Organic Process Research & Development **2004**, *8*, 229−237

# Transesterification of 2-*o*-Benzylglycerol with Vinyl Acetate by Immobilized Lipase: Study of Reaction and Deactivation Kinetics

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**Abstract:**

**The transesterification of 2-*o*-benzylglycerol with vinyl acetate has been studied in dichloromethane using free and immobilized lipase from *Pseudomonas cepacia* with particular emphasis on the effect of the pertinent variables and kinetic aspects of the reaction. The activity of the immobilized lipase was found to be maximum in the temperature range of 30−50 °C, and it retained about one-third of the initial activity up to the third cycle after repeated use. The kinetics of the reaction was analysed by the so-called Ping Pong Bi Bi mechanism, and the model parameters were estimated by nonlinear regression analysis using Marquard's technique. The immobilized lipase, which had the activity in general, 3 times greater than that of the free lipase was deactivated by both temperature and vinyl acetate. The deactivation was found to be insignificant up to a certain level of vinyl acetate concentration above which, however the lipase activity fell rapidly. The deactivation kinetics was analysed by using a mathematical model which accounts for a series type of reaction mechanism.**

## Introduction

The use of enzymes in organic media, well recognized for preparative-scale enantioselective transesterification, has been of interest in technological perspectives, but limited due to poor stability, high cost, and difficulty in handling.[1] Immobilized enzymes are advantageous for practical processes[2] as these can be reused for continuous operation under mild reaction conditions.[3] Therefore, extensive studies have been made on the use of immobilized enzyme for various reactions.[4−6]

Like most other enzymes, lipases are water soluble, but unlike most enzymes, they act on oil−water interfaces, which makes a two-phase system mandatory for the reactions they catalyse. This two-phase system contains one water-rich phase and an organic phase composed of a water-immiscible organic solvent.

The universally acknowledged enzyme deactivation is the unfolding of the protein molecule.[7] This unfolding, with or without subsequent conformational changes,[8] requires the molecule in question to have freedom of movement. Therefore, the locking of the enzyme molecule into a rigid conformation with physical constraints on its mobility (by immobilization) would permit easy unfolding, and thus would alter its susceptibility to denaturing agents such as solvent and temperature. All proteins, including lipases, are vulnerable to temperature-induced deactivation. This prevents their use at higher temperatures which may be desirable from an industrial point of view. Enhancing enzyme thermostability constitutes a major research thrust today, especially in light of the fact that thermostable enzymes are also more resistant to the deleterious effects of pH and organic solvents. A prerequisite for this is the elucidation of deactivation kinetics. In this contribution, we report a comprehensive kinetic study of 2-*o*-benzylglycerol with vinyl acetate catalysed by *Pseudomonas cepacia* lipase by varying parameters such as lipase concentration, reaction temperature, and substrate concentration ratio on the reaction rate. The reaction product chiral-acetyl-2-*o*-benzylglycerol, (*R*) or (*S*) is a very useful building block for the preparation of enantiomerically pure, biologically active molecules such as phospholipids, PAF (platelet aggregation factor), phospholipase A2 inhibitors, sphinoglycolipids, etc.[9] The compound can be advantageously synthesized by transesterification of prochiral 2-*o*-benzylglycerol with vinyl acetate (Scheme 1) catalysed by free and immobilized lipase from *Pseudomonas cepacia*. In this reaction, 2-*o*-benzylglycerol undergoes acetylation by vinyl acetate to give (*R*)- and (*S*)-2-*o*-benzylglycerol-1-acetate and vinyl alcohol. No product inhibition is considered in this reaction as the byproduct vinyl alcohol tautomerizes to acetaldehyde which, being volatile, prevents the reverse reaction. The monoacetates undergo further acetylation to give 2-*o*-benzylglycerol-1,3-diacetate. In this reaction, the rate of formation of diacetate from the *R*-isomer as the substrate is greater than that from the *S*-isomer[9] which was

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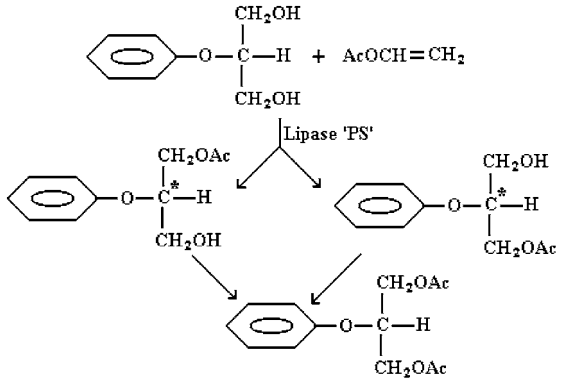
(9) Wang, Y. F.; Lalonde, J. J.; Momongan, M.; Bergbreiter, D. E.; Wong, C. H. Lipase Catalysed Irreversible Transesterification using Enol Esters as Acylating Reagents: Preparative Enantio and Regioselective Synthesis of Alcohols, Glycerol Derivatives, Sugars and Organometallics. *J. Am. Chem. Soc.* **1988**, *110*, 7200−7205.

10.1021/op030040y CCC: $27.50 
Published on Web 02/13/2004

**Scheme 1**




also inferred from the results presented in our previous communication.[10] Lipase PS can stereoselectively form the *S*-isomer, and thus, the formation of 2-*o*-benzylglycerol-1,3-diacetate is reasonably lower than that of 2-*o*-benzylglycerol-1-acetate and is neglected for the kinetic study. At the same time complementary study has also been made on the deactivation of this immobilized lipase to understand the implication of the system for process applications. In the whole study we used dichloromethane as the solvent media as we found that this solvent gives the highest initial rate of reaction, enantiomeric excess, and selectivity of monoester formation; the probable explanation was furnished for the observed enantioselectivity behaviour as reported in our previous communication.[10]

## Experimental Section

**Reagents.** Free and immobilized lipases from *Pseudomonas cepacia* were obtained from Amano Pharmaceutical Co., Nogoya, Japan (Lipase PS and Lipase PS-C "Amano II"). The lipase PS-C "Amano II" is immobilized on chemically modified ceramic particles of size 200 $\mu$m with a loading of 2 g of crude lipase in 2 g of ceramic particle. The unit activities of the free and immobilized lipase were 34 IU/mg solid and 70 IU/mg solid, respectively. 2-*o*-Benzylglycerol and vinyl acetate were procured from Fluka.The solvents and molecular sieves were obtained from CDH Pvt Ltd., New Delhi, India. The solvents were purified by distillation and dried over 3Å molecular sieves prior to use.

## Analytical Method

The concentration of 2-*o*-benzylglycerol-1-acetate was determined by HPLC using high-pressure liquid chromatography (Waters) equipped with U6k universal Injector, Waters 510 pumps, Waters 486 tunable absorbance detector coupled with Waters 746 data module and a $\mu$-Porasil HPLC column (300 mm × 3.9 mm i.d.) packed with $\mu$-porasil particles of size 10 $\mu$m, 125 Å manufactured by Waters U.S.A. (part no. WAT 027477). Elution was conducted with a hexane: 2-propanol (80:20) mixture, and the flow rate was adjusted to 1.0 mL/min, under a pressure of 205 psi. Chart speed and attenuation of the integrator were 1.0 cm/min and 64, respectively. Quantitative data were obtained with an integrator after calibration with the standard, 2-*o*-benzylglycerol-1-acetate.

**Procedure.** Standard 2-*o*-benzylglycerol-1-acetate was prepared by the same procedure reported in our previous communication.[10]

The kinetic experiments were carried out in a 50-mL round-bottom flask in which the reaction mixture was agitated vigorously with a magnetic stirrer. The temperature, lipase, and substrate concentrations were varied systematically from 10 to 35 °C, 5 to 15 mg/mL and 50 to 200 mmol, respectively, using dichloromethane as the solvent as this was found to be the optimum for this reaction.[10,11] The solvent was dried over 3Å molecular sieve before use. Aliquots of the samples were withdrawn at regular interval of time and analysed by HPLC. The initial reaction rates were calculated from the conversion versus time profiles corresponding to the first 10% conversion below which the profiles were found to be linear. The rate was expressed as the amount of substrate converted per unit time and weight of the lipase (mmol $min^{-1}$ $g^{-1}$).

For studying deactivation kinetics of the immobilized lipase, 50 mmol of 2-*o*-benzylglycerol and 100 mmol of vinyl acetate were mixed with 15 mg/mL lipase in 10 mL of solvent at 30 °C in a closed glass reactor of 50 mL capacity and stirred with a magnetic stirrer. The speed of agitation was maintained at 200 rpm, and it was found that, at this speed, the reaction rate was no longer a function of the agitation speed, implying that external mass transfer influences were overcome. At higher speeds mechanical abrasion of the beads was observed. This procedure, along with the specified substrate concentrations, temperature, etc. was followed in all cases, unless stated otherwise.

Preincubation experiments were carried out by incubating the immobilized beads with a specified quantity of vinyl acetate and/or otherwise at different temperatures and for a specific duration. After this, vinyl acetate and 2-*o*-benzylglycerol were added to make vinyl acetate to 2-*o*-benzylglycerol ratio 2:1 and then the reaction carried out as usual. This was the general procedure followed in all the experiments conducted for deactivation study at specified substrate concentration and temperature. To examine the effect of water on deactivation, 1 g of bead was incubated in 5 mL of water at various temperatures in the range of 30−60 °C, and the mass was added to a mixture of 2-*o*-benzylglycerol (50 mmol)−vinyl acetate mixture (100 mmol). For analysis of the samples, 0.5 mL of sample from the reaction mixture was withdrawn periodically at a regular interval of time and analysed by HPLC. The percentage activity was calculated on the basis of conversion obtained in 1 h, with the conversion of nonpreincubated substrates at 30 °C being considered as equivalent to 100% activity.

**Determination of Lipase Activity.** For determining the lipase activity, the reaction was conducted with free or immobilized lipase for a fixed period of time after which

(10) Hazarika, S.; Dutta, N. N.; Goswami, P. Lipase Catalysed Transesterification of 2-*o*-Benzylglycerol with Vinyl Acetate: Solvent Effect. *Chem. Eng. J.* **2003**, *94*, 1−10.

(11) Hazarika, S.; Goswami, P.; Dutta, N. N. Ethyl Oleate Synthesis by Porcine Pancreatic Lipase. *Chem. Eng. J.* **2002**, *54*, 61−68.

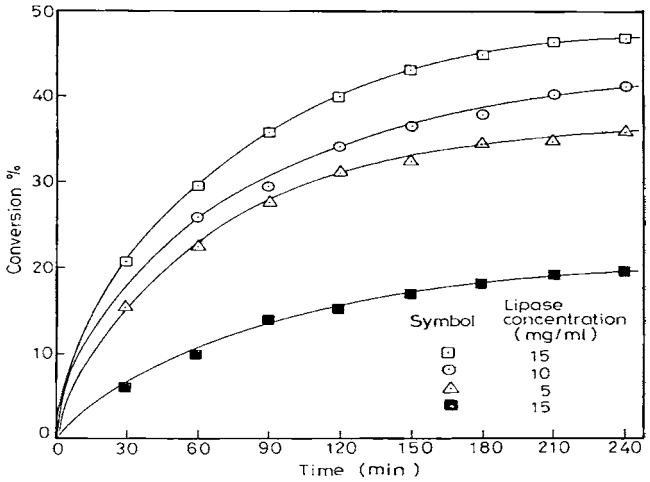



***Figure 1.*** **Effect of lipase concentration on conversion versus time profile. Solid circle: free lipase. Open circle: immobilized lipase. [2-*o*-Benzylglycerol] = 50 mmol, [vinyl acetate] = 100 mmol.**

the reaction mixture was diluted with dichloromethane and analysed by HPLC. A blank run was conducted in a similar way but without addition of the lipase. One unit of lipase activity was defined as the amount of lipase that released 1 $\mu$mol of 2-*o*-benzylglycerol-1-acetate per minute under the experimental conditions. The activities of free and immobilized lipases were thus found to be 1243.31 and 177.02 units, respectively.

## Results and Discussions

**Effect of Lipase Concentration.** Figure 1 shows the typical time course of conversion based on 2-*o*-benzylglycerol as the substrate at various lipase concentrations at a fixed substrate concentration. It is evident from the figure that the % conversion increases with increasing lipase concentration. In the lower conversion range, conversion increases almost linearly, and this linear relationship is consistent with a kinetically controlled enzymatic reaction. However, linearity alone does not rule out the possibility of mass transfer limitations on the reaction rate.[12] It is apparent that the immobilized lipase gives higher overall conversion than that of the free lipase, an observation identical to that reported for other lipase-catalysed reactions.[13−15] The high activity of immobilized lipase may be attributed to hyperactivation of lipase upon contact with the adsorbed media and also the fact that, during immobilization by adsorption from crude lipase preparation, only the purified form of the lipase is adsorbed, an observation identical to that of *Candida cylindracea* lipase immobilized on nylon support used for the esterification and hydrolysis reactions on membrane surfaces.[13,14] The effect of lipase concentration on conversion is quite evident from Figure 1, which shows that a 3-fold increase in lipase concentration results is around 15% increase in conversion.

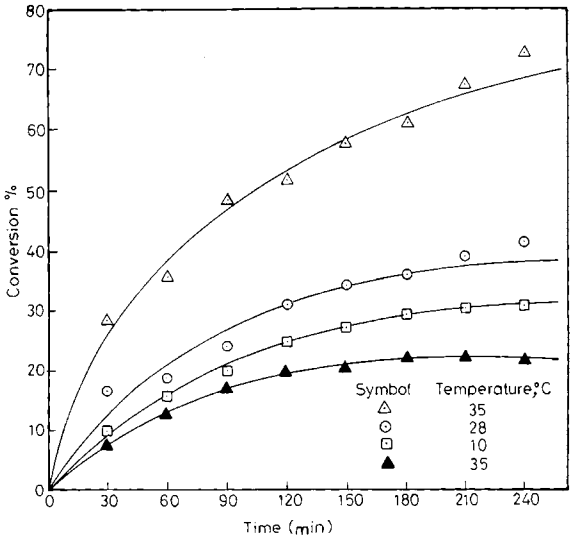



***Figure 2.*** **Effect of temperature on conversion versus time profile. Solid circle: free lipase. Open circle: immobilized lipase. [2-*o*-Benzylglycerol] = 50 mmol, [vinyl acetate] = 100 mmol.**

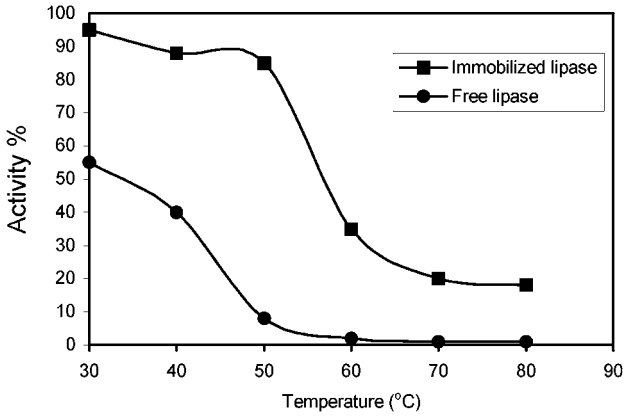



***Figure 3.*** **Residual activity of free and immobilized lipase as a function of incubation time. [2-*o*-Benzylglycerol] = 50 mmol, [vinyl acetate] = 100 mmol. Lipase = 15 g/mL.**

**Effect of Temperature and Substrate Concentration.** The effect of temperature on time course of conversion of the substrate is shown in Figure 2 which pertains to experimental data generated with a lipase concentration of 15 mg/mL. In Figure 2 the time course of conversion for free lipase at 35 °C is also shown, and it is evident that the catalytic activity of the free lipase at 35 °C is less than that of the immobilized lipase at all the temperatures studied in this work. Figure 3 shows the catalytic activity of free and immobilized lipase with temperature. It is apparent that the immobilized lipase was inactivated at temperature above 80 °C, while the free lipase was found to be inactivated at 50 °C. Thus, the immobilization not only improved catalytic activity but also resulted in an altered sensitivity to temperature, an observation identical to that reported in the literature for the esterification and hydrolysis reactions, too.[13,14]

Figures 4 and 5 show the effect of substrate concentration on the time course of conversion. It is apparent from Figure

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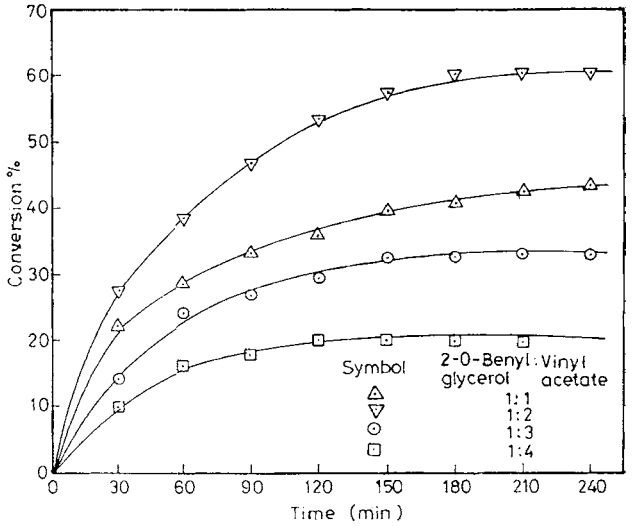


***Figure 4.*** **Effect of substrate concentration ratio on conversion versus time profile. Lipase = 15 mg/mL, [2-*o*-benzylglycerol] = 50 mmol. [Vinyl acetate]: △ = 50 mmol, ▽ = 100 mmol, ○ = 150 mmol, □ = 200 mmol.**

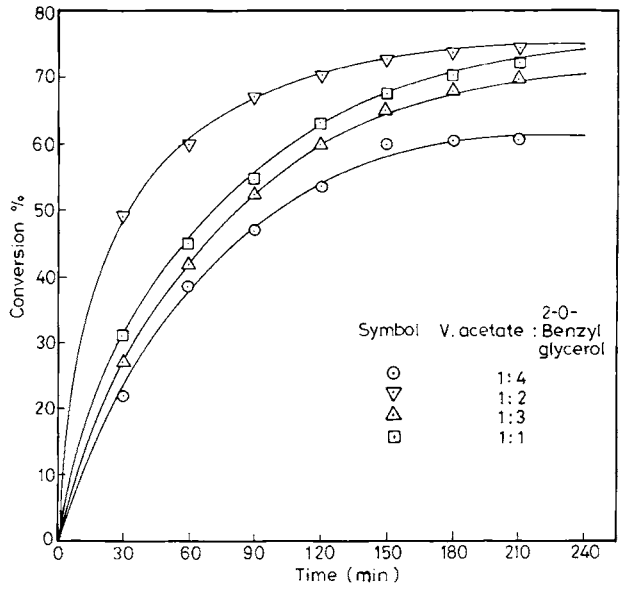


***Figure 5.*** **Effect of substrate concentration ratio on conversion versus time profile. Lipase = 15 mg/mg, [vinyl acetate] = 100 mmol. [2-*o*-Benzylglycerol]: □ = 50 mmol, ▽ = 100 mmol, △ = 150 mmol, ○ = 200 mmol.**

4 that, in general, a 2-*o*-benzylglycerol-to-vinyl acetate molar ratio of 1:4 (50 mmol 2-*o*-benzylglycerol and 200 mmol vinyl acetate) gives lower conversion. It is interesting to note that the conversion profile for 2-*o*-benzylglycerol to vinyl acetate molar ratio of 1:2 gives the highest conversion as well as initial rate. However the stoichiometrically excess of vinyl acetate concentration favors the % conversion, but too high an excess of vinyl acetate excercises synergistically inhibitory effect on the lipase. From Figure 5, on increasing the concentration of 2-*o*-benzylglycerol at a fixed concentration of vinyl acetate, the initial rate decreases; the trend is, however, not uniform at all of the vinyl acetate concentrations studied in this work, and hence, the effect cannot be generalized.

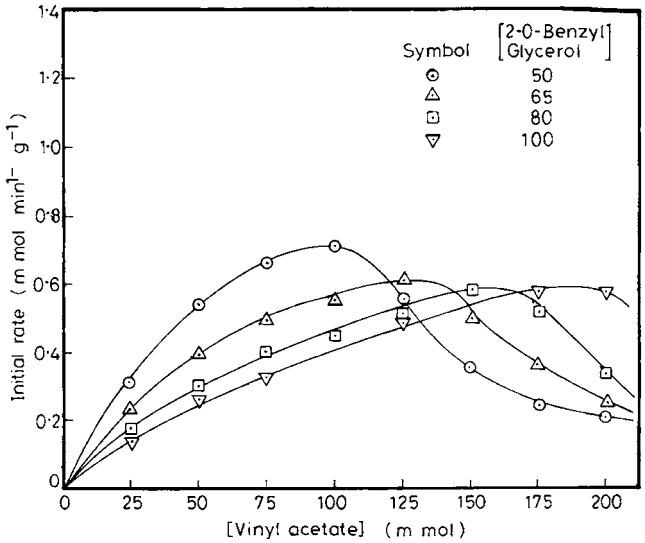


***Figure 6.*** **Initial velocity as a function of vinyl acetate concentration at a fixed concentration of 2-*o*-benzylglycerol. Lipase = 15 mg/mL.**

***Table 1.*** **Repeated use of free and immobilized enzyme[a]**

| lipase | no of use | concentration of 2-*o*-benzylglycerol (mmol) | retained activity(%) |
|---|---|---|---|
| free | first | 9.85 | 100 |
| | second | 5.23 | 0.52 |
| immobilized | first | 36.28 | 100 |
| | second | 35.33 | 97.4 |
| | third | 34.21 | 96.8 |

[a] Reaction conditions: lipase 15 mg/mL, 2-*o*-benzylglycerol 50 mmol, vinyl acetate 100 mmol, temperature 35 °C, reaction time 15 min.

With an increase in the concentration of vinyl acetate the % conversion as well as initial rate of the reaction increases up to a certain level (i.e., 100 mmol) after which the % conversion as well as initial rate of the reaction decreases as shown in Figure 6 which may be attributed to the inactivation of the lipase by the higher vinyl acetate concentration. As shown in Figure 6, for an increase of the vinyl acetate:2-*o*-benzylglycerol ratio to 2:1 at a vinyl acetate concentration of 100 mmol the maximum level of the initial reaction rate increases, but beyond this ratio no further increase is observed. Thus, the optimum vinyl acetate:2-*o*-benzylglycerol ratio appears to be 2:1.

**Reusability of the Immobilized Lipase.** The immobilized lipase was filtered off, washed with dichloromethane, and reused in three successive batches of experiments, and the activity was found to be unaffected even after the third use. While the activity of the immobilized lipase remained unaffected even after the third use, the free lipase activity droped almost 100% after the first batch of experiments, as shown in Table 1. It should be noted that, although these experiments provide a measure of the relative stability of the enzyme, the actual stability observed in a process would depend on other operating conditions.[13]

**Mechanism of the Reaction.** The reaction mechanism of the transesterification reaction has been elucidated by reciprocal plots of both initial rate and substrate (2-*o*-

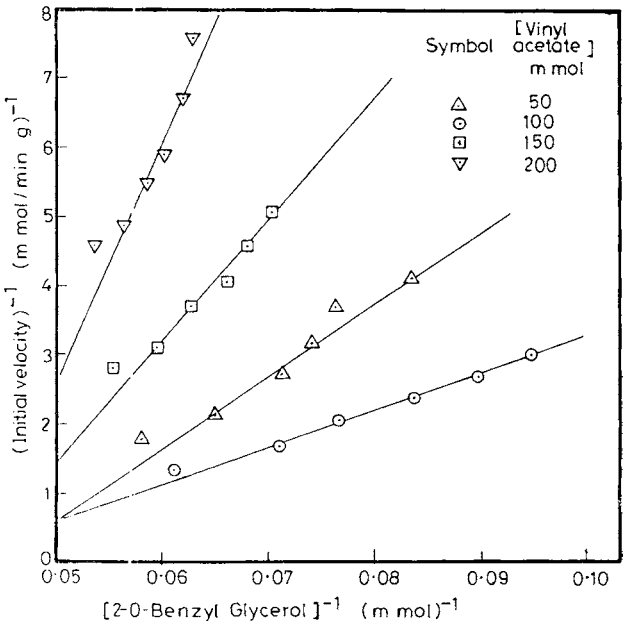


***Figure 7.*** **Reciprocal of initial velocity of the transesterification reaction as a function of reciprocal of 2-*o*-benzylglycerol concentration.**

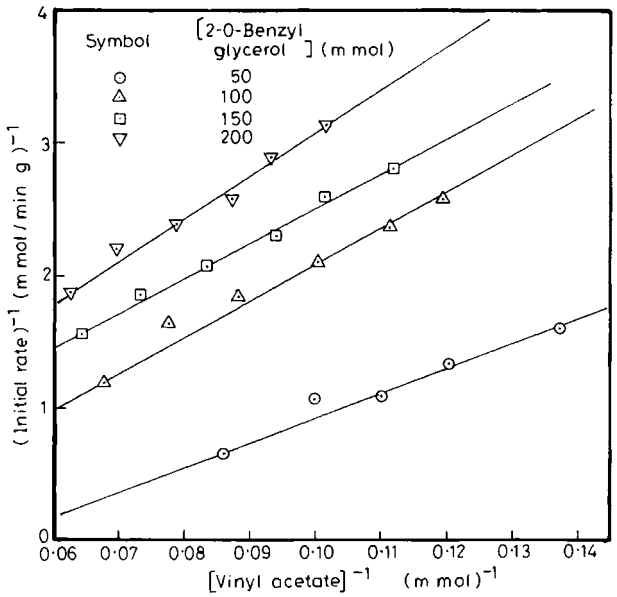


***Figure 8.*** **Reciprocal of initial velocity of the transesterification reaction as a function of reciprocal of vinyl acetate concentration.**

benzylglycerol) concentrations as shown in Figure 7. It is apparent that an increase of 2-*o*-benzylglycerol concentration at constant vinyl acetate concentration increases the initial rate. The observation of decrease in the initial rate with increase of vinyl acetate concentration apparently reflects the inhibiting effect of vinyl acetate. However, there are some exceptions in the curve where the vinyl acetate concentration is fixed at 50 mmol. At this concentration of vinyl acetate the initial rate is lower than that obtained at a vinyl acetate concentration of 100 mmol. Thus, an increase of vinyl acetate concentration up to a certain range at a fixed concentration of 2-*o*-benzylglycerol concentration increases the initial rate. The highest initial rate was obtained when the vinyl acetate concentration is 100 mmol and the 2-*o*-benzylglycerol concentration is 50 mmol. Thus, the optimum ratio of vinyl acetate to 2-*o*-benzylglycerol for this transesterification reaction is 2:1. Similar results were obtained when the 2-*o*-benzylglycerol concentration is varied from 50 mmol to 200 mmol at a fixed concentration of vinyl acetate, as shown in Figure 8. The plots in Figure 7 at low vinyl acetate concentration tend to be parallel, whereas at high vinyl acetate concentration, the slopes of the lines appear to increase, and the intercepts tend to reach a limiting value equivalent to $1/V_{max}$. This behaviour is typical of the so-called Ping Pong Bi Bi mechanism in which only one substrate is bound to the enzyme at any time to form the substrate−enzyme complex. As soon as one product is formed and released, the other substrate binds to the modified enzyme (substituted form of free enzyme) to form the second product.[16] According to this mechanism the initial velocity ($v$) equation is

$$v = \frac{V_m[G][V]}{K_{mV}[G] + K_{mG}[V] + [G][V]} \quad (1)$$

where, $V_m$ represents the maximum velocity, [G] and [V] are the concentrations of 2-*o*-benzylglycerol and vinyl acetate, respectively, and $K_{mV}$ and $K_{mG}$ are the affinity constants of vinyl acetate and 2-*o*-benzylglycerol, respectively.

***Table 2.*** **Kinetic comparison of free, immobilized, and inactivated lipase**

| lipase | cycle | $V_m$ (mmol min$^{-1}$ g$^{-1}$) | $K_{mG}$ | $K_{mV}$ | SD |
|---|---|---|---|---|---|
| immobilized | first | $5.97 \times 10^2$ | 69.22 | $2.28 \times 10^4$ | 0.76 |
| | second | $3.45 \times 10^2$ | 81.67 | $5.22 \times 10^4$ | 0.71 |
| | third | $3.25 \times 10^2$ | 90.39 | $8.65 \times 10^4$ | 0.74 |
| free | first | $1.22 \times 10^2$ | $21.05 \times 10^{-2}$ | $4.71 \times 10^5$ | 0.23 |
| | second | 0.03 | $1.25 \times 10^{-3}$ | $12.0 \times 10^2$ | 0.21 |

The kinetic parameters determined from Figures 7 and 8 for the immobilized lipase are shown in Table 2, wherein the parameters for free lipase are also shown. It is apparent also from the values of these parameters that the immobilized lipase is more active than the free lipase. In general, some enzymes on porus particles of discrete sizes exhibit low activity due to mass transfer limitations and interfacial deactivation.[17] The internal diffusion effect has not been tested in detail due to the unavailability of the catalyst in different sizes, but it may be expected that this effect is also insignificant with the particle size used in the present system. This aspect has been semiquantatively analysed from the classical theory of internal diffusion effect in heterogeneous catalysts[18] and by considering two well-known parameters, namely catalytic effectiveness factor ($\eta$) and thiele modulus ($h_o$) which can be defined by the following equation,

$$\text{effectiveness factor, } \eta = \frac{3}{r}\left(\frac{D_e}{k_1 \rho A_g}\right) \quad (2)$$

(16) Segel, I. H. *Biochemical Calculations*, 2nd ed., John Wiley & Sons: New York, 1976.

and

$$\text{thele modulus, } h_o = r\left(\frac{\rho A_g V_m}{D_e[S]}\right) \quad (3)$$

where $D_e$ is diffusivity ($cm^2\ s^{-1}$), $r$ is radius of the particle (cm), $A_g$ is surface area of the particle ($cm^3$), $k_1$ is rate constant ($s^{-1}$), $\rho$ is density of the particle ($g\ cm^{-3}$), and [S] is concentration of 2-*o*-benzylglycerol (mmol). The value of diffusivity ($D_e$) was estimated from the Wilke−Chang correlation.[19] The values of $r$, $A_g$, and $\rho$ were taken from Amano catalogue and $V_m$ was taken from our previous communication.[10] The numerical values of $\eta$ and $h_o$ were $4.4412 \times 10^{-5}$ and 1.0008 respectively, implying negligible internal diffusion effect.

**Deactivation of Lipase.** *Theory*. Deactivation refers to the decrease in enzyme activity with time. Thus, a study of deactivation kinetics is highly essential for the proper design of enzyme bioreactor.

Generally, a first-order kinetics is used for characterization of irreversible deactivation from the native state, N, to the denatured state, D, for the reaction,

$$N \xrightarrow{k_1} D \quad (4)$$

Now,

$$\text{rate} = -\frac{d[N]}{dt} = k_1[N] \quad (5)$$

where $t$ is the time of exposure to denaturant, $k_1$ is the first-order rate constant, and [N] is the amount of native form N at time $t$.

Integrating eq 5

$$\ln([N]/[N]_o) = -k_1 t \quad (6)$$

where $[N]_o$ is the amount of native enzyme initially present.

From eq 6, it follows that the plots of $\ln([N]/[N_o])$ against deactivation time should be linear. Considering the complexity of the enzyme molecule and the extremely high number of bonds and other interactions that can be disrupted, it is expected that a simple first-order mechanism would not suffice to explain the denaturation patterns obtained. A more complex mechanism would, therefore, be expected. To this end, various multistep series as well as parallel deactivation models have been proposed by Henley and Sadana.[20,21]

This can be derived from first principles as described by Gianfreda et al.[22] The native form, N, is assumed to go through a series of intermediates, I, which are in equilibrium with each other, to a final state, D, with or without residual activity.

$$N \xrightarrow{k_1} I \xrightarrow{k_2} D \quad (7)$$

This rate-limiting steps of both of these transitions are assumed to obey irreversible first-order kinetics. Another assumption is that, initially, only the native form exists so that $[I] = [D] = 0$ at $t = 0$.

By resolving this situation in terms of specific activities of the various forms of enzyme, the following equation has been derived

$$\gamma = \alpha_2 + e^{-k_1 t}\left(1 + \frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_2}{k_2 - k_1}\right) + e^{-k_2 t}\left(\frac{k_1(\alpha_2 - \alpha_1)}{k_2 - k_1}\right) \quad (8)$$

where, $\alpha_1$ and $\alpha_2$ are the ratios of the specific activities of the intermediate and the denatured states to the native one, respectively, and $\gamma$ is the ratio of the overall rate of reaction to the specific activity of the native form. All these are normalized with respect to the amount of enzyme, $[n_o]$. $k_1$ and $k_2$ are the rate constant for the first and second steps, respectively.

If the intermediate is very stable so that $k_2$ has a low value, then the equation is given as,

$$\gamma = \alpha_1 + e^{-k_1 t}(1 - \alpha_1) \quad (9)$$

However, deactivation in the present case was found to be affected by both temperature and vinyl acetate concentration. To incorporate the effect of vinyl acetate, the above model was modified suitably as,

$$N + V \xrightarrow{k_1'} I + V \quad (10)$$

and the rate is given by,

$$\frac{d[N]}{dt} = -k_1'[N][V] \quad (11)$$

where $k_1'$ is the second-order rate constant.

For a given vinyl acetate concentration, this leads to the expression

$$\gamma = \alpha_1 + e^{-k'_1[V]t}(1 - \alpha_1) \quad (12)$$

The effect of vinyl acetate on residual activity may be taken into account by substituting $\alpha_1$ with a linear expression, $(m + n[V])$, where $m$ and $n$ are constants. It may be noted at this stage that this substitution is purely empirical. It signifies correlation of substrate concentration and the constants used. Hence eq 12 becomes,

$$\gamma = (m + nV) + e^{-k'_1 t}[1 - (m + nV)] \quad (13)$$

Since $k_1 = k_1'[V]$, the eq 13 may be simplified to

$$\gamma = (m + n[V]) + e^{-k_1 t}[1 - (m + n[V])] \quad (14)$$

which was the final form of the model used to correlate the experimental data.


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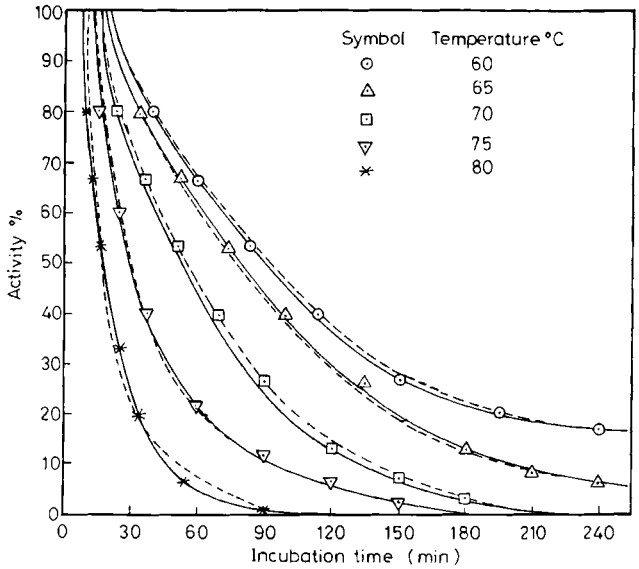


***Figure 9.*** **Residual activity of the lipase as a function of incubation time at various temperatures. Lipase = 15 mg/mL, [2-*o*-benzylglycerol] = 50 mmol, [vinyl acetate] = 100 mmol.**

*Effect of Temperature on Deactivation.* The effect of temperature on deactivation is shown in Figure 9 from which it is apparent that the activity of immobilized lipase decreases up to 80% at 60 °C within 4 h, but at 70 °C the lipase becomes deactivated within 3 h. At 75 °C, the deactivation is faster and occurs within 2.5 h, whereas at 80 °C, the enzyme activity is lost by 90% within 30 min. In the presence of excess vinyl acetate at 50 °C, the enzyme lost its activity by about 10% at 15 min and was completely deactivated within around 1.5 h. When the ratio of 2-*o*-benzylglycerol and vinyl acetate concentrations was 1:2, maximum initial rate was observed at 50 °C. A similar effect was also observed for immobilized lipozyme for esterification of lauric acid with butanol.[23] This greater stability of the enzyme can be explained by considering the fact that the hydrophobicities of both substrates will impart a fairly low concentration of these substrates in the enzyme contained in the organic phase.[21] Thus, during the course of the reaction, the deleterious effect of vinyl acetate and 2-*o*-benzylglycerol on the enzyme can be avoided.

There was no adverse effect on the enzyme when 2-*o*-benzylglycerol was added at 50 °C. After 1 h, the incubated mixture was cooled to 30 °C, 50 mmol of 2-*o*-benzylglycerol was added to it, and the reaction was carried out as usual. There was no difference in the conversion or rate obtained with the preincubation experiment. When the reaction was carried out at 50 °C instead of 30 °C, the conversion was slightly lower. Hence, 2-*o*-benzylglycerol itself has no effect on enzyme deactivation unlike that reported for deactivation of lypozyme by butanol,[23] but the observed deactivation may be attributed to the increase of temperature and vinyl acetate concentration. By using Table 3 the values of model parameters at different temperatures were determined and are shown in Table 4. The activation energy value for the

***Table 3.*** **Effect of preincubation of the lipase containing different amounts of vinyl acetate at different temperatures**

| amount of vinyl acetate (mmol) | time of incubation (at different temperature) required for loss of 20% activity (min) | | | | |
|---|---|---|---|---|---|
| | 60 °C | 65 °C | 70 °C | 75 °C | 80 °C |
| 200 | 40 | 35 | 25 | 15 | 10 |
| 225 | 30 | 28 | 18 | 12 | 8 |
| 250 | 28 | 20 | 12 | 10 | 7 |
| 275 | 25 | 15 | 10 | 9 | 5 |
| 300 | 20 | 14 | 9 | 6 | 3 |

***Table 4.*** **Effect of temperature on deactivation[a]**

| temperature (°C) | $k_1$ ($\times 10^{-2}$ min$^{-1}$) | $\alpha_1$ ($10^{-1}$) | rms |
|---|---|---|---|
| 60 | 3.73902 | −0.357230 | 0.0028630 |
| 65 | 2.06692 | −0.370138 | 0.0029890 |
| 70 | 9.80021 | −1.973870 | 0.0030341 |
| 75 | 6.62204 | −2.080760 | 0.0067577 |
| 80 | 6.69817 | −0.858458 | 0.0078090 |

[a] Amount of glycerol = 100 mmol. Amount of vinyl acetate = 200 mmol

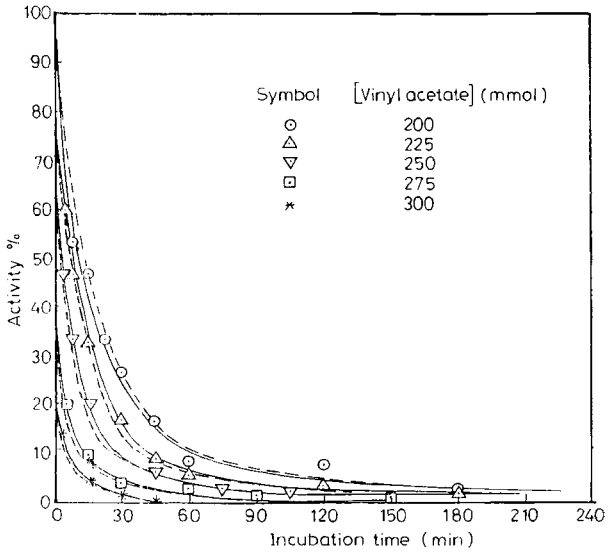


***Figure 10.*** **Residual activity of the lipase as a function of incubation time at different vinyl acetate concentrations. Lipase = 15 mg/mL, [2-*o*-benzylglycerol] = 50 mmol.**

deactivation reaction estimated from the so-called Arrhenius plot was found to be 108.92 kJ/mol, whereas for Porcine pancreatic and lipozyme lipases, the values were found to be 196 kJ/mol and 168 kJ/mol, respectively.[23,24]

*Effect of Vinyl Acetate Concentration.* The effect of vinyl acetate concentration on deactivation is shown in Figure 10 which indicates that there is a fast decline in catalytic activity with increase of vinyl acetate concentration studied in the range of 200−300 mmol. It is apparent from Figure 10 that when vinyl acetate concentrations are increased three times the % activity of the lipase decreased rapidly. In this range the lipase gets saturated with vinyl acetate, and the enzyme activity is lost due to the more hydrophilic nature of vinyl acetate. With 200 mmol of vinyl acetate, deactivation started

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***Table 5.*** **Effect of vinyl acetate concentration on deactivation[a]**

| concentration of vinyl acetate (mmol) | $k_1$ ($10^{-1}$ min$^{-1}$) | $\alpha_1$ ($10^{-2}$) | $m$ | $n$ (ml mol$^{-1}$) | rms |
|---|---|---|---|---|---|
| 200 | 5.62781 | 5.6278 | 0.571024 | −0.002480 | 0.0028630 |
| 225 | 1.46787 | 14.6787 | 0.665741 | −0.002010 | 0.0029890 |
| 250 | 1.96284 | 19.6242 | 0.651522 | −0.002070 | 0.0030341 |
| 275 | 4.73388 | 48.6438 | 0.652329 | −0.002065 | 0.0067577 |
| 300 | 11.0957 | 110.9577 | 0.665388 | −0.020190 | 0.0078090 |

[a] Amount of glycerol = 100 mmol.

at 15 min, and total deactivation was obtained when incubated with 300 mmol of vinyl acetate. This observation may be explained from the solubility consideration described in the literature.[23] When the enzyme-containing phase is not saturated with vinyl acetate, some of the enzyme molecules can retain catalytic activity. Beyond this saturation concentration, the lipase is totally exposed to the deleterious effect of vinyl acetate, which is reflected in the rapid fall in activity in this region. However, when the vinyl acetate and 2-*o*-benzylglycerol were present in sufficient quantity, the enzyme-containing phase is not saturated by vinyl acetate, and the enzyme is unaffected by vinyl acetate. As a result acetylation occurs very rapidly. Table 5 shows the values of the model parameters at different vinyl acetate concentrations, indicating a definite effect on lipase activity. It may be inferred that there is deactivation attributable to product, as the vinyl alcohol formed in the reaction tautomerizes to acetaldehyde, which, being volatile,[9] does not take part in the complexation reaction.

*Effect of Water on Deactivation.* At 55 °C, the activity was lost by 75% in 15 min of incubation, but at 60 °C, the activity is lost by 65% for the same incubation time. However, at 30 °C for 15 min of incubation, loss of activity was found to be only 5%.

For studying the combined effects of water and vinyl acetate, 100−200 mmol of the latter was added to the incubation mixture of 1 g of beads with 5 mL of water at 50 °C. After 1 h, it was cooled to 30 °C, and then a sufficient amount of 2-*o*-benzylglycerol was added to the reaction mixture which was stirred at 30 °C. It was found that the residual activity of the enzyme decreases to 26% at 15 min incubation, whereas the activity decreased to 75% when water was used alone (Figure 11). Comparing these data with those reported in the literature,[25] it may be inferred that the residual activity of the enzyme decreased largely in the presence of both the aqueous and organic phases and that this decrease was lower than that observed for a single aqueous phase.

**Mathematical Model Simulation and Validation from Experimental Results.** The kinetics of deactivation of the immobilized enzyme in the presence of vinyl acetate was studied by fitting various models given in the theory. Data obtained from Table 3 were used for this purpose. A Gauss−Newtonian algorithm for optimization of parameters by

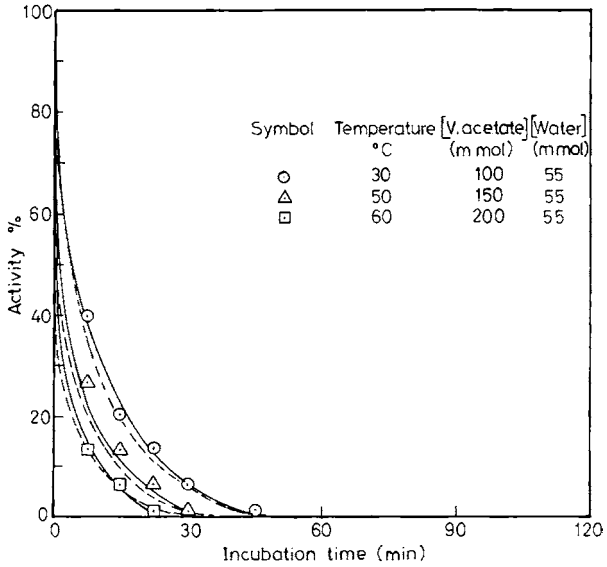


***Figure 11.*** **Residual activity of the lipase as a function of incubation time at a fixed water (55 mmol) and different vinyl acetate concentrations. Lipase = 15 mg/mL, [2-*o*-benzylglycerol] = 50 mmol.**

nonlinear regression was employed for the models of eqs 8 and 9, and it was found that the latter model yielded a better fit of experimental data. The effect of vinyl acetate concentration was analysed from eq 14. It may be noted that a number of other empirical substitutions for $\alpha_1$ were made, but the best results were obtained with eq 14. In Figures 9−11, the experimental and simulated values of activity versus time are shown, and it appears that the agreement between experimental and simulated results can be considered reasonably good. A similar substitution was made by Henley and Sadana[20,21] and Gandhi et al.[23] for the mathematical analysis of enzyme deactivation by NADP on glucose-6-phosphate-dehydrogenase deactivation and lipozyme deactivation by butanol and temperature. With this model, the correlation between the experimental and predicted values was found to be reasonable. Tables 4 and 5 show the values of the model parameters at different temperatures and vinyl acetate concentrations, respectively. The agreement between experimental and simulated results can be considered reasonable good.

The first-order dependence of the lipase concentration on the deactivation rate, d[N]/d$t$, was established by independent simulation of the model derived from. The use of the suggested model is also justified by the experimental nature of the activity versus time curves as shown in Figure 9. The use of a series mechanism, rather than a parallel one, may be considered valid as there is no evidence for hypothesizing the presence of more than one enzyme form. The exponential shape of the activity versus time curves shows a tendency toward activity stabilization, justifying the use of a two-step mechanism [eq 14] rather than three-step mechanism [eq 8].

## Conclusions

The transesterification of 2-*o*-benzylglycerol with vinyl acetate catalysed by free and immobilized lipases was affected by reaction conditions such as lipase concentration,

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temperature, substrate concentration, etc., and the reaction was found to conform to the Ping Pong Bi Bi mechanism. The activity of immobilized and free lipase from *Pseudomonas cepacia* was studied through the transesterification of 2-*o*-benzylglycerol and vinyl acetate, and it was found that the activity of immobilized lipase was higher than the free lipase. The deactivation of the immobilized lipase was also studied, and it was found that the concentration of vinyl acetate has profound effect on lipase deactivation. The results on deactivation kinetics of the immobilized lipase were interpreted with a suitable mathematical model. High catalytic activity of the enzyme could be inferred from the relatively low value of the activation energy obtained for the reaction.


## Acknowledgment

We are grateful to O. M. Sahoo for his assistance in the development of the computer program required to solve the proposed mathematical model.

Received for review September 1, 2003.

OP030040Y