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OPTIMIZATION OF THE IMMOBILIZATION PROCESS OF β-GALATOSIDADE BY COMBINED ENTRAPMENT-CROSS-LINKING AND THE KINETICS OF LACTOSE HYDROLYSIS

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Abstract - The immobilization of *Aspergillus oryzae* β-galactosidase was achieved by entrapment in sodium alginate and gelatin and cross-linking with glutaraldehyde. The optimal concentrations of the aforementioned variables in the immobilization process were determined using an orthogonal central composite design with an orthogonal axial value of 1.35313. The concentrations of alginate, gelatin and glutaraldehyde that provided the greatest enzymatic activity were 6.60%, 4.05% and 3.64% (w/v), respectively. The stability of the immobilized enzyme under the optimal conditions was evaluated through daily activity assays. After 25 uses, a 20% decrease in the enzymatic activity was observed, indicating that the immobilization process could be used to produce a stable biocatalyst. This study investigates the influence of lactose and product concentrations on kinetic reaction hydrolysis. The concentration ranges for the studied variables were 10 to 56 g/L for lactose and 0 to 11.5 g/L for glucose and galactose. Only galactose presented a competitive inhibitory effect.
Keywords: Alginate; *Aspergillus oryzae*; β-galactosidase enzyme; Entrapment; Optimization, Gelatin.

INTRODUCTION

Compared to soluble forms, immobilized enzymes are more stable and can be easily separated from the reaction medium and the final product, leading to a significant decrease in enzyme consumption. To enhance the competitiveness of immobilized enzymes for technical applications, the cost of immobilization must be minimized (Tischer and Kasche, 1999; Ladero *et al*., 2000; Haider and Hussain, 2007; Haider and Hussain, 2009).

Lactose hydrolysis by lactase has two main biotechnological applications: the first is in the utilization of whey because both glucose and galactose have greater fermentation potential in comparison with lactose; the second is for the consumption of dairy products by lactose-intolerant persons (Ladero *et al*., 1998; Roy and Gupta, 2003). Lactose is a sugar that is found in milk and cheese whey, and the hydrolysis of lactose has been the subject of intense research over the last two decades because a large number of people cannot properly digest lactose due to a lack or inactivity of intestinal β-galactosidase (Ladero *et al*., 1998; Nakkharat and Haltrich, 2006).

The immobilization of β-galactosidase has been achieved through a variety of different methods, including physical adsorption, gel entrapment and covalent binding (Kierstan and Coughlan, 1991; Gaur *et al*., 2006). Moreover, a large number of immobilization processes and supports have been used (Carpio *et al*., 2006). The choice of support is

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determined by the conditions of the desired application and the method used to immobilize the enzyme (Cao, 2005).

Entrapment in alginate gel is one of the simplest methods of immobilization; however, the technique is limited by the stability and porosity of the gel, which can lead to enzyme leakage (Ates and Mehmetoglu, 1997; Parizia and Foster, 1983; Mammarella and Rubiolo, 2009). Thus, new methods of enzyme immobilization that present high enzymatic activity and good physical chemical properties are highly desirable. An approach to circumvent this leaching problem consists of cross-linking the enzyme and gelatin with glutaraldehyde, which forms an insoluble structure. Glutaraldehyde treatment also stabilizes the alginate beads and is commonly used as a cross-linking agent in combination with other composites such as gelatin (Guisan *et al*., 2006; Haider and Hussain, 2007; Guisan *et al*., 2007; Kosseva *et al*., 2009). The molecular microenvironment of the enzyme can be modified to increase the stability of the enzyme in the composite. In covalent immobilization methods, reagents that bind to both the enzyme and the support alter the local environment of the enzyme. Reagents that are capable of binding the support and the enzyme can be classified as small molecules (such as amino acids or other amines) or macromolecules (bovine albumin, gelatin and polyethylene glycol) (Cao, 2005). Under mild conditions, gelatin can cross-link with glutaraldehyde to form an aldimine bond; as a result, only the lysine residues of the protein are affected (Tanriseven and Dogan, 2002). Thus, the hydrolysis reaction occurs inside the beads, and the substrate penetrates into the matrix by mass transfer.

Lactase derived from *Aspergillus oryzae* is safe for applications in the food and pharmaceutical industry and *Aspergillus oryzae* has a history of safe usage (Kosseva *et al*. 2009). Various studies on the immobilization of β-galactosidase derived from fungi have been published (Gaur *et al*, 2006; Prashanth and Mulimani, 2005; Neri *et al*., 2009), and *Aspergillus oryzae* has been subjected to numerous tests (Parizia and Foster, 1983; Haider and Hussain, 2009; Zeng *et al*., 2009; Guidini *et al*., 2010; Illanes *et al*., 2010). Galactose has an inhibitory effect on the activity of beta-galactosidase, which is important in the design of enzymatic reactors (Ozdural *et al*., 2003). Beta-galactosidase from different sources has been found to exhibit competitive or mixed inhibition by galactose (Ladero *et al*., 1998; Haider and Hussain, 2007).

The objectives of the present study were to optimize the immobilization procedure of *Aspergillus oryzae* β-galactosidase in sodium alginate, gelatin and glutaraldehyde, which was employed as a reticulating agent, and to study the kinetics of lactose hydrolysis in terms of lactose and product concentrations.

MATERIALS AND METHODS

Enzyme

Aspergillus oryzae β-galactosidase (3.2.1.23) was obtained from the Sigma Chemical Co. The enzyme is available in the form of a white powder and its activity for lactose hydrolysis is 9 units per mg. The unit of activity (U) is equal to 1 μmol of lactose per minute at 30°C and a pH of 4.5.

Enzyme Activity Determination

The catalytic activity of β-galactosidase for both forms was determined using the initial rates method. The unit of the specific activity of the free enzyme (U_F) was defined as the grams of glucose produced per liter per minute per milligram of protein ($g_{glucose}/L.min.mg_{protein}$). The enzyme was diluted to 1% (w/v) in pH 4.5 acetate buffer and contained 0.14 mg/mL of protein, which was determined using Lowry's method (Lowry *et al*., 1951). The immobilized enzyme activity (U_I) was defined as the grams of lactose consumed per minute per m^3 of immobilized biocatalyst ($g_{lactose}/(min.m^3cat)$). The glucose produced by the reaction was determined according to the glucose-oxidase method. The volume of biocatalyst particles was determined by measuring the displacement of the fluid volume using a pycnometer.

Enzyme Immobilization

a) Experimental Design

An orthogonal central composite design (CCD) was proposed to optimize the alginate, gelatin and glutaraldehyde concentrations in the immobilization process, which consisted of two levels and three variables. The CCD contained three replicates at the central point and 6 experiments at each axial point for a total of 17 experiments. To achieve the orthogonal CCD, an α-value of 1.35313 (axial points) was used. The influences of alginate (X_1 %), gelatin (X_2%) and glutaraldehyde (X_3%) concentrations were studied in the range of 1 to 6.75% for alginate and 0 to 8.05% for gelatin and glutaraldehyde. The initial values of these variables

were obtained through preliminary tests and based on previous results from the literature (Bódalo *et al*., 1991; Ates and Mehmetoglu, 1997; Becerra *et al*., 2001; Tanriseven and Dogan, 2002; Haider and Hussain, 2007; Haider and Hussain, 2008).

b) Immobilization Procedure

The enzyme was immobilized in technical grade sodium alginate and PA gelatin as described by Tanriseven and Dogan (2002). A suspension of sodium alginate and gelatin was diluted with water to achieve a final mass of 40 g. The suspension was heated to 80°C until the alginate was completely dissolved. Subsequently, the suspension was cooled to 40ºC, and 10 mL of a solution of 10% (w/v) β-galactosidase was added to achieve a final mass of 50 g. Using a peristaltic pump, the suspension was dripped into a solution of 0.05 M $CaCl_2$ and glutaraldehyde under magnetic stirring to form immobilized biocatalyst spheres with an average diameter of 4.4 mm that were resistant to the reaction medium conditions. The biocatalyst was stored in a solution of 0.05 M $CaCl_2$ for at least 12 hours at 4°C before the initial activity of the immobilized enzyme was determined. In each activity assay, the biocatalyst was washed with 0.01 M acetate buffer (pH 4.5). After each use, the immobilized enzyme was washed and stored in the same buffer solution. Enzyme activities that were determined immediately after the dropping and after hardening of the gel particles were essentially the same. The enzymatic activity in the supernatant after the enzyme immobilization was negligible for all immobilization assays.

Influence of Lactose Concentration on the Activity of Free and Immobilized β-Galactosidase

The relationship between β-galactosidase enzyme activity and substrate concentration (lactose) was determined experimentally using the initial reaction rates procedure in a range of substrate concentrations from 10 to 100 g/L at 35°C and pH 4.5 in an acetate buffer (10^{-1} M) for both forms. All of the experiments were performed in duplicate.

The hydrolysis reactions were performed in a reactor containing 50 mL of buffered lactose solution at the appropriate conditions of lactose concentration, pH and temperature. The assays were conducted in a reactor with 60 mm diameter with a 25 mm magnetic stirrer. This speed was sufficient to maintain the immobilized biocatalysts in suspension and did not cause its rupture. For the immobilized form of β-galactosidase, 15 cm^3 of the immobilized enzyme beads were added to the reactor. For the soluble form of β-galactosidase, 0.5 mL of a solution (1% w/v) of the enzyme was added to the buffered solution.

Influence of the Initial Concentration of Lactose, Glucose and Galactose on the Free Enzyme Activity

A central composite design (CCD) was used to analyze the combined influence of the concentration of the reaction products (glucose and galactose) and the substrate (lactose) in the hydrolysis of lactose by β-galactosidase in the free form. The CCD contained three replicates at the center point and 6 experiments at each axial point for a total of 17 experiments. To achieve the orthogonal CCD, an α-value of 1.35313 was used.

The concentration ranges for the studied variables were 10 to 56 g/L for lactose and 0 to 11.5 g/L for glucose and galactose based on preliminary studies and the works of Ladero *et al*. (1998, 2000) and Portaccio *et al*. (1998). Substrate inhibition was not verified in this range of lactose concentration. The experiments were performed at 35°C in an acetate buffer at pH 4.5, and activities were determined using the initial reaction rates procedure. All of the experiments were carried out in duplicate.

Influence of the Initial Concentration of Galactose on Free and Immobilized Enzyme Activity

Based on the results of the previous experiments, it was concluded that glucose had no significant influence on the activity of free β-galactosidase; therefore, only the influence of the initial concentration of galactose on the enzyme activity was studied. This study was performed according to Portaccio *et al*. (1998) and was used to determine the enzyme activities for lactose concentrations (S) in a range of 5 to 100 g/L in the presence of galactose concentrations (I) in a range of 0 to 10 g/L for free and immobilized β-galactosidase as shown in Table 1.

The experiments were performed at 35°C with 50 mL of substrate solution of the desired concentrations in an acetate buffer at pH 4.5. The study used 0.5 mL of 1% (w/v) enzyme solution in an acetate buffer and different sets of immobilized biocatalysts (one for each substrate concentration) using the previously optimized immobilization conditions. All of the experiments were carried out in duplicate. The experimental results for the reaction rate were fitted to the kinetic models of competitive and non-competitive inhibition using the *Statistica* 7.0 software.

Table 1: Experiments for the kinetic model study of enzyme inhibition for free and immobilized enzyme

Exp.	Lactose (g/L)	Galactose (g/L)	Exp.	Lactose (g/L)	Galactose (g/L)
1	5	0	16	60	0
2	5	2.5	17	60	2.5
3	5	5	18	60	5
4	5	7.5	19	60	7.5
5	5	10	20	60	10
6	20	0	21	80	0
7	20	2.5	22	80	2.5
8	20	5	23	80	5
9	20	7.5	24	80	7.5
10	20	10	25	80	10
11	40	0	26	100	0
12	40	2.5	27	100	2.5
13	40	5	28	100	5
14	40	7.5	29	100	7.5
15	40	10	30	100	10

RESULTS AND DISCUSSIONS

Optimization

The central composite design permitted the identification of the optimal conditions for β-galactosidase immobilization, including the concentrations of alginate, gelatin and glutaraldehyde. After the experimental matrix was defined, the experiments were conducted and the initial activity (Y) of the immobilized enzyme was obtained as shown in Table 2.

A multiple regression analysis was conducted on the experimental data and parameters with a significance level greater than 10% were neglected. The variables that displayed a significance level lower than 10% in Student's t-test were the most relevant to the model. The results indicated that the square of the alginate concentration (X_1^2), the alginate concentration (X_1), the square of the gelatin concentration (X_2^2) and the square of glutaraldehyde concentration (X_3^2) were significant as shown in Equation (1):

$$Y = 984.304 + 60.996X_1 + 81.638X_1^2 - 42.505X_2^2 - 45.946X_3^2 \quad (1)$$

where X_1 , X_2 and X_3 are the coded values of the concentrations of alginate, gelatin and glutaraldehyde, respectively, according to Table 1. The coefficient of determination (R^2) of the equation was 89%, indicating that the experimental data were adequately adjusted and that the aforementioned variables were related to the activity of the enzyme. Therefore, 89% of the variability of the experimental data was explained by the empirical equation. To validate the model, three experiments were performed under optimal conditions and the activities obtained by the tests were 1190.5 U_I, 1257.6 U_I and 1180.4 U_I. From the model (Equation (1)), the maximum activity was 1195.9 U_I. To illustrate the effects of the variables on enzymatic activity, the response surfaces relating pairs of variables were constructed and are presented in Figures 1, 2 and 3.

Table 2: Experimental enzymatic activities in the immobilization process where X_1, X_2 and X_3 are the concentrations of alginate, gelatin and glutaraldehyde, respectively.

	Codified value (Real value)			
Exp.	X_1	X_2	X_3	Activity (UI)
1	-1(1.75%)	-1(1.05%)	-1(1.05%)	956.4±78.4
2	-1(1.75%)	-1(1.05%)	+1(7%)	873.6±65.5
3	-1(1.75%)	+1(7%)	-1(1.05%)	934.2±80.3
4	-1(1.75%)	+1(7%)	+1(7%)	898.3±61.3
5	+1(6%)	-1(1.05%)	-1(1.05%)	1021.8±78.7
6	+1(6%)	-1(1.05%)	+1(7%)	1095±88.7
7	+1(6%)	+1(7%)	-1(1.05%)	1059.8±63.7
8	+1(6%)	+1(7%)	+1(7%)	1005.7±82.9
9	- α(1%)	0 (4.03%)	0(4.03%)	1083.6±85.6
10	+α(6.75%)	0 (4.03%)	0(4.03%)	1181.4±100.4
11	0 (3.875%)	- α(0%)	0(4.03%)	865.8±65.8
12	0 (3.875%)	+ α(8.05%)	0(4.03%)	944.9±65.2
13	0 (3.875%)	0 (4.03%)	- α(0%)	888±67.5
14	0 (3.875%)	0 (4.03%)	+α(8.05%)	915.8±64.1
15	0 (3.875%)	0 (4.03%)	0(4.03%)	971.4±82.6
16	0 (3.875%)	0 (4.03%)	0(4.03%)	993.6±79.5
17	0(3.875%)	0 (4.03%)	0(4.03%)	990.9 ±73.64

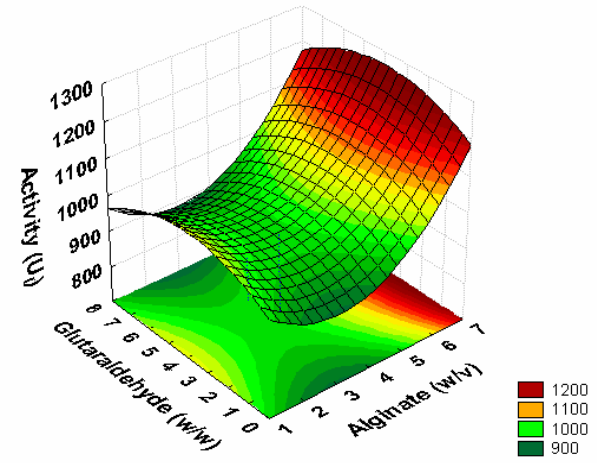

Figure 1: Response surface diagram of the enzymatic activity as a function of the alginate and gelatin concentrations

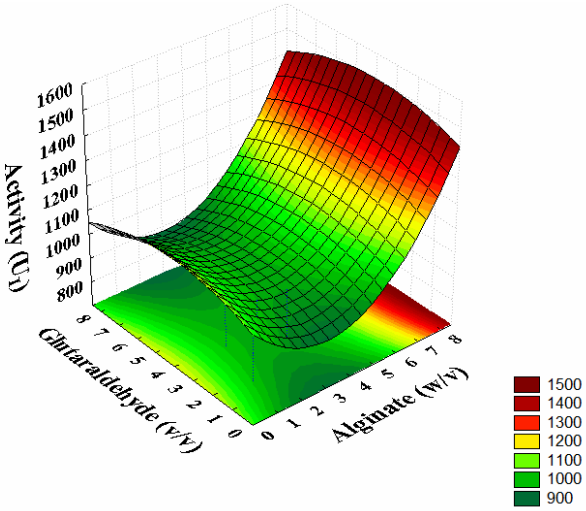

Figure 2: Response surface diagram of the enzymatic activity as a function of the alginate and glutaraldehyde concentrations

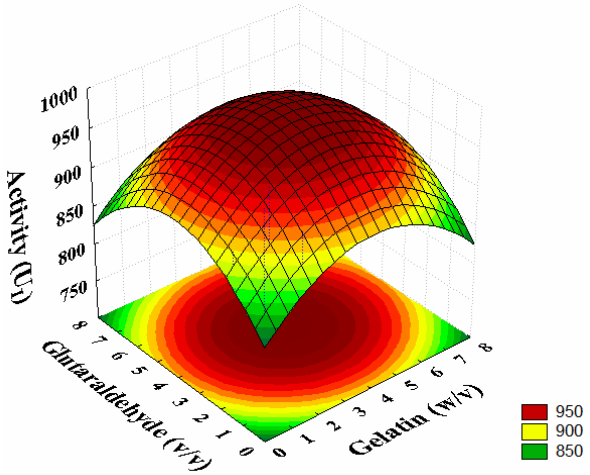

Figure 3: Response surface diagram of the enzymatic activity as a function of the glutaraldehyde and gelatin concentrations

From the complete form of Equation (1), an algorithm was implemented in Maple V (release 4) to calculate the optimal point of enzyme immobilization and to maximize enzymatic activity. The actual concentrations of the variables at the maximization point were as follows: X_1 = 6.60% alginate (w/v); X_2 = 4.05% gelatin (w/v) and X_3 = 3.64% glutaraldehyde (v/v). Under these conditions, the immobilization yield, defined as the ratio of the enzyme activity of the immobilized enzyme to the total activity of the soluble enzyme used (Emregul *et al*., 2006, Nogales *et al*., 2006), was 30%. A further analysis of the results shown in Figures 1, 2 and 3, as well as in Table 2, revealed that the maximum enzyme activity was obtained in experiment 10, in which the alginate concentration was 6.75% and the glutaraldehyde and gelatin concentrations were 4.03%. Note that these values are very close to those obtained at the optimal point of maximum activity.

To study the stability of the immobilized enzyme under the optimized conditions, enzymatic activity was measured daily and the activity of the immobilized biocatalyst under optimized conditions decreased by only 20% after 25 uses. In addition, the half-life of the immobilized enzyme was 12.8 hours at 53°C, with a activation energy for the thermal deactivation process of 72.03 kcal/mol. These results confirmed that the optimized conditions led to high enzyme retention in the support. Therefore, compared to similar processes reported in the literature, the proposed process of

immobilization produced a sufficient and stable biocatalyst at a low cost.

Influence of Lactose Concentration on the Activity of Free and Immobilized β-Galactosidase

The experimental reaction rates were fitted to the Michaelis-Menten model in a lactose concentration range of 10 to 100 g/L for free and immobilized enzymes. Through a non-linear regression, which was performed using Statistica 7.0 using the numerical method of Levenberg-Marquardt, the values of the parameters with significant values at 5% (p-value <0.05) were estimated. The V_m and K_m values were 2.56 ± 0.3 U_F and 17.83 ± 0.9 g/L (52.13 mM), respectively, (R^2 = 96.16%) for the free enzyme. For the immobilized form, these parameters were 1032.07 ± 51.6 U_I and 20.63 ± 1.0 g/L (60.3 mM), respectively (R^2 = 90%). Similar values of the K_m parameter for both enzyme forms were observed, showing that the free and immobilized enzyme forms presented similar affinities for the substrate.

Influence of the Initial Concentrations of Lactose, Glucose and Galactose on Free Enzyme Activity

After defining the central composite design (CCD) matrix, the experiments were conducted and the initial activities (Y) of the free enzyme were determined as shown in Table 3.

A multiple regression analysis was conducted on the experimental data and parameters with a significance level greater than 10% were neglected. These results are presented in Equation (2) (R^2 = 96.9%).

$$Y = 1.137 + 0.417X_4 + 0.127X_4^2 + 0.168X_5 - 0.322X_6 + 0.141X_6^2 - 0.144X_4X_5 + 0.126X_4X_6 - 0.162X_5X_6 \quad (2)$$

where X_4 , X_5 and X_6 are the coded concentrations of lactose, glucose and galactose, respectively. Figures 4, 5 and 6 represent the respective response surfaces.

Figure 4 shows that the region of maximum activity was found for higher levels of lactose concentration, which was expected because the maximum concentration of lactose was approximately 50 g/L, which is a value that does not reach the range of substrate inhibition. Under these conditions, enzyme activity was independent of glucose concentration; therefore, glucose did not affect the maximization of enzymatic activity response, only the lactose concentration. Figure 5 illustrates the influence of the galactose concentration on enzymatic activity. A range of highest enzyme activity is observed for low levels of galactose and lower activities are found at higher galactose concentrations. Figure 6 shows the inhibitory effect of galactose, and the highest activities occurred with higher concentrations of glucose and low concentrations of galactose. Figures 4 and 6 show a clear increase in activity with glucose concentration, suggesting that glucose may be acting as an activator of enzyme activity. Therefore, only galactose had an inhibitory effect on the hydrolysis of lactose by β-galactosidase in its soluble form.

Table 3: Experimental enzymatic activities as a function of the initial concentrations of lactose, glucose and galactose for the free enzyme.

Exp.	Real value (Codified value)			Activity (UF)
	X_4 Lactose (g/L)	X_5 Glucose(g/L)	X_6 Galactose (g/L)	
1	16 (-1)	1.5 (-1)	1.5 (-1)	0.918±0.036
2	16 (-1)	1.5 (-1)	10 (+1)	0.283±0.012
3	16 (-1)	10 (+1)	1.5 (-1)	2.001±0.076
4	16 (-1)	10 (+1)	10 (+1)	0.597±0.033
5	50 (+1)	1.5 (-1)	1.5 (-1)	1.919±0.089
6	50 (+1)	1.5 (-1)	10 (+1)	1.668±0.079
7	50 (+1)	10 (+1)	1.5 (-1)	2.305±0.11
8	50 (+1)	10 (+1)	10 (+1)	1.525±0.05
9	10 (- α)	5.75 (0)	5.75 (0)	0.914±0.051
10	56 (+ α)	5.75 (0)	5.75 (0)	1.838±0.11
11	33 (0)	0 (- α)	5.75 (0)	1.177±0.052
12	33 (0)	11.5 (+ α)	5.75 (0)	1.413±0.066
13	33 (0)	5.75 (0)	0 (- α)	1.828±0.071
14	33 (0)	5.75 (0)	11.5 (+ α)	0.975±0.054
15	33 (0)	5.75 (0)	5.75 (0)	1.064±0.06
16	33 (0)	5.75 (0)	5.75 (0)	0.975±0.05
17	33 (0)	5.75 (0)	5.75 (0)	1.053±0.05

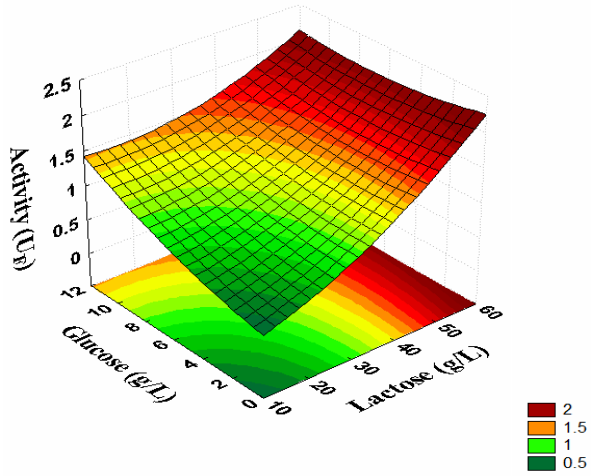

Figure 4: Response surface for the activity as a function of the variables lactose and glucose concentrations.

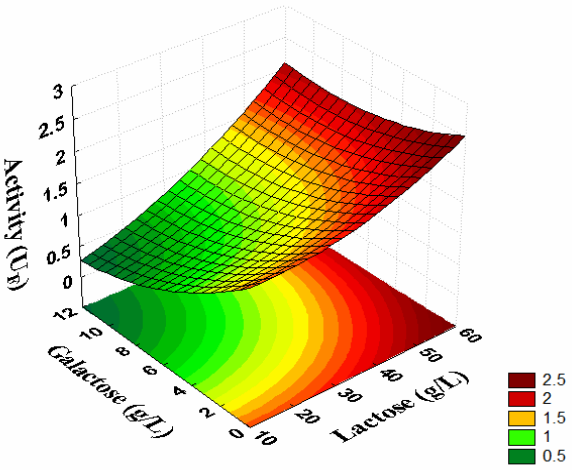

Figure 5: Response surface for the activity as a function of the variables lactose and galactose concentrations.

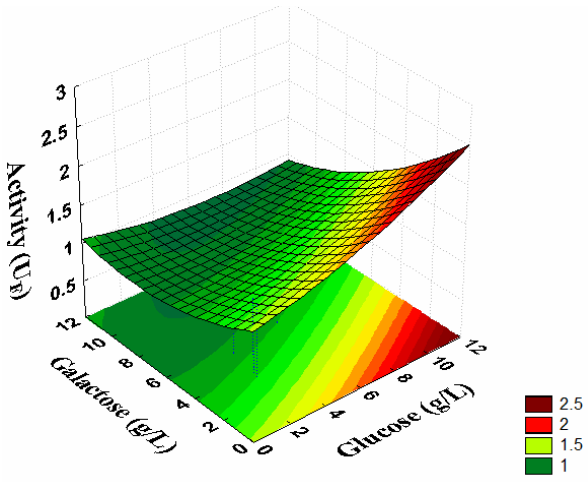

Figure 6: Response surface for the activity as a function of the variables glucose and galactose concentrations.

Hatzinikolaou *et al*. (2005) used β-galactosidase from *Aspergillus niger* to hydrolyze lactose in acid cheese whey permeate and found that only galactose acted as a competitive inhibitor. Jurado *et al*. (2002; 2004) also concluded that galactose acted as a competitive inhibitor for *Kluyveromyces fragilis* β-galactosidase during the hydrolysis of lactose. According to Ladero *et al*. (2000), several authors also have observed competitive inhibition by galactose using different sources of β-galactosidase.

Influence of the Initial Concentration of Galactose on Free and Immobilized Enzyme Activity

An analysis of the results of the influence of the reaction products showed that galactose has a significant inhibitory effect on β-galactosidase activity. The experimental results for the reaction rate of lactose hydrolysis by free and immobilized β-galactosidase in the presence of galactose were fitted by non-linear regression to the kinetic models of competitive and non-competitive inhibition using *Statistica* 7.0 through the Levenberg-Marquardt method. The choice of inhibition model that was fitted to the experimental results was based on the physical meaning of its parameters, which were a significance level of less than 10% by Student's t-test, a higher coefficient of determination (R^2) and the lowest sum of squares deviations. Table 4 presents the fitted parameters with the corresponding Student's t-test, the coefficients of determination and the sum of squares deviations for the effect of inhibition caused by galactose.

Table 4: Kinetic parameters in the presence of galactose as an inhibitor

Inhibition model I=Galactose		Vm (U*)	Km (mM)	Ki (mM)	$\sum(V - V_M)^2$	R^2
Competitive (free)	Parameters	2.4	51.49	5.64	0.255	96.35%
	p-level	0	0.0001	0		
Noncompetitive (free)	Parameters	3.14	100.4	23.27	0.200	97.34%
	p-level	0	0	0		
Competitive (immobilized)	Parameters	633.6	48.8	53.33	97917.9	82.7%
	p-level	0	0.001	0.07		
Noncompetitive (immobilized)	Parameters	821.0	102.3	290.5	80312.8	87.4%
	p-level	0	0	0.12		

U*: UF for free and UI for immobilized enzyme

For the free enzyme, the non-competitive inhibition model had the highest R^2 value and the lowest sum of squares deviations and all of the parameters were statistically significant. However, the values of the parameters V_m and K_m were very different when compared with the values obtained by analyzing the influence of the concentration of lactose in the absence of inhibitors, which was then adjusted by the Michaelis-Menten model. In the non-competitive inhibition model, the V_m value does not reach that obtained by the Michaelis-Menten model without an inhibitor; therefore, the value of V_m of 3.14 U_F was considered to be very high when compared with the value of 2.56 U_F that was previously obtained. In the competitive inhibition the V_m value can achieve the value of V_m of the Michaelis-Menten model, which was actually observed. Based on this analysis, the model that best fitted the data was the competitive inhibition by galactose, which showed K_m and K_i values of 51.49 and 5.64 mM, respectively. Hatzinikolaou *et al*. (2005) found K_m and K_i values of 31.8 and 6.56 mM, respectively, for the model of competitive inhibition by galactose for β-galactosidase from *Aspergillus niger*. Mateo *et al*. (2004), using β-galactosidase from *Kluyveromyces lactis*, found competitive inhibition by galactose and obtained K_m and K_i values of 3.6 and 758 mM, respectively.

For the enzyme in its immobilized form, the non-competitive inhibition model showed the highest R^2 and lowest sum of squares deviations, but the parameter K_i showed a p-value greater than 10%, which made the model statistically invalid. Therefore, the best-fit model was competitive inhibition by galactose, confirming the same result obtained for the enzyme in its free form. The K_m and K_i values were 48.8 and 53.33 mM, respectively, for the competitive inhibition model. This same model for inhibition by galactose is found in the work of Portaccio *et al*. (1998), in which the enzyme β-galactosidase from *Aspergillus oryzae* was immobilized on chitosan beads and nylon membrane. This study showed that the value of K_i for the immobilized form was much higher than for the free enzyme, indicating that there was a reduction of the inhibition by galactose in immobilized biocatalysts. It has been recently shown that immobilization can reduce enzyme inhibition (Pessela *et al*. 2007, Mateo *et al*. 2007).

CONCLUSIONS

The results of this study demonstrated that the optimal conditions for the immobilization of β-galactosidase were 6.60% alginate (w/v), 4.05% gelatin (w/v) and 3.64% glutaraldehyde (v/v). The immobilized enzyme obtained under the optimized conditions maintained 80% of its initial activity after 25 uses. For the studied lactose concentrations, which ranged from 10 to 100 g/L, there was no substrate inhibition for the enzyme in its immobilized form. The Michaelis-Menten kinetic model with competitive inhibition by galactose was the best-fit model for the experimental results of the reaction rate for the hydrolysis of lactose by free and immobilized β-galactosidase. The values of K_m and K_i for the free enzyme were 51.49 and 5.64 mM, respectively, and 48.83 and 53.33 mM, respectively, for the immobilized enzyme. Glucose did not significantly influence β-galactosidase activity.

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