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β-Galactosidase of *Aspergillus oryzae* immobilized in an ion exchange resin combining the ionic-binding and crosslinking methods: Kinetics and stability during the hydrolysis of lactose

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ABSTRACT

In this work, the hydrolysis kinetics of lactose by *Aspergillus oryzae* β-galactosidase was studied using the ionic exchange resin Duolite A568 as a carrier. The enzyme was immobilized using a β-galactosidase concentration of 16 g/L in pH 4.5 acetate buffer and an immobilization time of 12 h at 25 ± 0.5 °C. Next, the immobilized β-galactosidase was crosslinked using glutaraldehyde concentration of 3.5 g/L for 1.5 h. The influence of lactose concentration was studied for a range of 5–140 g/L, and the Michaelis–Menten model was fitted well to the experimental results with V_m and K_m values of 0.71 U and 35.30 mM, respectively. The influence of the product galactose as an inhibitor on the hydrolysis reaction was studied. The model that was best fitted to the experimental results was the competitive inhibition by galactose with V_m, K_m and K_i values of 0.77 U, 35.30 mM and 27.44 mM, respectively. The influence of temperature on the enzymatic activity of the immobilized enzyme was studied in the range of 10–80 °C, in which the temperature of the maximum activity was 60 °C, with an activation energy of 5.32 kcal/mol of lactose, using an initial concentration of lactose of 50 g/L in a pH 4.5 sodium acetate buffer solution. The thermal stability of the immobilized biocatalyst was determined to be in the range 55–65 °C. The first-order model described well the kinetics of thermal deactivation for all the temperatures studied. The activation energy of thermal deactivation from immobilized biocatalyst was 66.48 kcal/mol with a half-life of 8.9 h at 55 °C.

1. Introduction

Lactose is a component of milk and whey, and its utilization is rather limited due to its low solubility and indigestibility in many people [1]. The hydrolysis of lactose is a promising process in the food industry for the development of new products with no lactose in their composition. The total elimination of lactose in milk is required to solve the problem of lactose intolerance in a large percentage of the world population (approximately 70%) [2]. The β-galactosidase enzyme of *Aspergillus oryzae* (E.C. 3.2.1.23) has received special interest for use in hydrolyzing whey lactose due its optimum acidic pH and high thermal stability in addition to producing an isomolecular mix of glucose and galactose [3,4].

β-Galactosidase can be used in two forms: in the soluble enzyme form, which is normally used in batch processes and the immobilized form, which is used preferably in continuous operation [5,6]. Immobilization has been a widely employed technique in industrial applications of enzymes for enhancing stability, imparting reusability and making enzyme-based processes cost-effective and viable [7]. The use of immobilized enzymes in industry can be economically more viable than that of the free enzymes; immobilization allows the reutilization of enzymes, provides control of the catalytic process, provides high selectivity, allows large quantities of substrate to be processed, permits continuous operation and increases enzyme stability [8–10].

Many types of carriers and techniques have been used for the immobilization of β-galactosidase. The use of magnetic carriers allows the enzyme to be quickly separated at the end of the reaction, reducing the operating cost in the separation of the immobilized enzyme from the medium [11,12]. The use of ionic exchange resins as carriers for immobilization is used often in the food industry, involving ionic and electrostatic interactions between ions of the protein and ions of opposite charge from the resin [13,14].

Glutaraldehyde activation of the carriers is one of the widely used techniques to immobilize enzymes. The methodology is quite simple and efficient, and in some instances, it even improves

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enzyme stability by multipoint or multisubunit immobilization. Moreover, glutaraldehyde has also been widely used to introduce intermolecular crosslinking in proteins or to modify adsorbed proteins on aminated supports [15].

Several authors have studied the performance of various reactions in the hydrolysis of the lactose components of whey and milk, which employed several types of reactors and sources of β-galactosidases. Almost all of these authors selected a kinetic model based on the Michaelis–Menten mechanism, which takes into account competitive inhibition by galactose during the hydrolysis of lactose [2,16].

In the work of Guidini et al. [17] crosslinking an enzyme adsorbed in Duolite A568 with glutaraldehyde enhanced the catalytic activity when the enzyme was used repeatedly in various cycles.

In the work of Guidini et al. [17] was verified that the crosslinking with glutaraldehyde of this enzyme adsorbed in Duolite A568 enhanced the catalytic activity when used repeatedly in various cycles.

The purpose of this work was to study the reaction kinetics of lactose hydrolysis by β-galactosidase from *A. oryzae* immobilized on the ionic exchange resin, Duolite A568, followed by crosslinking with glutaraldehyde. The influence of the concentration of lactose, glucose and galactose on the enzymatic activity as well as the influence of temperature on the activity and stability of the immobilized enzyme were analyzed.

2. Experimental

2.1. Material

β-Galactosidase (E.C. 3.2.1.23) from *A. oryzae* was obtained from Sigma. The exchange resin used in this work, Duolite A568, was kindly donated by Dow Brasil S.A. Duolite A568 is a highly porous, granular, weakly basic, anionic exchange resin, based on crosslinked phenol–formaldehyde polycondensate with functional groups of tertiary amine. Its hydrophilic structure combined with a controlled pore size distribution makes it a suitable resin for use as an enzyme carrier in many bioprocessing applications. According to Rohm Hass company, the ionic strength, pore volume, pore size and particle size of Duolite A568 are designed for optimum immobilization of enzymes used in the starch and fat industry as well as other industries. All other reagents were of analytical grade.

2.2. Immobilization and determination of the activity from the immobilized enzyme

The Duolite A568 resin was initially washed with distilled water and activated with 10 volumes of 1 M hydrochloric acid for each volume of resin with an agitation of 50 rpm for 30 min. Following the acid treatment, the resin was washed again with distilled water and added to a 1 M sodium hydroxide solution under the same dilution rate and agitation conditions used for the acid treatment. Finally, the resin was washed in distilled water.

The immobilization procedure used in this study was that developed by Guidini et al. [17] in which were optimized the conditions of adsorption of enzyme on the resin and the crosslinking process with glutaraldehyde. A volume of 10 mL of enzymatic solution at a concentration of 16 g/L in a pH 4.5 acetate buffer was incubated with 0.5 g of the activated resin, Duolite A568, under agitation of 50 rpm in a rotary incubator at 25 ± 1 °C for 12 h. Afterwards, the immobilized enzyme was washed with the pH 4.5 acetate buffer and mixed with 2.5 mL of 3.5 g/L of glutaraldehyde solution for the crosslinking reaction for 1.5 h at 25 ± 1 °C.

The enzymatic activity of the lactose hydrolysis reaction by the immobilized β-galactosidase was determined by the initial rate method, and the formed glucose was measured by the glucose-oxidase method [18]. The reactions were conducted in a mixture reactor containing 100 mL of lactose solution, under magnetic agitation, while the pH and temperature were defined for each experiment. The immobilized enzyme particles were kept in a fixed stainless steel basket during the reaction, placed on top of the reactor. The unit of activity (U) was defined as the grams of glucose produced per liter, per minute, per gram of immobilized enzyme. All of the experiments were conducted in duplicate.

2.3. Influence of the initial concentration of lactose on the activity of the immobilized β-galactosidase

This study showed the influence of the initial lactose concentration on the catalytic activity of immobilized β-galactosidase. The enzyme was immobilized in accordance with the procedure described by Guidini et al. [17]. The enzymatic activity was determined in a pH 4.5 acetate buffer with the concentration of lactose ranging from 5 to 140 g/L at 35 ± 1 °C, using the procedure of the initial rate of reaction.

2.4. Influence of the initial concentration of lactose, glucose and galactose on immobilized β-galactosidase activity

A central composite design (CCD) was proposed with the purpose of qualitatively analyzing the simultaneous influence of the concentration of the substrate, lactose and reaction products, glucose and galactose, on the enzymatic hydrolysis reaction. For these experiments, the enzyme was immobilized and subsequently submitted to the crosslinking process under optimized conditions in accordance with the work of Guidini et al. [17]. The activities were determined by the initial rate of reaction method. The concentration ranges for the studied variables were based on the works from Santos et al. and Fernandes et al. [16,19]. The experiments were performed in a pH of 4.5 at 35 ± 1 °C in the range of a lactose concentration of 8.82–56.18 g/L, glucose from 0 to 23 g/L and galactose from 0 to 23 g/L.

2.5. Influence of the initial galactose concentration on the immobilized β-galactosidase activity

From the experimental results from the CCD, a study of the influence of galactose concentration on enzymatic activity was performed. The enzyme was immobilized with subsequent crosslinking under optimum conditions in accordance with work from Guidini et al. [17]. In these experiments, the adopted range of the initial lactose concentration was 5–60 g/L, and for galactose, it was 0–22.5 g/L at pH 4.5 at 35 ± 1 °C. This study was based on the work of Portaccio et al. [20], who analyzed the influence of galactose on the β-galactosidase activity.

The activities were determined by the initial rate of reaction method. The experimental results of the reaction rate were fitted to the kinetic models of competitive inhibition, noncompetitive inhibition, uncompetitive inhibition, linear mixed inhibition and partially noncompetitive inhibition using Statistica® 7.0 software and the numerical method of Levenberg–Marquardt [21].

2.6. Influence of temperature on the activity of the immobilized β-galactosidase

The purpose of this study was to evaluate the influence of temperature on the activity of the immobilized β-galactosidase and to determine the activation energy of the reaction from lactose hydrolysis. In this experiment, the optimized conditions were

adopted in accordance with the work of Guidini et al. [17], except that the enzyme concentration was 5 g/L. This procedure was used to evaluate the behavior of the immobilized enzyme with respect to temperature. The experiments were performed using a lactose concentration of 50 g/L in pH 4.5 acetate buffer and a reaction medium temperature range from 10 to 80 ± 1 °C. The enzymatic activities were determined by the initial rate of reaction method. To determine the activation energy of the hydrolysis reaction, the results of the enzymatic activity as a function of temperature (thermal activation step) were fitted to the Arrhenius model using the Origin® 7.0 software.

2.7. Study of the thermal stability of the immobilized β-galactosidase

With the aim of studying the thermal stability of the immobilized β-galactosidase enzyme, the samples were placed into 50 mL of pH 4.5 acetate buffer and incubated in a thermostatic bath ranging in temperature from 55 to 65 °C. For each temperature of incubation, the sets of particles from the immobilized biocatalyst were removed at appropriate intervals of time (2 in 2 min at 65 ± 1 °C, 4 in 4 at 63 ± 1 °C, 7 in 7 min at 60 ± 1 °C, 13 in 13 min at 57 ± 1 °C, 20 in 20 min at 55 ± 1 °C). These samples were cooled quickly in an ice bath, and the residual activity was determined by the initial rate of reaction with an initial concentration of lactose of 50 g/L, pH 4.5 at 35 ± 1 °C. The results of the residual enzymatic activity as a function of incubation time for each temperature were fitted to first order thermal deactivation models and in series in a single step by the numerical method of Levenberg–Marquardt [21] using the Statistica® 7.0 software to determine the kinetic parameters for the best fit [22].

3. Results and discussions

3.1. Influence of the initial lactose concentration on kinetics of the immobilized β-galactosidase

The experimental results of the initial rate of reaction from the lactose hydrolysis in the range of 5–140 g/L by β-galactosidase from immobilized *A. oryzae* were determined in accordance with Section 2.3.

The experimental results were adjusted to the Michaelis–Menten model with a good approximation of the experimental points to those fitted by the model. A determination coefficient (R^2) equal to 0.98 was obtained and in the lactose concentration studied was not verified substrate inhibition. Through a nonlinear regression using the Statistica® 7.0 software and employing the numerical method of Levenberg–Marquardt [21], the values of the kinetic parameters V_m and K_m were estimated to be 0.71 U and 35.30 mM, respectively, which were significant at 5% (p value < 0.05).

The K_m value determined in this work was 35.30 mM, near the value found by Tanrisevan and Dogan [23], which was 51 mM. These authors immobilized β-galactosidase from *A. oryzae* in fibers composed of alginate and gelatin hardened with glutaraldehyde. Haider and Husain [24] obtained a K_m value of 5.18 mM for a calcium alginate-entrapped, crosslinked Con A–β galactosidase.

3.2. Influence of the concentration of glucose and galactose on the reaction kinetics of the immobilized β-galactosidase

This study aimed to analyze qualitatively if the products of the reaction, glucose and galactose, inhibited the enzymatic reaction. The experimental results of the activity of the immobilized enzyme as a function of the initial concentrations of lactose, glucose and galactose, determined in accordance with Section 2.4, are shown in Table 1.

Table 1
Experimental results of enzymatic activity as a function of lactose, glucose and galactose concentrations according the CCD, for real and codified values.

Experiments	Real value (codified value)			
	Lactose (g/L)	Glucose (g/L)	Galactose (g/L)	Activity (U^*)
1	15.00 (−1)	3.00 (−1)	3.00 (−1)	0.37
2	15.00 (−1)	3.00 (−1)	20.00 (1)	0.19
3	15.00 (−1)	20.00 (1)	3.00 (−1)	0.76
4	15.00 (−1)	20.00 (1)	20.00 (1)	0.48
5	50.00 (1)	3.00 (−1)	3.00 (−1)	1.00
6	50.00 (1)	3.00 (−1)	20.00 (1)	0.23
7	50.00 (1)	20.00 (1)	3.00 (−1)	1.17
8	50.00 (1)	20.00 (1)	20.00 (1)	0.79
9	8.82 (−α)	11.50 (0)	11.50 (0)	0.31
10	56.18 (α)	11.50 (0)	11.50 (0)	0.65
11	32.50 (0)	0.00 (−α)	11.50 (0)	0.30
12	32.50 (0)	23.00 (α)	11.50 (0)	0.83
13	32.50 (0)	11.50 (0)	0.00 (−α)	1.05
14	32.50 (0)	11.50 (0)	23.00 (α)	0.73
15	32.50 (0)	11.50 (0)	11.50 (0)	0.78
16	32.50 (0)	11.50 (0)	11.50 (0)	0.70
17	32.50 (0)	11.50 (0)	11.50 (0)	0.75

$U^* = (g_{glucose}/L \min g_{resin})$

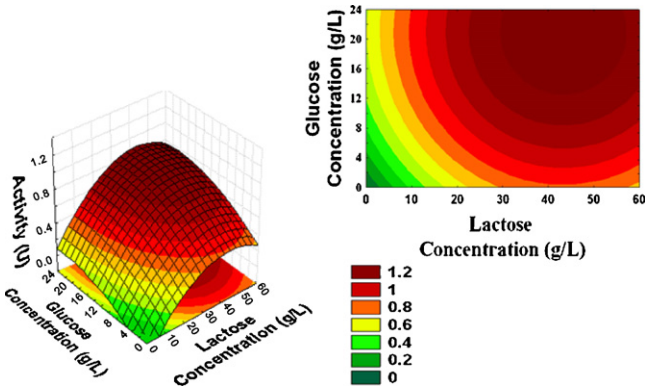

Fig. 1. Response surface from the influence of lactose and glucose on the enzymatic activity.

From the results of the enzymatic activity, the response surfaces were constructed in accordance with Figs. 1–3. The experimental results of the enzymatic activity were fitted by multiple regression using the Statistica® 7.0 software, obtaining Equation 1

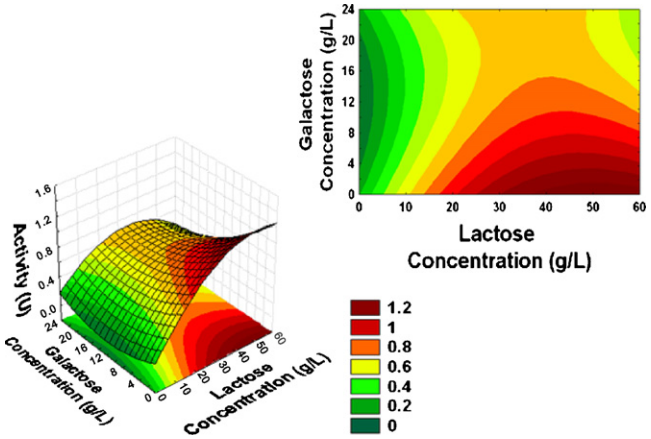

Fig. 2. Response surface from the influence of lactose and galactose on the enzymatic activity.

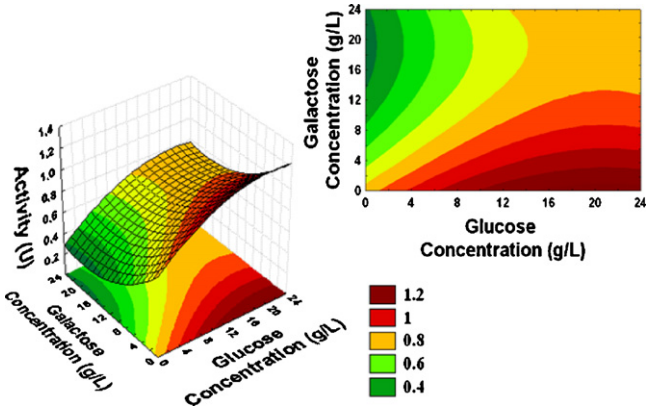

Fig. 3. Response surface from the influence of galactose and glucose on the enzymatic activity.

with the codified significant variables (x_1 = lactose concentration; x_2 = glucose concentration; x_3 = galactose concentration) for the activity of the immobilized enzyme y.

$$y = 0.731 + 0.159x_1 - 0.128x_1^2 + 0.182x_2 - 0.082x_2^2 - 0.175x_3 + 0.096x_3^2 - 0.086x_1x_3 \quad (1)$$

The parameters from a level of significance smaller than 10% ($p < 0.1$) were considered significant. The determination coefficient (R^2) was 0.95, which indicated an adequate adjustment to the experimental data in obtaining the activity of the immobilized enzyme and showed that 95% of the data variability was explained by the empirical equation proposed.

Figs. 1 and 2 show that the highest activity is near the lactose concentration of 50 g/L. In Fig. 1, it may be noted that the glucose did not interfere on the maximum activity. As shown in Figs. 2 and 3, galactose acts as an inhibitor because an increase in its concentration results in a decrease in the enzymatic activity. This feature is noted for both the surface galactose/lactose and for the surface galactose/glucose. Özdural et al. [25] verified that galactose competitively inhibits β-galactosidase from *A. oryzae* that is immobilized on Duolite A568 using a packed bed reactor. Jurado et al. [26], using β-galactosidase from *Kluyveromyces fragilis,* and Hatzinikolaou et al. [27], using β-galactosidase from *Aspergillus niger,* demonstrated that galactose is a competitive inhibitor.

3.3. Influence of the galactose concentration on the activity of the immobilized β-galactosidase

Analyzing the results of the experiments from the CCD concerning the influence of the initial concentration of lactose, glucose and galactose on enzyme activity, it can be conclude that galactose significantly inhibited the immobilized β-galactosidase.

In this study, the influence of the galactose concentration on the activity of immobilized β-galactosidase was analyzed. The experimental results of the enzymatic activity, determined in accordance with Section 2.5, in the presence of galactose were adjusted by a nonlinear regression using the Statistica® 7.0 software. The Levenberg–Marquardt method [21] was used, employing the kinetic models of competitive inhibition, noncompetitive inhibition, uncompetitive inhibition, linear mixed inhibition and partially noncompetitive inhibition. The adopted value of 35.30 mM for K_m was determined in Section 3.1. In Table 2, the adjusted parameters are shown with their analysis from Student's t-test, the determination coefficients and the sum of the squares of the deviations to demonstrate the inhibitory effect of galactose.

Based on the results of Table 2, the parameters with a significance level less than 5% in the Student's t-test analysis were considered significant. For the choice of the best inhibition model, the statistical and physical measurements from the parameters of the highest value of the determination coefficient R^2 and the lowest value of the sum of the squared residuals were considered. Analyzing the linear mixed inhibition model, it was verified that the K_i parameter does not present physical measurement. With this information, this type of inhibition was eliminated as the ideal fit for modeling the inhibition by galactose. For the partially noncompetitive model, it was observed that no physical significance existed for the β parameter. The uncompetitive and noncompetitive models of inhibition, despite a physical and statistical significance, were eliminated as the choice of best fit of inhibition because they presented higher values of the sums of the squared residuals and because the R^2 values were smaller than those for competitive inhibition. Thus, the model that best fit was the competitive inhibition by galactose. The K_m and K_i values obtained in this work were 35.30 mM and 27.44 mM, respectively. In other studies, galactose inhibition was found. Portaccio et al. [20] immobilized β-galactosidase (*A. oryzae*) on chitosan and nylon supports. In the work of these researchers, competitive inhibition by galactose was found with K_m values of 110 and 150 mM and K_i of 5 and 30 mM for the immobilized enzyme on chitosan and nylon supports, respectively. Carrara and Rubiolo [28] immobilized β-galactosidase from *K. fragilis* on chitosan beads by glutaraldehyde; they found competitive inhibition by galactose with a K_m value of 43.6 mM and K_i value of 51.9 mM.

3.4. Influence of temperature on the activity and thermal stability of the immobilized β-galactosidase

The obtained results in triplicate for the study of the influence of temperature on the enzymatic activity are illustrated in Fig. 4. The temperature range studied was from 10 to 80 °C; the level of activity increased until reaching a temperature near 60 °C, at which the highest activity occurred. Above this temperature, the enzymatic activity presented a sharp drop, becoming essentially inactive at 80 °C. Tanrisevan and Dogan [23] immobilized β-galactosidase

Table 2
Parameters of the kinetic models studied in the presence of galactose as the inhibitor.

Inhibition models		V_m (U)	K_i (mM)	α	β	$\Sigma(V - V_{model})^2$	R^2
Competitive	Parameters	0.77	27.44	–	–	0.04	0.98
	p-level	0	0	–	–		
Non competitive	Parameters	0.76	114.77	–	–	0.12	0.93
	p-level	0	0	–	–		
Acompetitive	Parameters	0.73	94.94	–	–	0.19	0.89
	p-level	0	0	–	–		
Linear mixed	Parameters	1	−40657311	23421	–	0.39	0.76
	p-level	0	0	0	–		
Partially non competitive	Parameters	1	10271522	–	−43066	0.15	0.92
	p-level	0	0	–	0		

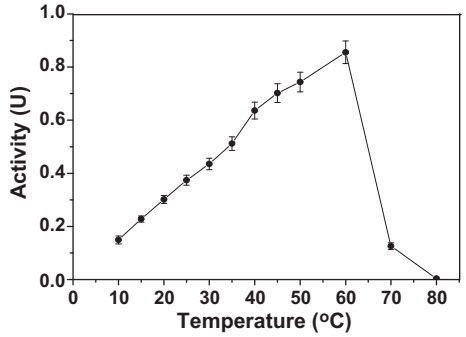

Fig. 4. Influence of temperature on the enzymatic activity of the immobilized β-galactosidase on the lactose hydrolysis.

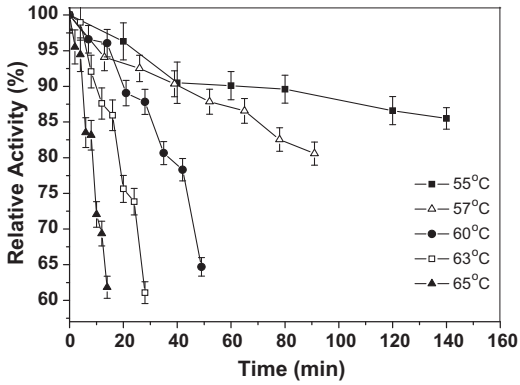

Fig. 5. Activity relative as a function of incubation time for the temperatures of 55–65 °C.

from *A. oryzae* in fibers composed of alginate and gelatin hardened with glutaraldehyde and found a maximum activity at 50 °C and verified a drop of 73% regarding the initial activity at 70 °C.

The results of the enzymatic activity as a function of temperature (thermal activation step) were fitted to the Arrhenius model using the Origin® 7.0 software. With the linear fit, the determination coefficient of 0.98 and the activation energy value of the reaction of 5.32 kcal/mol from lactose were obtained. This low activation energy of the reaction indicates that a slight increase in the reaction medium temperature will result in a large increase in the reaction rate. In the work of El-Masry et al. [29], the β-galactosidase from *A. oryzae* was immobilized in two types of nylon membranes, and lactose was used as a substrate. The values of the activation energy found were 5.6 and 6 kcal/mol.

Freitas [30] studied the influence of temperature on the activity of β-galactosidase from *A. oryzae* in the free form on the lactose hydrolysis. In this work, for the temperature range studied from 10 to 70 °C, the thermal activation step occurred in the range of 10–55 °C, with 55 °C having the highest activity. From this temperature, the enzyme deactivated rapidly, becoming completely inactive at 70 °C. The immobilization of the enzyme did not interfere in the activity profile in relation to the temperature of the reaction medium. The temperature range of the maximum enzymatic activity cited in the literature for the free β-galactosidase varies from 50 to 60 °C [7,13,23,29].

The activity, which is a function of incubation time relative to the temperature studied, is presented in Fig. 5.

Fig. 5 shows the strong dependence of the stability of the immobilized enzyme on the temperature. It is observed that for the temperature of 65 °C in 14 min, there was a drop of 38.2% from activity in relation to the initial activity. For the temperature of 55 °C, after 140 min, a drop of 14.5% in the activity occurred, demonstrating that for lower temperatures, the immobilized biocatalyst is more resistant to the loss of activity. These results can be compared with those of Haider and Hussain [3], who immobilized β-galactosidase from *A. oryzae* in an alginate-entrapped, crosslinked con A–β galactosidase complex and obtained a drop of 41% from the activity after 6 h of incubation at 60 °C.

In this work, the experimental results of the residual enzymatic activity were fitted to the model of thermal deactivation in the first order and enzymatic deactivation in series in a single step by the numerical method of Levenberg–Marquardt [21] using the Statistica® 7.0 software. Table 3 shows the results of the determination coefficients for each equation, the sum of the squared deviations, with the respective adjusted parameters and significance analysis using the Student's *t*-test test and adopting similar significant parameters as those with significance levels smaller than 10%.

Table 3
Adjustments of the parameters for the thermal deactivation of immobilized β-galactosidase.

Temperature (°C)	Model	K_d	α_1	R^2	$\Sigma(V - V_{model})^2$
		p-value	*p*-value		
65	1*	0.030	–	0.97	0.007
		0			
	2*	0.221 e^{-4}	−1168.6	0.98	0.005
		0.999	0.999		
63	1*	0.012	–	0.97	0.004
		0			
	2*	0.114 e^{-4}	−937.4	0.98	0.003
		0.999	0.999		
60	1*	0.005	–	0.97	0.002
		0			
	2*	0.970 e^{-5}	−516.69	0.98	0.002
		0.999	0.999		
57	1*	0.002	–	0.98	0.001
		0			
	2*	0.009	0.672	0.99	0.001
		0.067	0.001		
55	1*	0.001	–	0.90	0.003
		0			
	2*	0.018	0.847	0.98	0.007
		0.008	0		

1* – model of enzymatic deactivation from first order, 2* – model of enzymatic deactivation in series in a single step.

Table 4
Half-life for each temperature using the model of thermal deactivation of the first order.

Temperature (°C)	k_d (min^{-1})	R^2	$t_{1/2}$ (min)	$t_{1/2}$ (h)
65	0.030	0.97	23.03	0.38
63	0.012	0.97	58.24	0.97
60	0.005	0.97	126.02	2.1
57	0.002	0.98	288.79	4.8
55	0.001	0.90	533.15	8.9

Observing the results obtained for the modeling of thermal deactivation in the temperatures studied, it has highest temperatures (65, 63 and 60 °C), that the best fit was obtained by applying first order deactivation kinetics. The model of deactivation in series in a single step was impractical for the adjustment in these temperatures because of the presented negative values of parameter α_1 and the significance level greater than 10%, which is not significant by the Student's *t*-test. For the temperatures of 57 and 55 °C, the model of deactivation in series in a single step was adjusted better to the experimental data with higher values of R^2 and lower values of the sum of the squared residuals. When all of the temperatures studied were considered, the values of k_d showed inconsistent physical measurements because according to the Arrhenius equation, the higher the temperature is, the greater the value of the thermal deactivation constant. Thus, the constants of thermal deactivation (k_d) of the model from the first order were used for all of the temperatures studied in the calculation of half-life, as presented in Table 4.

In the work of Freitas [30], the half-life calculated using the first order thermal deactivation model for β-galactosidase from *A. oryzae* in the free form at 65, 63, 61, 57 and 55 °C were 2.40, 11.68, 13.59, 57.70 and 177.70 min, respectively. These results clearly show the enzyme in the immobilized form has higher thermal stability compared to the free enzyme. In the work of Gaur et al. [7], β-galactosidase from *A. oryzae* was immobilized by adsorption on celite, covalent binding to chitosan or crosslinking with glutaraldehyde; in this study, half-life at 55 °C of 18, 14.17 and 17.37 h were obtained for chitosan immobilization, celite immobilization and glutaraldehyde crosslinking, respectively. For 65 °C, these values were 0.79 and 1.07 h for the immobilization in chitosan and crosslinking, respectively. A strong dependence on the thermal stability of the enzyme on the method of immobilization was verified.

The results of k_d, according to Table 4, were fitted to the Arrhenius model using the *Origin*® 7.0 software. The linear fit obtained reached a determination coefficient of 0.99. Thus, the activation energy obtained from the process of thermal deactivation was 66.48 kcal/mol. This value was smaller than that relative to the free enzyme cited by Freitas [30], which was 88.14 kcal/mol for the activation energy from the process of thermal deactivation for the same enzyme. This finding suggests the immobilized enzyme in the present work is more stable for an increased temperature in the process of lactose hydrolysis in relation to the same enzyme in the free form.

The work of Guidini et al. [17] also observed a greater stability in storage and reusability. The cross-linking process did not imply in decrease of activity in relation to the enzyme adsorbed. The immobilized enzyme activity, without glutaraldehyde addition, was 51% of its initial activity after 30 uses, while the cross-linked immobilized retained 90% in the same conditions. The immobilized enzyme reticulation using glutaraldehyde was efficient, due to the increase in enzyme stability, the increase in the number of uses and the pH range to be used on the enzyme application. The results concerning to the influence of storage time showed that the activity kept on 100% after 3 months of storage in pH 4.5 and 4 °C.

4. Conclusions

In the range of the lactose concentration studied, 5–140 g/L, there was no inhibition by the substrate for the immobilized enzyme. The kinetic parameters adjusted to the Michaelis–Menten model of V_m and K_m were, respectively, 0.71 U and 35.30 mM. In the kinetic study, the glucose did not inhibit the activity of β-galactosidase immobilized in Duolite A568. The kinetic model that was better adjusted to the experimental results of the reaction rate of the lactose hydrolysis by immobilized β-galactosidase was the Michaelis–Menten model with competitive inhibition by galactose. The values of the parameters V_m, K_m and K_i were 0.77 U, 35.30 mM and 27.44 mM, respectively. In the range studied, the temperature at which the immobilized enzyme presented higher activity was 60 °C. The value of the activation energy of the reaction of lactose hydrolysis by immobilized β-galactosidase was 5.32 kcal/mol of lactose consumed. For the kinetics of the thermal deactivation of the immobilized enzyme, the first order thermal deactivation model best described all the temperatures studied, while the model of deactivation in series in a single step only adjusted to the temperatures of 55 and 57 °C. The activation energy from process of thermal deactivation of the immobilized β-galactosidase was 66.48 kcal/mol with a half-life of 8.9 h at 55 °C.

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