CHIRAL RESOLUTION OF *(R,S)*-1-PHENYLETHANOL USING IMMOBILIZED LIPASES IN BATCH STIRRED TANK AND RECIRCULATED PACKED BED REACTORS

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To my beloved parents, husband and especially my sons, Yee Henn and Yee Jie.

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ABSTRACT

This study investigated the enantioselective esterification of *(R,S)*-1-phenylethanol in isooctane. Lauric acid was used as acyl donor in the acyl transfer reaction. Six commercial immobilized lipases; Lipase PS-C, Lipase Sol-Gel-Ak, Chirazyme L2, c.-f., C2, lyo, Chirazyme L2, c.-f., C3, lyo, ChiroCLEC-CR and ChiroCLEC-PC were screened for their resolution activities. Lipases from *Pseudomanas cepacia* (ChiroCLEC-PC) and *Candida antarctica Lipase B* (Chirazyme L2, c.-f., C3, lyo) showed higher resolution activities and therefore used in the subsequent study. The kinetic studies were carried out in a batch stirred tank reactor. The enzyme activity and enantioselectivity were determined by varying the enzyme loadings, substrate concentrations from 25 - 250 mM, chain length of fatty acids from C12 – C18, organic solvents with logP value from 1.4 - 4.5, water contents from 0 – 0.5 %v/v and reaction temperatures from 25 – 50 ^{o}C. Both enzymes showed the highest activity at the ratio of alcohol to acid 1:3 in isooctane at 35 ^{o}C. Both enzymes are also highly selective toward the *(R)*-enantiomer of 1-phenylethanol with the enantioselectivity value, E > 200. The resolution achieved enantiomeric excess of substrate, ee_s up to 97 % when molecular sieve 3Å was added into the reaction mixture. A series of reaction progress curves were used to develop the kinetic model using MATLAB. The rate equation was derived based on the principle of mass action law with steady state assumption. The reaction follows Ping-Pong Bi-Bi mechanism with the inhibition of substrates and water. A similar reaction was carried out in a recirculated packed bed reactor. The performance of the enzymes was reduced in this reactor. The decrease was mainly due to poor bed permeability and compaction. A decrease of about 38 – 58 % in term of volumetric productivity was observed as compared to batch stirred tank reactor. However, the productivity of Chirazyme L2, c.-f., C3, lyo (2.74 g/day/g biocatalyst) was much higher than the productivity obtained in the synthesis of *(R)*-monobenzoyl glycerol (0.94 g/day/g biocatalyst) using the same enzyme in packed bed reactor reported by Xu *et al.* [246]. The enzymes performance also also reduced in the five fold scaled up reactor compared to the small scale recirculated packed bed reactor. The problems of channelling effect and immobilized enzyme particles compaction exacerbated the enzymes performance in the scaled up of recirculated packed bed reactor.

ABSTRAK

Kajian ini menyelidik tindakbalas pengesteran terhadap *(R,S)*-1-feniletanol secara enantioselektif dalam isooktana. Asid laurik digunakan sebagai penderma asil dalam tindakbalas perpindahan asil. Enam jenis lipase tersekatgerak komersil seperti Lipase PS-C, Lipase Sol-Gel-Ak, Chirazyme L2, c.-f., C2, lyo, Chirazyme L2, c.-f., C3, lyo, ChiroCLEC-CR and ChiroCLEC-PC disaring kesesuaiannya dalam resolusi ini. Di antara enzim ini, lipase daripada *Pseudomanas cepacia* (ChiroCLEC-PC) dan *Candida antarctica* Lipase B (Chirazyme L2, c.-f., C3, lyo) digunakan dalam kajian seterusnya. Kajian kinetik dijalankan dalam satu reaktor berkelompok. Aktiviti dan enantiopilihan enzim ditentukan dengan mengubah nilai kuantiti enzim, kepekatan substrak dari 25 – 250 mM, kepanjangan rantai karbon asid lemak dari C12 – C18, pelarut organik bernilai logP dari 1.4 – 4.5, kandungan air dari 0 – 0.5 %v/v dan suhu tindakbalas dari 25 – 50 ^{o}C. Kedua-dua enzim ini menunjukkan aktiviti yang tertinggi dalam nisbah alkohol kepada asid, 1:3 dalam isooktana pada 35 ^{o}C. Enzim-enzim tersebut sangat memilih terhadap *(R)*-enantiomer daripada 1-feniletanol dengan nilai enantiopilihan, E > 200. Resolusi ini mencapai enantiomerik lebihan substrak, ee_s sehingga 97 % apabila penapis molekul 3Å ditambahkan dalam larutan tindakbalas. Satu siri lengkuk perkembangan tindakbalas digunakan untuk membangunkan model kinetik menggunakan MATLAB. Persamaan kadar diterbitkan berdasarkan prinsip hukum tindakan jisim dengan andaian keadaan mantap. Tindakbalas ini mengikuti mekanisme Ping-Pong Bi-Bi dengan rencatan kedua-dua substrak dan air. Tindakbalas yang sama dijalankan dalam satu reaktor lapisan terpadat jenis edaran semula. Pencapaian enzim menurun dalam reaktor itu. Penurunan ini terutamanya disebabkan oleh masalah ketelapan dan kemampatan enzim dalam turus. Penurunan sebanyak 38 – 58 % produktiviti isipadu berlaku berbanding dengan reaktor berkelompok. Walaubagaimanapun, produktiviti bagi Chirazyme L2, c.-f., C3, lyo (2.74 g/hari/g biomangkin) adalah jauh lebih tinggi daripada produktiviti yang diperolehi oleh Xu *et al.* [246] dalam sintesis *(R)*-monobenzoil gliserol (0.94 g/hari/g biomangkin) menggunakan enzim yang sama dalam reaktor lapisan terpadat. Prestasi enzim juga menurun dalam reaktor yang dibesarkan skalanya sebanyak lima kali ganda berbanding dengan reaktor bersaiz kecil. Masalah kesan saluran dan pemadatan zarah enzim tersekatgerak mengurangkan prestasi enzim dalam reaktor lapisan terpadat jenis edaran semula yang diperbesarkan skalanya.

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LIST OF ABBREVIATIONS AND SYMBOLS

1-PhE	-	1-phenylethanol
A	-	Peak area of chromatogram
BSTR	-	Batch stirred tank reactor
d	-	Diameter of enzyme particle
E	-	Enantioselectivity or enantiomeric ratio
ee_s	-	Enantiomeric excess of substrate
ee_p	-	Enantiomeric excess of product
g/g	-	Weight per weight
k_n	-	Elementary rate constant
LA	-	Lauric acid
L	-	Large
M	-	Medium
P_R	-	*(R)*-enantiomer of product
P_S	-	*(S)*-enantiomer of product
Q	-	Flow rate of solution
R	-	Alkyl or aryl group
Re	-	Reynolds number
RPBR	-	Recirculated packed bed reactor
r	-	Radius of enzyme particle
S_R	-	*(R)*-enantiomer of substrate
S_S	-	*(S)*-enantiomer of substrate
V	-	Volume of solution
v/v	-	Volume per volume
w/v	-	Weight per volume
subscript $_i$	-	Initial reaction time
subscript $_t$	-	t minute of reaction time

ξ	-	Conversion
ρ	-	Density of solvent
μ	-	Viscosity of solvent
ε	-	Voidage
τ	-	Residence time
ϕ	-	Thiele modulus

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CHAPTER 1

INTRODUCTION

1.1 Introduction to the Overall Study

The development of chirotechnology is currently getting much attention, especially from pharmaceutical and fine chemical industries. This ever-increasing trend of utilizing enzyme chirality in biotransformation is mainly due to the stability and selectivity of enzymes [1,2].

Enzymes, especially lipases are relatively stable in organic media. Although the idea of enzyme working in nonaqueous system media goes against the conventional practice, the reaction schemes have been confirmed by several researches over the past few years [2,3,4,5,6]. Furthermore, the performance of lipases is much better in organic solvents than in aqueous media [7]. Many organic substances such as fatty acids and lipids are also well dissolved in organic solvents. The reactions involving these organic substances are difficult to carry out in aqueous media.

In more recent years, several studies showed that enzyme selectivity, especially enantioselectivity could be further enhanced in organic media [8]. This finding along with the high market requirement for enantiopure chiral compounds has intensified the study on the enantioselectivity of enzymes.

The enantioselectivity of enzymes is used to prepare enantiomerically enriched compounds. Among the enzymatic methods, kinetic resolution is the

simplest and the most economical approach [9]. The method is based on the difference in the transformation rate of one enantiomer over the other. The theoretical yield of the transformation is 50 %.

Kinetic resolution is usually used to prepare chiral alcohols from racemic compounds. This is because chiral alcohols are versatile and important synthons for the preparation of complex chemical substances. The resolution is carried out either via esterification or transesterification. Esterification requires an acid, whereas transesterification requires an ester as acyl donor in the acyl transfer reaction. The selection of a suitable acyl donor is very important in the resolution. It enhances not only the reaction rate, but also enzyme enantioselectivity.

Many researches have used vinyl ester as acyl donor [9,10,11,12,13] in the resolution. This activated ester is more reactive and makes the reaction irreversible. However, the liberated by-product, acetaldehyde may inactivate the enzymes.

The use of conventional acyl donor such as long chain fatty acid would not create such a problem. Long chain fatty acids are the natural substances of lipases. The only by-product is water in the esterification reaction. Water may promote the reverse reaction toward hydrolysis direction. However, a proper control of water activity would reduce the problem. Furthermore, a small amount of water is required for enzyme activation. Therefore, enzyme could maintain its active conformation throughout the reaction.

A comparable result was obtained when lauric acid was used as acyl donor in the resolution of *(R,S)*-1-phenylethanol. The resolution could achieve the enantiomeric excess of substrate up to 97 % if molecular sieve 3Å was added into the reaction solution. The high performance of fatty acid as acyl donor in the secondary alcohol resolution has also been reported by several researchers [14,15,16,17,18]. The results are comparable with the result obtained when the other types of acyl donor such as vinyl acetate [9,10,11,13] and anhydride [19,20] were used in the resolution.

The precise mechanism involves in the lipase-catalyzed reaction is still unclear. However, the enantiopreference of enzyme can be recognized by substrate mapping using Kazlauskas rule [21]. For example, *(R,S)*-1-phenylethanol has a large phenyl group and a small methyl group in its molecular structure. The compound has a significant difference in the size of the substituents. Therefore, it can be resolved efficiently using high enantioselectivity of lipases.

In this study, the efficiency of lipases in the resolution was determined by kinetic analysis. Kinetic studies were carried out using the data obtained from batch stirred tank reactor. The concentration of substrates was varied at the fixed reaction conditions.

Similar reaction was also carried out in a recirculated packed bed reactor. The performance of the enzymes was compared in both batch stirred tank reactor and recirculated packed bed reactor. The enzyme performance decreased in term of initial reaction rate, productivity and equilibrium time in recirculated packed bed reactor. The reactor was then scaled up for the resolution.

A series of reaction progress curves at different substrate concentrations was used to develop a kinetic model. The mathematical model was written into program file using MATLAB. The mechanism of the resolution was Ping-Pong Bi-Bi mechanism with the inhibition of substrates and water.

1.2 Objective and Scopes of the Study

The objective of this research was to study an enzymatic resolution of *(R,S)*-1-phenylethanol via enantioselective esterification with lauric acid catalyzed by immobilized lipases in isooctane in a recirculated packed bed reactor.

In the preliminary study, the resolution reaction was carried out in a batch stirred tank reactor. Kinetic studies were carried out by varying enzymes and substrates concentration at the fixed reaction conditions. The other parameters such

as chain length of fatty acid, organic solvent, reaction temperature, water content and glycerol effect were also investigated in order to understand the behaviour of the enzymes. A kinetic model was developed using a series of reaction progress curves by MATLAB. This kinetic study is essential to obtain the mechanistic information of enzyme reaction.

The similar reactions were carried out in a recirculated packed bed reactor. The performance of enzymes in the reactor was investigated and compared with the batch stirred tank reactor. The recirculated packed bed reactor was then scaled-up to the preparative scale. The performance of enzymes in the resolution was also compared between the scaled up and the small scale recirculated packed bed reactor.

1.3 Identification of Research Problem

The chemical method for the preparation of optically active 1-phenylethanol requires heavy metal catalyst, namely Ruthenium (II) complexes and lithium aluminium hydride complexes in the asymmetric reduction of acetophenone [22,23]. In addition to the negative impact on the environment, this method also unable to produce chiral 1-phenylethanol with sufficient optical purity (48 % ee_p) compared to the enzymatic approach.

Although enzyme aminoacylases [24,25] and NADH-dependent phenylacetaldehyde reductases [26] had successfully been used, lipases are still considered as the most suitable enzyme for preparing enantiomerically pure 1-phenylethanol. Lipases, especially from the genera of *Pseudomonas* can produce 1-phenylethanol with high enantiomeric excess, >99 % [10,12,13]. Lipase-catalyzed reaction could be carried out in a wide variety of reaction conditions. They require no cofactor and readily available at low cost.

The asymmetric reduction of prochiral ketones and the enantioselective oxidation of single enantiomer are another two possible microbial methods of chiral alcohol preparations [27]. The yeast-mediated reduction required the regeneration of

coenzyme NAD(P)H and hence energy sources must be added to the system. Furthermore, only about 10 % of acetophenone was converted to 1-phenylethanol as catalyzed by yeast cells [27]. On the other hand, the enantioselective oxidation required an oxygen sources for the reaction. The reaction could produce *(R)*-1-phenylethanol with sufficient optical purity (> 90%) only after 80 hours of continuous production.

Most of the studies on the enantioselective resolution of racemic secondary alcohols are focused on aliphatic secondary alcohols such as 2-octanol [28,29,30,31,32,33,34,35] and terpenic alcohol especially menthol [6,36,37,38,39,40,41,42,43,44,45,46,47,48,49,50,51]. Much less study is concentrated on sterically aromatic secondary alcohol such as 1-phenylethanol [9,10,11,13]. Therefore, the steric restriction of enzyme active site is rarely studied.

The majority of reactions involved in the resolution are transesterification. Vinyl acetate was used as acyl donor [9,10,11,12,13,52,53]. Even though vinyl acetate is more reactive, the reaction can produce acetaldehyde as by-product. This volatile acetaldehyde has been proven may cause enzyme deactivation, especially when lipases from *Candida rugosa* and *Geotrichum candidum* were used [54]. However, only a few papers studied on the enantioselective esterification of 1-phenylethanol using lauric acid as acyl donor [14,15,16,18].

Until now, a relatively less effort has been spent on the development of bioreactor for enzymatic reaction in organic media. Many studies were focused on small scale and in batch mode. The scale-up from a small laboratory scale to a preparative scale needs intensive studies on the underlying reaction and transport processes. Therefore, a quantitative understanding of the reaction and enzyme reactor is necessary for preparative scaling up of the packed bed reactor.

1.4 Importance of the Study

The importance of this study, primary lies on the premise that there is an advantage in producing natural products from natural sources using enzymatic method. Product that is produced from enzymatic reaction is considered natural and is perceived to have better quality, thereby enhancing its economic value [55,56,57]. The substrates used in this chiral esterification are derived from plants. The first substrate, 1-phenylethanol is an essential oil from *Humulus lupulus* [58], *Tagetes minuta, Tagetes erecta* and *Tagetes patula* [59], olive oil [60] and chestnut honey [61]. The second substrate, lauric acid is a middle chain fatty acid that can be obtained from palm kernel oil (40-52%) and coconut oil (44-52%).

The aim of this study is to diversify the application of lauric acid. Instead of using toxic acyl donor such as vinyl acetate and anhydride, lauric acid was used to resolve the racemic alcohol into its enantiomers. High reaction rate as well as high enantiomeric excess could be obtained by using lauric acid. Furthermore, water is the only by-product, which is easier to be handled as compared to the hazardous by-product, namely acetaldehyde.

Another valuable output from this research is the wide application of optically active 1-phenylethanol in industries. The optically active 1-phenylethanol is used as chiral building block and synthetic intermediate in fine chemical, pharmaceutical and agrochemical industries [19,62,63]. In pharmaceutical industry, 1-phenylethanol is used as ophthalmic preservative [64]. This chiral compound may also inhibit cholesterol intestinal adsorption and thus decrease high cholesterol level [60].

The other application area of the enantiomers is in chemical analysis. Both the *(R)*- and *(S)*-enantiomer of 1-phenylethanol are used as chiral reagent for the determination of enantiomeric purity [65], for the resolution of acid [66] and for the asymmetric opening of cyclic anhydrides and expoxides [65]. It is also used as auxiliary in butadienes for asymmetric Diels-Alder reaction [66].

Since *(R)*-1-phenylethanol contains mild floral odour, it is used as hyacinth-like fragrance in cosmetic industries [67]. It is also used as perfumery ingredient [68]. Moreover, *(R)*-1-phenylethanol can be used in Solvatochromic dye [69].

The study of chiral esterification of *(R,S)*-1-phenylethanol is also essential in providing basic knowledge of enzyme reaction in organic media. The knowledge is useful in predicting the enzyme performance towards more bulky aromatic secondary alcohols. The simplest example is 1-phenylethanol's homologes such as 1-phenylpropanol and 1-phenylbutanol. Thus, this study is an important step to produce more complicated structural chiral compounds.

The study on the effects of solvent polarity and chain length of fatty acid in combination with the kinetic modelling would provide new knowledge of substrate and enzyme interactions. The understanding of the interactions is essential in protein engineering in order to control enzyme activity for synthetic biocatalyst. Thus, this knowledge is useful for creating tailor-made biocatalysts for specific applications.

The knowledge of the resolution behaviour in the recirculated packed bed reactor is important for the preparation of optically active 1-phenylethanol in a larger scale production. This is because packed bed system is readily scaled up using commercially available large radius columns. This study paves the way for the investigation of continuous production of high yield and purity of 1-phenylethanol. Therefore, a better understanding of the process would lead to a better design of enzyme reactor and reaction conditions.

a better understanding of the behaviour of enzymatic resolution. This understanding is essential for process prediction and optimization.

7.2 Recommendations for future study

Several recommendations are suggested for future study on the enzymatic resolution of *(R,S)*-1-phenylethanol with lauric acid in organic media. Firstly, a wider range of organic solvents should be tested for the resolution. It is important to determine a specific logP value that can alter the enantioselectivity of enzymes. The value indicated that the catalytic confirmation of enzymes started to change at certain polarity level of solvents. This finding is crucial in improving the optical purity of product.

A reliable method to continuously control the water activity at the optimal level during the reaction needs to be developed. This can be carried out by directly adding a suitable salt hydrate pair into the reaction mixture. However, the effects of the salt on the reaction as well as on the enzyme itself have to be studied in detail.

The addition of molecular sieves into the reaction mixture has been proven to improve the reaction conversion. Nevertheless, it is suggested to do some modifications on the reactor configuration in order to allocate molecular sieves in a proper way. A certain amount of molecular sieves can be kept in a bag and hang it in the middle of reaction solution. This method can reduce the problem of abrasion on the molecular sieves by the stirrer.

In the packed bed reactor system, molecular sieves can be packed in another column after the enzyme column. The water produced after the reaction will pump together with the reaction solution through the molecular sieves column for water removal. The efficiency of molecular sieves is dependent on the flow rate of solution. In addition to flow rate, the quantity of molecular sieves required in the reaction has to be determined.

It is recommended to carry out the resolution catalyzed by ChiroCLEC-PC in a micro-scale recirculated packed bed reactor. The enzymes can be packed in this micro-scale reactor column without the need for packing materials. Hence, a real behaviour of the enzymes in the packed bed reactor system can be studied. The complexity because of the integration of enzymes with packing material can be eliminated.

In determining the reaction mechanism, it was found that there are two possible mechanisms can represent the reaction. The effort of optimization the value of elementary rate constants of Ping-Pong Bi-Bi mechanism with the irreversible inhibition of *(R,S)*-1-phenylethanol, lauric acid and water has been done. Although the difference between the mechanisms is only the reversibility step of water inhibition, the behaviour of the enzymes was greatly different in these two mechanisms. Hence, an intensive work should be carried out to compare the difference between the mechanisms. This will definitely improve the knowledge of enzyme behaviour in the resolution in organic media.

This study assumed *(R,S)*-1-phenylethanol as one chemical compound represented by the alphabet of capital B in the kinetic modelling process. However, one of the most important parameters, namely enantioselectivity is not considered in the modelling process. The model based on the elementary catalytic steps is actually essential for the prediction of enantiomeric ratio value. This is because the present methods of E value determination are only of limited accuracy [148]. In order to take this kinetic parameter into account, the *(R)*- and *(S)*-enantiomer of the racemic alcohol may consider as two different chemical compounds. Now, the reaction becomes a tri-bi reaction. The resolution could achieve 100 % conversion of *(R)*-enantiomer thereotically. The presence of *(S)*-enantiomer does not inhibit the resolution can be also determined from the model. Therefore, the enantioselectivity value can be calculated from the rate ratio of *(R)*- and *(S)*-enantiomer of the alcohol.

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