0273-2289/05/126/0049/$30.00

Enzymatic Kinetic of Cellulose Hydrolysis

Inhibition by Ethanol and Cellobiose

Rui M. F. Bezerra* and Albino A. Dias

CETAV-Departamento de Engenharia Biológica e Ambiental, Universidade de Trás-os-Montes e Alto Douro, Apartado 1013, 5001-911 Vila Real, Portugal, E-mail: bezerra@utad.pt

Received December 14, 2004; Revised April 7, 2005; Accepted April 13, 2005

Abstract

The ethanol effect on the *Trichoderma reesei* cellulases was studied to quantify and clarify this inhibition type. To determine inhibition parameters of crude cellulase and purified exoglucanase Cel7A, integrated Michaelis–Menten equations were used assuming the presence of two inhibitors: cellobiose as the reaction product and ethanol as a possible bioproduct of cellulose fermentation.

It was found that hydrolysis of cellulose by crude enzyme follows a model that considers noncompetitive inhibition by ethanol, whereas Cel7A is very slightly competitively inhibited. Crude cellulase is much more inhibited ($K_{iul} = K_{icl} = 151.9$ mM) than exoglucanase Cel7A ($K_{icl} = 1.6 \times 10^{15}$ mM). Also, calculated inhibition constants showed that cellobiose inhibition is more potent than ethanol inhibition both for the crude enzyme as well as exoglucanase Cel7A.

Index Entries: Cellulase kinetics; ethanol inhibition; exoglucanase Cel7A; integrated Michaelis–Menten equations; kinetic with two inhibitors.

Introduction

Cellulose is the world's most plentiful renewable biomolecule and could be the basis for the eventual development of biomass conversion systems for alternative fuel production *(1,2)*.

The fungus *Trichoderma reesei* produces, like other cellulose-degrading fungi, a set of cellulolytic enzymes that cooperate synergistically. The cellulolytic system of *T. reesei* is made up of two exoglucanases (EC 3.2.1.91) Cel7A (CBHI) and Cel6A (CBHII), and at least five endoglucanases (EC 3.2.1.4) Cel7B (EGI), Cel5A (EGII), Cel12A (EGIII), Cel61A

*Author to whom all correspondence and reprint requests should be addressed.

(EG IV), and Cel45A (EGV). Of these, Cel7A is the most important sugar-producing enzyme comprising 60 to 75% of the cellulases in a *T. reesei* filtrate *(3,4)*.

Previous works show that cellulases inhibition by ethanol follows a noncompetitive inhibition pattern for ethanol concentrations less than 4 *M*. When the ethanol concentration is increased, the enzyme is denatured. Ethanol also interferes with enzyme (manly cellobiohydrolases) adsorption to cellulose and modifies the cooperative effect between cellobiohydrolases and endoglucanases *(5,6)*. Low ethanol concentrations (less than 4 *M*) do not affect β-glucanase activity. Indeed, a slight increase in activity can be observed *(7,8)*. When compared to other fermentation products, Takagy *(9)* considered that ethanol, glucose, and butanol have a similar inhibition potential and classified them as "strong" inhibitory compounds. However, Holtzaple et al. *(6)* concluded that ethanol inhibition is less potent than glucose. Also, the conversion of cellobiose to ethanol can reduce the inhibition of cellulases by a factor of 16 *(6)*. Nevertheless, ethanol inhibition presents a potential problem in the process of simultaneous saccharification and fermentation of cellulosic materials *(10)*.

Despite the insoluble nature of cellulose, experimental results strongly suggest the feasibility of using integrated Michaelis–Menten equations *(11,12)* in the cellulase kinetics *(13–18)* and other heterogeneous reactions such as lipid hydrolysis *(19)*. Kinetic analysis with integrated Michaelis–Menten equations also permits the determination of inhibition constants (K_i) when more than one reaction product inhibits the enzyme *(20,21)*. In this situation:

$$\sum_{j=1}^{n} 1/K_{ij} = 1/K_i$$

where K_{ij} is the inhibition constant for one product and K_i is the inhibition constant for all products.

The main objective of this work was to elucidate the pattern of ethanol inhibition on cellulose hydrolysis taking into account the mandatory simultaneous presence of cellobiose.

Materials and Methods

Enzyme Preparation and Chemicals

Celluclast 1,5 L, a commercial cellulase slurry from *T. reesei*, was kindly provided by Novo Nordisk A/S (Copenhagen, Denmark). It was used both as a crude enzyme after dialyfiltration (Amicon stirred cell, 10,000 cut-off) and as a source of Cel7A exoglucanase. Purification of this enzyme was accomplished according to methodology previously described *(18)*. The purified enzyme Cel7A exoglucanase was electrophoretically homogenous because a single band appeared in polyacrylamid gels (sodium dodecyl sulfate polyacrylamide gel electrophoresis; SDS-PAGE). Avicel, carboxymethyl-

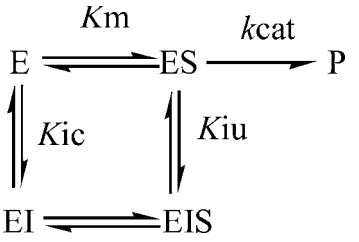

Fig. 1. Mixed linear (total) inhibition model *MI* where: E, enzyme; ES, enzyme substrate complex; EIS, enzyme substrate inhibitor complex; EI, enzyme inhibitor complex; P, product; K_m, Michaelis–Menten constant; K_{ic}, K_{iu}, inhibition constants, k_{cat}, rate constant. A model without inhibition *WI* can be obtained by simplification of previous model assuming K_{ic}, K_{iu}, as infinite. Thus it is possible to obtain the following linear (total) inhibition models: competitive inhibition *CI* ($K_{iu} = \infty$); noncompetitive inhibition *NCI* ($K_{ic} = K_{iu} = \infty$); uncompetitive inhibition *UI* ($K_{ic} = \infty$).

cellulose, cellobiose and absolute ethanol were purchased from Merck. Other chemicals used in this study were of reagent grade.

Enzyme Activities

The enzymatic activities (CMCase, Avicelase and aril-β-glucosidase) were measured according to the IUPAC-Biotechnology Commission procedures. The reducing sugars released were determined by dinitrosalicyclic acid (DNS) or the Somogy-Nelson method, using cellobiose as a standard *(18)*.

Kinetic Assays

Kinetic studies were performed at 40°C in 50 m*M* citrate buffer, pH 4.8 with sodium azide 0.01% and monitored for 47 h (22 sampling points for each run). Different experiments were carried out with four concentrations of substrate (Avicel) 5.0, 2.5, 0.25, and 0.025% (w/v) which were equivalent to 154.2, 77.1, 7.7, and 0.77 m*M* potential cellobiose, six concentrations of Cel7A (4, 10, 42, 100, 417, and 1000 μg/mL), and three concentrations of crude enzyme (10, 100, and 1000 μg/mL). The assays were performed at ethanol concentrations 0.0, 0.43, 0.86, and 1.72 *M*. Identical experiments with added initial cellobiose (7.5 and 15.0 m*M*) without ethanol, were performed at the 5.0 and 2.5% (w/v) substrate concentrations and enzyme (crude and Cel7A) concentrations of 100 and 1000 μg/mL.

For each assay, a complete progress curve was analyzed using the non linear regression method DUD (doesn't use derivatives) of SAS software (SAS Institute Inc.) and the kinetic constants for the models presented in Fig. 1 were determined. All experimental points were used, except those with ratios [S] (μg/mL)/[E] (μg/mL) smaller than 6.5 which were not considered *(18)*.

Results and Discussion

Theoretical Framework

The kinetic models to be developed must recognize the simultaneous presence of two inhibitors (ethanol and cellobiose) as sugar is a reaction product of cellulose hydrolysis. In order to study the enzymatic kinetic with these two inhibitors it was necessary to formulate models assuming that ethanol and cellobiose compete to the same inhibition sites of the enzyme. More complex models such as models that assume nonproductive enzyme substrate complexes, parabolic inhibition, enzyme denaturation, or even hyperbolic inhibition were not considered as previous work has shown that they are not the principal constraints in enzymatic cellulose hydrolysis *(18)*.

Linear mixed inhibition (*MI*) includes all of the common types of inhibition and will therefore be taken as a general case (Fig. 1), although some inhibition constants can tend to infinity, meaning that they are irrelevant. This methodology has already been explained in previous works *(18,23)* and can be described by the rate equation:

$$v = \frac{V[So]}{K_m(1 + [I]/K_{ic}) + [So](1 + [I]/K_{iu})}$$

(with the symbols explained in Table 1 and Fig. 1).

Given [*EP*] as the concentration of the enzyme-cellobiose complex and [*EA*] as the concentration of the enzyme-ethanol complex, we can define [*EI*] = [*EP*] + [*EA*].

Similarly we can define [*I*] as ethanol concentration [*A*] plus cellobiose concentration [*P*] ([*I*] = [*A*] + [*P*])

It is assumed that the inhibitor concentration [I] is equal to the product concentration [P] plus the ethanol concentration [A]. Thus, when the ethanol concentration is not present [I] = [P]. Then in a similar way, with competitive inhibition we can define an ethanol inhibition constant K_{icl} and a cellobiose inhibition constant K_{ic} (*see* Fig. 1 and Table 1). The obtained equation is

$$v = \frac{dP}{dt} = \frac{1}{K_m/(V[So])(1 + [P]/K_{ic} + [A]/K_{icl}) + 1/V(1 + [P]/K_{iu} + [A]/K_{iul})}$$

This equation can be integrated assuming the substrate concentration constant, where the subscript "o", e.g., So and Po, means initial concentration at t = 0.

$$V\int_0^t dt = \left(\left(\frac{K_m}{[So]} + 1 + \frac{[A]}{K_{iul}} + \frac{K_m[A]}{[So]K_{icl}}\right)\int_{P_0}^{P_t} dP + \left(\frac{K_m}{[So]K_{ic}} + \frac{1}{K_{iu}}\right)\int_{P_0}^{P_t} [P]dP\right)$$

$$t = \frac{1}{V}\left\{\left(\frac{K_m}{So} + \frac{K_m[A]}{So\ K_{icl}} + \frac{[A]}{K_{iul}} + 1\right)(Pt - Po) + \left(\frac{K_m}{2So\ K_{ic}} + \frac{1}{2K_{iu}}\right)(Pt^2 - Po^2)\right\}$$

Table 1
Nomenclature

Abbreviation	Terminology
A	ethanol
E	free enzyme
EI	enzyme inhibitor complex
EII	enzyme inhibitor complex for the second inhibitor
EIS	enzyme substrate inhibitor complex
ES	enzyme substrate complex
ES'	enzyme substrate complex (nonproductive)
$f_{0.95}$	denoting the point of the Fpa,pb (F distribution) curve with area 0.95 (to its right)
I	all inhibitors
k_{cat}	catalytic constant (per hour)
K_{ic}	competitive inhibition constant (m*M*) to cellobiose
K_{icl}	competitive inhibition constant (m*M*) to ethanol
K_{iu}	uncompetitive inhibition constant (m*M*) to cellobiose
K_{iul}	uncompetitive inhibition constant (m*M*) to ethanol
K_m	Michaelis constant (m*M*)
n	experimental points
P	reaction product (cellobiose)
p_A, p_B	parameters
Po	initial product
P_t	product at time t (min)
R^2	determination coefficient
S	substrate
SSE	sum of squares error
t	time (min)
V_{max}	maximum velocity
w	quotient used to test the significance of the improvement of different models that are interconvertible by addition or elimination of parameters by comparison of the F-value

The other four models are simplifications of this one (*MI*), assuming that different constants (K_{iu}, K_{ic}, K_{iul}, K_{icl}) tend to infinity. For example, if K_{iu} and K_{iul} is negligible ($K_{iu} = \infty$ and $K_{iul} = \infty$) the model obtained is a *CI* model (competitive inhibition) both for cellobiose and ethanol. Otherwise, if K_{ic} and K_{icl} is negligible ($K_{ic} = \infty$ and $K_{icl} = \infty$) the model obtained is *UCI* (uncompetitive inhibition) both for cellobiose and ethanol inhibitors. When $K_{ic} = K_{iu} \neq \infty$ and $K_{icl} = K_{iul} \neq \infty$, the inhibition obtained is *NCI* (noncompetitive inhibition). When all inhibition constants tend to infinity the model obtained is *WI* (without inhibition). At the end there is the possibility that the inhibition is a mixed linear (total) inhibition (*MI*) and so $K_{ic} \neq K_{iu} \neq \infty$ and $K_{icl} \neq K_{iul} \neq \infty$. In this case it is still possible that one inhibitor be predominantly of one type and the other predominantly of any other inhibition type, as shown by the constant values obtained for the parameters K_{ic}, K_{iu}, K_{icl}, and K_{iul} *(23)*.

In the reactions where two inhibitors are reaction products, Hsu *(24)* showed that if there is no initial inhibitor the two constants, K_{ic} and K_{icl}, as well as K_{iu} and K_{iul}, are in a lumped form and can not be determined. Since in the present study ethanol is not a reaction product and experiments with initial cellobiose have been carried out, we can assume that the inhibition constants for ethanol and cellobiose are not lumped.

Discrimination and Obtained Constants

As previously demonstrated *(25)*, discrimination between any two models, for example, A and B with $\boldsymbol{p_A}$ and $\boldsymbol{p_B}$ parameters, are separately fitted to the same experimental points $\boldsymbol{n}$ and if the model $\boldsymbol{B}$ were an extension of the simpler model **A**, the significance of improvement obtained by the addition of the new parameters $(p_B - p_A)$ can be tested by comparing the quotient w:

$$w = \frac{(SSE_A - SSE_B)\,(n - p_B)}{(p_B - p_A)SSE_B}$$

When $\mathbf{w - f\text{-}value} > 0$ the more complex model should be preferred, if not then a simpler model cam be applied. The *f*-values are obtained from statistical tables (F distribution, $f(p_B - p_A,\ n - p_B)$) at the desired level of probability ($f_{0.95}$) knowing $p_B - p_A$ (number of parameters added) and $n - p_B$ (degrees of freedom) for these models (Tables 2 and 3).

With exoglucanase Cel7A, it is only relevant to compare *WI* with *CI* model and *CI* with *MI* model (Table 2) because the inspection of SSE (Table 4) shows that *CI* model is better than any other with the same number of parameters. The $\boldsymbol{w - f\text{-}value}$ comparison between models (Table 2) shows that *CI* should be preferred when compared with *WI* or *MI* models. Furthermore, K_{ic} and K_{icl} values obtained by mixed linear inhibition (*MI* model) support this conclusion (Table 4). In fact, these constants show that they are the most important because K_{iu} and K_{iul} are so large that they can be eliminated from model *MI*.

With the crude enzyme there are two models that give a $\boldsymbol{w - f\text{-}value}$ less than zero (Table 3), meaning that the simpler model should be preferred rather than complex models. These two models (*CI* and *NCI*) have the same parameters but the SSE values demonstrate that the *NCI* model is better. Thus, ethanol (and cellobiose) inhibit non competitively the crude cellulase with the constants in Table 5. Calculated inhibition constants show strong differences between the inhibition power of ethanol and cellobiose in reactions catalyzed with exoglucanase Cel7A ($K_{icl} = 1.6 \times 10^{15}$ m*M* vs $K_{ic} = 0.035$ m*M*) as well as with the crude enzyme ($K_{iul} = K_{icl} = 151.9$ m*M* vs $K_{iu} = K_{ic} = 0.05$ m*M*). With the crude enzyme the same methodology gives values that point to a much more potent noncompetitive inhibition by ethanol ($K_{iul} = 151.9$ m*M*) when compared with competitive inhibition obtained with Cel7A ($K_{iul} = 1.6 \times 10^{15}$ m*M*). Ethanol is a much more effective inhibitor for crude enzyme than for exoglucanase Cel7A. The effect of ethanol in the

Table 2
Summary of the $w - f_{0.95}$ Values in Assays With the Cel7A

Models a/b	SSE_A	SSE_B	n	p_A	p_B	w	$p_B - p_A$	$n - p_B$	$f_{0.95}$	$w - f_{0.95}$
WI/CI	3670408	172637	1043	2	4	10525.510	2	1039	3.00	10522.510
CI/MI	172637	172638	1043	4	6	–0.003	2	1037	3.00	–3.003

The models are explained at Fig. 1; *see also* the text.

Table 3
Summary of the $w - f_{0.95}$ Values in Assays With the Cellulase Crude Enzyme

Models a/b	SSE_A	SSE_B	n	p_A	p_B	w	$p_B - p_A$	$n - p_B$	$f_{0.95}$	$w - f_{0.95}$
WI/CI	210482	74097	864	2	4	791.47	2	860	3.00	788.47
CI/MI	74097	73659	864	4	6	2.55	2	858	3.00	–0.45
NCI/MI	72813	73659	864	4	6	–4.93	2	858	3.00	–7.93

The models are explained at Fig. 1, *see also* the text.

Table 4
Summary of the Obtained Constants
and Statistical Parameters in Assays With Cel7Aa

	WI	CI	NCI	UCI	MI
K_m (m*M*)	9.6	21.1	184×10^3	25.2	21.4
K_{ic} (m*M*)		0.035	0.035		0.035
K_{icl} (m*M*)		1.5×10^{15}	6.5×10^{19}		2.8×10^8
K_{iu} (m*M*)			0.035	0.478	5.9 × 10
K_{iul} (m*M*)			6.5×10^{19}	1.5×10^8	6.5×10^{12}
k_{cat} (h^{-1})	2	12	9.4×10^3	6	12
SSE	367408	172637	176153	250726	172638
R^2	0.374	0.706	0.701	0.573	0.706
p	2	4	4	4	6
n	1043	1043	1043	1043	1043

The models are explained in Fig. 1; *see also* the text.

Table 5
Summary of the Obtained Constants
and Statistical Parameters in Assays With Cellulose Crude Enzyme

	WI	CI	NCI	UCI	MI
K_m (m*M*)	16	3394	846	6158	1731
K_{ic} (m*M*)		0.015	0.05		0.085
K_{icl} (m*M*)		52.1	151.9		227.9
K_{iu} (m*M*)			0.05	0.020	0.02
K_{iul} (m*M*)			151.9	6.3	15.6
k_{cat} (h^{-1})	5	2.5×10^3	2.2×10^3	2.0×10^3	2.9×10^3
SSE	210482	74097	72813	141785	73659
R^2	0.562	0.846	0.849	0.705	0.847
p	2	4	4	4	6
n	864	864	864	864	864

The models are explained in Fig. 1; *see also* the text.

reaction with crude enzyme is shown in Figs. 2 and 3 with obtained constants it is possible to predict cellulose hydrolysis (Fig. 2).

Our results with the crude enzyme are in line with Ooshima et al. *(5)* who reported a non competitive inhibition of *T. viride* cellulase complex by ethanol. In contrast, purified Cel7A exoglucanase is very slightly competitively inhibited by ethanol.

The present study shows that cellobiose is a strong competitive inhibitor of exoglucanase Cel7A and a noncompetitive inhibitor of crude enzyme. Ethanol can change the cellulose hydrogen bond or the enzyme tertiary structure due to the more pronounced nucleophilic effect of ethanol with respect to water. Furthermore, ethanol can activate several enzymes, namely β-glucosidase, and interfere with its K_m and K_{ic} *(7,8)* and could help to explain the difference between crude enzyme and Cel7A exoglucanase.

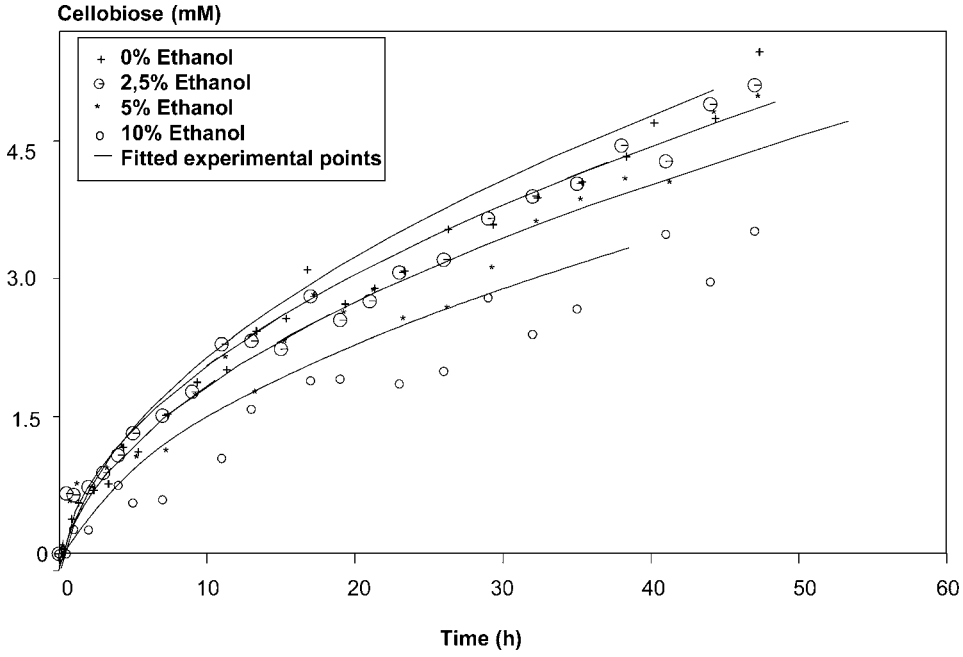

Fig. 2. Experimental points and fitted curves of cellulose hydrolysis with crude enzyme (10 μg/mL). The model utilized in fitted curves was *NCI*. The model with tabulated constants (Table 4) allowed to fit beyond the figure, values of all substrate/enzyme ratios up to 6.5 (864 experimental points).

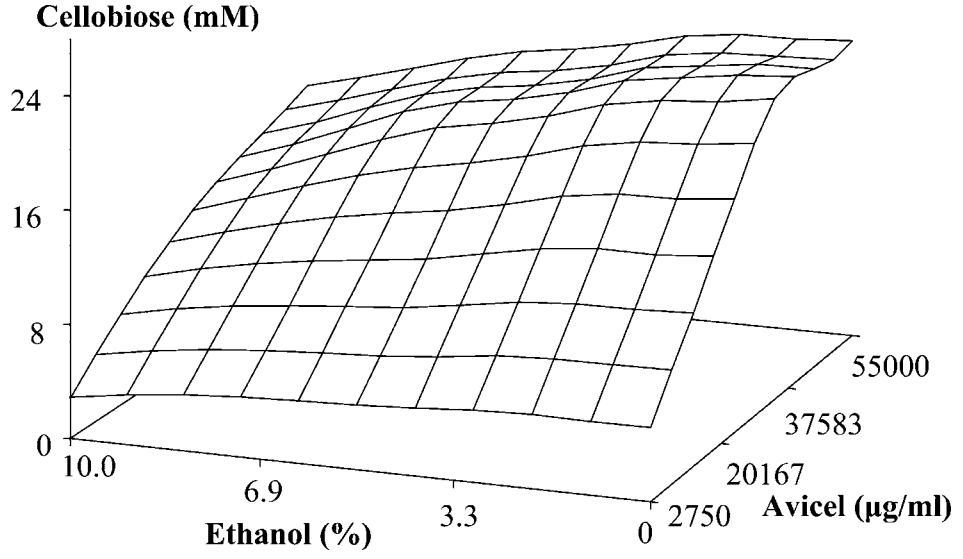

Fig. 3. Surface obtained from experimental results that predict the variation of the three axes variables. The ethanol concentration is 0.0, 0.043, 0.86, and 1.72 *M* with crude enzyme (100 μg/mL) and cellulose 0.77, 7.7, 77.1, and 154.2 m*M*. The product concentration corresponds to the hydrolysis at the end of 47 h.

These results quantitatively confirm that the conversion of cellobiose to ethanol (e.g., in reactors SSF) has a positive effect on cellulose hydrolysis which can be ascribed to the small, inhibitory power of ethanol when compared with cellobiose inhibition.

Conclusions

Hydrolysis of cellulose by purified Cel7A follows a model that considers competitive inhibition by both cellobiose and ethanol with the following parameters: K_m = 9.6 m*M*, K_{ic} = 0.035 m*M* and $K_{icl} = 1.5 \times 10^{15}$ m*M*. The value of the K_{icl} constant pointed out a very weak inhibition by ethanol. Hydrolysis of cellulose by crude enzyme follows a model that looks at noncompetitive inhibition by ethanol with the following parameters: K_m = 846.3 m*M*, $K_{ic} = K_{iu}$ = 0.05 m*M*; $K_{icl} = K_{iul}$ = 151.9 m*M*. Ethanol is a much more effective inhibitor of the crude enzyme than of exoglucanase Cel7A. Ethanol has an inhibition constant much greater than the calculated value for cellobiose.

References

1. Pettersson, P. O., Eklund, R., and Zacchi, G. (2002), *Appl. Biochem. Biotechnol.* **98–100,** 733–746.
2. Sun, Y. and Cheng, J. (2004), *Biotechnol. Prog.* **20,** 698–705.
3. Eriksson, T., Karlsson, J., and Tjerneld, F. A. (2002), *Appl. Biochem. Biotech.* **101,** 41–60.
4. Rabinovich, M. L., Melnick, M. S., and Bolovova, A. V. (2002) *Appl. Biochem. Microbiol.* **38,** 305–321.
5. Ooshima, H., Ishitani, Y., and Harano, Y. (1985), *Biotechnol. Bioeng.* **27,** 389–397.
6. Holtzapple, M., Cognata, M., Shu, Y., and Hendrickson, C. (1990), *Biotechnol. Bioeng.* **36,** 275–287.
7. Kennedy, J. F. and Cabral, J. M. S. (1987), in *Biotechnology, vol. 7a* (Rehm, H. J. and Reed, G., eds.), pp. 253–391.
8. Caldini, C., Bonomi, F., Pifferi, P. G., Lanzarini, G., and Galante, Y. M. (1994), *Enzyme Microb. Technol.* **16,** 286–291.
9. Takagi, M. (1984), *Biotechnol. Bioeng.* **XXVI,** 1506–1507.
10. Wu, Z. and Lee, Y. Y. (1997), *Biotechnol. Lett.* **19,** 977–979.
11. Orsi, B. A. and Tipton, K. F. (1979), *Methods Enzymol.* **63,** 159–183.
12. Duggleby, R. G. (1986), *Biochem. J.* **235,** 613–615.
13. Howell, J. A. and Stuck, J. D. (1975), *Biotechnol. Bioeng.* **17,** 873–893.
14. Hsu, T.-A. and Tsao, G. T. (1979), *Biotechnol. Bioeng.* **21,** 2235–2246.
15. Hsu, T.-A., Gong, C.-S., and Tsao, G. T. (1980), *Biotechnol. Bioeng.* **12,** 2305–2320.
16. Pereira, A. N. (1987), PhD Thesis, Purdue University, West Lafayette, IN.
17. Nidetzky, B., Hayn, M., Macarron, R., and Steiner, W. (1993), *Biotechnol. Lett.* **15,** 71–76.
18. Bezerra, R. M. F. and Dias, A. A. (2004) *Appl. Biochem. Biotechnol.* **112,** 173–184.
19. Jones, E. and Weatherley, L . R. (2003), *J. Chem. Technol. Biotechnol.* **78,** 194–198.
20. Foster, R. J. and Niemann, C. (1955) *J. Am. Chem. Soc.* **77,** 1886–1892.
21. Philo, R. D. and Selwyn, M. J. (1973) *Biochem. J.* **135,** 525–530.
22. Wood, T. M. and Bhat, K. M. (1988), *Methods Enzymol.* **160,** 87–113.
23. Bezerra, R. M. F. (1999) *J. Med. Biochem.* **3,** 9–16.
24. Hsu, T.-A. (1979), PhD Thesis, Purdue University, West Lafayette, IN.
25. Mannervik, B. (1982), *Methods Enzymol.* **87C,** 370–391.