated in Pectin-Alginate Hydrogel and Hydrolysis of Whey Lactose and Whole Milk

Maykon J. Paiva,[a] Fabrício C. Paula-Elias,[a] Luciana A. Pereira,[a] Solange C. Carreiro,[a] Erika C. Vieira-Almeida,[b] Ezequiel M. Silva,[c] Giancarlo S. Dias,[d] Michelle A. C. Xavier,[a] Sergio A. Morales,[a] Rafael F. Perna[d] and Alex F. Almeida*[,a]

[a]*Departamento de Engenharia de Alimentos, Universidade Federal do Tocantins, Av. NS 15, ALCNO-14, 77001-090 Palmas-TO, Brazil*

[b]*Departamento de Farmácia, Universidade de Gurupi, Av. Rio de Janeiro, 1585, 77403-090 Gurupi-TO, Brazil*

[c]*Departamento de Engenharia de Bioprocessos e Biotecnologia, Universidade Federal do Tocantins, Rua Badejós, Chácaras 69-72, Zona Rural, 77404-970 Gurupi-TO, Brazil*

[d]*Instituto de Ciência e Tecnologia, Universidade Federal de Alfenas, Rod. José Aurélio Vilela, 11999, km 533, 37715-400 Poços de Caldas-MG, Brazil*

The operational conditions of β-galactosidase encapsulated in pectin-alginate hydrogel were investigated as well as its catalytic activity in the hydrolysis of lactose from whey and whole milk. β-Galactosidase encapsulated in pectin-alginate hydrogel showed the best results to yields and diffusional effect and pH range. Thermostability showed considerable income gain for half-life (1.7-fold), activation energy of denaturation (31.6-fold), activation of denaturation (217.3-fold at 40 ºC; 266.8-fold at 50 ºC; 345.6-fold at 60 ºC), and entropy of activation of denaturation (–42.0 J mol^{-1} K^{-1}). Reusability of encapsulated β-galactosidase was observed in 8-cycles for the milk whey lactose hydrolysis (51.9%) and 7-cycles for whole milk (55.6%), and the lactose hydrolysis was observed in 13% in milk whey and 10.3% in whole milk after 10 cycles. These results revealed an industrial application potential of β-galactosidase encapsulated in pectin-alginate hydrogel for lactose hydrolysis of milk whey and whole milk processes.

Keywords: enzyme encapsulation, lactase, thermostability, reusability, lactose hydrolysis

Introduction

A wide variety of fresh dairy products contain a considerable amount of lactose, including milk, fermented products like yogurt and sour cream desserts such as cakes and biscuits, as well as whey products. Furthermore, foods like chocolate, coffee drinks and baked goods are also not lactose-free.[1] However, the large number of lactose intolerant people demands a higher offer of lactose-free or lactose-reduced dairy products that can offer important gains in people's nutrition and health. This has motivated an increase in research about the conversion of lactose on the enzyme β-galactosidase (β-D-galactoside-galactohydrolase, E.C. 3.2.1.23), also called lactase. Specifically, lactase catalyzes the hydrolysis of a glycosidic link in lactose molecule, releasing glucose and galactose (molecules easily absorbed by the intestines).[2] The world market of this enzyme was estimated at about US$ 217 million in 2020 and is expected to reach US$ 298 million in 2025, registering a Compound Annual Growth Rate (CAGR) of 6.5%.[3]

The fast activity loss of free lactase is the bottleneck to its practical application. Alternatively, the immobilization of lactase can enhance its stability even at harsh reaction conditions (pH and temperature). Also, the use of immobilized enzymes facilitates its separation after reaction and its reuse, enhances the product quality and decreases production costs, which are important issues for enzyme application in food and pharmaceutical industries.[4,5] Also, immobilized lactase could allow the continuous conversion of lactose in packed bed reactors.[6,7] Nevertheless, the performance of these biocatalysts

*e-mail: alexfernando@uft.edu.br
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during reaction depends on the immobilization technique, support material, enzyme load and reaction parameters used. Lactase has been immobilized by entrapment, physical adsorption on water-insoluble carriers, covalent bonding, crosslinking, microencapsulation and bio-affinity. Particularly, immobilization of enzymes by entrapment has shown important advantages such as low cost, simplicity and applicability to numerous enzymes. Also, this technique uses renewable biopolymers as matrixes such as alginate, chitosan and cellulose, etc., which also are abundant, non-toxic, biocompatible and biodegradable.[5]

The gelation of alginate (an anionic polysaccharide copolymer) by the addition of Ca^{2+} ions allows the entrapment of proteins, enzymes and cells.[5,8-12] Nevertheless, pellets of calcium alginate containing entrapped enzymes can show low mechanical resistance, limit the substrate diffusion to the enzyme active sites and show leaching of the enzyme during consecutive reaction cycles.[13] These problems can be reduced by using matrixes made from several biocompatible polysaccharides, such as chitosan, gelatin, starch and pectin.[5,14] Hang *et al.*[7] reported that the entrapment of *Aspergillus niger* inulinase in Ca-alginate-gelatin improved the enzyme reuse and its thermal stability for the production of fructose in a packed bed reactor. Similarly, *Aeromonas caviae* MTCC 7725 carboxymethylesterase entrapped in chitosan-coated Ca-alginate showed higher stability and remaining activity than the free enzyme in several organic solvents.[15] The immobilized enzyme also showed higher storage stability and was reused by five reaction cycles.[15]

Furthermore, the stability of encapsulated enzymes can be improved by previous crosslinking using chemical agents such as glutaraldehyde or glyoxal.[13] Currently, glutaraldehyde is the crosslinking agent most used for the synthesis of biocatalysts because it is bi-functional and capable of polymerization. When this compound is added to a protein solution, it promotes the chemical aggregation of the enzyme or introduces intramolecular crosslinks into the protein structure, improving its stability.[16] This work aimed to study the biochemical properties of commercial β-galactosidase from *Aspergillus oryzae* crosslinked with glutaraldehyde and entrapped in Ca-alginate modified with citrus pectin, as well as its catalytic activity in the hydrolysis of lactose from whey and whole milk.

Experimental

Materials

A. oryzae β-galactosidase 10000 FCC ALU (Deslac Lactase®) was provided by Maxinutri Laboratory of Brazil (Arapongas, Paraná, Brazil). Sodium alginate was purchased from Dinâmica Química Contemporânea Ltda (Indaiatuba, São Paulo, Brazil). Citrus pectin was provided by CP Kelco (Limeira, São Paulo, Brazil). Whey lactose and milk were provided from Jandira Comércio de Produtos Alimentícios LTDA (Gurupi, Tocantins, Brazil). Glutaraldehyde and epichloridine were purchased from Sigma-Aldrich (São Paulo, São Paulo, Brazil). Chitosan was provided by the company Nativa (Gurupi, Tocantins, Brazil). Carboxymethylcellulose, sodium salt, with low viscosity was purchased from the company Merck (São Paulo, São Paulo, Brazil). Bovine serum albumin and glucose oxidase-peroxidase, GOPOD kit were purchased from the Sigma-Aldrich (São Paulo, São Paulo, Brazil). All chemicals employed were of analytical grade and used without any further purification.

Encapsulation of β-galactosidase

Firstly, the Na-alginate aqueous solution (2.0% m/v) was mixed and stirred vigorously with soluble β-galactosidase (1:1 v/v) (total volume = 40 mL). In sequence, the drip of the as-prepared mixture in 0.2 M calcium chloride solution under constant stirring formed the alginate spheres, which remained at rest for 20 min in the calcium chloride solution. Finally, the encapsulated enzyme was separated, washed with distilled water, and maintained at 10 ºC for 12 h. Calcium chloride solutions and washing water were reserved and used for enzymatic analysis and protein content.

Modification of β-galactosidase encapsulation

β-Galactosidase cross-linking

β-Galactosidase was cross-linked using 25% glutaraldehyde solution as follows: β-galactosidase was added in 25% (v/v) glutaraldehyde at a final concentration of 2.5% (v/v) and kept stirring for 3 h at 25 °C. The cross-linked enzyme was added and mixed in the sodium alginate solution and stirred vigorously (final volume = 40 mL). The encapsulation steps were performed as previously described.

Modification of Ca-alginate for β-galactosidase encapsulation

Modifications of Ca-alginate were performed using the adjuncts carboxymethylcellulose (CMC), or chitosan (Chi), or citrus pectin (Pec), or sodium sulfate. The adjuncts were added at 1% (m/v), individually, to the alginate solution. The solutions were stirred vigorously to completely dissolve. The soluble and cross-linked enzymes were added to the modified Ca-alginate solutions and stirred vigorously

(final volume = 40 mL). The encapsulation steps were performed as previously described.

Enzyme activity assays

The enzyme activity assays were performed with the incubation of 0.1 mL of soluble or 0.1 g of encapsulated enzyme in the following medium: 2% m/v lactose solution in 0.2 mM McIlvaine buffer (pH 4.5) at 35 ºC (final volume 50 mL). The optimum enzymatic activity of β-galactosidase in McIlvaine buffer (pH 4.5) is well-stablished in the literature.[17] At regular time intervals, the samples taken were immersed in 100 ºC water bath for 1 min to terminate the reaction. The enzymatic activity of both enzymatic reaction assays was estimated by the amount of glucose released, quantified at 510 nm in a solution formed by 10 µL of appropriately diluted medium reaction and 1 mL test reagent (glucose oxidase-peroxidase, GOPOD kit, Sigma-Aldrich). β-Galactosidase activity was determined by the initial lactose hydrolysis rates and one unit of activity (U) was defined as gram of glucose produced *per* milliliter of the medium *per* minute *per* gram/volume of enzyme.[18]

Estimation of total proteins concentration

Bradford method using bovine serum albumin (0-2.0 mg mL^{-1}) as a standard was carried out to determine the total proteins concentration.[19] The analysis was performed in triplicate.

Encapsulation parameters

The encapsulation efficiency (EE) and yield (EY), encapsulated enzyme activity, and diffusional effect of encapsulation were calculated using equations 1, 2, 3, and 4, respectively.

$$EE = \frac{C_i V_i - C_f V_f}{C_i V_i} \quad (1)$$

$$EY\,(\%) = \frac{\text{encapsulated enzyme activity}}{\left(\text{initial soluble enzyme activity}\right) - \left(\text{enzyme activity in water filtered}\right)} \times 100 \quad (2)$$

$$\text{Encapsulated enzyme activity} = \frac{\text{enzyme activity}}{\text{pellets of encapsulation}} \quad (3)$$

$$\eta = \frac{\nu_{imm}}{\nu_{free}} \quad (4)$$

where C_i is initial concentration of protein, V_i is initial volume of enzyme, C_f is concentration of protein in water filtered, V_f is total volume, and ν_{imm} and ν_{free} represent the rates of the reaction of immobilized and free enzyme, respectively, under identical conditions.

Biochemical properties of soluble and encapsulated enzyme

Optimum temperature and pH activities

The optimum temperature and pH activities were determined for soluble and immobilized β-galactosidase. The temperature enzymatic assays were performed in the range of 10 to 70 °C with intervals of 5 °C (with the same reaction medium previously described). The pH enzymatic assay activity was performed in the range of 3.0 to 8.0, with 0.5 intervals. The enzymatic activity was quantified as previously described.

Thermal stability of soluble and encapsulated β-galactosidase enzyme and determination of apparent thermodynamics parameters

The thermal stability was evaluated at 40, 50 and 60 °C in medium containing only McIlvaine buffer (pH 4.5). The reaction extended for 1440 min, and the aliquots taken at regular time intervals were submitted to the residual activities analysis. The first-order thermal denaturation constant (k_D, in min^{-1}) was estimated by Sadana and Henley[20] model (equation 5).[20,21]

$$\frac{A}{A_0} = (1-\alpha)\exp(-k_D t) + \alpha \quad (5)$$

where t is the incubation time of the enzyme (min), α is a dimensionless parameter, and A and A_o are final and initial residual activity, respectively.

The thermal denaturation activation energy (E_D, in kJ mol^{-1}) was determined by linearized Arrhenius equation (equation 6).

$$\ln(k_D) = \ln(\theta) - \left(\frac{E_D}{R}\right)\left(\frac{1}{T}\right) \quad (6)$$

where θ is the Arrhenius frequency of the collision factor, R is the universal gas constant (8.314 J mol^{-1} K^{-1}) and T is the absolute temperature (K).

The biocatalyst half-lives ($t_{1/2}$, in min) and the variation of enthalpy (ΔH_D, in kJ mol^{-1}), Gibbs energy (ΔG_D, in kJ mol^{-1}) and entropy (ΔS_D, in kJ mol^{-1} K^{-1}) of activation of denaturation were determined using equations 7, 8, 9 and 10, respectively.[20-23]

$$t_{1/2} = -\left(\frac{1}{k_D}\right)\ln\left[\frac{(0.5-\alpha)}{(1-\alpha)}\right] \quad (7)$$

$$\Delta H_D = E_D - RT \quad (8)$$

$$\Delta G_D = (-RT)\ln\left(\frac{k_D h}{k_b T}\right) \quad (9)$$

$$\Delta S_D = \frac{(\Delta H_D - \Delta G_D)}{T} \quad (10)$$

where h is the Planck constant (11.04×10^{-36} J min) and k_b is the Boltzmann constant (1.38×10^{-23} J K^{-1}).

Effect of salts on enzyme activity

The manganese sulfate ($MnSO_4$), chloride sodium (NaCl), calcium chloride ($CaCl_2$), and magnesium sulfate ($MgSO_4$) effects were evaluated with the addition of 10 mM solution into the reaction medium. The enzyme activity was determined under optimum experimental conditions for soluble and encapsulated enzyme.

Obtention of Michaelis-Menten kinetic parameters

The enzymatic activity of soluble and encapsulated enzyme was obtained in different lactose concentrations (0.0 to 70 g L^{-1}). The Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) were estimated by the adjustment of Lineweaver-Burk linearization.[24]

Reuse and hydrolysis of whey lactose and whole milk

One hundred milligram of encapsulated β-galactosidase were used successively (10 cycles) in hydrolysis reactions of milk whey and whole milk. At the end of each cycle (15 min), the pellets were removed from the tube reaction (50 mL of whey or whole milk at 40 and 45 ºC for encapsulated and soluble enzyme, respectively) and washed with McIlvaine buffer (pH 4.5). The enzymatic activity was quantified as previously described. The modified phenol-sulfuric method was used to determine the amount of lactose in milk.[25]

The lactose hydrolysis of whey and whole milk was calculated by equation 11.

$$\text{Lactose hydrolysis}(\%) = \frac{C_{glu} MM_{lac}}{Ci_{lac} MM_{glu}} \times 100 \quad (11)$$

where C_{glu} is glucose concentration, MM_{lac} is molecular mass of lactose, Ci_{lac} is initial concentration of lactose, MM_{glu} is molecular mass of glucose.

Results and Discussion

β-Galactosidase encapsulation

The encapsulation of *A. oryzae* β-galactosidase under different conditions is shown in Table 1. The encapsulation efficiency in Ca-alginate showed values above 74.0%; therefore, the results presented for the enzyme encapsulation yield (EY = 3.19%) and specific activity (SA = 0.71 U mg^{-1}) suggest that there was a diffusional barrier involving the movement of the substrate into the pellets and the output of the product to the external environment. Based on the diffusion factor (η) it was observed that β-galactosidase encapsulated in Ca-alginate had a diffusion limitation of substrate/product by the polymer during the hydrolysis process (η = 0.07). The results can be explained once enzyme encapsulation reduces the volume available for substrate entry (exclusion effect) and increases the path for substrate movement (obstruction effect).[26]

In order to reduce the diffusion effects in the encapsulation of β-galactosidase, modifications of

Table 1. Hydrogel evaluation for commercial β-galactosidase encapsulation

	Total protein / (mg g^{-1})	Specific activity / (U mg^{-1})	EE / %	EY / %	η
Ca-Alg	1.23 ± 0.08	0.71 ± 0.01	74.42 ± 4.93	3.19	0.07
Ca-Alg/CMC	0.72 ± 0.08	7.65 ± 0.64	43.01 ± 5.29	43.68	0.43
Ca-Alg/Chi	1.09 ± 0.15	5.81 ± 1.14	48.64 ± 6.78	30.54	0.47
Ca-Alg/Pec	0.94 ± 0.13	5.66 ± 1.93	53.29 ± 7.79	32.58	0.60
Ca-Alg/Na_2SO_4	0.85 ± 0.17	4.94 ± 0.50	49.87 ± 6.35	24.26	0.30
Ca-Alg/Lac-Crosslinked	1.52 ± 0.09	3.82 ± 0.35	64.21 ± 3.94	40.69	0.50
Ca-Alg/CMC/Lac-Crosslinked	0.98 ± 0.15	6.36 ± 0.82	50.28 ± 7.81	32.14	0.48
Ca-Alg/Chi/Lac-Crosslinked	1.04 ± 0.05	4.34 ± 0.12	54.66 ± 2.94	22.80	0.35
Ca-Alg/Pec/Lac-Crosslinked	0.61 ± 0.00	11.97 ± 0.22	30.83 ± 0.31	59.03	1.20
Ca-Alg/Na_2SO_4/Lac-Crosslinked	0.80 ± 0.05	6.92 ± 0.56	54.18 ± 3.52	42.50	0.60

EE: encapsulation efficiency; EY: encapsulation yield; η: diffusional effect; Ca-Alg: calcium alginate; Lac: lactase; Chi: chitosan; Pec: citrus pectin; CMC: carboxymethylcellulose.

Ca-alginate with adjuvants have been proposed to form networks and ensure greater stability of the system. The addition of carboxymethyl cellulose, chitosan, citrus pectin, and sodium sulfate in combination with alginate increased the recovery of specific activity compared to Ca-alginate. The highest encapsulation yields were observed with Ca-Alg/CMC and Ca-Alg/Pec/Lac-Crosslinked (43.68 and 59.03%, respectively). The encapsulation efficiency reduced considerably with the addition of adjuvants compared to Ca-alginate; therefore, the values for diffusional effect (η) indicate that the alginate modification reduced the diffusion limitation of the substrate/product during the lactose hydrolysis process. The Ca-Alg/Pec/Lac-Crosslinked matrix showed the best results for diffusional effect (η = 1.20) showing that the use of the cross-linked enzyme and the modification of the alginate with citrus pectin favored the diffusion of the substrate/product through the matrix. The addition of adjuvants used in the encapsulation of various enzymes reduced the diffusion effects and improved the conditions of experimental ones in several operating systems.[27,28] Glutaraldehyde has been used extensively as an activator/stabilizer in the immobilization of β-galactosidase in various polymeric matrices. Satar *et al*.[29] reported in the review work that the transposition stability of glutaraldehyde in immobilized β-galactosidase systems is more expressive than other aldehydes, promoting greater operational stability, in addition to being more effective in the hydrolysis of milk lactose in continuous and batch reactors.

Biochemical properties of soluble and encapsulated β-galactosidase

Biochemical properties of encapsulated β-galactosidase were determined using the Ca-Alg/Pec/Lac-Crosslinked matrices as they have the smallest diffusion effects, highest immobilization yield and specific activity.

Optimal pH of soluble and encapsulated enzyme

Figure 1 shows the variation of enzymatic activity in relation to the pH for the soluble and encapsulated enzyme. The optimal pH of activity was different for the soluble (pH 5.0) and encapsulated (pH 6.5) enzyme. Ca-Alg/Pec/Lac-Crosslinked presented a pH range between 5.0 and 6.5 (88-100%), while the soluble enzyme showed a pH range of 4.5 to 5.5 (ca. 90%). Above the optimum activity ranges, Ca-Alg/Pec/Lac-Crosslinked showed a sharp reduction in enzyme activity with activity between 15% (pH 2.5) and 26% (pH 8.0). Similar behavior has been also observed in other immobilization processes and matrices of β-galactosidase. For example, the immobilization in graphite promoted a change in the optimal pH to alkaline value (pH 7.7) when compared with the soluble enzyme (pH 6.6).[30] The optimal pH for immobilized (cross-linked chitosan with glutaraldehyde) and free β-galactosidase was 2.8 and 4.4, respectively.[31] Also, a reduction in the optimum pH from 6.0 to 5.3 was observed after the immobilization of the enzyme in Eupergit® C.[32] This improvement in immobilized β-galactosidase activity is related to the immobilization process where the enzyme is resistant to acid-base denaturation since it can alter the enzyme's microenvironment and consequently the hydrolytic activity.[33]

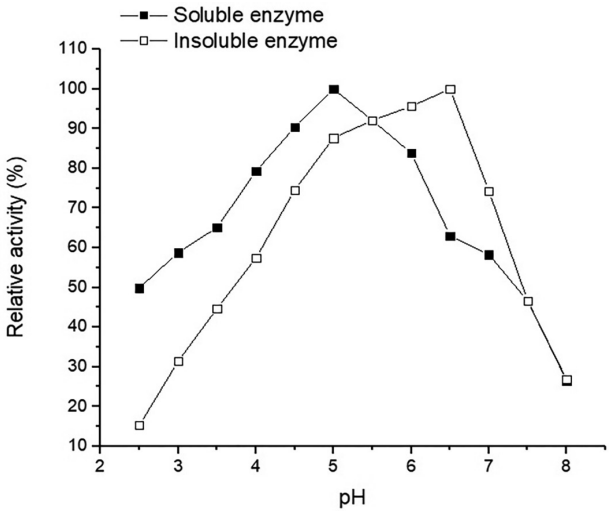

Figure 1. Effect of pH on soluble (■) and encapsulated (□) β-galactosidase. The maximum activity for soluble (10 U mL^{-1}) and immobilized β-galactosidase (8 U g^{-1}) were defined as 100% of relative activity.

Optimal temperature of soluble and encapsulated enzyme

The enzymatic activity of soluble and encapsulated β-galactosidase in different temperatures is displayed in Figure 2. The greatest enzymatic activity of the soluble enzyme was observed at 45 °C, while for the encapsulated enzyme it was at 40 °C. The soluble enzyme showed higher activity values between 35 and 45 °C, with a sharp drop above 45 °C, while Ca-Alg/Pec/Lac-Crosslinked showed greater activity in the higher temperature ranges. Ladero *et al*.[34] observed optimal activity at 40 °C for β-galactosidase from *Kluyveromyces fragilis* soluble and immobilized on silica-alumina. Satar and Ansari[35] also did not observe a change in the optimal temperature (50 °C) of soluble *Cicer arietinum* β-galactosidase immobilized on agarose functionalized with glutaraldehyde. Enzymatic reactions are accelerated by increasing the temperature, within the range in which the enzyme is stable and maintains its full activity.[36] The increase in temperature reaction raises the number of collisions between enzyme and substrate,

increasing the enzymatic activity. This increase in activity ends when the denaturation process begins, which in turn is caused by temperature conditions, when the collisions become disordered, tending to disrupt the molecular interactions of the enzymatic structure, decreasing its catalytic activity.[37]

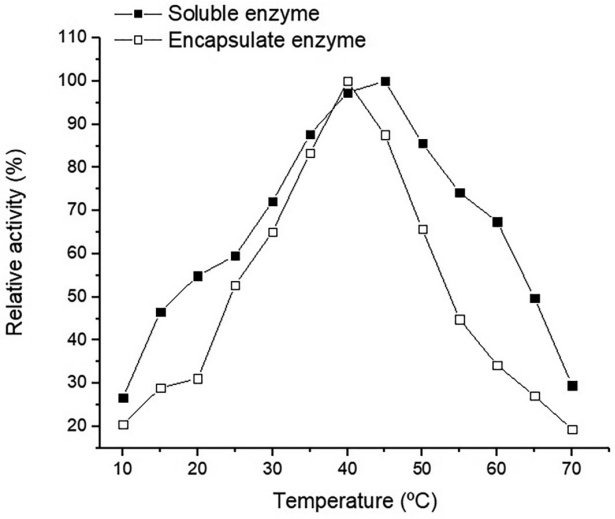

Figure 2. Effect of temperature on soluble (■) and encapsulated (□) β-galactosidase. The maximum activity for soluble (10 U mL^{-1}) and immobilized β-galactosidase (8 U g^{-1}) were defined as 100% of relative activity.

Thermal stability of soluble and immobilized enzyme activity

Figure 3 shows the inactivation rate of soluble (Figure 3a) and immobilized (Figure 3b) β-galactosidase from *A. oryzae* at different incubation temperatures. At the end of the reaction time, the residual activity of soluble and immobilized enzymes was 17.10% (1.48 U g^{-1}) and 18.90% (1.79 U g^{-1}) at 40 ºC, respectively. In addition, the residual activity values decreased with increasing temperature (50 and 60 ºC) and incubation time, indicating the thermal denaturation of the biocatalyst (Figure 3). The effect of immobilization on thermal stability has been demonstrated by several studies[1,38-40] associating the increased stability with the immobilization process due to the enzyme bonds to the matrix.

The same behavior was detected in thermal stability of β-galactosidase from *Kluyveromyces lactis.* In addition, the enzyme from *A. oryzae* was more thermally stable.[38] As the commercial β-galactosidase was thermally stable only at 30, 35 and 40 °C, presenting an activity reduction of 19.44% at 45 ºC after 80 min and complete inactivation after 40 min at 50 °C.[41]

The soluble and immobilized β-galactosidase thermodynamics parameters are shown in Table 2. The half-life ($t_{1/2}$) for soluble enzyme was about 1.2-fold higher at 40 ºC (145.20 min) than that of the biocatalyst evaluated at 50 ºC (125.43 min) and 60 ºC (128.26 min). On the hand other, the $t_{1/2}$ values for immobilized β-galactosidase were 244.95, 120.00 and 24.78 min at 40, 50 and 60 ºC, respectively. The higher $t_{1/2}$ values for encapsulated enzyme than soluble enzyme at 40 ºC indicates higher reaction rates for a longer time, allowing its industrial processes application.[21,24,42]

Table 2 also shows that the E_D value of encapsulated β-galactosidase (96.07 kJ mol^{-1}) was significantly higher (about 31.6-fold) than that soluble enzyme (3.04 kJ mol^{-1}), suggesting more thermostability and heat resistance to the immobilized biocatalyst. E_D values indicate the amount of energy required to denature a protein (enzyme). The results obtained for the encapsulated β-galactosidase show that a greater input of activation energy is necessary to reach

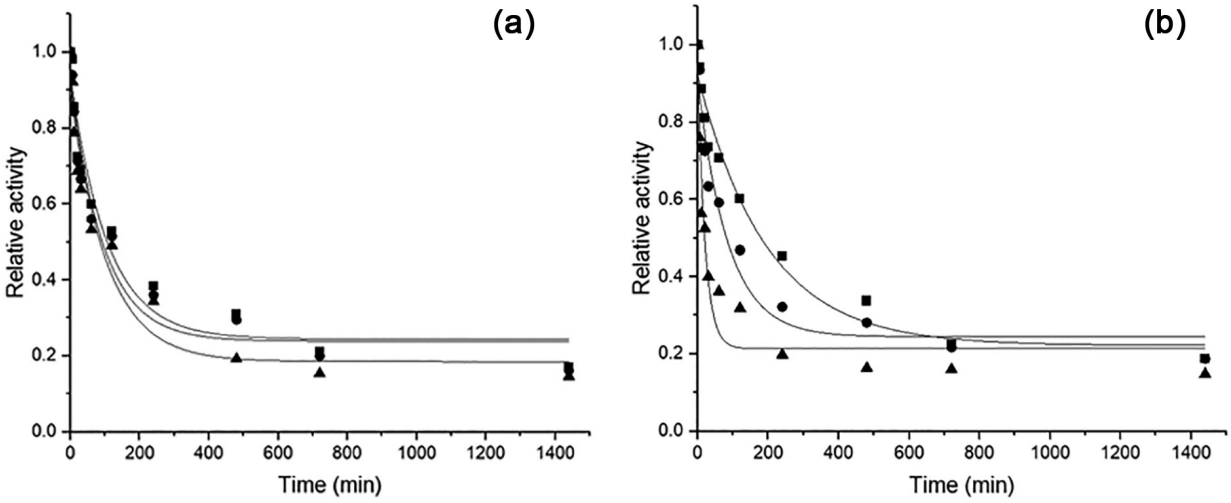

Figure 3. Thermal stability of soluble β-galactosidase (a) and immobilized β-galactosidase (b) over 24 h of incubation at different temperatures: 40 ºC (■), 50 ºC (●) and 60 ºC (▲). Continuous line: Sadana-Henley[20] thermal inactivation model fitted to the experimental data. The maximum activity for soluble (10 U mL^{-1}) and immobilized β-galactosidase (8 U g^{-1}) were defined as 100% of relative activity.

Table 2. Thermodynamic parameters of soluble and immobilized β-galactosidase incubated at different temperatures

Parameter	β-Galactosidase	Temperature / ºC		
		40	50	60
R^2	soluble	0.94	0.94	0.93
	immobilized	0.96	0.93	0.92
k_D / min^{-1}	soluble	8.90×10^{-3}	10.44×10^{-3}	9.52×10^{-3}
	immobilized	5.13×10^{-3}	11.67×10^{-3}	47.31×10^{-3}
$t_{1/2}$ / min	soluble	145.20	125.43	128.26
	immobilized	244.95	120.00	24.78
E_D / (kJ mol^{-1})	soluble		3.04	
	immobilized		96.07	
ΔH_D / (kJ mol^{-1})	soluble	0.43	0.35	0.27
	immobilized	93.46	93.38	93.30
ΔG_D / (kJ mol^{-1})	soluble	105.27	108.28	111.97
	immobilized	106.70	107.98	107.53
ΔS_D / (J mol^{-1} K^{-1})	soluble	–334.77	–334.00	–335.31
	immobilized	–42.27	–45.19	–42.73

R^2: coefficient of determination; k_D: thermal denaturation constant; $t_{1/2}$: biocatalyst half-life; E_D: activation energy; ΔH_D: enthalpy variation of the activation of denaturation; ΔG_D: variation of Gibbs energy of the activation of denaturation; ΔS_D: variation of entropy of the activation of denaturation.

its thermal denaturation, indicating greater enzymatic thermostability.[24,43]

Similarly, the enthalpy of activation of denaturation (ΔH_D) of the immobilized enzyme was 217.3, 266.8 and 345.6-fold greater than that of soluble biocatalyst. The positive and higher ΔH_D values associated with the E_D values indicate that immobilization of β-galactosidase benefitted the enzymatic thermostability.[23,24,43]

Table 2 also shows the ΔS_D and ΔG_D values. Encapsulation of β-galactosidase in Ca-Alg/Pec/Lac-Crosslinked increased the ΔS_D (about 7.7 times) than that soluble enzyme. These results show that with enzyme encapsulation the hydrophobic interactions can be strengthened and stabilize the three-dimensional structure of the protein molecule, leading to negative ΔS_D values, since the enzyme resistance to unfolding because of stronger hydrophobic interactions overcomes the enzyme tendency to fall apart due to weakened polar interactions at high temperatures.[24,44,45]

On the other hand, positive ΔG_D values were obtained for soluble and immobilized β-galactosidase, suggesting more amount of enzyme in the native state than denatured state at the equilibrium condition.[34,43] In addition, these values indicate that the thermal denaturation of the enzyme is a non-spontaneous process.[21,23,24]

Effect of salts on soluble and encapsulated enzyme activity

Table 3 shows the results for the effect of adding salts in the reaction medium on the enzymatic activity of soluble and encapsulated β-galactosidase. Salts can act as enzymatic cofactors and accelerate an enzymatic reaction as well as act as inhibitors for some enzymes.[32] The addition of 10 mM $MgSO_4$ to the reaction medium increased the activity by 195.8% for Ca-Alg/Pec/Lac-Crosslinked and by 155.7% for the soluble enzyme. Magnesium acts as a cofactor or activator of more than 300 enzymes, among which are the enzymes hexokinase, glycokinase and β-galactosidase. This ion is considered an activator that accelerates enzyme reaction rates by promoting the active state of the substrate or enzyme.[46]

Table 3. Effect of salts on activity of soluble and encapsulated β-galactosidase

Salt	Relative activity / %	
	Soluble β-galactosidase	Encapsulated β-galactosidase
Control	100.00 ± 1.10	100.00 ± 1.05
$MnSO_4$	115.48 ± 1.25	150.00 ± 1.75
$CaCl_2$	73.36 ± 0.95	96.98 ± 0.78
NaCl	96.05 ± 0.84	117.35 ± 1.15
$MgSO_4$	150.76 ± 1.35	195.75 ± 1.85

$MnSO_4$ also showed activation of the soluble and encapsulated enzyme in 115.5 and 150.0%, respectively. Manganese could act both as an enzyme activator and as a constituent of metalloenzymes. NaCl showed an increase in enzymatic activity of 117.35% for the encapsulated enzyme. For the free enzyme, there was a reduction in enzyme activity (96.05%). $CaCl_2$ did not influence the enzyme activity for

the encapsulated enzyme, while for the free enzyme, there was a reduction in enzyme activity (73.36%).

Enzyme kinetic

The determination of K_m and V_{max} was performed in different concentrations of lactose (0 to 70 g L^{-1}) to measure the enzymatic activity of the soluble and encapsulated enzyme. The K_m constant of Ca-Alg/Pec/Lac-Crosslinked was 70.0 mmol L^{-1} and for the soluble enzyme 13.78 mmol L^{-1}. The maximum activity for the immobilized enzymes was 1.78-fold higher compared to the free enzyme (V_{max} = 23.98 mmol L^{-1} min^{-1}). These results reflect the presence of the diffusional effect during the immobilization process, influencing the kinetic results due to experimental conditions.

The increase in K_m values in relation to those found for the soluble enzyme can be explained by decreasing the enzyme's affinity for the substrate, which may be related to factors linked to the enzyme confinement process and the difficulty of the substrate to access the active sites of the enzyme.[47] Kumar *et al.*[48] observed an increase of approximately 2-fold in the K_m values for urease immobilized in alginate and chitosan and the V_{max} values maintained without significant changes. The authors attributed this change in K_m value mainly to the restriction of substrate diffusion caused by the alginate matrix. The matrix often prevents the free diffusion of the substrate and, therefore, the substrate takes longer to get to the catalysis site.

Reusability of encapsulated β-galactosidase and hydrolyze of milk whey lactose and whole milk

The reusability of the enzyme is essential to reduce production costs in industrial applications.[48] The reusability of encapsulated β-galactosidase from *A. oryzae* was carried out to determine the potential of hydrolysis of milk whey lactose and whole milk and the recycle of the encapsulated enzyme (Figure 4). The encapsulated enzyme presented better retention of hydrolytic activity using milk whey lactose, maintaining 30.0% of hydrolysis after 5 cycles (Figure 4a). The reusability of this enzyme in milk whey lactose was found in 8 cycles (51.9%) (Figure 4b). Encapsulated β-galactosidase showed lower hydrolysis values for whole milk lactose compared to whey lactose. The reusability for this condition was found in 7 cycles, but with a sharper decline among encapsulated enzyme reuses. Commercial β-galactosidase NOLA™ Fit 5500 encapsulated into alginate capsules showed reuse of 6 cycles retaining 20% initial activity.[49] The loss of activity was associated with longer cycle time required to hydrolyze more than 97% of the lactose. A β-galactosidase immobilized in modified gum arabic hydrogel showed different yields in the hydrolysis of standard lactose (52.79% ± 0.85) and UHT milk lactose (93.92% ± 1.05) after 350 min of reaction.[50] In this sense, the encapsulated β-galactosidase can produce lactose-free/low-lactose foods and maintain its initial enzymatic activity.

Conclusions

The use of pectin-alginate hydrogel for β-galactosidase encapsulation from *A. oryzae* improved the immobilization parameters of enzyme and operational parameters. The thermodynamic parameters obtained suggest that the immobilization improved the thermostability of the biocatalyst. The results of thermostability of β-galactosidase from *A. oryzae* soluble and encapsulated are novel and represent an important contribution to

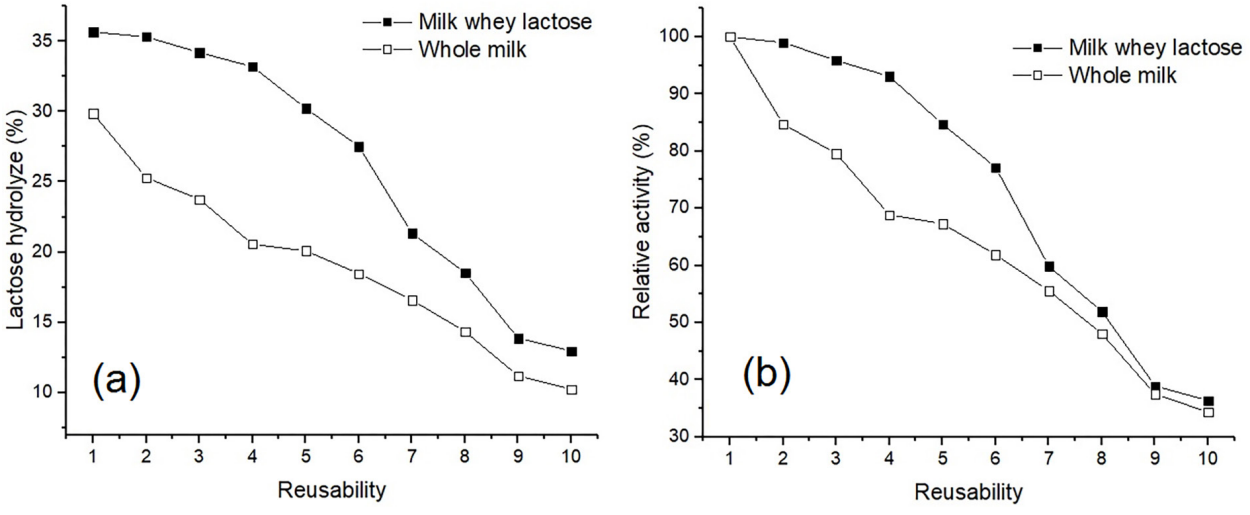

Figure 4. Lactose hydrolysis (a) and reusability (b) of encapsulated β-galactosidase in milk whey lactose and whole milk.

research about the immobilization of this enzyme and its biotechnological application. The enzyme encapsulated showed the possibility of reuse in up to 8 cycles for the milk whey lactose hydrolysis and 7 cycles for whole milk lactose hydrolysis will greatly reduce the operating costs of milk processing industries. So, the encapsulated β-galactosidase can produce lactose-free/low-lactose foods maintaining its initial enzymatic activity.

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Author Contributions

Maykon J. Paiva was responsible for the conceptualization, methodology, validation, formal analysis, investigation, data curation, writing-original draft, visualization; Fabrício C. Paula-Elias for the data curation, validation, methodology; Luciana A. Pereira and Erika C. Vieira-Almeida for the writing- review and editing; Solange C. Carreiro for the laboratory infrastructure, data curation, writing review; Ezequiel M. Silva for the experimental design of encapsulation, data curation, writing original draft; Giancarlo S. Dias and Michelle A. C. Xavier for the writing review - original draft and final; Sergio A. Morales and Rafael F. Perna for the methodology (thermal stability assay), data curation, validation, and writing original draft; Alex F. Almeida for the resources, data curation, writing - review and editing, supervision, funding acquisition.

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