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Thermal Stability and Energy of Deactivation of Free and Immobilized Amyloglucosidase in the Saccharification of Liquefied Cassava Starch

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ABSTRACT

Amyloglucosidase from Novo (Copenhagen, Denmark) was immobilized in controlled pore silica particles with the silane-glutaraldehyde covalent method. Thermal stability of the free and immobilized enzyme (IE) was determined with 30% (w/v) α-amylase liquefied cassava starch, pH 4.5, temperatures from 35 to 75°C. Free amyloglucosidase maintained its activity practically constant for 240 min and temperatures up to 50°C. The IE has shown higher stability retaining its activity for the same period up to 60°C. Half-life for free enzyme was 20.6, 6.44, 2.07, 0.69, and 0.24 h for 55, 60, 65, 70, and 75°C, respectively, whereas the IE at the same temperatures had half-lives of 116.4, 30.88, 8.52, 2.44, and 0.73 h. The energy of thermal deactivation was thus 50.6 and 57.6 kcal/mol, respectively for the free and IE, confirming stabilization by immobilization.

Index Entries: Cassava starch; amyloglucosidase; immobilized enzyme; stability; half-life.

INTRODUCTION

Cassava starch, known as "fécula" in Brazil, is a plentiful, renewable, cheap resource that may be used for the production of modified starches of large application into textile and paper industries. It has also been considered as a source of glucose syrups produced by saccharification with amyloglucosidase. These syrups could be used directly in the food industry, or converted to ethanol of high quality to be used in perfumes or alcoholic beverages such as liqueurs and spirits. Fuel ethanol that is used

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in Brazil in anhydrous form for blending with gasoline, or straight as in the azeotropic ethanol-water mixture used in ethanol cars, could be produced from cassava starch.

The potential of these applications have stimulated the development of a research program in our department aimed at studying the saccharification of liquefied cassava starch with free and immobilized amyloglucosidase *(1)*. The research program included: modeling of batch reactors with free enzyme *(2)* and of fixed- and fluidized-bed reactors with immobilized enzyme *(3,4)*, hydrodynamics *(5)*, axial dispersion *(6)*, internal *(4)* and external *(3)* mass transfer, and operational stability of the immobilized enzyme *(7)*; characterization of Novo Nordisk amyloglucosidase with respect to its activity in the saccharification of preliquefied cassava starch at different pHs and temperatures *(8)*; and determinations of the density, viscosity, and initial glucose contents of the Novo α-amylase liquefied starch, measured as a function of total dried matter in solution *(5)*.

This article covers experimental determination, of free and immobilized Novo Nordisk amyloglucosidase thermal deactivation at temperatures from 35 to 75°C during the saccharification of α-amylase preliquefied cassava starch.

DEACTIVATION HYPOTHESIS AND MODELING

As temperature is raised in a reaction catalyzed by free or immobilized enzyme, two opposing effects are observed: The enzyme activity increases with temperature, and this increases the reaction rate, but with higher temperatures the enzyme stability decreases by thermal denaturation and this reduces the concentration of active enzyme that reduces the reaction rate *(9–11)*. Usually in the range of 30 to 50°C, activation of the enzyme prevails and the enzymatic activity increases with temperature but above that, enzyme denaturation overtakes activation, and enzymatic activity begins to decline. Industrial processes that use enzyme are usually set at 60°C to avoid microbial contamination, and because of that the knowledge of the rate of deactivation of the enzyme at different temperatures is an important consideration.

The energy of activation of the thermal denaturation reaction, or as it is usually called: energy of deactivation, can be obtained from thermal stability data obtained by carrying out an experiment for various temperatures, in which the enzyme is incubated for a certain time within specified conditions, and then its residual activity is assayed. It is normally assumed that enzyme thermal denaturation is a reaction with the rate of enzyme deactivation (r_d) being first order in relation to the concentration of the active enzyme (E):

$$r_d = - K_d E \tag{1}$$

and the deactivation constant (K_d) being a function of temperature as given by the Arrhenius equation:

$$K_d = K_d^\circ \exp(-E_d/RT) \quad (2)$$

where E_d is the energy of deactivation, R the universal gas constant (1.987 cal/mol K), and T the absolute reaction temperature. It should be clearly understood that the energy of deactivation is the preferred name for the energy of activation of the deactivation reaction.

For a batch reactor of constant liquid density the rate of reaction equals the time derivative of the concentration, and therefore it follows from equation *(1)* that:

$$(dE/dt) = -K_d E \quad (3)$$

which integrated with the initial condition: $E = E_0$ for $t = 0$, gives:

$$E = E_0 \exp(-K_d t) \quad (4)$$

where E_0 is the initial active enzyme concentration, and t is time elapsed during reaction.

When the enzyme is present in catalytical quantities, that is, in low concentration, the residual enzyme activity (A_r) is directly proportional to the concentration of the active enzyme (E):

$$(A_r/A_0) = (E/E_0) \quad (5)$$

where A_0 is the initial enzyme activity observed with the initial enzyme concentration (E_0).

Combining equations *(4)* and *(5)*, the residual enzyme activity results as:

$$A_r = A_0 \exp(-K_d t) \quad (6)$$

This result is known as the exponential decay model. Therefore, by plotting residual activity data in the form of log of A_1/A_0 against time, the deactivation constant (K_r) is obtained as the angular coefficient of the adjusted straight line.

From equation *(2)*, and, as observed experimentally, it can be seen that the deactivation constant increases with temperature. Values obtained for K_d for various test temperatures are plotted in the form of Arrhenius plot, that is log of K_d against the inverse of absolute temperature, yielding the energy of deactivation (E_d), as the angular coefficient of the adjusted straight line, times R, the universal gas constant.

In a given set of experimental conditions, another important parameter related to enzyme stability is the enzyme half-life $(t_{1/2})$, that corresponds to the period of time taken by the residual enzyme activity to decrease to

50% of its initial value. From equation *(6)*, it results that the half-life ($t_{1/2}$) can be calculated by:

$$t_{1/2} = -1n\ (0.5)/K_d = 0.693/K_d \quad (7)$$

showing that $t_{1/2}$ is inversely proportional to the deactivation constant (K_d).

The energy of deactivation (E_d) of most enzymes is normally within 47 to 96 kcal/mol, clearly higher than the energy of activation (E_a) that is normally smaller than 25 kcal/mol *(12)*. E_a is the activation energy associated with the normal reaction catalyzed by the enzyme in which the substrate is transformed to products. These different ranges for the energy of activation and energy of deactivation, have as result the observed fact that enzymes are activated at lower temperatures and deactivated at high temperatures. This is a direct consequence of the rule that applies to competing reactions with different energies of activation, namely: high temperatures favor reactions with high energies of activation *(13)*.

MATERIALS AND METHODS

Enzyme

Aspergillus niger amyloglucosidase kindly donated by Novo (AMG 150L, with 130 mg of protein/mL) was used as free enzyme in solution and immobilized.

Support

Controlled pore silica (CPS) was supplied by Corning Glass Works (Corning, NY) with a particle mean diameter of 0.436 mm, average pore size of 37.5 nm, and internal porosity of 56.6%.

Substrate

The substrate was cassava starch (Copagra-PR) at a final concentration of 30% (w/v), liquefied with α-amylase (Thermamyl 120L, Novo) in the presence of 70 ppm $CaCl_2$, pH 6.0, for 1 h at 95 to 100°C. After cooling to room temperature, the pH of this solution was adjusted to 4.5 with sodium acetate buffer to a final concentration of 0.02 *M*. The DE (dextrose equivalent) of this solution was measured with the DNS method *(14)*, giving 38.6.

Enzyme Immobilization

Amyloglucosidase was immobilized in CPS with the silane-glutaraldehyde covalent method of Weetall *(15)* that consists in the following steps: silanization of the support with 0.5 (v/v) γ-aminopropyltrietoxisilane for 3 h at 75°C; wash the silanized support with distilled water and dry it for 15 h at 105°C; activate the dried silanized support with a solution of 2.5% (v/v) glutaraldehyde, pH 7.0 for 45 min at 20°C; wash with water; contact the activated support with the enzyme solution for 15 h at 20°C; wash

the immobilized enzyme with water and stock it in buffer solution in a refrigerator for later use *(15,16)*. The immobilized amyloglucosidase was kept in 0.02 *M* acetate buffer, pH 4.5, at 4°C.

Thermal Deactivation Test

Free or immobilized amyloglucosidase was incubated at the following temperatures: 35, 40, 45, 50, 55, 60, 65, 70, and 75°C, in the substrate solution, for a period of 4 h. In the case of the thermal deactivation test with the enzyme free in solution 10 mL of the substrate solution contained 6.2 mg of protein/mL, and each 40 min a 1 mL sample of the incubated enzyme was taken and the residual enzymatic activity was determined. For the immobilized enzyme (IE), seven stainless steel baskets containing 1,000 g wet weight of IE were used, six being immersed in 200 mL of substrate at the temperature of the thermal deactivation test, and one used for determining the initial enzymatic activity (A_0). Each 40 min, one of the baskets with the incubated IE was taken for determining the residual activity. The value of the humidity of the immobilized enzyme, which was necessary to calculate the exact IE dry weight in the basket, was determined in a parallel experiment at 105°C.

Determination of Amyloglucosidase Activity

The residual enzymatic activity was determined by the method of initial velocities *(10)*. Samples of free or immobilized enzyme taken from the thermal deactivation test were put in contact with 50 mL of the substrate solution in a batch reactor maintained at the selected temperature, and aliquots were taken at regular intervals for determining the glucose produced by the saccharification reaction. Total reaction time was 30 min in this test to give maximum substrate conversion below 30%, usually much lower than this, and 0.5-mL samples were collected every 5 min. In these conditions the rate of glucose production as a function of time (μmol/min) is linear, and the residual enzyme-specific activity is obtained by dividing this rate by the quantity of protein present (mg). Therefore the residual specific activity was expressed in μmol/(min/mg of protein), that is, U/mg of protein.

Enzyme Activity

One unit (U) of enzyme activity corresponds to the quantity of enzyme that produces 1 μmol of glucose/min with the aforementioned substrate at the specified temperature.

Assay Methods

The glucose produced in the test for determination of the enzymatic activity was measured by the orthotoluidine method *(17)*, and the total protein contents of the enzymatic solution was assayed by the method of Lowry et al. *(18)*.

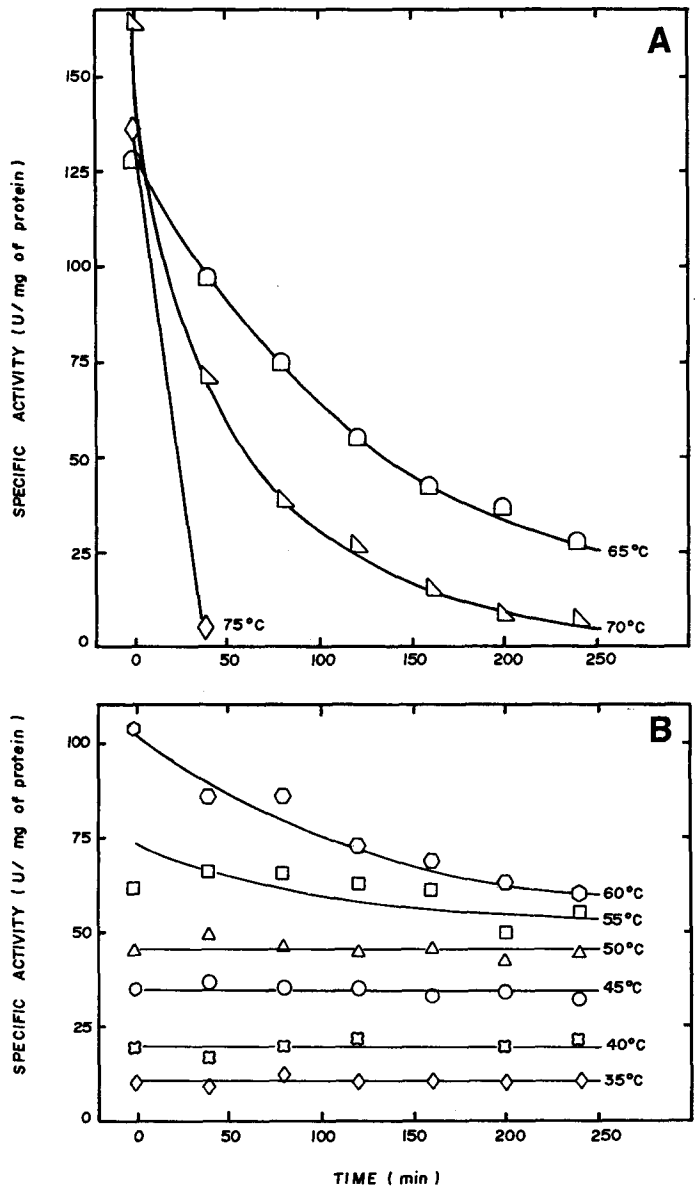

Fig. 1. Thermal deactivation data for free Novo Nordisk amyloglucosidase incubated in liquefied cassava starch, 30% (w/v), pH 4.5. Each point in the figure corresponds to the amyloglucosidase residual activity measured with 1 mL of enzyme taken from the thermal deactivation test and added to 50 mL of substrate solution giving 0.124 mg of protein/mL of solution.

RESULTS AND DISCUSSION

Immobilized amyloglucosidase was produced offering 4.48 mg/g of protein of support, and 4.12 were retained, giving an enzyme yield of 92.0%, with 22.4 U/mg of protein at 45°C.

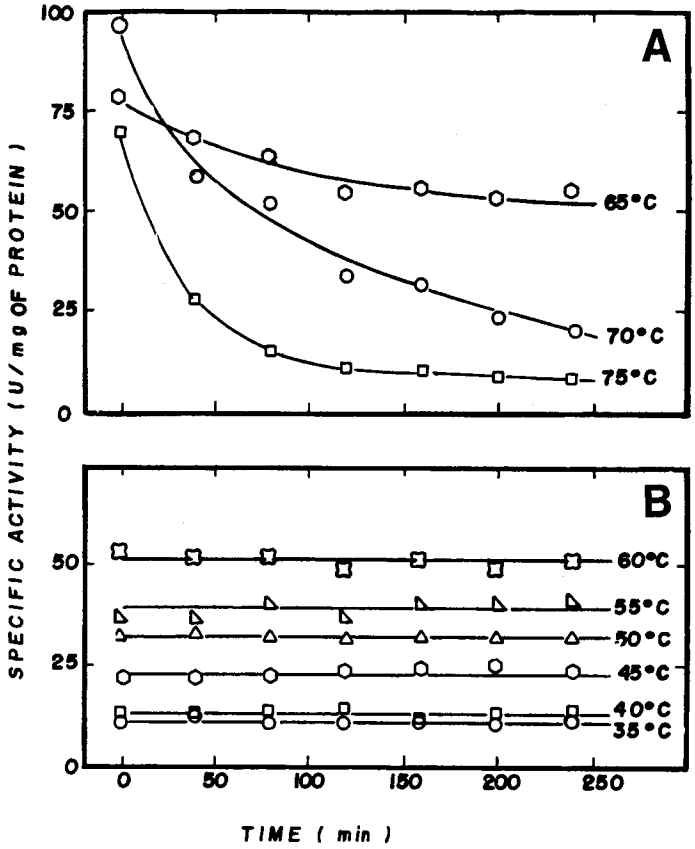

Fig. 2. Thermal deactivation data for Novo Nordisk amyloglucosidase immobilized in controlled pore silica, and incubated in liquefied cassava starch, 30% (w/v), pH 4.5. Each curve corresponds to the residual activity of the IE, measured with six baskets incubated in 200 mL of substrate solution at the specific temperature. The IE had 4.12 mg/g of protein of support and each basket contained about 0.564 g of IE dry weight.

Experimental data obtained from the thermal deactivation test for free amyloglucosidase are shown in Fig. 1, and for the immobilized enzyme in Fig. 2. It can be observed that the immobilized enzyme loses very little of its activity in the period of 4 h at the temperatures of 35 to 60°C, whereas for the free enzyme this applies only for 35 to 50°C. The free enzyme thermal denaturation at 60°C is already quite noticeable, retaining only 59% of its activity after 4 h in contact with the substrate. At the higher temperatures of 65 to 75°C, the denaturation of the free amyloglucosidase is much more pronounced than that of the immobilized enzyme.

Equation *(6)* adjusted to data obtained at the higher temperatures gave the results shown in Table 1, in which the half-lives were calculated with equation *(7)*. Missing values in Table 1 reflect the difficulties of obtaining good fit at these points. For 55°C, the IE deactivation is too slow, and experimental errors are greater than the observed reduction in activity.

Table 1
Exponential Decay Model, Equation *(6)*, Adjusted to Thermal Deactivation of Novo Nordisk Amyloglucosidase Thermally Denatured in Liquefied Cassava Starch 30% (w/v), pH 4.5, at Different Temperatures

Temperature (°C)	Free Enzyme			Immobilized Enzyme		
	k_d (h^{-1})	r	$t_{1/2}$ (h)	k_d (h^{-1})	r	$t_{1/2}$ (h)
75	-	-	-	1.1484	0.9943	0.60
70	0.8712	0.9943	0.80	0.2017	0.9928	3.44
65	0.3764	0.9960	1.84	0.0933	0.9988	7.43
60	0.1297	0.9765	5.34	0.0229	0.9933	30.24
55	0.0287	0.8742	24.1	-	-	-

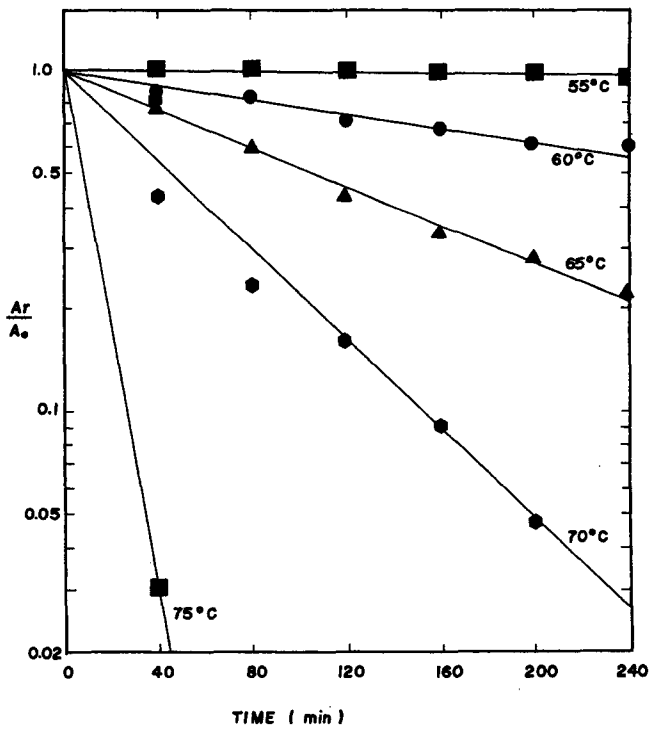

Fig. 3. Free Novo Nordisk amyloglucosidase thermal deactivation data compared with the exponential decay model, equation *(6)*.

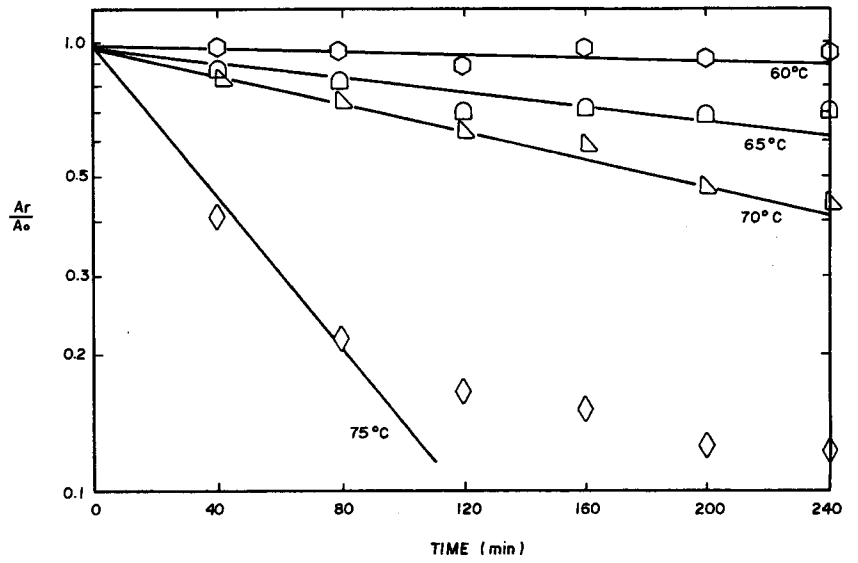

Fig. 4. Immobilized Novo Nordisk amyloglucosidase thermal deactivation data compared with the exponential decay model, equation *(6)*.

For 75°C, the free enzyme deactivation is too fast, and only one point was not enough to give a good measurement of K_d.

Figures 3 and 4 compare the exponential decay model with the deactivation data obtained at the higher temperatures for free and immobilized enzyme, respectively. It can be observed that there is a general good fit. Exception was seen for 75°C which is a difficult experiment owing to the fast deactivation observed at this temperature. The good fit lends support to the application of the exponential decay model for the thermal deactivation of Novo Nordisk amyloglucosidase thermally denatured in liquefied cassava starch solutions.

If industrial cassava saccharification would be run at 60°C as practiced by the corn industry in the batch process with free amyloglucosidase *(19)*, then equation *(6)* with K_d for 60°C taken from Table 1 predicts that at the end of reaction, within 48 h, practically all of the enzyme would be thermally deactivated, namely: 99.8%. After 24 h, 95.6% of the amyloglucosidase is already deactivated. Therefore, in the 48-h process, during the last 24 h, very little active enzyme is present. This suggests the staged addition of the enzyme to improve the process.

Figure 5 compares the Arrhenius plot of the deactivation constant (K_d) for free and immobilized amyloglucosidase. Equation *(2)* adjusted for this data gives:

free amyloglucosidase:

$$K_d = 1.771 \times 10^{32} \exp(-50{,}604/R\,T);\ r = 0.993 \qquad (8)$$

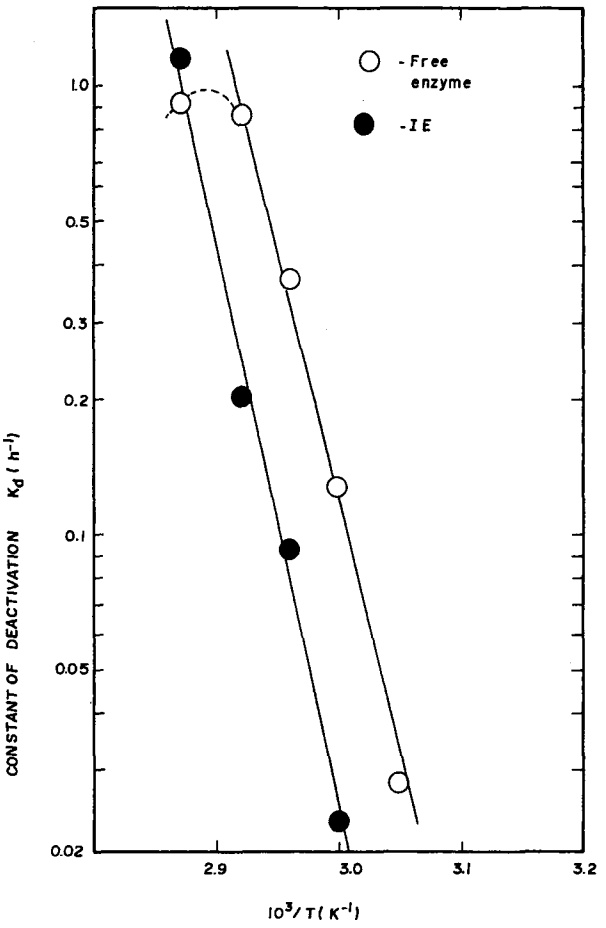

Fig. 5. Deactivation constant as a function of the inverse of absolute temperature (Arrhenius plot), for free and immobilized Novo Nordisk amyloglucosidase.

immobilized enzyme:

$$K_d = 1.409 \times 10^{36} \exp(-57{,}587/RT); r = 0.989 \quad (9)$$

Therefore, the experimentally observed energy of deactivation (E_d) is approx 50.6 and 57.6 kcal/mol, respectively, for the free and immobilized Novo Nordisk amyloglucosidase thermally denatured in the liquefied cassava-starch substrate. There is a 13.8% increase in the energy of deactivation of the immobilized enzyme, confirming that immobilization confers more stability to the enzyme as observed by previous work *(20–26)*. Also the value for the energy of deactivation of the free enzyme in contact with the liquefied cassava starch is comparable to results obtained with maltose: E_d = 50 kcal/mol *(27)*, and soluble starch: E_d = 54.2 kcal/mol *(28)*, and E_d = 57 kcal/mol *(20)*.

Table 2
Comparison of Experimental and Predicted Values for Novo Nordisk Amyloglucosidase Half-Life ($t_{1/2}$) in the Saccharification of Liquefied Cassava Starch 30% (w/v), pH 4.5

Temperature (°C)	Free Enzyme $t_{1/2}$ (h)		Immobilized Enzyme $t_{1/2}$ (h)	
	Experimental	Predicted	Experimental	Predicted
75	-	0.24	0.60	0.73
70	0.80	0.69	3.44	2.44
65	1.84	2.07	7.43	8. 52
60	5.34	6.41	30.24	30.88
55	24.1	20.6	-	116.4

Equations *(8)* and *(9)* allow one to calculate predicted values for the deactivation constant and then, with equation *(7)*, the predicted half-life is obtained. Experimental and predicted values for amyloglucosidase half-life are compared in Table 2. Agreement is satisfactory, and predicted half-lives deviate from the experimental values, on average, 16%.

CONCLUSIONS

1. Novo Nordisk amyloglucosidase when thermally denatured in a solution of liquefied cassava starch, 30% (w/v), pH 4.5, is stable up to 50°C for a period of 4 h.
2. The same enzyme, immobilized in controlled pore silica by the silane-glutaraldehyde covalent method, is stable up to 60°C, in the same conditions.
3. For higher temperatures, the enzyme deactivation reasonably follows the exponential decay model and half-lives are satisfactorily predicted with equation *(7)* and *(8)* or *(9)*, for free or immobilized enzyme, respectively.
4. The immobilized amyloglucosidase is more stable than the free enzyme, and their energy of deactivation is 57.6 and 50.6 kcal/mol, respectively.

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