Brazilian Journal
of Chemical
Engineering

ISSN 0104-6632
Printed in Brazil
www.abeq.org.br/bjche

Vol. 36, No. 04, pp. 1403 - 1417, October - December, 2019
dx.doi.org/10.1590/0104-6632.20190364s20190235

STABILIZATION STUDY OF TETRAMERIC *Kluyveromyces lactis* β-GALACTOSIDASE BY IMMOBILIZATION ON IMMOBEAD: THERMAL, PHYSICO-CHEMICAL, TEXTURAL AND CATALYTIC PROPERTIES

Adriano Gennari[1], Francielle Herrmann Mobayed[1], Ruan da Silva Rafael[1], André Luis Catto[2], Edilson Valmir Benvenutti[3], Rafael C. Rodrigues[4], Raul Antonio Sperotto[5], Giandra Volpato[6] and Claucia Fernanda Volken de Souza[1,7*]

[1] Universidade do Vale do Taquari, Programa de Pós-Graduação em Biotecnologia, Laboratório de Biotecnologia de Alimentos, Lajeado, RS, Brasil. ORCID: 0000-0002-2965-8135; ORCID: 0000-0003-4829-969X; ORCID: 0000-0002-9195-0137; E-mail: claucia@univates.br - ORCID: 0000-0001-7708-3762
[2] Universidade do Vale do Taquari, Centro de Ciências Exatas e Tecnológicas, Lajeado, RS, Brasil. ORCID: 0000-0001-5024-436X
[3] Universidade Federal do Rio Grande do Sul, Instituto de Química, Porto Alegre, RS, Brasil. ORCID: 0000-0002-7889-3625
[4] Universidade Federal do Rio Grande do Sul, Instituto de Ciência e Tecnologia de Alimentos, Grupo de Biotecnologia, Bioprocessos e Biocatálise, Porto Alegre, RS, Brazil. ORCID: 0000-0001-8592-850X
[5] Universidade do Vale do Taquari, Centro de Ciências Biológicas e da Saúde, Programa de Pós-Graduação em Biotecnologia, Lajeado, RS, Brasil. ORCID: 0000-0003-1602-6101
[6] Instituto Federal de Educação, Ciência e Tecnologia do Rio Grande do Sul, Campus Porto Alegre, Curso de Biotecnologia, Porto Alegre, RS, Brasil. ORCID: 0000-0002-2524-8868
[7] Universidade do Vale do Taquari, Centro de Ciências Exatas e Tecnológicas, Programa de Pós-Graduação em Biotecnologia, Lajeado, RS, Brasil.

(Submitted: May 6, 2019 ; Revised: June 24, 2019 ; Accepted: July 24, 2019)

Abstract - We investigated the immobilization of a tetrameric *Kluyveromyces lactis* β-galactosidase (EC: 3.2.1.23) (KL-Gal) on Immobead 150 using different support modification strategies. Immobead support was modified using an acid solution of H_2SO_4:HNO_3 (3:1) (Immobead-Ac) or 5 % (v/v) glutaraldehyde (Immobead-Glu). Its unmodified form (Immobead) was also tested. Immobilization yields and efficiencies were evaluated by testing protein loads from 10 to 200 mg.g^{-1} support. The thermal, physico-chemical, textural and catalytic properties of the supports (modified and unmodified) and their derivatives (Immobead-KL-Gal, Immobead-Ac-KL-Gal and Immobead-Glu-KL-Gal) were analyzed. The highest immobilization yields and efficiencies were achieved with a protein load of 100 mg.g^{-1} support. Surface and pore areas of the Immobead support were greatly decreased after modification. Michaelis constant of the immobilized β-galactosidase increased in the derivatives. Maximum velocity decreased approximately 2.8 times for Immobead-KL-Gal and Immobead-Glu-KL-Gal, and approximately 1.4 times for Immobead-Ac-KL-Gal. In batch processes, the three derivatives could be reused successfully at least 15 times, maintaining high residual enzymatic activity during the lactose hydrolysis (in both cheese whey and milk). The tetrameric *K. lactis* β-galactosidase immobilized on Immobead supports via the tested treatments was stabilized and is an alternative tool for lactose hydrolysis in the dairy industry.

Keywords: Glutaraldehyde; Acid solution; Batch hydrolysis; Yeast.

* Corresponding author: Claucia Fernanda Volken de Souza - E-mail: claucia@univates.br

INTRODUCTION

Interest in biocatalytic processes has been increasing due to the growing demand for biotransformations with high specificity in the fine chemical industries, such as pharmaceuticals, food and agrochemicals (Faber, 2011; Flickinger, 2010). However, these biocatalysts generally have high industrial costs, and their recovery at the end of reaction processes is not economically viable. Moreover, the structural stability of some enzymes during biochemical reactions is also challenging (Kawaguti et al., 2006; Panesar et al. 2010). Enzyme immobilization is a promising tool to overcome these limitations, providing many benefits, such as convenient separation from the product, possible enhancement of stability under both storage and operational conditions, and maintenance of the enzyme performance after continuous reuses (Sheldon, 2007; DiCosimo et al., 2013; Liese and Hilterhaus, 2013).

For these purposes, many enzymes have been immobilized using different protocols, such as adsorption, covalent attachment, chemical aggregation, microencapsulation, and entrapment (Sun et al., 2010; Sharifi et al., 2018). In addition to the immobilization technique, selection of support material is also of vital importance for enzyme immobilization. Inert polymers and inorganic materials are generally used as carrier matrices for enzyme immobilization (Datta et al., 2013). Immobead 150 is an epoxy support with a high surface area that is formed by cross-linked copolymers of methacrylate bearing oxirane groups (Mateo et al., 2007). Given the enzyme carrier requirements of large surface area, narrow pore size distribution, well-defined pore geometry, and thermal/mechanical stability, this polymer is a promising candidate for enzyme immobilization (Hartmann, 2005; Yiu and Wright, 2005), and has been employed to immobilize different enzymes by taking advantage of its capacity to adsorb or form covalent linkages (Mateo et al., 2007; Alagöz et al., 2016; Matte et al., 2016; Harini et al., 2017).

β-Galactosidase catalyzes lactose hydrolysis into glucose and galactose (Vasiljevic and Jelen, 2001). An important industrial application of this enzyme is to provide lactose-free products for consumption by lactose-intolerant individuals (Haider and Husain, 2009; Novalin et al., 2005). This enzyme can be obtained from a wide variety of microorganisms, plants, and animals, and the optimum catalytic properties, pH and temperature are source-dependent (Klewicki, 2007; Yi et al., 2011; Zeuner et al., 2016).

β-Galactosidase from *Kluyveromyces lactis* is an intracellular enzyme consisting of 1,024 amino acids. Its crystal structure reveals a tetrameric protein with a molecular weight of ~ 118 kDa per subunit (Mahoney, 2002; Pereira-Rodríguez et al., 2012). This enzyme is most commonly used to hydrolyze milk lactose, sweet whey and neutral pH dairy products, since its optimum pH is 6.5-7.0 (Spohner et al., 2016). In addition, its Generally Recognized As Safe (GRAS) status makes this enzyme one of the preferred β-galactosidases for food and pharmaceutical industrial applications (Panesar et al., 2010). Monomeric β-galactosidases, such as found in *Aspergillus oryzae*, have been immobilized and stabilized on several materials for application in catalytic hydrolysis reactions (Neri et al., 2009; Kang et al., 2015; Gennari et al., 2018; Križnik et al., 2018; Zaak et al., 2018). For multimeric enzymes, as reported previously (Fernández-Lafuente et al., 1999; Akhtar et al., 2002; Hudmon, 2005), dissociation of the subunits may become a relevant issue when simulating industrial scale processes under very mild experimental conditions. Thus, any strategy that provides quaternary stabilization for multimeric enzymes is important to improve the stability of these enzymes under dissociating conditions (Fernandez-Lafuente, 2009).

We studied the immobilization of *Kluyveromyces lactis* β-galactosidase on Immobead 150 supports. Stabilization of this multimeric enzyme was examined and compared after immobilization on unmodified Immobead (via epoxy groups), or on Immobead modified with glutaraldehyde or by an acid mixture. Thermal, physico-chemical, and textural characteristics of the modified or unmodified supports and their derivatives were evaluated. Biocatalysts were further characterized in terms of optimum catalytic conditions, kinetic parameters and thermal/operational stability.

MATERIALS AND METHODS

Materials

Enzyme β-galactosidase from *K. lactis* (Lactomax Pure, EC: 3.2.1.23) (KL-Gal) was purchased from Prozyn Company (São Paulo, Brazil). Skim milk powder and cheese whey powder were purchased from BRF - Brasil Foods S.A. (Rio Grande do Sul, Brazil). Whey permeate powder was provided by Arla Foods (Córdoba, Argentina). Enzymatic kit for glucose determination was acquired from Labtest (Minas Gerais, Brazil). Coomassie (Bradford) Protein Assay Kit, support Immobead 150, ortho-nitrophenyl-β-D-galactopyranoside (ONPG), and other chemicals were purchased from Sigma Aldrich Co. (St. Louis, USA). All other reagents were of analytical grade.

Methods

Support pretreatments

Based on Gennari et al. (2018), the Immobead 150 (Immobead) support was pretreated using two different

strategies: modification of the surface groups with glutaraldehyde (Immobead-Glu), and oxidation of the surface groups with an acid solution (Immobead-Ac). To prepare Immobead-Glu, 1 g of Immobead was added to 10 mL of ethylenediamine (EDA, 1 M, pH 10), and the mixture was incubated for 12 h with gentle stirring at room temperature. Then, modified support was washed thoroughly with distilled water and dried at room temperature. Subsequently, 50 mg of EDA-activated Immobead was mixed with 4 mL of glutaraldehyde (5 %, v/v) and stirred for 8 h. Immobead was also treated with an acid solution (Immobead-Ac). Briefly, 50 mg of support was mixed with 4 mL of 1 M H_2SO_4 and 1 M HNO_3 (3:1, v/v), and maintained at 150 rpm for 8 h. The treated supports were recovered by centrifugation (1,000 *g* for 5 min at 4 °C), washed with ultra-pure water, and stored in sodium phosphate buffer (100 mM, pH 7.0) until further use.

Immobilization of β-galactosidase on Immobead

Immobilization was carried out on both unmodified (Immobead) and modified (Immobead-Glu and Immobead-Ac) supports. The enzyme KL-Gal was dissolved in corresponding volumes of sodium phosphate buffer (1 M, pH 7.0) to obtain concentrations equivalent to 10-200 mg protein.g^{-1} support. Briefly, each Immobead preparation was mixed with the enzyme solution and shaken for 24 h at 30 °C. Supernatant of each mixture was used for protein and activity determination to monitor the immobilization effect. Immobilized β-galactosidase was separated by centrifugation (5,000 *g* for 5 min at 4 °C), and derivatives formed by reaction of the enzyme with unmodified (Immobead-KL-Gal) or modified (Immobead-Ac-KL-Gal and Immobead-Glu-KL-Gal) supports were washed twice with sodium phosphate buffer (100 mM, pH 7.0). Immobilization yield and efficiency, and activity recovery were calculated by using Eq. 1, 2, and 3, respectively, according to Sheldon and Van Pelt (2013).

$$\text{Yield}(\%) = \frac{\text{Immobilized activity}}{\text{Initial activity}} \times 100 \quad (1)$$

$$\text{Efficiency}(\%) = \frac{\text{Observed activity}}{\text{Immobilized activity}} \times 100 \quad (2)$$

$$\text{Activity Recovery}(\%) = \frac{\text{Observed activity}}{\text{Initial activity}} \times 100 \quad (3)$$

Immobilized activity is the one that remains in the enzyme solution after immobilization by subtracting this activity from the initial activity. Observed activity is the one measured in the particles after immobilization. Immobilization yield and efficiency, activity recovery, and derivative activity were analyzed using One-Way ANOVA, and an F test was performed to evaluate the significance of the model. Differences in Tukey test were considered significant at the 95 % level ($p<0.05$), using Statistica 13.1 software.

Determination of β-galactosidase activity

Activities of free and immobilized β-galactosidase were assayed using the ONPG method. A dilute enzyme solution was added to an ONPG solution (18 mM in 100 mM sodium phosphate buffer, pH 7.0, with 5 mM magnesium chloride) at 37 °C. The reaction was conducted for 2 min and stopped adding 1.5 mL of sodium carbonate (2 M, pH 9.8). Activity was measured as the increase in the absorbance corresponding to the release of o-nitrophenol (molar extinction coefficient 4,500 $L.mol^{-1}.cm^{-1}$ at 415 nm, pH 7.0) from the ONPG hydrolysis. One unit of specific activity was defined as the amount of enzyme required to hydrolyze 1 mmol of ONPG per min at pH 7.0 and 37 °C. Protein concentrations were determined using the Bradford method (Bradford, 1976).

Thermal and physico-chemical study of support and derivatives

Unmodified and modified supports, along with derivatives, were studied by infrared spectroscopy (IR) using a Shimadzu/IRAffinity-1 spectrometer. Infrared spectrum was obtained using KBr pellets at room temperature with 45 cumulative scans and resolution of 4 cm^{-1}. Thermogravimetric analysis (TGA) curves of the unmodified (Immobead) and modified (Immobead-Ac and Immobead-Glu) supports and their derivatives (Immobead-KL-Gal, Immobead-Ac-KL-Gal, and Immobead-Glu-KL-Gal) were obtained with a Perkin-Elmer TGA 4000 instrument using a heating rate of 5 °C.min^{-1} in a temperature range of 25-550 °C under a nitrogen atmosphere. The specific heat capacities (determined by differential scanning calorimetry (DSC)) of the unmodified and modified Immobead, and also of the derivatives, were measured with the Perkin-Elmer model 4000 instrument. Around 5.4 mg of sample was introduced into a DSC aluminum crucible with 40 μL capacity. Temperature varied from 25 to 400 °C with a 5 °C.min^{-1} heating ramp, a nitrogen gas flow of 20 mL.min^{-1} and a gas pressure of 3.4 bar.

Textural characterization

Textural characterization was performed using N_2 adsorption-desorption isotherms at the boiling point of liquid N_2, with a Tristar Kr II 3020 Micromeritics instrument. Samples (Immobead, Immobead-Ac, Immobead-Glu, Immobead-KL-Gal, Immobead-Ac-KL-Gal, and Immobead-Glu-KL-Gal) were previously degassed at 120 °C under vacuum for 12 h. The specific surface area was determined by the Brunauer, Emmett

and Teller (BET) multipoint technique, and the pore size distribution was obtained by the Barret-Joyner-Halenda (BJH) and density functional theory (DFT) methods (Webb and Orr, 1997).

Biocatalyst properties

Different buffer systems were employed to study the optimal pH for the free and immobilized β-galactosidases. Activities of the free and immobilized forms were measured at 37 °C in 100 mM sodium phosphate and acetate buffers with pH ranging from 5.5 to 8.5. Optimal temperature was evaluated by varying the temperature from 4 to 55 °C in sodium phosphate buffer (100 mM, pH 7.0). The activity recorded at the optimum pH or temperature was taken as 100 % activity, and the activities obtained with the other conditions were expressed as percentages of this optimum activity.

The Michaelis constant (K_M) and maximum reaction rate (V_{max}) were calculated for free and immobilized β-galactosidase using the Michaelis-Menten model and Lineweaver-Burk linearization. For this purpose, different concentrations of ONPG (3-18 mM) in 100 mM sodium phosphate buffer, pH 7.0, were employed during the enzymatic reaction. Turnover numbers (k_{cat}) of free and immobilized β-galactosidase were calculated by the relation of the maximum velocity and the total enzyme concentration in the reaction. The catalytic specificity constant was calculated as the ratio between the turnover number (k_{cat}) and the Michaelis constant (K_M).

Thermal stability of free and immobilized β-galactosidases was determined in terms of enzyme activity loss upon incubation at different temperatures, according to Rosolen et al. (2017). Samples were maintained for certain periods of time at different temperatures (55, 58, 60 and 63 °C). Thermodynamic parameters were calculated according to the simplified deactivation model described by Kikani and Singh (2015).

The activation energy (E_a) for the inactivation process was calculated using nonlinear regression and considering the Arrhenius plot (Eq. 4):

$$k = A \cdot e^{-\frac{Ea}{R \cdot T}} \tag{4}$$

where k (min^{-1}) is the kinetic deactivation constant, A (min^{-1}) is the pre-exponential factor, E_a ($J.mol^{-1}$) is the activation energy, R (8.314 $J.mol^{-1}.K^{-1}$) is the universal gas constant, and T (K) is the temperature.

Half-life ($t_{1/2}$) of the enzyme is defined as the time required to lose half of the initial enzyme activity. Half-life was estimated by using Eq. 5:

$$t_{1/2} = \frac{\ln 2}{k} \tag{5}$$

where $t_{1/2}$ (min) is the half-life, and k (min^{-1}) is the kinetic deactivation constant.

The stabilization factor (SF) at a specific temperature was determined as the ratio between the half-life of each derivative and the half-life of free β-galactosidase.

The Gibbs free energy of thermal inactivation (ΔG) was calculated using different temperatures from the first-order rate constant of the inactivation process (Eq. 6):

$$\Delta G = -R \cdot T \cdot \ln\left(\frac{k \cdot h}{k_B \cdot T}\right) \tag{6}$$

where R (8.314 $J\ mol^{-1}.K^{-1}$) is the universal gas constant, T (K) is the temperature, k (min^{-1}) is the kinetic deactivation constant, h (6.6262×10^{-34} J.s) is the Planck constant, and k_B ($1.3806 \times 10^{-23}\ J.K^{-1}$) is the Boltzmann constant.

Activation enthalpy (ΔH) was calculated from the activation energy ($\Delta H = E_a - RT$), and the entropy (ΔS) was calculated from the enthalpy and Gibbs free energy of activation ($T\Delta S = \Delta H - \Delta G$).

Operational stability

Reusability of the immobilized β-galactosidase was determined by the hydrolysis of four different solutions: lactose, whey permeate, cheese whey, and skim milk (all prepared to 5 % w/v lactose). Immobilized enzyme (100 mg) was incubated with 10 mL of each solution at 4 °C and 150 rpm for 30 min. Then, the enzyme was separated by centrifugation (5,000 *g* for 5 min at 4 °C), and its activity was assayed according to Section 'Determination of β-galactosidase activity'. Supernatant was used to determine the lactose hydrolysis by glucose formation using a Glucose Oxidase kit. After the activity assay, the enzyme was washed, and fresh substrate solution was added to initiate the next round. Residual activity of each derivative after the first cycle was taken as 100 % activity. The hydrolysis degree of each derivative after the first cycle was defined as 100 % hydrolysis, and the hydrolysis percentages of the following cycles (relative hydrolysis) were expressed in relation to the first cycle. Free β-galactosidase was submitted to one hydrolysis cycle in the same reaction conditions of immobilized enzyme, and the results are shown in absolute values of hydrolysis yield.

RESULTS AND DISCUSSION

Immobilization of β-galactosidase on Immobead

Initially, the enzyme load used for immobilization on each support (modified and unmodified) (Fig. 1) was varied. The immobilization yield and efficiency,

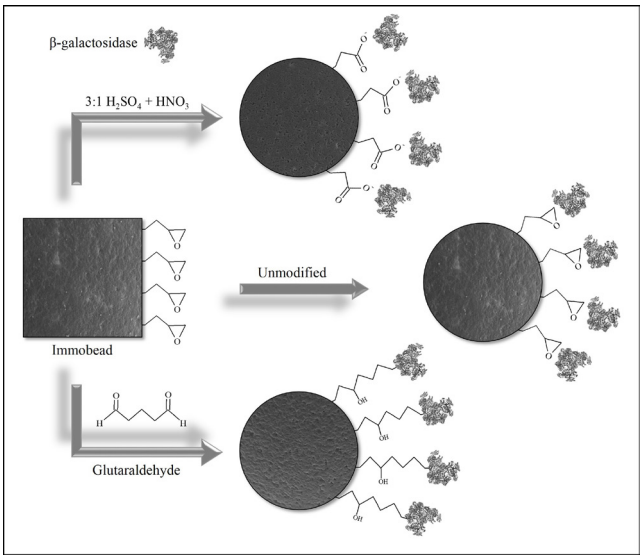


Figure 1. Scheme for β-galactosidase immobilization on Immobead. Scanning Electron Microscope (SEM) images of support modified by an acid solution, glutaraldehyde, and in its unmodified form.

activity recovery, and derivative activity of *K. lactis* β-galactosidase on Immobead are presented in Table 1.

Immobilization yield was found to be 100 % with a protein load of 10 $mg.g^{-1}$ support. A protein load higher than 100 $mg.g^{-1}$ support results in a significant yield reduction. All three immobilization strategies resulted in efficiencies of at least 60 %. The highest activity recovery was obtained for the Immobead-KL-Gal derivative. It was observed that the increase to 200 mg of protein results in expressive reductions of the activity recovery and derivative activity ($U.g^{-1}$ of support) for all derivatives. Although the glutaraldehyde treatment presented the lowest efficiency among the different treatments, its yields remained 100 % up to a loading of 100 mg of protein. Immobead-Glu-KL-Gal presented the highest yield among the examined protocols. Glutaraldehyde can interact covalently with ε-amino groups from Lys in the β-galactosidase enzyme. Even though their pK_as > 10.5, a small percentage of these amines are unprotonated at lower pH, enough to react with glutaraldehyde, driving the acid-base equilibrium to deprotonation of these groups for further reaction (Migneault et al., 2004). Conversely, in Immobead-KL-Gal, immobilization first occurs via hydrophobic adsorption between the enzyme and the hydrophobic surface of the support; subsequent covalent interactions occur between the terminal amino group of the enzyme and the epoxy groups of the support surface, which may also form additional linkages with the amino groups of enzyme Lys side chains. On the Immobead-Ac-KL-Gal derivative, immobilization occurred via nucleophilic attack on the oxirane groups of Immobead support. This acid oxidation treatment was followed by stabilization of the carbocation through electrostatic interactions with the negative charges in the β-galactosidase side chains (Balasubramanian and Burghard, 2005).

The enzyme β-galactosidase from *Aspergillus oryzae* was also immobilized on Immobead by Gennari et al. (2018). The three support preparations showed no more than 89 % of yield to a load of 100 mg protein.g^{-1} support. On the other hand, authors obtained efficiencies higher than 84% for all derivatives. Such differences observed with the present study could be attributed to the enzyme quaternary structure, since *A. oryzae* β-galactosidase is monomeric, while *K. lactis* enzyme is tetrameric. High immobilization yields were obtained by Wahba and Soliman (2018) when *A. oryzae* β-galactosidase was covalently bound to carrageenan beads activated with whey protein and glutaraldehyde. In these conditions, the obtained immobilization yield was 90.46 %. However, the immobilized β-galactosidase activity recovery was only 34.80 %. Such low activity level is probably due to the extensive glutaraldehyde cross-linking which can severely distort the active sites, inactivating the enzyme. Immobilization of a multimeric β-galactosidase from *K. lactis* on modified Arabic gum-based hydrogel showed that the immobilization protein capacity was approximately 242 $mg.g^{-1}$ support using acetate buffer (pH 4.0) (Wolf et al., 2018). Although using a higher protein load than our study, immobilization efficiencies were only around 8%. According to Wolf et al. (2018), these results were influenced by destabilization of the hydrophilic three-dimensional network of the hydrogel and the enzyme isoelectric point.

Considering the enzyme concentrations and the immobilization yield, efficiency, activity recovery and derivative activity results, preparations with protein loads of 100 $mg.g^{-1}$ support were selected for evaluation of thermal, physico-chemical, textural and biocatalytic properties, along with operational stability.

Thermal and physico-chemical study of treated Immobead supports and their derivatives

The TGA curves of Immobead, Immobead-Ac and Immobead-Glu showed a three-step weight loss (Fig. 2A). For Immobead, the steps were: 1) from room temperature to 100 °C (approximately 5 % w/w), assigned to the thermal desorption of water physically adsorbed on the support surface (Poppe et al., 2013); 2) 100-350 °C (60 % w/w), attributed to oxidative decomposition of Immobead (Matte et al., 2017); and 3) 350-700 °C (30 % w/w), related to the complete oxidation of the polymer and the oxygen-containing

Table 1. β-Galactosidase immobilized on Immobead: Protein loading study of the different support preparations.

Protein load ($mg.g^{-1}$ support)	Immobead-KL-Gal				Immobead-Ac-KL-Gal				Immobead-Glu-KL-Gal			
	Yield (%)	Efficiency (%)	Activity recovery (%)	Derivative activity (U/g)	Yield (%)	Efficiency (%)	Activity recovery (%)	Derivative activity (U/g)	Yield (%)	Efficiency (%)	Activity recovery (%)	Derivative activity (U/g)
10	100.00 ± 0.00 ^{A,a}	83.78 ± 5.78 ^{A,a}	83.78 ± 5.78 ^{A,a}	227.89 ± 15.73 ^{A,c}	100.00 ± 0.00 ^{A,a}	81.12 ± 6.08 ^{A,a}	81.12 ± 8.08 ^{A,a}	220.64 ± 21.97 ^{A,d}	100.00 ± 0.00 ^{A,a}	79.20 ± 5.06 ^{A,a}	79.20 ± 7.06 ^{A,a}	215.41 ± 19.22 ^{A,e}
20	100.00 ± 0.00 ^{A,a}	84.00 ± 1.55 ^{A,a}	84.00 ± 1.55 ^{A,a}	456.97 ± 8.43 ^{A,c}	98.67 ± 0.07 ^{B,a}	82.47 ± 3.48 ^{A,a}	81.38 ± 3.47 ^{AB,a}	442.68 ± 18.68 ^{AB,cd}	100.00 ± 0.00 ^{A,a}	71.17 ± 6.46 ^{B,ab}	71.17 ± 6.46 ^{B,ab}	387.14 ± 35.16 ^{B,de}
30	97.14 ± 0.73 ^{AB,ab}	77.55 ± 4.35 ^{AB,a}	75.36 ± 4.77 ^{A,a}	614.94 ± 38.94 ^{A,bc}	93.68 ± 2.77 ^{B,a}	79.36 ± 5.15 ^{A,a}	74.40 ± 6.24 ^{A,ab}	607.09 ± 50.96 ^{A,c}	100.00 ± 0.00 ^{A,a}	68.21 ± 0.52 ^{B,ab}	68.21 ± 0.52 ^{A,ab}	556.62 ± 4.28 ^{A,d}
50	95.85 ± 2.47 ^{AB,ab}	79.92 ± 1.27 ^{A,a}	76.62 ± 2.74 ^{A,a}	1,042.02 ± 37.32 ^{A,b}	92.21 ± 4.39 ^{B,ab}	81.64 ± 1.81 ^{A,a}	75.33 ± 5.08 ^{AB,ab}	1,024.45 ± 69.12 ^{AB,b}	100.00 ± 0.00 ^{A,a}	65.39 ± 4.49 ^{B,ab}	65.29 ± 4.49 ^{B,ab}	889.37 ± 61.00 ^{B,c}
100	93.02 ± 1.73 ^{B,ab}	79.58 ± 4.01 ^{A,a}	74.09 ± 7.61 ^{A,a}	2,015.14 ± 207.11 ^{A,a}	84.49 ± 4.05 ^{C,ab}	74.18 ± 3.26 ^{AB,a}	62.75 ± 5.59 ^{A,b}	1,706.93 ± 152.10 ^{A,a}	100.00 ± 0.00 ^{A,a}	61.45 ± 5.82 ^{B,b}	61.45 ± 5.82 ^{A,b}	1,671.49 ± 158.34 ^{A,b}
200	55.09 ± 2.64 ^{B,c}	81.51 ± 4.82 ^{A,a}	45.03 ± 6.32 ^{A,b}	2,449.37 ± 343.64 ^{A,a}	44.12 ± 2.82 ^{C,c}	73.97 ± 4.17 ^{A,a}	35.35 ± 5.04 ^{A,c}	1,922.92 ± 274.43 ^{A,a}	75.55 ± 5.38 ^{A,b}	60.17 ± 3.49 ^{B,b}	45.46 ± 2.60 ^{A,c}	2,468.56 ± 141.47 ^{A,a}

Each value represents the mean ± standard deviation of three independent experiments performed in triplicate. Different uppercase letters indicate that immobilization parameter is statistically different ($p<0.05$) for the different derivatives. Different lowercase letters indicate that immobilization parameter is statistically different ($p<0.05$) for the different protein loads for each derivative.

oxirane groups (Matte et al., 2017). The unmodified support (Immobead) and modified with glutaraldehyde (Immobead-Glu) showed similar curve profiles. The Immobead curve presented higher temperature stability until ~350 °C, while Immobead-Glu was more stable even at higher temperatures. Different behavior was verified for Immobead-Ac on the weight loss curve, as this treatment decreased the support stability by displacement of the second mass loss step to 240 °C. Most likely, the use of H_2SO_4 and HNO_3 was responsible for such reduction, indicating that treatment with this mixture could be more damaging to the Immobead than other modifiers.

Derivatives (Immobead-KL-Gal, Immobead-Ac-KL-Gal and Immobead-Glu-KL-Gal) and free enzyme (Free KL-Gal) curves are presented in Fig. 2B. After immobilization, derivatives exhibited an additional weight loss stage from ~320 to 450 °C, which can be attributed to the decomposition of the β-galactosidase residual groups. Immobead-KL-Gal and Immobead-Glu-KL-Gal maintained similar profiles, but the use of glutaraldehyde seemed to provide more resistance to weight loss. Immobead-Ac-KL-Gal was more affected by increasing temperatures, as observed in the absence of the enzyme (Fig. 2A). Free KL-Gal was stable until around ~250 °C; after that the enzyme rapidly decomposed.

Thermal properties of free β-galactosidase and Immobead support and its derivatives were investigated by DSC (Fig. 2C and D). The three treated supports (Fig. 2C) showed an endothermic peak at approximately 70 °C, which corresponds to the energy required to desorb water (Kittur et al., 2002). DSC thermograms of Immobead and Immobead-Glu presented a second endothermic peak at approximately 320 and 350 °C, respectively, which are likely related to Immobead decomposition. This observed higher stability against decomposition of Immobead-Glu corroborates the results of the TGA curves. Immobead-Ac showed an exothermic peak at ~210 °C, assigned to the oxidation caused by H_2SO_4 and HNO_3, followed by an endothermic peak at ~230 °C, probably related to changes on the Immobead structure resistance caused by the acid treatment.

DSC results of the derivatives (Fig. 2D) showed an increase in the peak intensity of ~70 °C, probably caused by enzyme denaturation added to the water removal energy. Another endothermic peak verified at ~200 °C was likely due to residual glycerol of commercial enzyme (Sandu et al., 2015). Immobead-Ac-KL-Gal kept the exothermic peak at 210 °C and showed other variation peaks at higher temperatures. Peak profiles of Immobead-KL-Gal and Immobead-Glu-KL-Gal were uniform, but presented differences in their heat flow. Immobead 150 was also studied in DSC analysis by Poppe et al. (2013), who described a process of multipoint covalent immobilization of lipases. These authors verified different curves for native and modified support, and also for derivatives. These different degradation kinetics were attributed to structural changes that occurred on the support surface during the process of exchanging epoxy to aldehyde groups. β-Galactosidase thermograms were reported by Soto et al. (2017), who studied the folding/unfolding enzyme process due to interactions with the immobilization additives Triton X-100 and ethanol. The circular dichroism study of the enzyme secondary structure related to temperature showed

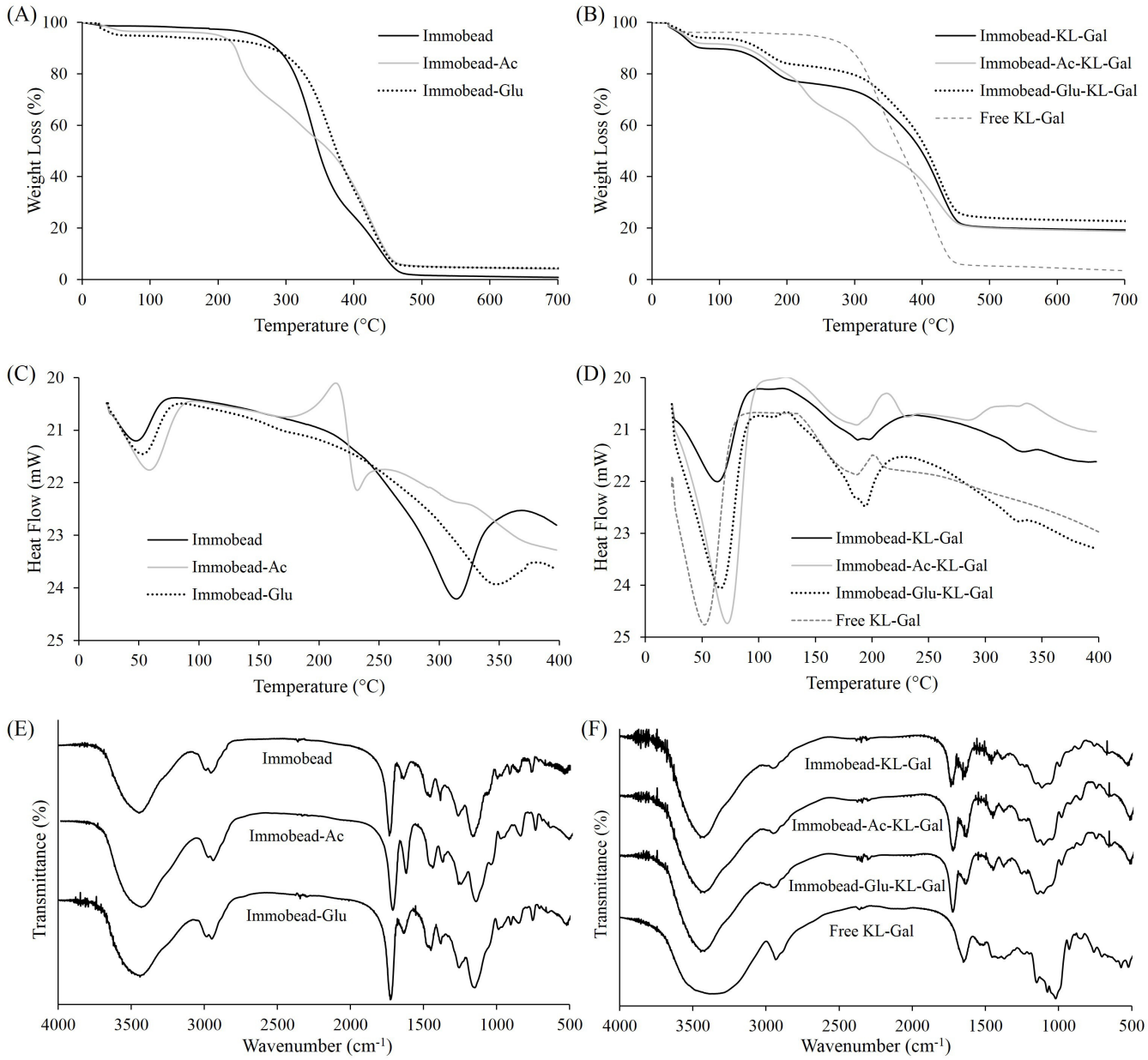


Figure 2. Structural studies: (A) TGA curves of unmodified and modified Immobead (Immobead, Immobead-Ac and Immobead-Glu); and (B) derivatives (Immobead-KL-Gal, Immobead-Ac-KL-Gal and Immobead-Glu-KL-Gal) and free enzyme (Free KL-Gal); (C) DSC thermograms of unmodified and modified Immobead (Immobead, Immobead-Ac and Immobead-Glu), and (D) derivatives (Immobead-KL-Gal, Immobead-Ac-KL-Gal and Immobead-Glu-KL-Gal) and free enzyme (Free KL-Gal); and (E) FT-IR spectra of unmodified and modified Immobead (Immobead, Immobead-Ac and Immobead-Glu), and (F) derivatives (Immobead-KL-Gal, Immobead-Ac-KL-Gal and Immobead-Glu-KL-Gal) and free enzyme (Free KL-Gal).

that temperature increases lead to featureless spectra, which indicate unfolding of the protein. According to the authors, these observations are in agreement with the DSC thermogram, which showed the denaturation temperature of β-galactosidase around 75 °C.

IR spectra, presented in Fig. 2E, were similar for the three support treatments (Immobead, Immobead-Ac and Immobead-Glu). The spectra exhibited a broad band with a maximum around ~3500 cm^{-1} due to O-H stretching, as well as stretching bands for other functional groups, such as C=O (at 1727 cm^{-1}), epoxy COC (at 1230 cm^{-1}), and CO (at 1077 cm^{-1}) (Mondal et al., 2017). The spectrum for free KL-Gal (Fig. 2F) presented a band at approximately 1640 cm^{-1}, as well as a group of peaks between 1200 and 1000 cm^{-1}. In the IR spectra of derivatives, changes among the band ratios of ~1750 and ~1640 cm^{-1} are important to note when comparing unmodified and modified Immobead (Fig. 2E). These changes demonstrate the presence of the β-galactosidase in Immobead-KL-Gal, Immobead-

Ac-KL-Gal and Immobead-Glu-KL-Gal. In addition, derivatives demonstrated different band profiles between 1500 and 1200 cm^{-1}.

Textural characteristics of the Immobead preparations

The specific surface area, pore diameter and volume of the unmodified (Immobead) and modified (Immobead-Ac, Immobead-Glu) supports and their derivatives (Immobead-KL-Gal, Immobead-Ac-KL-Gal, and Immobead-Glu-KL-Gal) obtained from nitrogen adsorption-desorption isotherms are summarized in Table 2. The modified supports have decreased specific surface areas and pore volumes (Table 2) compared to those of the unmodified Immobead. These changes could be explained by pore surface modification with glutaraldehyde (Caldas et al., 2017), partial blocking of pore entrances by glutaraldehyde arms bonded to the Immobead-Glu support (Wine et al., 2007), or introduction of carboxyl groups onto the Immobead by oxidation with the acid solution in Immobead-Ac (Balasubramanian and Burghard, 2005).

The N_2 isotherms are shown in Fig. 3A. Immobilization of β-galactosidase on the supports changes the isotherm profile when compared to unmodified support without enzyme. A decrease in the microporosity (Fig. 3B), as well as in the mesoporosity (Fig. 3C), can be clearly seen and are likely due to blocking of the support pores by immobilized enzyme (Matte et al., 2017). The observed reductions in the textural characteristics of the supports can be related to the protein load used to construct the derivatives (100 mg protein.g^{-1} support). Matte et al. (2017) immobilized a lipase from *Thermomyces lanuginosus* on Immobead 150 by multipoint covalent attachment, and verified a reduction in the specific surface area of the support from 137 to 63 $m^2.g^{-1}$ after immobilization.

Biocatalyst properties

Dependence of immobilized β-galactosidase catalytic activity on pH and temperature was compared with the free enzyme. Fig. 4 shows pH and temperature activity profiles for free and immobilized β-galactosidase. Immobilized enzyme showed no change in its optimum pH profile (Fig. 4A), but exhibited a broadening in its basic pH activity profile

Table 2. Textural evaluation by surface area and pore volume of unmodified and modified Immobead supports and their derivatives.

Derivative	BET Surface Area ($m^2.g^{-1}$)	BJH Pore Volume ($cm^3.g^{-1}$)
Immobead	189.0	0.368
Immobead-Ac	148.0	0.338
Immobead-Glu	129.0	0.328
Immobead-KL-Gal	17.1	0.047
Immobead-Ac-KL-Gal	13.2	0.042
Immobead-Glu-KL-Gal	11.8	0.035

* BET: Brunauer, Emmett and Teller; BJH: Barret-Joyner-Halenda.

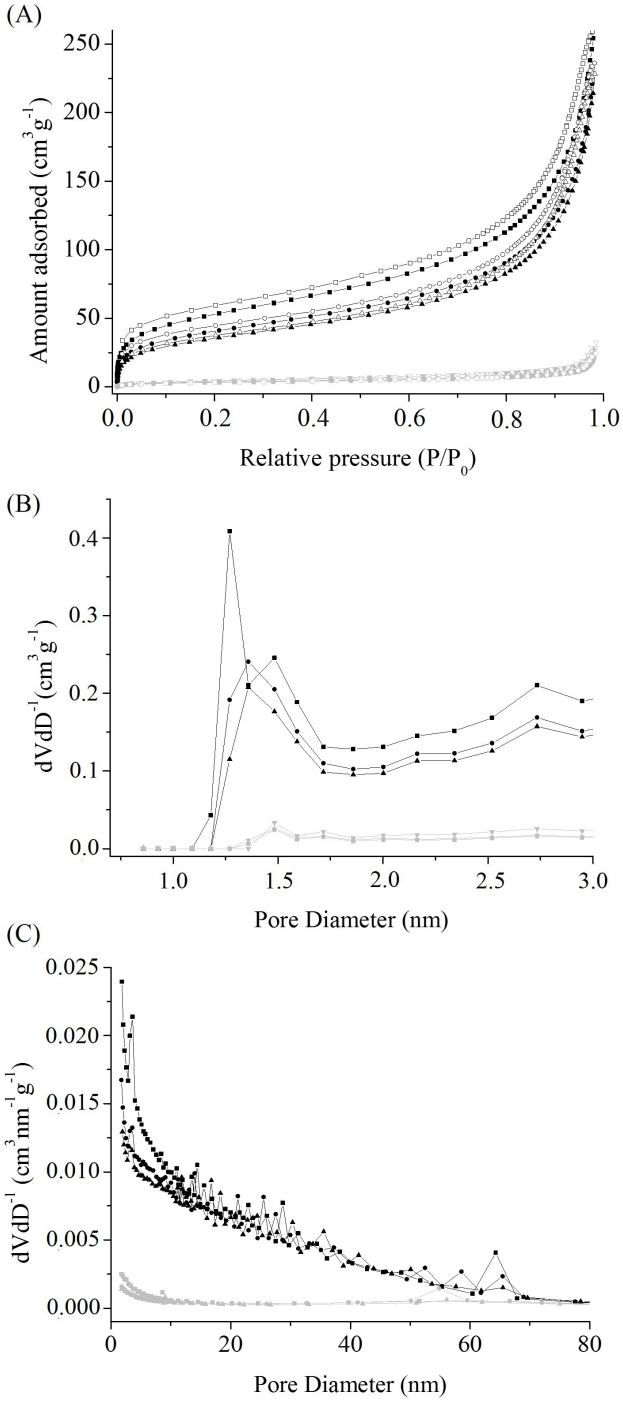


Figure 3. Textural characterization of the supports: (A) N_2 adsorption (■,●,▲) and desorption (□,○,△) isotherms. (B) DFT micropore size distribution. (C) BJH-pore size distributions of (■) Immobead, (●) Immobead-Ac, (▲) Immobead-Glu, (■) Immobead-KL-Gal, (●) Immobead-Ac-KL-Gal, and (▲) Immobead-Glu-KL-Gal.

compared with the native enzyme. Such difference could be related to easier alteration/distortion of the tertiary structure of free enzyme compared with the immobilized one in highly acidic and basic solutions (Neri et al., 2008). Activity of free and immobilized enzymes decreased markedly when pH was reduced from 7.0 to 5.5, and almost no enzymatic activity was detected below pH 6.0. This behavior can be attributed to dissociation of β-galactosidase subunits from their tetrameric form, which promotes enzyme denaturation and inactivation (Fernandez-Lafuente, 2009).

The activity of free and immobilized enzymes increased with temperature and reached maximum activity at 40 °C. At higher temperatures, all derivatives and the free enzyme lost almost 90 % of their activity, indicating that elevated temperature leads to enzyme denaturation and rupture of the both β-galactosidase polypeptide chain forms (Ansari et al., 2013). According to Ansari and Husain (2011), as the enzyme catalytic activity depends on the conformational structure of the protein, even minor alterations in the tertiary structure can result in activity loss. Moreover, no differences in the optimum pH and temperature conditions after enzyme immobilization were found by Ansari et al. (2013), who studied the immobilization of *K. lactis* β-galactosidase on glutaraldehyde-modified multiwalled carbon nanotubes.

Working with the same enzyme, Souza et al. (2018) evaluated the effect of *K. lactis* β-galactosidase (LAC) complexation with the polysaccharides sodium alginate (ALG) or l-carrageenan (CA) on the enzyme catalytic properties. The authors verified that, despite the optimum pH and temperature being the same for immobilized or free enzyme forms, the derivative stability (LAC/ALG and LAC/CA) decreased when compared with the free LAC. The lower activities observed in the complexes were attributed to the high affinity between lactase and the polysaccharides, which hampers the access of the substrate to the active site.

Table 3 shows that enzyme immobilization resulted in increased K_m and decreased V_{max} values for all the derivatives. In this process, interactions with Immobead could induce some distortions in the β-galactosidase structure, reducing enzyme activity (Pal and Khanum, 2012). Furthermore, this effect is also attributed to a reduced transport of the substrate and products into and out of the modified Immobead (Dwevedi and Kayastha, 2009). Although the ionic interaction in Immobead-Ac-KL-Gal resulted in the lowest affinity with Immobead, it presented the highest V_{max}, k_{cat} and catalytic specificity constant among the evaluated treatments. The covalent bond in Immobead-Glu-KL-Gal presented higher enzyme affinity with the substrate than with the other derivatives, but its velocity was reduced 2.5-fold. The hydrophobic interactions of the support with the enzyme might play an important role in covalent immobilization and lead to changes in the enzyme structure or performance. In addition, hydrophobic interactions may alter the acidity constants of key substrate binding groups of the enzyme (Chaplin and Bucke, 1990). Changes in

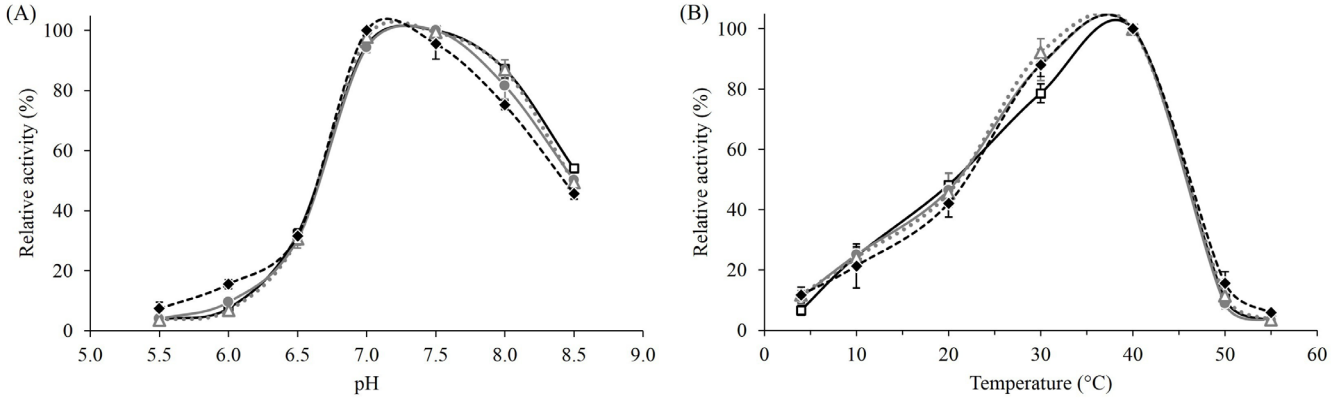


Figure 4. (A) pH and (B) temperature activity profiles for soluble and immobilized β- galactosidase. Relative activity refers to the activity/maximum activity. (□) Free KL-Gal; (●) Immobead-KL-Gal; (△) Immobead-Ac-KL-Gal and (◆) Immobead-Glu-KL-Gal.

Table 3. Kinetic parameters for soluble β-galactosidase and enzyme immobilized on the three prepared Immobead forms.

Derivative	K_m (mM)	V_{max} (mM.min^{-1})	k_{cat} (min^{-1})	Catalytic specificity constant (min^{-1} mM^{-1})
Free KL-Gal	2.33 ± 0.08	30.74 ± 1.73	184.41	79.12
Immobead-KL-Gal	5.97 ± 0.28	11.05 ± 0.38	89.52	15.00
Immobead-Ac-KL-Gal	7.68 ± 1.33	22.39 ± 3.74	214.39	27.91
Immobead-Glu-KL-Gal	4.89 ± 0.09	12.00 ± 0.29	117.16	23.96

the kinetic parameters of the derivatives compared to those of free β-galactosidase were also reported by Wahba (2017), who verified an increase in k_m from 72.61 mM to 98.19 mM, and a decrease in V_{max} from 22.99 to 9.51 μmol.min^{-1}.mg^{-1} enzyme after the immobilization of *A. oryzae* β-galactosidase on treated calcium pectinate beads.

Thermal (Table 4) and thermodynamic (Table 5) parameters of the free and immobilized β-galactosidases were examined to investigate the applicability of the immobilized enzymes at the industrial scale. Thermal inactivation constant (*k*) (Table 4) decreased after immobilization of the three derivatives. As a result, the $t_{1/2}$ values of Immobead-KL-Gal, Immobead-Ac-KL-Gal and Immobead-Glu-KL-Gal were higher than those of the free enzyme at all evaluated temperatures. Immobead-Glu-KL-Gal was the most stable derivative, showing that covalent binding improved rigidity and resistance to unfolding during heat treatment more than other immobilization interactions (Banjanac et al., 2016). Half-life values of free and immobilized β-galactosidase decreased according to the increase in temperature. Through the kinetic deactivation constant and $t_{1/2}$ values, Gennari et al. (2018) also verified that Immobead treatment with glutaraldehyde provided, in general, the best enzymatic stability to immobilized *A. oryzae* β-galactosidase. The differences in the half-life values found by Gennari et al. (2018) in comparison with our study are probably due to the different enzyme three-dimensional forms.

Increase in thermal stability was also verified by Eskandarloo and Abbaspourrad (2018), using functionalized glass beads to immobilized *A. oryzae* β-galactosidase for galacto-oligosaccharides production. Studying the enzyme thermal stability, the authors verified a higher sensitivity of free enzyme when compared with the derivative. This characteristic was attributed to reduced conformational flexibility of immobilized β-galactosidase, and to covalent bonds provided by glutaraldehyde between support and enzyme molecules.

Sensitivity of the reaction rate to temperature was determined by the Arrhenius activation energy (E_a) for free and immobilized β-galactosidases. No difference was found between the two enzyme forms (Table 5). The Gibbs free energy (ΔG) was higher for Immobead-KL-Gal, Immobead-Ac-KL-Gal and Immobead-Glu-KL-Gal than for free enzyme, which shows that, in all these cases, interactions with Immobead increase thermal stability. Similarly, according to the half-life values, treatment with glutaraldehyde stabilized the enzyme structure. Enthalpy (ΔH), which represents the heat variation of the enzyme when incubated at the tested temperatures, decreased after the immobilization process. The lower enthalpy of the derivatives indicates that the number of non-covalent bonds broken was reduced. Furthermore, the enthalpy of the enzyme showed a temperature dependence: as the temperature increased, a decrease in enthalpy was observed. Entropy of the system also changed, according to the values for free and immobilized β-galactosidases. Thermodynamic parameters indicated that Immobead treatments followed by β-galactosidase immobilization contributed to reduce the susceptibility of the enzyme to denaturation (Aguiar-Oliveira and Maugeri, 2011). Working with another epoxy support, da Silva Campello et al. (2012) immobilized *K. lactis* β-galactosidase on Eupergit® C, and verified the same trend of our study in the ΔH and ΔS values, which increased after the immobilization process.

Operational stability

Reuse potential of immobilized β-galactosidase in the lactose hydrolysis of lactose, permeate, cheese whey, and milk solutions was examined (Fig. 5). The absolute values of hydrolysis yields were around 50 % in the first cycle (corresponding to 13.16 g.L^{-1} of

Table 4. Thermal inactivation properties of free and immobilized β-galactosidase.

Derivative	55 °C			58 °C			60 °C			63 °C		
	k (min^{-1})	$t_{1/2}$ (min)	SF	*k* (min^{-1})	$t_{1/2}$ (min)	SF	*k* (min^{-1})	$t_{1/2}$ (min)	SF	*k* (min^{-1})	$t_{1/2}$ (min)	SF
Free KL-Gal	0.97	0.71	-	1.65	0.42	-	1.99	0.35	-	2.23	0.31	-
Immobead-KL-Gal	0.68	1.02	1.43	1.26	0.55	1.30	1.56	0.44	1.27	1.91	0.36	1.17
Immobead-Ac-KL-Gal	0.94	0.74	1.03	1.52	0.46	1.09	1.76	0.39	1.13	1.88	0.37	1.19
Immobead-Glu-KL-Gal	0.46	1.50	2.11	0.60	1.16	2.77	0.75	0.93	2.67	1.78	0.39	1.25

SF: Stabilization factor.

Table 5. Thermodynamic stability of free and immobilized β-galactosidase.

Derivative	E_a	55 °C			58 °C			60 °C			63 °C		
		ΔG	ΔH	ΔS	ΔG	ΔH	ΔS	ΔG	ΔH	ΔS	ΔG	ΔH	ΔS
Free KL-Gal	2.61	91.84	-0.11	-280.34	91.25	-0.14	-276.09	91.29	-0.16	-274.62	91.83	-0.18	-273.83
Immobead-KL-Gal	2.49	92.81	-0.24	-283.68	91.98	-0.27	-278.68	101.06	-0.28	-304.33	92.25	-0.31	-275.48
Immobead-Ac-KL-Gal	2.50	91.92	-0.23	-280.93	91.47	-0.25	-277.11	91.63	-0.27	-275.97	92.30	-0.29	-275.59
Immobead-Glu-KL-Gal	2.61	93.87	-0.12	-286.55	94.05	-0.15	-284.58	102.72	-0.16	-308.95	92.46	-0.19	-275.72

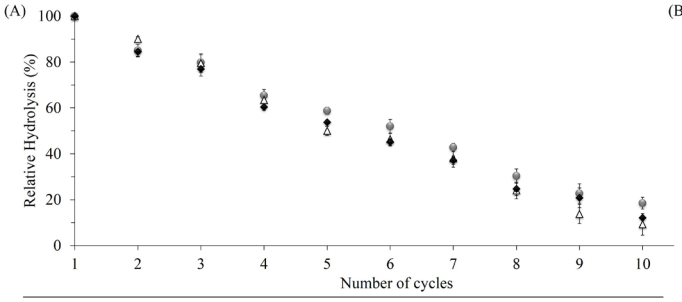
E_a (kJ.mol^{-1}); ΔG (kJ.mol^{-1}); ΔH (kJ.mol^{-1}); ΔS (J.mol^{-1}.K^{-1}).

glucose) for all solutions and tested derivatives. The free KL-Gal hydrolyzed around 40, 62, 81, and 93 % of the substrate in lactose, whey permeate, cheese whey, and skim milk solutions, respectively, in the same reaction conditions (4 °C and 150 rpm for 30 min). The results showed that immobilized preparations maintained approximately 50 % of their initial activity after being reused five times in a lactose solution (graph Fig. 5A), and ten times in permeate (graph Fig. 5B). The recovered activity values of Immobead-KL-Gal, Immobead-Ac-KL-Gal and Immobead-Glu-KL-Gal in hydrolyzed permeate (table Fig. 5B) after the 10th cycle were almost 30 % higher than those in lactose solution (Table Fig. 5A). For cheese whey and milk, enzyme operational stability of the three derivatives was higher, showing approximately 40 % of lactose hydrolysis after 15 reuse cycles (graphs Fig. 5C and Fig. 5D).

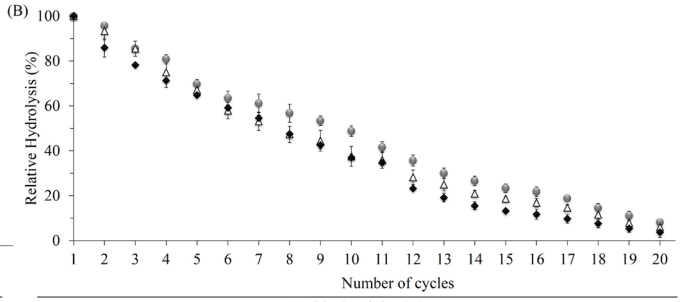
Previous studies have reported that galactose, one of the end products of β-galactosidase-catalyzed lactose hydrolysis, competitively inhibits enzyme activity (Ustok et al. 2010; Ansari and Husain, 2011). The same trend was observed in lactose hydrolysis (graphs Fig. 5A to Fig. 5D) and in residual activity of the enzyme (Tables Fig. 5A to Fig. 5D).

Using the same support of this work, Gennari et al. (2018) evaluated the reusability of *A. oryzae* β-galactosidase immobilized on Immobead. Hydrolysis was also performed in four different substrate solutions (lactose, permeate, cheese whey, and milk) for 30 min. The derivatives presented high reusability in lactose solution and low in milk, the opposite of our results. Such behavior was caused by different optimum pH of each enzyme, around 4.5 and 7.0 for β-galactosidases of *A. oryzae* and *K. lactis*, respectively (Todorova-Balvay et al., 2007; Pereira-Rodríguez et al., 2012).

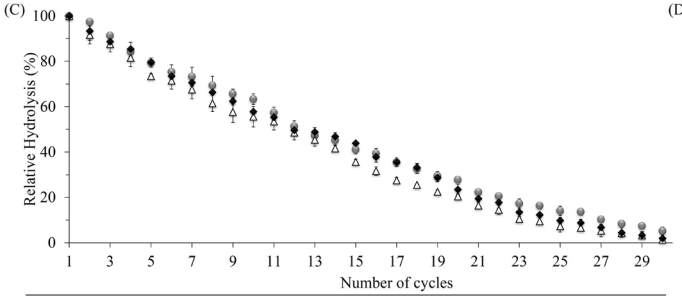
Reusability of tetrameric β-galactosidase in lactose hydrolysis was also evaluated by de Albuquerque et al. (2018). Investigating the optimal conditions for lactulose synthesis promoted by a *K. lactis* β-galactosidase immobilized on chitosan, the derivative presented only 20 % of lactose conversion after 6 reuse cycles. According to the authors, this effect may be related to the fact that not all of the enzyme subunits were attached to the support surface.



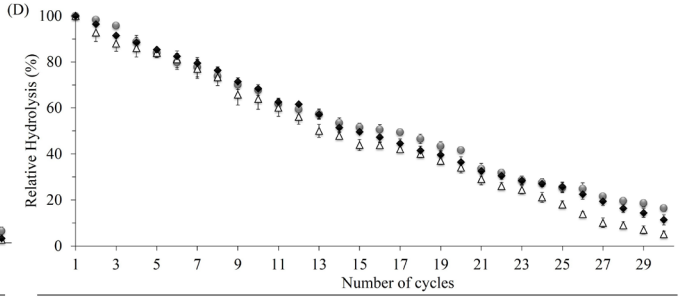
Residual activity (%)				
Derivative	Number of cycles			
	1	3	5	10
Immobead-KL-Gal	100.00	86.34	61.45	18.57
Immobead-Ac-KL-Gal	100.00	90.22	52.60	11.79
Immobead-Glu-KL-Gal	100.00	85.58	57.59	14.73



Residual activity (%)					
Derivative	Number of cycles				
	1	5	10	15	20
Immobead-KL-Gal	100.00	68.74	55.37	26.48	11.54
Immobead-Ac-KL-Gal	100.00	66.45	40.12	22.17	10.43
Immobead-Glu-KL-Gal	100.00	66.39	43.43	19.75	9.86



Residual activity (%)							
Derivative	Number of cycles						
	1	5	10	15	20	25	30
Immobead-KL-Gal	100.00	80.32	62.65	42.58	28.65	19.55	9.54
Immobead-Ac-KL-Gal	100.00	75.34	57.12	39.56	19.54	10.38	3.68
Immobead-Glu-KL-Gal	100.00	79.84	59.84	43.84	24.83	16.84	5.73



Residual activity (%)							
Derivative	Number of cycles						
	1	5	10	15	20	25	30
Immobead-KL-Gal	100.00	84.54	69.67	56.86	42.65	25.90	19.52
Immobead-Ac-KL-Gal	100.00	82.63	63.65	46.98	37.56	19.54	12.45
Immobead-Glu-KL-Gal	100.00	84.88	70.34	52.54	39.15	26.32	16.64

Figure 5. Reusability of immobilized β-galactosidase for lactose hydrolysis. The activity determined during the first cycle was taken as the reference (100 %) for the calculation of the remaining activity after each use. Lactose hydrolysis was performed in solutions of (A) lactose, (B) whey permeate, (C) cheese whey, and (D) skim milk, all reconstituted to contain 5 % w/v of lactose. Symbols: (●) Immobead-KL-Gal; (△) Immobead-Ac-KL-Gal, and (◆) Immobead-Glu-KL-Gal.

The combination of short reaction times and high yields demonstrates the ability of the β-galactosidase biocatalysts constructed herein to be used in batch hydrolysis processes. However, to design an industrial process, improvements that facilitate packed-bed reactor processes are still needed.

CONCLUSION

Immobilization of tetrameric *K. lactis* β-galactosidase on Immobead revealed stable enzyme-support interactions. The modifications to Immobead evaluated confer different characteristics to the biocatalyst. The highest immobilization yields and efficiencies were achieved with a protein load of 100 $mg.g^{-1}$ support. Optimum pH and temperature of the enzyme were not modified after immobilization, but the thermal stability of all the derivatives was higher than the free enzyme. Kinetic parameters of β-galactosidase were impacted by the binding with the support. The enzyme showed great stability in the reusability assays in different substrate solutions and could be reused 15 times for lactose hydrolysis in milk and cheese whey, maintaining approximately 40 % of its initial activity. These results show that *K. lactis* β-galactosidase was stabilized by the immobilization protocols examined, demonstrating new possibilities for application in studies simulating industrial processes using fluidized and packed-bed reactors.

ACKNOWLEDGMENTS

We would like to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) for the scholarships, and Universidade do Vale do Taquari - Univates for the financial support granted for this research. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

NOMENCLATURE

Ac	Acid solution of H_2SO_4:HNO_3 (3:1)
ANOVA	Analysis of variance
BET	Brunauer, Emmett and Teller
BJH	Barret-Joyner-Halenda
DFT	Density functional theory
DSC	Differential scanning calorimetry
EDA	Ethylenediamine
Glu	Glutaraldehyde 5 % (v/v)
Immobead	Unmodified Immobead
Immobead-Ac	Immobead modified using an acid solution of H_2SO_4:HNO_3 (3:1)
Immobead-Glu	Immobead modified using glutaraldehyde 5 % (v/v)
IR	Infrared spectroscopy
KL-Gal	*Kluyveromyces lactis* β-galactosidase
ONPG	Ortho-nitrophenyl-β-D-galactopyranoside
SEM	Scanning Electron Microscope
TGA	Thermogravimetric analysis

Symbols

A (min^{-1})	Pre-exponential factor
E_a ($J.mol^{-1}$)	Energy of activation
h (6.6262×10^{-34} J.s)	Planck constant
k (min^{-1})	Kinetic deactivation constant
k_B (1.3806×10^{-23} $J.K^{-1}$)	Boltzmann constant.
k_{cat}	Turnover numbers
K_M	Michaelis constant
R (8.314 $J.mol^{-1}.K^{-1}$)	Universal gas constant
T (K)	Temperature
$t_{1/2}$ (min)	Half-life
V_{max}	Maximum reaction rate
ΔG	Gibbs free energy of activation
ΔH	Enthalpy of activation
ΔS	Entropy of activation

REFERENCES

Aguiar-Oliveira, E., Maugeri, F. Thermal stability of the immobilized fructosyltransferase from *Rhodotorula* sp. Brazilian J. Chem. Eng., 28, 363-372 (2011). https://doi.org/10.1590/S0104-66322011000300002

Akhtar, M. S., Ahmad, A., Bhakuni, V. Guanidinium chloride- and urea-induced unfolding of the dimeric enzyme glucose oxidase. Biochem., 41, 3819-3827 (2002). https://doi.org/10.1021/bi0116700

Alagöz, D., Çelik, A., YIldIrIm, D., Tükel, S. S., Binay, B. Covalent immobilization of Candida methylica formate dehydrogenase on short spacer arm aldehyde group containing supports. J. Mol. Catal. B Enzym., 130, 40-47 (2016). https://doi.org/10.1016/j.molcatb.2016.05.005

Ansari, S. A., Husain, Q. Immobilization of *Kluyveromyces lactis* β galactosidase on concanavalin A layered aluminium oxide nanoparticles - Its future aspects in biosensor applications. J. Mol. Catal. B Enzym., 70, 119-126 (2011). https://doi.org/10.1016/j.molcatb.2011.02.016

Ansari, S. A., Satar, R., Chibber, S., Khan, M. J. Enhanced stability of *Kluyveromyces lactis* β-galactosidase immobilized on glutaraldehyde modified multiwalled carbon nanotubes. J. Mol. Catal. B Enzym., 97, 258-263 (2013). https://doi.org/10.1016/j.molcatb.2013.09.008

Balasubramanian, K., Burghard, M. Chemically functionalized carbon nanotubes. Small., 1, 180-192 (2005). https://doi.org/10.1002/smll.200400118

Banjanac, K., Carević, M., Ćorović, M., Milivojević, A., Prlainović, N., Marinković, A., Bezbradica, D. Novel β-galactosidase nanobiocatalyst systems for application in the synthesis of bioactive galactosides. RSC Adv., 6, 97216-97225 (2016). https://doi.org/10.1039/C6RA20409K

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72, 248-254 (1976). https://doi.org/10.1016/0003-2697(76)90527-3

Caldas, E. M., Novatzky, D., Deon, M., Menezes, E. W. de, Hertz, P. F., Costa, T. M. H., Arenas, L. T., Benvenutti, E. V., Pore size effect in the amount of immobilized enzyme for manufacturing carbon ceramic biosensor. Microporous Mesoporous Mater., 247, 95-102 (2017). https://doi.org/10.1016/j.micromeso.2017.03.051

Chaplin, M. F., Bucke, C. The preparation and kinetics of immobilised enzymes. Enzym. Technol., 80-136 (1990).

Da Silva Campello, G., Trindade, R. A., Rěgo, T. V., Burkert, J. F. de M., Burkert, C. A. V. Immobilization of β-galactosidase from *Kluyveromyces lactis* on eupergit® c and properties of the biocatalyst. Int. J. Food Eng., 8 (2012). https://doi.org/10.1515/1556-3758.2760

Datta, S., Christena, L. R., Rajaram, Y. R. S. Enzyme immobilization: an overview on techniques and support materials. Biotech., 3, 1-9 (2013). https://doi.org/10.1007/s13205-012-0071-7

de Albuquerque, T. L., Gomes, S. D. L., D'Almeida, A. P., Fernandez-Lafuente, R., Gonçalves, L. R. B., Rocha, M. V. P. Immobilization of β-galactosidase in glutaraldehyde-chitosan and its application to the synthesis of lactulose using cheese whey as feedstock. Process Biochem., 73, 65-73 (2018). https://doi.org/10.1016/j.procbio.2018.08.010

DiCosimo, R., McAuliffe, J., Poulose, A. J., Bohlmann, G. Industrial use of immobilized enzymes. Chem. Soc. Rev., 42, 6437 (2013). https://doi.org/10.1039/c3cs35506c

Dutra Rosolen, M., Gennari, A., Volpato, G., Souza, C. F. V. de, Biocatalytic characterization of *Aspergillus oryzae* β-galactosidase immobilized on functionalized multi-walled carbon nanotubes. Biocatal. Biotransformation., 35, 260-268 (2017). https://doi.org/10.1080/10242422.2017.1323886

Dwevedi, A., Kayastha, A. M., Stabilization of β-galactosidase (from peas) by immobilization onto Amberlite MB-150 beads and its application in lactose hydrolysis. J. Agric. Food Chem., 57, 682-688 (2009). https://doi.org/10.1021/jf802573j

Eskandarloo, H., Abbaspourrad, A. Production of galacto-oligosaccharides from whey permeate using β-galactosidase immobilized on functionalized glass beads. Food Chem., 251, 115-124 (2018). https://doi.org/10.1016/j.foodchem.2018.01.068

Faber, K. Biotransformations in Organic Chemistry, 4th ed., Springer-Verlag, 2011. https://doi.org/10.1007/978-3-642-17393-6

Fernández-Lafuente, R., Rodríguez, V., Mateo, C., Penzol, G., Hernández-Justiz, O., Irazoqui, G., Villarino, A., Ovsejevi, K., Batista, F., Guisán, J. M., Stabilization of multimeric enzymes via immobilization and post-immobilization techniques. J. Mol. Catal. - B Enzym., 7, 181-189 (1999). https://doi.org/10.1016/S1381-1177(99)00028-4

Fernandez-Lafuente, R. Stabilization of multimeric enzymes: Strategies to prevent subunit dissociation. Enzyme Microb. Technol., 45, 405-418 (2009). https://doi.org/10.1016/j.enzmictec.2009.08.009

Flickinger, M. C. Encyclopedia of Industrial Biotechnology: Bioprocess. Bioseparation, and Cell Technology, 2010. https://doi.org/10.1002/9780470054581

Gennari, A., Mobayed, F. H., Rafael, R. da S., Rodrigues, R. C., Sperotto, R. A., Volpato, G., Souza, C. F. V. de. Modification of Immobead 150 support for protein immobilization: Effects on the properties of immobilized *Aspergillus oryzae* β-galactosidase. Biotechnol. Prog., 34, 934-943 (2018). https://doi.org/10.1002/btpr.2652

Gennari, A., Mobayed, F. H., Volpato, G., Souza, C. F. V. de. Chelation by collagen in the immobilization of *Aspergillus oryzae* β-galactosidase: A potential biocatalyst to hydrolyze lactose by batch processes. Int. J. Biol. Macromol., 109, 303-310 (2018). https://doi.org/10.1016/j.ijbiomac.2017.12.088

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. Chem. Eng. Process. Process Intensif., 48, 576-580 (2009). https://doi.org/10.1016/j.cep.2008.02.007

Harini, T., Basetty, S., Jasti, L. S., Lavanya, K., Fadnavis, N. W., Recovery of functionally active trypsin inhibitor (SBTI) and lipoxygenase (LOX) from soymilk via multimodal technique. React. Funct. Polym., 121, 45-50 (2017). https://doi.org/10.1016/j.reactfunctpolym.2017.10.009

Hartmann, M. Ordered mesoporous materials for bioadsorption and biocatalysis. Chem. Mater., 17, 4577-4593 (2005). https://doi.org/10.1021/cm0485658

Hudmon, A. A Mechanism for Ca^{2+}/Calmodulin-Dependent Protein Kinase II Clustering at Synaptic and Nonsynaptic Sites Based on Self-Association. J. Neurosci., 25, 6971-6983 (2005). https://doi.org/10.1523/JNEUROSCI.4698-04.2005

Kang, I., Bang, H. J., Kim, I. H., Choi, H. D., Kim, B. H., Synthesis of trans-10,cis-12 conjugated linoleic acid-enriched triacylglycerols via two-step lipase-catalyzed esterification. LWT - Food Sci. Technol., 62, 249-256 (2015). https://doi.org/10.1016/j.lwt.2015.01.041

Kawaguti, H. Y., Manrich, E., Sato, H. H., Production of isomaltulose using Erwinia sp. D12 cells: Culture medium optimization and cell immobilization in alginate. Biochem. Eng. J., 29, 270-277 (2006). https://doi.org/10.1016/j.bej.2006.01.006

Kikani, B. A., Singh, S. P., Enzyme stability, thermodynamics and secondary structures of α-amylase as probed by the CD spectroscopy. Int. J. Biol. Macromol., 81, 450-460 (2015). https://doi.org/10.1016/j.ijbiomac.2015.08.032

Kittur, F. S., Prashanth, K. V. H., Sankar, K. U., Tharanathan, R. N., Characterization of chitin, chitosan and their carboxymethyl derivatives by differential scanning calorimetry. Carbohydr. Polym., 49, 185-193 (2002). https://doi.org/10.1016/S0144-8617(01)00320-4

Klewicki, R. Formation of gal-sorbitol during lactose hydrolysis with β-galactosidase. Food Chem., 100, 1196-1201 (2007). https://doi.org/10.1016/j.foodchem.2005.10.064

Križnik, L., Vasić, K., Knez, Ž., Leitgeb, M. Hyper-activation of ß-galactosidase from *Aspergillus oryzae* via immobilization onto amino-silane and chitosan magnetic maghemite nanoparticles. J. Clean. Prod., 179, 225-234 (2018). https://doi.org/10.1016/j.jclepro.2018.01.117

Liese, A., Hilterhaus, L. Evaluation of immobilized enzymes for industrial applications. Chem. Soc. Rev., 42, 6236 (2013). https://doi.org/10.1039/c3cs35511j

Mahoney, R. R. Enzymes Exogenous to Milk in Dairy Technology: b-D-Galactosidase, in: Encycl. Dairy Sci., Elsevier, 2002, 907-914. https://doi.org/10.1016/B0-12-227235-8/00146-2

Mateo, C., Palomo, J. M., Fernandez-Lorente, G., Guisan, J. M., Fernandez-Lafuente, R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme Microb. Technol., 40, 1451-1463 (2007). https://doi.org/10.1016/j.enzmictec.2007.01.018

Matte, C. R., Bordinhão, C., Poppe, J. K., Benvenutti, E. V., Costa, T. M. H., Rodrigues, R. C., Hertz, P. F., Ayub, M. A. Z., Physical-chemical properties of the support immobead 150 before and after the immobilization process of lipase. J. Braz. Chem. Soc., 28, 1430-1439 (2017). https://doi.org/10.21577/0103-5053.20160319

Matte, C. R., Bordinhaõ, C., Poppe, J. K., Rodrigues, R. C., Hertz, P. F., Ayub, M. A. Z. Synthesis of butyl butyrate in batch and continuous enzymatic reactors using *Thermomyces lanuginosus* lipase immobilized in Immobead 150. J. Mol. Catal. B Enzym., 127, 67-75 (2016). https://doi.org/10.1016/j.molcatb.2016.02.016

Migneault, I., Dartiguenave, C., Bertrand, M. J., Waldron, K. C. Glutaraldehyde: Behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. Biotechniques., 37, 790-802 (2004). https://doi.org/10.2144/04375RV01

Mondal, M. K., Mukherjee, S., Saha, S. K., Chowdhury, P., Babu, S. P. S. Design and synthesis of reduced graphene oxide based supramolecular scaffold: A benign microbial resistant network for enzyme immobilization and cell growth. Mater. Sci. Eng. C., 75, 1168-1177 (2017). https://doi.org/10.1016/j.msec.2017.02.136

Neri, D. F. M., Balcão, V. M., Carneiro-da-Cunha, M. G., Carvalho, L. B., Teixeira, J. A. Immobilization of β-galactosidase from *Kluyveromyces lactis* onto a polysiloxane-polyvinyl alcohol magnetic (mPOS-PVA) composite for lactose hydrolysis. Catal. Commun., 9, 2334-2339 (2008). https://doi.org/10.1016/j.catcom.2008.05.022

Neri, D. F. M., Balcão, V. M., Dourado, F. O. Q. Oliveira, J. M. B. Carvalho, L. B., Teixeira, J. A. Galactooligosaccharides production by β-galactosidase immobilized onto magnetic polysiloxane-polyaniline particles. React. Funct. Polym., 69 246-251 (2009). https://doi.org/10.1016/j.reactfunctpolym.2009.01.002

Novalin, S., Neuhaus, W., Kulbe, K. D. A new innovative process to produce lactose-reduced skim milk. J. Biotechnol., 119, 212-218 (2005). https://doi.org/10.1016/j.jbiotec.2005.03.018

Pal, A., Khanum, F. Covalent immobilization of xylanase on the surface of alginate-glutaraldehyde beads decreases the 'catalytic efficiency' but provides 'low temperature stabilization' effect. J. Biochem. Technol., 3, 409-413 (2012).

Panesar, P. S., Kumari, S., Panesar, R. Potential Applications of Immobilized β-Galactosidase in Food Processing Industries. Enzyme Res., 1-16 (2010). https://doi.org/10.4061/2010/473137

Pereira-Rodríguez, Á., Fernández-Leiro, R., González-Siso, M. I., Cerdán, M. E., Becerra, M., Sanz-Aparicio, J. Structural basis of specificity in tetrameric *Kluyveromyces lactis* β-galactosidase. J. Struct. Biol., 177, 392-401 (2012). https://doi.org/10.1016/j.jsb.2011.11.031

Poppe, J. K., Costa, A. P. O., Brasil, M. C., Rodrigues, R. C., Ayub, M. A. Z. Multipoint covalent immobilization of lipases on aldehyde-activated support: Characterization and application in transesterification reaction. J. Mol. Catal. B Enzym., 94, 57-62 (2013). https://doi.org/10.1016/j.molcatb.2013.05.017

Sandu, T., Sarbu, A., Damian, C. M., Patroi, D., Iordache, T. V., Budinova, T., Tsyntsarski, B., Yardim, M. F., Sirkecioglu, A. Functionalized bicomponent polymer membranes as supports for covalent immobilization of enzymes. React. Funct. Polym., 96, 5-13 (2015). https://doi.org/10.1016/j.reactfunctpolym.2015.09.001

Sharifi, M., Robatjazi, S. M., Sadri, M., Mosaabadi, J. M., Covalent immobilization of organophosphorus hydrolase enzyme on chemically modified cellulose microfibers: Statistical optimization and characterization. React. Funct. Polym., 124, 162-170 (2018). https://doi.org/10.1016/j.reactfunctpolym.2018.01.019

Sheldon, R. A., Pelt, S. van. Enzyme immobilisation in biocatalysis: why, what and how. Chem. Soc. Rev., 42, 6223-6235 (2013). https://doi.org/10.1039/C3CS60075K

Sheldon, R. A. Enzyme immobilization: The quest for optimum performance. Adv. Synth. Catal., 349, 1289-1307 (2007). https://doi.org/10.1002/adsc.200700082

Soto, D., Escobar, S., Guzmán, F., Cárdenas, C., Bernal, C., Mesa, M. Structure-activity relationships on the study of β-galactosidase folding/unfolding due to interactions with immobilization additives: Triton X-100 and ethanol. Int. J. Biol. Macromol., 96, 87-92 (2017). https://doi.org/10.1016/j.ijbiomac.2016.12.026

Souza, C. J., Garcia-Rojas, E. E., Favaro-Trindade, C. S. Lactase (β-galactosidase) immobilization by complex formation: Impact of biopolymers on enzyme activity. Food Hydrocolloid., 83, 88-96 (2018). https://doi.org/10.1016/j.foodhyd.2018.04.044

Spohner, S. C., Schaum, V., Quitmann, H., Czermak, P., *Kluyveromyces lactis*: An emerging tool in biotechnology. J. Biotechnol., 222, 104-116 (2016). https://doi.org/10.1016/j.jbiotec.2016.02.023

Sun, J., Jiang, Y., Zhou, L., Gao, J. Immobilization of *Candida antarctica* lipase B by adsorption in organic medium. N. Biotechnol., 27, 53-58 (2010). https://doi.org/10.1016/j.nbt.2009.12.001

Todorova-Balvay, D., Stoilova, I., Gargova, S., Vijayalakshmi, M. A. An efficient two step purification and molecular characterization of β-Galactosidases from *Aspergillus oryzae*. J. Mol. Recognit., 19, 299-304 (2007). https://doi.org/10.1002/jmr.788

Ustok, F. I., Tari, C., Harsa, S. Biochemical and thermal properties of β-galactosidase enzymes produced by artisanal yoghurt cultures. Food Chem., 119, 1114-1120 (2010). https://doi.org/10.1016/j.foodchem.2009.08.022

Vasiljevic, T., Jelen, P. Production of β-galactosidase for lactose hydrolysis in milk and dairy products using thermophilic lactic acid bacteria. Innov. Food Sci. Emerg. Technol., 2, 75-85 (2001). https://doi.org/10.1016/S1466-8564(01)00027-3

Wahba, M. I., Soliman, T. N. Whey protein isolate for the preparation of covalent immobilization beads. Biocatal Agric Biotechnol., 14, 328-337 (2018). https://doi.org/10.1016/j.bcab.2018.04.003

Wahba, M. I. Chitosan-glutaraldehyde activated calcium pectinate beads as a covalent immobilization support. Biocatal. Agric. Biotechnol., 12, 266-274 (2017). https://doi.org/10.1016/j.bcab.2017.10.016

Webb, P. A., Orr, C. Analytical Methods in Fine Particle Technology, Micromeritics Ins. Corp., 1997.

Wine, Y., Cohen-Hadar, N., Freeman, A., Frolow, F. Elucidation of the Mechanism and End Products of Glutaraldehyde Crosslinking Reaction by X-Ray Structure Analysis. Biotechnol. Bioeng., 98, 711-718 (2007). https://doi.org/10.1002/bit.21459

Wolf, M., Gasparin, B. C., Paulino, A. T. Hydrolysis of lactose using β-d-galactosidase immobilized in a modified Arabic gum-based hydrogel for the production of lactose-free/low-lactose milk. Int. J. Biol. Macromol., 115, 157-164 (2018). https://doi.org/10.1016/j.ijbiomac.2018.04.058

Yi, S. H., Alli, I., Park, K. H., Lee, B. H., Overexpression and characterization of a novel transgalactosylic and hydrolytic β-galactosidase from a human isolate Bifidobacterium breve B24, N. Biotechnol., 28, 806-813 (2011). https://doi.org/10.1016/j.nbt.2011.07.006

Yiu, H. H. P., Wright, P. A., Enzymes supported on ordered mesoporous solids: a special case of an inorganic-organic hybrid. J. Mater. Chem., 15, 3690-3700 (2005). https://doi.org/10.1039/b506090g

Zaak, H., Sassi, M., Fernandez-Lafuente, R. A new heterofunctional amino-vinyl sulfone support to immobilize enzymes: Application to the stabilization of β-galactosidase from *Aspergillus oryzae*. Process Biochem., 64, 200-205 (2018). https://doi.org/10.1016/j.procbio.2017.09.020

Zeuner, B., Nyffenegger, C., Mikkelsen, J. D., Meyer, A. S. Thermostable β-galactosidases for the synthesis of human milk oligosaccharides. N. Biotechnol., 33, 355-360 (2016). https://doi.org/10.1016/j.nbt.2016.01.003