Role of Diffusion in Biocatalytic Polytransesterification

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The solvent-free enzyme-catalyzed polytransesterification of divinyl adipate and 1,4-butanediol yielding high molecular-weight polyesters was studied. This heterogeneous system is characterized by initial exothermicity, a 10^4-fold increase in viscosity, and complex kinetics involving parallel reactions and variable reaction volumes. Herein a semitheoretical analysis of solvent-free polytransesterification was developed to further refine understanding of the role of diffusion, especially how molecular weight and polydispersity evolve during solvent-free biocatalytic polytransesterification. The evolution of polydispersity observed during the polymerization was attributed to diffusion, and therefore internal diffusion limitations were assessed experimentally. The analysis demonstrated that the system is initially under weak diffusional control, which is strengthened by the initial exothermicity of the reaction. At molecular weights over 5,000 Da, the system experienced severe mass-transfer resistance due to chain entanglements. Reduced enzyme specificity with increasing chain length, enzyme deactivation, and vinyl hydrolysis dampen the diffusional constraints toward the end of the polymerization such that the system could return to slight diffusional or kinetic control on exceeding molecular weights of 20,000 Da.

Introduction

A number of nonaqueous biotransformations have been studied and standard equations to aid in assessing the role of diffusion for such systems have been developed (Kamat et al., 1992). In this article, we have used a semitheoretical approach to further refine our understanding of the role of diffusion in the enzyme-catalyzed polytransesterification of divinyl adipate (DVA) and 1,4-butanediol (BD) under solvent-free conditions. Polyesters, which form an important class of polymers (Goodman, 1988), contain carboxylate ester groups in the repeat unit and have been produced enzymatically by self-condensation of hydroxy-carboxylic acids (Knani et al., 1993) or polycondensation of a dicarboxylic acid or its derivatives with a diol (Okumura et al., 1984; Brazwell et al., 1989; Margolin et al., 1987; Binns et al., 1993). Enzyme-catalyzed polytransesterification is effective at moderate temperatures and is specific with respect to the reactants and reaction site (Kline et al., 1998). We have been studying the synthesis of poly(butylene adipate) from DVA and BD condensation using a commercial immobilized lipase preparation (Novozym 435). Reaction kinetics have been reported for both solvent and solvent-free systems (Chaudhary et al., 1997, 1998a; Kline et al., 2000).

The solvent-free system for the polymerization of DVA and BD has several unique features when compared to traditional stepwise polycondensation. Complex kinetics involving side reactions, a variable volume due to the release of acetaldehyde, and nonisothermal conditions resulting from an initial exothermic heat of reaction complicate our understanding of the system (Chaudhary et al., 1998a). One would expect substrate and product diffusion to influence the system performance, since the enzyme, lipase B derived from *Candida*

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antarctica, has been immobilized on an acrylic resin carrier. In the solvent-free system, the physicochemical properties of the reaction environment change as the polymer grows, and hence we have investigated the role of diffusion over the time course of the reaction. Diffusion is dependent on the initial conditions of the reaction mixture and the weight-average molecular weight and polydispersity of the polyester, which are in turn dependent on the reaction time and rates.

Conventional understanding of polymer chemistry predicts that the polydispersity of the polymer usually increases with the degree of polymerization (Odian, 1991). Theoretically, at nearly complete conversion of the reactants, the degree of polymerization for stoichiometrically balanced linear condensation reactions is very high and the polydispersity approaches a value of two. In reality, PDI can be higher than predicted theoretically when the system involves parallel side reactions, which have a tendency to form molecular chains with unreactive end groups. High values of PDI can also result when the reaction is not at complete equilibrium. For the biocatalytic polytransesterification of DVA and BD in organic solvents, the dispersity of the polymer was found to increase with the extent of reaction (Chaudhary et al., 1995).

In contrast, PDI was found to proceed through a maximum, reaching a value of 3 to 5 at intermediate molecular weights of 1,000–6,000 Da for Novozym 435 catalyzed solvent-free polymerizations (Kline et al., 2000). Such an increase in polydispersity during biocatalytic polyester synthesis could result from insufficient and heterogeneous mass transfer in the melt. Indeed, if growing chains are not removed efficiently from the catalyst surface, they could further extend at the expense of the shorter chains in the bulk environment, thus broadening dispersity. The unusual evolution of polydispersity seems to indicate that the role of diffusion is changing with time during polytransesterification. Herein we use a combination of experimental results and theory to determine how mass-transfer limitations and reaction kinetics can explain the evolution of molecular weight and PDI as the reaction proceeds. The theoretical analysis was extended to predict the effects of initial exothermicity, reduction in enzyme activity for longer chains, thermal deactivation of the enzyme, and loss of vinyl functional groups by the parallel reaction of hydrolysis on the role of diffusion.

Materials and Methods

Materials

HPLC grade tetrahydrofuran (THF) and 1,4-butanediol (BD, 99+% purity) were purchased from Aldrich Chemicals Inc. (Milwaukee, WI). Divinyl adipate (DVA) was obtained as a kind of gift from Union Carbide Chemicals and Plastic Co., Inc. (Danbury, CT). Novozym 435, which is a preparation of triacylglycerol hydrolase (E.C.3.1.1.3) derived from *C. antarctica* lipase B cloned and expressed in *Aspergillus oryzae*, then immobilized onto acrylic resin, was a kind gift from Novo Nordisk Bioindustrials, Inc. (Denmark).

Reaction procedure

Most experiments were performed using a 70 mmol equimolar mixture of DVA and BD, preheated to a given reaction temperature (50 to 70° C), then charged to a preheated mechanically stirred reactor equipped with a water jacket (Kline et al., 2000). The desired quantity of Novozym 435 (0.5 to 5% by weight) was added and the system was sealed. Samples were withdrawn over time to follow the extent of polymerization.

Analysis procedures

Molecular Weight and Polydispersity. Sample aliquots taken from the reactor were dissolved in THF (0.33 to 1 w/v % concentration) and analyzed by gel permeation chromatography to determine polymer molecular weight and dispersity. The Waters 150CV gel permeation chromatograph (GPC) uses THF as the mobile phase at 1.0 mL/min flow rate and a temperature of 35° C. The GPC was equipped with three columns to achieve separation in the molecular-weight range of 500–30,000 Da. The first two mixed-porosity columns are PLgel Mixed-E columns from Polymer Laboratories. The third column is a Waters Ultrastyragel column with a 500-Å pore size. The GPC was calibrated using 11 polystyrene standards in the molecular-weight range of 580–66,000 Da. The use of polystyrene to calibrate various types of polymers for GPC analysis is an established practice and it is understood that the resulting molecular weights from these analyses are overestimated by as much as 25%.

Viscosity. Sample viscosity was measured at 60°C using a Brookfield DVIII viscometer using Spindle 18 equipped with a 7-mL sample adapter. The temperature of the polyester was maintained using a circulating water bath.

Electron Microscopy. Electron microscopy of enzyme samples was performed by the Electron Microscopy facility in the Department of Material Science and Engineering at the University of Pittsburgh using a Philips XL-30 Field Emission Gun. Both dry samples and methanol suspensions of Novozym 435 were used for analysis.

Water Content. A Fisher Scientific Coulomatic K-F Titrimeter (Model 447) was used to measure the moisture content of the monomers and enzyme. While monomers with the highest purity were used in order to reduce the initial level of moisture in the reaction, the main source of water at the beginning of the reaction was the enzyme. No attempts to control the water level in the reaction either from the enzyme or from the occurrence of side reactions were made.

Enzyme Activity. Lipase activity was determined titrimetrically using a Sigma diagnostic kit 800-B. Immobilized enzyme (10 mg) was incubated in a shaker with 3 mL of substrate for 2 h at 50°C. The amount of fatty acid produced was determined to estimate the enzyme activity, which was expressed as Sigma-Tietz Units/mg.

Results and Discussion

In a catalytic reaction involving a porous solid catalyst, chemical reaction, bulk diffusion, and pore diffusion are the three basic parameters that can control the rate (Satterfield, 1980). Typically, at high temperatures, intrinsic kinetics control the reaction, whereas at lower temperatures, bulk diffusion or pore diffusion play predominant roles, giving rise to nonlinear reactant and product concentration gradients in the bulk liquid and across the catalyst particle. Since both kinetic and diffusional factors vary with time and molecular weight

of the polyester in the case of solvent-free biocatalytic polytransesterification, an interesting situation occurs even at constant temperature. Initially, reaction kinetics are faster than the usual enzyme-catalyzed reactions in organic solvents due mainly to increased substrate concentrations. Further, because of the elimination of solvent, the overall mass-transfer rate at zero reaction time should be less than that in a solvent system, resulting in diffusional limitations at the onset of the polymerization. However, both intrinsic kinetics and mass-transfer rates vary significantly as the reaction proceeds.

In this article, we seek to map how these two rates change over the course of the reaction and then to show how these relate to the resulting molecular weight and polydispersity. In order to accomplish these tasks, the following steps were taken. First, dimensionless numbers were developed to represent both the kinetic and mass-transfer rates during the reaction. The ratio of these two numbers provided a way to assess diffusional and kinetic control. Second, it was necessary to determine whether the solvent-free DVA-BD polytransesterification was diffusionally or kinetically limited at $t = 0$. Third, the influence of other factors, such as specificity, that had been neglected in the analysis up to this point were then taken into account.

Development of a kinetic reduction coefficient

Polymerization of divinyl adipate and 1,4-butanediol is a polycondensation reaction where vinyl alcohol is eliminated as the small molecule in each step. The vinyl alcohol tautomerizes to acetaldehyde evaporating at temperatures above 20°C. Due to this continual transformation and removal of byproduct, the "equilibrium" shifts in the forward direction. The tautomerization reaction is vigorously exothermic (−30 kJ/mol) and the initial heat liberation can increase the reaction temperature by several degrees, giving rise to nonisothermal conditions. The effect of this exothermicity on reaction temperature is dependent on the reactor configuration (Kline et al., 2000).

We have hypothesized that the overall reaction has three phases: initial fast transesterification, intermediate chain extension, and finally a slow polymerization. At the end of the third phase, no further polymerization occurs, since the vinyl groups disappear and most of the oligomers have acid end groups. After the initial fast phase of polymerization, the second phase can be represented by a classic stepwise condensation reaction, which follows pseudo-second-order kinetics:

$$-dC_t/dt = k_2 C_t^2, \tag{1}$$

where C_t is the concentration of the functional groups at a given time, and k_2 is the second-order rate constant. Assuming an equal number of end groups for condensation polymerization, the extent of reaction (p), number average degree of polymerization (X_n), initial end-group concentration (C_i), and reaction time (t) are related by the following equations (Odian, 1991):

$$X_n - 1 = C_i k_2 t \tag{2}$$

$$p = (X_n - 1)/X_n. \tag{3}$$

The final stage of DVA-BD polytransesterification follows zero-order kinetics (Kline et al., 2000).

Our first task was to predict theoretically how the rate of polymerization would decrease as a result of the changing chain length. A kinetic reduction coefficient (K_r) was defined as $\{K_r = (C_t/C_i)^2 = (1-p)^2\}$. Similarly to our solvent-based analysis, the extent of reaction, p, was calculated using the following expressions (Chaudhary et al., 1997):

$$p = (X_w - 1)/(X_w + 1) \tag{4}$$

$$X_w = M_w/200, \tag{5}$$

where X_w is the weight-average degree of polymerization, M_w is the weight-average molecular weight, and 200 is the molecular weight of one repeat unit of poly(butylene adipate) (refer to Table 1). The values in the first row of Table 1 represent the initial conditions at time = 0. When relating K_r to a reduction in the rate of polymerization, a constant value of rate constant (k_2) is assumed under a given set of conditions and changes in the concentration of functional groups due to side reactions are not included.

To better represent the actual biocatalytic polytransesterification, it is essential to account for additional parameters such as exothermicity, enzyme specificity, enzyme deactivation, and vinyl hydrolysis. Exothermicity will tend to increase the rate of reaction by increasing k_2, whereas the other parameters will decrease the rate. Based on the temperature profile of the exothermic reaction, the enhancement effect of exothermicity is significant only during the onset of polymerization, though changes in the intrinsic enzyme activity will play a role throughout the reaction. Vinyl hydrolysis will decrease the coefficient K_r by decreasing the concentration of the functional groups (C_t), whereas enzyme specificity and activity primarily affect the rate constant (k_2).

While the values in Table 1 neglect the role of the aforementioned factors, we have assessed their impact and incorporated them into the analysis later. We have previously described apparent kinetics and exothermicity effects for solvent-free DVA-BD polytransesterification (Chaudhary et al., 1997; Chaudhary et al., 1998a). Earlier studies have shown that the activity of Novozym 435 is molecular-weight dependent and this type of enzyme specificity results in an exponential reduction in the reaction rate with an increase in the

Table 1. Effect of Degree of Polymerization on Rate of Conversion

Degree of Polymerization (X_w)	Molecular Weight (M_w)	Conversion (p)	$K_r = (C_t/C_i)^2$
1	200	0.000	1.0
3	600	0.500	2.50×10^{-1}
5	1,000	0.667	1.11×10^{-1}
10	2,000	0.818	3.30×10^{-2}
15	3,000	0.875	1.60×10^{-2}
20	4,000	0.905	9.00×10^{-3}
25	5,000	0.923	5.93×10^{-3}
50	10,000	0.961	1.52×10^{-3}
100	20,000	0.980	4.00×10^{-4}
150	30,000	0.987	1.70×10^{-4}
200	40,000	0.990	1.00×10^{-4}

number of repeat units (Chaudhary et al., 1998b). Thermal deactivation kinetics of the enzyme during the polymerization were also studied previously (Kline et al., 2000). These studies formed the basis from which one can analyze the influence of various factors on the role of diffusion in the solvent-free biocatalytic polymerization of DVA and BD.

Development of a mass-transfer reduction coefficient

Knowing that the rate of diffusion is a time-based factor in the biocatalytic polytransesterification of DVA and BD, the reduction in this rate was predicted using a semitheoretical approach based on experimental data. The most important parameter that influences the mass-transfer rate is the diffusion coefficient of the substrate in the reaction mixture. The diffusivity of substrates in immobilized enzyme systems, membrane reactors, and biofilms have been studied in processes such as biotransformations, wastewater treatment, and biosensing (Wisemann et al., 1994; Lyons et al., 1996; Aksu and Bulbul, 1998; Debacker and Baron, 1994). In most cases, however, the diffusion of small molecules such as dissolved oxygen and organic substrates were studied (Amos et al., 1994). To our knowledge, self-diffusion of an enzyme substrate that varies in molecular weight with time has not been considered.

The mass-transfer rate for a fixed oligomer can be represented by

$$-dC_t/dt = k_L a[\Delta C], \tag{6}$$

where $k_L a$ is the mass-transfer coefficient for bulk and pore diffusion (L/s) based on effective diffusivity, and $[\Delta C]$ is the average concentration gradient of that particular polymer chain at time t (s). Since a mixture of various chain lengths and end groups exists in the system, the overall rate of diffusion will be dependent on the average molecular weight and polydispersity at that instant. We describe herein a simplified approach involving theoretical prediction of diffusivity from experimental data. The ratio of diffusivity at a given time (D_t) to initial diffusivity (D_i) was used to calculate a mass-transfer reduction coefficient ($M_t = D_t/D_i$) in an analogous manner to the kinetic reduction coefficient (K_r).

A sharp decrease in polymer diffusivity is expected to occur above a particular molecular weight. This transition in behavior takes place at the critical molecular weight of the polymer. Therefore, before an adequate estimation of diffusivity can be calculated, the critical molecular weight, which depends on the identity of the polymer, must be determined. Group interaction modeling can be utilized to predict this value based on the structure and composition of any polymer through the following equation (Porter, 1995):

$$M_{cr} \approx 0.26\, M\, E_{coh}/N_c^2, \tag{7}$$

where M is the molecular weight of the repeat unit, E_{coh} is the cohesive energy in J/mol, and N_c is the number of degrees of freedom in the polymer backbone. These values are known for a wide variety of polymers. The critical molecular weight for poly(butylene adipate) based on this equation ($M = 200$, $E_{coh} = 76{,}000$ J/mol, and $N_c = 24$) is approximately 6,900 Da, which closely coincides with the reported critical molecular weight of 6,000 Da (Van Krevelen, 1990). Estimation of diffusivity determined using separate relationships above and below the critical molecular weight will be explained in the subsequent two sections.

Diffusivity Below M_{cr}. Various configurations for polymers, including the elastic dumbbell, the chainlike model, and the general bead-rod-spring model have been developed in order to derive a diffusion equation. In all cases, an inverse relationship between diffusivity and viscosity results (Bird et al., 1977), and therefore it is acceptable to estimate the diffusivity of a polymer below its critical molecular weight using the Stokes-Einstein equation for self-diffusion (Bird et al., 1960):

$$D = kN^{(1/3)}T/2\pi\mu_A V_A^{(1/3)}, \tag{8}$$

where D is the diffusivity in cm^2/s, k is the Boltzmann constant, N is Avogadro's number, T is the temperature in K, μ_A is the viscosity of the polymer in poise, and V_A is the molar volume of the polymer in cm^3/mol. This can be further justified by realizing that chain entanglement can be neglected below the critical molecular weight (Van Krevelen, 1990; Bueche, 1962; Fox et al., 1956). Therefore, the Stokes-Einstein equation (Eq. 8) simply equates the polymer to large spheres that diffuse through a field of spheres of the same size. Our analysis does not include the effect of countercurrent diffusion due to acetaldehyde, since this flux is only relevant in the first few minutes of reaction. The molar volume of the repeat unit of poly(butylene adipate) was obtained from Kopp's law and V_A at a particular molecular weight was then calculated using this base value (Satterfield and Sherwood, 1963). Thus, if data are collected for melt viscosity of the polyester at a desired temperature, one can estimate the change in diffusivity during the polymerization.

Although a polymer is essentially a mixture of chains with varying lengths, the longer species in the mixture possess more influence on both the physicochemical properties and weight-average molecular weight (M_w) of the polymer. Hence, melt viscosity of a polymer is usually correlated to M_w or X_w using a suitable logarithmic function (Fox et al., 1956). For linear aliphatic polyesters, a relationship exists between the square root of the molecular weight and the melt viscosity (Flory, 1940). Therefore, melt viscosity of the biocatalytically produced poly(butylene adipate) was measured using several samples with average molecular weight ranging from 800 Da to 5,500 Da (Figure 1) to obtain the following equation:

$$\log\ \mu = 0.04(M_w)^{0.5} - 2.27. \tag{9}$$

Polymers with very high PDI values ranging from 3 to 6 were used to estimate viscosity in an effort to mimic the reaction composition when lower molecular-weight polymers were present. If polymer with lower PDI were used, we suspect that it would possess higher viscosity, but further investigation is necessary to confirm this hypothesis. The viscosity-dependent diffusion coefficient was estimated from Eqs. 8 and 9 for molecular weights below M_{cr}. The mass-transfer reduction coefficient, M_r, was calculated using these diffusion coefficients (Table 2). The first row in Table 2 corresponds to the initial conditions in the solvent-free polymerization.

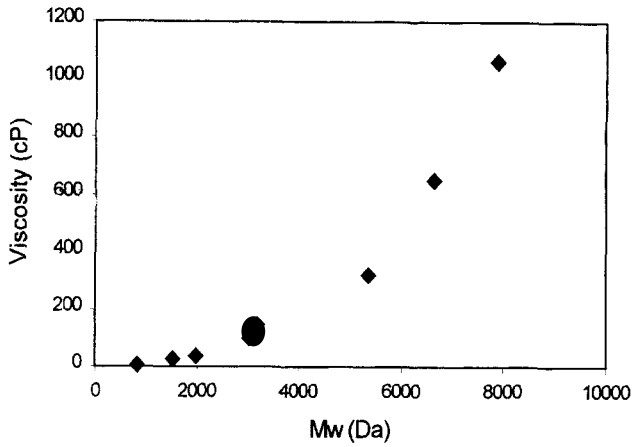

Figure 1. Experimentally measured melt viscosity (in cp) of polyester at 60°C as a function of weight-average molecular weight.

Diffusivity Above M_{cr}. When polymers achieve molecular weights greater than their critical molecular weight, they can no longer be modeled as spheres using the Stokes-Einstein equation (Eq. 8). Chain entanglement must be taken into account, and it is intuitive that diffusivity and viscosity will be more strongly dependent on polymer molecular weight. It has been shown that viscosity is proportional to $\sim M_w^{3.4}$ and diffusivity is inversely proportional to $\sim M_w^3$. Therefore, to estimate a self-diffusion coefficient for polyesters above ~ 6,000 Da, a more appropriate relationship must be used.

Similar equations for the self-diffusion coefficient have been developed separately based on the principles of Brownian motion (Bueche, 1962) and reptation theory (Doi and Edwards, 1986), such that

$$D = AnL^2/\tau_P, \tag{10}$$

where A is a constant, n is the number of repeat units in the polymer chain, L is the length of the repeat unit, and τ_P is the terminal relaxation time. Equation 10 can then be expressed in terms that can be calculated using group interaction modeling (Porter, 1995):

$$D = B(L^2/\tau)(n/Z^3), \tag{11}$$

Table 2. Effect of Degree of Polymerization on Diffusivity

Deg. of Polymer. (X_w)	Molec. Wt. (M_w)	Visc. (cp)	V_a (cm³/mol)	Diffus. (D) (cm²/s×10⁷)	M_r = (D_t/D_i)
1	200	2.0	251.2	4.90×10^{-6}	1.0
3	600	5.1	753.6	1.30×10^{-6}	2.70×10^{-1}
5	1,000	10	1,256	5.80×10^{-7}	1.20×10^{-1}
10	2,000	33	2,512	1.40×10^{-7}	2.90×10^{-2}
15	3,000	83	3,768	4.80×10^{-8}	9.80×10^{-3}
20	4,000	180	5,024	2.00×10^{-8}	4.10×10^{-3}
25	5,000	360	6,280	9.30×10^{-9}	1.90×10^{-3}
50	10,000	—	—	1.70×10^{-14}	3.50×10^{-9}
100	20,000	—	—	4.20×10^{-15}	8.60×10^{-10}
150	30,000	—	—	1.90×10^{-15}	3.90×10^{-10}
200	40,000	—	-	1.10×10^{-15}	2.20×10^{-10}

Table 3. Parameters to Calculate Self-Diffusion Coefficients Above M_{cr}

Parameter	Value
C_∞	5.25*
L	12.895 Å**
θ_1	293 K
T_g	205 K†
M_s	42 Da††

*The characteristic ratio for aliphatic polyesters ranges between ~ 5.25 and 6.5 (Kurata and Tsunashima, 1989). The value of the ratio increases with increasing polymer chain length.
**The length of a repeat unit was determined using molecular modeling with Chem 3D. Energy minimization for the value given was via MM2.
†The T_g given is reported as an experimental value (Porter, 1995).
††M_s is calculated for polymers with trans-configurations as three methylene groups, and this value has been shown to be suitable for aromatic polymers, poly(ethylene), and poly(carbonate) (Porter, 1995).

where B is a constant, τ is the characteristic relaxation time for a repeat unit, and Z is defined as M_w/M_s, where M_s is the molecular weight of a characteristic oscillating segment in a polymer chain. We have chosen to use reptation theory for our calculations. Therefore, in terms of reptation theory, B is equivalent to $C_\infty/1.5$, where C_∞ is the characteristic ratio of the polymer. The relaxation time of a repeat unit is defined by the following relationship (Porter, 1995):

$$\tau = (h/2\pi k\theta_1)\exp\left[(1{,}280 + 50\ln\theta_1)/(T - T_g - 50)\right], \tag{12}$$

where h is Planck's constant, k is the Boltzmann constant, θ_1 is the reference temperature for skeletal vibrations parallel to the chain axis in kelvin, T is the reaction temperature in kelvin, and T_g is the glass transition temperature of the polymer in kelvin. Table 3 lists the known or estimated parameters for poly(butylene adipate) necessary to calculate the self-diffusion coefficient, D, by Eq. 11.

The self-diffusion coefficient based on reptation theory was calculated using the values in Table 3 for molecular weights above 6,000 Da. These diffusivity coefficients as well as the initial diffusivity of the polymer repeat unit calculated from the Stokes-Einstein equation (Table 2) were used to calculate M_r.

Control regimes during biocatalytic polytransesterification

In Tables 1 and 2, K_r and M_r reflect the changing kinetic and mass-transfer rates with time in biocatalytic polytransesterification. These reduction coefficients predict that both rates will decrease by several orders of magnitude. In fact, K_r/M_r must be calculated in order to determine which process limits the reaction at a given time in the reaction. The resulting apparent Damköhler number can be plotted as a function of the degree of polymerization (Figure 2) (Bailey and Ollis, 1986). Since the initial values of K_r and M_r are equivalent to one by definition, only comments on general trends in the figure can be made at this time.

Figure 2 shows that a sharp increase in K_r/M_r occurs when the degree of polymerization is greater than 25, which can be attributed to the onset of chain entanglement in the bulk environment. At molecular weights of 10,000 Da and greater, the calculated kinetic and mass-transfer reduction coefficients clearly show that K_r is much greater than M_r and that

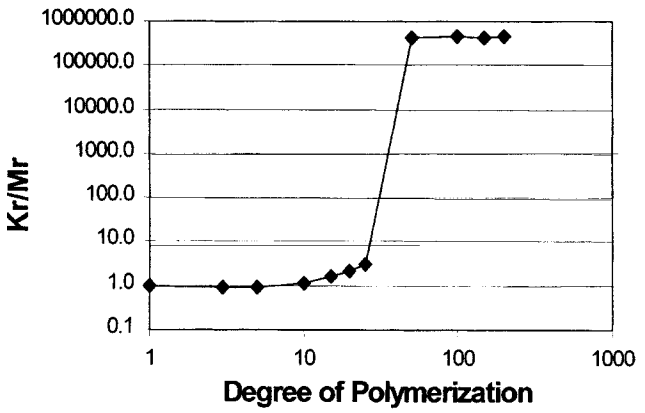

Figure 2. Dependence of calculated M_r/K_r as a function of degree of polymerization.

the increase in K_r/M_r strongly indicates that the control regime has switched from kinetic to diffusional control. It must be determined whether the reaction is initially under kinetic control and remains so until higher molecular weights are achieved, or if the reaction is diffusionally controlled at the onset of polymerization. Furthermore, it would be worthwhile at this point to distinguish between the two possible types of mass-transfer limitations. We suggest that the majority of initial and intermediate diffusional limitations can be attributed to both pore and bulk diffusion. At higher molecular weights, however, pore diffusion no longer contributes significantly and external diffusion is responsible for any mass-transfer limitation.

In order to estimate whether kinetics or diffusion initially control the reaction, we can determine the ratio of intrinsic rate of reaction to mass-transfer rate, which we define as resultant rate (R). In general, when R is significantly greater than one, the resistance to mass transfer is large and the system is under diffusional control. Conversely, a value much smaller than one indicates that the rate of reaction is greater than the mass-transfer rate, resulting in kinetic control. Resultant rates were evaluated for two solvent-free systems with different conditions using the experimentally measured rate constants and estimated mass-transfer coefficients for pore diffusion (Table 4). The values for the solvent-free system reflect that the kinetic process is slightly faster than the internal pore diffusion process at the start of the reaction. Similar calculations performed for a biocatalytic polytransesterification in tetrahydrofuran at 50°C and an enzyme concentration of 5 g/L resulted in a resultant rate of approximately one, indicating that the rates of reaction and pore diffusion are evenly matched.

We have estimated how K_r/M_r changes with chain length (Tables 1 and 2) and now have some idea of the initial value of R (Table 4). These data can be combined to calculate the resultant rate as a function of average molecular weight. Thus, one can predict the prevailing control regime during the solvent-free polytransesterification of DVA and BD. For the reaction of DVA and BD at 60°C with 2% enzyme, R gradually increased from 30, for a repeat unit of 200, to 94, for a polymer of 5,000 Da. At molecular weights greater than 5,000 Da, R increases by several orders of magnitude. These results indicate that there is weak diffusional control in the initial and intermediate stages of the reaction, whereas at higher molecular weights, several mass-transfer limitations are present. It is important to consider that this behavior was predicted by only considering pseudo-second-order kinetics and physicochemical properties affecting diffusion. In reality, additional factors such as exothermicity, vinyl hydrolysis, enzyme specificity, and thermal degradation can alter the kinetics and hence the controlling step of the reaction. We comment below on the effect of each of these factors.

Initial exothermicity

During the initial stage of biocatalytic polytransesterification, which only lasts for a few minutes, R will change with the degree of polymerization due to the exothermic tautomerization of vinyl alcohol to acetaldehyde. Qualitatively, exothermicity will increase the rate of reaction and R such that, initially, stronger diffusional control would exist. Figure 3 shows the calculated resultant rate as a function of molecular weight for the nonisothermal reaction with an initial temperature of 50°C and 5% enzyme. The effect of exothermicity was estimated by first calculating the initial resultant rate using the previously obtained pseudo-second-order rate constant of 10.1×10^{-3} l/mol·s·g-catalyst (Chaudhary et al., 1998a). Since this rate constant is greater than the isothermal rate constant by a factor of 35, the initial R value for the exothermic reaction is 35 times greater than that of the isothermal reaction. This rate then decreases over the next 30 min until the temperature can be maintained. Therefore, enhancement factors were estimated using the experimentally observed temperature profile for the 5% enzyme reaction (Chaudhary et al., 1997) and a normalization factor of 35.

As expected, the nonisothermal conditions that exist as a result of the initial exothermicity cause an increase in the value of R. If, as we have suggested, the reaction is only slightly controlled by diffusion when R ranges from approxi-

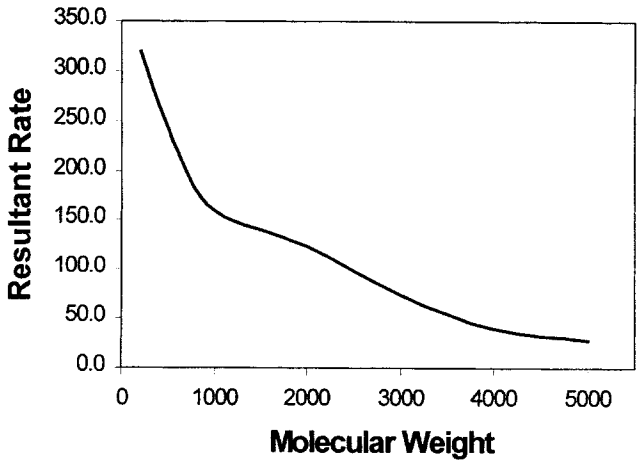

Figure 3. Effect of initial exothermicity (nonisothermal conditions) on calculated resultant rate (R) as a function of weight-average molecular weight.

Table 4. Kinetic and Mass-Transfer Parameters for Determination of Initial Conditions

Parameter	50°C 5% Enzyme	60°C 2% Enzyme
Pseudo-second-order rate const. (k, L/mol·s·g)	2.8×10^{-4}	8.1×10^{-4}
Init. functional group conc. (C_i, mol/L)	7.1	7.1
Enzyme conc. ([E], g/L)	50	20
Pseudo-first-order rate const. (k'_1, L/s)*	0.10	0.12
Initial diffusivity (D_i, cm^2/s)	4.9×10^{-6}	4.3×10^{-6}
Mass-transfer coeff. (k_{sl}, cm/s)**	2.0×10^{-3}	1.8×10^{-3}
Interfacial area (a, cm^2/cm^3 dispersion)†	5.5	2.2
Volumetric mass-transfer coeff. ($k_{sl}a$, L/s)	0.011	0.004
Resultant rate (R)††	**9.1**	**30**

*Pseudo-first-order rate constant $(k'_1) = k * C_i * [E]$.
**The mass-transfer coefficient is estimated from the dimensionless Galilei number and the particle Reynolds number, which are calculated from the physicochemical properties of the reaction mixture. Initially, it is assumed that the reaction mixture consists predominantly of oligomer with one repeat unit (molecular weight = 200 Da). Novozym 435 is used as the catalyst.
†Dispersion of the enzyme in the reaction environment is estimated from enzyme holdup and particle size.
††Resultant rate $(R) = k'_1/k_{sl}a$.

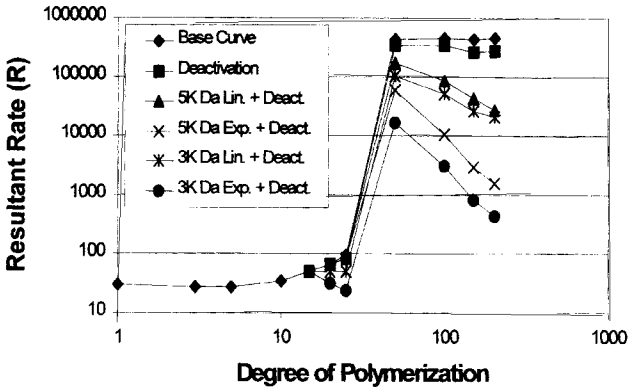

Figure 4. Cumulative effect of enzyme specificity loss and deactivation on overall profile of resultant rate (R) as a function of average molecular weight.

mately 1 to 100, then the exothermic effect of the nonisothermal reaction with 5% enzyme increases initial mass-transfer resistance significantly. Weak mass-transfer limitations are then established and maintained in the molecular-weight region of 3,000 to 5,000 Da. It is important to note that Figure 3 shows a general trend, but the actual numbers will depend on the initial reaction temperature as well as the enzyme concentration and activity.

Enzymatic factors

To demonstrate the effect of various enzymatic factors on the resultant rate, a base curve representing the isothermal reaction at 60°C with 2% enzyme was first generated using Figure 2 and the initial value of R from Table 4 (refer to Figure 4). As mentioned previously, this base theoretical curve suggests that the polymerization will be strongly controlled by diffusion when the molecular weight is above 10,000 Da. However, enzyme deactivation, vinyl hydrolysis, and enzyme specificity were neglected when this curve was generated.

Deactivation. Irreversible thermal deactivation of enzyme occurs during the polytransesterification, decreasing the rate of transesterification and any undesirable side reactions. One expects that the extent of enzyme deactivation during the polytransesterification would be a function of reaction time, not the degree of polymerization. However, the deactivation of the enzyme was investigated in the monomers as well as in the polymer, and it was demonstrated that different deactivation kinetics occur based on the environment (Kline et al., 2000). Therefore, enzyme deactivation may have some dependence on polymer chain length. Previously, it was determined that enzyme inactivation becomes significant after 0.5 h. Since this time interval generally produces 5,000–10,000-Da polyester, enzyme deactivation kinetics were only superimposed on the theoretical resultant rate curve at degrees of polymerization of 25 and greater (Figure 4). The curve representing deactivation was produced by multiplying the base resultant rates by a factor between 0.5 and 1.0. These factors were obtained using the previously measured relationship for enzyme deactivation in the polyester at 60°C (Kline et al., 2000). Since molecular weights greater than 30,000 Da were not achieved, the deactivation factors for $DP \geq 150$ were estimated.

Catalytic Hydrolysis. Loss of vinyl groups by hydrolysis will also contribute to a reduction in both intrinsic reaction rate and R. It was reported earlier that the initial hydrolysis rate was lower by a factor of 100 in the solvent-based polymerization of DVA and BD (Chaudhary et al., 1998b). In the solvent-free system, one would expect a lesser extent of hydrolysis at the onset of polymerization due to the low solubility of water and the immiscible nature of the monomers. As the polymerization continues, however, a homogeneous liquid phase is formed. Production of polar acid end groups via hydrolysis is expected to increase the solubility of water in the oligomer mixture, enhancing the hydrolysis. Further, water molecules are mainly associated with the enzyme and are located in the vicinity of the active site. Therefore, at a fixed enzyme concentration, the rate of hydrolysis should be independent of water concentration and directly proportional to the concentration of vinyl groups. Regeneration of water by the simultaneous esterification reaction between acid and alcohol end groups will also assist further hydrolysis. Thus, the rate of hydrolysis will decrease to a lesser extent than the rate of polytransesterification as the reaction progresses. If it is assumed that the vinyl hydrolysis reaction is initially 100 times slower, hydrolysis should gain significance when the rate of transesterification drops by a factor of 100 or more. This situation corresponds to a stage when $DP \cong 25$ as indicated by K_r in Table 1. Hence, the detrimental effect of vinyl hydrolysis is expected to occur above 5,000 Da.

Specificity. Unlike enzyme deactivation or vinyl hydrolysis, the specific activity of Novozym 435 is solely a function of molecular weight. In a previous solvent-based model, the intrinsic activity (nonequal reactivity) was reduced by logarith-

mic scaling factors applied with every addition of the repeat unit, to account for the chain-length-dependent specificity. By using a scaling factor of approximately 2.5, the experimental data fit reasonably well with the model (Chaudhary et al., 1998b). For a solvent-free system, the initial rate constant for the enzymatic polytransesterification is taken as unity. The reduction in the rate constant above the chosen critical molecular weights of 3,000 Da and 5,000 Da were calculated using both a linear function as well as an exponential function (power 2.5). In other words, the enzyme reactivity was assumed to decrease for oligomers longer than 15 or 25 repeat units, respectively.

The effect of enzyme specificity on the resultant rate has also been incorporated into Figure 4 so that the cumulative effect of enzyme deactivation and specificity can be visualized. With the assumption of a linear reduction in the enzyme reactivity, the resultant rate is still significantly above 100, indicating that the reaction is still mass-transfer limited. When an exponential relationship between the chain length and reactivity is assumed, the resultant rate decreased to a range of 400 to 1,500, depending on the critical molecular weight used. While these values are still high enough to imply diffusional control, it is important to note that the detrimental effect of vinyl hydrolysis at higher molecular weights has not been included in Figure 4. Therefore, these simulated data suggest that a transitional control regime, in which both diffusional and kinetic factors are significant, can be reestablished upon reaching molecular weights above 30,000 Da. The experimental results show that exponential reduction in enzyme reactivity above the critical molecular weight of 5,000 Da is a realistic assumption.

Pore diffusion

After establishing that mass-transfer limitations are important throughout solvent-free biocatalytic polymerization of DVA and BD, an attempt was made to distinguish between the impacts of pore and bulk diffusion in the reaction. Because the concentration of monomers is very high, pore diffusion is expected to play a crucial role at the onset of polymerization. Therefore, a series of experiments was conducted to assess its importance.

Pore diffusion in biological systems involving enzymes or microorganisms can be investigated for a variety of situations, using basic chemical engineering principles. Previous work in our group reported the use of a classic approach to theoretically predict the level of enzyme activity in an anhydrous organic solvent or supercritical fluid at which the reaction would become diffusionally limited (Kamat et al., 1992). The limiting value of the intrinsic enzyme activity per unit surface area was estimated to be 0.01 cm/s. Due to the lower diffusivity in a solvent-free system, one would expect a lower limit for the

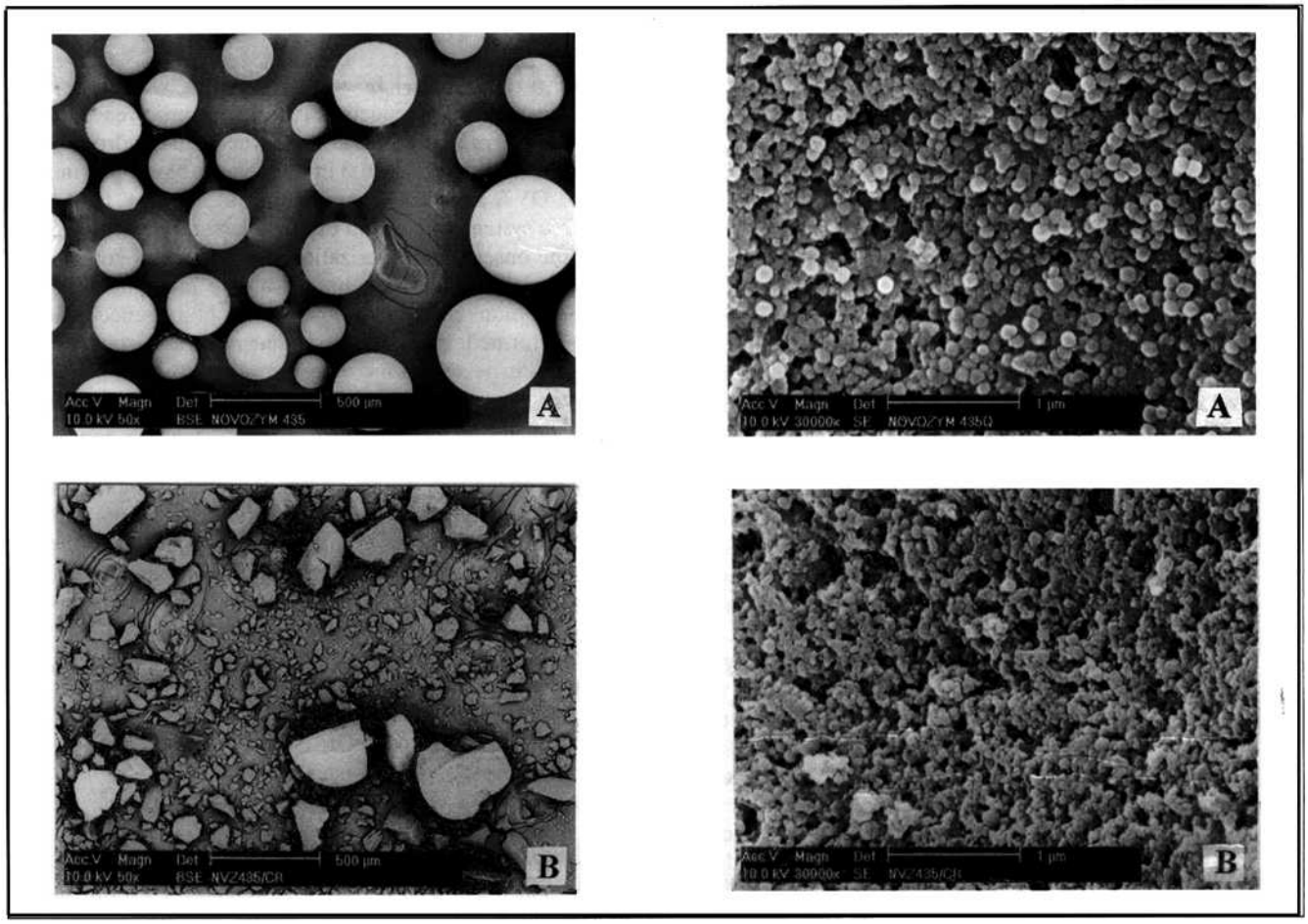

Figure 5. Electron micrograph of (A) standard and (B) crushed Novozym 435.

specific activity, above which the system will have diffusion limitations. For a typical solvent-free polytransesterification, the specific enzyme activity (pseudo-first-order rate constant/specific interfacial area) ranged from 0.018 cm/s to 0.055 cm/s (Table 4). Thus, a severe pore-diffusion limitation may exist, even at the onset of solvent-free DVA-BD polymerization.

One way to reduce the effect of pore diffusion is to decrease the particle size of the catalyst, thereby increasing the fraction of enzyme at the catalyst external surface. The commercial immobilized enzyme, Novozym 435 (average diameter 0.55 ± 0.14 mm), was separated to generate samples having different particle sizes. The smallest particles (0.05–0.12 mm) were obtained by crushing the enzyme. The morphologies of the commercial Novozym 435 in standard and crushed forms were analyzed via electron microscopy (Figure 5). At a magnification of 50, the standard enzyme showed spherical particles with significant variation in size, whereas the crushed particles were irregular in shape and size. A magnification of 30,000 revealed a uniform microporous structure with similar pore sizes for both samples. Therefore, the only difference between standard and crushed enzyme is that the latter form has an increased amount of external surface area.

Isothermal reactions were conducted at 50°C using 2% enzyme varying enzyme particle size. Figure 6 shows how the molecular weight of the polyester changes as a function of the enzyme bead size. It was observed that the polymerization was faster, and higher molecular-weight polyesters were produced, with smaller particles. We reported earlier that, at 2–5% enzyme concentration, the standard enzyme produced polyester with a molecular weight of 15,000–20,000 Da in 2 to 4 h between 50 and 60°C. Interestingly, polyester with 23,400-Da molecular weight was obtained in just one hour when 5% crushed enzyme was used at 60°C (Kline et al., 2000). These results and Figure 6 suggest that the effect of internal diffusion was reduced significantly when smaller enzyme particles were used.

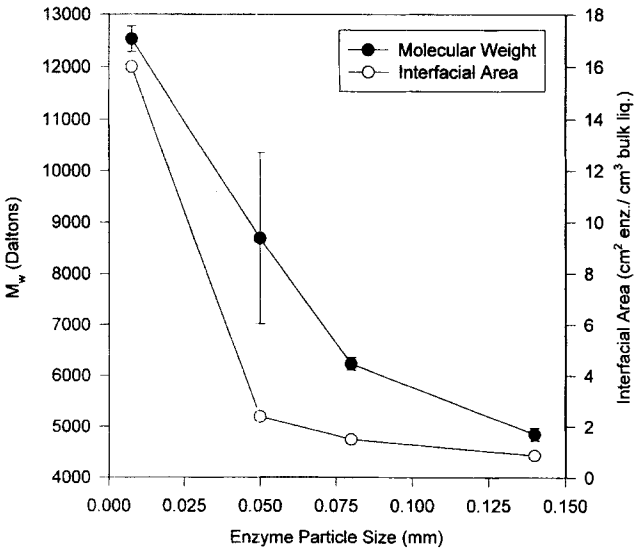

Figure 6. Effect of enzyme particle size on experimentally observed molecular weight.

Reactions were performed at 50°C using 2% enzyme for 2 h.

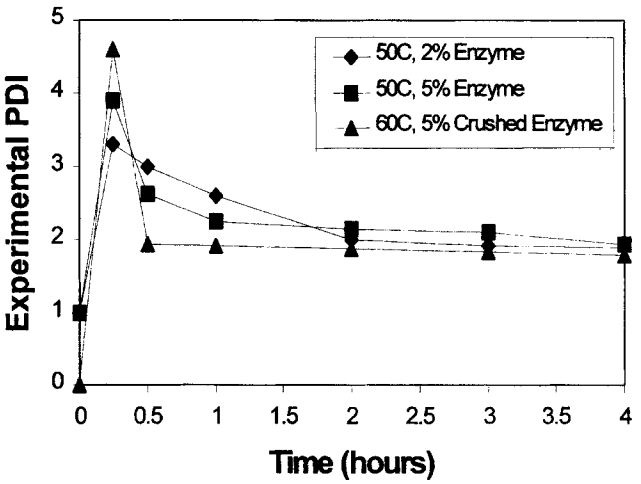

Figure 7. Variation in experimentally observed PDI as a function of polymerization time.

Given all that we now know, it is clear that once the polymer molecular weight exceeds about 5,000 Da, the pore of Novozym 435 will become clogged and the polymer inside will no longer be available to return to the bulk polymer. Indeed, we predict that at this point only the exterior of the catalyst particle will be relevant in further chain extension of the bulk substrate. If this is the case, then chain extension in the core and surface of the bead will now proceed at different rates in effectively separate phases.

Evolution of PDI during biocatalytic polytransesterification

The evolution of PDI over time is dependent on enzyme concentration and particle size (Figure 7). One would expect that the faster kinetics at higher enzyme concentration should result in more pronounced diffusion limitations and produce polyesters with increased dispersity. Indeed, at a fixed temperature with the standard enzyme, 5% enzyme concentration resulted in a polymer with higher PDI than obtained with 2% enzyme. We have, however, reported an unusual evolution of PDI during solvent-free transesterification (Kline et al., 2000). PDI was found to go through a maximum (PDI > 3) at intermediate molecular weights of 1,000–6,000 Da. Interestingly, the maximum value of PDI was observed exactly in that range of molecular weight where we expect internal diffusion and chain entanglement to prevent the core of the enzyme bead from participating in the reaction. The initial presence of diffusion limitations would cause concentration gradients to form across the enzyme particles, resulting in formation of oligomers with a wide variation in chain lengths, thus broadening the dispersity. After a molecular weight of about 6,000 Da is attained, the pores become clogged and external diffusion becomes rate limiting. This is supported by reaction data. Bulk polymer was decanted from the enzyme, and the enzyme beads were crushed, placed in THF, and agitated in an effort to extract the intrapore polymer. While the bulk polymers reached higher molecular weights in the range of 10,000 to 15,000 Da and possessed low PDI, the pore polymers were higher in polydispersity and molecular weight was less than 6,000 Da. The elimination of

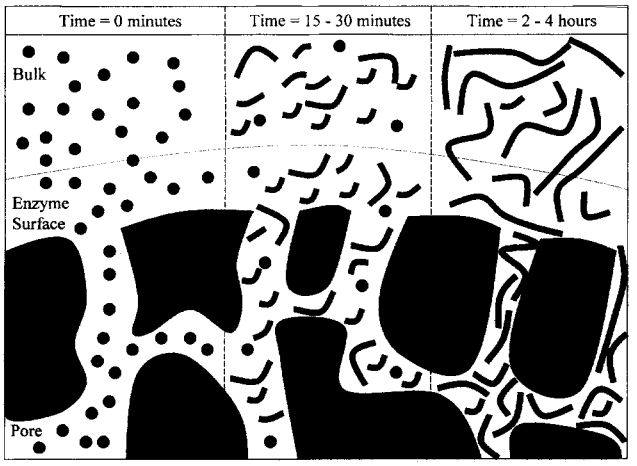

Figure 8. Evolution of molecular weight and PDI in bulk environment and enzyme pores.

core-enzyme participation seems to cause a fast convergence of PDI, which should resemble the kinetically predicted value. Figure 8 summarizes what we believe occurs during the polymerization as well as our hypothesis to explain the evolution of molecular weight and PDI.

Conclusion

It is clear that the biocatalytic polytransesterification of DVA and BD using Novozym 435 proceeds through two "rough" spots in terms of molecular-weight limitations. The first resistance occurs when the pores of the enzyme particles become clogged as a result of polymer chain entanglements and can no longer contribute to the reaction. At this point, there will be a sudden reduction in enzyme concentration. Therefore, it is intuitive that the reaction efficiency would be augmented by exposing a greater amount of enzyme to the surface of the bead. The achievement of theoretical PDI also appears to depend on this switch from weak to strong diffusional control exhibited at molecular weights of 5,000–10,000 Da. After polymer with molecular weights of 5,000 Da have been produced, a second obstruction takes place due to other factors such as hydrolysis of vinyl groups, enzyme deactivation, and reduced enzyme specificity. It is difficult to overcome these issues if molecular weights greater than 30,000–40,000 Da are desired. We are now in a position to engineer the catalyst preparation to address these issues further.

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