

## Ethyl oleate synthesis by *Porcine pancreatic* lipase in organic solvents

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Received 23 October 2000; accepted 5 February 2001

### Abstract

The *Porcine pancreatic* lipase catalysed esterification of oleic acid with ethanol was studied in 10 different solvents with constant initial water content in the reaction mixture. The initial rates of the esterification reaction were attempted to correlate with such solvent properties as hydrophobicity ( $\log P$ ), water solubility ( $S_w$ ), dielectric constant, electron pair acceptance and donation index (expressed as  $E_T^N + DN^N$ ), polarisability etc. While significantly good linear correlations with  $\log P$  and  $\log S_w$  were obtained, the correlations with the other properties were found to be inferior. The kinetics of the reactions was found to conform to the so-called Ping-Pong-Bi-Bi model with ethanol inhibition effect and the estimated model parameters exhibited statistically significant correlation with  $\log P$  consistent to its correlation with the initial rate. Assuming that organic solvents do not interfere with the lipase–substrate binding process nor with the catalytic mechanism, the contribution of substrate–solvent interactions to enzyme kinetics was accounted for by replacing the substrate concentrations of the intrinsic kinetic equations by thermodynamic activities. The values of the corrected intrinsic parameters ( $K_m$ ,  $k_{sp}$ ) and the maximal rate ( $V_{max}$ ) were found to be nearly equal for all the media. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** *Porcine pancreatic* lipase; Ethyl oleate; Esterification

### 1. Introduction

Ethyl oleate is useful as biological additive, PVC plasticiser, water resisting agent and for hydraulic fluid. Due to several advantages of enzyme catalysis in organic solvents [1], the synthesis of ethyl oleate and other esters of oleic acid have been studied by exploiting the catalytic activities of lipases isolated from various microbial sources, such as *Mucor miehei* [2–7], *Chromobacterium viscosum* [8,9], *Rhizopus oligosporus* [10], *Penicillium simplicissimum*, *Rhizopus delemar*, *Rhizopus arrhizus* [11], *Candida rugosa*, *Rhizomucor miehei* and *Pseudomonas fluorescens* [12]. One of the cheap and commercially available nonmicrobial enzymes is *Porcine pancreatic* lipase which has high thermostability and activity in anhydrous reaction mixtures as demonstrated for esterification and transesterification reactions [13,14].

The potential advantages of using enzymes in nonaqueous media include substrate specificity [15], regioselectivity [16], and increased solubilities of organic substrates [17]. However, enzymes used predominantly in organic media require some water to achieve good catalytic activity [18]. In esterification reactions, the initial activity of lipase some-

times exhibits an optimum value at a certain water content in the reaction medium [19]. Solvents including water, participate in enzyme catalysis because they interact with substrates as well as and products (solvation and desolvation) [20]. The enzymes do not dissolve in most of the commonly used organic solvents and hence catalysis takes place in dispersed media, for which the efficiency depends on the amount of enzyme. Enzyme catalysis and nonaqueous media are not incompatible entities as demonstrated by catalytic activity for enzymes when dispersed in organic solvents, but substantial differences have been made between reaction rate, maximal velocity and substrate affinity determined in water and in various organic solvents.

The effect of solvent is highly dependent on the nature of the enzyme and the solvent. However, it appears that there is no difference in dependence of solvent properties with enzymatic activity of lipolytic and nonlipolytic enzymes [20], thus, inferring the generality of enzymatic catalysis in organic solvents.

In spite of the importance of lipase catalysis in organic solvents, systematic investigation of the effect of solvents and their properties (i.e. hydrophobicity, water solubility, dielectric constant, polarisability etc.), on rate and selectivity of esterification, transesterification and hydrolysis reactions has been reported only for a few cases [21–24]. In this paper, we present a comprehensive study on the effect of solvents on esterification of oleic acid with ethanol using *Porcine*

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pancreatic lipase which has hitherto been not reported in literature.

## 2. Experimental

The *Porcine pancreatic* lipase (with a specific activity 70 U/mg protein) and ethanol were procured from SRL Pvt. Ltd., Mumbai, India. Oleic acid (*cis*-9-octadecanoic acid) of 97% purity and ethyl oleate of chromatographic standard and the solvents of analytical grades were procured from CDH Pvt. Ltd., New Delhi, India. The esterification reactions were carried out in a 250 ml round bottom flask by mixing the reaction mass with a turbine type impeller at a speed of 250 rpm. The reaction temperature was maintained at 30 °C by putting the sealed reaction flask in a beaker where constant temperature water was circulated from a Julabo circulator.

Experiments on solvent effect were carried out under optimised reaction conditions with 25 mmol acid and 25 mmol alcohol dissolved in 50 ml of water saturated solvent. The lipase content of the reaction media was 10 mg/ml. Aliquots of sample were drawn periodically and analysed in a GC (Varian 3700 GC equipped with a FID) using a mixed phase 4% OV101 + 6% OV210 coated on chromosorb W HP 80/100 mesh packed in a S.S. column (50 cm × 1/8 in. i.d.). The column was programmed with an initial temperature of 120 °C for 4 min, then raising it at 2 °C/min to a final temperature of 230 °C. The injector and detector temperatures were maintained at 230 and 240 °C, respectively. Chart speed of the recorder was 0.25 cm/min. The samples were diluted with the solvent to a known level and the enzyme was filtered out for performing the GC analysis. All the experiments were conducted in duplicate and the reproducibility was found to be ±5%.

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 per unit weight of lipase (expressed as mmol/min g). All the kinetic experiments were carried out at 30 °C using only *n*-hexane as the solvent. The substrate and lipase concentrations were maintained at 10–85 mM and 10 mg/ml, respectively.

## 3. Results and discussions

In the present study, we used water saturated solvent throughout keeping water activity in the system essentially close to unity. The water formed in the reaction is assumed to have no influence on the reaction rate as the rate measurements were made at reasonably low conversion. The amount of water formed corresponding to the conversion taken for calculation of initial rate was estimated to be written 3% of the water solubility value in the solvent. This increase of water content will not significantly change the water activity. It may, therefore, be presumed that experiments conducted under such a condition of water activity control would not deter from meaningful interpretation of the solvent effect in the present system. In fact, the effect of water activity on lipase catalysed reactions will be realised differently in different solvents and should be studied separately [25,26].

### 3.1. Initial rate versus solvent properties

The pertinent properties of the solvents used for this study are listed in Table 1. These solvents were selected on the basis of their hydrophobicity ( $\log P$ ) values which lie in between 0.45 and 3.5 as the activity and stability of the enzymes have been reported to be optimal in this range of  $\log P$  [23,27]. The  $\log P$  value has been proposed as a quantitative measure of solvent polarity [29] and the enzyme activity for lipase catalysed reactions in general, increases with increasing hydrophobicity of the solvent [24,29]. The relation of initial reaction rate with  $\log P$  values of the solvent is shown in Fig. 1 from which it is apparent that the enzyme activity increases almost linearly with an increase of  $\log P$  and that

Table 1  
Pertinent properties of the solvent used in this study<sup>a</sup>

S. no.	Solvent	$\log P$	$\log S_w$	$E_T^N$	$DN^N$	Dielectric constant	Polarisability (in units of $10^{-24}$ )
1	Acetonitrile	-0.33	Miscible	-	-	37.50	6.33
2	Acetone	-0.23	Miscible	-	-	20.70	4.40
3	Dichloromethane	0.60	-0.84	0.321	0.03	8.90	6.48
4	Ethyl acetate	0.70	0.21	-	-	6.00	9.70
5	Ethyl ether	0.85	-0.24	0.120	0.49	4.34	10.20
6	Chloroform	2.00	-1.12	0.260	0.10	4.81	9.50
7	Toluene	2.50	-1.80	0.096	0	2.38	11.80
8	Carbon tetrachloride	3.00	-1.93	0.090	0	2.24	11.20
9	Cyclohexane	3.20	-2.25	-	-	2.00	11.00
10	Hexane	3.50	-2.39	0.074	0	1.88	11.90

<sup>a</sup>  $\log P$  is the logarithm partition coefficient in the octanol-water system ([28]),  $\log S_w$  is the logarithm of the saturated solubility of water in the solvent on molar basis,  $E_T^N$  is the normalised electron pair acceptance index and  $DN^N$  is the normalised Gutmann donor number ([24]); dielectric constant and polarisability values were taken from [45].

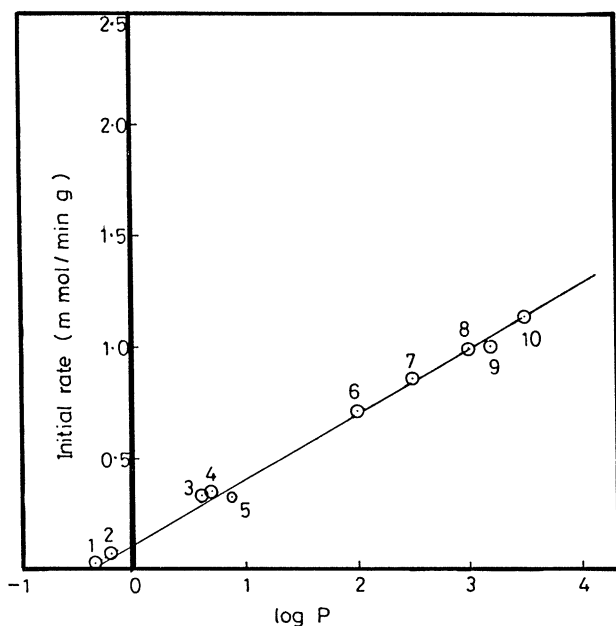


Fig. 1. Initial reaction rate as a function of solvent hydrophobicity: [Eth] = 2.5 mM; [Ol] = 2.5 mM; lipase = 10 mg/ml; temperature = 30 °C.

the lipase can catalyse the esterification in a wide variety of solvents. The correlation between initial rate ( $r$ ) and  $\log P$  can be represented by

$$r = 0.3004(\log P) + 0.09 \quad (1)$$

with a correlation coefficient of 0.99 which may be considered highly significant. The present observation of increased lipase activity with hydrophobicity of the solvent is similar to those reported for enantioselective esterification of 2-chloropropionic acid with *n*-butanol by *Candida cylindracea* [30,31] and racemic glycidol with butyric acid [32] by *Porcine pancreatic lipase*. A good correlation of  $\log P$  and mole fraction of ester at equilibrium was observed also for *Chromobacterium viscosum* lipase catalysed esterification of decanoic acid with glycerol [9]. This correlation of the lipase activity with  $\log P$  reflects in the extent to which the solvent molecule can enter the relatively polar phase around the enzyme and hence contact it. For the esterification of butyric acid with propanol in immobilised *Pseudomonas cepacia* lipase at constant water activity, the relation of initial rate and  $\log P$  was found to be sigmoidal in nature [33].

The esterification of diol lactone precursor (s)-(+)-[6-(R)-[2-8(s),6(R)-dimethyl-1',2',6',7',8',8'A-(R)-hexahydro-naphthol) ethyl]-4(R)-hydroxy-3,4,5,6-tetrahydro-2-II-pyran-2-one diol lactone]] with 2-methyl butyric acid catalysed by *Candida rugosa* lipase immobilised on a nylon support was found to exhibit rate versus  $\log P$  relationship [20] identical to that obtained in the present work. However, no such correlation could be obtained when hexane mixtures comprising different co-solvents were used. This probably implies that presence of co-solvent not only affects the enzyme activity, but also may impact the controlling mechanism.

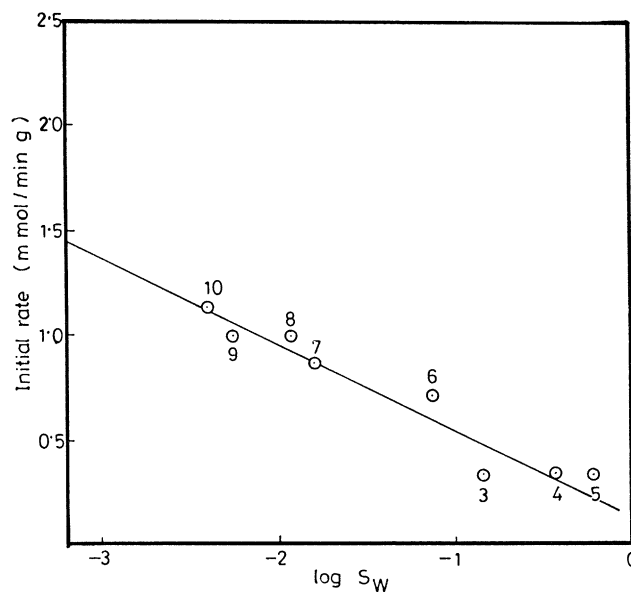


Fig. 2. Initial reaction rate as a function of the water solubility of the solvent: [Eth] = 2.5 mM; [Ol] = 2.5 mM; lipase = 10 mg/ml; temperature = 30 °C.

The water solubility of the solvent has been recognised as the most useful index of the solvent polarity for correlating the rates of esterification reactions [24]. Fig. 2 shows the correlation of the solubility of water (molar basis) in the solvent ( $\log S_w$ ) with initial rate of the reaction and the relationship could be deduced as

$$r = -0.4167(\log S_w) + 0.1333 \quad (2)$$

with a correlation coefficient of 0.97 which may also be considered significant. Acetone and acetonitrile are highly miscible with water and hence the initial rates of the reaction in both the solvents (not shown in the figure) are significantly lower. It may, therefore, be inferred that solvents with low water solubility favour the esterification reaction, the observation being akin to that reported for other lipase catalysed reactions [24].

Nonpolar solvents are also chosen from the well accepted rules for the effects on biocatalyst activity [20]. For predicting performance of the reaction media using polarity as the criteria, there is also other fundamental basis, which seems to rely on the donor–acceptor interactions of the solvent including hydrogen bonding capability. Solvation of water requires both donation and acceptance of hydrogen bonds (or electron pairs) or other dipole–dipole interactions. Accordingly, an attempt has been made to correlate the initial rate with the sum of the normalised electron pair acceptance index ( $E_T^N$ ) and Gutmann's donor number ( $DN^N$ ), and the correlation is shown in Fig. 3. The correlation for the present system seems to be rather weak in comparison to that reported by Velivety et al. [24] who established a good correlation between water solubility and ( $E_T^N + DN^N$ ) of several organic solvents for which these values are

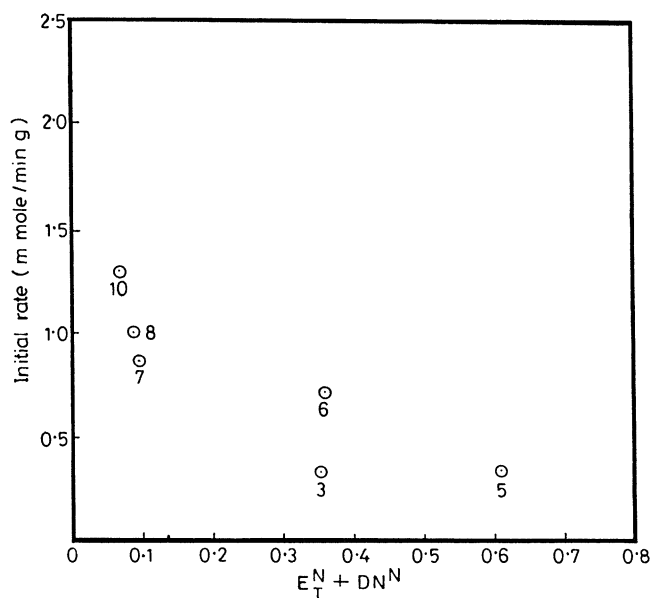


Fig. 3. Initial reaction rate as a function of  $(E_T^N + DN^N)$ : [Eth] = 2.5 mM; [Ol] = 2.5 mM; lipase = 10 mg/ml; temperature = 30 °C.

available and arrived at a conclusion that the contributions of electron pair acceptance and donation are roughly equal.

It was also deduced that the hydrogen bond donating and accepting capacity of the solvent determines both water solubility and esterification reaction equilibrium in that solvent. The weak correlation reflected in Fig. 3 may be attributable to the lack of data points for four solvents whose  $E_T^N + DN^N$  values are not known. Indeed, the values of this parameter for many solvents are not known and are considered uncertain in many cases. However, the observed trend on decrease of initial rate with  $(E_T^N + DN^N)$  may be considered reasonable and explained from the solvation effect of the ester which involves electron pair acceptance from two oxygen atoms. In the acid and alcohol together, there are three oxygen atoms requiring this type of interactions and two hydrogen atoms capable of interacting with electron pair donors. The differential solvation which would be expected to affect the equilibrium position involves additional acceptor and donor interactions. Solvents capable of imparting either or both of these interactions would favour hydrolysis product rather than esterification product. Therefore, the present finding on the effect of  $(E_T^N + DN^N)$  appears to be reasonable.

We have attempted to correlate the initial rate with another important solvent property called polarisability which measures the ability of a solvent to stabilise the charge of a dipole in solution. It is a function of dielectric constant and refractive index which are easily measurable and hence, known for most of the solvents. As such no good relation was obtained between initial rate and the polarisability (not shown). When combined with  $\log P$  and expressed in terms of  $\log P$  divided by polarisability, relatively representative effect of this parameter on initial rate could be deduced as shown in Fig. 4, wherein the relation of initial rate with

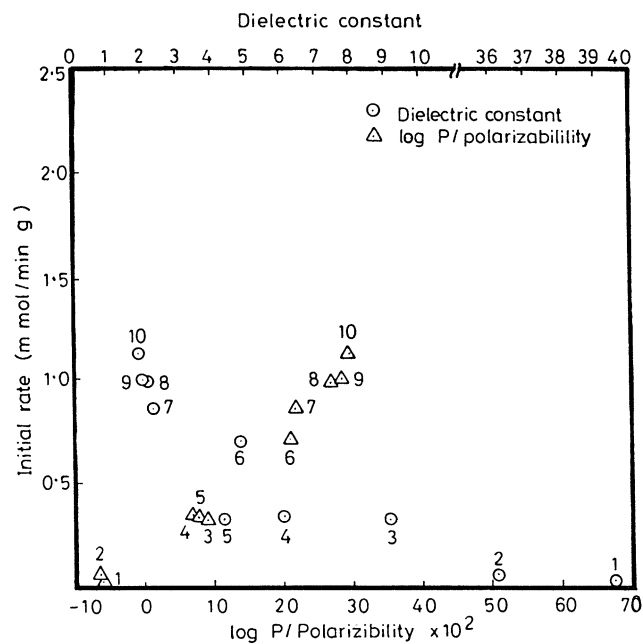


Fig. 4. Initial reaction rate as a function of dielectric constant and polarisability: [Eth] = 2.5 mM; [Ol] = 2.5 mM; lipase = 10 mg/ml; temperature = 30 °C.

dielectric constant is also shown. The rate tend to increase with  $\log P$ /polarisability linearly, but the estimated correlation coefficient of the linear relationship was found to be less than 0.95 implying again a weak correlation in contrast to that observed for esterification of dodecanol with decanoic acid using the same lipase [4]. As evident from Fig. 4, the exponential decrease of rate with dielectric constant also does not represent a statistically sound correlation, but the observed trend of increasing and decreasing rates appears to be reasonable.

The variation of esterification rate with  $\log S_w$  and  $(E_T^N + DN^N)$  perhaps indicate the role of the bulk behaviour of the solvent and functional group with specific interaction often referred to as “chemical” effects by liquid state theories as suggested for other esterification reactions too [34].

### 3.2. Lipase activity

Solvent effect has been analysed also from a probable relationship between enzyme activity and substrate partitioning on solvation. Different solvents would exhibit different abilities to solvate the substrate and, thus, may influence the thermodynamic activity of the substrate, the measured enzyme activity [35,36] and partition coefficients of substrates as well as the products. The implication is that solvent selection for biocatalysis under nearly anhydrous conditions would depend on the substrates and the catalyst type [37].

*Porcine pancreatic* lipase has been found to exhibit high affinity for various alcohols in esterification and transesterification reactions [38]. In order to assess the effect of solvents, the  $V_{max}$  and  $K_m$  values of the Michaelis–Menten

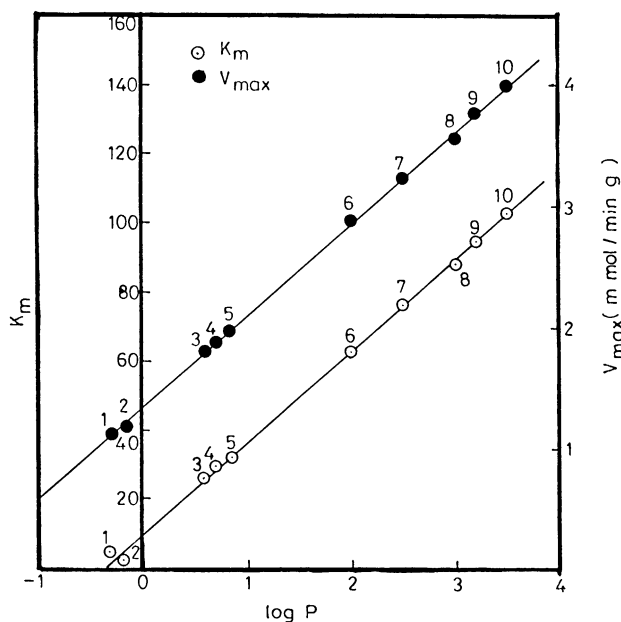


Fig. 5.  $K_m$  and  $V_{max}$  as a function of  $\log P$ .

kinetic equation have been evaluated and plotted against  $\log P$  values (Fig. 5). The root mean square error for estimation of  $V_{max}$  and  $K_m$  was found to be within  $\pm 5\%$  for all the solvents studied and may be considered reasonable. Statistically significant correlations are obtained as represented by the following empirical equations:

$$K_m = 27.125(\log P) + 8.1 \quad (3)$$

$$\text{and } V_{max} = 0.804(\log P) + 1.2 \quad (4)$$

with correlation coefficients of 0.99 for both the equations. This finally provides an idea about affinity of the lipase, however, since substrate inhibition for ethanol was also observed, measurements were necessarily carried out at optimum ethanol concentration so as to eliminate the inhibition effect and keep specificity constant ( $V_{max}/K_m$ ) essentially unaltered. The linear correlation between the Michaelis–Menten kinetic parameters and  $\log P$  may be considered phenomenologically consistent with the rate versus  $\log P$  correlation shown in Fig. 1.

*Porcine pancreatic* and *Pseudomonas* lipase are known to act via Ping-Pong-Bi-Bi mechanism [39] and the apparent kinetic parameters for one substrate depend on the thermodynamic activity of the other substrate in support of which specific rate equations exist as shown below.

$$v = \frac{V_{max}^{app} c_{Eo} \times a_{Eth}^-}{K_{m,Eth}^{app} + a_{Eth}^-} \quad V_{max}^{app} = \frac{V_{max}^{int}}{1 + (K_{m,OI}^{int}/a_{OI}^-)} \quad (5)$$

$$K_{m,Eth}^{app} = \frac{K_{m,Eth}^{int}}{(1 + K_{m,OI}^{int}/a_{OI}^-)}$$

where,  $v$  is the initial velocity,  $c_{Eo}$  the total enzyme concentration,  $a^-$  the activity,  $V_{max}^{app}$  the apparent maximal velocity,

$K_{m,Eth}^{app}$  the apparent Michaelis constant and the superscript ‘int’ refers to the respective intrinsic parameters.

Due to the existence of solvent substrate interaction, the ‘availability of substrate to the enzyme’ forms part of the overall kinetic parameters observed and the contribution of this substrate will vary with the nature of the solvent. A comparison of enzyme behaviour in different solvents obviously needs correction for these contributions to enable assessment of real effects of solvents on the enzyme. It is believed that the substrate specificity of enzymes stems from their ability to utilise the free energy of binding with substrates to facilitate the reaction [40,41]. Since the net binding energy is the difference between the binding energies of the substrate with the enzyme and with water [41], an alternative approach to changing substrate specificity would be the replacement of water with another reaction medium.

Evaluation of solvent effect on the performance of an enzyme may also rely on correction of the kinetic parameters for substrate–solvent interactions with a concomitant comparison of their values. The contribution of substrate solvent interaction to enzyme kinetics can be accounted for by replacing the substrate concentration with the thermodynamic activity in the rate equation. This is considered valid when the organic solvents are assumed to have no interference with the binding process. The initial rate of the esterification reaction, at a constant oleic acid concentration and variable ethanol concentration was found to be the highest in hexane and lowest in acetonitrile. However, the nature of variation of initial rate versus activity for all the solvents appears to be identical as shown in Fig. 6. In case of hexane as the solvent, the initial rate tends to decrease at higher concentration of

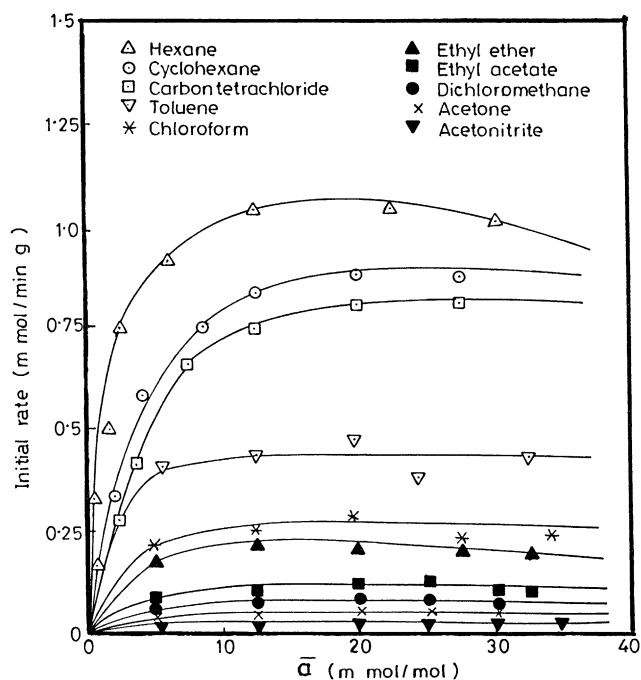


Fig. 6. Initial reaction rate as a function of activity of the solvent.

Table 2

Specificity constants ( $k_{sp} = V_{max}/K_m$ ) of *Porcine pancreatic lipase* suspended in various solvents for esterification of oleic acid and ethanol<sup>a</sup>

Solvent	$k_{sp,OH}$ (ml min <sup>-1</sup> gm <sup>-1</sup> )	Int $k_{sp,OH}$ (mmol min <sup>-1</sup> gm <sup>-1</sup> )	$k_{sp,COOH}$ (ml min <sup>-1</sup> gm <sup>-1</sup> )	Int $k_{sp,COOH}$ (mmol min <sup>-1</sup> gm <sup>-1</sup> )
Acetonitrile	$3.492 \times 10^{-2}$	$7.525 \times 10^{-2}$	$6.062 \times 10^{-2}$	$8.695 \times 10^{-2}$
Acetone	$2.380 \times 10^{-2}$	$2.339 \times 10^{-2}$	$3.211 \times 10^{-2}$	$3.230 \times 10^{-2}$
Dichloromethane	$2.101 \times 10^{-2}$	$2.201 \times 10^{-2}$	$3.101 \times 10^{-2}$	$3.133 \times 10^{-2}$
Ethyl acetate	$2.223 \times 10^{-2}$	$2.206 \times 10^{-2}$	$3.230 \times 10^{-2}$	$3.142 \times 10^{-2}$
Ethyl ether	$1.903 \times 10^{-2}$	$2.124 \times 10^{-2}$	$2.340 \times 10^{-2}$	$3.117 \times 10^{-2}$
Chloroform	$1.612 \times 10^{-2}$	$2.429 \times 10^{-2}$	$2.010 \times 10^{-2}$	$3.016 \times 10^{-2}$
Toluene	$1.257 \times 10^{-2}$	$2.309 \times 10^{-2}$	$2.000 \times 10^{-2}$	$3.008 \times 10^{-2}$
Carbon tetrachloride	$0.902 \times 10^{-2}$	$1.999 \times 10^{-2}$	$1.980 \times 10^{-2}$	$2.983 \times 10^{-2}$
Cyclohexane	$0.676 \times 10^{-2}$	$0.650 \times 10^{-2}$	$1.580 \times 10^{-2}$	$2.310 \times 10^{-2}$
Hexane	$0.425 \times 10^{-2}$	$0.550 \times 10^{-2}$	$0.990 \times 10^{-2}$	$2.222 \times 10^{-2}$

<sup>a</sup> The parameters were obtained from the experimental data at constant oleic acid concentrations in Fig. 6. The ordinary and intrinsic specificity constants are related to each other via  $k_{sp} = \gamma \times k_{sp}^{int}$ , where  $\gamma$  is the activity coefficient determined by ASOG method.

ethanol due to inhibition effect as will be discussed further in a Section 3.3. It is believed that the surface of the enzyme only participates in catalysis. Since the nature of the solvent and the substrate could affect the available catalytic surface, the differences in the amount of available catalytic surface may contribute to variation in reaction rates. The specificity constant ( $k_{sp} = V_{max}/K_m$ ) values determined by nonlinear regression of data presented in Fig. 6 through use of Eq. (5) are shown in Table 2. Neglecting the value obtained for hexane with solvent and comparing those for the others, it may be inferred that the variation is less in case of intrinsic than for the apparent values. Similar effects of solvents on catalytic activity of lipase has been reported also for other hydrolysis, esterification and transesterification reactions [20].

### 3.3. Reaction mechanism

For detail kinetic study, *n*-hexane has been considered as the only solvent as it provides adequate activity of the lipase and is less toxic in comparison to the others. The reaction mechanism has been elucidated by a plot of reciprocal of both initial rate and substrate (oleic acid) concentration as shown in Fig. 7. It is apparent that an increase of oleic acid concentration at a constant ethanol concentration increases the initial rate. The observation of decrease in initial rate with increase of ethanol concentration apparently reflects the ethanol inhibition effect. Similar inhibition effect was also observed for the *Mucor miehei* catalysed synthesis of ethyl oleate [2–5] and ethyl myristate [42] using immobilised lipase in *n*-hexane and in supercritical CO<sub>2</sub>, respectively. The plots in Fig. 7 at low ethanol concentration tend to be parallel, whereas at high ethanol 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 with ethanol inhibition effect for which the initial velocity equation is [43,44]

$$\frac{V}{V_{max}} = \frac{[OI][Eth]}{K_{m(OI)}[Eth](1 + [Eth]/k_i) + K_{m(Eth)}[OI] + [Eth][OI]} \quad (6)$$

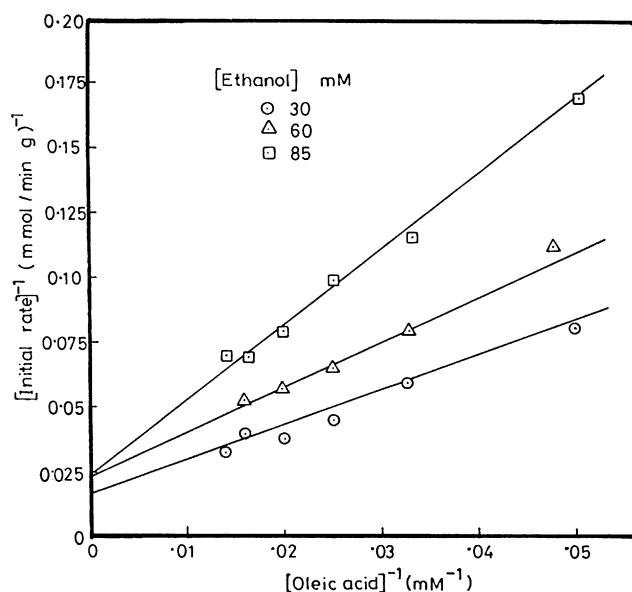


Fig. 7. Reciprocal coordinate representation of lipase activity on oleic acid and ethanol concentrations: lipase = 10 mg/ml; temperature = 30 °C.

with [OI] and [Eth] representing the initial molar concentration of oleic acid and ethanol, respectively,  $K_{m(OI)}$  and  $K_{m(Eth)}$  are the respective affinity constants,  $k_i$  is the inhibition constant of ethanol and  $V_{max}$  is the maximum reaction rate. The kinetic parameters,  $V_{max}$  and  $K_{m(Eth)}$  were estimated from the data presented in Fig. 7, whereas the values of  $K_{m(OI)}$  and  $k_i$  were obtained from Fig. 8. For better accuracy, the values of  $K_{m(Eth)}$ ,  $K_{(OI)}$ ,  $k_i$  and  $V_{max}$  were computed from the equation for reaction velocity by numerical parameter identification using the Gauss–Newton algorithm of error minimisation and their values are given in Table 3, wherein the literature values for immobilised lipase are also shown. From the data, it seems that *Porcine pancreatic lipase* may be considered somewhat less active than *Mucor miehei* for ethyl oleate synthesis. This may be contradicted if  $K_{m(OI)}$  and  $K_{m(Eth)}$  values are considered for a discussion. In dispersed system, the diffusional limitation can be considered

Table 3  
Kinetic parameters for lipase catalysed ethyl oleate synthesis

Lipase	Solvent	$V_{\max}$ (mmol min <sup>-1</sup> gm <sup>-1</sup> )	$K_{m(OI)}$ (mM)	$K_{m(Eth)}$ (mM)	$k_i$ (mM)	Reference
<i>Porcine pancreatic</i>	<i>n</i> -Hexane	4.0	66	103	20	This work
<i>Mucor miehei</i> <sup>a</sup>	<i>n</i> -Hexane	23.0	450	600	60	[3]
<i>Mucor miehei</i> <sup>a</sup>	SCCO <sub>2</sub>	14.0	170	1600	65	[3]
<i>Mucor miehei</i> <sup>a</sup>	<i>n</i> -Hexane	5.70	120	190	40	[2]
<i>Mucor miehei</i> <sup>b</sup>	<i>n</i> -Hexane	5.33	–	–	43	[42]
<i>Mucor miehei</i> <sup>b</sup>	SCCO <sub>2</sub>	8.32	–	–	120	[42]
<i>Rhizomucor miehei</i> <sup>a</sup>	SCCO <sub>2</sub>	0.65	–	6.24	20.98	[46]

<sup>a</sup> Immobilised.

<sup>b</sup> Ethyl myristate synthesis.

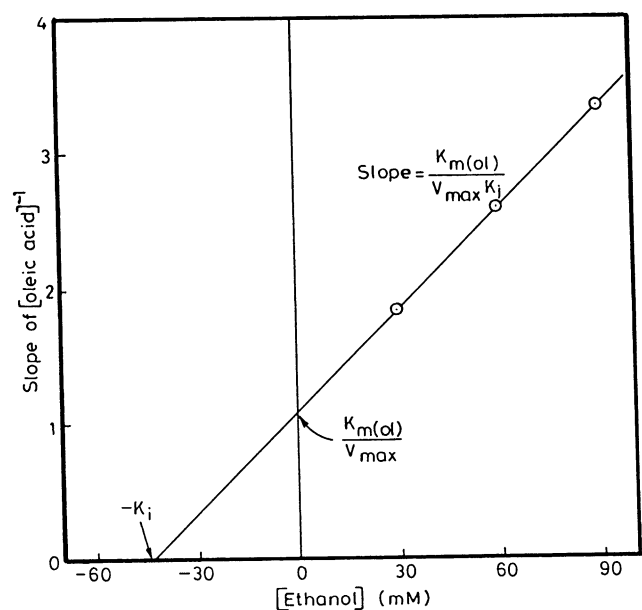


Fig. 8. Slope of [oleic acid]<sup>-1</sup> as a function of ethanol concentration: lipase = 10 mg/ml; temperature = 30 °C.

negligible and this would predict lower affinity of oleic acid as well as ethanol, i.e. lower  $K_{m(OI)}$  and  $K_{m(Eth)}$  values. If *Mucor miehei* lipase catalysed ethyl oleate synthesis in dispersed system is being considered, one would expect higher  $K_{m(OI)}$  and  $K_{m(Eth)}$  values. Thus, considering the  $K_{m(OI)}$  and  $K_{m(Eth)}$  values, one could justifiably infer that *Mucor miehei* and *Porcine pancreatic* lipases are not comparable in their activities for ethyl oleate synthesis. In Table 3, data on ethyl myristate synthesis by immobilised lipase of *Mucor miehei* in *n*-hexane as well as SCCO<sub>2</sub> [42] are also shown. The data indicate nearly identical ethanol inhibition effect of both the lipases. It may, thus, be presumed that the esterification reaction studied in this exhibits Ping-Pong-Bi-Bi mechanism with ethanol inhibition effect. Our data seem to be insufficient to suggest such a mechanism but in support of the same, the reported results on esterification reaction of butyric acid with glycidol (2,3-epoxypropanol) using *Porcine pancreatic* lipase may be referred to. This reaction was well described by the Ping-Pong-Bi-Bi mechanism involving formation of

ternary complex of alcohol, acid and enzyme which apparently does not account for the inhibition effect [17,44].

#### 4. Conclusion

The effect of solvent on esterification of oleic acid with ethanol has been studied in a dispersed system of *Porcine pancreatic* lipase. The initial reaction rates correlate well with hydrophobicity and water solubility of the solvents, whereas the correlations with the other solvent properties such as electron pair acceptance and donation indices, dielectric constant and polarisability were found to be inferior. However, the observed trend in variation of rates with these parameter may be considered reasonable. Assuming that organic solvents interfere neither with the enzyme–substrate binding process nor with the catalytic mechanism, the contribution of substrate–solvent interactions to enzyme kinetics was accounted for by replacing the substrate concentrations in the equations with thermodynamic activities and this transformation appears to affect the affinity parameters ( $K_m$ ,  $k_{sp}$ ) only. Thus, the values of the corrected intrinsic parameters ( $K_m^{int}$ ,  $k_{sp}^{int}$ ) and the maximal rate ( $V_{max}$ ) were found to be equal. The kinetics of the reaction in *n*-hexane as the solvent was found to conform to Ping-Pong-Bi-Bi mechanism with ethanol inhibition effect.

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