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Comparison of Catalytic Properties of Free and Immobilized Cellobiase Novozym 188

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Abstract

The enzyme cellobiase from Novo was immobilized in controlled pore silica particles by covalent binding with the silane-glutaraldehyde method with protein and activity yields of 67 and 13.7%, respectively. The activity of the free enzyme (FE) and immobilized enzyme (IE) was determined with 2 g/L of cellobiose, from 40 to 75°C at pH 3.0–7.0 for FE and from 40 to 70°C at pH 2.2–7.0 for IE. At pH 4.8 the maximum specific activity for the FE and IE occurred at 65°C: 17.8 and 2.2 micromol of glucose/(min · mg of protein), respectively. For all temperatures the optimum pH observed for FE was 4.5 whereas for IE it was shifted to 3.5. The energy of activation was 11 kcal/mol for FE and 5 kcal/mol for IE at pH 4.5–5, showing apparent diffusional limitation for the latter. Thermal stability of the FE and IE was determined with 2 g/L of cellobiose (pH 4.8) at temperatures from 40 to 70°C for FE and 40 to 75°C for IE. Free cellobiase maintained its activity practically constant for 240 min at temperatures up to 55°C. The IE has shown higher stability, retaining its activity in the same test up to 60°C. Half-life experimental results for FE were 14.1, 2.1, and 0.17 h at 60, 65, and 70°C, respectively, whereas IE at the same temperatures had half-lives of 245, 21.3, and 2.9 h. The energy of thermal deactivation was 80.6 kcal/mol for the free enzyme and 85.2 kcal/mol for the IE, suggesting stabilization by immobilization.

Index Entries: Immobilized enzyme; cellobiase; cellobiose; thermal stability; energy of activation; energy of deactivation.

Introduction

The search for new, renewable raw materials as energy sources useful in biotechnology has led to increasing interest in the study of enzymatic

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hydrolysis of cellulosic biomass. However, lignocellulose is difficult to hydrolyze because cellulose is closely associated with hemicellulose and lignin, as well as other biomass constituents *(1)*.

Cellulose is a linear polymer of D-glucose units linked by 1,4-β-D-glucosidic bonds. The enzyme system for the conversion of cellulose to glucose comprises endo-1,4-β-glucanase, cellobiohydrolase, and β-glucosidase (cellobiase). Cellulolytic enzymes in conjunction with β-glucosidase act sequentially and cooperatively to degrade crystalline cellulose to glucose. The cellobiase is generally responsible for the regulation of the entire cellulolytic process and is a rate-limiting factor during enzymatic hydrolysis of cellulose, since both endoglucanase and cellobiohydrolase activities are often inhibited by cellobiose. Thus, the cellobiase not only produces glucose from cellobiose but also reduces cellobiose inhibition, allowing the cellulolytic enzymes to function more efficiently *(2)*.

Cellobiose hydrolysis rates depend on both reaction conditions and catalyst activity; therefore, the knowledge of the cellobiase thermal deactivation through time is a prerequisite to obtain useful design equations *(3)*.

Because the substrate of cellobiase, i.e., cellobiose, is water soluble, an immobilized (water-insoluble) cellobiase preparation could be used to supplement commercial cellulase/cellulose mixtures *(4)*. Advantages are the possibility of using continuous processes with insoluble enzymes, facilitation of enzyme recovery, and in some cases modification of the properties of the enzyme. It has been claimed that immobilization of an enzyme on an insoluble support will result in an enhanced stability against the denaturing effect of heat. However, there seems to be no clear-cut correlation between the observed stability and the way in which the enzyme was fixed to the support. Immobilization may thus result in stabilization, destabilization, or no effect at all *(5)*.

The technology of immobilized enzyme (IE) offers technical and economical advantages, such as the following *(6,7)*:

1. Enzyme consumption is reduced, since once immobilized the enzyme can be used for a much longer period than in the soluble form.
2. IE can lead to preferred continuous processes that may use either fixed or fluidized-bed reactors.
3. It is possible to use higher enzyme dosage per volume of reactor than in the soluble enzyme process, and this contributes to high reaction rates and, consequently, small reactor sizes.
4. These technical advantages allow a reduction in the process operational and capital costs, if the IE half-life is sufficiently long.

This article covers experimental determination of the activity, energy of activation, thermal stability, and energy of deactivation of free and immobilized Novo Nordisk cellobiase.

Materials and Methods

Substrate

The substrate was cellobiose from Sigma (St. Louis, MO), and it contained a very low level contamination of glucose (0.133% [w/w]).

Enzyme

Novozym 188, a β-glucosidase produced by the microorganism *Aspergillus niger*, containing 170 mg/mL of protein and a specific activity of 9.5 micromol of glucose/(min·mg of protein) at 50°C and pH 4.8 was kindly supplied by Novo Nordisk (Copenhagen, Denmark).

Carrier

The carrier used for immobilization was controlled pore silica (CPS), a gift from Corning Glass Works, having a mean pore size of 37.5 nm and average particle diameter of 0.351 mm.

Enzyme Immobilization

Cellobiase was immobilized in CPS by the covalent method of Weetall *(8)* with the following steps:

1. Silanization of the carrier, with a 0.5% (v/v) solution of γ-aminopropyltrietoxisilane, for 3 h at 75°C.
2. Washing with distilled water and drying for 15 h at 105°C.
3. Activation with a 2.5% (v/v) solution of glutaraldehyde (pH 7.0) for 45 min at 20°C.
4. Washing with water.
5. Contact of the activated carrier (50 g) with a solution of the enzyme (250 mL, 20 mg of protein/mL) for 15 h at 20°C.
6. Washings of the IE with distilled water and stocking under sodium acetate buffer (0.2 *M*), pH 4.5, at 4°C.

Procedure for Assaying Enzymatic Activity

Activity tests were conducted using the method of initial rate *(9)*, using a jacketed glass batch thermo-controlled reactor, equipped with magnetic stirring. A volume of 20 mL of cellobiose solution containing 1 mg/mL of sodium benzoate was incubated at the current pH and temperature with the enzyme β-glucosidase at a concentration of 95 µL of enzyme/L of solution, whereas for the immobilized cellobiase, 0.06 g of dry wt IE was used inside a stainless steel screen basket. Half-milliliter samples were collected at 3-min intervals for a period of 18 min and were boiled and stocked at 4°C for later glucose assay.

Analytical Methods

Glucose was assayed with the enzymatic method GOD-PAP *(10)*, and protein was measured according to the method of Lowry et al. *(11)* using bovine serum albumin as protein standard.

Specific Activity as a Function of pH and Temperature

The specific activity of the free enzyme (FE) and IE was determined using a 2 g/L solution of cellobiose (5.85 m*M*) at 40–70°C and pH 3.0–7.0 for FE and pH 2.2–7.0 for IE. This span of pH values was covered with the McIlvaine buffer *(12)* at a final concentration of 10 m*M*.

Enzyme Activity as Function of Temperature

The increase in activity as temperature is raised in an enzymatic reaction is modeled with the Arrhenius equation:

$$V = V_0 \exp(-E_a/RT) \quad (1)$$

in which E_a = energy of activation (cal/mol); R = universal law gas constant (1.987 cal/mol K); T = absolute temperature (K); V = enzymatic activity measured by the initial rate of reaction (µmol of glucose/[min·mg of protein]); and V_0 = Arrhenius preexponential constant.

The Arrhenius equation applies only for temperatures below that in which thermal denaturation of the enzyme becomes severe. From Eq. 1, it can be seen that the Arrhenius plot of log of activity (V) as a function of the inverse of the absolute temperature (T) results in a straight line in which the angular coefficient multiplied by R gives the energy of activation (E_a). For the higher temperatures where thermal deactivation becomes too fast, the experimental points in the Arrhenius plot deviate from the straight line, giving lower activities and shaping the curve with a maximum, associated with the optimum temperature for maximum activity. At this point, the gains in activity given by higher temperatures are offset by decreased concentration in active enzyme, caused by thermal denaturation *(6,9,13)*.

Enzyme Thermal Stability

The thermal stability of the FE and IE was determined at pH 4.8 and temperatures from 40 to 70°C for FE and 40 to 75°C for IE. The enzyme was incubated at the specified temperature and pH in a 2 g/L solution of cellobiose, and samples were collected every 40 min to measure the residual enzymatic activity at 50°C and pH 4.8. For the FE, at 70°C sampling time was shortened to 5-min intervals because of the rapid thermal denaturation observed at this temperature.

Energy of Thermal Deactivation

With the data obtained for the thermal deactivation of cellobiase, the energy of thermal deactivation of the enzyme was calculated. This follows the assumption that enzyme thermal denaturation is a reaction in which the rate of enzyme deactivation (r_d) is first order in relation to the concentration of the active enzyme (E):

$$r_d = -K_d E \quad (2)$$

and the deactivation constant (K_d) is a function of temperature as given by the following Arrhenius equation *(14)*:

$$K_d = K_d^0 \exp(-E_d/RT) \tag{3}$$

in which E_d is the energy of thermal deactivation (cal/mol), and R and T are defined as for Eq. 1.

For a batch reactor of constant liquid density, the rate of reaction equals the time derivative of the concentration, and therefore it follows from Eq. 2 that

$$\frac{dE}{dt} = -K_d E \tag{4}$$

which integrated gives:

$$E = E_0 \exp(-K_d t) \tag{5}$$

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

When the enzyme is present in catalytic quantities, i.e., in low concentrations, the residual enzyme activity (A_r) is directly proportional to the concentration of the active enzyme (E):

$$\frac{A_r}{A_0} = \frac{E}{E_0} \tag{6}$$

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

By combining Eqs. 5 and 6, the residual enzyme activity results as follows:

$$A_r = A_0 \exp(-K_d t) \tag{7}$$

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

From Eq. 3, and as observed by experiment, 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 an Arrhenius plot, i.e., ln of K_d against the inverse of absolute temperature (T), yielding the energy of deactivation (E_d), as the angular coefficient of the adjusted straight line times R, the universal gas constant.

It is of interest to calculate also the enzyme half-life ($t_{1/2}$), i.e., the time period necessary for the residual enzymatic activity to decrease to half of its initial value. If the enzyme thermal denaturation follows Eq. 7, then there is an inverse relation between the half-life of the enzyme and the deactivation constant *(15)*:

$$t_{1/2} = \ln 0.5/(-K_d) = 0.693/K_d \tag{8}$$

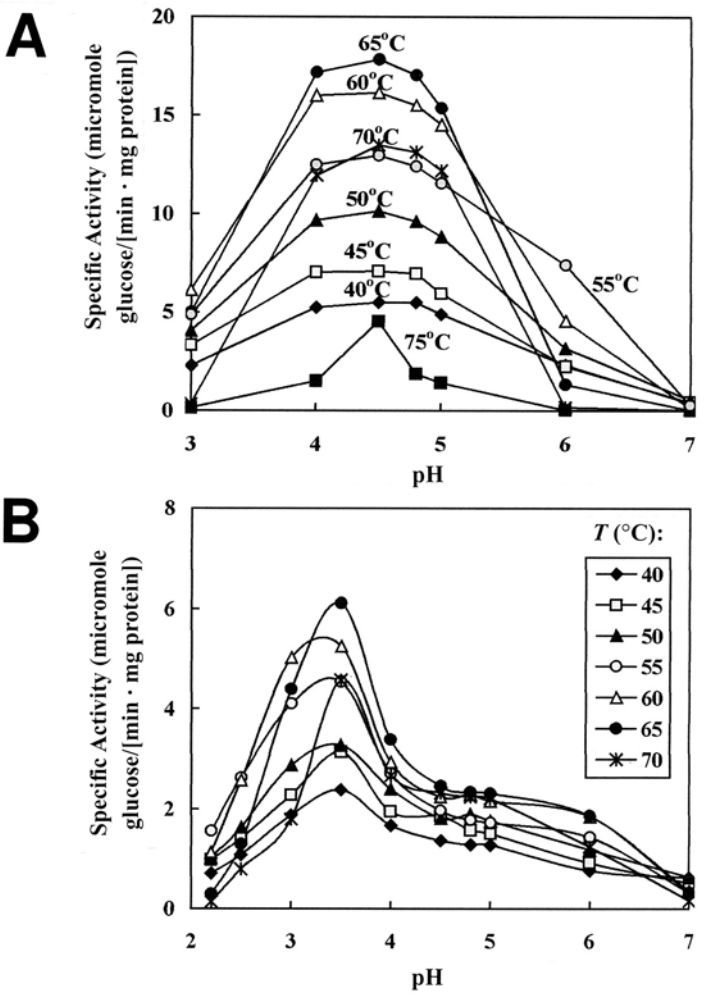

Fig. 1. Specific activity as a function of *T* (°C) and pH (2 g/L of cellobiose as substrate) for **(A)** free Novozym cellobiase at a concentration of 95 µL/L, pH 3.0–7.0; and **(B)** immobilized Novozym cellobiase at a concentration of 0.06 g of dry wt IE/20 mL of substrate, pH 2.2–7.0.

Results and Discussion

Immobilization

The quantity of protein fixed on the CPS support on immobilization was 74.9 mg of protein/g of dry support, representing a protein yield of 67% (i.e., 67% of the theoretical maximum that corresponds to the total enzyme offered for immobilization).

Activity as a Function of Temperature and pH

Figure 1A shows the specific activity of the Novo free cellobiase as a function of pH and temperature. Figure 1B gives the same kind of results but for the IE.

Table 1
Comparison of Energy of Activation for Enzymatic Hydrolysis of Cellobiose with Novozym 188 Cellobiase for Different pH Values for FE and IE[a]

pH	$V = V_0 \exp(-E_a/RT)$	Energy of activation (kcal/mol)
	Free enzyme	
3.0	$V = 1.627 \times 10^7 \exp(-9782/RT)$, $r = 0.9917$	9.78
4.0	$V = 6.961 \times 10^8 \exp(-11{,}633/RT)$, $r = 0.9993$	11.64
4.5	$V = 5.266 \times 10^8 \exp(-11{,}435/RT)$, $r = 0.9972$	11.44
4.8	$V = 2.610 \times 10^8 \exp(-11{,}005/RT)$, $r = 0.9985$	11.01
5.0	$V = 7.747 \times 10^8 \exp(-11{,}766/RT)$, $r = 0.9948$	11.77
6.0	$V = 1.225 \times 10^8 \exp(-15{,}495/RT)$, $r = 0.8830$	15.50
7.0	$V = 3.163 \times 10^8 \exp(-7037/RT)$, $r = 0.9806$	7.04
	Immobilized enzyme	
2.2	$V = 4.752 \times 10^6 \exp(-9773/RT)$, $r = 0.9600$	9.78
2.5	$V = 7.422 \times 10^6 \exp(-9790/RT)$, $r = 0.9662$	9.79
3.0	$V = 4.419 \times 10^7 \exp(-10{,}588/RT)$, $r = 0.9937$	10.59
3.5	$V = 1.058 \times 10^6 \exp(-8086/RT)$, $r = 0.9820$	8.09
4.0	$V = 2.200 \times 10^4 \exp(-5889/RT)$, $r = 0.9927$	5.89
4.5	$V = 3.824 \times 10^3 \exp(-4931/RT)$, $r = 0.9973$	4.93
4.8	$V = 3.455 \times 10^3 \exp(-4881/RT)$, $r = 0.9526$	4.88
5.0	$V = 3.220 \times 10^3 \exp(-4861/RT)$, $r = 0.9748$	4.86
6.0	$V = 1.864 \times 10^6 \exp(-9192/RT)$, $r = 0.9978$	9.19

[a]Calculated by Fitting Eq. 1 to the activity data of Figs. 1A,B (V = specific activity of the enzyme, V_0 = Arrhenius constant, E_a = energy of activation of the enzyme).

In Fig. 1A maximum activities were observed in the pH range 4.0–4.8. The maximum activity, 17.8 micromol of glucose/(min·mg of protein), was found at 65°C and pH 4.5. These optimum conditions are in exact agreement with those of Woodward et al. *(4)* and Dekker *(16)*. For the IE, Fig. 1B shows that the optimum pH is observed at pH 3.5 for all temperatures studied in this experiment, and the optimum temperature was 65°C in the pH range 3.5–6.0. The activity at 65°C was 6.1 micromol of glucose/(min·mg of protein) at pH 3.5. Disregarding the shift in pH, the activity yield on immobilization is low: 34.0%. For the same pH (pH 4.5), the activity yield is lower: 13.7%. FE data can also be found in an earlier publication *(17)*; the objective here is the comparison of the free and immobilized cellobiase.

Comparison of Fig. 1A,B shows that the immobilization of cellobiase in CPS by the covalent method shifts the optimum pH of the enzyme about 1.0 points, from 4.5 to 3.5. Similar results were obtained by Bergamasco et al. *(18)* for immobilized invertase in CPS: the pH optimum was shifted from 5.0 to 4.5.

The energy of activation was calculated by fitting Eq. 1 to the activity data of Fig. 1A,B, and the hydrolysis rate can be written in the Arrhenius form as presented in Table 1 for FE and IE.

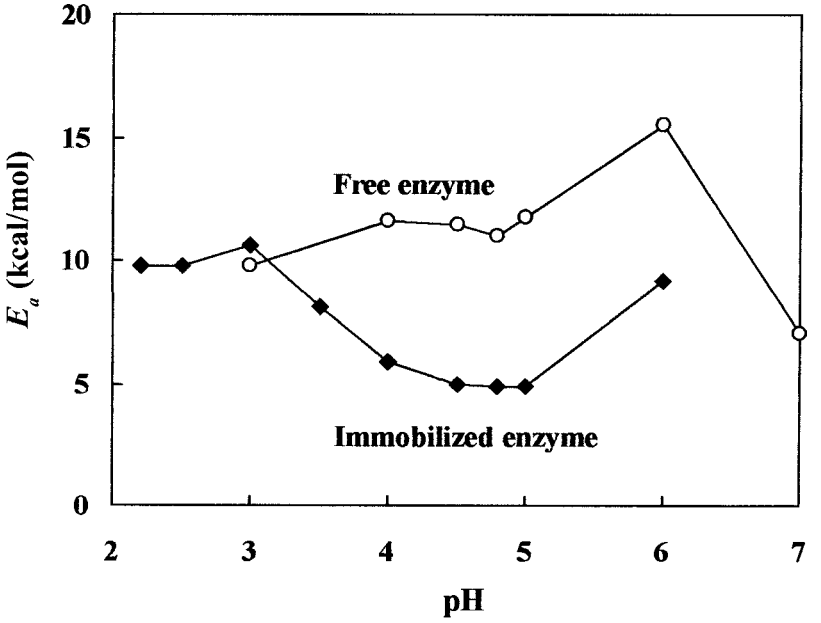

Fig. 2. Comparison of the energy of activation as a function of pH, for free and immobilized Novozym cellobiase, 2 g/L of cellobiose, 95 µL of enzyme/L for FE, and 0.06 g of dry wt IE/20 mL of substrate for IE.

The plot of E_a as a function of pH in Fig. 2 reveals a dependence of the energy of activation on pH and shows that E_a for the IE is generally lower. Figure 2 shows that the energy of activation for the hydrolysis of cellobiose with FE is about 11 kcal/mol in the pH range 4.5–5.0, in accordance with published data for the same enzyme *(16,19,20)*, which show values for the energy of activation in the range of 10–12.5 kcal/mol. In the same range of values, other researchers have found equivalent results for cellobiases produced by different microorganisms *(21–23)*. Figure 2 also shows that in the same pH range, the energy of activation for the IE is about 5 kcal/mol. Therefore, the IE energy of activation observed is half that of the FE, demonstrating an apparent diffusional limitation with the IE *(22,24,25)*. Bisset and Sternberg *(22)* immobilized β-glucosidase of *Aspergillus phoenicis* on chitosan and obtained 11.95 and 7.93 kcal/mol for FE and IE, respectively. They also interpreted their results as a diffusional limitation in the case of the IE.

The experimental results obtained with the thermal stability test for FE are shown in Fig. 3A, and for IE in Fig. 3B. It can be observed in Fig. 3A that up to 55°C, and a thermal denaturation period of 240 min, the FE maintained its activity practically constant, whereas at 70°C the enzyme was almost totally deactivated in 40 min. The IE (Fig. 3B) has shown higher stability, maintaining its activity in a similar test up to 60°C; at 70°C, 40% of its activity was retained after 240 min.

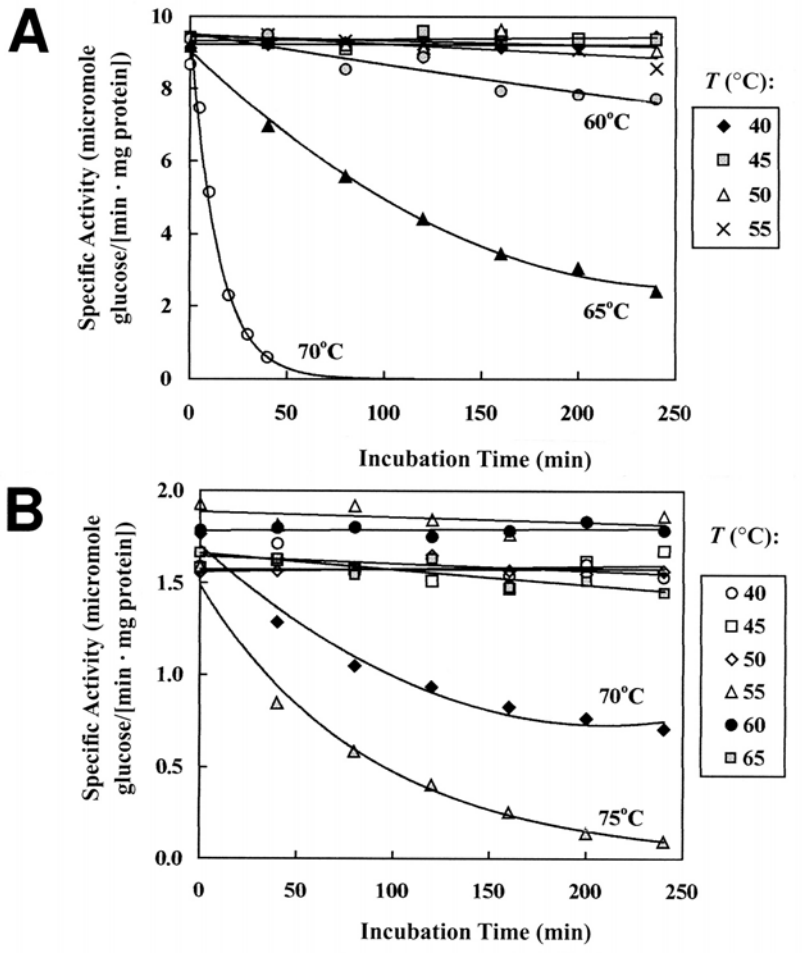

Fig. 3. Residual enzymatic activity incubated in 2 g/L of cellobiose at pH 4.8 for **(A)** free Novozym cellobiase at a concentration of 95 µL/L and **(B)** immobilized Novozym cellobiase at a concentration of 0.06 g of dry wt IE/20 mL of substrate.

Residual activity data for temperatures from 55 to 70°C for FE and from 60 to 75°C for IE were applied to Eq. 7, giving the experimental results for the deactivation constant (K_d) and half-lives ($t_{1/2}$) shown in Table 2 (first K_d and $t_{1/2}$ column). Figure 4 compares the Arrhenius plot of the deactivation constant (K_d) for free and immobilized cellobiase. Equation 3 adjusted for these data gives:

Free cellobiase

$$K_d = 5.728 \times 10^{51} \exp(-80{,}573/RT) \qquad r = 0.9789 \tag{9}$$

Immobilized cellobiase

$$K_d = 3.175 \times 10^{53} \exp(-85{,}238/RT) \qquad r = 0.9885 \tag{10}$$

Table 2
Comparison of Deactivation Constant (K_d) and Half-Life ($t_{1/2}$) Values for Free and Immobilized Cellobiase Novozym 188, Adjusted for a Single Temperature and for All Temperatures[a]

T (°C)	Fitting equation $y = \ln(A_r/A_0) = -K_d t + C_1$ (t = time [h], C_1 = constant)	Adjusted for a single temperature		Adjusted for all temperatures	
		K_d (h^{-1})	$t_{1/2}$ (h)	K_d (h^{-1})	$t_{1/2}$ (h)
	Free enzyme				
		Eq. 7	Eq. 8	Eq. 9	Eq. 8
55	$y = -0.0192t + 0.0238$, $r = 0.8348$	0.0192	36.09	0.0124	80.65
60	$y = -0.0492t + 0.0158$, $r = 0.9773$	0.0492	14.09	0.0789	8.78
65	$y = -0.3288t + 0.0452$, $r = 0.9969$	0.3288	2.11	0.4771	1.45
70	$y = -4.1678t + 0.0910$, $r = 0.9969$	4.1678	0.166	2.7382	0.253
	Immobilized enzyme				
		Eq. 7	Eq. 8	Eq. 10	Eq. 8
60	$y = -0.00283t + 0.0104$, $r = 0.8273$	0.00283	244.9	0.0038	182.4
65	$y = -0.0326t + 0.0086$, $r = 0.9404$	0.0326	21.26	0.0255	27.18
70	$y = -0.2412t + 0.1080$, $r = 0.9691$	0.2412	2.87	0.1620	4.28
75	$y = -0.6991t + 0.013$, $r = 0.9986$	0.6991	0.991	0.9756	0.710

[a]The enzyme was incubated in 2 g/L of cellobiose at pH 4.8.

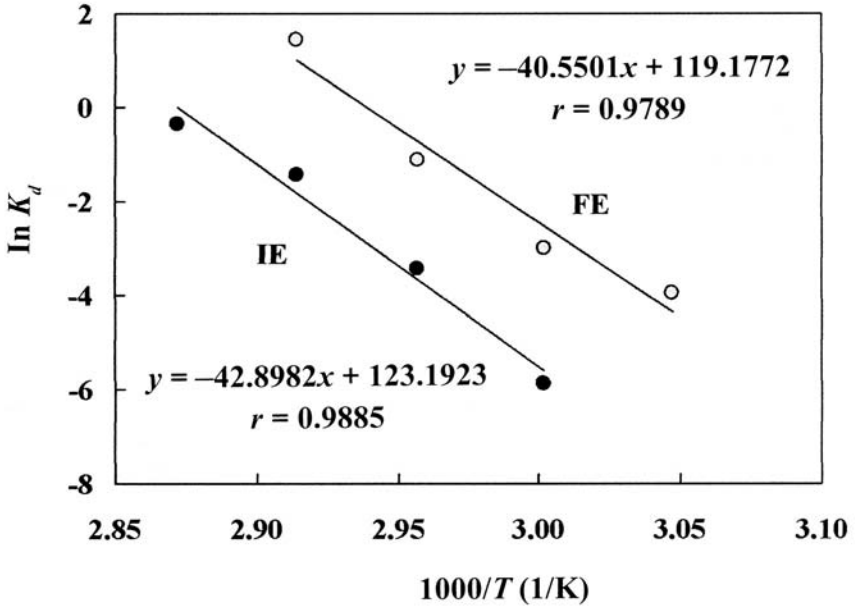

Fig. 4. Arrhenius plot for the energy of thermal deactivation of FE (95 µL/L) and IE (0.06 g of dry wt IE/20 mL of substrate), incubated in 2 g/L of cellobiose at pH 4.8.

Thus, the experimentally observed energy of deactivation (E_d) is approx 80.6 kcal/mol for FE and 85.2 kcal/mol for IE. There is a 5.8% increase in the energy of deactivation for IE, demonstrating that immobilization increases enzyme stability.

From Eqs. 9 and 10 the values of the deactivation constant, adjusted for all temperatures, were obtained and, then, with Eq. 8, the adjusted half-life. The results are shown in Table 2 (second K_d and $t_{1/2}$ column). Comparison of the experimental values obtained for $t_{1/2}$ shows that the IE half-lives are 18.8 times greater on average than half-lives shown by FE, confirming that immobilization confers more stability to the enzyme. The ratio of the half-lives decreases with temperature from 20.8 at 60°C to 16.9 at 70°C. Note that thermal stabilization may, on occasion, be an apparent result. Under mild conditions only the external layer of IE may be working. As deactivation sets in, deep layers of the IE become active, replacing the deactivated ones. However, the same methodology of immobilization as used here was employed with very low enzyme loading (4.12 mg of protein/g of support) with amyloglucosidase *(15)* and stabilization by immobilization was observed. We believe the same phenomenon is occurring here.

Bisset and Sternberg *(22)* obtained the following half-lives for the cellobiase derived from *A. phoenicis* QM 329 using 7.5 m*M* cellobiose as substrate at pH 4.8: 216 h (55°C), 8.6 h (60°C), 0.5 h (65°C), and 0.04 h (70°C). By comparing these results with the experimental half-lives for free enzyme presented in Table 2, it can be observed that cellobiase Novozym 188 is more stable than the enzyme derived from *A. phoenicis* QM 329 at temperatures between 60 and 70°C.

Conclusion

During the immobilization of the enzyme in CPS with the silane-glutaraldehyde method, 67% of the offered enzyme was fixed on the support. The method gives a relatively low activity yield: 13.7% at 65°C and pH 4.5. For all temperatures the enzyme showed optimum activities in the pH range of 4.0–4.8 for FE and about pH 3.5 for IE. Maximum activity was 17.8 micromol of glucose/(min·mg of protein) at 65°C and pH 4.5 for FE and 6.1 micromol of glucose/(min·mg of protein) at 65°C and pH 3.5 for IE. The energy of activation of the enzyme was 11.0 kcal/mol for FE and 5 kcal/mol for IE, at pH values from 4.5 to 5.0, demonstrating an apparent diffusional limitation for the IE.

Cellobiase Novozym 188 when thermally denaturated free in solution in a 2 g/L cellobiose solution (pH 4.8) was stable up to 55°C for a period of 4 h. Immobilized in CPS by the silane-glutaraldehyde covalent method, the same enzyme was stable up to 60°C, under the same conditions.

The enzyme thermal deactivation followed reasonably the exponential decay model, giving $K_d = 5.728 \times 10^{51} \exp(-80{,}573/RT)$ for FE and $K_d = 3.175 \times 10^{53} \exp(-85{,}238/RT)$ for IE. Finally, the immobilized cellobiase showed half-lives almost 20 times greater on average than the half-lives

observed with the FE, suggesting that immobilization confers more stability to this enzyme.

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