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Optimization of invertase immobilization by adsorption in ionic exchange resin for sucrose hydrolysis

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Abstract

This work presents as a main objective to study the immobilization process of yeast invertase by adsorption in the ion exchanging resin Duolite A-568 for invert sugar production. Initially, a kinetic study of the soluble form of the enzyme was carried out. At the sequence was studied the immobilization process of yeast invertase in the weakly exchanging anionic resin Duolite A-568. The influences of the pH, enzyme concentration and temperature in the enzyme immobilization were analyzed through a central composite design (CCD). The results indicated that the retention of the catalytic activity in immobilization was strongly dependent of these variables, being maximum in a pH value of 5.0, with an enzyme concentration of 12.5 g/L (1.875 g of protein per liter) and temperature of 30 °C. The simultaneous influence of pH and temperature on the free and immobilized invertase activity was also studied through a CCD.

Keywords: Invert sugar; Immobilized invertase; Exchanging anionic resin

1. Introduction

Invertase or β-D-fructofuranosidase (E.C.3.2.1.26) is an enzyme widely used in the food and drink industry. Enzymatic hydrolysis of sucrose by invertase results in the cleavage of α-1,4-glycosidic bonds of sucrose, resulting in the formation of an equimolar mixture of glucose and fructose, known as invert sugar. The later is sweeter and easer to incorporate in industrial preparations than is granular sucrose [1–3]. Invert sugar is widely used in the production of non-crystallising creams, jams, artificial honey and in confectionary industry and to a lesser extent in the industrial production of liquid sugar [4]. Invertase also catalyzes transfer reactions with other acceptors beside water, resulting in the formation of oligosaccharides consisted of units of glucose and fructose [5].

The enzyme invertase has been found in bacteria, fungi, insects, mammals and vegetables, but the main industrial source are the yeasts. Yeast invertase has a molecular weight of 270 kDa and an isoelectric point between 3.4 and 4.4 and do not require cofactors for its activation [6,5,2].

The enzymatic sucrose hydrolysis processes utilizes invertase in their free or immobilized forms. There are many benefits in using the immobilized enzyme in comparison with their soluble form, such as the reutilization of the heterogeneous biocatalyst, costs reductions and an improvement in the process control. The invertase catalyzed hydrolysis in its free and immobilized forms produces high quality syrup with low concentrations of 5-hydroxymethyl-2-furfural (HMF) and without color development compared to the colored version obtained through acid hydrolysis [7–9].

The use of free enzyme in industrial processes is restricted due to its high cost, to its recovery difficulty at the end of the process and also its instability. The application of the enzyme in its immobilized form may have some advantages in relation to its free form, such as cost reduction, possibility of better process control, continuous operation and stability increases, however some alterations in the kinetic properties of the enzyme can be verified as well [10–12].

The immobilization process choice for a given enzyme depends on the process essential factors, such as the substrate used, types of reactions and the reactor configurations,

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demanding an adequate project to attend to the reaction needs. One of the main factors is the selection of an adequate support for the enzyme fixation. Thus the chosen method must attend two necessities, the catalytic, expressed in productivity, efficiency, stability and selectivity and the non-catalytic, which is control related and down-streaming process [13,9].

Invertase has been immobilized on a variety of support materials, by different methods, for invert sugar production [3,4,14,15]. Among these supports, the most important are polymers [16].

Many immobilization methods have been tried, ranging from covalent attachment to adsorption or physical entrapment. Among the various immobilization techniques available, adsorption may have a higher commercial potential than other methods because the adsorption process is simpler, less expensive, retains a high catalytic activity, and most importantly, the support could be repeatedly reused after inactivation of the immobilized enzyme [17].

The enzyme immobilization in ionic exchanging resins is carried out through in a simple procedure when compared with others immobilization methods. It basically involves ionic and electrostatic interactions between the protein ions and the opposing ions from the resin. Although the linking forces between enzyme and support are stronger than in adsorption, the immobilization conditions are moderate, the conformationals alterations are small, resulting in an elevated enzymatic activity. As disadvantage of the method, there is the possibility of the enzyme unfastening of carrier out on variations of pH and ionic force of the medium. Even so, there are many advantages, such as support recovery, low cost and support availability. The nature of ion exchanging resins is complex, most of than being polymers. Active ions are cations in a cationic exchanger and anions in an anionic exchanger [18–20].

While enzyme immobilization has been studied for a number of years, the appearance of published research and review papers indicate a continued interest in this area, more than 550 enzyme immobilization-related papers have been published in 2005 [2].

In this work the yeast invertase immobilization process in the Duolite A-568 resin was studied and a kinetic study of sucrose hydrolysis by free and immobilized invertase was carried out.

2. Experimental

2.1. Material

Yeast invertase (β-D-fructofuranosidase, E.C.3.2.1.26) was purchased from Sigma. The weakly basic ionic exchanging resin, Duolite A-568 was acquired from Rohm Hass. The other reagents used were of analytical grade.

2.2. Activity determination of free and immobilized enzyme

The enzymatic activity was determined through the initial rates of sucrose hydrolysis reaction method, thus being determined the formed reduced sugar by the DNS method [21]. The reactions with the free enzyme were conducted in a mixture mini-reactor, with 100 mL of useful volume, at the initial concentrations of sucrose, pH and temperature defined for each experiment, under magnetic agitation. The immobilized enzyme was contained in a stainless steel basket and its catalytic activity determined by the same procedure used for the free enzyme.

The unit of enzyme activity in the experiments of free enzyme (U_F) was defined as gram of reducing sugar produced per liter, per minute, per gram of invertase Sigma powder and for the immobilized enzyme (U_i) was defined as gram of reducing sugar produced per liter, per minute, per gram of support.

Protein determination measurements were performed by Lowry's method [22].

2.3. The effect of sucrose concentration in the activity of free and immobilized invertase

The study of the substrate concentration influence in the enzymatic activity was carried out, determining the initial rates of the hydrolysis reaction, with the initial concentration of sucrose ranging from 2 to 500 g/L, in a temperature of 30 °C and a pH of 4.5 for the free enzyme, with an invertase concentration of 0.01 g/L. For immobilized invertase the temperature and pH were 40 °C and 5.5, respectively.

2.4. Immobilization process

The resin was previously washed with distilled water and than activated with a 1 M hydrochloric acid solution, with a 1 M sodium hydroxide solution and than washed again with distilled water and a 4.5 pH acetate buffer. The invertase immobilization in the Duolite A-568 ionic exchanging resin was carried out by incubating 10 mL of an invertase solution with 0.5 g of resin during 24 h, in the conditions defined by Table 1, under a 60 rpm

Table 1
Central composite design of the temperature, pH and enzyme concentration effect in the immobilization process

Experiments	Real valor (codified valor)		
	Temperature (°C)	pH	Enzymatic concentration (g/L)[a]
1	14 (−1)	3.5 (−1)	3.0 (−1)
2	14 (−1)	3.5 (−1)	17.0 (+1)
3	14 (−1)	6.5 (+1)	3.0 (−1)
4	14 (−1)	6.5 (+1)	17.0 (+1)
5	40 (1)	3.5 (−1)	3.0 (−1)
6	40 (1)	3.5 (−1)	17.0 (+1)
7	40 (1)	6.5 (+1)	3.0 (−1)
8	40 (1)	6.5 (+1)	17.0 (+1)
9	27 (0)	5.0 (0)	10.0 (0)
10	27 (0)	5.0 (0)	10.0 (0)
11	27 (0)	5.0 (0)	10.0 (0)
12	9 (−α)	5.0 (0)	10.0 (0)
13	45 (+α)	5.0 (0)	10.0 (0)
14	27 (0)	3.0 (−α)	10.0 (0)
15	27 (0)	7.0 (+α)	10.0 (0)
16	27 (0)	5.0 (0)	0.5 (−α)
17	27 (0)	5.0 (0)	19.5 (+α)

[a] (g invertase Sigma powder)/L—1 mg of invertase Sigma powder presented 0.15 mg of protein.

Table 2
Central composite design of the temperature and pH effect in the free invertase enzymatic activity

Experiments	Real valor (codified valor)	
	Temperature (°C)	pH
1	30 (−1)	3 (−1)
2	70 (+1)	3 (−1)
3	30 (−1)	6 (+1)
4	70 (+1)	6 (+1)
5	50 (0)	4.5 (0)
6	50 (0)	4.5 (0)
7	50 (0)	4.5 (0)
8	27 (−α)	4.5 (0)
9	73 (+α)	4.5 (0)
10	50 (0)	2.8 (−α)
11	50 (0)	6.2 (+α)

agitation in shaker. After the immobilization, the resins were washed with a 4.5 pH acetate buffer.

The influences of the enzyme concentration, pH and immobilization temperature were analyzed by using a central composite design (CCD) with three central replicas totalizing 17 experiments. The comprehended variables range is shown in Table 1.

2.5. Temperature and pH effect in the free and immobilized enzyme activity

The study of the joint effect of the temperature and pH in the immobilized and free enzyme activity was carried out by using a central composite design with three central replicas, as shown on Tables 2 and 3, respectively, by using the Statistica® 5.0 software. The enzymatic activity were obtained by the initial rates of sucrose hydrolyses reaction method, in a mixture mini-reactor containing 100 mL of a 50 g/L sucrose solution. The experiments with the free enzyme were carried out using an acetate buffer. With the immobilized enzyme, when the pH ranged from 2.7 to 5.1, it was used an acetate buffer and when it ranged from 7.0 to 7.5 it was used a citrate–phosphate buffer.

Table 3
Central composite design of the temperature and pH effect in the immobilized invertase enzymatic activity

Experiments	Real valor (codified valor)	
	Temperature (°C)	pH
1	17 (−1)	3.0 (−1)
2	17 (−1)	7.2 (+1)
3	70 (+1)	3.0 (−1)
4	70 (+1)	7.2 (+1)
5	13 (−α	5.1 (0)
6	74 (+α	5.1 (0)
7	44 (0)	2.7 (−α
8	44 (0)	7.5 (+α
9	44 (0)	5.1 (0)
10	44 (0)	5.1 (0)
11	44 (0)	5.1 (0)

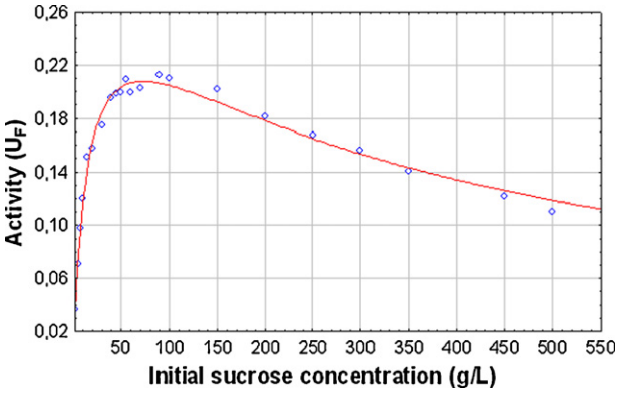

Fig. 1. Initial sucrose concentration influence in the enzymatic activity.

3. Results and discussion

3.1. Sucrose concentration effect in the free invertase and immobilized activity

The experimental results of reaction rate as a function of the initial sucrose concentration for free invertase were adjusted by substrate inhibition model and the parameters were determined by non-linear regression. The found V_m (maximal activity) value was 0.0803 M/min and the K_m (Michaelis–Menten constant) and K_i (inhibition constant) values were 45.2 mM and 1.06 mM, respectively.

The kinetic parameters for free invertase obtained in this work are in agreement with those presented in the literature [6,23,24,16], reported, respectively, values of 59.0, 62.3, 25.91 and 26.6 mM for K_m.

The correlation obtained from the model with the experimental data was $R^2 = 0.98$ and a comparison between the experimental and predicted data is presented in Fig. 1. It can be observed that for sucrose concentrations higher than 50 g/L, the substrate inhibition became quite sensitive.

The experimental results of immobilized invertase activity, according immobilization process of item 1.3.2, were also fitted to the inhibition by the substrate model. The fitting reached a determination coefficient of 0.93 and a comparison between the experimental and the predicted data can be

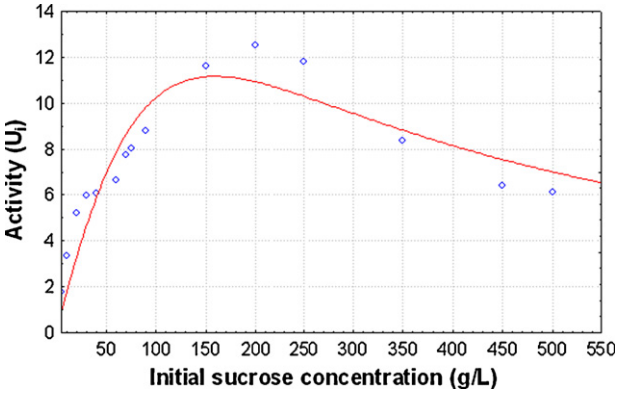

Fig. 2. Profile of sucrose concentration influence in the immobilized enzymatic activity.

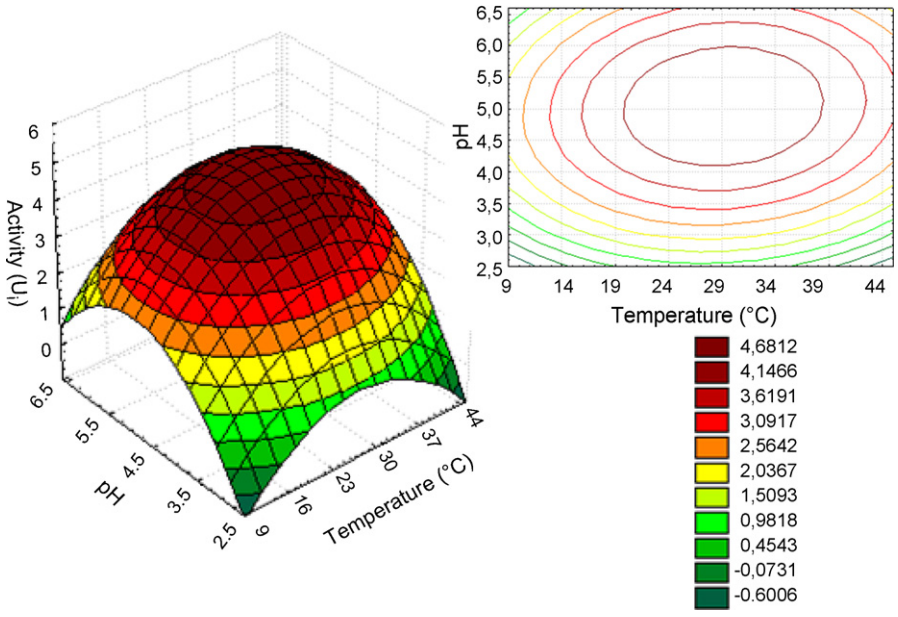

Fig. 3. Response surface of the temperature and pH influence in the immobilized invertase activity.

seen on Fig. 2. The V_m value found was 0.047 M/min and the K_m and K_i values were 176 mM and 1.08 M, respectively.

The Michaelis–Menten model constants for free and immobilized invertase obtained in this work were 45.2 and 176 mM, respectively. The K_m value of immobilized invertase was approximately 3.9-fold higher than that of free invertase. The increase in K_m value can be attributed to the diffusional resistances to mass transfer.

Similar results were reported previously in the literature. For example, Rebros et al. [15] reported an increase in K_m of 3.6 times higher for invertase immobilized in polivinil alcohol beads. The K_m value obtained by Sanjay and Sugunan [16], for invertase adsorbed in montmorillonite K-10 was 14-fold higher than that of free counterpart Osman et al. [25] reported an increase in the K_m of 19 times higher for immobilized invertase related the free form. On the other hand, Tomotani and Vitolo [20] obtained similar values of K_m for free and immobilized invertase in Dowex 1 × 4, suggesting that the mass transfer resistances are negligible.

3.2. Optimized immobilization conditions

The response surface methodology allows verifying through a lesser number of experiments the influence of diverse variables in the results, as well as optimizing them.

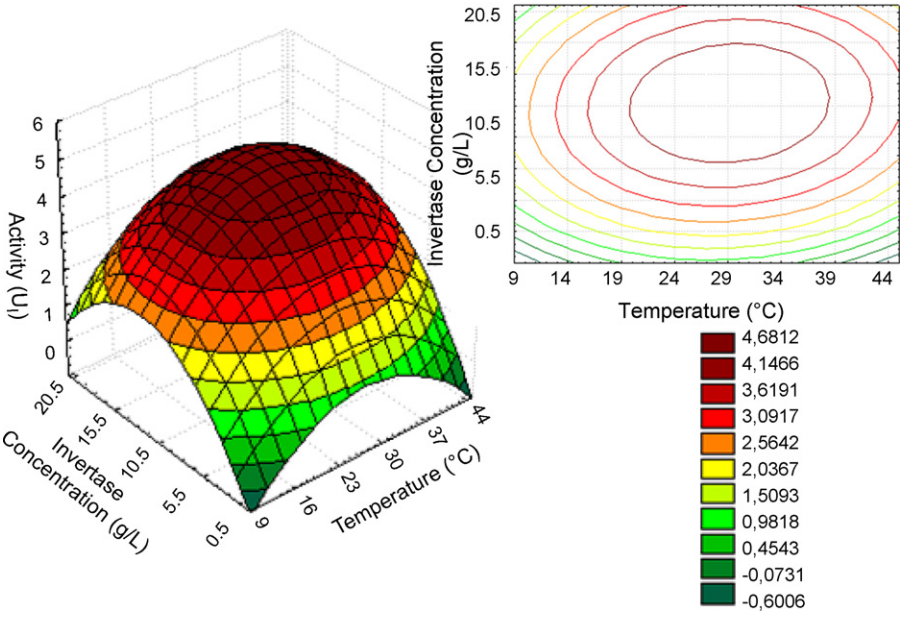

Fig. 4. Response surface of the invertase concentration and the enzyme immobilization temperature influence in the immobilized invertase activity.

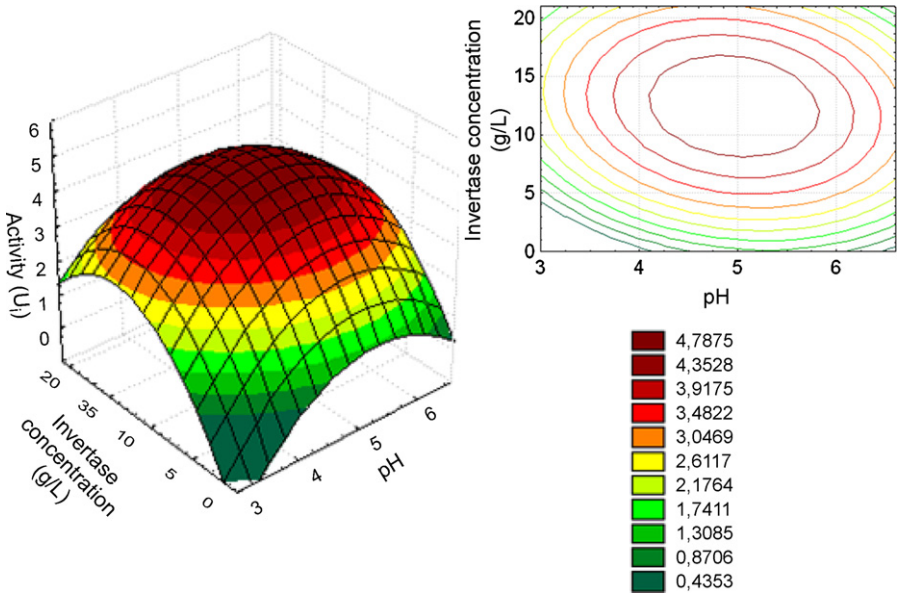

Fig. 5. Response surface of the invertase concentration and pH influence in the immobilized invertase activity.

The central composite design made possible to find the best conditions in terms of temperature, pH and enzymatic concentration at of the studied range, to immobilize invertase in the Duolite A-568 resin. Moreover, the influences of these variables in the immobilization as well as the interactions between these effects were confirmed. The model's most relevant variables which presented the lowers significance levels on the t of Student test were the enzyme concentration on the quadratic term (x_3^2) and isolated (x_3), and the quadratic terms of the temperature (x_1^2) and pH (x_2^2).

As a form to illustrate the variable effects on the enzymatic activity response, the response surfaces relating the variables in pairs are presented in Figs. 3–5. It is observed that there is in all surfaces a stationary point corresponding to the point of maximum response in all surfaces.

With the data obtained by the experimental planning, a multiple regression was made. The parameters with significance level higher than 10% were neglected. The adjusted model ($R^2 = 0.89$) is given by the Eq. (1), in which the temperature, pH and the enzymatic concentration are represented by x_1, x_2 and x_3, respectively. With the implementation of an algorithm to calculate the maximum point of enzymatic activity, by using the model complete equation, the codified values that maximized the response were obtained. After eliminating the non-significant parameters, Eq. (1) was obtained. As the objective of this work is to increase the enzymatic activity (y), the found values that

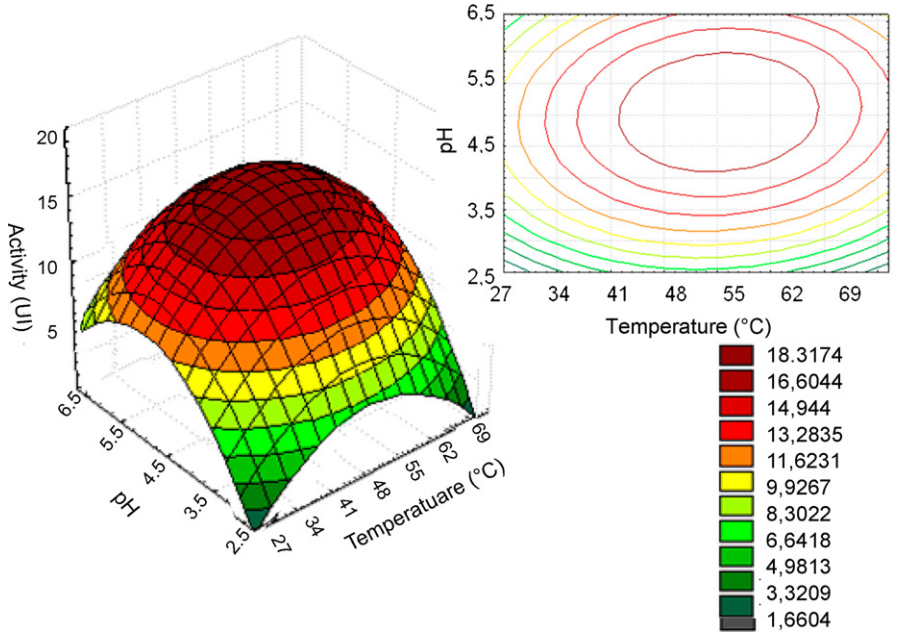

Fig. 6. Response surface of the temperature and pH influence in the free invertase activity.

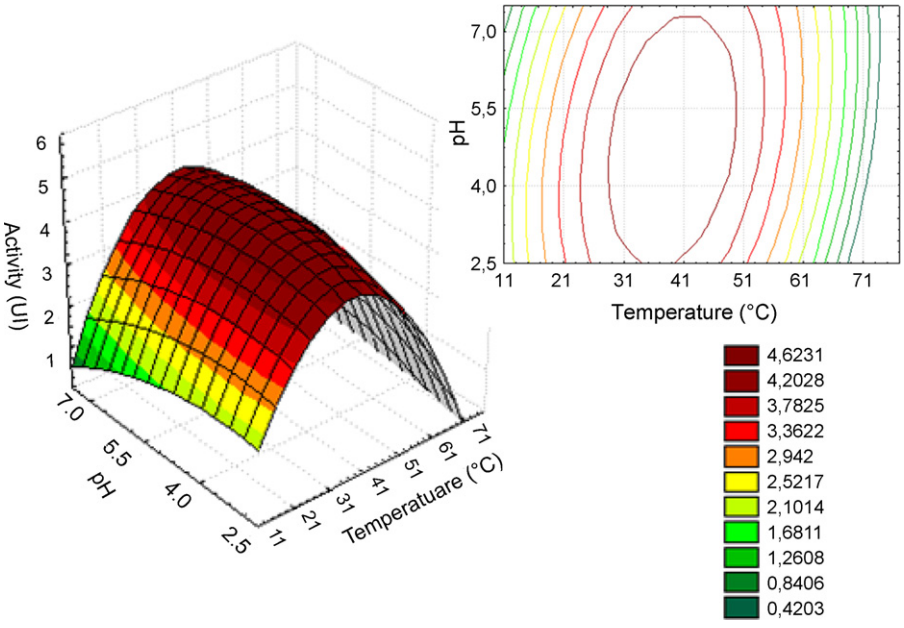

Fig. 7. Response surface of the temperature and pH influence in the immobilized invertase activity.

maximized the response were temperature of 29 °C, pH of 5.0 and enzymatic concentration of 12.5 g/L of the Sigma powder enzyme, or 1.875 g of protein per liter. One milligram of invertase Sigma powder presented 0.15 mg of protein determined by Lowry method [22].

$$y = 2.276 + 0.394x_3 - 0.489x_1^2 - 0.6512x_2^2 - 0.553x_3^2 \quad (1)$$

To validate the model, experiments were performed under optimal conditions given by the CCD method and the average activity obtained in these tests was 4.606 U_i and the activity calculated by the Eq. (1) was 4.704 U_i, showing a good agreement between experimental and calculated values.

3.3. Influence of pH and temperature in the enzymatic activity of the free and immobilized enzyme

In order to determine the best operational conditions in the sucrose hydrolysis with soluble invertase process, a two variable central composite design was made (temperature and pH), totalizing 11 experiments, 2^2 experiments inquiring for linear model, 3 central points and 4 axial points in a α distance from the central point. The value of $\alpha = 1.1474$ was calculated so that the CCD was orthogonal, a planning where the matrix of variance and covariance is diagonal and the estimated parameters are not correlated. The response surfaces generated by the experiments showed in Tables 2 and 3 are presented in Figs. 6 and 7, respectively, and it can be observed that the stationary point is a maximum point at the surface.

The study of the influence of the variables pH and temperature using free invertase resulted in a model obtained by multiple regression with all the significant coefficients represented in Eq. (2), x_1 and x_2 being the codified variables for temperature and pH, respectively. The most significant parameters in the enzymatic activity, obtained with the hypotheses tests by using the t of Student statistics, were the temperature and pH quadratic term. The interaction effects between temperature and pH (x_1x_2) and isolated pH (x_2) presented a higher than 10% significance level, thus being neglected. With the same procedure previously cited, the codified values that maximized the enzymatic activity were obtained, being a temperature of 47 °C and a 4.7 pH. A correlation coefficient (R^2) of 0.84 indicates an adequate adjustment of the experimental data in the response enzymatic activity, showing that 84% of the data variability was explained by the proposed empirical equation.

$$y = 18.119 - 2.775x_1 - 9.702x_1^2 - 5.794x_2^2 \quad (2)$$

To validate the model, experiments were performed under optimal conditions and the result for the average activity, which was given by 17.38 U_F, was compared to the activity calculated by the model (Eq. (2)), which was 18.21 U_F.

The simultaneous influence of pH and the temperature in the immobilized invertase enzymatic activity, after the multiple regression, resulted in the model described by the Eq. (3). In this model, the parameters with the significance level of the t of Student test lower than 10%, the relevant data, were the quadratic temperature term (x_1^2), its isolated term (x_1) and the temperature/pH interaction (x_1x_2). With the aid of an algorithm it was obtained a temperature of 40 °C and the pH of 4.9, values that maximized the activity of the immobilized enzyme. The coefficient of correlation (R^2) of 0.98 indicates an adequate adjustment of the experimental data in the enzymatic activity response.

$$y = 4.557 - 0.812x_1 - 2.624x_1^2 - 0.324x_2^2 + 0.465x_1x_2 \quad (3)$$

To validate the model, experiments were performed under conditions of optimum point and found the average activity of 4.82 U_i and the activity calculated by Eq. (3) was 4.63 U_i, presenting a good agreement between this values.

The curves in Figs. 6 and 7 for free and immobilized invertase are very different in appearance. The surface response of the activity for the free enzyme presents a well-defined point respect to pH, whereas for the immobilized form it appears that in the optimum temperature, the pH does not influence significantly the activity. This fact shows the importance of simultaneous analysis of temperature and pH in the activity. But in the two forms of invertase the temperature exerted strong influence on the enzymatic activity.

The effect of temperature on enzyme reactions is intimately related to the composition of the medium. The optimum temperature for free enzyme was 7 °C higher than for the immobilized invertase, probably due to thermal effects on the microenvironment of the enzyme. This result is in agreement with the work of Tomotani and Vitolo [20], which suggests that aspects of the transfer of heat between the environment and the particles of resin can lead to an overheating at the particle surface and consequently the thermal deactivation of the enzyme.

The pH value of the maximum enzymatic activity was practically the same for the free and immobilized enzyme. These results oppose those of Tomotani and Vitolo [20], who immobilized the invertase in a Dowex 1×4 anionic resin, and verified that the pH that maximized the activity of the immobilized enzyme was lower than the one that maximized the free enzyme.

With the use of the CCD was possible decrease by 30%–49% the number of experiments necessary to determine the optimum values of temperature and pH of the free and immobilized invertase compared with the work of Danisman et al. [26] and Gómez et al. [27], respectively. Moreover, the application of statistical planning allows simultaneous analysis of the influence of the two variables in the response required.

4. Conclusion

From the data presented, it can be concluded that:

The sucrose hydrolysis by free and immobilized invertase follows substrate inhibition kinetics. The K_m value of immobilized invertase was approximately 3.9-fold higher than that of free invertase. The increase in K_m value can be attributed to the diffusional resistances to mass transfer.

The experimental conditions that maximized the invertase catalytic activity in the immobilization process were the rate of the 20 mL of enzyme solution at 12.5 g/L (1.875 g of protein per liter) by gram of resin, at pH 5.0 in the immobilization medium of and a temperature of 29 °C. In these conditions, the enzyme activity of immobilized invertase was 4.704 U_i.

The values of pH and temperature determined by the CCD that implied in the maximum enzymatic activity for the free enzyme were 4.7 and 47 °C, respectively.

For invertase immobilized in Duolite A-568, the pH and temperature values that implied in the maximum enzymatic activity, according to the CCD, were 4.9 and 40 °C, respectively.

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