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Modeling the Catalytic Activity and Kinetics of Lipase (Glycerol-Ester Hydrolase)

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In order to design industrial scale reactors and proceises for multi-phase biocatalytic reactions, it is essential to understand the mechanisms by which such systems operate. To illustrate how such mechanisms can be modeled, the hydrolysis of the primary ester groups of triglycerides to produce fatty acids and monoglycerides by lipase (glycerol-ester hydrolase) catalysis has been selected as an example of multiphase biocatalysis. Lipase is specific in its behavior such that it can act only on the hydrolyzed (or emulsified) part of the substrate. This follows because the active center of the enzyme is catalytically active only when the substrate contacts it in its hydrolyzed form. In other words, lipase acts only when it can shuttle back and forth between the emulsion phase and the water phase, presumably within an interphase or boundary layer between these two phases. In industrial applications lipase is employed as a fat splitting enzyme to remove fat stains from fabrics, in making cheese, to flavor milk products, and to degrade fats in waste products. Effective use of lipase in these processes requires a fundamental understanding of its kinetic behavior and interactions with substrates under various environmental conditions. Therefore, this study focuses on modeling and simulating the enzymatic activity of the lipase as a step towards the basic understanding of multi-phase biocatalysis processes.

Key words: lipase, modeling and simulation, biocatalysis process, enzymatic activity

INTRODUTION

Treatment by biocatalysis is an appealing alternative to the traditional organic chemical processing of water insoluble materials for numerous reasons. For instance, unlike conventional organic chemical processes, biocatalysts function under moderate environmental conditions: normally at temperatures of 20-100°C, pH values of 4-10 and at atmospheric pressure.

The industrial utilization of enzymes or biocatalysts to catalyze reactions comprising water-insoluble organic reactants and products has so far been limited to a small number of specific biotransformations. This follows because a large number of compounds of interest to the chemical and biological industries have low solubilities in aqueous solutions. Chemical conversion of many of these compounds (such as cholesterol, 1,7-octadiene, methyl acetate, triglyceride, oleic acid, glycerol) is possible, but the concentrations of many of these reactants and/or products are much lower than those commonly used in chemical reactions.

However, it is possible to operate a multi-phase biocatalytic reactor in which either a solid substrate or an organic phase is present along with water, and which occupies a large proportion of the reactor volume. Very often the organic phase includes the reactant alone or the reactant dissolved in a water-immiscible organic solvent. Such systems can overcome one of the major drawbacks of biological catalysts: the need to work in relatively dilute aqueous solutions which results in the requirement for water removal as a major downstream recovery step.

Biocatalysts may be employed in various forms depending on the reaction to be catalyzed. The biocatalyst may be one or more free enzymes, either dissolved in the aqueous phase, or immobilized. Similarly, cells or parts of cells may be used either free in suspension or immobilized [1].

It must be emphasized that the mass transfer of the reactants and products across the interfaces of the different phases is of primary importance to the performance of these reactions, since this transfer may be the rate limiting step. The extent of the resistance to reactant and/or product mass transfer across the phases depends upon the respective mass transfer coefficients and the interfacial area.

In order to design reactors and processes for the industrial utilization of multi-phase biocatalytic reactions, it is important to understand the mechanisms by which such systems operate. To illustrate how those possible mechanisms can be incorporated into a process model, the example of the hydrolysis of the primary ester groups of triglycerides to produce fatty acids and monoglycerides by lipase (glycerol-ester hydrolase) catalysis has been selected as an example case for a multi-phase biocatalytic reaction. Lipase, an enzyme used to react with solids, is employed as a fat splitting enzyme to remove fat stains from fabrics, to accelerate maturation in cheese, to flavor milk products, and to degrade fats in industrial waste product applications. Lipase is so specific in its behavior, because it can only act on the hydrolyzed (or emulsified)

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part of the solid substrate (first phase), since the active center of the enzyme is reactive only when the enzyme contacts the emulsified form of the substrate [2]. That is, the reaction does not occur unless the hydrolyzed form of the substrate(second phase) is available in solution. Lipase acts at the interface between the solid organic phase and aqueous phase (third phase) [3]. If a very insoluble substrate, such as triolein, is used to create an emulsion, a typical Michaelis-Menten enzymatic curve starting from the origin is obtained. With a more soluble substrate such as methyl butyrate, on the other hand, no activity is shown at low concentrations and a Michaelis-type curve starts from the point at which the aqueous phase is saturated with ester. This is illustrated in Fig. 1 (in which the concentration of the methyl butyrate is normalized to its saturation concentration) with the lack of activity for concentrations less than the with the lack of activity for concentrations less than the saturation concentration of the ester. Therefore, unlike conventional soluble systems, here the yield is inversely proportional to the aqueous phase saturation concentration, so that the enzymatic activity rises as the concentration falls.

MODELING

The hydrolysis of triglyceride by lipase to give short-chain fatty acids and monoglycerides can be used to illustrate just how to modify the completely soluble model to generalize it to a multi-phase model. Since lipase hydrolyzes emulsified substrates, substrates in true solutions are not attacked by such enzymes. Lipase is adsorbed by the emulsified substrate and the rate of reaction is a function of the number of enzyme molecules adsorbed at the interface [4]. This premise is also supported by Ollis [5] in his observation that the experimental data of Sarda and Desneulle [6], shown in Fig. 1, indicated clearly that purified pancreatic lipase preparations provide activity only when a substrate-water interphase is present (as for the "insoluble" portion of the graph in Fig. 1).

In modeling the multi-phase enzymatic reaction system, it is assumed that initially the substrate (triglyceride or long chain fat) is introduced in its insoluble form (S_I). As soon as the substrate is added to the vigorously mixed system, it starts to hydrolyze (emulsify) at a rate determined by its rate constant k. There are two mechanisms acting on the hydrolyzed

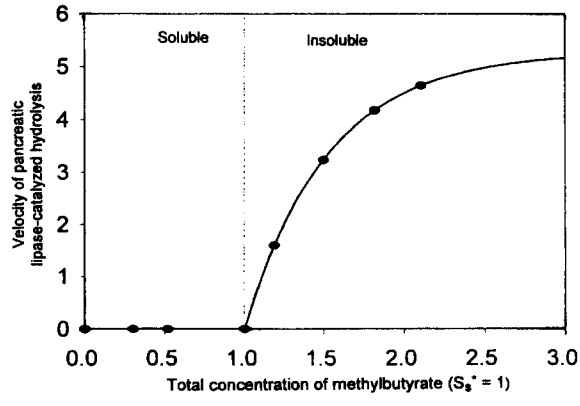
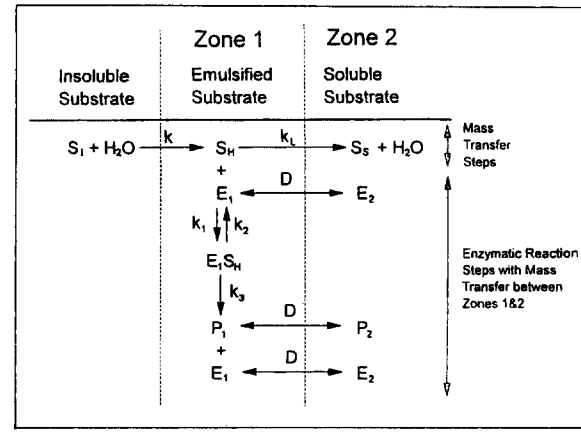


Fig. 1. Pancreatic lipase-catalyzed hydrolysis of methyl butyrate (after Sarda and Desnuelle, 1958).

form of the substrate. First, the substrate is solubilized until it reaches its saturation concentration (S_s^*) in Zone 2 at a rate determined by its liquid phase mass transfer coefficient (k_L). The second mechanism is the enzymatic activity of lipase on the hydrolyzed (or emulsified) part of the substrate, which is described by the enzymatic reaction rate constants (k_1, k_2 and k_3). These two mechanisms (mass transfer and chemical kinetics) occur concurrently until the aqueous saturation concentration of the substrate is reached. After this point, the enzymatic activity of lipase is the controlling mechanism on the hydrolyzed substrate. In other words, S_I is constantly decreasing until it is completely hydrolyzed; when S_s^* is reached S_H becomes completely available to the lipase. This system is represented by the following enzymatic reaction mechanism;



Here the step:

$$S_I + H_2O \xrightarrow{k} S_H$$

is the emulsion step in which insoluble substrate becomes hydrolyzed substrate.
The step:

$$S_H \xrightarrow{k_L} S_s$$

is the solubilization of hydrolyzed substrate and is characterized by the liquid phase mass transfer coefficient (k_L) which is very difficult to obtain experimentally. There are, however, well-established emprical evaluation methods in the literature (e.g., [7]). Here, however k_L is normalized to 1, for simplicity for this early qualitative study. The enzymatic reaction occurs with the lipase (E) reacting with the hydrolyzed substrate (S_H). Although E_1 and E_2 are the same constituents physically, the subscripts are used to denote the zone in which the enzyme is present. Since lipase is active only in zone 1, E_1 refers to its "active" form in zone 1(the emulsion pase) and E_2 is the "inactive" enzyme in the non-reactive (soluble substrate) phase or zone 2.

The mixing pattern of a reactor can also have a profound effect on the efficiency and the mechanistic representation of the system. Weinstein and Adler [8] defined micromixing as the mixing component which specifies the variation in residence times experienced by molecules flowing through the system. Micromixing

specifies the environment experienced by molecules during their passage through the system. Weinstein and Adler [8] further stated that macromixing alone is generally a suitable mixing criterion for design, scale-up and mathematical modeling. Thus, it has been assumed here that micromixing in the reaction vessel is insignificant (as it is in general for all first order reaction systems). That is , the reacting media is a macrofluid consisting of aggregates of single fluid molecules and the dominant mixing pattern is the macromixing type in the reaction vessel.

In a reactor filled with a macrofluid, each aggregate acts as a separate reactor and the rate of product formation is the same in all aggregates [9]. Consequently, in such a system, the enzyme is fully or partly adsorbed on the hydrolyzed part of the substrate molecule and there exists a boundary (a few micron-thick water layer) between a single enzyme-substrate (ES) molecule and the rest of the water phase. Accordingly Barman [4] proposed that lipase is adsorbed by its emulsified substrate and that the initial rate of reaction is a function of the number of enzyme molecules adsorbed at the interface.

Tanner *et al.* [10] developed a two CSTR's in series model to study a batch system with imperfect mixing characteristics. By utilizing this model, the physical system described earlier can be represented by two separate CSTR's with equal volumes. The modified two zone system is depicted in Fig. 2.

The two-zone model can easily be extended to a three-zone system as visualized in Fig. 2 by adding an insoluble zone within Zone 1 and more physically by the schematic in Fig. 3.

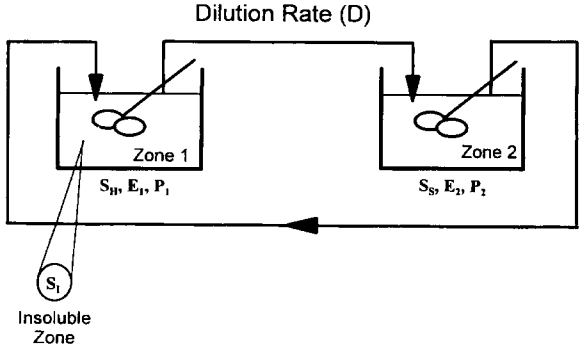


Fig. 2. Representation of the batch system as two CSTR's in series.

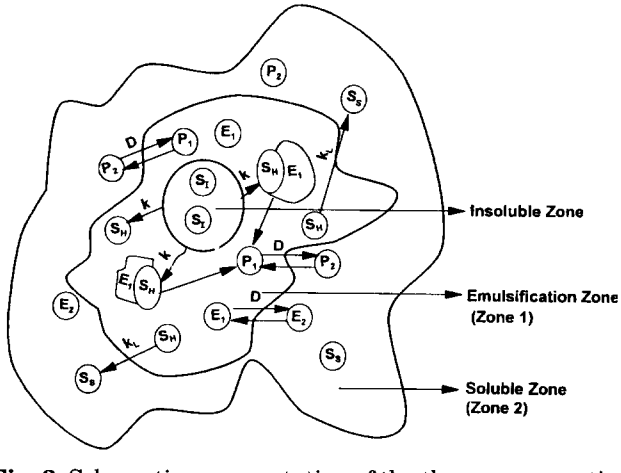


Fig. 3. Schematic representation of the three-zone reaction system.

In the three-zone system described in Fig. 3 the emulsification is the first step which takes place in zone 1. E and P are transferred between zone 1 and zone 2 and schematically described by the dilution rate D defined as F/V, where F is the volumetric flow rate and $V=V_1=V_2$. Formation of the ES complex takes place only in zone 1. E and P in zone 1 are denoted by subscripts 1 and 2, when they are in zones 1 and 2, respectively. The hydrolyzed substrate in zone 1 is partitioned by mass transfer to the soluble form, S_S, in zone 2. In addition, the hydrolyzed substrate is converted to the product by the enzymatic action of lipase.

According to the law of mass action for the enzymatic reactions and the flow system, the differential equations governing the mass balance of the system can be written as follows for the three zones;

Insoluble Zone

$$\frac{dS_I}{dt} = -kS_I$$

Zone 1

$$\frac{dS_H}{dt} = kS_I - k_L(S_s^H - S_s) - k_1(E_1)(S_H)$$

$$\frac{dP_1}{dt} = k_3(E_1S_H) + D(P_2 - P_1)$$

$$\frac{dE_1}{dt} = -k_1(E_1)(S_H) + (k_2 + k_3)(E_1S_H) + D(E_2 - E_1)$$

$$\frac{d(E_1S_H)}{dt} = k_1(E_1)(S_H) - (k_1 + k_3)(E_1S_H)$$

Zone 2

$$\frac{dS_s}{dt} = k_L(S^*{}_s - S_s)$$

$$\frac{dP_2}{dt} = D(P_1 - P_2)$$

$$\frac{dE_2}{dt} = D(E_1 - E_2)$$

Here by definition:

$$S_T \equiv S_I + S_H + S_S$$

and

$$P_T \equiv P_1 + P_2$$

These combined substrate and product terms will be used to depict the simulated results.

RESULTS AND DISCUSSION

The differential equations descrbing the system are solved simultaneously for different kinetic and operating parameters by the digital computer program TUTSIM.

To begin to validate this model, experimental data of the activity of the lipase is qualitatively compared with the simulation results. The experimental data of Sarda and Desnuelle [6] for the kinetic activity of pancreatic lipase are shown in Fig. 1. As previously dis-

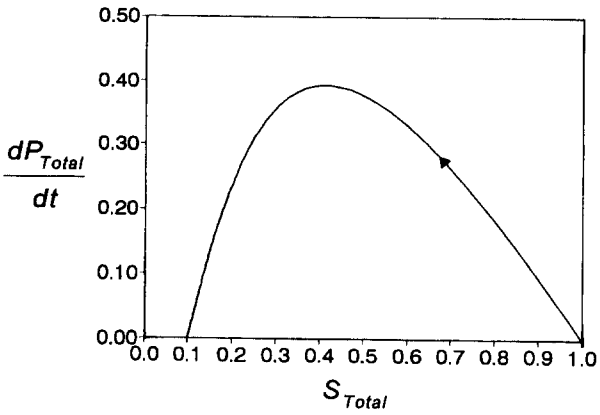


Fig. 4. Specific activity of lipase with the substrate saturation concentration of 0.1; time direction is indicated by the arrow (S_s^*=0.1 $(S_I)_0$=1, $(E_I)_0$=2, k=1, k_1=1, k_2=1, k_3=1, D=1, Δt=0.01).

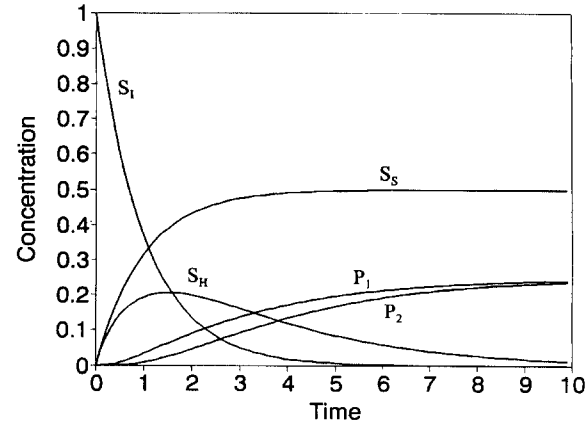


Fig. 5. Substrate utilization and product formation in the system (S_s^*=0.5, $(S_I)_0$=1, $(E_I)_0$=2, k=1, k_1=1 k_2=1 k_3=1, D=1, Δt=0.001).

cussed, the special behavior of lipase can be described as inactive in contact with the soluble form of substrate and active in contact with the hydrolyzed substrate (insoluble form). Therefore, until the hydrolyzed (or emulsified) form of the substrate is available in the system no enzymatic activity is observed, as shown in Fig. 1.

A typical simulation of the differetial equation model is shown in Fig. 4.

This simulation is qualitatively similar to the experimental data shown in Fig. 1, except the rate goes to zero for high values of S_T (unlike in Figure 1) Leaving off the early time transient portion of the simulated results (it is generally difficult to measure that rapid domain of the curve) leads to essentially the same qualitative response as depicted in Fig. 1.

As can be seen from Fig. 4, the activity of lipase is zero below the saturation value of the substrate. That is, when there is no hydrolyzed substrate in the system, lipase catalysis does not proceed any further. At this point all the substrate in the system is soluble substrate and its concentration is S_s^*. After generating these curves, it can be stated that the specific enzymatic activity of lipase can be qualitatively described with this simple model. In other words the model is qualitatively consistent with the data used for the comparison. Modifications of the model will be required to more closely match the detailed measurements for this solid dissolution lipase system.

Now that the simulated model has been shown to be qualitatively consistent with actual lipase data, further simulations are carried out. Typical substrate utilization and product formation versus time curves and rate of product formation versus hydrolyzed substrate concentration of the system are shown in Fig. 5, respectively.

From Fig. 5, it is clear that the insoluble form of the substrate, S_I, (the form which we introduced to the system) decreases to zero, at which it is completely hydrolyzed to S_H, its decomposition product. S_H in emulsified substrate zone is decreased by two simultaneous mechanisms : mass transfer to the soluble zone and the lipase action to form product, P. Once the saturation concentration of the substrate is reached, no further mass transfer to the soluble zone, zone 2, takes place and the lipase catalyzed reaction is the only mechanism depleting S_H. Since product formation occurs only in zone 1, the product is observed in zone 2 somewhat later in time than in zone 1. Product is transferred from zone 1 by convection (as determined by the dilution rate term in the model). It should be noted in Fig. 5 that as the saturation concentration of the substrate increases, concentration of the products, P_1 and P_2, decreases while the rate of formation of the soluble substrate increases. This supports the data in Fig. 1 that the product formation by the lipase activity increases as the solubility of the substrate decreases.

There are five constants used to describe the lipase system, namely reaction rate constants k, k_1, k_2, and k_3 and the mass transfer coefficient k_L. There are well established theoretical calaulation methods to estimate the liquid phase mass transfer coefficient, k_L [7]. Since rate constants are normalized, a constant value of 1 is used for k_L. The rate constants are then perturbed relative to k_L in the sensitivity analysis. Since it is quite hard to determine all of these kinetic constants, a sensitivity analysis is performed to compare the relative importance of these constants within the model. In each simulation in the sensitivity analysis, one of the constants was varied while the others were kept constant.

From the sensitivity analysis, it appears that the solid dissolution rate constant (k) has clear importance for values up to 10 (10 times the mass transfer coefficient, k_L) and could be the rate limiting step in the reaction if the stirring in the insoluble zone is too low. This means that increasing the stirring speed or increasing the surface area of the solid is likely to siginificantly affect the rate of formation of product in the system around a particular base-line value of these variables. Similarly, all the remaining constants (especially k_3) have significant effect on the system and each one of these could represent a rate-limiting step. Enhancing these constants (such as by increasing the temperature up to the denaturation temperature) is likely to speed up the rate of reaction.

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NOMENCLATURE

S_I : Insoluble substrate concentration (mass. volume^{-1})

S_s : Soluble substrate concentration (mass. volume $^{-1}$)
S_s^* : Substrate saturation concentration (mass. volume $^{-1}$)
S_H : Hydrolyzed substrate concentration (mass. volume $^{-1}$)
S_T : Total substrate concentration=S_I+S_H+S_s (mass. volume $^{-1}$)
E_1 : Active enzyme concentration in zone 1 (mass. volume $^{-1}$)
E_2 : Inactive enzyme concentration in zone 2 (mass. volume $^{-1}$)
ES : Concentration of enzyme-Substrate complex (mass. volume $^{-1}$)
P_1 : Product concentration in zone 1 (mass. volume $^{-1}$)
P_2 : Product concentration in zone 2 (mass. volume $^{-1}$)
P_T : Total product concentration=P_1+P_2 (mass. volume $^{-1}$)
D : Dilution rate (time $^{-1}$)
k_L : Liquid phase mass transfer coefficient (time $^{-1}$)
k, k_1, k_2, k_3: Reaction rate constants (time $^{-1}$)

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