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Kinetic and Mass Transfer Parameters of Maltotriose Hydrolysis Catalyzed by Glucoamylase Immobilized on Macroporous Silica and Wrapped in Pectin Gel

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Abstract

Kinetic and mass transport parameters were estimated for maltotriose hydrolysis using glucoamylase immobilized on macroporous silica and wrapped in pectin gel at 30°C. Free enzyme assays were used to obtain the intrinsic kinetic parameters of a Michaelis-Menten equation, with product inhibition by glucose. The uptake method, based on transient experimental data, was employed in the estimation of mass transfer parameters. Effective diffusivities of maltotriose in pectin gel were estimated by fitting a classical diffusion model to experimental data of maltotriose diffusion into particles of pectin gel in the absence of silica. The effective diffusivities of maltotriose in silica were obtained after fitting a bidisperse model to experimental data of maltotriose hydrolysis using glucoamylase immobilized in silica and wrapped in pectin gel.

Index Entries: Maltotriose hydrolysis; kinetic parameters; effective diffusivities; macroporous silica; pectin gel.

Introduction

This work was part of a project that studied an unconventional continuous bioreactor configuration for the simultaneous hydrolysis and fermentation of liquefied starch designed to produce ethanol using

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glucoamylase immobilized on silica and baker's yeast entrapped in pectin gel. In this process, the enzyme is covalently linked on controlled pore silica after silanization (with γ-aminopropylethoxysilane) and activation of the support with glutaraldehyde. The silica, containing enzyme, is later coimmobilized with baker's yeast in 4-mm-diameter spherical particles of pectin gel. This biocatalyst may be used in a continuous fixed-bed reactor *(1,2)*. To simulate this reactor, the reaction kinetic and mass transport parameters of the immobilized enzyme must be known. The process substrate, liquefied starch, is a mixture of glucose, maltose, maltotriose, maltotetraose, and maltopentaose. Ono et al. *(3)* report that maltose and maltotriose have the slowest rate of hydrolysis in this process. The present study focuses on the second substrate. The hydrolysis of maltose has been studied previously *(4,5)*.

Immobilization causes a deliberate restriction on the mobility of enzymes and cells, which can affect the mobility of solutes. These phenomena may reduce the apparent reaction rate and, consequently, may decrease the process efficiency, when compared to that for soluble enzymes *(6)*. A reduced apparent reaction rate can also result from external diffusion restrictions on the surface of supports. To prevent this effect, a batch system with vigorous agitation was used. Although silica particles are extremely fragile with respect to shear stress, the pectin gel that surrounds these particles protects them against the action of the stirrer. The uptake method, used here, is a classic methodology for the estimation of effective diffusivities in gel beads. It employs whole beads of the biocatalyst, preserving the shape they possess in the actual industrial process.

This approach, however, is transient in nature and requires a careful design of the experiments in order to minimize errors related to the time of sampling *(7)* and to the presence of adsorptive effects. Solute adsorption can lead to different concentrations inside and outside the support, therefore disguising the actual diffusion effects. This phenomenon, if present, must be taken into account for solutes that might interact with the gel by ionic or adsorptive forces. Gonçalves et al. *(5)*, however, observed that the adsorption of glucose, maltose, and lactose in pectin gel was not significant for the conditions used during the reaction experiments. Consequently, adsorption effects were not considered here.

The main purpose of the present study was to determine the kinetic parameters of maltotriose hydrolysis and effective diffusivities of maltotriose in macroporous silica containing immobilized enzyme wrapped in citric pectin gel. It was necessary to perform preliminary assays with free glucoamylase to estimate intrinsic kinetic parameters for maltotriose hydrolysis, under the operational conditions found in the bioreactor. It was assumed that only one amino group of the enzyme was bound to the activated support during the immobilization process. Therefore, the immobilization would not change significantly the conformation of the enzyme. So intrinsic and inherent kinetics would be essentially the same. This hypothesis was based on the reactivity of the amino and aldehyde

groups present, respectively, in the enzyme and on the support. In this work, the immobilization reaction was proceeded at pH 4.2. Acidic pH favors the reactivity of aldehyde groups, but at low pH few amino groups will be uncharged and able to act as nucleophiles in the immobilization reaction. That low concentration prevents the formation of multipoint bonds between the same enzyme molecule and the activated support *(8)*.

Materials and Methods

Chemicals

Soluble starch (mol wt = 29,400) was purchased from Merck, and maltotriose was purchased from Omega. All other chemicals were of laboratory grade, from commercial suppliers.

Enzyme

Glucoamylase from *Aspergillus niger* was donated by Novo Nordisk Brazil (Araucária, Paraná, Brazil) (activity: 180 U/mL).

Supports

High-porosity silica, with an average diameter of 170 μm, porosity of 0.57, and mean pore diameter of 270 Å (measured by N_2 desorption, ASAP 2000; Micrometrics, Araraquara, São Paulo, Brazil) and low methoxylation citric pectin (type 8002) were donated by Braspectina do Brasil (Limeira, São Paulo, Brazil).

Enzyme Activity

Enzyme activity was evaluated by measuring the glucose released during hydrolysis of starch, 40 g/L in 100 m*M* acetate buffer, pH 4.2. The difference between enzymatic activities of the supernatant before and after immobilization was used to assess the silica enzyme loading. One unit of enzyme activity was defined as the amount of enzyme that yields 1 g of glucose/(L·h) from 40 g/L of soluble starch at 60°C and pH 4.2.

Enzyme Immobilization on Silica (5)

Silica was silanized with a solution of γ-aminopropyltriethoxysilane (0.5 [v/v]) at pH 3.3 and 75°C for 3 h with a liquid-solid ratio of 3.0 mL/g. Then, the catalyst was washed with water and acetone. It was later dried until no further change in weight occurred.

The support was activated with glutaraldehyde (in a 2.5% sodium hydrogenophosphate buffer, 0.1 *M* and pH 7.0) for 1 h at 20–25°C, with a liquid-solid ratio of 3.0 mL/g. It was washed again and reacted with the enzyme solution for 36 h at 20–25°C, pH 4.2, under stirring.

Preparation and Characterization of Biocatalyst

Six grams of citric pectin was added to 88 g of water and 6 mL of acetate buffer (1 *M* at pH 4.2). A known amount of silica containing immobilized

enzyme was added to the resulting solution and trickled into 0.2 *M* $CaCl_2$ under mild stirring. The gel particles were then cured at 4°C for 18–24 h. Before each run, the mean particle diameter was calculated using a picnometer, with a sample of 500 spherical gel particles. The gel porosity was obtained by gravimetric analysis and its density using a picnometer.

Analytical Methods

Glucose was determined by the glucose-oxidase method (GOD-PAP; Merck) and maltotriose was measured by reducing sugar analysis *(9)*.

Experimental Procedure

A batch reactor with magnetic stirring was used in all the experiments described herein. Enzymatic hydrolysis was carried out in 100 m*M* acetate buffer solution, pH 4.2, at 30°C. For all assays, 0.1-mL samples were taken periodically. The stirring was high enough to avoid the effects of external mass transport resistance. The stirring was increased until the concentration vs time curve of the product was no longer influenced by this variable.

Initial reaction rates of maltotriose hydrolysis were performed in the absence of glucose (product) in order to estimate the Michaelis-Menten parameters $V_m = k_3 E_t$ and K_m using linear regression (classic Lineweaver-Burk method *[10]*) and nonlinear regression (Marquardt algorithm). All other assays were performed in a batch reactor.

Mathematical Models

Three different models were used in this work: a homogeneous model to estimate intrinsic kinetic parameters *(5)*, a diffusion model *(11)* for the effective diffusion coefficient of maltotriose in pectin gel, and a diffusive-reactive model *(4)* (bidisperse model) to estimate the effective diffusivity of maltotriose in silica wrapped in pectin gel in the presence of reaction. The diffusion and bidisperse models assume negligible external mass transfer resistance, invariant effective diffusivities, enzyme homogeneously immobilized on the silica, and constant temperature. Solute concentrations are based on the volume of the gel available for diffusion (volume of pores).

Intrinsic Kinetics of Maltotriose Hydrolysis

A Michaelis-Menten equation with competitive product inhibition by glucose was used to model the kinetics of maltotriose hydrolysis (Eq. 1):

$$-R_M = \nu R_G = \frac{k_3 E_t C_M}{K_m [1 + (C_G/k_i)] + C_M} \quad (1)$$

This is a simplified, lumped-parameter model of the hydrolysis.

Homogeneous Model

The mass balance for the batch reactor with free enzyme is as follows:

$$\frac{dC_{M1}}{dt} = -R_M \tag{2}$$

$$\frac{dC_{G1}}{dt} = \nu R_G \tag{3}$$

The ordinary differential Eqs. 2 and 3 were solved numerically, using the algorithm described earlier *(12)*. These equations were used to estimate the inhibition constant, k_i, in Eq. 1, using a maximum likelihood method *(13,14)*. This routine takes into account random errors, with normal distribution, in all measured variables (except time, which is assumed to be error free). The experimental variance is 0.25 $(g/L)^2$ for glucose. Since the analytical method to measure maltotriose is less precise, only glucose data were used to estimate the kinetic parameters (k_3, K_m, k_i).

Diffusion Model

The fundamental equations are as follows:

Solute mass balance at the surface of the gel beads for component *i*:

$$\frac{dC_{il}}{dt} = \frac{\partial C_i}{\partial t}\bigg|_{\zeta=1} = -\frac{3}{R_p^2}\frac{(1-\varepsilon_r)}{\varepsilon_r}\varepsilon_{gp}D_{e,ig}\frac{\partial C_i}{\partial \zeta}\bigg|_{\zeta=1} \tag{4}$$

*i.c.: $t = 0$, $C_i = C_i(0)$

Solute mass balance inside the gel for component *i*:

$$\frac{\partial C_i}{\partial t} = \frac{1}{R_p^2}\frac{D_{e,ig}}{\zeta^2}\frac{\partial}{\partial \zeta}\left(\zeta^2\frac{\partial C_i}{\partial \zeta}\right) \tag{5}$$

i.c.: $t = 0$, $C_i = (0)$; **b.c.1: $\zeta = 0$, $\partial C_i/\partial\zeta = 0$; †b.c.2: $\zeta = 1$, $C_i(\zeta) = C_{il}$.

Equations 4 and 5 were solved numerically. The problem was discretized in the space coordinate using orthogonal collocation *(15)*, using Jacobi polynomials, $P^{(0,1/2)}$. Tests with different numbers of collocation points showed that five internal nodes gave accurate results.

When the solution is approximated by polynomials, the order of the problem is reduced. The resulting set of differential ordinary equations (which have time as independent variable) was solved numerically *(12)*. In all simulations, the volume retrieved when each sample is taken (0.1 mL) was accounted for. The fitting parameters in these equations were the effec-

*i.c., initial conditions; **b.c.1, boundary condition 1; †b.c.2, boundary condition 2.

tive diffusion coefficients. Again, a maximum likelihood approach was employed *(13,14)*.

Bidisperse Model

The fundamental equations are as follows:

Solute mass balance at the surface of the gel beads for component *i*:

$$\frac{dC_{il}}{dt} = \frac{\partial C_i}{\partial t}\bigg|_{\zeta=1} = -\frac{3}{R_p^2}\frac{(1-\varepsilon_r)}{\varepsilon_r}\varepsilon_p D_{e,ig}\frac{\partial C_i}{\partial \zeta}\bigg|_{\zeta=1} \tag{6}$$

i.c.: $t = 0$, $C_i = C_i(0)$

Solute mass balance inside the gel for component *i*:

$$\varepsilon_{gp}\frac{\partial C_{ig}}{\partial t} = \frac{\varepsilon_p}{R_p^2}\frac{De_{ig}}{\zeta^2}\frac{\partial}{\partial \zeta}\left(\zeta^2\frac{\partial C_{ig}}{\partial \zeta}\right) - \frac{3\varepsilon_S}{R_S^2}\left(\frac{\varepsilon_p - \varepsilon_{gp}}{\varepsilon_S - \varepsilon_{gp}}\right)\left(De_{iS}\frac{\partial C_{iS}}{\partial \zeta_S}\right)\bigg|_{\zeta=1} \tag{7}$$

i.c.: $t = 0$, $C_i = (0)$; b.c.1: $\zeta = 0$, $\partial C_{ig}/\partial\zeta = 0$; b.c.2: $\zeta = 1$, $C_{ig}(\zeta) = C_{il}$.

Solute mass balance inside the silica for component *i*:

$$\varepsilon_S\frac{\partial C_{iS}}{\partial t} = \frac{\varepsilon_S}{R_S^2}\frac{D_{e,iS}}{\zeta_S^2}\frac{\partial}{\partial \zeta_S}\left(\zeta_S^2\frac{\partial C_{iS}}{\partial \zeta_S}\right) - R_M \tag{8}$$

i.c.: $t = 0$, $C_{iS} = (0)$; b.c.1: $\zeta = 0$, $\partial C_{iS}/\partial\zeta = 0$; b.c.2: $\zeta = 1$, $C_{iS}(\zeta_S) = C_{ig}(\zeta)$.

Equations 6–8 were solved numerically, using the same approach described in the previous section. Effective diffusion coefficients were obtained using a direct search procedure.

Results and Discussion

Kinetic Parameters of Maltotriose Hydrolysis

To estimate the kinetic parameters of maltotriose hydrolysis, initial rates and transient assays were used. The initial rates of reaction were obtained after a linear regression of glucose concentration (product of this reaction) vs time for different initial concentrations of maltotriose ($S^0 = 1.78$, 4.52, 9.04, and 19.77 g/L). Values of V_m and K_m obtained from a Lineweaver-Burk plot (Fig. 1) were the initial guesses for a nonlinear, least-squares, Marquardt algorithm (Fig. 2). Finally, transient assays were performed to estimate k_i using the maximum likelihood method (Fig. 3). Table 1 shows the resulting parameters, together with some other values from the literature. The last ones correspond to different conditions and sub-

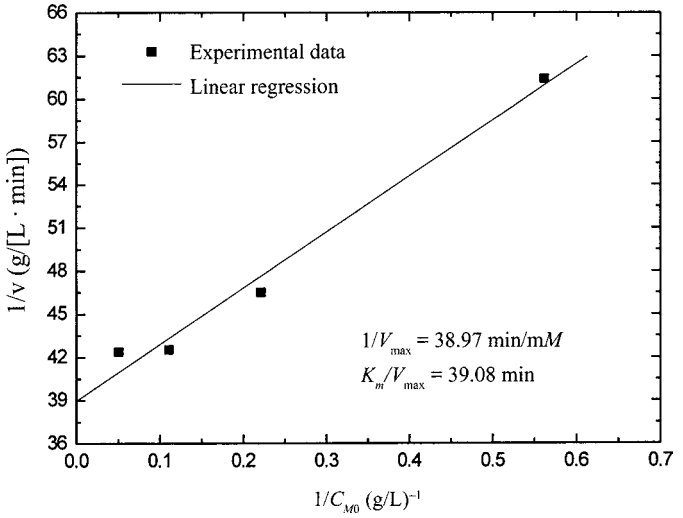

Fig. 1. Lineweaver-Burk diagram for maltotriose hydrolysis using free glucoamylase at pH 4.2 and 30°C.

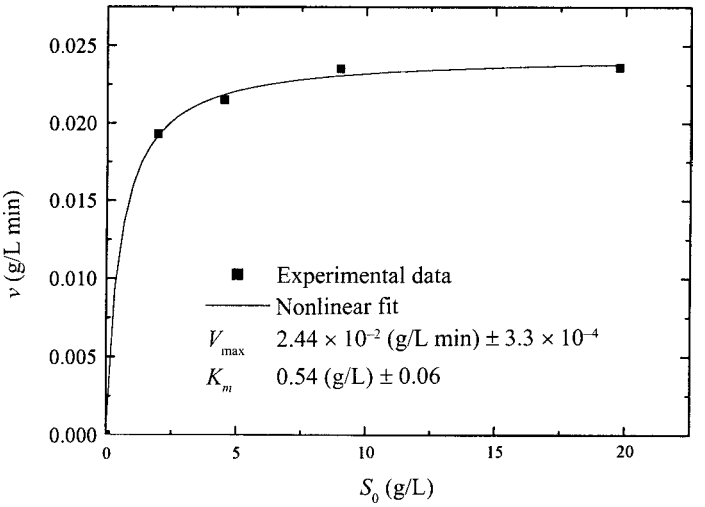

Fig. 2. Nonlinear fitting to maltotriose hydrolysis using free glucoamylase at pH 4.2 and 30°C.

strates, but their order of magnitude is in the same range. Although a lumped and simplified model was used to represent the hydrolysis reaction, it can be seen that K_m values, in molar basis, decrease sharply from maltose to starch, as already expected, and that k_3 values show the correct tendency *(3)*. A sharp increase in this parameter from maltose to starch was expected. Since the fitted model represents the experimental data well, it can be used in the simulation of the global process, in which the biocatalyst contains enzyme and microorganism coimmobilized in pectin

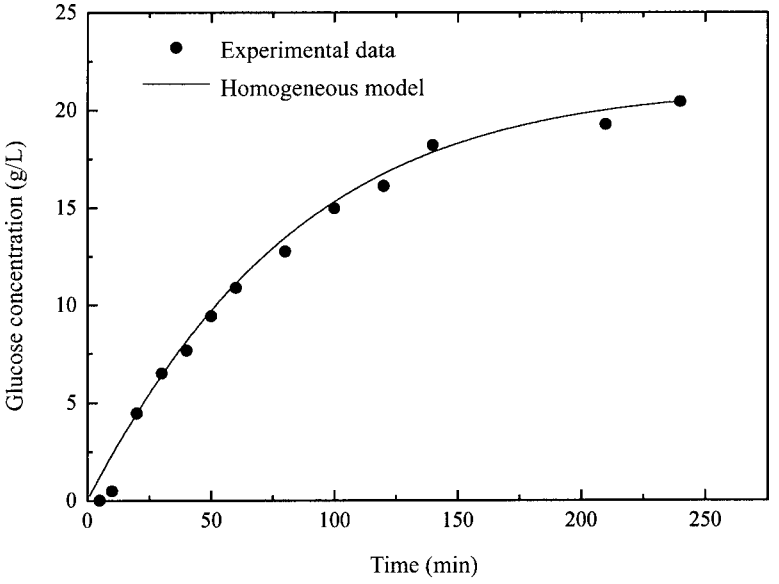

Fig. 3. Glucose concentration formed by maltotriose hydrolysis at pH 4.2 and 3°C using free enzyme (0.19 U/mL) and C_{M0} = 25.76 g/L.

Table 1
Kinetic Parameters of Maltotriose, Maltose, and Starch Hydrolysis Using Glucoamylase at pH 4.2[a]

Parameter	Maltotriose, 30°C (this study)	Maltose, 30°C *(5)*	Starch, 30°C *(16)*	Maltotriose, 15°C *(13)*
K_m (g/mL)	$4.5 \times 10^{-4} \pm 6 \times 10^{-5}$	6.1×10^{-4}	5.0×10^{-4}	1.0×10^{-4}
K_m (M)	8.9×10^{-4}	17.8×10^{-4}	0.17×10^{-4}	2.0×10^{-4}
k_3 (g/[U·s])	$2.1 \times 10^{-5} \pm 3 \times 10^{-7}$	0.9×10^{-5}	3.7×10^{-5}	—
k_i (g/mL)	$5.2 \times 10^{-4} \pm 1 \times 10^{-6}$	1.2×10^{-1}	3.6×10^{-4}	—

[a]Confidence interval of 95%.

gel. Because the literature reports that glucoamylase has a multichain mechanism and the release of maltose was not considered here, more experiments measuring maltose and glucose concentrations are needed to confirm the obtained results.

The constants in Table 1 will be used to describe the kinetics of the immobilized enzyme; in other words, intrinsic and inherent rates are considered equal. This is a reasonable hypothesis, since immobilization was carried out at pH 4.2.

Under immobilization conditions, the low concentration of uncharged amine groups prevents the formation of multipoint bonds between the same enzyme molecule and the activated support *(8)*.

Table 2
Effective Diffusion Coefficients in Pectin Gel Beads at 30°C

Substrate/gel	Effective diffusivity ($\times 10^6$ cm^2/s)	Molecular diffusion coefficient in water ($\times 10^6$ cm^2/s)[a]
Maltotriose/citric pectin (this work)	4.19 ± 0.1	4.63
Maltose/citric pectin *(4)*	4.49	5.98
Glucose/citric pectin *(4)*	5.29	
Glucose/κ-carrageenan *(17)*	3.83	6.29

[a]Calculated using Wilke-Chang method *(14)*.

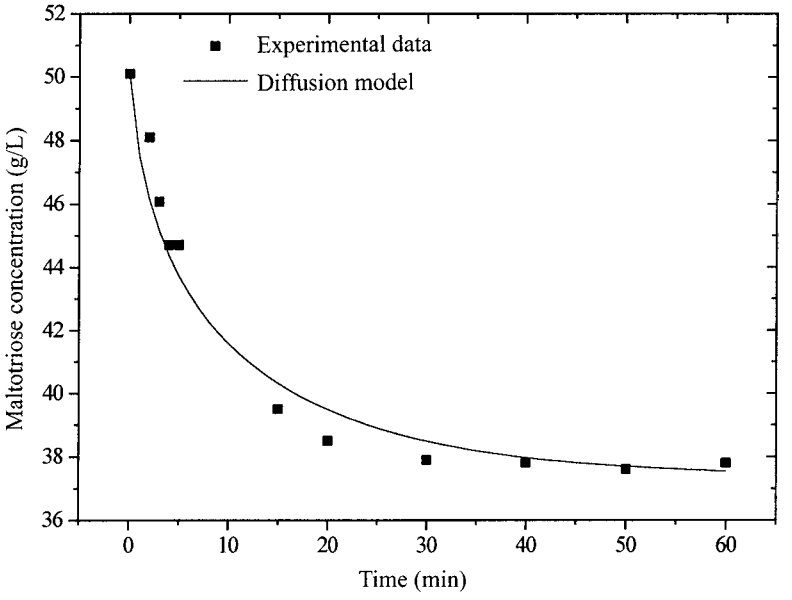

Fig. 4. Maltotriose diffusion into pure pectin particle when D_p = 0.21 cm and ε_{gp} = 0.97 at 30°C.

Consequently, there will be minimum conformational changes in the glucoamylase tertiary structure owing to the immobilization.

Effective Diffusion Coefficient of Maltotriose in Pectin Gel

The effective diffusion coefficient of maltotriose in pectin gel was estimated by fitting the diffusion model (Eqs. 4 and 5) to experimental data of maltotriose diffusion into particles of pectin gel in the absence of silica. Table 2 compares the effective diffusion coefficients of maltotriose, maltose, and glucose in two different gels with their respective molecular diffusion coefficients in water.

The fitting of the model for the maltotriose diffusion experiments is shown in Fig. 4. We can observe that effective diffusivities are slightly

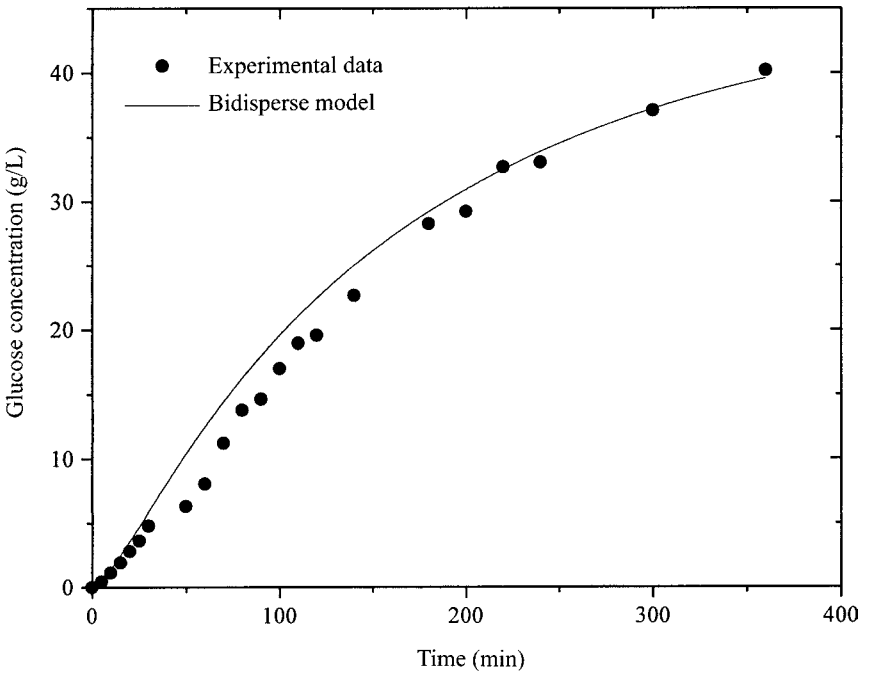

Fig. 5. Glucose production during maltotriose hydrolysis using glucoamylase immobilized on silica and wrapped in pectin gel at 30°C and pH 4.2.

Table 3
Effective Diffusivities
of Different Substrates in Silica and in Pectin Gel at 30°C

Substrate	Effective diffusivity in gel (cm^2/s)	Effective diffusivity in silica (cm^2/s)
Glucose	5.29×10^{-6} *(8)*	0.55×10^{-6} *(5)* 1.08×10^{-6}[b] 1.77×10^{-9}[c]
Maltose	4.49×10^{-6} *(8)*	0.50×10^{-6} *(5)*
Maltotriose	4.18×10^{-6}[a]	0.45×10^{-6}[a]

[a]Values estimated in this work.
[b]Silica porous diameter = 116 Å *(18)*.
[c]Silica porous diameter = 74 Å *(18)*.

lower than the molecular coefficient. This is an expected result, because the gel matrices have large-diameter pores.

Effective Diffusion Coefficient of Maltotriose in Macroporous Silica

Hydrolysis of maltotriose was performed using glucoamylase immobilized on silica and wrapped in pectin gel. The bidisperse model was used to estimate the effective diffusivity of maltotriose in the silica pores using the $D_{e,mg}$ obtained previously. The results are portrayed in Fig. 5 and the

estimated coefficient is given in Table 3. The estimated coefficient was compatible with other researchers' values and one order of magnitude smaller than the molecular diffusion coefficient in water (Table 3). A good representation of the experimental data is observed. The small, systematic deviation of the model from the empirical data may be caused by the assumption that there is no release of maltose during the hydrolysis, as already discussed.

Conclusion

The estimated kinetic parameters for maltotriose hydrolysis catalyzed by gluoamylase and its effective diffusion coefficients in pectin gel and silica agreed with other researchers' data *(5,11,17,18)*. The bidisperse model represents the experimental data well, and it can be used in the simulation of the global process, in which the biocatalyst contains enzyme and microorganism coimmobilized in pectin gel.

Acknowledgments

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Nomenclature

C_G = glucose concentration (g/mL)
C_M = maltotriose concentration (g/mL)
$D_{e,mg}$ = effective diffusivity of maltose (or component *i*) in the gel (cm^2/s)
$D_{e,mS}$ = effective diffusivity of maltose (or component *i*) in the silica (cm^2/s)
E_t = enzyme reactor load ($U/cm^3_{reactor}$)
k_3 = kinetic constant (g/[U·s])
k_i = product inhibition constant (g/mL)
K_m = Michaelis-Menten constant (g/mL)
R_G = glucose rate of formation (g/[mL·s])
R_M = maltotriose rate of hydrolysis (g/[mL·s])
R_P = particle radius (cm)
$V_m = k_3E_t$ = maximum rate (g/[mL·s])

Subscripts

i = component
l = liquid
g = pectin gel
S = silica
ε_{gp} = pure gel porosity
ε_p = biocatalyst porosity

ε_r = bed porosity
ε_S = silica porosity
ς = a dimensional radius
ν = stoichiometric coefficient (mass base)

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