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Modeling Cellobiose Hydrolysis with Integrated Kinetic Models

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Abstract

The enzyme cellobiase Novozym 188, which is used for improving hydrolysis of bagasse with cellulase, was characterized in its commercial available form and integrated kinetic models were applied to the hydrolysis of cellobiose. The specific activity of this enzyme was determined for pH values from 3.0–7.0, and temperatures from 40–75°C, with cellobiose at 2 g/L. Thermal stability was measured at pH 4.8 and temperatures from 40–70°C. Substrate inhibition was studied at the same pH, 50°C, and cellobiose concentrations from 0.4–20 g/L. Product inhibition was determined at 50°C, pH 4.8, cellobiose concentrations of 2 and 20 g/L, and initial glucose concentration nearly zero or 1.8 g/L. The enzyme has shown the greatest specific activity, 17.8 U/mg, at pH 4.5 and 65°C. Thermal activation of the enzyme followed Arrhenius equation with the Energy of Activation being equal to 11 kcal/mol for pH values 4 and 5. Thermal deactivation was adequately modeled by the exponential decay model with Energy of Deactivation giving 81.6 kcal/mol. Kinetics parameters for substrate uncompetitive inhibition were: Km = 2.42 mM, V_{max} = 16.31 U/mg, Ks = 54.2 mM. Substrate inhibition was clearly observed above 10 mM cellobiose. Product inhibition at the concentration studied has usually doubled the time necessary to reach the same conversion at the lower temperature tested.

Index Entries: Cellobiase; cellobiose; kinetic modeling; thermal stability; energy of deactivation; energy of activation.

Introduction

About 12 billion liters of ethanol per year are produced in Brazil from sugar cane. This operation generates a great excess of by-product sugarcane bagasse. Approximately 75% of this bagasse are used for energy

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co-generation *in situ*, and a small portion of it is sold as solid fuel for other industries, or as fodder. The residual bagasse (25%) that presently finds no economical application could possible be used for the production of fermentable sugars, which could be used in term for the production of more ethanol or other chemical products.

The enzymatic hydrolysis of cellulose and cellulosic substrates to glucose is a multistep process catalyzed by the three major components of the cellulase complex, endoglucanases, exoglucanases, and β-glucosidases, working in a synergistic mechanism *(1,2)*. The enzyme β-glucosidase (EC 3.2.1.21), also known as cellobiase, hydrolyses β-glucosidic bonds of cellobiose producing two molecules of glucose. In cellulose hydrolysis cellobiase is essential for the conversion of the intermediate cellobiose, which is a strong inhibitor of the other cellulase components. The action of β-glucosidase on cellobiose also shows substrate inhibition *(3,4)*. Since the substrate of cellobiase, that is cellobiose, is water soluble, this enzyme can be used for enhancement of cellulose hydrolysis, in the form of immobilized enzyme, usually in a column adjacent to the main reactor *(5,6)*.

Cellobiose hydrolysis rates depend on both reaction conditions and catalyst activity *(3)*; therefore, the knowledge of the β-glucosidase thermal deactivation and the best reaction conditions for its work, as pH, temperature and substrate or product inhibition is a prerequisite to obtain useful design equations. This work builds upon previous kinetic attempts that have addressed mostly initial rate kinetics and considers the presence of substrate and product inhibition in six alternative integrated kinetic models. The objective is to compare the integrated kinetics of each case with experimental data covering cellobiose conversions from zero to close to 100% conversion. For industrial purposes, it would be desirable that the cellobiose hydrolysis integrated kinetic model were be able to describe accurately the conversion as a function of time, from time zero to nearly complete conversion of the substrate.

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 U/mg of protein, was kindly supplied by Novo Nordisk (Denmark).

One unit (U) of enzymatic activity corresponds to the quantity of cellobiase that produces 1 μmol of glucose/min at pH 4.8, 50°C and other test conditions, shown at the following procedure.

Procedure for Assaying Enzymatic Activity

Activity tests were carried out using the method of initial rate *(7)*, using a jacketed glass batch thermo-controlled reactor equipped with magnetic stirring. A volume of 20 mL of cellobiose solution, buffered with acetate buffer at 50 m*M*, pH 4.8 and containing 1 mg/mL of sodium benzoate was incubated at the test temperature with the enzyme β-glucosidase, at the concentration of 95 µL of enzyme/L of solution. Half-milliliter samples were collected with an interval of 3 min, for a period of 18 min, boiled, and stocked at 4°C for later glucose assay.

Analytical Methods

Glucose was assayed with the enzymatic method GOD-PAP *(8)*, and protein was measured according to Lowry et al. *(9)* using BSA as protein standard.

Specific Activity as a Function of pH and Temperature

The specific activity of cellobiase was determined for pH from 3–7 and temperatures from 40–75°C, using a 0.2% (w/v) solution of cellobiose (5.85 m*M*). This span of pH values was covered with the McIlvaine buffer *(10)* at the final concentration of 10 m*M*.

Arrhenius Energy of Activation

The data on specific activity as a function of temperature was used to calculate the Energy of Activation based on Arrhenius equation (Eq. 1):

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

where: E_a = Energy of Activation (cal/mol); R = Universal law gas constant (1.987 cal/gmol K); T = Absolute temperature (K); V_0 = Arrhenius pre-exponential constant; and V = Initial rate of reaction (µmol of glucose/[min/mg enzyme]).

Enzyme Thermal Stability

The thermal stability of cellobiase was determined at pH 4.8 and temperatures in the range of 40–70°C. The enzyme was incubated at the specified temperature and pH, in a 0.2% (w/v) solution of cellobiose and samples were collected each 40 min to measure the residual enzymatic activity. 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 the reaction that thermally denatures the enzyme is first order and obeys the Arrhenius relation *(11)*:

$$K_d = K_d^0 \exp(-E_d/RT) \quad (2)$$

$$A_r = A_0 \exp(-K_d t) \quad (3)$$

where: A_0 = Initial enzymatic activity (U/mg of protein); A_r = Residual enzymatic activity (U/mg of protein); E_d = Energy of Thermal Deactivation (cal/mol); K_d = Deactivation constant (h^{-1}); and t = time (h)

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 this initial value. If the enzyme thermal denaturation follows Eq. 3, then there is an inverse relation between its half-life and the deactivation constant:

$$t_{1/2} = \ln 0.5/(-K_d) = 0.693/K_d \quad (4)$$

Initial Rate of Hydrolysis and Substrate Inhibition Test

The effect of substrate concentration on the rate of hydrolysis was studied at 50°C, pH 4.8, using cellobiose solutions from 0.4–20 g/L (1.2–58.5 m*M*). From this data the kinetic parameters *Km*, *Ks*, and V_{max} were obtained as described in the item for kinetic modeling.

Cellobiose Conversion Tests in the Batch Reactor

With the aim of modeling the hydrolysis of cellobiose, measuring product inhibition and observing the enzyme thermal deactivation for long periods of reaction, hydrolysis was followed up to nearly 100% cellobiose conversion, in the presence and absence of exogenously added product (glucose) at the start of the test.

An orbital shaker fitted with a 500 mL Erlenmeyer flask was used for the conversion tests. Each Erlenmeyer contained 250 mL of the reaction mixtures shown at Table 1, which also presents the reaction conditions. Short sampling intervals (3–5 min) were used at the beginning of the reaction and they were increased with reaction time. At the end of the whole reaction period, the residual enzymatic activity was measured.

To the data on cellobiose conversion as a function of time, six alternative integrated kinetic models were fitted. These models are now described.

Kinetic Models for Cellobiose Enzymatic Hydrolysis

The general reaction scheme for cellobiose hydrolysis is shown in Fig. 1. The equation for reaction velocity was obtained by the method of rapid equilibrium *(12)* using the definitions for the equilibrium constants shown at Fig. 1. The reaction velocity is Eq. 5:

$$V = -dS/dt = (1/2)\, dG/dt = (1/2)\,(2\, Kcat\, [ES]) = Kcat\, [ES] \quad (5)$$

Dividing the left side of Eq. 5 by $[E_T]$, the total enzyme concentration, and the right side by the sum that gives the total enzyme concentration (Fig. 1), and substituting the concentration of the enzyme forms using the equilib-

Table 1
Substrate Reaction Mixtures and Conditions for the Conversion Tests

Substrate mixtures				Reaction conditions		
Cellobiose (g/L)	Added glucose (g/L)	Conservant sodium benzoate (1 g/L)	Cellobiase enzyme (μL/L)	Temperature (°C)	pH	Duration (h)
2	0	1	95	40 and 50	4.8	30
2	1.8	1	95	40 and 50	4.8	30
20	0	1	95	40 and 50	4.8	100 and 73

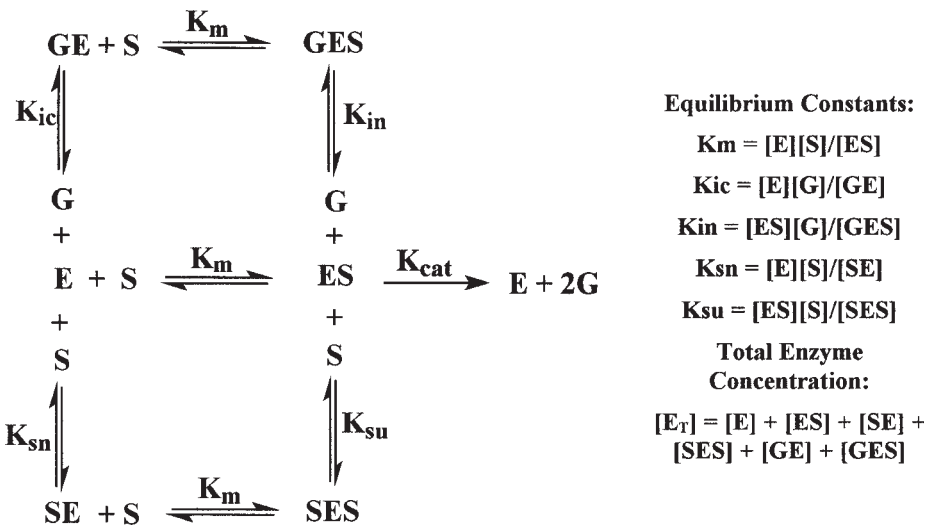

Fig. 1. General scheme for the enzymatic hydrolysis of cellobiose showing noncompetitive inhibition by product and substrate (E, free enzyme; ES, the productive complex of enzyme-substrate; SE, the nonproductive complex of enzyme-substrate; G, glucose (product); GE, glucose-enzyme complex; GES, product-enzyme-substrate complex; Km, dissociation constant for the complex [ES]; Kic, dissociation constant for complex GE; Kin, dissociation constant for the complex [GES]; Ksn, dissociation constant for the complex [SE]; Ksu, dissociation constant for the complex [SES]; SES, nonproductive substrate-enzyme-substrate complex; S, substrate (cellobiose) concentration.

rium constants shown at Fig. 1, the general expression for the reaction velocity follows Eq. 6:

$$V = (V_{max}\, S)/[S\,(1 + G/Kin) + Km\,(1 + S/Ksn + G/Kic) + S^2/Ksu] \quad (6)$$

where: $V_{max} = K_{cat}[E_T]$, G, and S are the concentrations of glucose and substrate, respectively. Other constants are defined in Fig. 1.

Table 2 shows various simplified forms of Eq. 6, which arise as the kinetic constants assume special values depending on the particular kinetic

Table 2
Equations Derived from the General Eq. 6
for the Velocity of Reaction for Various Kinetic Models with Different Inhibition Conditions

Type of inhibition		Inhibition constant					
Substrate	Product	*Km*	*Kic*	*Kin*	*Ksn*	*Ksu*	Equation for velocity of reaction
Uncompetitive	$G \rightarrow 0$, initial velocity	*Km*	∞	∞	∞	*Ks*	$V = (S\ V_{max})/(S + Km + S^2/Ks)$
	Competitive	*Km*	*Ki*	∞	∞	*Ks*	$V = (S\ V_{max})/[S + (1 + G/Ki)\ Km + S^2/Ks]$
	Noncompetitive	*Km*	*Ki*	*Ki*	∞	*Ks*	$V = (S\ V_{max})/[S + (1 + G/Ki)\ Km + S\ G/Ki + S^2/Ks]$
	Uncompetitive	*Km*	∞	*Ki*	∞	*Ks*	$V = (S\ V_{max})/[S\ (1 + G/Ki) + Km + S^2/Ks]$
Noncompetitive	$G \rightarrow 0$, initial velocity	*Km*	∞	∞	*Ks*	*Ks*	$V = (S\ V_{max})/(S + Km + S\ Km/Ks + S^2/Ks)$
	Competitive	*Km*	*Ki*	∞	*Ks*	*Ks*	$V = (S\ V_{max})/[S + (1 + S/Ks + G/Ki)\ Km + S^2/Ks]$
	Noncompetitive	*Km*	*Ki*	*Ki*	*Ks*	*Ks*	$V = (S\ V_{max})/[S\ (1 + G/Ki) + (1 + S/Ks + G/Ki)\ Km + S^2/Ks]$
	Uncompetitive	*Km*	∞	*Ki*	*Ks*	*Ks*	$V = (S\ V_{max})/[S\ (1 + G/Ki) + Km\ (1 + S/Ks) + S^2/Ks]$
No Inhibition	No Inhibition	*Km*	∞	∞	∞	∞	$V = (S\ V_{max})/(S + Km)$

model, namely uncompetitive or noncompetitive for the substrate, and competitive, noncompetitive or uncompetitive for the product.

Determination of Km, V_{max}, *and* Ks

In the initial velocity tests, product concentration is very low, i.e., $G \rightarrow 0$, and Eq. 6 reduces to the two cases of substrate inhibition with $G \rightarrow 0$ shown in Table 2. The equation for reaction velocity in these cases can be rearranged into a parabolic form, namely:

Uncompetitive inhibition by substrate with $G \rightarrow 0$:

$$S/V = (Km/V_{max}) + (1/V_{max})\, S + (1/Ks\, V_{max})\, S^2 \quad (7)$$

Noncompetitive inhibition by substrate with G → 0:

$$S/V = (Km/V_{max}) + (1/V_{max})\,(1 + Km/Ks)\, S + [1/(Ks\, V_{max})]\, S^2 \quad (8)$$

Equations 7 and 8 were fitted to the data from the Initial Rate of Hydrolysis and Substrate Inhibition on Test, using the computer program Statistica® (Statsoft, Inc.). This fitting allowed to obtain the values of the kinetic constants *Km*, *Ks*, and V_{max} for each case of substrate inhibition. Equation 7 is due to Haldane *(7,13)*.

Integrated Forms of the Kinetic Models

Substituting in Eq. 6, $S = S_0(1 - X_A)$, and $G = Gi + 2\, S_0\, X_A$, that is, the substrate (*S*) and product concentration (*G*) as a function of the substrate conversion (X_A), and simplifying results Eq. 9:

$$\begin{aligned} dX_A/dt = {} & [V_{max}(1 - X_A)]/\{S_0(1 - X_A)\,[1 + (Gi + 2\, S_0\, X_A)/Kin)] + \\ & Km\,[1 + S_0(1 - X_A)/Ksn + (Gi + 2\, S_0\, X_A)/Kic] + S_0^2(1 - X_A)^2/Ksu\} \end{aligned} \quad (9)$$

Integrating Eq. 9 for the case of a batch isothermal reactor of constant volume gives:

$$\begin{aligned} t = {} & \{(S_0/V_{max})\,[1 + Gi/Kin + Km/Ksn + S_0/Ksu - 2\, Km/Kic]\}\, X_A - \\ & \{(Km/V_{max})\,[1 + Gi/Kic + 2\, S_0/Kic]\}\, \ln(1 - X_A) - \\ & \{(S_0^2/V_{max})\,[-1/Kin + \tfrac{1}{2}\, Ksu]\}\, X_A^2 \end{aligned} \quad (10)$$

Equation 10 can be written in the compact general form of Eq. 11, in which the parameters α, β, and γ are defined by Eqs. 12–14:

$$t = \alpha X_A - \beta \ln(1 - X_A) - \gamma\, X_A^2 \quad (11)$$

$$\alpha = (S_0/V_{max})\,[1 + Gi/Kin + Km/Ksn + S_0/Ksu - 2\, Km/Kic] \quad (12)$$

$$\beta = (Km/V_{max})\,[1 + Gi/Kic + 2\, S_0/Kic] \quad (13)$$

$$\gamma = (S_0^2/V_{max})\,[-1/\mathrm{Kin} + \tfrac{1}{2}\, Ksu] \quad (14)$$

$$X_A = \tfrac{1}{2}\,(G - Gi)/S_0 \quad (15)$$

where: $Gi = Gc + Ga$; *Ga* = concentration of exogenously glucose added at time zero; *Gc* = concentration of glucose that originates from low level contamination of the substrate; S_0 = Real initial concentration of cellobiose, discounted the low level contamination of glucose and *t* = reaction time.

Table 3
Equations Derived from the General Eq. 11
for the Parameters α, β, and γ for Various Kinetic Models with Different Inhibition Constants

Type of inhibition								
Substrate	Product	α		β		γ		
Uncompetitive	$G \rightarrow 0$	$(S_0/V_{max})\,[1 + S_0/Ks]$	(16)	(Km/V_{max})	(17)	$S_0^2/(2\,V_{max}\,Ks)$	(18)
	Competitive	$(S_0/V_{max})\,[1 + S_0/Ks - 2\,Km/Ki]$	(19)	$(Km/V_{max})\,[1 + Gi/Ki + 2\,S_0/Ki]$	(20)	$(S_0^2/(2\,V_{max}\,Ks)$	(21)
	Noncompetitive	$(S_0/V_{max})\,[1 + Gi/Ki + S_0/Ks - 2\,Km/Ki]$	(22)	$(Km/V_{max})\,[1 + Gi/Ki + 2\,S_0/Ki]$	(23)	$(S_0^2/V_{max})\,[\frac{1}{2}\,Ks - 1/Ki]$	(24)
	Uncompetitive	$(S_0/V_{max})\,[1 + Gi/Ki + S_0/Ks]$	(25)	(Km/V_{max})	(26)	$(S_0^2/V_{max})\,[\frac{1}{2}\,Ks - 1/Ki]$	(27)
Noncompetitive	$G \rightarrow 0$	$(S_0/V_{max})\,[1 + Km/Ks + S_0/Ks]$	(28)	(Km/V_{max})	(29)	$S_0^2/(2\,V_{max}Ks)$	(30)
	Competitive	$(S_0/V_{max})\,[1 + Km/Ks + S_0/Ks - 2\,Km/Ki]$	(31)	$(Km/V_{max})\,[1 + Gi/Ki + 2\,S_0/Ki]$	(32)	$[S_0^2/2\,V_{max}Ks]$	(33)
	Noncompetitive	$(S_0/V_{max})\,[1 + Gi/Ki + Km/Ks + S_0/Ks - 2\,Km/Ki]$	(34)	$(Km/V_{max})\,[1 + Gi/Ki + 2\,S_0/Ki]$	(35)	$(S_0^2/V_{max})\,[\frac{1}{2}\,Ks - 1/Ki]$	(36)
	Uncompetitive	$(S_0/V_{max})\,[1 + Gi/Ki + Km/Ks + S_0/Ksu]$	(37)	(Km/V_{max})	(38)	$(S_0^2/V_{max})\,[\frac{1}{2}\,Ks - 1/Ki]$	(39)
No inhibition	No inhibition	(S_0/V_{max})	(40)	(Km/V_{max})	(41)	—	

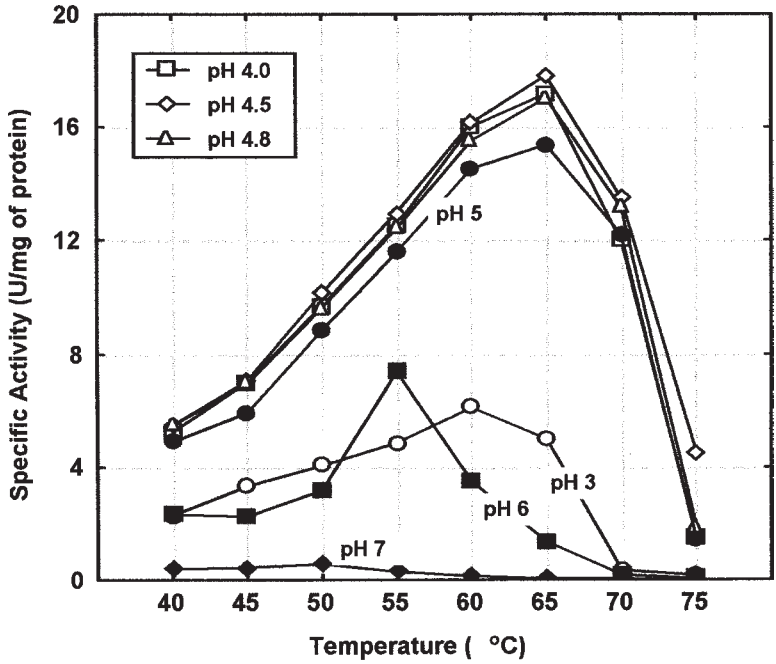

Fig. 2. Specific activity as a function of temperatures and pH for Novozym cellobiase at the concentration of 95 µL/L in 2 g/L cellobiose.

The integrated general Eq. 11 can be simplified for each case of inhibition model, in which the kinetic constants assume special values, and α, β, and γ take the forms as shown in Table 3, Eqs. 16–41.

Comparison of the Kinetic Models and Reactor Data

First, Eq. 11 was fitted to the data obtained in the Cellobiose Conversion Tests in the Batch Reactor, using the non-linear fitting procedure available in the computer program Statistica® (Statsoft, Inc.). This gave values of α, β, and γ for each experimental condition used with the reactor test.

Secondly, the values of α, β, and γ and values of *Km*, V_{max}, and *Ks* were introduced in the defining Eqs. 16–41 for α, β, and γ (Table 3) and this allowed to calculate the other kinetic parameters: *Ki* and *Kcat*.

Results and Discussion

Activity as a Function of Temperature and pH

Figure 2 shows the specific activity as a function of temperature and pH. It can be seen that maximum activities were observed in the pH range 4.0–5.0. The maximum activity, 17.84 U/mg of protein, was found at 65°C, pH 4.5. These optimum conditions are in exact agreement with those of Woodward et al. *(14)* and Dekker *(15)*.

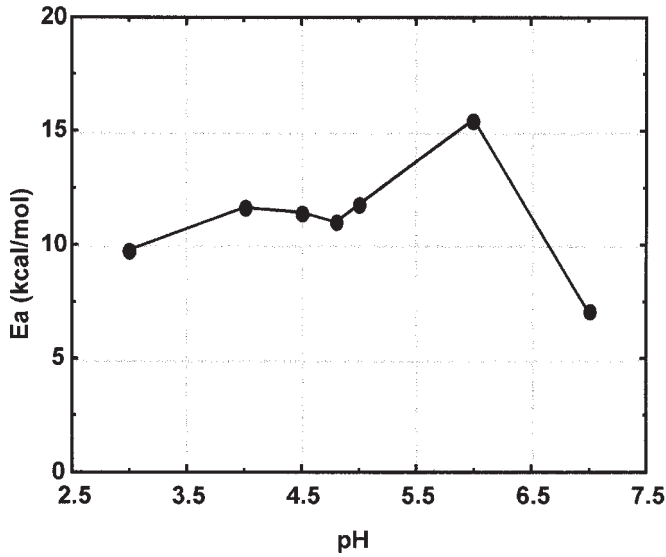

Fig. 3. Energy of activation for the enzymatic hydrolysis of cellobiose (2 g/L), 95 µL enzyme/L of solution.

Figure 3 shows that the Energy of Activation for the hydrolysis of cellobiose is about 11 kcal/mol in the pH range 4.0–5.0, in accord with published data for the same enzyme *(15–17)*, which show values for the Energy of Activation in the range of 10–12.5 kcal/mol. In the same range of values, other authors have found equivalent results for cellobiases produced by different microorganisms *(18–20)*. For pH 4.8 the hydrolysis rate can be written in the Arrhenius form as Eq. 42:

$$V = 2.61 \cdot 10^{8} \exp [-11005/RT],\ r = 0.9985 \qquad (42)$$

Thermal Stability of the Enzyme

The experimental results obtained with the test of thermal stability are shown in Fig. 4. It can be observed that up to 50°C, and a thermal denaturation period of 240 min, the enzyme Novozym 188 looses very little of its initial activity, whereas at 70°C the enzyme is almost totally deactivated in 40 min.

Residual activity data, for temperatures in the range of 55 to 70°C, was applied to Eq. 3 giving the results for the deactivation constant (K_d) show in Table 4. These values of K_d in turn applied Eq. 2, as shown in Fig. 5, gives the energy of Thermal Denaturation of the enzyme: 81.6 kcal/mol, and:

$$K_d = 2.5233 \cdot 10^{52} \exp [-81574.3/RT],\ r = 0.9803 \qquad (43)$$

This result shows that this enzyme is relatively more stable than amyloglucosidase (E_d = 50.6 kcal/mol, *[21]*, or invertase (E_d = 64.9 kcal/mol, *[22]*).

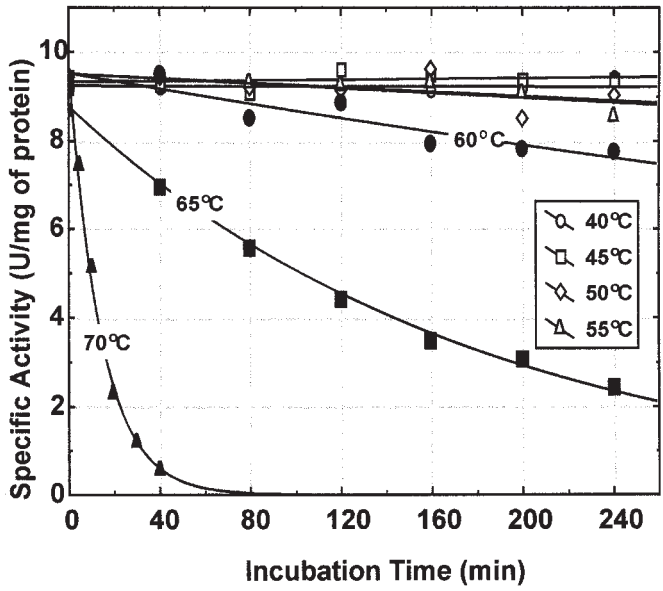

Fig. 4. Residual enzymatic activity for Novozym cellobiase 95 µL/L incubated in 2 g/L cellobiose at pH 4.8.

Table 4
Thermal Deactivation Constant Values (K_d) Obtained for the Enzyme β-Glucosidase at Different Temperatures

	Experimental[a]		From fitted equation[a]		
Temperature (°C)	K_d (Eq. 3) (h^{-1})	$t_{1/2}$ (Eq. 4) (h)	K_d (Eq. 43) (h^{-1})	$t_{1/2}$ (Eq. 4) (h)	$t_{1/2}$ experimental[b] (h)
55	0.0180	38.51	0.0117	59.240	216.00
60	0.0480	14.44	0.0766	9.050	8.60
65	0.3300	2.10	0.4735	1.460	0.50
70	4.1700	0.17	2.7769	0.250	0.04

[a]This work, β-glucosidase Novozym 188 incubated in cellobiose 5.8 mm, pH 4.8.
[b]β-glucosidase from *Aspergillus phoenicis* QM 329 incubated in cellobiose 7.5 mM, Bisset and Sternberg *(19)*.

Table 4 presents values obtained for the enzyme half-life calculated with Eq. 4. It is also shown for comparison the half-lives determined by Bisset and Sternberg *(19)* for cellobiose from another source. This comparison allows concluding that Novozym 188 for T ≥ 60°C is a more thermostable enzyme than that of *Aspergillus phoenicis* QM 329.

Effect of Substrate Initial Concentration

Figure 6 shows that at substrate concentrations above 10 m*M* the hydrolysis of cellobiose is inhibited by the substrate. The parameters *Km*,

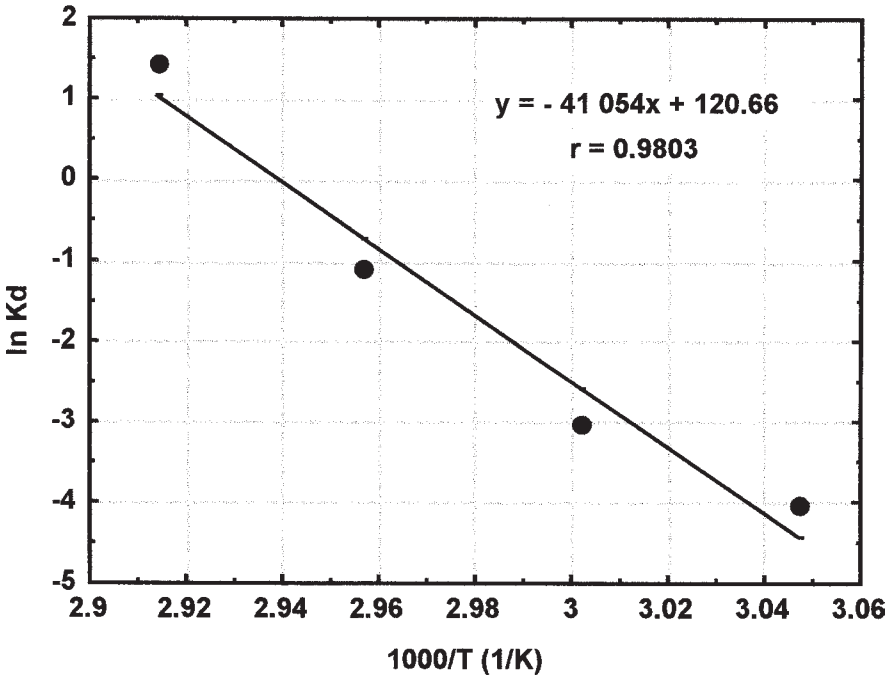

Fig. 5. Arrhenius plot for the energy of thermal deactivation of Novozym cellobiase (95 µL/L) incubated in 2 g/L cellobiose at pH 4.8.

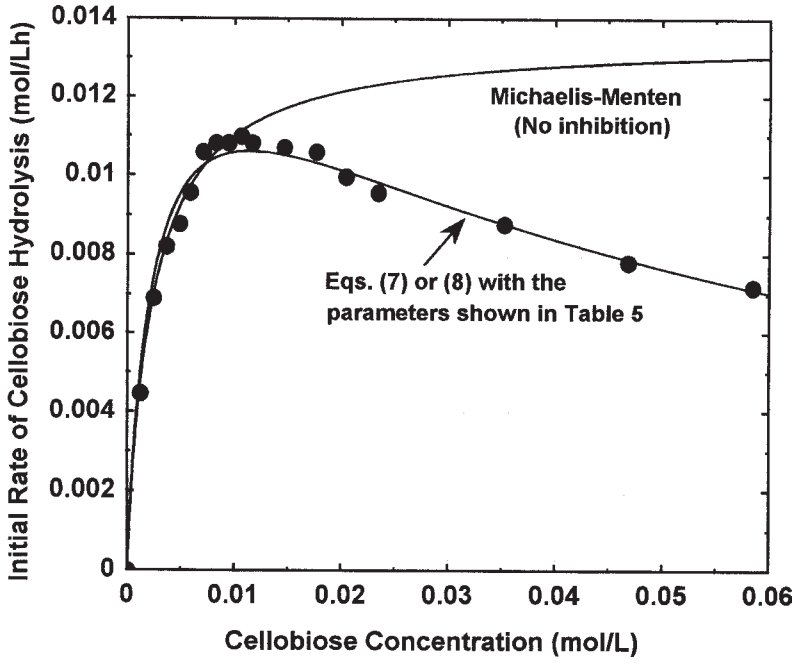

Fig. 6. Initial rate of cellobiase. (Reaction conditions: 50°C, pH 4.8, 95 µL of enzyme/L of solution.)

Table 5
Kinetic Parameters for Enzymatic Hydrolysis of Cellobiose

Author	*Km* (m*M*)	V_{max} µmol glucose/ (min. mg of protein)	*Ks* (m*M*)
This work (pH 4.8, 50°C)			
Uncompetitive substrate	2.42	16.30	54.2
Noncompetitive substrate	2.54	17.10	51.7
Beltrame et al. *(16)*	2.70	1.70	43.0
Dekker *(15)*	5.63	33.74	—
Grous et al. *(24)*	1.66	—	43.4

V_{max}, and *Ks* were calculated from this data as described in Materials and Methods yielding the values presented in Table 5. This table also presents other values from literature, showing order of magnitude agreement. It can be observed that considering either uncompetitive or noncompetitive substrate inhibition, the values are very close. The inhibition caused by the substrate reduces by 45% the initial rate of hydrolysis at the substrate concentration of 20 g/L (58.5 m*M*).

Conversion of Cellobiose as a Function of Time

Figures 7 and 8 show that the addition of glucose together with the substrate at the beginning of the reaction reduces the rate of hydrolysis demanding much greater times for higher conversions. For example, at 40°C (Fig. 7) without added glucose the time needed to reach conversion equal to 98% is about 6 h whereas with added glucose (10 m*M*) this time increases to 12 h. At 50°C, Fig. 8, the same effect is observed but with shorter times, 4 and 8 h, respectively.

Figure 9, which plots the conversion data for high cellobiose concentration (20 g/L, 58.5 m*M*) at two different temperatures, shows that a conversion as high as 92% is reached in this case, only after 80 h at 40°C and 50 h at 50°C.

The residual activity of cellobiase in the conversion tests with high substrate concentration (Fig. 9) has show that even after 100 h at 50°C or 73 h at 40°C, no detectable loss of activity could be seen. This demonstrates clearly the protecting effect of the substrate that reduces the rate of thermal deactivation of this enzyme. As already seen in regard to Fig. 4, at a substrate concentration 10 times lower (2 g/L, 5.8 m*M*) there is a small, but measurable, loss of activity after only 4 h at 40 or 50°C.

The six curves shown in Figs. 7–9, are the result of fitting the integrated kinetic equation, Eq. 11 to the conversion data as described in Materials and Methods. The kinetic parameters derived from the values obtained for α, β, and γ in each of the inhibition models are shown in Table 6. Although the quality of the data in Figs. 7–9 is very good, astonishing results are seen in Table 6.

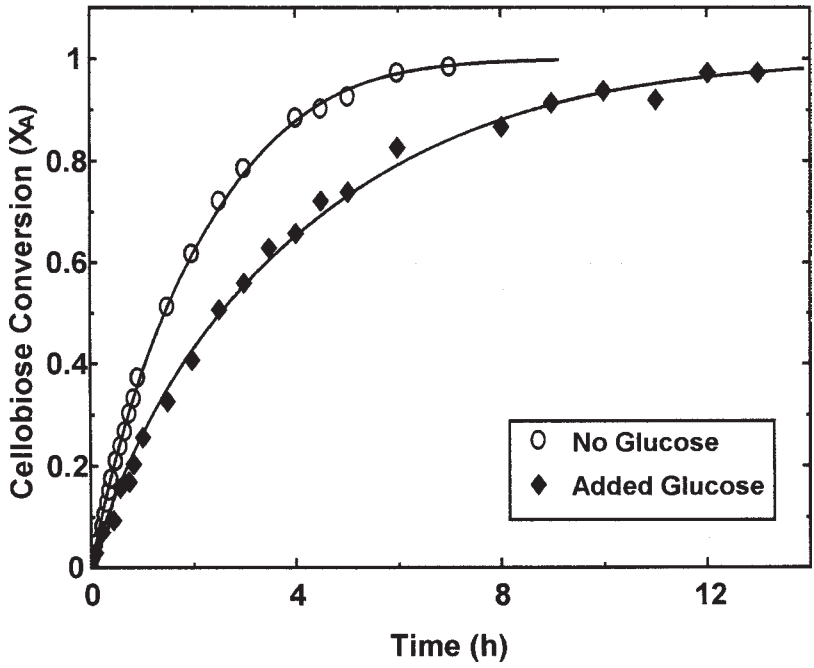

Fig. 7. Enzymatic conversion of cellobiose to glucose in a batch reactor at the following conditions: 2 g/L cellobiose, 95 µL/L of Novozym cellobiase, 40°C, pH 4.8, no added glucose, and 1.8 g/L of added glucose (10 m*M*).

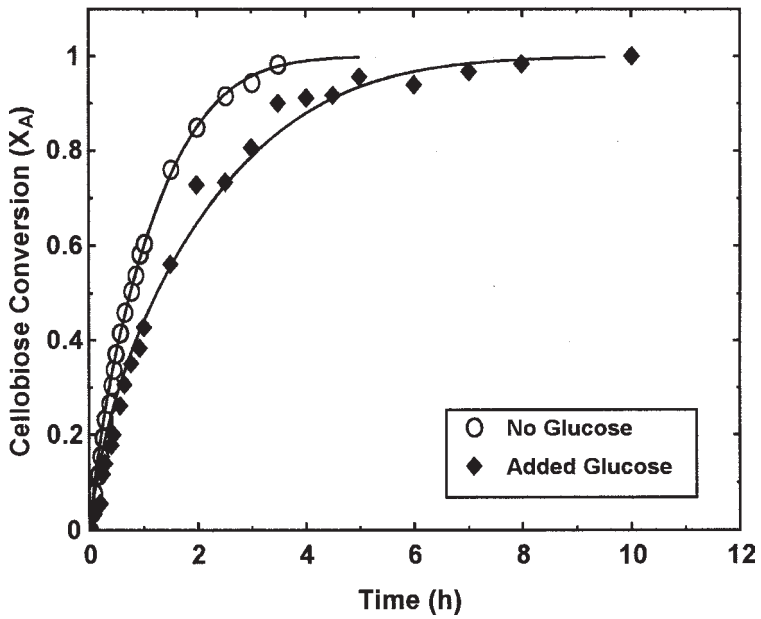

Fig. 8. Enzymatic conversion of cellobiose to glucose in a batch reactor at the following conditions: 2 g/L cellobiose, 95 µL/L of Novozym cellobiase, 50°C, pH 4.8, no added glucose, and 1.8 g/L of added glucose (10 m*M*).

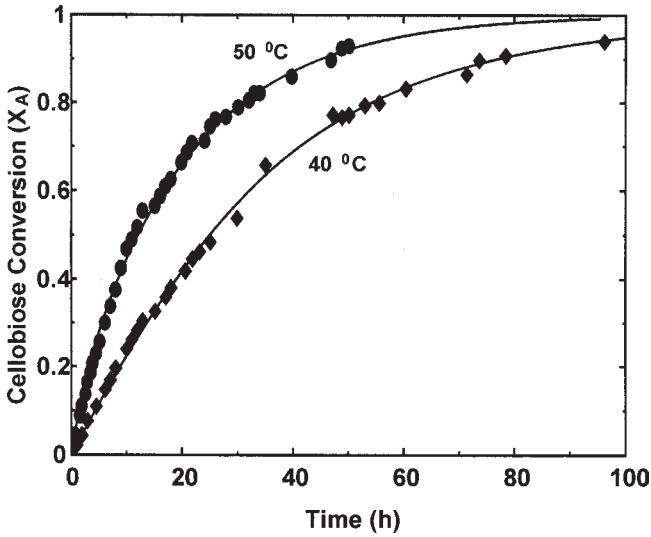

Fig. 9. Enzymatic conversion of cellobiose to glucose in a batch reactor at the following conditions: 20 g/L cellobiose, 95 µL/L of Novozym cellobiase, 40 and 50°C, and pH 4.8, no added glucose.

Very little coherence is seen in the same parameter values for *Ki*, and *Kcat*, obtained either from α or β. From γ the values of *Kcat* obtained also differ from V_{max}/E_t = 158.6 mmol of glucose/(h · mL of enzyme) for uncompetitive substrate and 166.3 mmol of glucose/(h · mL of enzyme) for noncompetitive substrate. Even more baffling is the negative values that result in some of the curves for *Ki* or *Kcat*, because these constants cannot be negative. Similar physical inconsistencies have already been found for starch hydrolysis *(23)* and there we suspected that the origin of the problem was the great complexity of starch hydrolysis, which is a multisubstrate kinetic problem. However, in the cellobiase case, the reaction seems to be reasonably simple: a single substrate and single product, small molecular sizes, the enzyme is very stable, and even so the same kind of physical inconsistencies have appeared when the integrated kinetic models were fitted to the experimental data covering conversions from reaction time zero to nearly 100% conversion.

It appears then that the hypotheses used to derive the simple inhibition models shown in Table 2, are not sufficient to describe the whole course of kinetics of this kind of reaction from time zero to 100% conversion. Either these hypotheses break down at higher conversions or new hypotheses have to be added. It could be for example that, the different combinations of substrate and product concentrations might have a complex effect of inhibition or activation of the enzyme *(16)*.

Therefore, for the purpose of satisfactorily modeling cellobiose hydrolysis for the whole course of the reaction, and in view of the potential industrial application, new fundamental studies should be directed to

Table 6
Values of the Kinetic Constants, Obtained by Fitting the Alternative Inhibition Integrated Kinetic Models, to the Conversion Data of Figs. 7–9 (Temperature 50°C, pH 4.8)

(S_0) (m*M*)	Gi (m*M*)	α (h)	β (h)	γ (h)	Ki (m*M*)	Ki (m*M*)	Kcat (mmol/h mL enz.)	Ki (m*M*)	Ki (m*M*)	Kcat (mmol/h mL enz.)	Ki (m*M*)	Ki (m*M*)	Kcat (mmol/h mL enz.)
					Competitive product			Noncompetitive product			Uncompetitive product		
Uncompetitive substrate					(Eq. 19)	(Eq. 20)	(Eq. 21)	(Eq. 22)	(Eq. 23)	(Eq. 24)	(Eq. 25)	(Eq. 27)	(Eq. 26)
5.84	0.0239	0.2181	0.6696	–0.7528	8.83	3.64	–4.39	8.78	3.64	126.4	–0.044	2.92	37.70
5.84	10.4800	–0.2779	1.3146	–1.9159	2.63	3.05	–1.73	–3.11	3.05	59.6	–5.740	1.17	19.20
58.4	0.1478	–6.9089	18.9913	–9.3466	1.24	0.99	–35.40	1.20	0.99	3,836	–0.038	19.80	1.33
Noncompetitive substrate					(Eq. 31)	(Eq. 32)	(Eq. 33)	(Eq. 34)	(Eq. 35)	(Eq. 36)	(Eq. 37)	(Eq. 39)	(Eq. 38)
5.84	0.0239	0.2181	0.6696	–0.7528	8.88	3.70	–4.60	8.86	3.70	124.0	–0.042	2.79	39.90
5.84	10.4800	–0.2779	1.3146	–1.9159	2.65	3.08	–1.81	–2.82	3.08	58.9	–5.470	1.11	20.30
58.4	0.1478	–6.9089	18.9913	–9.3466	1.25	1.00	–37.10	1.22	1.00	3,797	–0.037	18.90	1.41

investigate what are the unaccounted phenomena that invalidate the application of the presently available models in their integrated form.

Conclusions

1. For cellobiose hydrolysis with Novozym 188 cellobiase, the optimum conditions gives an activity of 17.84 U/mg of protein for pH 4.5 and temperature 65°C, respectively.
2. For all temperatures the enzyme has shown optimum activities in the pH range of 4.0–5.0.
3. The Energy of Activation of the enzyme is 11.0 kcal/mol at pH 4.8, and the hydrolysis rate can be expressed in the Arrhenius form: $V = 2.61 \cdot 10^8 \exp [-11005/RT]$.
4. The enzyme is very stable and its thermal deactivation follows reasonably the exponential decay model giving: $K_d = 2.5233 \cdot 10^{52} \exp [-81574.3/RT]$
5. Substrate and product inhibitions were experimentally observed, the latter having a strong effect on lengthening the course of reaction to reach high conversions.
6. Integrated kinetic models including inhibition of either competitive or noncompetitive type for glucose, and uncompetitive or noncompetitive for cellobiose gave inconsistent values for the kinetic parameters, such as *Ki* and *Kcat*.
7. New fundamental studies seem necessary to unveil the unknown phenomena that preclude the application of the integrated kinetic models to the hydrolysis of cellobiose.

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