0273-2289/96/5758—0617$7.25

Modeling Cassava Starch Saccharification with Amyloglucosidase

GISELLA MARIA ZANIN* AND FLÁVIO FARIA DE MORAES

State University of Maringá,
Chemical Engineering Department, 87020-900 Maringá, PR, Brazil

ABSTRACT

A solution of α-amylase liquefied cassava starch, 30% (w/v), was saccharified with amyloglucosidase at 45°C, pH 4.5, in a batch reactor in the presence and absence of added glucose. Reactor conversion results were modeled with a multisubstrate model that considers intermediate dextrins of starch hydrolysis, reversibility of some reactions, substrate and product inhibition, and competition among dextrins and isomaltose formation. Kinetic parameters were obtained from initial velocity saccharification tests at different starch concentrations and from the literature. The model can represent well the saccharification of cassava starch even in the presence of a great excess of glucose (100 g/L), added to test its capability.

Index Entries: Cassava starch; amyloglucosidase; saccharification; modeling.

Nomenclature: C_A, liquefied starch concentration, g/L; C_{Ao}, initial starch concentration, 300 g/L; C_g, glucose concentration, g/L; C_{ga}, concentration of added glucose, g/L; C_{gi}, glucose concentration at the start of saccharification, g/L; E, enzyme concentration, mL of enzyme stock solution/L of substrate solution; f, ratio of molecular weights for the anhydroglucose unit in starch and glucose, f = 162/180 = 0.9; G, glucose molar concentration, mol/L; G_2, maltose molar concentration, mol/L; G_3, maltotriose molar concentration, mol/L; G_4, susceptible oligosaccharides molar concentration, mol/L; G_6, resistant oligosaccharides molar concentration, mol/L; G_I, isomaltose molar concentration, mol/L; G_{eq}, G_{2eq}, G_{3eq}, G_{Ieq} equilibrium molar concentration for glucose, maltose, maltotriose, and isomaltose, respectively, mol/L; k_{cat}, reaction rate constant related to product formation, mol/(h·mL of enzyme); K_{eq2}, K_{eq3}, K_{eqI}, equilibrium constants for maltose (mol/L), maltotriose (mol/L), and isomaltose (L/mol), respectively; K_i, product (glucose) inhibition constant, mol/L; K_{m2}, K_{m3}, K_{m4}, K_{m6}, Michaelis-Menten constants for maltose, maltotriose, susceptible oligosaccharides, and resistant oligosaccharides, respectively, mol/L; K_S, substrate inhibition constant, mol/L; M_G, molecular weight of glucose, 180 g/gmol; n, average degree of polymerization, dimensionless; r_2, r_3, r_4, r_6, r_I, rate of reaction for maltose, maltotriose, susceptible oligosaccharides, resistant oligosaccharides, and isomaltose, respectively, mol/(L·h); t, reaction time, h; V, initial rate of glucose production, g/(L·h); V_{IM}, second-order rate constant for isomaltose, L^2/(mol·h·mL of enzyme); V_{m2}, V_{m3}, V_{m4}, V_{m6}, maximum velocity constants

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

associated with the reaction rate of maltose, maltotriose, susceptible oligosaccharides, and resistant oligosaccharides, respectively, mol/(h·mL of enzyme); X_A, conversion of liquefied starch to glucose, %; α, parameter in Eq. (22), $\alpha = (C_{Ao}/k_{cat})(1 - K_m/K_i + C_{Ao}/K_S)$, mL of enzyme·h/L; β, parameter in Eq. (22), $\beta = (K_m/k_{cat})(1 + C_{gi}/K_i + C_{Ao}/K_i)$, mL of enzyme·h/L; γ, parameter in Eq. (22), $\gamma = C_{Ao}^2/(2\,K_S\,k_{cat})$, mL of enzyme·h/L; τ_b, normalized reaction time, $\tau_b = E \cdot t$, mL of enzyme·h/L.

INTRODUCTION

In the south of Brazil, greatly purified cassava starch in powder form, known as "fécula," is produced in large quantities. This material has been considered as a renewable, clean source for the production of modified starches and basic chemicals, such as ethanol, which is used for motor fuel in Brazil.

Many starch applications begin with its depolymerization to glucose. Hence, a research program was set up to study the saccharification of "fécula" with commercial amyloglucosidase using the following process options: enzyme in soluble form or immobilized, and three types of reactors: batch, fixed, and fluidized bed *(1)*.

Reactor studies included bed hydrodynamics *(2)*, axial dispersion *(3)*, internal and external mass-transfer *(4)*, comparative reactor performances for conversion *(4)*, and enzyme half-life in actual process operating conditions *(5)*. The enzyme, NOVO amyloglucosidase, has been characterized with respect to its activity toward saccharification of preliquefied "fécula," at different pH and temperature values *(6)*. The liquefaction step was conducted with α-amylase, NOVO Termamyl, and the density, viscosity, and initial glucose contents were measured as a function of total dried matter in solution *(2)*. This information is useful for future design of the saccharification reactor. This article covers experimental determination of kinetic parameters, conversion data, and modeling of the batch-type saccharification reactor, operating with soluble enzyme.

STARCH HYDROLYSIS MODEL

Available kinetic models for starch hydrolysis with amyloglucosidase vary widely in their assumed simplifications and consequent complexity. One of the most elaborate *(7)* takes into consideration all intermediary reactions up to oligomer G_{10}, and consequently, its practical application is relatively complex. It needs a large number of parameters that have to be experimentally determined. On the other hand, some models are very simple, and their application is restricted to the operational conditions of their test *(4)*.

Developments by Marc and coworkers *(8–11)* have led to an intermediate complexity model *(11)* that achieves satisfactory representation of the main phenomena with a limited number of parameters.

This starch hydrolysis model was extensively validated with data taken by Marc *(11)* for different conditions of substrate concentration and glucose inhibition. Tests were also made starting with pure glucose and following the reverse synthesis of dextrins: maltose, maltotriose, and isomaltose. Experimental data and modeling agreed remarkably well, and the model has exibited all of the very important trends observed in the experimental data, such as:

1. The lingering hydrolysis reactions owing to the presence of resistant substrate molecules containing α-1,6 bonds;

2. The slowing down of hydrolysis as conversion increases owing to the reversibility of maltose and maltotriose reactions; and
3. The impossibility of reaching 100% conversion of starch to glucose because of the reversible reactions, including the synthesis of isomaltose at long reaction times.

The aim of this work was to adapt this already validated starch hydrolysis model for the specific application of cassava starch saccharification, measure experimentally the most important model parameters by independent initial velocity tests, and show that these same parameters describe successfully the long-run hydrolysis that is of interest to commercial applications.

Adaptation of Marc's Model *(11)* to Cassava Starch Saccharification

1. Since the substrate is starch that has been extensively hydrolyzed with α-amylase, the average degree of polymerization *(n)* of the remaining oligosaccharides with degree of polymerization >3 was assumed to be 5, based on Reilly's description of α-amylase action *(12)*.
2. These oligosaccharides are considered to be formed of two fractions: (a) one is more susceptible to hydrolysis, comprises approx 77% of the molecules, and contains the α-1,4 chemical bonds that are rapidly hydrolyzed. (b) The second fraction is resistant to hydrolysis, comprises the remaining 23% of the molecules, and contains the α-1,6 chemical bonds associated with branching that are hydrolyzed at a slower rate than the α-1,4 bonds *(11)*.
3. Saccharification proceeds through multiple reactions that occur simultaneously and are divided into three classes:
   a. Hydrolysis reactions of oligosaccharides with degree of polymerization *(n)* >3 are lumped together as a single class that is divided in the two aforementioned fractions—susceptible (G_4) and resistant (G_6):

$$\text{Susceptible oligos. } (G_4) + (n-3)\,H_2O \rightarrow \text{maltotriose } (G_3) + (n-3)\,\text{glucose (G)} \quad (1)$$

$$\text{Susceptible oligos. } (G_6) + (n-3)\,H_2O \rightarrow \text{maltotriose } (G_3) + (n-3)\,\text{glucose (G)} \quad (2)$$

   b. Hydrolysis of maltotriose (G_3) produces maltose (G_2) and is reversible:

$$\text{Maltotriose } (G_3) + H_2O \rightleftharpoons \text{maltose } (G_2) + \text{glucose (G)} \quad (3)$$

   c. Hydrolysis of maltose produces glucose(G) and is reversible:

$$\text{Maltose } (G_2) + H_2O \rightleftharpoons 2\,\text{glucose (G)} \quad (4)$$

4. Glucose can undergo condensation to isomaltose (G_1). This reaction is reversible:

$$2\,\text{glucose (G)} \rightleftharpoons \text{isomaltose } (G_1) \quad (5)$$

5. There is product inhibition (K_i) in the case of glucose (G), for reactions given by Eqs. (1)–(4).
6. There is substrate inhibition (K_S) by oligosaccharides with degree of polymerization >3, but not for maltotriose and maltose.
7. The various substrates (G_4, G_6, G_3, G_2) compete for the amyloglucosidase active site.
8. During saccharification, water in the reaction medium is in excess and therefore its concentration is assumed constant.

9. Thermal deactivation of the enzyme was not considered in this work, since at 45°C and 30% substrate concentration, it was observed that amyloglucosidase stability is high *(1)*.
10. The reaction medium is considered homogeneous and isothermal.

Hydrolysis Rate and Mass Balance Equations for a Batch Reactor

Given the aforementioned considerations, Eqs. (6)–(19) apply:

1. Hydrolysis rate for the susceptible oligosaccharides (r_4):

$$r_4 = V_{m4}\, E\, G_4/[K_{m4}\,(1 + G/K_i) + G_4 + G_2K_{m4}/ K_{m2} + G_3K_{m4}/K_{m3} + G_6K_{m4}/K_{m6} + /K_S] \quad (6)$$

2. Hydrolysis rate for the resistant oligosaccharides (r_6):

$$r_6 = V_{m4}\, E\, G_6/[K_{m6}\,(1 + G/K_i) + G_6 + G_2K_{m6}/ K_{m2} + G_3K_{m6}/K_{m3} + G_4K_{m6}/K_{m4} + /K_S] \quad (7)$$

3. Rate of consumption of susceptible (G_4) and resistant (G_6) oligosaccharides:

$$dG_4/dt = -r_4 \quad (8)$$

$$dG_6/dt = -r_6 \quad (9)$$

4. Rate of hydrolysis of maltotriose (r_3):

$$r_3 = V_{m3}\, E\, (G_3 - G_2\, G)/[K_{eq3}\, K_{m3}(1 + G/K_i) + G_3 + G_2K_{m3}/K_{m2} + G_4K_{m3}/K_{m4} + G_6K_{m3}/K_{m6}] \quad (10)$$

At equilibrium:

$$K_{eq3} = (G_{2eq}\, G_{eq})/G_{3eq} \quad (11)$$

5. Rate of hydrolysis of maltose (r_2):

$$r_2 = V_{m2}\, E\, (G_2 - G^2/K_{eq2})/[K_{m2}\,(1 + G/K_i) + G_2 + G_3K_{m2}/K_{m3} + G_4K_{m2}/K_{m4} + G_6K_{m2}/K_{m6}] \quad (12)$$

At equilibrium:

$$K_{eq2} = G_{eq}^2/G_{2eq} \quad (13)$$

6. Net rate of formation of maltotriose and maltose:

$$dG_3/dt = r_4 + r_6 - r_3 \quad (14)$$

$$dG_2/dt = r_3 - r_2 \quad (15)$$

7. Rate of formation of isomaltose (r_I) resulting from the α-1,6 con-densation of two molecules of glucose (Eq. [5]):

$$r_I = V_{IM}\, E\, (G^2 - G_I/K_{eqI}) \quad (16)$$

At equilibrium:

$$K_{eqI} = G_{Ieq}/G_{eq}^2 \quad (17)$$

$$dG_I/dt = r_I \quad (18)$$

8. Net rate of glucose formation:

$$dG/dt = (n - 3)\,(r_4 + r_6) + r_3 + 2\,(r_2 - r_I) \quad (19)$$

MATERIALS AND METHODS

Substrate

The substrate is cassava starch preliquefied and extensively hydrolyzed with NOVO Termamyl α-amylase, at 95°C for 1 h *(1,6)*.

Enzyme

For saccharification NOVO amyloglucosidase-AMG 200 L was used with an activity of 5358.4 U/mL at 45°C, pH 4.5, 30% (w/v) substrate concentration, and protein contents equal to 124.4 mg/mL of stock solution.

Reactor

The reactor used for saccharification tests was made of glass with a total volume of 500 mL and mounted on a water bath shaker with thermostatic control.

Initial Rate of Saccharification and Substrate Inhibition Test

The substrate solution was diluted to various concentrations (total of 20 from 2–300 g/L), and 50 mL of the diluted solutions were warmed to 45°C in a batch reactor. When this temperature was reached, a sample of 0.5 mL was taken for blank assays. Then 1 mL of the enzyme solution diluted 1:20 was added. Samples were taken from the reaction mixture at regular intervals, diluted 1:10, and boiled for 10 min. The samples were kept at 4°C until assayed for glucose. Total reaction time decreased from 30 min used for more concentred solutions down to 16 min for more diluted, so that the rate of reaction could be considered approximately constant. This test was used for the determination of K_{m4}, V_{m4}, and K_S.

Kinetics of Maltose Hydrolysis

To test for maltose kinetics, a similar experiment to the above-described experiment was conducted for maltose. In this case, concentrations varied from 2 to 50 g/L, and the enzyme stock solution was diluted 1:200. The results were used for determination of K_{m2} and V_{m2}.

Starch Conversion Tests in the Batch Reactor

With the objective of evaluating the proposed starch hydrolysis model, two tests were run with different total initial glucose concentration in the substrate solution: Test 1 had no glucose added, and test 2 had 100 g/L of glucose added. The other conditions remained the same, namely: volume of substrate solution (400 mL), starch concentration (300 mg/mL), amyloglucosidase stock solution added (0.4 mL), temperature (45°C), pH 4.5, duration of the test (30 h). Samples were taken at 5-min intervals in the beginning of the reaction and at longer intervals near its end. These samples were treated as described for the test on the initial rate of saccharification.

Conversion of the multiple substrates present in the liquefied starch solution (G_2, G_3, G_4, G_6) was calculated on a mass basis by the following equation:

$$X_A = 100\, f\, (C_g - C_{gi})/[C_{Ao} - f\, (C_{gi} - C_{ga})] \quad (20)$$

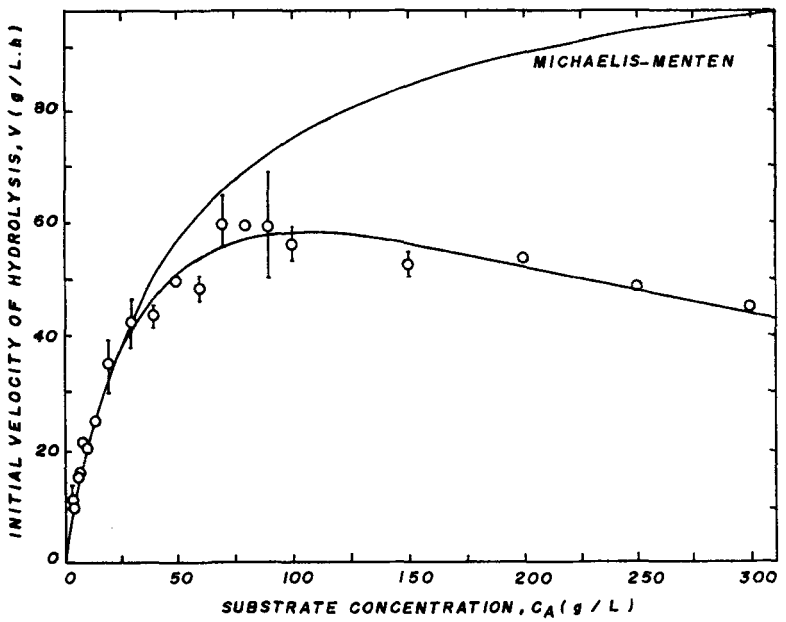

Fig. 1. Initial rate of glucose production (V), as a function of substrate concentration (C_A). Reaction conditions: 45°C, pH 4.5, 1 mL of stock enzyme/L of substrate solution.

Analytical Methods

The following assay methods were used: high performance liquid chromatography (HPLC) to determine the contents of the saccharides (G, G_2, G_3, and > G_3) in the liquefied starch, *ortho*-toluidine method *(13)* for glucose assay in samples taken at regular intervals from the reactor, and Lowry method *(14)* to assay protein contents.

RESULTS AND DISCUSSION

The HPLC analysis of the initial substrate solution has given the following concentrantions: 14.73 g/L glucose (G), 42.63 g/L maltose (G_2), 53.37 g/L maltotriose (G_3), 189.27 g/L oligosaccharides (G_4 + G_6).

Figure 1 presents the initial rate of glucose formation as a function of substrate concentration. It can be clearly seen that, for a concentration of hydrolyzed cassava starch above 30 g/L, there is substrate inhibition. Maximal rates of saccharification are observed around 90 g/L, whereas at 300 g/L (typical industrial starch concentration), the rate of saccharification is about 25% slower.

Since the product glucose was measured, and susceptible oligosaccharides (G_4) hydrolyze first, the molar initial rate of hydrolysis was calculated by:

$$V_{m4} = V/[(n - 3)\, M_{G4}\, E] \quad (21)$$

Where the factor $(n - 3)$ comes from the stoichiometry of Eq. (1).

Following Marc *(11)*, susceptible oligosaccharides concentration (G_4) was taken as 77% of the HPLC reading for oligosaccharides with degree of polymerization >3. Then the parameters K_{m4}, V_{m4}, and K_S were obtained by adjusting a polynomial of the second degree for (G_4/V_{m4}) as a function of G_4 *(15)*. The resulting parameters are given in Table 1.

Table 1
Kinetic Parameters for the Cassava Starch Hydrolysis Model

	Parameter	Parameter values
Equilibrium constants	K_{eq2}	200 mol/L[a]
	K_{eq1}	0.0544 L/mol[b]
	K_{eq3}	8 mol/L[a]
Michaelis-Menten constants	K_{m2}	0.0030 mol/L[d]
	K_{m3}	0.0264 mol/L[e]
	K_{m4}	0.0264 mol/L[d]
	K_{m6}	0.0264 mol/L[e]
Inhibition constants	K_i	0.040 mol/L[h]
	K_S	0.126 mol/L[d]
Maximum velocities		
	V_{m2}	0.0777 mol/h mL enzyme[d]
	V_{m3}	0.311 mol/h mL enzyme[f]
	V_{m4}	0.306 mol/h mL enzyme[d]
	V_{m6}	0.153 mol/h mL enzyme[g]
	V_{IM}	0.000109L^2/mol h mL enzyme[c]

[a]Values as given by Marc's work *(11)*.
[b]As in a, but corrected for temperature.
[c]As in b, but also corrected for greater enzyme activity.
[d]Experimentally determined in this work from initial velocity of hydrolysis.
[e]Following Marc's work *(11)*, it was assumed equal to K_{m4}.
[f]Assumed to be 4 times V_{m2}. Literature (11) values range from 1.3 to 6.4 times V_{m2}.
[g]Hydrolysis rate for resistant oligosaccharides was taken as half of that for susceptible oligosaccharides ($V_{m6} = V_{m4}/2$).
[h]The only adjustable parameter to fit data of Fig. 2.

Experimental results obtained for the initial rate of maltose (G_2) hydrolysis were used to determine K_{m2} and V_{m2}, and the observed behavior followed Michaelis-Menten kinetics. Then a linear least-square fit in the form of Hanes-Woolf *(16)* plot was used. Table 1 shows the computed values. To compare the data obtained in the conversion tests with the proposed model, further assumptions were used. These are included as footnotes to Table 1.

Table 1 contains the complete set of parameters used, whereas **Fig. 2** compares the conversion data points with modeling. Agreement is good, and the same set of parameters can represent equaly well both test situations: no glucose added to the liquefied starch and 100 g of glucose added. This confirms that parameters given in Table 1 are adequate for modeling cassava starch saccharification.

For comparison, the batch reactor conversion data were also adjusted to the simpler model of our earlier work *(4)*. This starch hydrolysis model is already general in the sense that it includes substrate and product inhibition. However, all dextrines are grouped in a single substrate. The integrated equation that relates normalized residence time (τ_b) and conversation (X_A) in a batch reactor is:

$$\tau_b = \alpha X_A - \beta \ln(1 - X_A) - \gamma X_A^2 \qquad (22)$$

giving Test 1, $\alpha = -0.12952$, $\beta = 0.31440$, and $\gamma = 0.14936$; and Test 2, $\alpha = 0.07947$, $\beta = 0.06981$, and $\gamma = -0.32489$.

The consequence of considering a single substrate and a single reaction while there are various substrates and reactions is that a negative value was obtained for

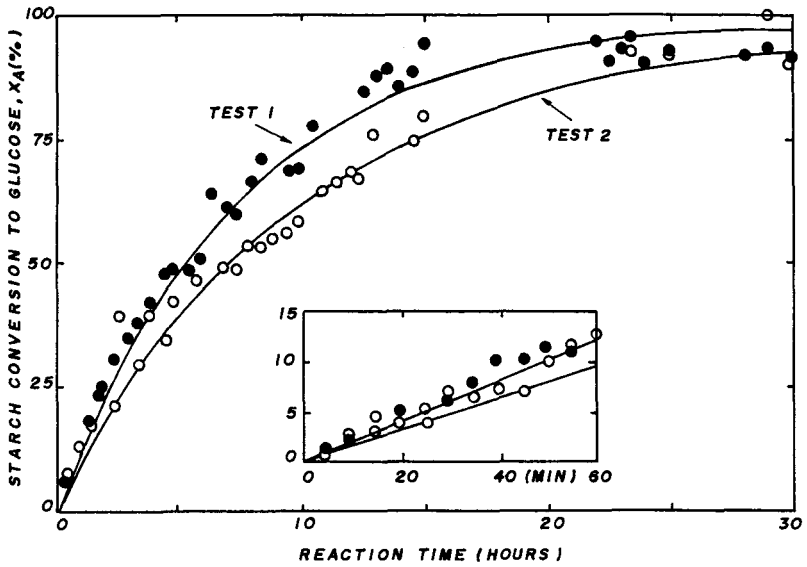

Fig. 2. Batch reactor saccharification data and modeling results. Reaction conditions: 45°C, pH 4.5, 1 mL of stock enzyme/L of substrate solution, liquefied starch concentration, 300 g/L. Test 1, no glucose added; test 2, 100 g/L of glucose added.

the parameter γ, implicating a negative value for k_{cat} that is not physically meaningful. Moreover, the glucose inhibition constant assumes different values if calculated either from the value of parameter α or β. It results also in different for each test. This confirms the need to use a model that takes into account a more detailed description of the complex kinetics of starch saccharification, such as the adaption of Marc's model.

The adapted model contains 11 equations (6 differential) that have been solved *(1)* with a special-purpose computer program based on the method of Runge-Kutta of 4th order. This program is avaliable on request. The model was also validated by its application to fixed- and fluidized-bed reactors, using immobilized enzyme and various test conditions. These results are being prepared for a subsequent publication.

Given the good fit obtained with the adapted model and the relative simplicity of using it, different case studies of potential industrial application can be easily run.

CONCLUSIONS

1. Marc's model adapted well to the description of the hydrolysis of cassava starch.
2. Although actual reactions in starch hydrolysis are in great number and are very complex, the assumed simplifications lead to a model of an intermediate complexity that is relatively easy to use.
3. Given the kinetic parameters, the adapted model is able to represent well the saccharification of liquefied starch even in the presence of glucose inhibition.
4. The present model inspires trustworthy predictions for different reaction conditions, allowing different case studies to be considered for industrial applications.

ACKNOWLEDGMENTS

This work was supported by CNPq, CONCITEC, FINEP, and the State University of Maringá.

REFERENCES

1. Zanin, G. M. (1989), Sacarificação de amido em reator de leito fluidizado com enzima amiloglicosidase imobilizada. Ph. D. Thesis, Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, Campinas-SP, Brazil.
2. Zanin, G. M. and de Moraes, F. F. (1984), Proceedings of XII Encontro sobre Escoamento em Meios Porosos, Maringá-PR, Brazil, **October, I,** 267–285.
3. Zanin, G. M., Neitzel, I., and de Moraes, F. F. (1993), *Appl. Biochem. Biotechnol.* **39/40,** 477–489.
4. Zanin, G. M., Kambara, L. M., Calsavara, L. P. V., and de Moraes, F. F. (1994), *Appl. Biochem. Biotechnol.* **45/46,** 627–639.
5. Zanin, G. M. and de Moraes, F. F. (1995), *Appl. Biochem. Biotechnol.* **51/52,** 253–262.
6. Zanin, G. M. and de Moraes, F. F. (1988), *Revista Microbiologia* **20,** 367–371.
7. Lee, D. D., Lee, G. K., Reilly, P. J., and Lee, Y. Y. (1980), *Biotechnol. Bioeng.* **22,** 1–17.
8. Marc, A., Duc, G., and Engasser, J. M. (1984), *Proceedings of Third European Congress on Biotechnology,* vol. II, Springer-Verlag, pp. 103–108.
9. Marc, A., Engasser, J. M., Moll, M., and Flayeux, R. A. (1983), *Biotechnol. Bioeng.* **25,** 481–496.
10. Beschkov, V., Marc, A., and Engasser, J. M. (1984), *Biotechnol. Bioeng.* **26,** 22–26.
11. Marc, A. (1985), Cinétique et modelisation de réacteurs á glucoamylase soluble et immobilisée. Docteur d'Etat thesis, Institute Polytechnique de Lorraine, France.
12. Reilly, P. J. (1985), in *Starch Conversion Technology,* Van Beynum, G. M. A. and Roels, J. A., eds., Marcel Dekker, New York, pp. 101–114.
13. Cooper, G. R. and McDaniel, V. (1970), *Clin. Chem.* **6,** 159–170.
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. J. (1975), *Biol. Chem.* **193,** 265–275.
15. Van Den Heuvel, J. C., and Beeftink, H. H. (1988), *Biotechnol. Bioeng.* **31,** 718–724.
16. Segel, I. H. (1975), *Enzyme Kinetics–Behavior and Analysis of Rapid Equilibrium and Steady–State Enzyme Systems,* John Wiley, New York, pp. 210,211.