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ORIGINAL ARTICLE

Esterification of lauric acid with lauryl alcohol using cross-linked enzyme crystals: Solvent effect and kinetic study

SUMBITA GOGOI[1], SWAPNALI HAZARIKA[1], P.G. RAO[2], & N.N. DUTTA[1]

[1]*Chemical Engineering Division, Regional Research Laboratory, Jorhat-785006, Assam, India and*
[2]*Director, Regional Research Laboratory, Jorhat-785006, Assam, India*

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Abstract
In this paper, we report a comprehensive kinetic study on esterification of lauric acid with lauryl alcohol catalyzed by commercial porcine pancreatic lipase (PPL) in the form of cross-linked enzyme crystals (CLEC) using glutaraldehyde as the cross linker. The stability of the CLEC was better than the immobilized enzyme for practical applications. Comparative studies using six different solvents having hydrophobicity (log p) values ranging from 0.70 to 3.50 revealed that the esterification reaction was favoured in hydrophobic solvents. The kinetics of the esterification reaction conformed with the so-called Ping-Pong–Bi-Bi mechanism with alcohol inhibition.

Keywords: *lipase, lauryl alcohol, CLEC, Glutaraldehyde*

Introduction

The hydrolysis and synthesis of esters using lipases has been studied for many years due to the inherent advantages of mild reaction conditions and reaction selectivity, particularly for the production of flavours and compounds for oil and fat industries (Chulalaksananukul et al. 1990). The use of organic solvents in such reactions can offer advantages such as improved substrate specificity, stereoselectivity, recoverability (Zaks & Klibanov 1986; Rubio et al. 1991) and low solubility of enzymes (Van Tol et al. 1995). However, a requirement for large-scale use is that the solvent must be able to dissolve high substrate concentrations to obtain high productivity (Parida & Dordick 1993). The reactivity of certain lipase catalyzed esterification and transesterification reactions in various organic solvents has been studied by Hazarika et al. (2002, 2003).

One of the factors for the successful application of enzymes is enzyme stability, which is often affected by the solvent in non-aqueous media. In the case of lipase, solvent-induced inactivation has been overcome by immobilization. Furthermore, immobilized enzymes are usually superior to crude enzyme for repeated usage. Among various immobilization methods, adsorption of the enzyme on an appropriate support matrix and subsequent lyophilization is simple but effective, retaining essential water molecules for enzyme activity and often significantly enhancing the enzyme activity in an organic solvent (Jeong et al. 2000).

However, cross-linked enzyme crystals (CLECs, microcrystals grown from aqueous solution and cross-linked with a bifunctional agent) have been shown to exhibit remarkable characteristics that are superior to both crude and conventionally immobilized enzyme (Persichetti et al. 1995) due to the greater stability, activity and regio- and stereoselectivity of biocatalysts (Lalonde et al. 1995; Khalaf et al. 1996) and simple recovery in the crystalline form. CLEC preparation requires crystallization of catalysts and then cross-linking of the highly pure micro-crystals with bi-functional reagents such as glutaraldehyde. After cross-linking, CLECs are insoluble in aqueous buffer and organic solvents, so they can be used in a variety of reaction media, then recovered and reused. CLECs also remain active after prolonged exposure to high temperatures.

Correspondence: N.N. Dutta, Chemical Engineering Division, Regional Research Laboratory, Jorhat-785006, Assam, India. Tel: (0376) 2370121 (O), 2370012 (O), 2370372 (R). Fax: (0376) 2370011. E-mail: sumbita_gogoi@yahoo.co.in

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In this paper, we report a comprehensive study of the kinetics of esterification of lauric acid with lauryl alcohol using CLEC-PPL and their stability in comparison to those of disperse system and immobilized catalyst. Solvent effects on the enzyme reactivity were also studied.

Materials and methods

Materials

Porcine pancreatic lipase (PPL) with specific activity 41 U mg^{-1} protein was from Sigma Chemicals, USA. Lauric acid, lauryl alcohol, solvents of analytical grade and celite 545 (particle size 20–45 microns) for immobilization were from CDH Pvt. Ltd., Mumbai, India. Glutaraldehyde (25%) was from Kemphasol, Mumbai, India.

Analytical methods

Lauric acid and lauryl laurate concentrations were determined by GC (Varian 3700, OV-17 column) with an oven temperature 150°C, injector temperature 230°C, flow rate of the nitrogen carrier gas 25 mL min^{-1}, detector temperature 230°C and injection volume 0.6 μL.

Experiments on solvent effects were carried out under optimized reaction conditions with 200 mM lauric acid and 350 mM lauryl alcohol dissolved in 10 mL of anhydrous solvent with 30 mg mL^{-1} CLEC-PPL. Samples were withdrawn at 30 minutes interval and analysed by GC. Initial reaction rates were calculated from conversion versus time profiles corresponding to the first 10% conversion, where the profiles were found to be linear.

All kinetic experiments were carried out using n-hexane as the solvent. The substrate and CLEC-PPL concentrations were maintained at 200–600 mM and 30mg mL^{-1} respectively. In all experiments the water concentration was maintained constant as determined with a Karl Fischer Titrator (Spectralab MA-101-B, Alfa Instruments, New Delhi). All experiments were conducted in triplicate and the reproducibility was found to be $\pm$5% and all data points represent average values.

The stability of the CLEC-PPL was examined in relation to that of crude and immobilized PPL by measuring their activities after incubation in hexane for several days. The reaction mixture consisted of 50 mM lauric acid and 150 mM lauryl alcohol dissolved in 10 mL of anhydrous hexane in the presence of 30 mg of each enzyme and incubated at 40°C with shaking.

Preparation of cross-linked enzyme crystals of PPL

PPL was crystallized by a batch method. One hundred milligrams of crude lipase was dissolved in 5 mL 50 mM phosphate buffer pH 7.0. Then 5 mL 1.0 M calcium acetate and 5 mL 30% dimethyl sulfoxide were added with stirring for 4 h at 25°C. The solution was kept at this temperature for 24 h, then the crystals formed were separated by centrifugation and washed with isopropyl alcohol.

The crystals were cross-linked in 5% (v/v) glutaraldehyde solution in 50 mM phosphate buffer pH 6.5 at 4°C for 6 h. After cross-linking, the crystals were filtered and washed three times with 20 mL of 0.02 M acetate buffer pH 4.5 by repeatedly adding fresh buffer solution to remove excess glutaraldehyde. The Cross-Linked enzyme crystals were characterized by FTIR (Perkin Elmer, system 2000) and stored at room temperature until required.

Preparation of immobilized enzyme

In the immobilization of PPL, celite 545 was used as support. The support powder (2.0 g) was added to 5 mL PPL solution containing approximately 10 000 U mL^{-1} enzyme and stirred with a magnetic stirrer at room temperature for 1 h. Then 20 mL of chilled acetone was added and the suspension filtered through a Buchner funnel. The immobilized enzyme was washed on the filter paper with another 20 mL of chilled acetone and dried in a vacuum desiccator for 4 h.

The amount of lipase immobilized was estimated from an analysis of lipase concentration in the aqueous phase with a UV-Visible Spectrophotometer (Shimadzu, model 6A) before and after immobilization, calculated from the equation

$$W = \frac{(C_i - C_f)V}{m_i} \quad (1)$$

where C_i and C_f are the initial and final concentration of lipase in mg L^{-1}, V is the volume of lipase solution (L) and m_i is the weight of adsorbent or immobilized media (g) taken initially. The enzyme loading was found to be 11.2 mg g^{-1} of solid support.

Preparation of standard lauryl laurate

The standard lauryl laurate was prepared by the thionyl chloride method. Thionyl chloride was added dropwise into a 500 mL round bottom flask containing 10 mmol of lauric acid and the reaction mixture stirred for 3 h. After removal of unreacted thionyl chloride, dichloromethane was added as a solvent, then alcoholysis of the acyl chloride with lauryl alcohol was carried out for 2 h. The mixture was refluxed to complete the reaction. the ester

Table I. Properties of the solvents used in this study[a].

Sl. No.	Solvent	log P	Log S_w	E_T^N	DN^N	Dielectric constant	Polarisability (in units of 10^{-24})
1	Ethyl acetate	0.70	0.21	–	–	6.00	9.70
2	Chloroform	2.00	−1.12	0.260	0.10	4.81	9.50
3	Toluene	2.50	−1.80	0.096	0	2.38	11.80
4	Carbon Tetrachloride	3.00	−1.93	0.090	0	2.24	11.20
5	Cyclohexane	3.20	−2.25	–	–	2.00	11.00
6	Hexane	3.50	−2.39	0.074	0	1.88	11.90

[a]log *P* is the logarithm of the partition coefficient in the Octanol-water system. Log S_w is the logarithm of the saturated solubility of water in the solvent on molar basis, E_T^N is the normalized Gutmann donor number. All the values of solvent properties were taken from Hazarika et al. (2002).

formed in the reaction mixture was separated by silica gel column chromatography (60–120 mesh) and the purified product concentrated in a vacuum rotatory evaporator (Buchi R-114, Switzerland). The purity and quantity of the ester was determined by GC (Varian 3700) and the structure verified by UV (Shimadzu, Japan), 60 MHz and 300 MHz NMR spectroscopy (Bruker, Germany). Mass spectra of the compound were recorded on a Bruker Daltonics LCMS (Germany).

Results and discussion

IR characterization of CLEC

The IR spectra for glutaraldehyde, crude PPL and CLEC-PPL were recorded. Before cross-linking, an IR absorption band corresponding to the aldehyde group of glutaraldehyde was observed at 1713.5 cm^{-1} and that of amino groups in the protein at 3388.3 cm^{-1}. After cross-linking, the IR stretching band for the aldehyde group disappeared and a band for the imine was observed at 1652.0 cm^{-1} which indicated that the lipase had been cross-linked with glutaraldehyde.

Effect of solvent on initial rate

The solvents used in this study were ethyl acetate, chloroform, toluene, carbon tetrachloride, cyclohexane and hexane. They were selected on the basis of their range of hydrophobicity (log P) values (Table I), which lie between 0.7 and 3.5, as the activity and stability of enzymes has been reported to be optimal in this range (Hazarika et al. 2002). The log P value is a quantitative measure of solvent polarity (Gobicza 1992) and the enzyme activity for lipase catalyzed reactions in general, increases with the hydrophobicity of the solvent. The relation of initial reaction rate with log P of the solvent is shown in Figure 1, from which it is apparent that the enzyme activity increases almost linearly with an increase in log P and that the CLEC-PPL can catalyze esterification in a wide variety of solvents. The correlation between initial rate and log P can be represented by the equation below

$$r = 0.0294(\log P) + 0.0067 \quad (2)$$

with a correlation co-efficient of 0.99. The correlation of CLEC-PPL activity with hydrophobicity of the solvent has been observed for esterification reactions catalyzed by free lipase (Hazarika et al. 2002; Klibanov & Zaks 1985). This correlation of the CLEC-PPL activity with log P reflects the extent to which solvents can enter the relatively polar phase around the enzyme and hence contact it.

The water solubility of the solvent has been recognized as the most useful index of solvent polarity for correlating the rates of esterification. Figure 2 shows the correlation of the solubility of water (molar basis) in the solvent (log S_W) with the initial rate of reaction. The relationship can be represented as

$$r = -0.0307(\log S_W) + 0.0321 \quad (3)$$

with a correlation co-efficient of 0.98. From the figure, it is evident that solvents with low water solubility favour the esterification reaction catalyzed by CLEC-PPL, similar to previous observations with lipase in dispersed systems (Valivety et al. 1991; Hazarika et al. 2002).

Nonpolar solvents would also be chosen based on the well accepted rules for the effects on biocatalytic

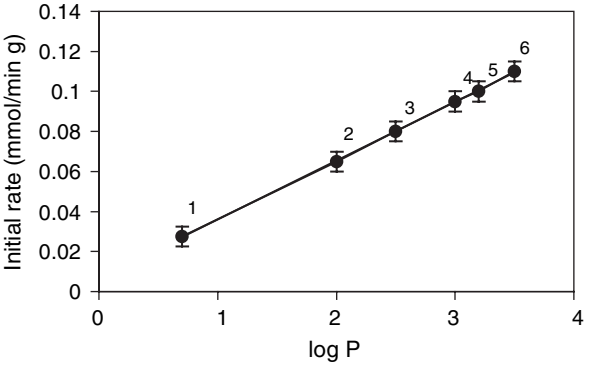

Figure 1. Initial rate as a function of solvent hydrophobicity. The reaction mixture consist of [Lauric acid] = 200 mM; [Lauryl alcohol] = 350 mM; lipase = 0.03 g mL^{-1}.

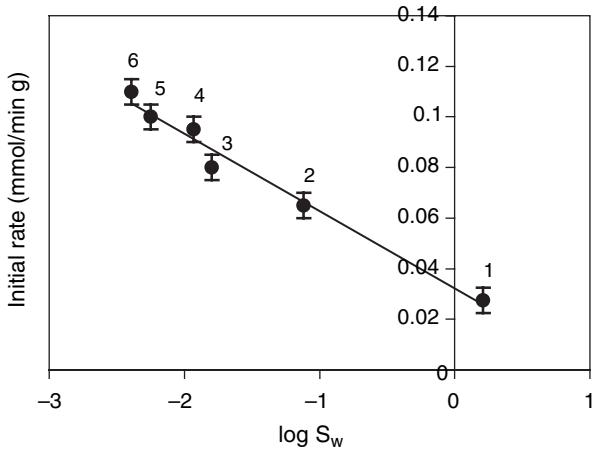

Figure 2. Initial rate as a function of water solubility of the solvent. The reaction mixture was the same as for Figure 1.

activity (Van Tol et al. 1995). For predicting performance of the reaction media using polarity as the criterion, the donor–acceptor interactions of the solvent including hydrogen bonding capability are also important. 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 normalized electron pair acceptance index (E_T^N) and Gutmann's donor number (DN^N) shown in Figure 3. The correlation for the present system seems to be rather weak in comparison to that reported by Valivety et al. (1991), who established a good correlation between water solubility and ($E_T^N + DN^N$) of several organic solvents for which these values are available and concluded that the contribution of electron pair acceptance and donation are roughly equal.

The hydrogen bond donating and acceptance capacity of the solvent determines both water solubility and esterification reaction equilibrium. The weak correlation shown in Figure 3 may be attributable to the lack of data points for two solvents whose ($E_T^N + DN^N$) values are not known; the values of this parameter for many solvents are not known and are considered uncertain in many cases. However, the observed trend of decrease in initial rate with ($E_T^N + DN^N$) is reasonable and may reflect the solvation 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 would be expected to affect the equilibrium position and involves additional acceptor and donor interactions. Solvents capable of imparting either or both of these interactions would favour hydrolysis over esterification. Therefore, the present finding on the effect of ($E_T^N + DN^N$) appears to be reasonable.

Solvent polarisability is another important property which represents the ability of a solvent to stabilize the charge of a dipole in solution by virtue of its dielectric constant. The values of solvent polarisability are known for all of the solvents used in this study, but an attempt to correlate the reaction rate with solvent polarisability revealed that the correlation was poor. However, the parameter log P divided by polarisability did correlate with initial rate (Figure 4), giving the relationship

$$r = 0.0035(\log P/\text{Polarisability}) + 0.0001 \qquad (4)$$

with a correlation co-efficient of 0.96. A similar correlation has been observed for other esterification reactions catalyzed by lipase (Valivety et al. 1991).

Since dielectric constant is a function of polarisability, we have attempted to correlate the initial rate with dielectric constant (Figure 5). It is apparent that the initial rate decreases with increasing dielectric constant of solvents, similar to the esterifica-

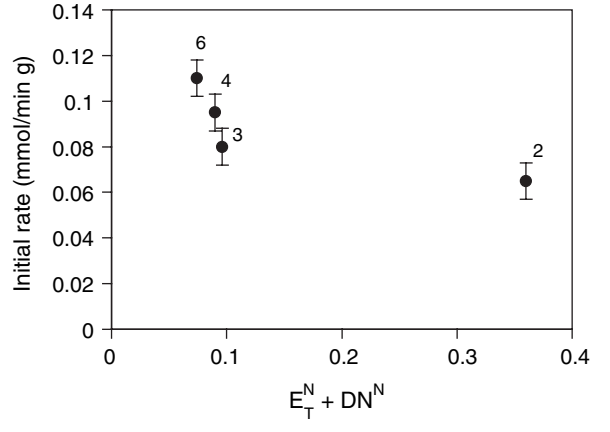

Figure 3. Initial rate as a function of ($E_T^N + DN^N$). The reaction mixture was the same as for Figure 1.

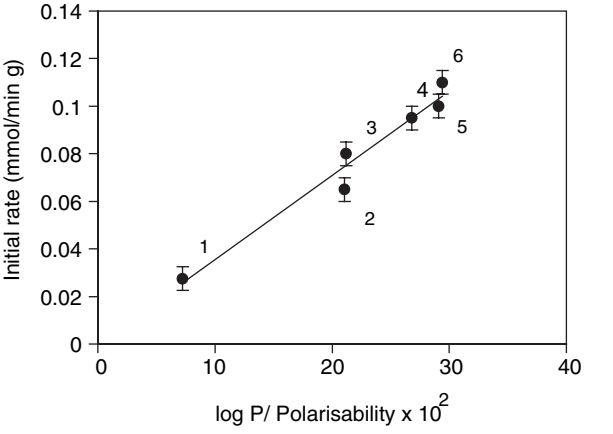

Figure 4. Initial rate as a function of polarisability. The reaction mixture was the same as for Figure 1.

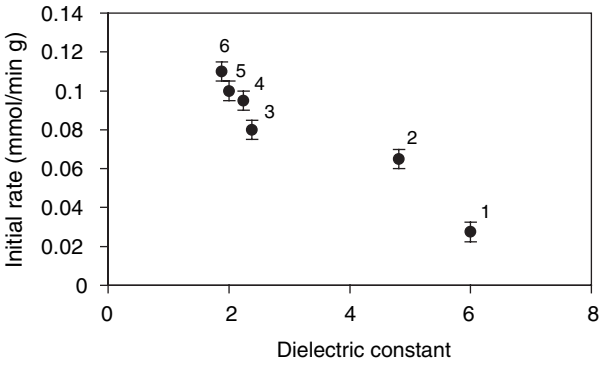

Figure 5. Initial rate as a function of dielectric constant. The reaction mixture was the same as for Figure 1.

tion of oleic acid and ethanol catalyzed by PPL (Hazarika et al. 2002), although the trend does not represent a statistically sound correlation.

Effect of enzyme concentration on initial rate

The kinetic study was done for the esterification reaction using equimolar concentrations of lauric acid and lauryl alcohol (200 mM each) varying the CLEC concentration from 10 mg mL^{-1} to 30 mg mL^{-1} at 30°C (Figure 6). An increase of CLEC concentration at constant substrate concentrations increased the initial velocity almost linearly in the low range of CLEC concentration and then the rate increases more slowly, reaching an asymptote at a CLEC concentration of 30 mg mL^{-1}. This implies that the reaction is kinetically controlled at low CLEC concentrations in agreement with the results of Hazarika et al. (2002) for esterification of oleic acid with ethanol catalyzed by free PPL.

Effect of substrate concentration on initial rate

The variation in initial rate of esterification as a function of lauryl alcohol concentration for various

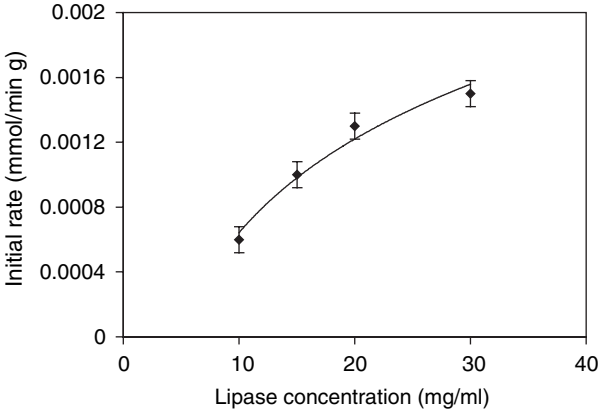

Figure 6. Initial rate as a function of lipase concentration. The reaction mixture consist of [Lauric acid] = 200 mM; [Lauryl alcohol] = 200 mM.

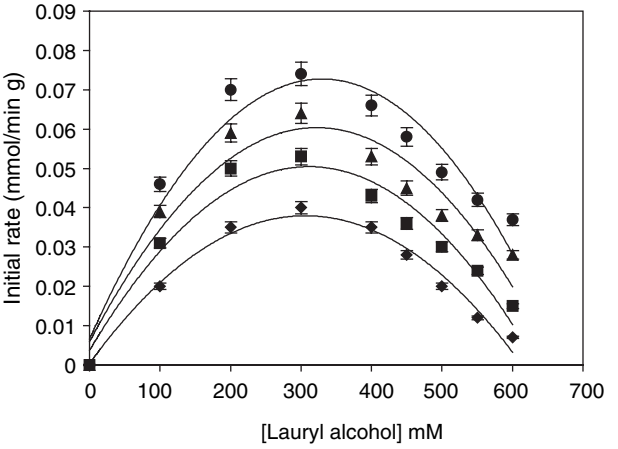

Figure 7. Initial rate as a function of lauryl alcohol concentration at various concentrations of lauric acid. [Lauric acid] (–◆–): 200 mM; (–■–): 300 mM; (–▲–): 400 mM; (–●–): 600 mM.

concentrations of lauric acid is shown in Figure 7. The initial velocity increased proportionally to a maximum, above which it decreased for all lauric acid concentrations tested.

Effect of temperature on initial rate

Kinetic analysis of the esterification reaction was studied over the temperature range 30°C to 50°C. The initial rate increased almost exponentially with reaction temperature as shown in Figure 8. A similar

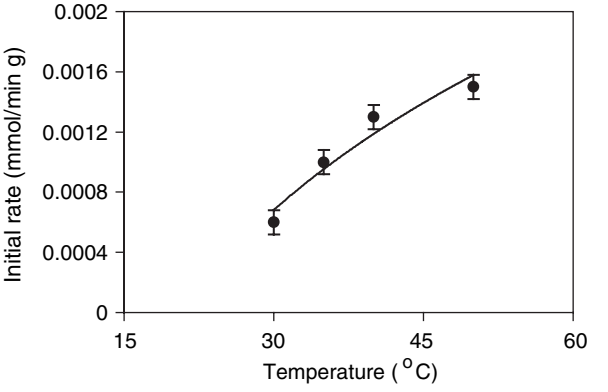

Figure 8. Initial rate as a function of temperature. The reaction mixture consist of [Lauric acid] = 200 mM, [Lauryl alcohol] = 350 mM, lipase = 0.03 g mL^{-1}.

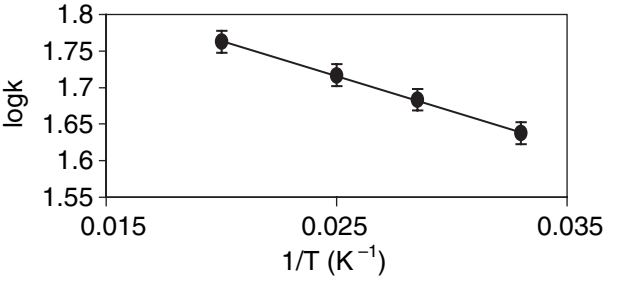

Figure 9. Activation energy curve based on data from Figure 8.

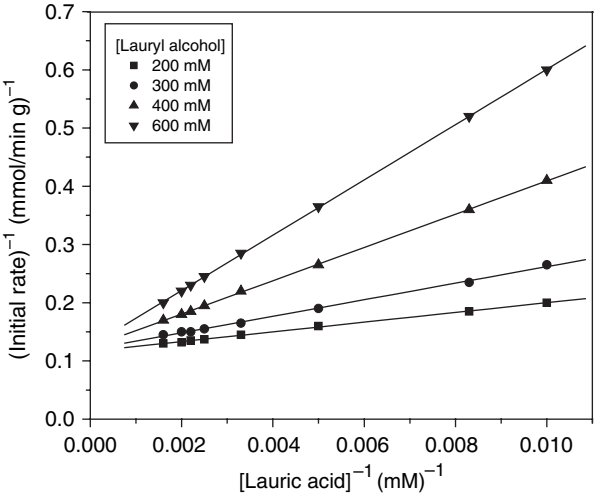

Figure 10. Reciprocal plot of lauric acid concentration and initial rate at various concentrations of lauryl alcohol.

observation was made by Harikrishna et al (2000) for *Rhizomucor miehei* lipase catalyzed synthesis of isoamyl butyrate.

The effect of temperature on the reaction rate constant (k) for the esterification of lauric acid with lauryl alcohol was studied under the same reaction conditions. The reaction rate constant (k) was found from the Arrhenius rate equation (Figure 9)

$$K = A\, e^{-Ea/RT} \tag{5}$$

The estimated value of the activation energy (E_a) was 183.81 J mol^{-1}. This value is lower than that obtained by Romero et al (2005) for immobilized *Candida antarctica* lipase catalyzed synthesis of isoamyl acetate which suggests a higher reactivity of CLEC together with higher stability.

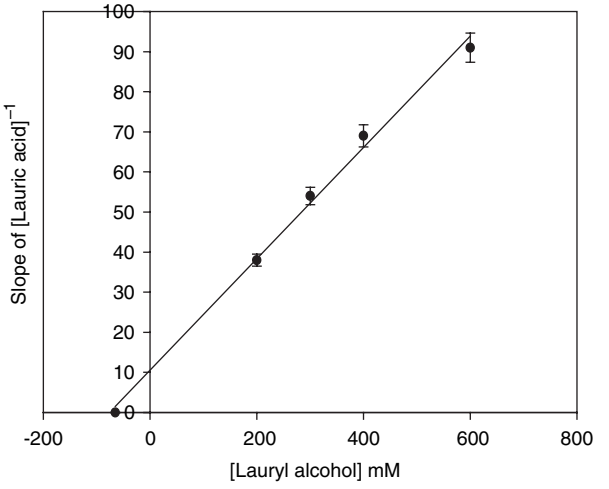

Figure 11. Slope of [Lauric acid]$^{-1}$ as a function of Lauryl alcohol concentration.

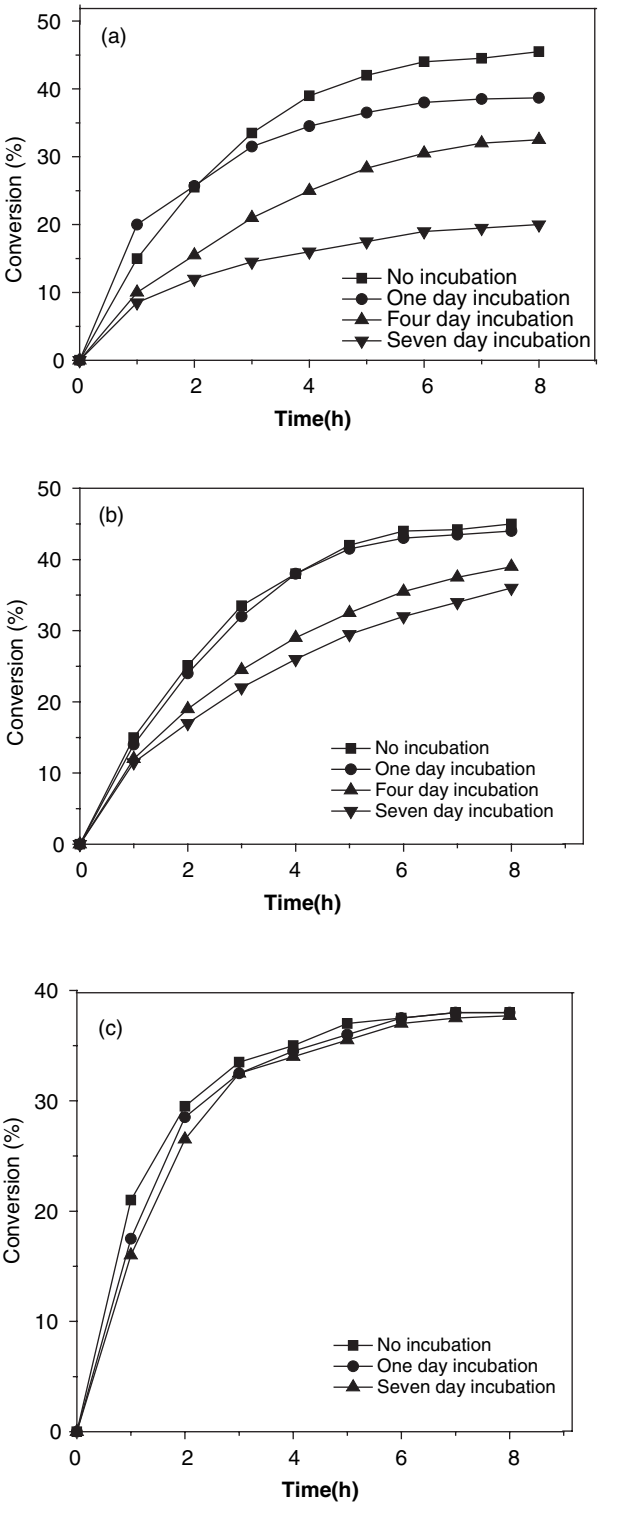

Figure 12. Stability of various forms of porcine pancreatic lipase in hexane. Conversion vs time profiles for various times of pre-incubation in hexane are plotted. The reaction mixture consisted of [Lauric acid] = 50 mM; [Lauryl alcohol] = 150 mM; solvent = 10 mL; lipase = 0.03 g mL^{-1}; temperature = 40°C.
(a) Crude enzyme. (b) Immobilised enzyme. (c) CLECs.

Table II. Kinetic parameters for lipase catalysed reactions.

Reaction product	Lipase	Solvent	V_{max} (mmol min^{-1} g^{-1})	$K_{m(acid)}$ mM	$K_{m(alc)}$ mM	K_i mM	Reference
Lauryl laurate	CLEC(PPL)	n-hexane	1.42	431	101	75	This work
Ethyloleate	*Porcine pancreas*	n-hexane	4.0	66	103	20	Hazarika et al. (2002)
Ethyloleate	*Mucor miehei*[a]	n-hexane	23.0	450	600	60	Marty et al. (1992)
Ethyloleate	*Mucor miehei*[a]	$SCCO_2$	14.0	170	1600	65	Marty et al. (1992)
Ethyloleate	*Mucor miehei*[a]	n-hexane	5.70	120	190	40	Chulalaksananukul et al. (1992)
Ethyl myristate	*Mucor miehei*[a]	n-hexane	5.33	–	–	43	Dumont et al. (1992)
Ethyl myristate	*Mucor miehei*[a]	$SCCO_2$	8.32	–	–	120	Dumont et al. (1992)
Ethyl myristate	*Rhizomucor miehei*[a]	n-hexane	11.72	3.03	3.06	6550	Harikrishna et al. (2001)

[a] immobilized.

Reaction mechanism and kinetics

For detailed kinetic study, n-hexane was used as the solvent as it exhibited low toxicity and gave adequate reactivity. The reaction mechanism was examined using a reciprocal plot of initial rate and substrate (lauric acid) concentration at fixed lauryl alcohol concentrations (Figure 10). It is apparent that an increase of lauric acid concentration at a constant lauryl alcohol concentration increases the initial rate. The decrease of initial rate with increase in lauryl alcohol concentration reflects lauryl alcohol inhibition. A similar effect has been observed for the synthesis of ethyl oleate catalyzed by *Mucor miehei* (Marty et al. 1992; Marty et al. 1997) and PPL (Hazarika et al. 2002) and for the synthesis of ethyl myristate (Dumont et al. 1992) using immobilized lipase in n-hexane as well as in supercritical CO_2. In Figure 10 the plots are found to be almost parallel at low lauryl alcohol concentration, whereas at high lauryl alcohol concentration, the slopes of the lines increase and the intercepts tend to reach a limiting value which is equivalent to $1/V_{max}$ indicating a behavior typical of a Ping-Pong–Bi-Bi mechanism with alcohol inhibition.

For this mechanism the initial velocity equation is represented as

$$\frac{V}{V_{max}} = \frac{[Acid][Alcohol]}{K_{m(acid)}[Alcohol](1 + [Acid]/K_i) + K_{m(alcohol)}[Acid] + [Alcohol][Acid]} \quad (6)$$

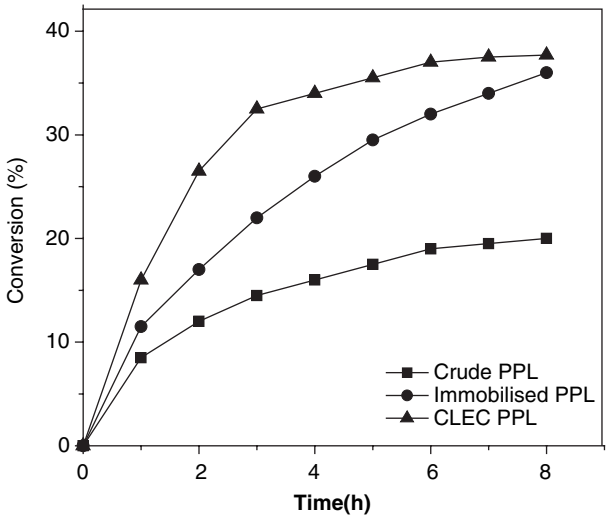

Figure 13. Conversion vs time profile for crude, immobilized and CLEC porcine pancreas lipase after 7 days incubation.

where [Acid] and [Alcohol] represents the initial molar concentrations of lauric acid and lauryl alcohol respectively. $K_{m(acid)}$ and $K_{m(alcohol)}$ are the respective affinity constants, K_i is the inhibition constant for lauryl alcohol and V_{max} is the maximum reaction rate. The kinetic parameters, V_{max} and $K_{m(alcohol)}$ were estimated from the data presented in Figure 10, and the values of $K_{m(acid)}$ and K_i were obtained from Figure 11. For better accuracy, the values of $K_{m(alcohol)}$, $K_{m(acid)}$, K_i and V_{max} were computed from the equation for reaction velocity by numerical parameter identification using a Gauss–Newton algorithm of error minimization with 5% mean deviation and their values are given in Table II, together with the literature values for immobilized and crude lipase. The results of the present study reveal a lower value of V_{max} in comparison to those obtained for other lipase catalyzed reactions. However, the values of $K_{m(acid)}$, $K_{m(alcohol)}$ and K_i

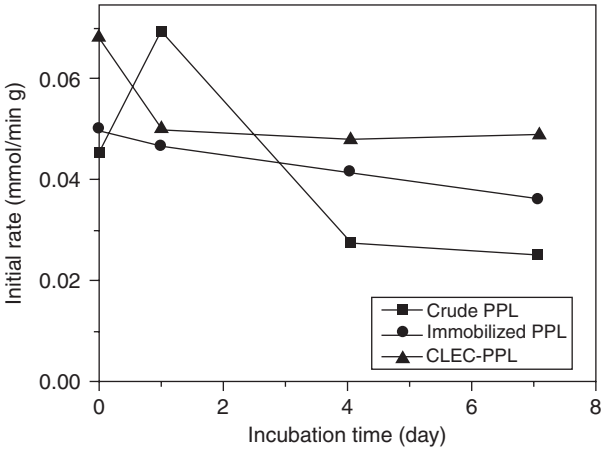

Figure 14. Initial rate as a function of incubation time.

are comparable to other systems except those obtained for isoamyl butyrate, probably due to the branched nature of the substrate (Harikrishna et al. 2001).

Stability of the enzyme

The batch stability test profiles are plotted in Figures 12 and 13. It is apparent that CLEC exhibits higher stability than immobilized, which is more stable than crude lipase. This may be attributed to glutaraldehyde cross-linking. The relatively low stability of immobilized lipase may be due to the weak interactions arising through physical adsorption. The higher reactivity of the CLEC may also reflect the higher stability, as suggested elsewhere (Jeong et al. 2000). This suggests that CLEC is the best option for practical application.

Figure 14 shows the initial rate versus incubation time for the three enzyme preparations. Although the activity of the dispersed system is initially high, it is inactivated more rapidly in the agitated reaction system than the immobilized and CLEC lipases.

Conclusion

The effect of solvents and kinetic parameters have been investigated for esterification of lauric acid with lauryl alcohol catalyzed by CLEC-PPL. The reaction rate using CLEC was found to correlate well with solvent properties such as hydrophobicity, water solubility and polarisability. The kinetics of reaction were consistent with a Ping-Pong–Bi-Bi model and the model parameters were estimated by regression analysis using the Gauss–Newton algorithm of error minimization. Lipase stability was also compared using dispersed, immobilized and CLEC system with the later exhibiting the highest stability and activity.

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