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Study on Kinetics and mechanism of reaction between 2-phenethyl alcohol and vinyl acetate catalyzed by cross-linked *Pseudomonas Cepacia* Lipase

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Abstract

The use of flavoring compounds in the food industry is now increasing day by day. The aim of the present study was to crystallization and cross-linking of lipase from Pseudomonas Cepacia and to study the kinetics of the synthesis of a rose flavor ester, 2-phenethyl acetate in an organic solvent system. The effect of different reaction co-ordinates such as enzyme concentration, temperature, substrate concentration were studied and found the maximum conversion in 50mg/ml enzyme concentration, temperature at 55^{0}C and substrate ratio of 1:4. The stability of the cross-linked lipase was observed in comparison to free lipase and retained 50% of original activity by cross linked lipase up to seven days of incubation.

Keywords: Lipase, 2-phenethyl acetate, transesterification.

Introduction

Lipases are one type of enzymes and known as triacylglycerol ester hydrolases (EC 3.1.1.3). There are various types of lipases obtained from bacterial, fungal, plant and animal sources. Generally lipases are used at commercial scale for ester production from esterification and transesterification reactions because of their higher stability and substrate specificity[1-3]. Lipases are widely used as enzymes in food industry, pharmaceutical sciences, chemical and detergent industries due to the reason that they can catalyze various types of reactions. In esterification and transesterification reactions, lipases are highly specific and stereoselective[4].

Non-aqueous enzymatic catalysis has vast applications in flavor, perfumery, pharmaceuticals and drug industries. Selection of solvent is one of the important factors for enzymatic reactions in non-aqueous medium. In such reactions, organic solvents facilitates the solubility of substrates. The organic solvents also makes easier the recovery of enzyme and product. Lipases can act at the interface between aqueous and non-aqueous phase[5]. In organic solvents, lipase can catalyze transesterification reactions and highly tolerable to different organic solvents. They can be used in a wide range of temperature and pH[6].

On the other hand, cross-linked enzyme crystals (CLECs) have the characteristic property that they are better to both crude and conventionally immobilized enzyme[7-9]. CLECs were prepared by crystallization of enzymes and then cross-linking of the micro-crystals with glutaraldehyde. After cross-linking, CLECs are not soluble even in aqueous buffer and organic solvents. So, they can be used in a various reaction media, can be recovered and reused. CLECs also retain their activity after exposure to high temperature[10].

The lipases from various sources are widely used enzymes, for the production of a number of important esters[7,11-16]. It is becoming important industrially due to stereoselectivity and substrate specificity in non-aqueous solvents[17,18]. 2-phenethyl acetate is a natural rose-like odoured volatile ester which is used as flavoring agents to cosmetics, soaps, foods and drinks[19,20]. The compound can be advantageously synthesized through catalytic transesterification reaction between 2-phenethyl alcohol and vinyl acetate using CLEC *Pseudomonas Cepacia* lipase. In this paper, a systematic study of kinetics of the transesterification reaction using CLEC-PCL has been reported.

Materials and methods

Lipase from *Pseudomonas cepacia* (specific activity of 40 U/mg) was obtained from FLUKA Chemicals Co. Switzerland. Vinyl acetate was obtained from MERCK-Schuchardt, Germany. 2-phenethyl alcohol was purchased from FLUKA Chemicals Co. Switzerland. All the solvents used in this study were purchased from CDH Pvt. Ltd., Mumbai, India.

Analytical methods: The substrates and the reaction product (2-phenethyl acetate) were analysed by Gas Chromatographic method using a Chemito model 1000 Gas Chromatography instrument coupled with a packed GC column, 10% OV-101 on chromosorb WHP (100/120), 2mx6.35mm od glass. The GC column oven temperature was maintained at 75^{0}C for 1 minute then programmed at the rate of 8^{0}C per minute up to 210^{0}C with a final hold time of 5 minutes. Quantitative evaluation was done by area normalization method using a GC data processor. Components in the reaction mixture were identified by comparing their retention times with the standard compounds.

The solvents as well as the substrates were distilled prior to use. The reaction mixture consisted of 2-phenethyl alcohol and vinyl acetate (50 mmol each) containing 30ml of anhydrous solvent. The amount of CLEC-PCL was 5mg/ml. The reaction was carried out in a 250ml round bottom flask with constant shaking at a speed of 150rpm and reaction temperature of 27^0C. Aliquots of the reaction mixture were collected at uniform time interval and the samples were analysed by Gas Chromatography. The initial reaction rates were calculated from the Gas Chromatogram[7].

The kinetic measurements were carried out in a standard reactor with vigorous stirring with a magnetic stirrer in a 100ml round bottom flask. The substrates and the lipase concentrations were varied systematically. The solvent used for the kinetic study was cyclohexane. The samples were collected from the reaction mixture at an uniform time interval of 30 minutes and analysed by GC[11].

The CLEC-PCL stability was determined in relation to that of crude lipase by calculating the activity of the enzyme as a function of incubation time, after storage in cyclohexane for several days[7,21]. The enzyme preparations (50mg each) were suspended in 4ml of cyclohexane and reaction mixture consisting of 50 mmol 2-phenethyl alcohol and 200 mmol vinyl acetate and incubated at 45^0C with constant stirring. The product concentrations were determined as discussed previously.

Preparation of cross-linked *Pseudomonas cepacia* lipase crystals: The cross-linked lipase crystals of PCL were prepared and characterized by the same method as mentioned in the previous communication[7].

Results and discussion

Characterization of CLEC: The CLEC prepared as per the method above was characterized for structural feature by FTIR Spectroscopy (Perkin Elmer, System 2000) which is discussed in our previous communication[7].

Effect of Reaction Parameters: Effect of CLEC-PCL Concentration: The variation of CLEC-PCL concentration was studied for the transesterification reaction. Figure-1 shows the typical conversion versus time profile at various substrate concentration ratios and CLEC-PCL concentrations. The substrate molar ratios were varied by changing the concentration of one substrate relative to the other at constant CLEC-PCL concentration. The conversion was calculated based on vinyl acetate as the substrate. It is seen from the figure that the 2-phenethyl alcohol to vinyl acetate molar ratio of 1:1 gives highest conversion and reaction rate at all the CLEC-PCL concentrations. The lowest

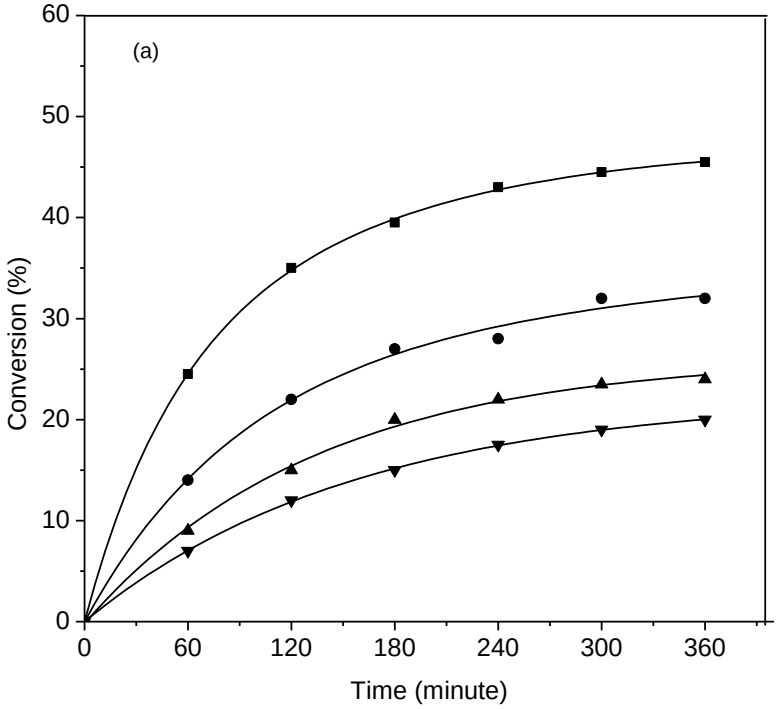

Figure-1: Effect of CLEC-PCL concentration on time and conversion at various substrate concentrations [CLEC-PCL] = 0.02g/ml Symbol: [2-Phenethyl alcohol] : [Vinyl acetate], ▪ 1 : 1, ● 2 : 1, ▲ 3 : 1, ▼ 4 : 1.

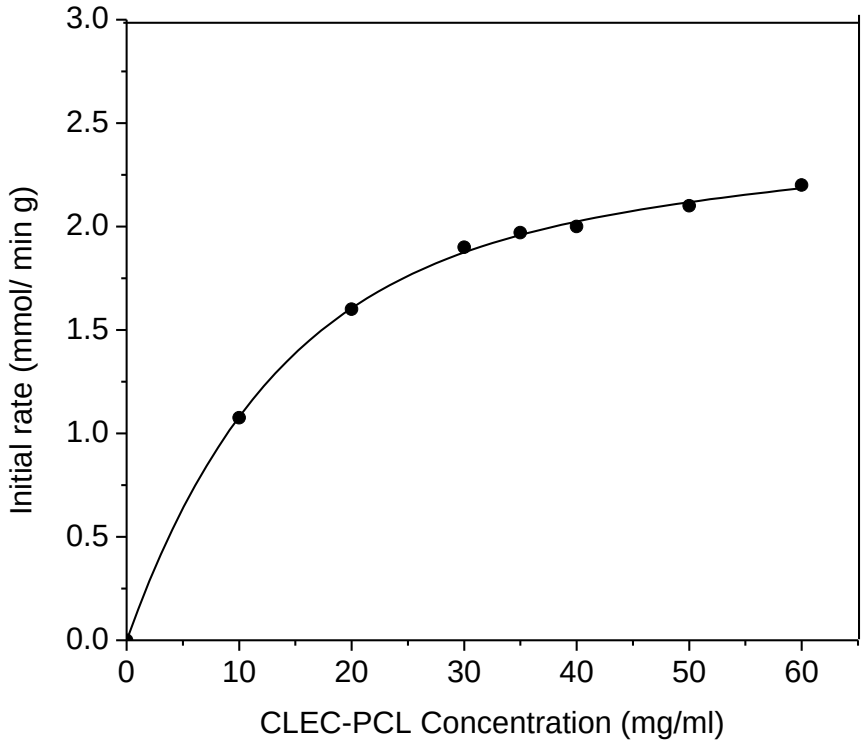

Figure-2: Effect of CLEC-PCL concentration on initial rate of transesterification reaction. [2-Phenethyl alcohol] = [Vinyl acetate] = 50 mmol Temperature = 27^0C, Solvent = Cyclohexane.

Reaction rate and conversion was obtained at a 2-phenethyl alcohol and vinyl acetate molar ratio of 4:1. It is also apparent from the figure that 2-phenethyl alcohol exercises an inhibitory effect on the enzyme.

The effect of lipase concentration on initial rate at an equimolar concentration of 2-phenethyl alcohol and vinyl acetate is shown in Figure-2. It is seen from the figure that with an increase of lipase concentration initial rate also increases almost linearly up to a lipase concentration of 10mg/ml and then exponentially at a lipase concentration of 50mg/ml, which is similar to a kinetically controlled enzymatic reaction at low lipase concentration. Above a lipase concentration of 50mg/ml, a distinct zone of lipase concentration effect was observed which is also considered typical of kinetically controlled enzymatic reaction[15]. This observation is identical to that reported for transesterification of (R,S)-1-phenylethanol with vinylacetate catalysed by immobilized Rhizopus Oryzae as Cross linked enzyme aggregrate[22] and *Candida antarctica* lipase in ionic liquid [bmim][BF_4][23].

Effect of Temperature: Temperature has important role on the activity and stability of lipase. Figure-3 shows the effect of temperature on conversion of the substrate at four different temperatures. It is seen from the figure that with increasing reaction temperature from 25^0C–55^0C, the conversion (%) increases. The initial rate of reaction at 55^0C was about 5 times faster than that at 25^0C, as evident from the Figure-4 which may be considered reasonable in as much as the temperature effect may be predicted by Arrhenius rate equation. This trend of increasing reaction rate with temperature indicates good thermo-stability of CLEC lipase. This result is similar to that observed by Heijden et al[24] and Pirozzi and Guido for *Candida antarctica* lipase catalysed transesterification reactions[25].

Effect of Substrate Concentration: Figure-5 and Figure-6 show the effect of substrate concentration on the time versus percentage conversion (%). It is seen from the Figure-5 that at a constant concentration of 2-phenethyl alcohol, with increase of vinyl acetate concentration, the percent conversion increases. However, at constant vinyl acetate concentration, an increase in the concentration of 2-phenethyl alcohol, decreases the percent conversion as evident from the Figure-6.

Figure-7 shows a systematic variation of initial rate at 55^0C with vinyl acetate concentration at a fixed CLEC concentration and 2-phenethyl alcohol concentration of 50 mmol, 75 mmol and 100 mmol. With an increase of concentration of vinyl acetate, the rate of the reaction increases at a fixed alcohol concentration. At an alcohol concentration of 100 mmol, highest conversion and initial rate was obtained. A systematic variation

of the alcohol concentration with initial rate was also studied at different vinyl acetate concentrations, as shown in Figure-8. It is seen from the figure that increase of alcohol concentration at a fixed vinyl acetate concentration, increases the reaction rate up to a critical alcohol concentration of 100 mmol. However, above this concentration the rates tend to decrease for all the vinyl acetate concentrations tested. Hence, 2-phenethyl alcohol may be considered as an inhibitor for the transesterification reaction. This type of inhibition was also reported for *Candida antarctica* lipase catalysed acyl transfer reaction[26] and an esterification reaction catalysed by free *Porcine Pancreas* lipase[13] and CLEC form of *Porcine Pancreas* lipase[7]. Identical inhibition was also observed for immobilized lipase catalyzed esterification reaction[11].

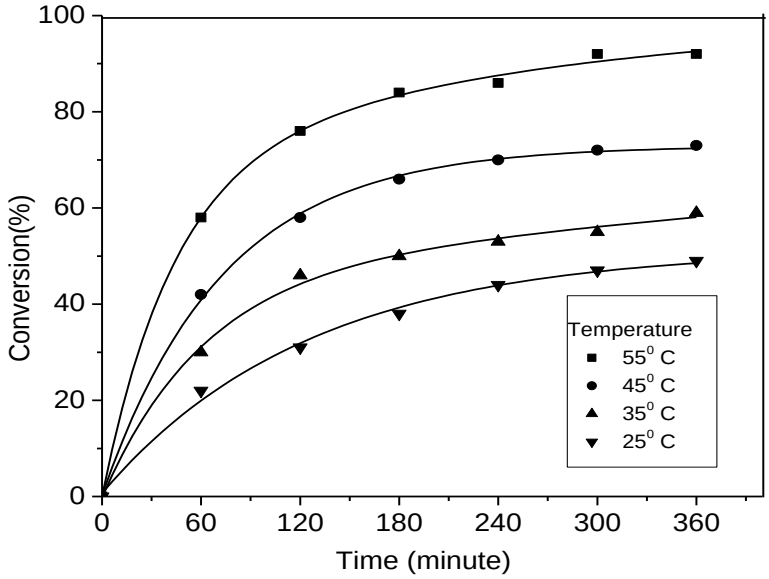

Figure-3: Temperature effect on time versus conversion profile of transesterification reaction catalysed by CLEC-PCL. [2-Phenethyl alcohol] = [Vinyl acetate] = 50 mmol [CLEC-PCL] = 50 mg/ml, Solvent = Cyclohexane.

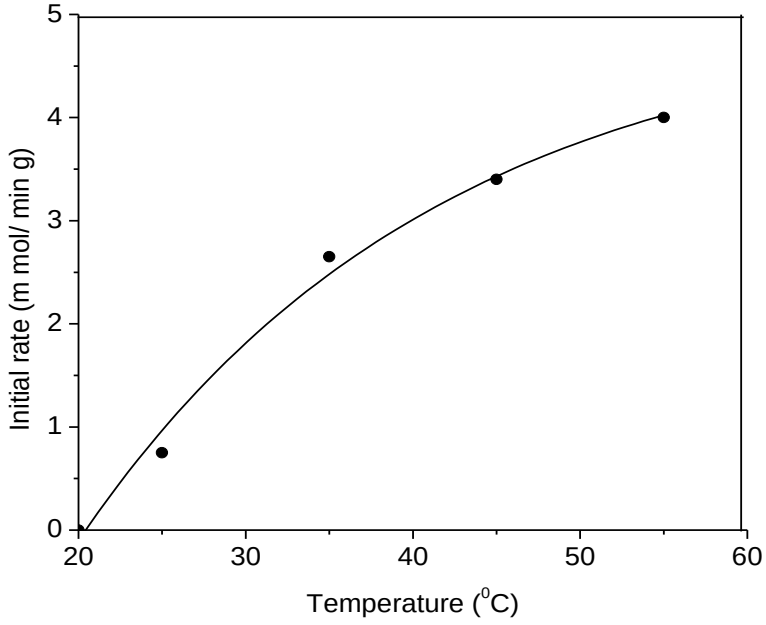

Figure-4: Temperature effect on initial rate of transesterification reaction catalysed by CLEC-PCL. Reaction condition: Same as in Figure-3.

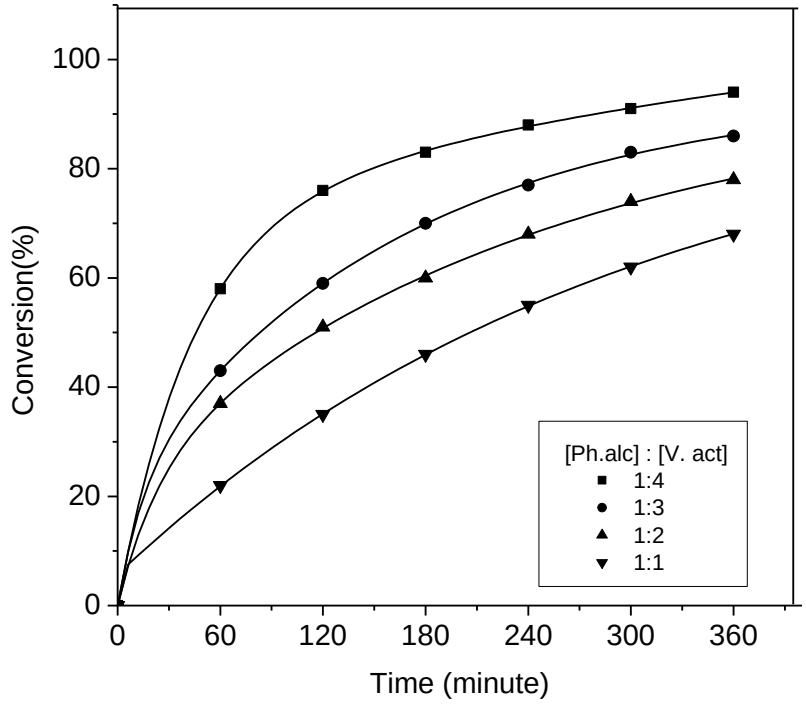

Figure-5: Substrate concentration effect on time versus conversion profile at fixed 2-phenethyl alcohol concentration. [CLEC-PCL] = 50 mg/ml, Temperature = 55^0C.

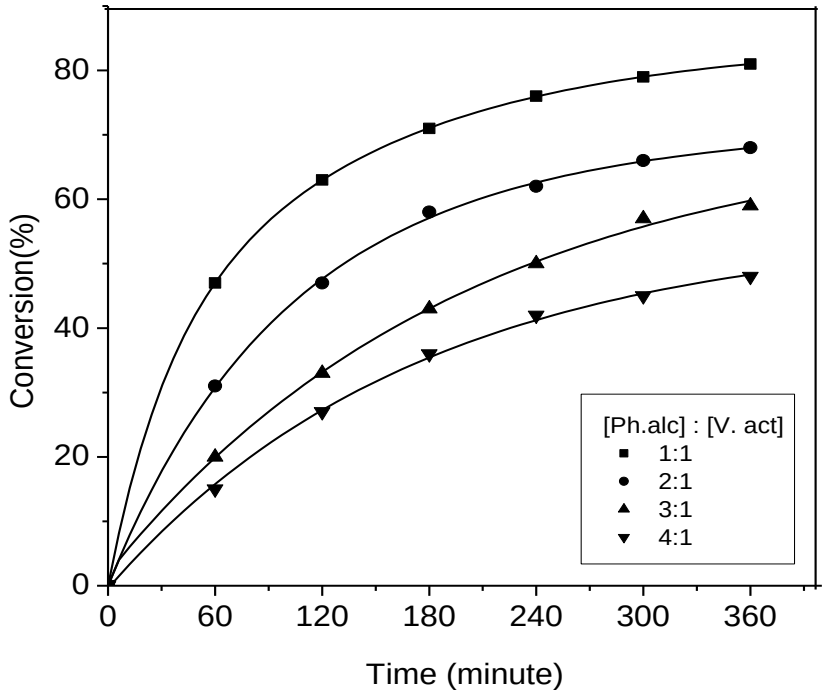

Figure-6: Substrate concentration effect on time versus conversion profile at fixed vinyl acetate concentration. Reaction condition: Same as in Figure-5.

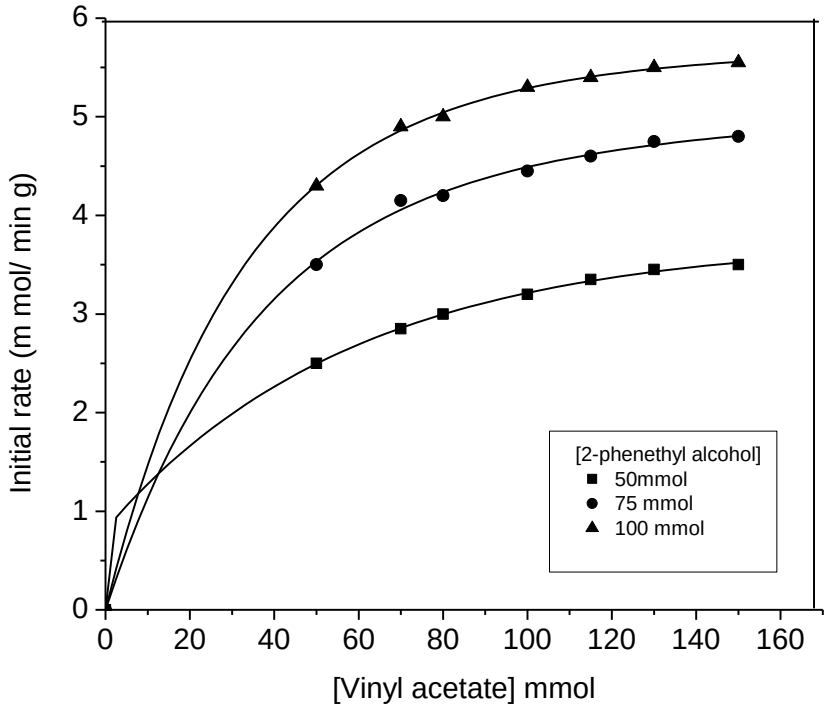

Figure-7: Vinyl acetate concentration effect on initial rate of transesterification reaction at constant 2-phenethyl alcohol concentration. Reaction condition: Same as in Figure-5.

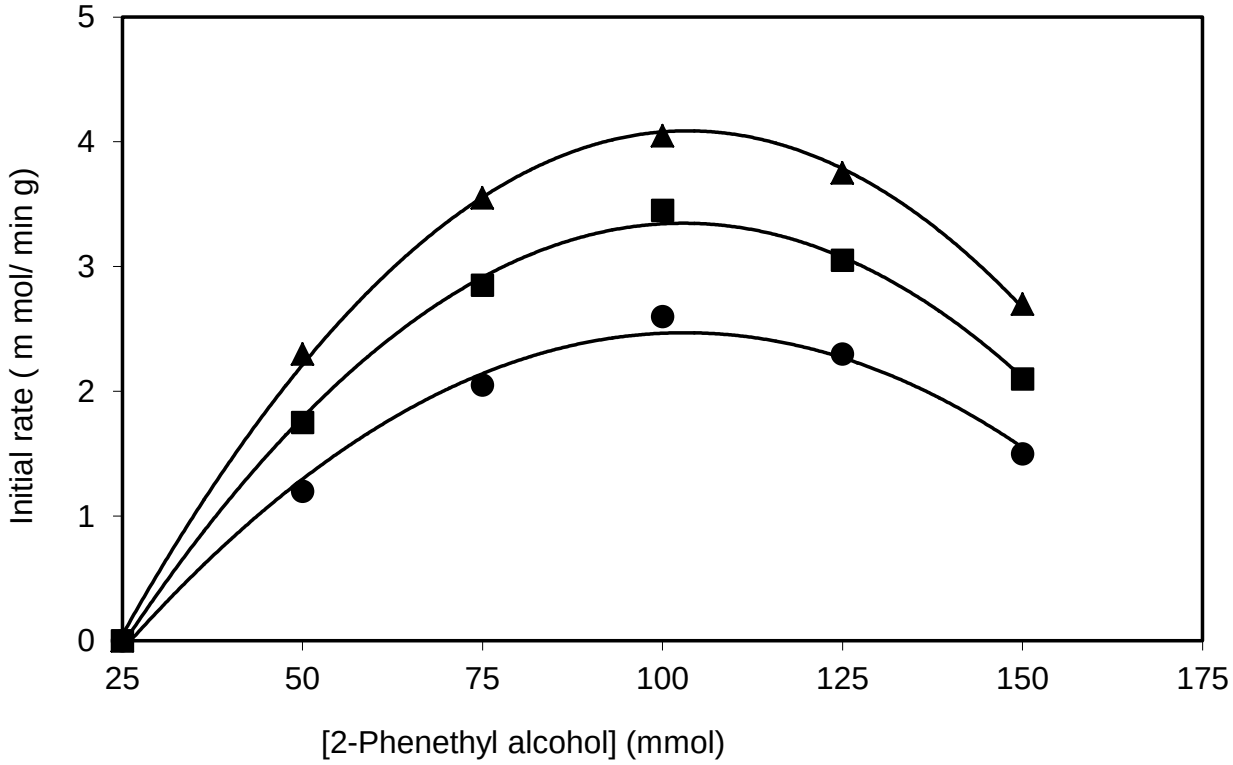

Figure-8: Effect of 2-phenethyl alcohol concentration on initial rate of transesterification reaction at constant vinyl acetate concentration. [Vinyl acetate] ●: 50 mmol, ■: 75 mmol and ▲: 100 mmol.

Validation of Kinetic Models: The mechanism of the reaction was examined by both the inverse plot of initial rate and concentration of vinyl acetate at different constant 2-phenethyl alcohol concentrations. The inverse plot of initial rate against to that of vinyl acetate concentration at different 2-phenethyl alcohol concentrations gives a set of straight lines as shown in Figure-9. With the increase of concentration of 2-phenethyl alcohol, the slopes of the lines increased and the intercepts tend to reach a limiting value of $1/V_{max}$. This behaviour indicates the Ping-Pong Bi-Bi mechanism with 2-phenethyl alcohol inhibition effect. Yadav and Trivedi reported similar inhibition by n-octanol and geraniol in the kinetics study of the transesterification with vinyl acetate catalysed by immobilized lipase[27]. Martinelle and Hult observed inhibition by (R)-2-octanol in the acyl transfer reaction of ethyl octanoate using *Candida antarctica* lipase B which also follow a Ping-Pong Bi-Bi mechanism[26]. The lipase catalysed esterification of levulinic acid with n-butanol follows Ping-Pong Bi-Bi mechanism with n-butanol inhibition effect[28].

Similar inhibition with this mechanism was also observed for transesterification of high oleic sunflower oil by butanol[29] and transesterification of cetronellol with vinyl acetate under microwave irradiation[30]. The hydrolysis of tetrahydrofurfuryl butyrate in heptane in a triphasic system catalysed by *Candida antarctica* lipase immobilized on macroporous polyacrylate resin was found to follow Ping-Pong Bi-Bi mechanism identical to that obtained in the present work[31]. For this reaction mechanism, the initial velocity equation is represented as

$$v = \frac{V_{max}[A][B]}{K_m^A[B]\left\{1 + \frac{[B]}{K_i^B}\right\} + K_m^B[A] + [A][B]} \quad (1)$$

Here [A] and [B] are the concentrations of the acetate and alcohol respectively, K_m^A and K_m^B are the Michaelis constants, V_{max} is the maximum reaction rate at saturation of the enzyme by both substrates and K_i^B is the inhibition constant.

The kinetic parameters were calculated by putting the experimental data to equation-1 by non-linear regression using computer programme following Gauss-Newton elimination written in GWBASIC. The kinetic parameters estimated are given in Table-1.

Stability of CLEC-PCL: The reaction profiles for stability test were plotted as shown in Figure-10. As evident from the figure, the initial activity is high for the crude enzyme before incubation. However, there was a loss of activity (~ 95%) in the first 24 h incubation time. The CLEC lipase was not very active initially. This enzyme preparation also undergoes initial loss of activity but then retained significant activity up to 7 days of incubation. After 7 days of incubation it retained half (~50%) of its original activity. It appears that both form of the lipase become inactivated in cyclohexane which seems to persist for the period of the incubation experiment[7,32]. However, it is seen that CLEC form of the lipase is by far the best option from practical applications in organic synthesis.

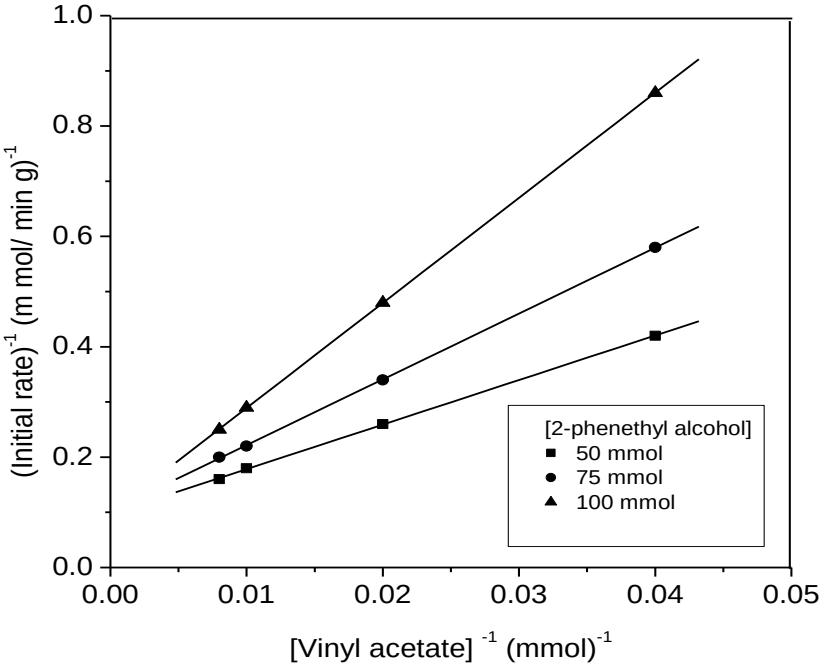

Figure-9: Plot of inverse of vinyl acetate concentration and initial rate of transesterification reaction at constant 2- phenethyl alcohol concentration.

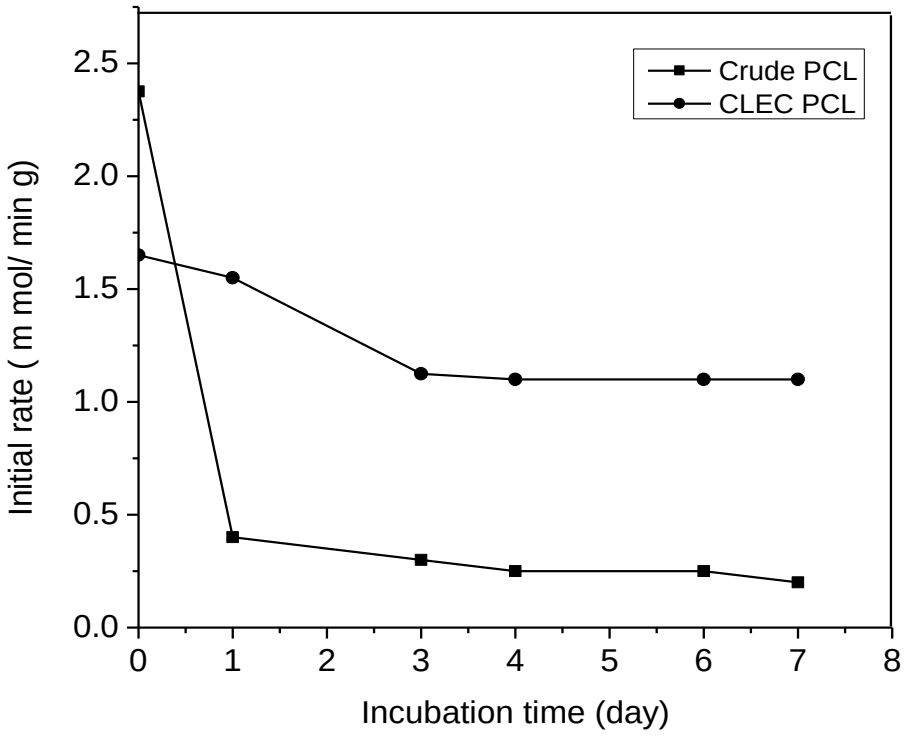

Figure-10: Stability test for CLEC-PCL in comparison to that of crude PCL in Cyclohexane. [2-phenethyl alcohol]: [Vinyl acetate] = 1:4. [CLEC-PCL] = 50mg, Temperature = 45^0C.

Table-1: Kinetic Parameters of Ping-Pong Bi- Bi Model for the Transesterification Reaction Catalysed by CLEC-PCL

Parameter	Value
V_{max} (mmol/ min g)	4.5
$K_m^{B}{}_{(alc)}$ (mM)	53.5
$K_m^{A}{}_{(acetate)}$ (mM)	44
K_i^{B} (mM)	20

Conclusion

The transesterification reaction studied in this paper was found to follow the Ping-Pong Bi-Bi model with 2-phenethyl alcohol inhibition effect. The parameters of this model were determined by regression analysis using the Gauss-Newton algorithm of error minimization. The activity and stability of the CLEC was compared to that obtained in dispersed system and exhibits the highest stability and activity.

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