Nonequal Reactivity Model for Biocatalytic Polytransesterification

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The lipase-catalyzed polytransesterification of divinyl adipate and 1,4-butanediol is a dynamic process controlled by the relative magnitudes of the rates of transesterification and hydrolysis as the polymerization proceeds. A mathematical model that describes the kinetics of the biocatalytic polytransesterification process is presented. Initial rate studies with model substrates in transesterification, hydrolysis, and esterification were used to provide the initial kinetic parameters that, when combined with the effect of enzyme specificity, were used to predict product compositions under different reaction conditions. A comparison of experimental data and model predictions shows that the product composition is strongly influenced by the selective nature of enzyme catalysis, and this model effectively predicts the results of a step condensation polymerization where reactivity depends on molecular weight.

Introduction

Linear polyesters are most commonly synthesized by stepwise condensation of carboxyl groups with hydroxyl groups (Kopnick et al., 1992). Reactions are either self-catalyzed or else external acid catalysts such as protonic acids, Lewis acids, titanium alkoxides, or dialkyl tin(IV) oxides are employed. The synthesis often involves heating a hydroxycarboxylic acid (A–B-type self-condensation) or a mixture of diol and dicarboxylic acid (AA–BB-type polycondensation) to temperatures at which esterification occurs. Since the polycondensation is limited by equilibrium, the water formed is usually removed from the reaction mixture. Diol dehydration (e.g., 1,4-butanediol to tetrahydrofuran) and cyclization are undesirable side reactions when strong acid catalysts are used. Strong acid catalysts also promote discoloration and product hydrolysis if they are not neutralized or removed from the product (Kopnick et al., 1992).

The beautiful selectivity of enzymes makes them attractive catalysts for processes where fewer side reactions can facilitate separations (Klibanov, 1986). Hydrolytic enzymes such as lipases, esterases, and proteases when placed in organic solvents catalyze ester syntheses reactions (Tirrell et al., 1994). Using difunctional esters (diesters) and alcohols (diols) as substrates, polyesters can be synthesized by successive transesterification (Margolin et al., 1987; Morrow et al., 1990). Polyesters have also been synthesized by lipase catalyzed self-condensation of a hydroxyacid (Ajima, 1985) or a hydroxyester (Knani et al., 1993) and "ring-opening" of lactones (Uyama and Kobayashi, 1993; MacDonald et al., 1995), followed by stepwise condensation.

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Polymerization

The practical synthesis of polymers requires an understanding of polymerization kinetics. The kinetics of polymerizations depend on the mechanism (Odian, 1981) and our limited understanding of enzyme-catalyzed polyester synthesis has made it difficult to explain and predict the products of polymerization.

Enzyme-catalyzed free-radical polymerizations have been studied extensively (Dordick et al., 1987; Rao et al., 1993). Dordick and colleagues have also developed a mathematical model that simulated the phenolic polymerization process and predicted the molecular weight and distribution as a function of monomer reactivity (Ryu et al., 1993). Since in this case, the enzyme does not play any role during chain propagation and termination steps, this model does not need to incorporate a detailed understanding of the role of the enzyme.

Lipase-catalyzed polytransesterification proceeds in a step-

wise manner with enzyme catalyzing alcoholysis throughout. Several investigators have followed the process of polymerization by monitoring the increase in molecular weight. We have also used matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (Chaudhary et al., 1996a) and reaction calorimetry (Chaudhary et al., 1997c) to follow changes in the polymerization mixture. In this report, we present for the first time a mathematical model that describes the kinetics of lipase-catalyzed AA–BB-type polytransesterification. We have clarified the role of lipase (as catalyst) in controlling the polymerization process by comparing the model predictions with experimental observations.

Since the early work of Flory, the kinetics of AA–BB-type polycondensations have been the target of intense research. The kinetic analysis (Flory, 1953) is greatly simplified by assuming that

1. The reactivities of both monomeric functional groups (hydroxyls of a diol or carboxyls of a diacid) are the same and that this reactivity does not change if the other end group is reacted.
2. The reactivity of a functional group is also independent of the size of molecule to which it is attached.

These simplifying assumptions, often referred to as *equal reactivity of functional groups*, make step polymerization identical to an analogous small molecule reaction such as esterification of acetic acid with ethanol. Both theoretical and experimental justifications have been offered (Bhide and Sudborough, 1925; Ueberreiter and Engel, 1977). Self-catalyzed step polymerization of the diacid/diol system is modeled using a third-order expression in [COOH]. When an external catalyst is used, the kinetics are described using a second-order model. At this time, it is vital to stress that very few exceptions to the "equal reactivity" rule have been reported for chemically catalyzed polycondensation systems (Goel et al., 1977), and fewer have been explained.

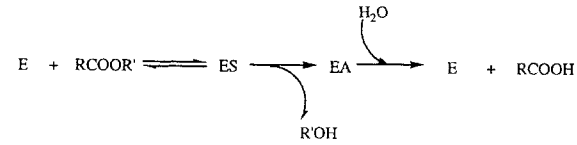
Having discussed the kinetics of nonenzymatic polycondensation, it is important to identify how the kinetic analysis will be affected when an enzyme is used as a catalyst. This will require both an understanding of the mechanism of transesterification process catalyzed by lipases in general, and determination of which of the assumptions mentioned earlier will not hold for enzyme-catalyzed processes.

Model Development

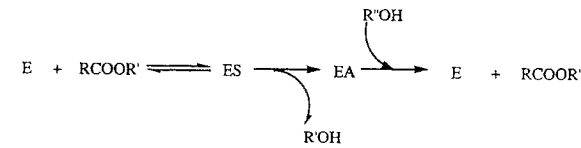
Transesterification mechanism

There is substantial evidence in the literature that serine proteases catalyze hydrolytic reactions by the acyl enzyme mechanism (Fersht, 1985; Fink and Bender, 1969; Whiting and Peticolas, 1994). In organic solvents, hydrolysis can be replaced by alcoholysis (more commonly referred to as transesterification), and investigations of the transesterification mechanism in nonaqueous media (Zaks and Klibanov, 1988; Chatterjee and Russell, 1993; Kamat, 1996) show that the acyl-enzyme mechanism is still valid (Adams et al., 1991; Chatterjee and Russell, 1992; Kanerva and Klibanov, 1989). For some lipases, a ping-pong bi-bi mechanism (Kamat, 1996) has also been established. Mechanistic models for enzyme-catalyzed hydrolysis and transesterification reactions follow.

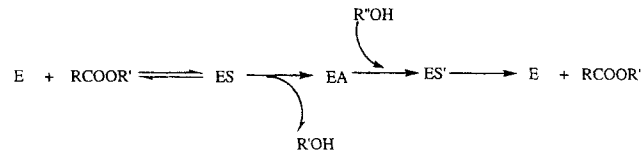
Serine Protease Catalyzed Hydrolysis of Esters (Acyl Enzyme Mechanism).



Alcholysis (Transesterification) of Esters (Acyl Enzyme Mechanism).



Alcholysis (Transesterification) of Esters (Ping-Pong Bi-Bi Mechanism).



The initial rate (v) of transesterification between substrates A and B follows the general form of

$$v = \frac{V_1[E_t][A][B]}{K_1[A] + K_2[B] + [A][B]}, \tag{1}$$

where V_1, K_1, and K_2 are functions of the microscopic rate constants of the elementary reaction steps and E_t is the concentration of enzyme (Cleland, 1963; Segel, 1975). We have also used this rate expression to describe the polytransesterification processes in polyester synthesis.

Enzymes are attractive biocatalysts because of their ability to discriminate between different types of substrates (Faber, 1995). Enzymes are selective to substrates containing different leaving groups (Zaks and Klibanov, 1986; Chaudhary et al., 1996b), functional groups in general (chemoselective) (Tawaki and Klibanov, 1993; Chinsky et al., 1989), and also exhibit regioselectivity (positional selectivity with identical functional groups (Kazandijian and Klibanov, 1991; Nicolosi et al., 1993). Indeed, the specificity of lipases has been exploited for synthetic applications in the food (Mojovic et al., 1993) and the pharmaceutical industry (Hughes et al., 1990). During the enzyme-catalyzed polytransesterification processes, substrates are continuously increasing in size, and changing their functionality. Therefore, the *equal reactivity* assumption may not be suitable for enzyme catalyzed processes.

Lipase-catalysis for polyester synthesis: experimental observations

Several attempts have been made previously to understand the role of lipases in AA-BB-type polyesterification reactions. Morrow and coworkers observed a faster disappearance of diol (1,4-butanediol) from the reaction mixture rela-

tive to bis(trichloroethyl) diester (Wallace et al., 1989), indicating that the dimer (hydroxyester) reacted faster with diol to form dihydroxy-capped trimer. In contrast with another substrate (bis(trifluroethyl) ester), they observed a higher loss of diester compared to 1,4-butanediol (Brazewell et al., 1995). Unequal amounts of ester and alcohol end groups in the system led them to conclude that trifluoroethyl groups were consumed by hydrolysis. Athavale also observed a faster disappearance of bis(2,3-butanedione monoxime) diester as compared to 1,4-butanediol (Athavale and Gaonkar, 1994). It is therefore possible that a hydroxyester may react preferentially with one of the starting monomers depending on the enzyme and substrates used.

Activated leaving groups have been used to increase the rate of slow biocatalytic reactions, and vinyl esters are particularly attractive since they not only accelerate the acyl transfer reaction but also shift the equilibrium to syntheses (Wang et al., 1988). This is required for very effective polytransesterification since equilibrium can limit the polyester molecular weight (Morrow, 1992; Linko et al., 1995).

We have analyzed lipase-catalyzed polytransesterification between divinyl adipate (DVA) and 1,4-butanediol (1,4-BD) in some depth. Following the polymerization in tetrahydrofuran, we observed that DVA disappeared from the reaction mixture at a faster rate than 1,4-BD (Chaudhary et al., 1997b). Although hydrolysis was an important side reaction consuming the diester, there were indications that higher reactivity of DVA with oligomers may be responsible for the faster disappearance of DVA.

Although the DVA/BD system is complex, our ability to use MALDI to dissect results of the polymerization provides a unique opportunity to develop a model that can be used not only to predict but also explain the observed behavior.

Nonequal reactivity model

To develop a nonequal reactivity model (NERM) to describe the kinetics of lipase-catalyzed polytransesterification, several assumptions were made:

1. There is no loss of enzyme activity within the time period studied.
2. The rate of esterification is negligible in comparison to the rate of transesterification and hydrolysis, and therefore the contribution of esterification rate to the polymer growth process can be neglected (see experimental results below).
3. The effect of enzyme specificity on the rate constants in Eq. 1 can be assessed by model substrate reaction kinetics and MALDI data from polymerized systems.
4. The redistribution (transesterification between ester end groups within a molecule) rate is negligible at the reaction temperatures used.
5. The effect of enzyme specificity on rate depends on chain length.

Biocatalytically synthesized polyesters comprise linear chains of different size. These chains can be classified based on the nature of functional end groups (Table 1). Based on this classification, it is possible to describe all the reaction steps that are occurring in the system:

$$A_i + B_j \rightarrow C_{i+j} \tag{2}$$

Table 1. Classification of Polymer Chains Present in the Reaction Mixture during Lipase-Catalyzed Polytransesterification Between Divinyl Adipate and 1,4-Butanediol

Nomenclature	End Groups
$A_{(i)}$, i = odd*	Both end groups are vinyl ester groups (*ee*)
$B_{(j)}$, j = odd	Both end groups are hydroxyl groups (*oo*)
$C_{(k)}$, k = even	One end group is vinyl ester and the other end group is hydroxyl group (*eo*)
$D_{(i)}$, i = odd	One end group is vinyl ester and other side end group is carboxylic group (*ea*)
$E_{(k)}$, k = even	One end group is carboxyl and other end group is hydroxyl group (*ao*)
$F_{(j)}$, j = odd	Both end groups are carboxyl groups (*aa*)

*i, j, and k correspond to the number of structural units within oligomer/polymer chains (e.g., A_1 = diester, B_1 = diol, C_2 = hydroxyester, D_1 = ester-acid, E_2 = hydroxyacid, and F_1 = diacid). Also for polycondensation reactions, two structural units = 1 repeat unit, therefore next diester = A_3, diol = B_3, hydroxyester = C_4, ester-acid = D_3, hydroxyacid = E_4, diacid = F_3, and so on.

$$A_i + C_k \rightarrow A_{i+k} \tag{3}$$

$$A_i + H_2O \rightarrow D_i \tag{4}$$

$$A_i + E_k \rightarrow D_{i+k} \tag{5}$$

$$B_j + C_k \rightarrow B_{j+k} \tag{6}$$

$$B_j + D_i \rightarrow E_{j+i} \tag{7}$$

$$C_k + C_l \rightarrow C_{k+l} \tag{8}$$

$$C_k + H_2O \rightarrow E_k \tag{9}$$

$$C_k + D_i \rightarrow D_{k+i} \tag{10}$$

$$C_k + E_l \rightarrow E_{k+l} \tag{11}$$

$$D_i + H_2O \rightarrow F_i \tag{12}$$

$$D_i + E_k \rightarrow F_{i+k}. \tag{13}$$

Clearly, chains can be formed and consumed by several possible routes, and a compilation of all the possible routes is given in Table 2. Using this information, mass balance equations for all the chains can be written. For example, the rate of change of the concentration of A_3 (at constant reaction volume) is given by

$$\frac{d(A_3)}{dt} = \Sigma(\text{formation rates for } A_3) - \Sigma(\text{disappearance rates for } A_3). \tag{14}$$

Similar equations can also be written for all species and this leads to a system of $3n$ simultaneous ordinary differential equations, where n is the number of structural units present in the longest hydroxy-ester chain present. Since we know the initial concentration of each species, the product composition can be obtained by simultaneous solution of these differential equations with time as an independent variable as long as information on specificity effects on rate constants is available.

Table 2. Disappearance Reactions for Various Chains and Products Formed as a Result of Condensation

Species	Reactions Removing the Species	Species Formed Due to Reaction
A_i	(i) $A_i + C_{n-p-1}$ (ii) $A_i + B_{n-p}$ (iii) $A_i + H_2O$ (iv) $A_i + E_{n-p-1}$ $(p < i,\ p = 1, 3, \ldots)$	(i) $A_{i+n-p-1}$ (ii) C_{i+n-p} (iii) D_i (iv) $D_{i+n-p-1}$
B_j	(i) $B_j + C_{n-q-1}$ (ii) $B_j + A_{n-q}$ (iii) $B_j + D_{n-q}$ $(q < j,\ q = 1, 3, \ldots)$	(i) $B_{n-q-1+j}$ (ii) C_{n-q+j} (iii) E_{n-q+j}
C_k	(i) $C_k + A_{n-r-1}$ (ii) $C_k + B_{n-r-1}$ (iii) $C_k + C_{n-r}$ (iv) $C_k + H_2O$ (v) $C_k + D_{n-r-1}$ (vi) $C_k + E_{n-r}$ $(r < n,\ r = 2, 4, \ldots)$	(i) $A_{k+n-r-1}$ (ii) $B_{k+n-r-1}$ (iii) C_{k+n-r} (iv) E_k (v) $D_{k+n-r-1}$ (vi) E_{k+n-r}
D_i	(i) $D_i + C_{n-p-1}$ (ii) $D_i + B_{n-p}$ (iii) $D_i + E_{n-p-1}$ (iv) $D_i + H_2O$ $(p < i,\ p = 1, 3, \ldots)$	(i) $D_{i+n-p-1}$ (ii) E_{i+n-p} (iii) $F_{i+n-p-1}$ (iv) F_i
E_k	(i) $E_k + A_{n-r-1}$ (ii) $E_k + C_{n-r}$ (iii) $E_k + D_{n-r-1}$ $(r < n,\ r = 2, 4, \ldots)$	(i) $D_{k+n-r-1}$ (ii) E_{k+n-r} (iii) $F_{k+n-r-1}$

Note: p, q, and r are the counters and n = size of the longest hydroxyester synthesized during the experiment.

The preceding system of simultaneous ordinary differential equations was solved using a fourth-order Runge-Kutta method (McCracken and Dorn, 1964; Carnahan et al., 1969). The algorithm for the procedure is described in Figure 1.

To conclude, we stress again that there are few chemical polycondensation systems where the *equal reactivity* assumption is not valid. For these cases, arbitrary changes in reactivity were used and theoretical predictions in changes of product distribution profiles were also made (Gupta et al., 1978, 1979). For our case, since MALDI-TOF results provide information on the composition of polyesters, we can identify quantitatively the role of enzyme specificity by comparison of experimental data with model predictions.

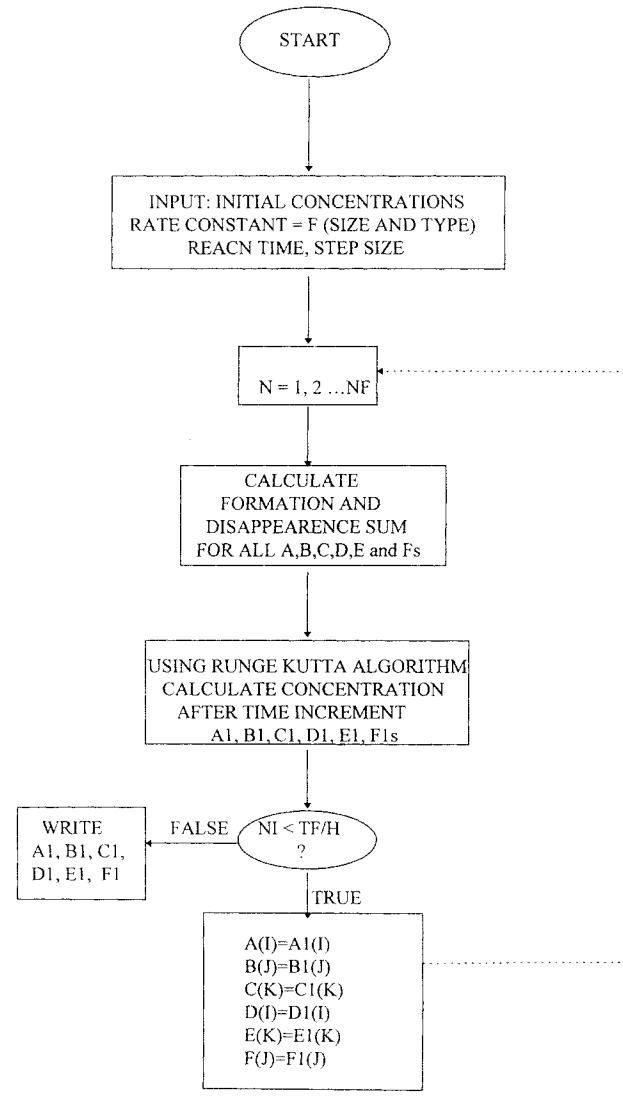


Figure 1. Algorithm for calculation of molecular weight and functionality profiles for lipase-catalyzed polytransesterification between divinyl adipate and 1,4-butanediol.

Experimental

Materials

Adipic acid (ADA), 1,4-butanediol (1,4-BD), dodecane, and tetrahydrofuran (HPLC and reagent grade) were purchased from Aldrich (St. Louis). Novozym-435 was obtained from Novo Nordisk Bioindustries (Denmark), and divinyl adipate (DVA) was a very kind gift from Union Carbide. All experiments were performed using enzyme from the same lot.

Reactions

All reactions were performed in 20-mL vials with DVA and 1,4-BD (250 to 700 mM) dissolved in the solvent. Typically, enzyme was dried over phosphorus pentoxide for 72 h and added to the reaction mixture, and after incubating for fixed periods at 22°C and shaking speed of 225 rpm, the mixture was filtered. Enzyme was thoroughly washed with tetrahydrofuran (THF) to capture any bound polymer. Filtrates and rinses were then pooled and the solvent was removed using a rotary evaporator.

Polyester analysis

Gel Permeation Chromatography. Gel permeation chromatography (GPC) measurements were performed using a Waters 150CV Gel Permeation Chromatograph. THF, at a flow rate of 1.0 mL/min (35°C), was used as mobile-phase solvent. The instrument was equipped with two ultrastyragel columns (from Waters) with pore sizes of 10^4 Å, 10^3 Å, and PL gel mixed-*E* columns (from Polymer laboratories) in series. About 10–12 polystyrene standards (obtained from Polymer Laboratories) within the molecular weight range of 162–11,600 were used for calibration.

Table 3. Isotopic Abundance Spectra of Chains Present in Biocatalytically Synthesized Polyester*

Polyester Chain	Theoretically Predicted Isotopic Abundance Spectra	Observed Isotopic Abundance Spectra
$C_{42}H_{68}O_{17}+Na^+$ (Ester–Alcohol)	867.4 (100), 868.4 (52), 869.44 (19), 870.44 (5)	867.2 (100), 868.2 (58), 869.3 (20), 870.2 (7)
$C_{44}H_{74}O_{18}+Na^+$ (Alcohol–Alcohol)	913.44 (100), 914.48 (55), 915.48 (16), 916.48 (5)	913.3 (100), 914.2 (59), 915.2 (18), 916.1 (8)
$C_{42}H_{68}O_{17}+Ag^+$ (Ester–Alcohol)	951.4 (96), 952.4 (48), 953.4 (100), 954.4 (46), 955.4 (18), 956.4 (4)	951.1 (90), 952.2 (55), 953.1 (100), 954.1 (44), 955.1 (16), 956.1 (5)
$C_{44}H_{74}O_{18}+Ag^+$ (Alcohol–Alcohol)	997.39 (90), 998.4 (48), 999.39 (100), 1,000.4 (48), 1,001.4 (13), 1,002.4 (4)	997.2 (87), 998.2 (35), 999.2 (100), 1,000.2 (35), 1,001.2 (12), 1,002.1 (5)
$C_{40}H_{62}O_{16}+Ag^+$ (Ester–Ester)	905.31 (93), 906.31 (39), 907.31 (100), 908.31 (45), 909.32 (13)	905.0 (56), 906.1 (45), 907.1 (100), 908.1 (45), 909.1 (60)

*Note that ester–acid and acid–acid chains were determined based on the molecular mass of peaks. Also an error of about 0.2–0.3 D during calibration is reflected in the position of the peaks.

Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry of Polyesters. A Fisons VG TOFspec SE spectrometer was used for the analysis of polyesters as described previously (Chaudhary et al., 1996a). Samples were prepared by mixing dithranol (10 mg/mL in THF), silver trifluoroacetate (1 mg/mL in THF), and polyester (10 mg/mL in THF) in the ratio of 8:1:1, respectively. Enough of this mixture was applied to the sample position to cover the 2.5-mm-diameter area (typically 0.3 μL). The spot was allowed to air dry without assistance. The instrument was calibrated with polystyrene ($M_p = 2{,}000$), angiotension-I ($M = 1{,}296$), and ACTH clip ($M = 2{,}466$). The presence of matrix and matrix-adduct peaks in the low-mass region prevents accurate determination of monomer and low oligomeric adducts below $M = 400$. Hence, matrix-assisted laser desorption/ionization time of flight (MALDI-TOF)-spectra were analyzed for chains with cationic adducts having mass values greater than 400, and comparison with the model predictions was also performed in that range.

In the analysis of polyesters by MALDI-TOF, two series of cationic adducts were detected for several of the polyester chains. These adducts had either sodium ($M + 23$) or silver ($M + 108$) as the cation component. The identity of these peaks was verified by comparing the observed isotopic abundance spectra with theoretical predictions (Table 3). The ionic yield of silver adducts was larger than that of sodium adducts, and hence peak heights of silver adducts were used to calculate the proportion of different chains in the polyester.

Initial-rate determination

Calibration curves were generated using different concentrations of all the monomers (DVA, 1,4-BD, and adipic acid) using the refractive index detector on GPC. These calibration curves were then used to determine the concentration of monomers for the initial-rate determination experiments as described below.

Transesterification between DVA and 1,4-BD. DVA and 1,4-BD at various concentrations (20–200 mM) were dissolved in 50 mL of THF. About 50 mg (1 mg/mL) of Novozym-435 was added to the reaction mixture. Samples (0.5 mL) were withdrawn within the first 10 min of reaction. Samples were diluted by addition of THF and then analyzed using gel permeation chromatography. Initial rate was determined by following the disappearance of the limiting substrate using the previously generated calibration curve.

Esterification between Adipic Acid (ADA) and 1,4-BD. An identical procedure to that described in (a) was used for the determination of esterification rate except that higher enzyme concentration (500 mg ~ 10 mg/mL) and longer time period (up to 3 h) were required to follow the esterification rate.

Hydrolysis of DVA. The rate of hydrolysis of DVA at varying enzymes (0.5 mg/mL to 10 mg/mL) and DVA concentrations (40–500 mM) was monitored using gas chromatography. No water was added to the reaction mixture, therefore the water for hydrolysis was that associated with the enzyme itself. Samples (0.2 μL) were manually injected into a Hewlett-Packard 5890 Series II gas chromatograph containing an HP-1 cross-linked capillary column (30 m × 0.53 mm × 1.0 μm) as described previously (Chaudhary et al., 1997b). A standard curve for DVA (generated using dodecane as an internal standard) was obtained and unknown concentrations were determined.

Data analysis

Regression and correlation coefficients for kinetic models were calculated using STATISTICA for Windows, version 4.3, Statsoft Inc. The enzyme–kinetics models were fitted to initial rate data using a nonlinear estimation routine that minimizes the sum of the squares of difference between the observed and predicted values using a quasi-Newton procedure.

Results and Discussion

Experimental kinetics

Initial Rate Analysis: Accuracy of Kinetic Models. For single-substrate reactions, it is common yet somewhat inappropriate to rearrange the Michaelis–Menten equation to determine kinetic constants by double-reciprocal plots. Although, a large number of reactions involving more than one substrate can be analyzed using multiple double-reciprocal plots (Marty et al., 1992; Kamat et al., 1995), in such analyses low regression coefficients of plots at different stages of analysis are usually not accounted for and errors in slope and intercept values may be propagated. It is therefore more appro-

priate to use nonlinear curve-fitting procedures to test the validity of kinetic models for reactions with multiple substrates. We have therefore followed a semiempirical procedure to obtain values of kinetic constants for the selected model expressions.

Using plots of initial rate vs. substrate concentration, we analyzed the *qualitative* dependence of initial rate with concentration of substrates. We then ensured that the acyl enzyme kinetic model accounted for at least 80% of the variability in the data ($R^2 \geq 0.8$) and calculated rate constants by a nonlinear curve-fitting procedure as described in the data-analysis section. For nontransesterification processes we used the simplest possible model, which accounted for 80% of the variability in the data.

Transesterification Rate. The effect of DVA and 1,4-BD concentration on initial rate is shown in Figure 2. As expected, the rate of reaction increases with concentration of DVA and 1,4-BD. When initial-rate values are plotted vs. DVA concentration (at constant concentration of 1,4-BD), a linear increase in initial rate with DVA concentration was observed followed by a saturating-type behavior (plots not shown). A similar trend was also observed with varying concentration of 1,4-BD (at constant DVA concentration). Based on this behavior, an empirical model that will incorporate both of these trends will have the following general form:

$$\text{Rate} \propto \left(\frac{[\text{DVA}]}{\text{const}+[\text{DVA}]}\right)\left(\frac{[\text{BD}]}{\text{const}+[\text{BD}]}\right). \qquad (15)$$

The rate model for the transesterification process ($R^2 = 0.812$) is given by

$$\nu = \frac{V_1 * [E_t] * [\text{DVA}] * [\text{BD}]}{K_1 * [\text{DVA}] + K_2 * [\text{BD}] + [\text{DVA}] * [\text{BD}]}, \qquad (16)$$

where $V_1 = 236.22$ mM/min g enzyme: $K_1 = 209.8$ mM; $K_2 = 526.8$ mM; E_t in gms; ν in mM/min enzyme; and [DVA] and [BD] in mM.

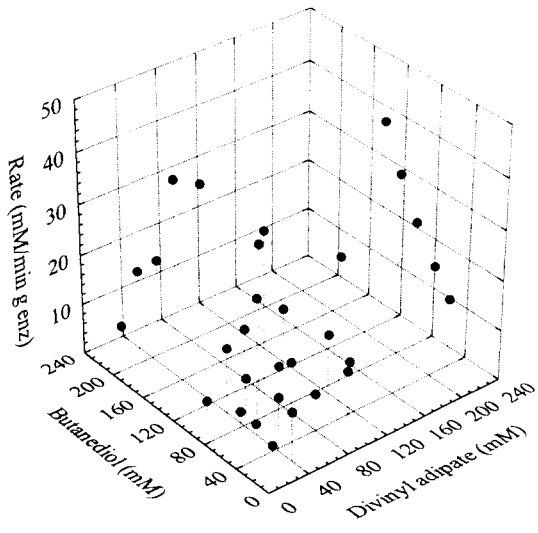


Figure 2. Initial rate of lipase-catalyzed transesterification between divinyl adipate and 1,4-butanediol (solvent = THF; temperature = 22°C).

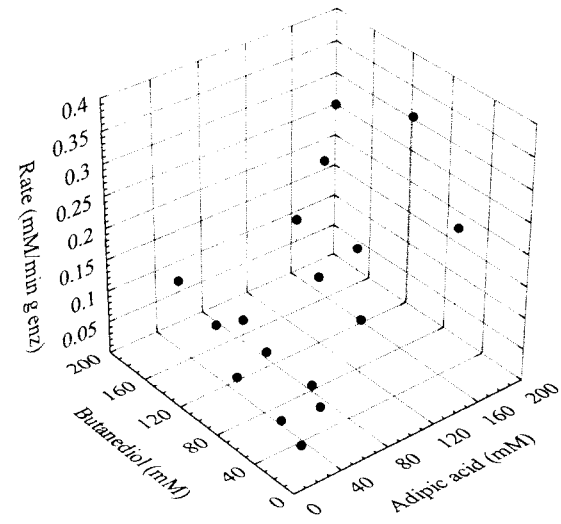


Figure 3. Initial rate of lipase-catalyzed esterification rate between adipic acid and 1,4-butanediol (solvent = THF; temperature = 22°C).

Esterification Rate. A plot of esterification rate vs. ADA and 1,4-BD concentration is shown in Figure 3. Once again, the esterification rate increases with concentration of ADA and 1,4-BD with the rate being more sensitive to ADA concentration than 1,4-BD. When initial rates are plotted against 1,4-BD concentration at constant ADA concentration, a saturating-type behavior with 1,4-BD concentration is also observed (plot not shown). A similar plot with ADA concentration gives a linear relationship over the ADA concentration range studied (plot not shown). The information obtained from the preceding analysis suggests that the simplest empirical model used to describe esterification rate will have the following form:

$$\nu \propto ([\text{ADA}]) * \left(\frac{[\text{BD}]}{\text{const}+[\text{BD}]}\right). \qquad (17)$$

A model ($R^2 = 0.91$) that explains our rate data is

$$\nu = \frac{V_1 * [E_t][\text{ADA}] * [\text{BD}]}{K_1 + [\text{BD}]}, \qquad (18)$$

where $V_1 = 21.78 \times 10^{-4}$ min^{-1} (g enz)$^{-1}$ and $K_1 = 21.44$ mM; ν in mM/min and E_t is in gms and [ADA] and [BD] in mM.

Hydrolysis Rate. The hydrolysis rate is a function of enzyme and DVA concentration as shown in Figure 4. When initial-rate values are plotted against enzyme concentration (at constant concentration of DVA), a linear relationship with enzyme concentration was observed with the line passing through the origin. Slopes of these lines changed with DVA concentration and showed saturating-type behavior with DVA concentration. This trend can be explained using the following expression:

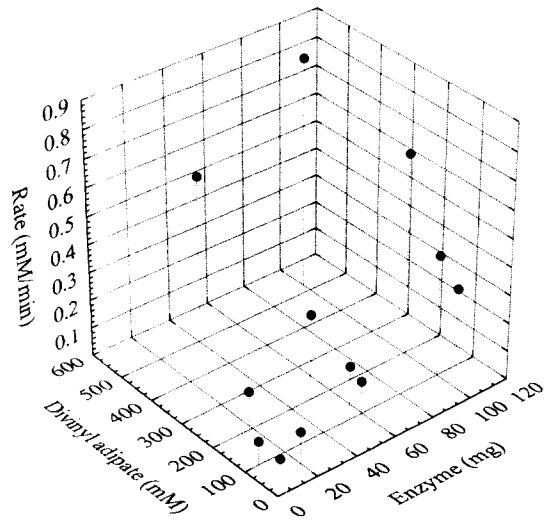


Figure 4. Initial rate of lipase-catalyzed hydrolysis rate at different concentrations of enzyme and divinyl adipate (solvent = THF; temperature = 22°C).

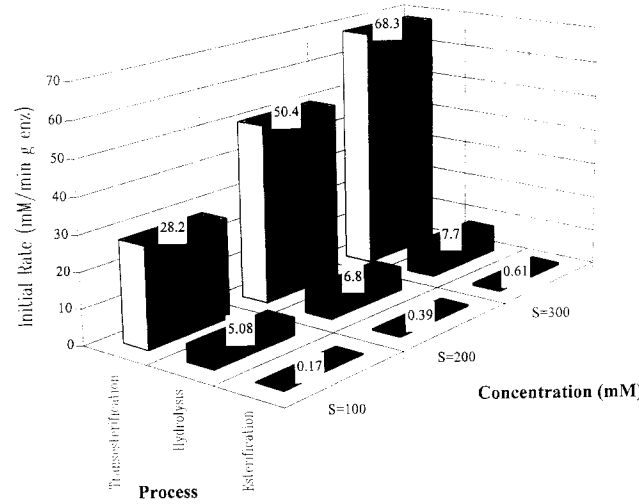


Figure 5. Comparison of rates of different types of processes occurring in biocatalytic polytransesterification (solvent = THF; temperature = 22°C).

$$\nu \propto ([E_t]) * \left(\frac{[\mathrm{DVA}]}{\mathrm{const} + [\mathrm{DVA}]} \right). \tag{19}$$

The model expression in Eq. 19 can describe hydrolysis kinetics relatively well ($R^2 = 0.958$):

$$\nu = \frac{V_1 * [E_t] * [\mathrm{DVA}]}{K_1 + [\mathrm{DVA}]}, \tag{20}$$

where $V_1 = 10.38$ mM/min g enzyme and $K_1 = 104.25$ mM; E_t in grams; ν in mM/min; [DVA] in mM).

Reaction Relative Rates. Using Eqs. 16, 18, and 20, we have calculated the initial rates of transesterification, hydrolysis, and esterification at different concentrations of substrates, assuming equimolar concentrations. For comparison, these rates are shown in Figure 5. It is important to note that within the concentration range used, the initial rate of esterification is negligible as compared to transesterification, and therefore its contribution to the polymer growth process can be neglected as postulated in assumption 2.

It is also interesting to note that the relative rate of transesterification to hydrolysis increases from 5.55 to 8.8 with an increase in substrate concentration. A similar increase (from 0.033 to 0.079) is also observed for the relative rates of esterification and hydrolysis. The relative rate of transesterification to esterification also changes from about 112 to 166 as the substrate concentration is lowered from 300 mM to 100 mM. Therefore, although the contribution of esterification to the polymer growth process is negligible ($< 1\%$) for concentration range described here, its contribution to polymerization rate will be important for reactions performed at higher concentrations of substrates. Indeed, a large concentration of substrates (in the complete absence of solvent) has resulted in increased rates of transesterification and esterification, and allowed us to develop a rapid, exothermic, and efficient biocatalytic process for synthesis of polyesters (Chaudhary et al., 1997c).

Effect of transesterification rate expression on different reactions

According to the transesterification expression (Eq. 15), the initial rate of reaction between DVA and 1,4-BD will be given as

$$\nu = \frac{V_1 * [E_t]}{\dfrac{526}{[\mathrm{DVA}]} + \dfrac{209}{[\mathrm{BD}]} + 1}. \tag{21}$$

For transesterification between a diester (acting as an ester moiety) and ester–alcohol (acting as an alcohol moiety), the initial rate will be

$$\nu = \frac{V_1 * [E_t]}{\dfrac{526}{[\mathrm{EE}]} + \dfrac{209}{[\mathrm{EO}]} + 1}, \tag{22}$$

and for the reaction between a diol (acting as an alcohol moiety) and ester-alcohol (acting as an ester moiety), the rate will be

$$\nu = \frac{V_1 * [E_t]}{\dfrac{526}{[\mathrm{EO}]} + \dfrac{209}{[00]} + 1}. \tag{23}$$

During the initial period, when low concentrations of ester–alcohol are present, the initial rate for a diester and ester–alcohol reaction will be $\sim V_1 * [\mathrm{EO}]/209$ (the contribution of other terms will be less significant due to high concentration of [DVA]). Similarly, the rate for an ester–alcohol and

Table 4

(a) Effect of Enzyme Concentration for Lipase-Catalyzed Polytransesterification Between Divinyl Adipate and 1,4-Butanediol (Temp. = 22°C, [DVA] = [BD] = 500 mM)

Time (h)	Molecular Weight (PDI)	
	E = 3.33 mg/mL	E = 13.3 mg/mL
0.5	—	1,781 (1.17)
1	1,584 (1.23)	2,465 (1.3)
2	1,650 (1.19)	3,273 (1.44)
3	2,126 (1.31)	3,400 (1.54)
4	1,863 (1.21)	3,599 (1.58)

(b) Effect of Substrate Concentration for Lipase-Catalyzed Polytransesterification Between Divinyl Adipate and 1,4-Butanediol (Temp. = 22°C, [E] = 10 mg/mL)

Time (h)	Molecular Weight (PDI)	
	250 mM	700 mM
0.5	1,312 (1.1)	1.578 (1.26)
1	1,942 (1.25)	2,104 (1.37)
2	2,229 (1.31)	2,777 (1.4)
3	2,516 (1.4)	3,264 (1.52)
4	2,800 (1.46)	4,369 (1.54)

(c) Effect of Substrate Stoichiometry on Lipase-Catalyzed Polytransesterification Between Divinyl Adipate and 1,4-Butanediol (Temp. = 22°C, [E] = 10 mg/mL, Limiting Substrate = 500 mM)

Time (h)	Molecular Weight (PDI)	
	DVA/BD = 1.2	BD/DVA = 1.2
0.5	1,703 (1.19)	1,359 (1.11)
1	2,378 (1.29)	1,597 (1.16)
2	3,200 (1.35)	1,957 (1.21)
3	2,905 (1.36)	2,151 (1.24)
4	3,393 (1.38)	2,222 (1.25)

alcohol–alcohol reaction will be $\sim V_1 * [EO]/526$. This suggests that DVA will disappear from the reaction mixture faster than BD during the initial phase, as was observed previously for short times (Chaudhary et al., 1997b). However, while qualitatively correct, the magnitude of the difference between BD and DVA consumption using these expressions is large, and must be corrected by using a suitable multiplying factor (> 1) for the reaction between a diol and ester–alcohol as shown later.

Polymerization Results. Reaction conditions such as substrate concentration, enzyme concentration, and substrate stoichiometry were found to be important in determining the molecular weight and functionality of polyesters synthesized by lipase-catalyzed polytransesterification between divinyl adipate and 1,4-BD. These parameters were varied for reactions performed in tetrahydrofuran, and their effects on polyester molecular weight and polydispersity index (determined using GPC) are shown in Table 4.

Molecular Weight. As expected, for equimolar concentrations of monomers, polyester molecular weight increases with both enzyme concentration (Table 4a) and substrate concentration (Table 4b). For reactions where the starting concentration of one of the monomers is different, higher molecular weights are obtained when DVA is the substrate in excess (Table 4c). This is consistent with our earlier observation that DVA has a higher reactivity with growing oligomers, and hence at any given time, higher DVA concentration leads to a relatively faster polymerization as compared to the case when a similar excess of 1,4-BD is used.

Functionality. Polyester end-group functionality is particularly important when polyesters are to be used as polyols in urethane manufacture. For a biocatalytic polytransesterification, the presence of a solvent such as tetrahydrofuran favors the release of water from the enzyme, and the subsequent hydrolysis of activated groups leads to the formation of acid end groups (Chaudhary et al., 1996a, 1997b).

Changes in overall functionality can be observed from MALDI-TOF analyses of the biocatalytically synthesized polyesters. The overall changes are a result of the change in the proportion of different types of species (ester–ester, ester–alcohol, alcohol–alcohol, ester–acid, acid–alcohol, and acid–acid). Since the final composition of different chains is guided by the kinetics and the reactivity of different substrates, we have evaluated the role of enzyme specificity in the following way.

Effect of enzyme specificity on rate constants

We have used the model to predict the composition of polyester products after 2 h of reaction under different conditions. The experimental data for polyester compositions are given in Table 5.

The experimentally determined rate constants for reactions of monomers with each other and with water are shown in Table 6. As mentioned previously, while traditional polycondensation kinetic analysis presumes that the rate constant for esterification is constant with respect to chain length, we cannot assume that this is the case for the analogous enzymatic process. Indeed, if one assumed that the rates of transesterification and hydrolysis are independent of the size and type of species ($V_L = V_1/L^x$, $x = 0$ and L = number of structural units in the reaction product, corresponding to V_1 =

Table 5. Composition of Polyesters Synthesized by Biocatalytic Polytransesterification Under a Different Set of Initial Conditions

Reaction Conditions			Polymer Composition					
DVA (mM)	BD (mM)	E_t (mg/mL)	*EE* (%)	*OO* (%)	*EO* (%)	*EA* (%)	*AA* (%)	*AO* (%)
600	500	10	44.0	3.6	13.7	19.0	10.6	9.3
500	600	10	18.5	37.7	10.1	10.8	10.8	11.8
500	500	3.33	40.9	21.2	15.6	11.3	5.0	6.0
500	500	13.33	30.0	23.0	12.0	10.2	9.9	12.6
250	250	10	23.6	29.7	6.3	14.2	13.8	12.4
700	700	10	30.5	30.6	12.3	10.2	9.7	6.7

Reaction time = 2 h; temperature = 22°C; solvent = tetrahydrofuran.

Table 6. Summary of Rate Constants for Initial Steps for Reactions in Biocatalytic Polytransesterification

Reaction	Rate Expression	Rate Constants
Transesterification	$\nu = \dfrac{V_1 * [E_t] * [\mathrm{DVA}] * [\mathrm{BD}]}{K_1 * [\mathrm{DVA}] + K_2 * [\mathrm{BD}] + [\mathrm{DVA}] * [\mathrm{BD}]}$	$V_1 = 236.22$ mM/min $K_1 = 209.8$ mM $K_2 = 526.8$ mM
Esterification	$\nu = \dfrac{\mathrm{V_1} * [\mathrm{E_t}][ADA] * [BD]}{\mathrm{K_1} + [BD]}$	$V_1 = 21.78 \times 10^{-4}$ min^{-1} (g enz)$^{-1}$ $K_1 = 21.44$ mM
Hydrolysis	$\nu = \dfrac{\mathrm{V_1} * [\mathrm{E_t}] * [\mathrm{DVA}]}{\mathrm{K_1} + [\mathrm{DVA}]}$	$V_1 = 10.38$ mM/min g enz $K_1 = 104.25$ mM

constant in Figure 6), then the model predicts that product would consist exclusively of acid–acid species after just 2 h. Our MALDI-TOF results (Table 5) show that this is clearly not the case, and therefore substantiate that the constant reactivity assumption must not be valid.

Given these results, we explored the ability for molecular-weight-dependent rate constants to provide an adequate description of the evolution of polymer molecular weight and end-group functionality in this enzyme-catalyzed polyesterification. Previous work in conventional polycondensation has shown that the rate constant decreases with molecular weight for very small molecules, but levels out quickly as size increases, forming the basis for Flory's well-known statistical analysis of polycondensation. Given that specificity plays a key role in governing enzymatic catalysis, we postulate that the various rate constants decrease with molecular size, yet do not approach their lower limit nearly as quickly as the analogous chemical process.

Given this hypothesis, we explored the utility of several rate constant-chain length profiles, as shown in Figure 6, by comparison of model predictions with experimentally determined acid–acid contents. As shown in Figure 6, a comparison was made between fixed-rate parameters (as described earlier, V_1 = constant) and parameters that decreased with chain length as (chain length)$^{-2}$ ($V_1(L) = V_1/L^{**}2$), and as (chain length)$^{-3}$ ($V_1(L) = V_1/L^{**}3$). The model-predicted extent of diacid production was then compared with the MALDI-generated experimental results. This calculation suggested that varying the rate parameters as (chain length)$^{-2.5}$ would provide the best results.

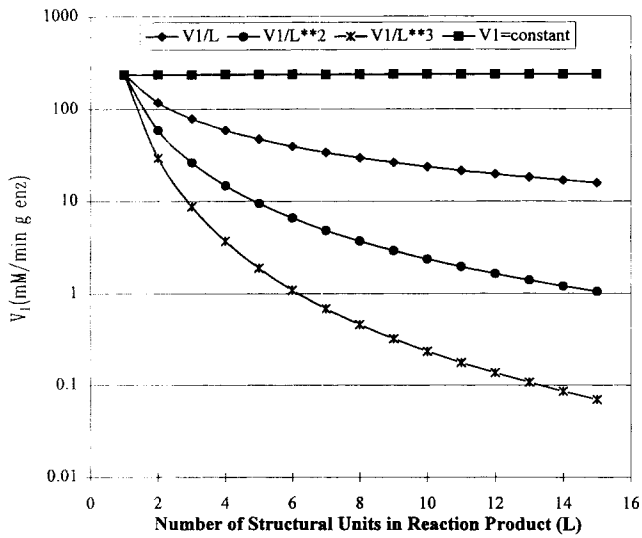


Figure 6. Selected assumptions for decrease in reactivity as a function of molecular weight.

Additionally, we also observed that the model overpredicted the composition of ester–ester species as compared to alcohol–alcohol species. As discussed before, this discrepancy is due to the form of transesterification rate expression and can be reduced by using a multiplying factor for rate between a diol and ester–alcohol. By comparison of the model predictions and experimental data, a multiplying factor of 2.4 was obtained.

Effect of substrate stoichiometry

The most important variable that can affect the polyester functionality in traditional synthesis is the stoichiometric ratio of monomers. In Figure 7, the composition of polyester obtained after changing the starting stoichiometry of substrates from 1 to 1.2 is shown along with the model predictions using the approach just described. It can be seen that model predictions agree remarkably well with the experimental data.

When divinyl adipate is used in excess, a higher proportion of ester–ester-type chains are formed. Similarly, when 1,4-BD is used in excess, a higher proportion of alcohol–alcohol chains are also present in the polyester product. Whereas altering stoichiometry is a straightforward approach for control of end-group functionality in chemically catalyzed polycondensation systems, our results and model predictions show that for both the cases just described (20% excess of either DVA or BD), residual hydrolysis leads to the production of more than 30% of acid-containing species. Since no hydrolysis is expected to occur, for a chemically catalyzed polytransesterification, the similar change in stoichiometry will only increase the proportion of the excess functional group (an ester or an alcohol) within the polyester chains.

Although acid production is a potential limitation when the polyester is used as a prepolymer for polyurethanes, our understanding of how the acid appears gives us the opportunity to design synthetic strategies that avoid acid production.

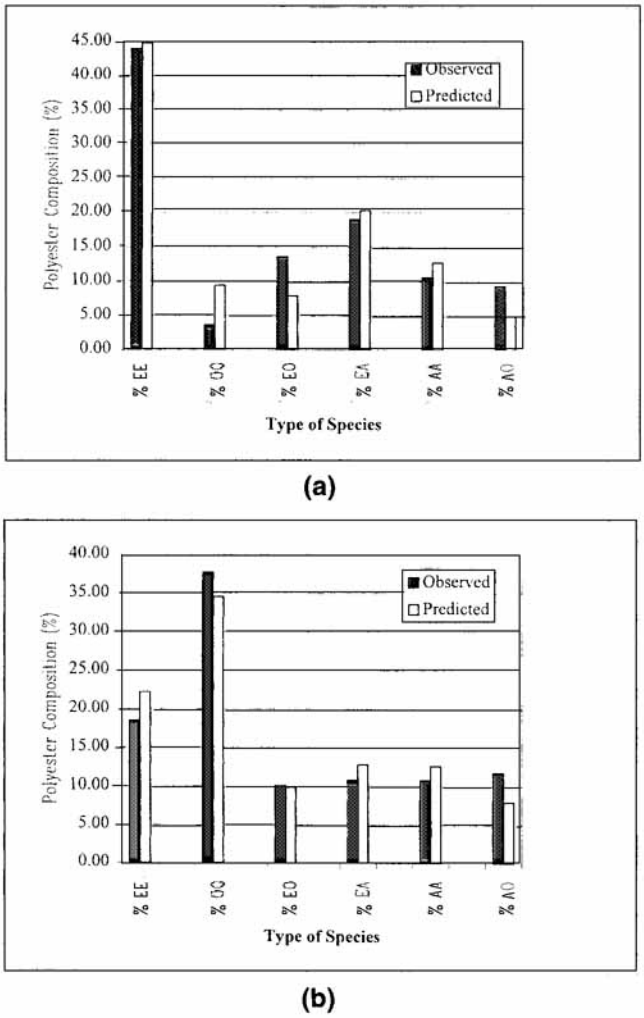


Figure 7. Effect of substrate stoichiometry on composition of biocatalytically synthesized polyester, and comparison with model predictions.

Temperature = 22°C; solvent = THF; limiting substrate concentration = 500 mM. (a) DVA/DB = 1.2. (b) BD/DVA = 1.2.

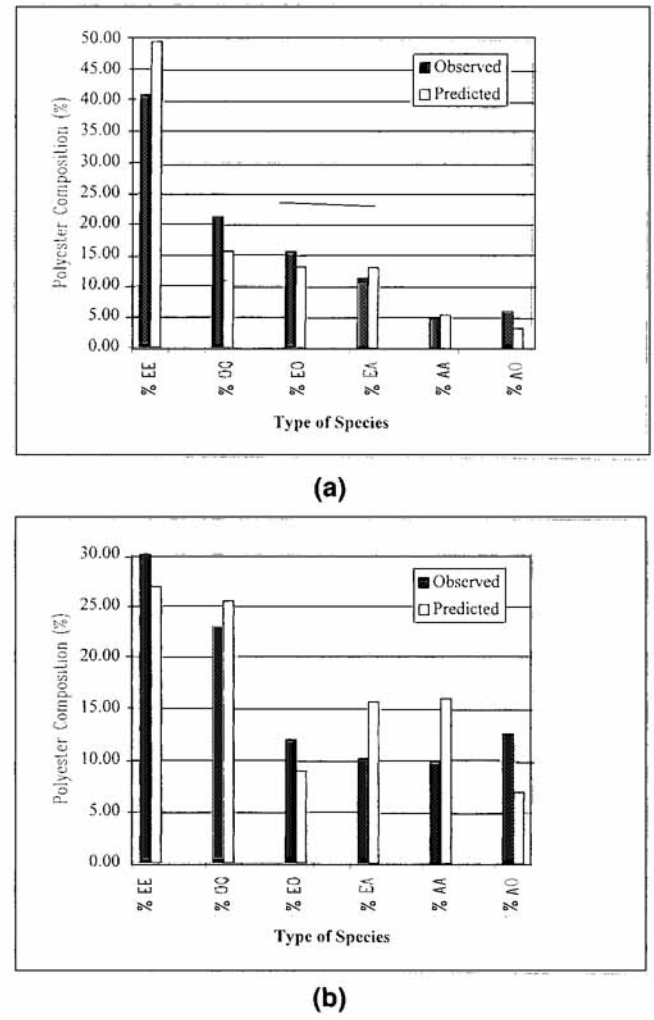


Figure 8. Effect of enzyme concentration on composition of biocatalytically synthesized polyester and comparison with model predictions.

Temperature = 22°C; solvent = THF; DVA = BD = 500 mM. (a) E = 3.33 mg/mL. (b) E = 13.33 mg/mL.

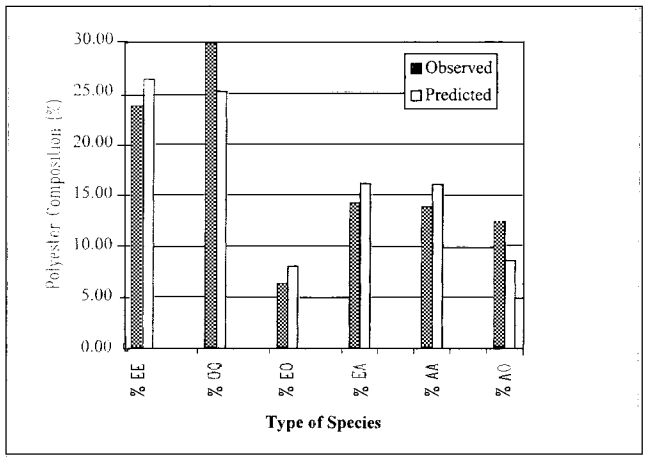
Effect of enzyme concentration

Previous studies have shown that the effect of enzyme concentration is not always predictable (Chaudhary et al., 1997b). Figure 8 shows the composition of polyesters at two enzyme concentrations. Increasing the concentration of enzyme leads to a decrease in the fraction of ester–ester species in the product (after 2 h), and increased proportion of acid-terminated species.

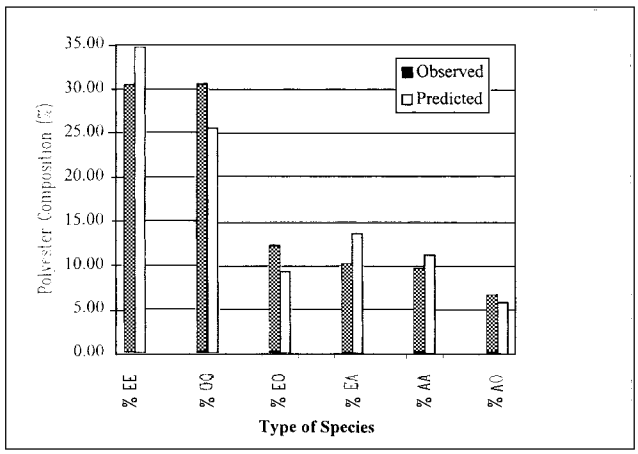
Because of the increase in the enzyme concentration, the initial rates of all the transesterification and hydrolysis reactions will increase. Whereas the ester-containing chains are consumed by direct hydrolysis, species containing alcohol end groups do not undergo direct hydrolysis. Also, the esterification rate is expected to be negligible, and therefore the chains possessing acid end groups will continue to build up in the product mixture. Therefore, the increase in hydrolysis rate because of the increase in enzyme concentration will affect the proportion of ester-containing chains to a greater extent than the chains possessing alcohol end groups.

Figure 9b also shows that at higher enzyme concentration, the model overpredicts the proportion of ester–acid and acid–acid chains in the polyester product. According to the model used to describe hydrolysis, the hydrolysis rate is proportional to the concentration of enzyme and the hydrolyzing substrate. It is important to note that a term for the concentration of water is not included in this rate expression. Since water for the hydrolysis reactions comes from the enzyme itself, its concentration should depend on the amount of enzyme used. This implies that rate constant V_1 for hydrolysis rate expression (Eq. 20) already includes the concentration of water.

As polymerization proceeds, there will be a decrease in the concentration of water. Therefore, a decrease in V_1 occurs as a result of increasing molecular weight as well as decreasing water concentration. Therefore, if we were to assume that the relative decrease in hydrolysis rate with an increase in molecular weight for hydrolysis is greater than the similar reactivity change for transesterification, then it is possible to



(a)



(b)

Figure 9. Effect of substrate concentration on composition of biocatalytically synthesized polyester and comparison with model predictions.

Temperature = 22°C; solvent = THF; $E = 0.15$ g. (a) DVA = BD = 250 mM. (b) DVA = BD = 700 mM.

explain the overprediction of acid-containing species observed here at higher a concentration of enzyme.

Effect of substrate concentration

Increasing the concentration of substrate from 250 mM to 700 mM leads to an increase in molecular weight and a decrease in the proportion of acid-ended chains in the polyester. The proportion of acid-ended species decrease by 13.8% with this change in initial concentration (Figure 9).

Using Eqs. 16 and 18, when substrate concentration is increased from 250 mM to 700 mM, the ratio of the initial rate of transesterification to hydrolysis will increase from 8.2 to 12.8 (approximately 50% increase). Therefore, the decrease in the proportion of acid-ended species is a result of a lower rate of direct hydrolysis of ester-containing polyester chains.

To summarize the results from the preceding experiments, the proportion of acid-containing chains decreases with increasing substrate concentration. Also, the proportion of hydroxyl-containing species can be increased by increasing the stoichiometric ratio of diol to diester as in conventional polycondensations. These results suggest that by combining both these strategies, that is, increasing the concentration of monomers and using higher stoichiometric ratios of diol to diester, a synthetic strategy for lipase-catalyzed synthesis of polyols can be developed.

Further refinements in the biocatalytic polytransesterification model

The polytransesterification kinetics model we have described could be improved by incorporating a more realistic effect of enzyme specificity on the rates of the reactions of interest. For example, a better fit of data from at least two sets of reaction conditions (at different stoichiometric concentrations of monomers) can be obtained by assuming that the decrease in reactivity for the hydrolysis reaction is higher than that for the transesterification by species containing acid end groups, which in turn is higher than that for transesterification reactions between species without any acid end groups.

However, since more than one set of assumptions may lead to favorable results, we have avoided the use of such a procedure to obtain an ideal, yet artificial fit between the experimental observations and model predictions. We will, however, continue to improve the predictable nature of our model by increasing the physical basis of our assumptions by estimating more rate constants and including polymerization results from many more experiments.

Conclusion

Reaction and substrate specificity is a hallmark of enzyme catalysis, and polyester composition is strongly influenced by this feature of biocatalysis. We have quantified the effect of hydrolysis side reactions on the composition of polyester. Both model predictions and experimental observations indicate that large decreases in enzyme specificity occur during the polytransesterification process. We presume that since the enzyme specificity profile will be a function of the source of lipase, it will also be possible to control end-group functionality and molecular weight by choice of catalyst. We have also shown herein that it is possible to tailor rationally these differences by solvent and reaction engineering (Chaudhary et al., 1997a).

Acknowledgments

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Literature Cited

Adams, K. A. H., S. H. Chung, and A. M. Klibanov, "Kinetic Isotope Effect Investigation of Enzyme Mechanism in Organic Solvents," *J. Amer. Chem. Soc.*, **112**(25), 9418 (1990).

Ajima, A., T. Yoshimoto, K. Takahashi, Y. Tamaura, Y. Saito, and Y. Inada, "Polymerization of 10-Hydroxydecanoic Acid in Benzene with Polyethylene Glycol-Modified Lipase," *Biotech. Lett.*, **7**(5), 303 (1985).

Athavale, V. D., and S. R. Gaonkar, "Enzymatic Synthesis of Polyesters by Lipase Catalyzed Polytransesterification," *Biotech. Lett.*, **16**(2), 149 (1994).

Bhide, B. V., and J. J. Sudborough, "Studies in Esterification," *J. Indian Inst. Sci.*, **8A**, 89 (1925).

Brazwell, E. M., D. Y. Filos, and C. J. Morrow, "Biocatalytic Synthesis of Polymers III. Formation of a High Molecular Weight

Polyester Through Limitation of Hydrolysis by Enzyme-bound Water and Through Equilibrium Control," *J. Poly. Sci., Part A: Poly. Chem.*, **33**, 89 (1995).
Carnahan, B., H. A. Luther, and J. O. Wilkes, *The Approximation of the Solution of Ordinary Differential Equations*, Wiley, New York (1969).
Chatterjee, S., and A. J. Russell, "Activity of Thiosubtilisin in Organic Solvents," *Biotechnol. Prog.*, **8**, 256 (1992).
Chatterjee, S., and A. J. Russell, "Kinetic Analysis of the Mechanism for Subtilisin in Essentially Anhydrous Organic Solvents," *Enzyme Microb. Technol.*, **15**, 1022 (1993).
Chaudhary, A. K., G. Critchley, A. Diaf, E. J. Beckman, and A. J. Russell, "Characterization of Synthetic Polymers Using Matrix-Assisted Laser Desorption/Ionization—Time of Flight Mass Spectrometry," *Macromol.*, **29**, 2213 (1996a).
Chaudhary, A. K., S. V. Kamat, E. J. Beckman, and A. J. Russell, "Control of Subtilisin Substrate Specificity by Solvent Engineering in Organic Solvents and Supercritical Fluoroform," *J. Amer. Chem. Soc.*, **118**, 12891 (1996b).
Chaudhary, A. K., E. J. Beckman, and A. J. Russell, "Biocatalytic Solvent-Free Polymerization to Produce High Molecular Weight Polyesters," *Biotechnol. Prog.*, **13**(3), 318 (1997a).
Chaudhary, A. K., E. J. Beckman, and A. J. Russell, "Biocatalytic Polyester Synthesis: Analysis of Evolution of Polymer Molecular Weight and End-Group Functionality," *Biotechnol. Bioeng.*, **55**(1), 227 (1997b).
Chaudhary, A. K., E. J. Beckman, and A. J. Russell, "Rapid Biocatalytic Polytransesterification: Reaction Kinetics in an Exothermic Reaction," *Biotech. Bioeng.*, in press (1998).
Chinsky, N., A. L. Margolin, and A. M. Klibanov, "Chemoselective Enzymatic Monoacylation of Bifunctional Compounds," *J. Amer. Chem. Soc.*, **111**, 386 (1989).
Cleland, W. W., "The Kinetics of Enzyme-Catalyzed Reactions with Two or More Substrates or Products," *Biochim. Biophys. Acta*, **67**, 104 (1963).
Dordick, J. S., M. A. Marletta, and A. M. Klibanov, "Polymerization of Phenols Catalyzed by Peroxidase in Non-Aqueous Media," *Biotechnol. Bioeng.*, **30**, 31 (1987).
Faber, K., *Biotransformations in Organic Chemistry — A Textbook*, Springer-Verlag, New York (1995).
Fersht, A., *Enzyme Structure and Mechanism*, Freeman, New York (1985).
Fink, A. L., and M. L. Bender, "Binding Sites for Leaving Groups and Added Nucleophiles in Papain-Catalyzed Hydrolyses," *Biochemistry*, **8**(12) (1969).
Flory, P. J., *Principles of Polymer Chemistry*, Cornell Univ. Press, Ithaca, NY (1953).
Goel, R., S. Gupta, and A. Kumar, "Rate of Condensation Polymerization for Monomers Having Reactivities Different from their Polymers," *Polymer*, **18**, 851 (1977).
Gupta, S. K., A. Kumar, and A. Bhargava, "Molecular Weight Distribution and Moments for Condensation Polymerization of Monomers Having Reactivity Different from their Homologues," *Polymer*, **20**, 305 (1978).
Gupta, S. K., A. Kumar, and A. Bhargava, "Molecular Weight Distributions and Moments for Condensation Polymerizations Characterized by Two Rate Constants," *Eur. Poly. J.*, **15**, 557 (1979).
Hughes, D. L., J. Bergan, J. Amato, M. Bhupathy, J. Leazer, J. McNamara, D. Sidler, P. Reider, and E. Grabowski, "Lipase-Catalyzed Asymmetric Hydrolysis of Esters Having Remote Chiral/Prochiral Centers," *J. Org. Chem.*, **55**, 6252 (1990).
Kamat, S. V., G. Critchley, E. J. Beckman, and A. J. Russell, "Biocatalytic Synthesis of Acrylates in Organic Solvents and Supercritical Fluids," *Biotechnol. Bioeng.*, **46**, 610 (1995).
Kamat, S., "Biocatalysis in Supercritical Fluids: Control of Enzyme Activity and Selectivity by Solvent Engineering," PhD Diss., Univ. of Pittsburgh, Pittsburgh, PA (1996).
Kanerva, L. T., and A. M. Klibanov, "Hammett Analysis of Enzyme Action in Organic Solvents," *J. Amer. Chem. Soc.*, **111**, 6864 (1989).
Kazandijian, R. Z., and A. M. Klibanov, "Regioselective Oxidation of Phenols Catalyzed by Polyphenol Oxidase in Chloroform," *J. Amer. Chem. Soc.*, **113**, 3166 (1991).
Klibanov, A. M., "Enzymes that Work in Organic Solvents," *Chemtech*, **6**, 354 (1986).
Knani, D., A. L. Gutman, and D. H. Kohn, "Enzymatic Polyesterification in Organic Media Enzyme-Catalyzed Synthesis of Linear Polyesters; I. Condensation Polymerization of Linear Hydroxyesters; II. Ring-Opening Polymerization of ϵ-Caprolactone," *J. Poly. Sci. Part A: Poly. Chem.*, **31**, 1221 (1993).
Kopnick, H., W. Brugging, and W. Kaminsky, "Polyesters," *Ulman's Encyclopedia of Industrial Chemistry*, Vol. A21, VCH, Weinheim, Germany, p. 227 (1992).
Linko, Y.-Y., Z.-L. Wang, and J. Seppala, "Lipase Catalyzed Linear Aliphatic Polyester Synthesis in Organic Solvent," *Enzyme Microb. Technol.*, **17**, 506 (1995).
MacDonald, R. T., S. K. Pulapura, Y. Y. Svirkin, R. A. Gross, D. Kaplan, J. Akkarra, G. Swift, and S. Wolf, "Enzyme-Catalyzed ϵ-Caprolactone Ring-Opening Polymerization," *Macromol.*, **73** (1995).
Margolin, A. L., J.-Y. Crenne, and A. M. Klibanov, "Stereoselective Oligomerizations Catalyzed by Lipases in Organic Solvents," *Tetra. Lett.*, **28**(15), 1607 (1987).
Marty, A., W. Chulaksananukul, R. M. Willemot, and J. S. Condoret, "Kinetics of Lipase-Catalyzed Esterification in Supercritical CO_2," *Biotechnol. Bioeng.*, **39**, 273 (1992).
McCracken, D. D., and W. S. Dorn, *Numerical Methods and Fortran Programming*, Wiley, New York (1964).
Mojovic, L., S. Siler-Marinkovic, G. Kukic, and G. Vunjak-Novakovic, "*Rhizopus arrhizus* Lipase-Catalyzed Interesterification of the Midfraction of Palm Oil to a Cocoa Butter Equivalent Fat," *Enzyme Microb. Technol.*, **15**, 438 (1993).
Morrow, C. J., "Biocatalytic Synthesis of Polyesters using Enzymes," *MRS Bull.*, 43 (1992).
Morrow, C. J., J. S. Wallace, G. M. Bybee, K. B. Reda, and M. E. Williams, "Biocatalytic Synthesis of Polyesters by Enzyme-Catalyzed Transesterifications in Organic Media," *Mat. Res. Soc. Symp. Proc.*, p. 174 (1990).
Nicolosi, G., M. Piattelli, and C. Sanfillippo, "Lipase-Catalyzed Regioselective Protection of Hydroxyl Groups in Aromatic Dihydroxyaldehydes and Ketones," *Tetra. Lett.*, **49**(5), 3143 (1993).
Odian, G., *Principles of Polymerization*, 2nd ed., Wiley, New York (1981).
Rao, A., V. T. John, R. D. Gonzalez, J. A. Akkara, and D. L. Kaplan, "Catalytic and Interfacial Aspects of Enzymatic Polymer Synthesis in Reversed Micellar Systems," *Biotechnol. Bioeng.*, **41**, 531 (1993).
Ryu, K., J. P. McEldoon, A. R. Pokora, W. Cyrus, and J. S. Dordick, "Numerical and Monte Carlo Simulations of Phenolic Polymerizations Catalyzed by Peroxidase," *Biotechnol. Bioeng.*, **42**, 807 (1993).
Segel, I. H., *Enzyme Kinetics*, Wiley-Interscience, New York (1975).
Tawaki, S., and A. M. Klibanov, "Chemoselectivity of Enzymes in Anhydrous Media is Strongly Solvent Dependent," *Biocatalysis*, **8**, 3 (1993).
Tirrell, J. G., M. J. Fournier, T. L. Mason, and D. A. Tirell, "Biomolecular Materials," *Chem. Eng. News*, 40 (1994).
Ueberreiter, K., and M. Engel, "Untersuchugen zur Kinetik der Polykondensation von Sebacoylchlorid mit α, ω-Alkanediolen," *Makromol. Chem.*, **178**, 2257 (1977).
Uyama, H., and S. Kobayashi, "Enzymatic Ring-Opening Polymerization of Lactones Catalyzed by Lipase," *Chem. Lett.*, 1149 (1993).
Wallace, J. S., and C. J. Morrow, "Biocatalytic Synthesis of Polymers: II. Preparation of $[AA\text{-}BB]_x$ Polyesters by Porcine Pancreatic Lipase Catalyzed Transesterification in Anhydrous, Low Polarity Organic Solvents," *J. Poly. Sci. Part A: Poly. Chem.*, **27**, 3271 (1989).
Wang, Y. F., J. J. Lalonde, M. Momongan, D. E. Bergbrietter, and C. H. Wong, "Lipase-Catalyzed Irreversible Transesterifications Using Enol Esters as Acylating Reagents: Preparative Enantio- and Regioselective Syntheses of Alcohols, Glycerol Derivatives, Sugars and Organometallics," *J. Amer. Chem. Soc.*, **110**, 7200 (1988).
Whiting, A. K., and W. L. Peticolas, "Details of the Acyl-Enzyme Intermediate and the Oxyanion Hole in Serine Protease Catalysis," *Biochemistry*, **33**, 552 (1994).
Zaks, A., and A. M. Klibanov, "Enzyme Catalysis in Non-Aqueous Solvents," *J. Biol. Chem.*, **263**, 3194 (1988).
Zaks, A., and A. M. Klibanov, "Substrate Specificity of Enzymes in Organic Solvents vs. Water is Reversed," *J. Amer. Chem. Soc.*, **108**, 2767 (1986).

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