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Immobilised lipase-catalysed resolution of (*R,S*)-1-phenylethanol in recirculated packed bed reactor

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Abstract

Six commercial immobilised lipases were screened for the resolution of *(R,S)*-1-phenylethanol in organic solvent. Among them, lipases from *Pseudomonas cepacia* (ChiroCLEC-PC) and *Candida antarctica* lipase B (Chirazyme L2, c.-f., C3, lyo) were used in the kinetic study of the resolution in batch stirred tank reactor (BSTR). Lauric acid was used as acyl donor in the acyl transfer reaction. This enzymatic resolution was carried out at 35 °C in isooctane. The enzyme activity as well as enantioselectivity was determined by varying substrates concentration from 25 to 250 mM, acyl length of fatty acid from C12 to C18, organic solvents with log *P* values from 1.4 to 4.5 and reaction temperature from 25 to 50 °C. An initial reaction velocity approach was used to determine the enzymes activities and a computer software, SELECTIVITY was used to calculate the enzyme enantioselectivity. The activity of ChiroCLEC-PC and Chirazyme L2, c.-f., C3, lyo are 1.4 kU/g and 1.0 U/g, respectively. The enzymes are highly selective toward the (*R*)-enantiomer of the chiral alcohol with the enantiomeric ratio, $E > 200$. A series of reaction progress curves was used to develop a kinetic model based on the principle of mass action law with steady-state assumption. The reaction follows a Ping–Pong Bi–Bi mechanism with substrate inhibition. The performance of Chirazyme L2, c.-f., C3, lyo in the resolution was also investigated in a recirculated packed bed reactor (RPBR). The enzyme performance in term of initial reaction rate was decreased 19% and the volumetric productivity was decreased 7% after 30 min of reaction time. The resolution was also required 350 min longer reaction time in order to achieve equilibrium. A comparable result could be obtained in a five-fold scaling up RPBR.

Keywords: Kinetic resolution; Immobilised lipase; Enantioselectivity; Recirculated packed bed reactor

1. Introduction

Recently, the preparation of enantiomerically enriched compounds has become a major research area in industries, especially pharmaceutical and fine-chemical industry. The high interest in producing optically active products is mainly due to the awareness of the physiological and ecological advantages of single enantiomers. The biological activities displayed by the enantiomeric pairs are greatly different. One enantiomer is usually provided the desired activity and the opposite enantiomer may be inactive or sometimes toxic.

Among the methods, kinetic resolution using enzyme as biocatalyst has gained much attention. The resolution of racemic compounds into individual enantiomer is more economical than asymmetric synthesis. This is because high concentration of substrate can be employed in kinetic resolution [1]. This method is simply based on the difference in the transformation rate of enantiomers.

The enzymatic approach is superior owing to the high enzyme activity as well as selectivity at mild reaction conditions. Furthermore, there is a rapid increase of market demand for natural chiral componds. Lipases from *Pseudomonas cepacia*, *Candida antarctica* and *C. rugosa* are the widely used enzyme for secondary alcohol resolution [2–6]. The enzymes accept abroad range of substrates while retaining high enantioselectivity for each. They are readily available at low cost and require no cofactor for reaction.

The acyl donor used in the acyl transfer reaction either as an acid in esterification or as an ester in transesterification. Activated esters are preferable in term of reaction rate and thermodynamic limitation. [7]. However, their disadvantages are required more tedious procedures to compensate.

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The most commonly employed activated ester is vinyl acetate. Acetaldehyde is the single by-product, which is volatile, thus making the reaction irreversible [8,9]. However, acetaldehyde is known to deactivate lipases from *C. rugosa* and *Geotrichum candidum* through the formation of Schiff bases with ϵ-amino groups of lysine residues [10]. This has limited the reliable use of vinyl ester as acylating reagent. Isopropenyl ester is the second most often used acyl donor [10]. Although this ester generates nonharmful acetone, it is less reactive than vinyl acetate [11].

Fatty acid is the conventional acyl donor. The only drawback is the side effect resulted by water. The produced water may associate with the enzyme molecules. This phenomenon may deactivate the enzymes and thereby decrease the value of equilibrium conversion. However, water is easy to be removed using molecular sieve without affecting the performance of the immobilised enzymes. The physical separation of substrate from the reaction solution is much easier in esterification than in transesterification [12].

Lauric acid was chosen as acyl donor in the resolution of 1-phenylethanol. This long chain fatty acid is natural substance for lipases. It can be obtained from renewable raw materials such as oils and fats. It is about 40–52% of lauric acid can be obtained from palm kernel oil and coconut oil. The exploitation of synthetic capability of lauric acid will promote sustainable chemistry [13]. Furthermore, the use of lauric acid in the resolution could achieve conversion near to the theoretical value, 50% with the enantioselectivity value more than 200. The result is compatible with the resolution using activated ester as acylating reagent. The performance of lauric acid as acyl donor in the resolution of the secondary alcohol was proven in the work of Okahata et al. [14,15].

The optically active 1-phenylethanol is used as chiral building block and synthetic intermediate in fine-chemical and pharmaceutical industries [16,17]. Especially (*R*)-1-phenylethanol is widely used as fragrance in cosmetic industry because it contains mild floral odour [18]. The other application includes Solvatochromic dye [19], ophthalmic preservative [20] and inhibitor of cholesterol intestinal adsorption [21].

This study investigated the behaviour of immobilised lipases for the enantioselective esterification of racemic 1-phenylethanol in a recirculated packed bed reactor (RPBR). Kinetic studies using initial velocity analysis were carried out by varying substrates concentration and reaction conditions in a batch stirred tank reactor (BSTR). The kinetic data was modeled using a high performance computing language, MATLAB. The reaction was found followed a Ping–Pong Bi–Bi mechanism with the inhibition of substrates and water. Based on the parameters determined from BSTR, the similar reaction was carried out in RPBR. The decrease of enzyme performance in RPBR is mainly due to the presence of mass transfer limitation in heterogeneous system. The performance of the enzyme was also investigated in the scaled up RPBR.

2. Materials and methods

2.1. Chemicals and enzymes

High purity grade of substrates, (*R,S*)-1-phenylethanol and lauric acid were purchased from Fluka (Switzerland). Isooctane was purchased from Merck (Germany). Several commercial immobilised enzyme lipases were obtained from suppliers as presented in Table 1.

2.2. Resolution of (R,S)-1-phenylethanol

Lauric acid (150 mM) and *(R,S)*-1-phenylethanol (50 mM) were dissolved in isooctane (25 ml). The reaction was started at 35 °C after added in the enzyme (ChiroCLEC-PC = 12.5 mg, Chirazyme L2, c.-f., C3, lyo = 250 mg). The solution was continuously stirred to ensure all the enzyme particles were homogeneously dispersed in the reaction medium. Samples (0.01 ml) were periodically withdrawn to analyse the time course of reaction. No reaction was detected in the absence of the enzyme.

2.3. Effect of substrates concentration

The effect of lauric acid and (*R,S*)-1-phenylethanol concentration were investigated by varying one of the substrates concentration, while the other was held constant. The lauric acid concentration was varied from 25 to 250 mM at the fixed (*R,S*)-1-phenylethanol concentration (50 mM). When the concentration of (*R,S*)-1-phenylethanol was varied from 25 to 250 mM, the lauric acid concentration was fixed at 150 mM.

Table 1
Commercial immbolised lipases

Commercial name	Microorganism	Supplier
Lipase PS-C	*P. cepacia*	Amano Pharmaceiticals Co., Japan
Lipase Sol-Gel-Ak	*C. cylindracea*	Fluka Chemie AG, Switzerland
Chirazyme L2, c.-f., C2, lyo	*C. antarctica*	Roche Molecular Biochemicals, Germany
Chirazyme L2, c.-f., C3, lyo	*C. antarctica*	Roche Molecular Biochemicals, Germany
ChiroCLEC-CR	*C. rugosa*	Altus Biologics Inc., USA
ChiroCLEC-PC	*P. cepacia*	Altus Biologics Inc., USA

2.4. Effect of single enantiomer

The single isomer, (*R*)-1-phenylethanol or (*S*)-1-phenylethanol (25 mM) was esterified by lauric acid (150 mM) separately in isooctane (10 ml) at 35 °C.

2.5. Effect of chain length of fatty acid

The effect of chain length of fatty acid on the reaction was investigated by using long chain fatty acids, namely lauric, myristic, palmitic and stearic acid as acylating agent in the racemate resolution.

2.6. Effect of location of phenyl alcohol

The location of phenyl group on either the alpha- or beta-carbon of the alcohol was investigated by using primary (2-phenylethanol) and secondary alcohol (1-phenylethanol) in the reaction. The alcohols esterified by lauric acid (150 mM) in isooctane (25 ml) at 35 °C.

2.7. Effect of organic solvent and temperature

The solvent effect was investigated by carrying out the reaction in different organic solvents. The solvents used were isooctane, heptane, hexane, cyclohexane, *tert*-butyl methyl ether and toluene. The influence of temperature on the reaction progress was investigated by varying the temperature in the range of 25–50 °C.

2.8. Resolution of (R,S)-1-phenylethanol in RPBR

The reaction mixture consisted of lauric acid (150 mM) and (*R,S*)-1-phenylethanol (50 mM) in isooctane (25 ml) were stirred in a water jacketed vessel. The solution was maintained at 35 °C and pumped upward through a thermostat jacketed column packed with enzymes (Chirazyme L2, c.-f., C3, lyo). The solution was recirculated until the reaction nearly reached completion.

2.9. Gas chromatography analysis

The time course of reaction was determined by a Shimadzu GC-17A gas chromatography equipped with a flame-ionisation detector (FID). The column was a chiral Beta-Dex^{TM} 120 fused-silica capillary column with the dimension 0.25 mm i.d. × 30 ml × 0.25 μm film thickness (Supelco, USA).

The temperature of injector and detector were maintained at 250 °C. The carrier gas was nitrogen with the total flow rate 104 ml/min at 100 kPa. The column temperature was kept at 120 °C for 15 min and then programmed to rise 40 °C/min to reach 190 °C. From 190 °C, the column temperature was increased to 220 °C at the rate of 2 °C/min and maintained at this temperature for 15 min. The retention times for (*R*)-1-pehnylethanol, (*S*)-1-phenylethanol, lauric acid and (*R*)-1-phenylethyl laurate were 12.33, 12.82, 25.57 and 44.86 min, respectively.

3. Results and discussion

3.1. Exploratory experiment of (R,S)-1-phenylethanol resolution

Six commercial immobilised lipases were screened for the kinetic resolution of (*R,S*)-1-phenylethanol with lauric acid at 35 °C in isooctane. Although the overall structure and serine triad of the lipases is similar, the degree of activity and substrate specificity differs widely. All the enzymes except Lipase PS-C and Lipase Sol-Gel-Ak were capable to catalyse the reaction. All of them show a good preference for (*R*)-1-phenylethanol, while (*S*)-1-phenylethanol remained unchanged.

ChiroCLEC-PC exhibited the highest performance for the resolution among the enzymes. The initial rate of reaction was 473.5 ± 10 μmol/min and the reaction reached equilibrium conversion at 45% after 100 min reaction. This cross-linking enzyme is more reactive because it involves protein purification during the formation of microcrystals.

Chirazyme L2, c.-f., C2, lyo and Chirazyme L2, c.-f., C3, lyo are lipase B from *C. antarctica*. They are noncovalently bonded to different insoluble organic polymer carriers. The enzyme with the carrier C3 was more reactive than C2 in the kinetic resolution of (*R,S*)-1-phenylethanol. The initial rate of the former was 1.2 times faster than the latter. This observation can be explained by the difference size of the enzyme beads. The lipase with the carrier C2 has a bigger diameter, 0.5 mm therefore it has higher internal mass transfer limitation than C3 carrier, 0.1 mm. The reaction progress curves reached equilibrium at 44% conversion after 250 min of reaction.

The performance of ChiroCLEC-CR in this resolution is the fourth after ChiroCLEC-PC, Chirazyme L2, c.-f., C3, lyo and Chirazyme L2, c.-f., C2, lyo. The reaction catalysed by ChiroCLEC-CR reached equilibrium only after 300 min. The result indicated that the *C. rugosa* lipase was capable in the discrimination of racemic alcohol. However, the performance of *C. rugosa* lipase was lower than lipases from *P. cepacia* and *C. antarctica*. ChiroCLEC-CR showed about 45% activity lower than ChiroCLEC-PC in the resolution, even though their microcrystals are covalently cross-linked by the bifunctiuonal reagent glutralaldehyde in the similar process. Therefore, the reactivity of *P. cepacia* lipase was higher than *C. rugosa* lipase in the resolution of aromatic secondary alcohol.

Lipase PS-C and Lipase Sol-Gel-Ak did not catalyse the reaction after stirred for two hours at 35 °C. The resolution of sterically alcohol could not be carried out by lipases immobilised on ceramic particles and in Sol-Gel-Ak. These lipases may be required higher temperature for catalysis but this is not economic in the preparative scale resolution.

3.2. Effect of lauric acid concentration

ChiroCLEC-PC and Chirazyme L2, c.-f., C3, lyo were used to catalyse the resolution in the following study. The results showed that the initial rates of the cross-linked crystalline lipase from *P. cepacia* showed higher activity than lyophilised lipase B from *C. antarctica* at all substrates ratios. They have similar curves which indicate the concentration of lauric acid, 150 mM is the optimum concentration for the resolution of (*R,S*)-1-phenylethanol (50 mM) in isooctane. In line with the work of Okahata et al. [22], substrate inhibition was observed when the concentration of lauric acid was over 150 mM.

An increase of lauric acid concentration may prevent the depletion of acyl donor at the enzyme active site. This would reduce the internal diffusion if the mass transfer limitation present. Another reason to use excess lauric acid is to shift the reaction toward synthesis direction since the reaction is reversible. This would simplify the kinetic analysis by reducing the reaction to pseudo-first order. According to Bakker et al. [11], secondary alcohol is a sluggish reactant, hence an irreversible reaction is a prerequisite for efficient kinetic resolution. The nonchiral compound usually presents in a molar excess over the chiral compound to secure the complete conversion of the reactive enantiomer. In the work of Okahata and Mori [14], the resolution of 1-phenylethanol (50 mM) required much more excess lauric acid (500 mM) as acyl donor catalysed by a lipid-coated lipase B from *P. fragi 22-39B* to achieve the enantioselectivity 250.

Another kinetic parameter investigated is enzyme enantioselectivity. The enantiomeric ratio of the enzymes was not affected by lauric acid concentration in the range of 25–250 mM. They are absolutely selective toward (*R*)-enantiomer with the *E*-value more than 200. This *E*-value was determined by a computer software, SELECTIVITY [23]. In contrast to the theoretical prediction that the concentration of lauric acid may alter the equilibrium constant of the reversible reaction which in turn influences the stereochemical behaviour of enzyme [12].

Since the chiral alcohol is the target compound, the enantiomeric excess of substrate (ee_s) value is determined instead of enantiomeric excess of product (ee_p). Indeed, the reaction conversion and the enantiomeric excess of the alcohol followed the same qualitative trends. The increase of ee_s was about twice of the conversion value. The reaction achieved equilibrium at the conversion near to 50%. The conversion value was never go beyond 50% as the enzymes are highly selective. The ee_s were 97 and 96% for Chiarzyme L2, c.-f., C3, lyo and ChiroCLEC-PC catalysed reactions, respectively, after removing the produced water by adding 10% (w/v) of molecular sieve 4 Å into the reaction mixture. The enantiomeric excess value is never reach 100%, although the reaction is reach completion [24]. This is because the equilibrium constant was infinite as a result of a large excess of acyl donor was employed.

3.3. Effect of (R,S)-1-phenylethanol concentration

The initial reaction rates were also increased with the increase of (*R,S*)-1-phenylethanol concentration. However, the note of increase was slow, nearly reaching a plateau value at 250 mM. The observation indicated that the available enzyme active sites were not enough for the increase amount of (*R,S*)-1-phenylethanol. Excessive (*R,S*)-1-phenylethanol in the reaction medium would restrict the mobility of alcohol bind to the enzyme complex. The enantioselectivity of the enzymes was not affected by the increase in alcohol concentration.

Another observation from the study was the decrease in final conversion value at high alcohol concentration. Enzyme deactivation might be occurred because the log *P* value for the alcohol is relatively low, 1.62. It is well established that enzymes exhibit optimum activity in organic solvents with the log *P* values between 2 and 4.

3.4. Effect of single enantiomer

The effect of single enantiomers on the reaction showed that both enzymes were highly selective toward (*R*)-1-phenylethanol. No reaction was observed for (*S*)-1-phenylethanol. The (*S*)-isomers may fit into the active sites at the nonproductive orientation because of the lack of catalytically essential hydrogen bond between alcohol oxygen and His-224 [25]. The final conversion of the reaction could achieve 48% regardless the existence of (*S*)-1-phenylethanol. This indicated that (*S*)-1-phenylethanol caused reversible inhibition to the enzymes.

The initial velocities of the reactions were 34.5 and 817.8 mM/(min g enzyme) for Chirazyme L2, c.-f., C3, lyo and ChiroCLEC-PC, respectively. When compared these values with the values of reaction rates in the presence of (*S*)-1-phenyleythanol, it was found that the reaction rate of single enantiomer was higher than racemic alcohol. It is about two-fold for *C. antarctica* lipase catalysed reaction and three-fold for *Pseudomanas cepacia* lipase catalysed reaction. Langrand et al. [26] explained that the rates of single enantiomers are only equal to the rate of racemic mixture if the total concentration is below the apparent Michaelis–Menten constant (K'_m). The values of K'_m and V'_{max} (apparent maximum reaction velocity) for the substrates are presented in Table 2. These kinetic parameters were determined by linearing the conversion curves. Owing to the presence of diffusional resistance in immobilised enzyme system, this method was used to determine the apparent kinetic parameters.

3.5. Effect of carbon chain of fatty acid

The initial velocities of reactions catalysed by Chirazyme L2, c.-f., C3, lyo and ChiroCLEC-PC are presented in Fig. 1. Both of the curves follow the similar trend with the increase of carbon number in the alkyl chain of fatty acids.

Table 2
Apparent kinetic parameters of substrates

Parameter	ChiroCLEC-PC		Chirazyme L2, c.-f., C3, lyo	
	Lauric acid	1-Phenylethanol	Lauric acid	1-Phenylethanol
K'_m (mM)	27.95	67.50	17.80	110.36
V'_{max} (mM/min)	1.56	3.02	2.20	6.59

The initial reaction rates were increased from lauric acid to palmitic acid, but decreased 3–4% only for stearic acid. The acyl binding site of the enzymes may be enough to accommodate acyl group up to the carbon number 16. Stearic acid has a relatively long acyl chain and may restrain the enzyme molecules from free rotation during catalysis. Another reason is the amphiphilic character of stearic acid, which may cause the partition of fatty acid between the enzyme particles and the bulk phase of organic solvent [27]. The overall result indicated that a longer chain length of fatty acid was more preferable for the reaction. This result in line with the observation reported that lipases from *C. cylindracea* favoured the esterification of menthol with longer chain length of fatty acid [28].

The activity of ChiroCLEC-PC in the resolution of 1-phenylethanol is about 20 times higher than Chirazyme L2, c.-f., C3, lyo. The difference in the reaction rate was also higher for ChiroCLEC-PC catalysed reactions at different carbon number of fatty acid. This observation may explain the distance between acyl and alcohol binding site of the enzyme. The alcohol binding sites of lipases from *P. cepacia* may be located near to their acyl binding site. Therefore, the carbon number of acyl donor had significantly affected the reaction rate.

However, the carbon number of fatty acids did not influence the enantioselectvities of the enzymes. As reported by Holmquist et al. [29], the enantioselectivity of enzyme was depended on the deacylation step. The variation in the chain length of acyl donor is only affected the rate of acylation step. This observation in contradictory to the finding of Ottoson and Hult [30] that the enantioselectivity of *C. antarctica* lipase B was strongly influenced by the achiral acyl chain length of vinyl ester in the transesterification of 3-methyl-2-butanol.

3.6. Effect of location of phenyl alcohol

The location of phenyl group at the α- or β-carbon of the alcohol is two different classes of alcohol. The primary alcohol of phenylethanol has an aromatic ring at the β-carbon and a hydroxyl group at the α-carbon. The secondary alcohol of phenylethanol has an aromatic ring at the α-carbon where the hydroxyl group of the alcohol located. Although the location of phenyl group of the alcohol is one carbon difference, the mechanism of the reaction is believed totally different. The difference in the reaction mechanism caused a great difference in the reaction rate. The experimental result showed that primary alcohol reacted much faster than secondary alcohol. It is about seven times higher for ChiroCLEC-PC catalysed reaction and five times for Chirazyme L2, c.-f., C3, lyo catalysed reaction. The observation was due to the steric hindrance of phenyl group located near to the hydroxyl group of secondary alcohol where the reactions were taking place.

3.7. Effect of organic solvent

The solvent effect is of practical importance for the application of lipase in racemate resolution. It is well known that the solvent polarity can affect the activity and enantioselectivity of enzyme.

The initial reaction rates increased with the enhancement of hydrophobicity of organic solvent for ChiroCLEC-PC catalysed reactions as shown in Fig. 2. The result is in good

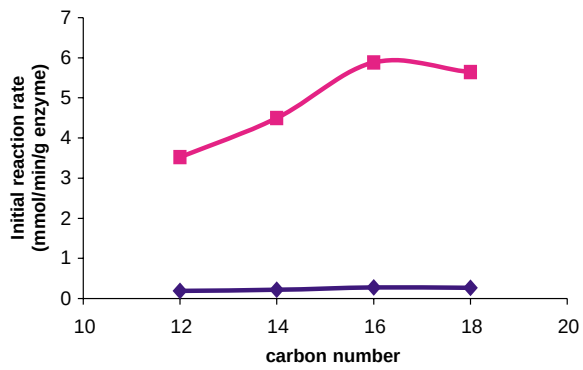

Fig. 1. Initial velocities of reactions catalysed by ChiroCLEC-PC (■) and Chirazyme L2, c.-f., C3, lyo (◆) at different carbon number.

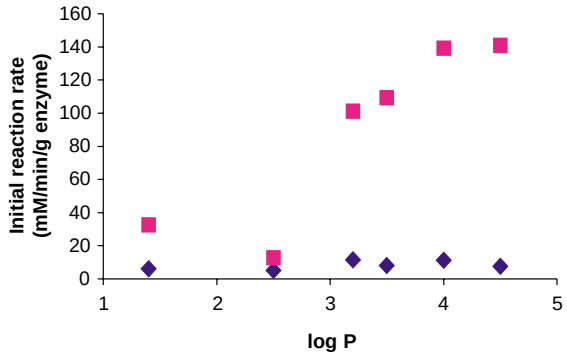

Fig. 2. Initial velocities of reactions catalysed by ChiroCLEC-PC (■) and Chirazyme L2, c.-f., C3, lyo (◆) in organic solvents with different log *P* values.

agreement with the S-shaped curve suggested for the biocatalysis in two phases system [31]. However, the performance of the enzyme was decreased significantly in toluene. 1-phenylethanol was more favourable to stay in the bulk phase of solvent instead of bound to the enzyme complex for the deacylation step. The π–π electron interaction on the benzene ring of toluene was more likely to attract the phenyl group of the alcohol.

Chirazyme L2, c.-f., C3, lyo behaved the same in the solvent of toluene. The catalytic activity of the enzyme in toluene was lower than the other solvents. Overall, no significant trend was noticed in the influence of solvent polarity and reaction rate for Chirazyme L2, c.-f., C3, lyo catalysed reactions.

The hydrophilic solvent may deactivate the enzyme in the way of distrupting the functional structure of enzyme or stripping off the essential water from the enzyme. This is because water has higher affinity in hydrophilic solvent rather than bound to the enzyme. For example, *tert*-butyl methyl ether exhibited low reaction rate because the solvent is more hydrophilic than the other hydrocarbon solvents. The catalytic activity of enzyme was decreased due to the lack of bound water to preserve the enzyme conformation flexibility. This structural mobility is necessary for its catalytic action.

Although the polarity of solvent greatly affects the catalytic activity, it seems not to influent the enzyme enantioselectivity. The enantioselectivities of both enzymes seem to be independent of organic solvent with the log *P*-value from 1.4 to 4.5.

3.8. Effect of temperature

The effect of temperature on the reaction rates of the enzymes is presented in Fig. 3. The results showed that the activity of crystallined lipase from *P. cepacia* was not much affected by temperature between 25 and 40 °C but significantly decreased at 50 °C. However, lyophilised lipase B from *C. antarctica* unable to exhibit its highest activity at low temperature. At high temperature, the enzymes were partially deactivated. The reaction rate was decreased above the optimal temperature at 35 °C.

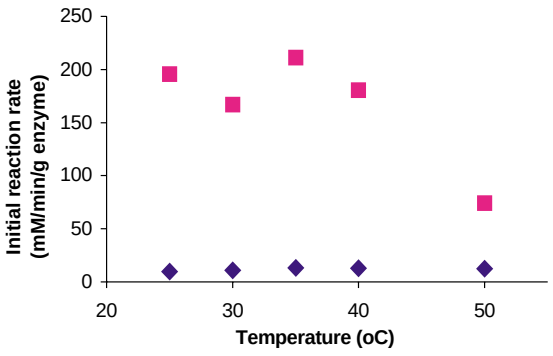

Fig. 3. Initial velocities of reactions catalysed by ChiroCLEC-PC (■) and Chirazyme L2, c.-f., C3, lyo (◆) at different temperature.

The enantioselectivities of the enzymes used in this study were also not affected by the change in temperature. The *E* values for the enzymes were maintained at more than 200 in the temperature range of 25–50 °C.

3.9. Kinetic modelling of reaction

The real enzymatic reaction mechanism is very complex and difficult to understand. It is enough to determine the plausible mechanism of reaction including reversibility and inhibition phenomena.

The possible mechanisms followed by Bi–Bi reaction are Ping–Pong, sequential ordered ternary complex and random ordered ternary complex [24]. Based on the experimental result of kinetic studies, a Ping–Pong Bi–Bi mechanism with the irreversible inhibition of (*R*,*S*)-1-phenylethanol, lauric acid and water was postulated. The schematic diagram of the mechanism is presented in Fig. 4.

A mathematical model for the resolution of (*R*,*S*)-1-phenylethanol was derived with the steady-state assumption using mass action law. The second order differential equation was solved by the nonstiff medium order (4–5) ordinary differential equation function from MATLAB. The program fits simultaneously a series of progress curves with a number of elementary rate constants by the numerical integration of the batch differential equations. Fig. 5 is the result of fitting at various (*R*,*S*)-1-phenylethanol concentration for Chirazyme L2, c.-f., C3, lyo catalysed reactions. The reaction progress was followed by monitoring the depletion of alcohol concentration. The model fits the experimental data satisfactory. This model was valid for the concentration range of substrates from 25 to 250 mM at the fixed reaction conditions. The program for the modelling is available upon request.

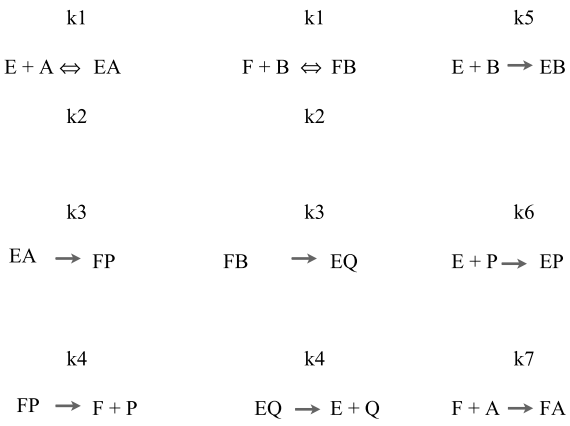

Fig. 4. Schematic diagram of Ping–Pong Bi–Bi mechanism. Where A: lauric acid; B: (*R*,*S*)-1-phenylethanol; P: water; Q: (*R*)-1-phenylethyl laurate; E: enzyme; F: enzyme complex; kn: elementary rate constant ($n = 1, 2, 3, \ldots$).

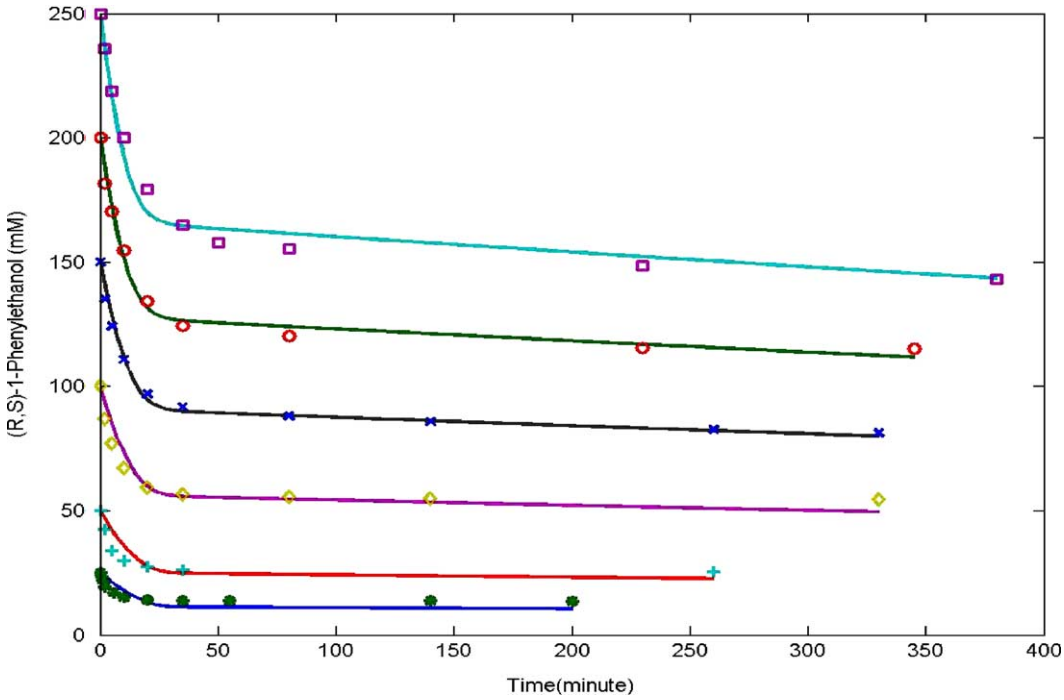

Fig. 5. Results of model fitting for Chirazyme L2, c.-f., C3, lyo catalysed reactions at various initial alcohol concentrations. Experimental data: 250 mM (□), 200 mM (○), 150 mM (×), 100 mM (◇), 50 mM (+) and 25 mM (●), model result (–).

3.10. (R,S)-1-Phenylethanol resolution in RPBR

The resolution was carried out in a RPBR using the reaction conditions determined from the BSTR. In this study, BSTR was assumed as an ideal reactor because the reaction solution was well stirred without the occurrence of mass transfer limitation in the heterogeneous system. The preference of RPBR for the resolution is mainly due to the advantages of the reactor over BSTR. The main advantages are the easy of enzyme reusability, reactor construction as well as operation. The problem of enzyme loose because of repeatedly transferring process in enzyme handling can be avoided.

Chirazyme L2, c.-f., C3, lyo was used to catalyse the resolution in the RPBR. This is because it has homogeneous particle size which can be packed in the reactor column. Furthermore, this enzyme did not swell in the reaction solution. The swelling of ChiroCLEC-PC could create high pressure in the packed column. Moreover, the required quantity of ChiroCLEC-PC was too little to be packed in the packed bed reactor. Therefore, ChiroCLEC-PC was not suitable to be used in RPBR even though it exhibited higher activity (1.4 kU/g) than Chirazyme L2, c.-f., C3, lyo (1 U/g).

The performance of Chirazyme L2, c.-f., C3, lyo for the resolution of 1-phenylethanol in BSTR and RPBR was compared in three important aspects such as initial reaction rate, volumetric productivity and equilibrium time. The initial reaction rate of the resolution was about 19% lower in RPBR (10.6 mM/(min g enzyme)) compared to BSTR (13.1 mM/(min g enzyme)). The productivity of the enzyme in RPBR (0.1959 g/(l min) or 3.86 nmol/(h g enzyme)) was decreased 7% after 30 min of reaction time. In the work of Xu et al. [32], the average productivity of packed bed reactor in the preparation of (*R*)-α-monobenzoyl glycerol catalysed by Chirazyme L2, c.f., C2, lyo were 169 μmol/(h g) biocatalyst in continuous mode and 201.08 in semicontinuous mode. A longer equilibrium time (380 min) was required in order to achieve the final conversion value 48%. The decrease of enzyme performance in RPBR is particularly due to mass transfer limitation, bed permeability and enzyme compaction.

3.11. Mass transfer study

Mass transfer study is very important to be carried out, especially when working with immobilised enzyme in organic media. The presence of mass transfer limitation in the reaction system may reduce the intrinsic enzyme activity as well as enzyme specificity. External mass transfer limitation was ruled out in this study because the reaction solution was pumped through the packed enzyme at the optimal flow rate (21.11 ml/min). While the existence of internal mass transfer limitation was investigated by using Thiele modulus.According to Blanch and Clark [33], the observable modulus for a spherical particle geometry can be represented by Eq. (1). This equation was converted into the actual Thiele modulus Eq. (2)

$$\varphi = \frac{V_{\text{obs}}}{D_{\text{eff}} S_{\text{o}}} \left(\frac{R}{3}\right)^2 \quad (1)$$

$$\varphi = \eta_I \left(\frac{\phi^2}{1+\beta} \right) \quad (2)$$

The constant β is assumed to be zero [34]. The diffusivity of (*R*,*S*)-1-phenylethanol (D_{eff}) was estimated from Wilke–Chang correlation to be 1.328×10^{-12} m^2/s.

After substituting the concentration of (*R*,*S*)-1-phenylethanol (S_o), 50 mM with correspond initial reaction rate (V_{obs}), 5.448×10^{-5} mol/(l s) with the particle size (*R*), 0.1×10^{-3} m, the values of φ and η_I are 0.9115 and 1, respectively. This means that substrate diffusion rate may present in the reaction system but not significant because the η_I value is about 1. The particle size of the enzymes is relatively small (0.1 mm), thus does not create an obvious gradient of substrate concentration within the particles.The observation was also interpreted by using the parameters such as reaction time constant, t_r (3) and diffusion time constant, t_d (4). The mass transfer coefficient, K_L, 2.6566×10^{-8} m/s was estimated from Eq. (5).

$$t_r = \frac{1 - PhE}{r} \quad (3)$$

$$t_d = \frac{D}{K_L^2} \quad (4)$$

$$K_L = \frac{2D}{d} \quad (5)$$

where *r* is the rate of reaction and *d* is the enzyme particle diameter.

Since the calculated t_d, 1882.10 s was about two-fold higher than t_r, 917.72 s, the reaction was partially influenced by mass transfer effect.

3.12. Stability of enzyme

Chirazyme L2, c.-f., C3, lyo shows a good operational stability in packed bed reactor. The catalytic efficiency of the enzyme was decreased after reused for ten reaction runs. The productivity decreased about 20% from 0.807 g/(l min) in the first run to 0.644 g/(l min) in the tenth run at the conversion below 25%. The enzyme was stable in high concentrated organic solution. The catalytic conformation of enzyme did not change because of the temperature employed and the chemicals used in the reaction.

3.13. Enzyme performance in scaled up reactor

The recirculated packed bed reactor was scaled up five-fold based on the basis of maintaining a similar superficial velocity throughout the bed and the length to diameter ratio of column. The volume of reaction solution and the quantity of enzyme used were increased proportionally with the reactor size.

The performance of Chirazyme L2, c.-f., C3, lyo in the preparative scale reactor was comparable with the small scale packed bed reactor. The productivity of the enzyme was linearly increased with the increase of conversion value. The progress curves of the reactors are nearly superimposed on each other (Fig. 6). However, the final conversion value of the resolution in the preparative scale was about 1% lower than in the small scale packed bed reactor. This is because of the difficulty in ensuring a good flow of solution throughout the bed in the larger scale packed bed reactor. The problems of channelling and enzyme compaction are always encountered in the preparative scale packed bed reactor. However, employing a low aspect ratio of column (length to diameter) could reduce the problems.

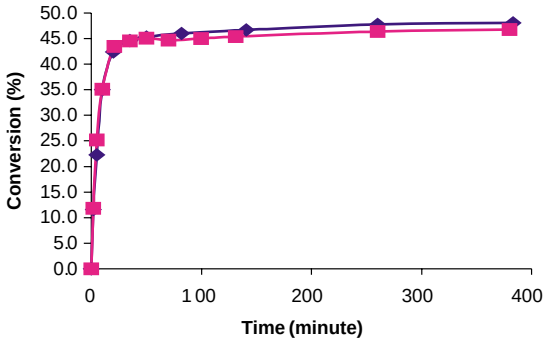

Fig. 6. Progress curves of small (◆) and preparative scale (■) RPBR.

4. Conclusions

The enantioselective esterification of (*R*,*S*)-1-phenylethanol catalysed by immobilised lipases from *P. cepacia* and *C. antarctica* were carried out in BSTR and RPBR. This reaction followed Ping–Pong Bi–Bi mechanism with the inhibition of substrates and water. The resolution of (*R*,*S*)-1-phenylethanol (50 mM) required 3 fold molars of nonchiral acyl donor in excess. The enantiomeric excess of substrate are 96 and 97% for ChiroCLEC-PC and Chirazyme L2, c.-f., c3, lyo catalysed reaction, respectively, when the formed water was removed. The enzymes are highly selective toward the (*R*)-enantiomer of the secondary alcohol with the enantioselectivity value >200. This E value was not affected by substrates concentration and reaction conditions. The performance of Chirazyme L2, c.-f., C3, lyo in this resolution was decreased in RPBR because of mass transfer limitation, bed permeability and enzyme compaction. A comparable result could be obtained in the preparative scale RPBR.

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