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MEMBRANE REACTOR**

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Performance of free *Candida antarctica* lipase B in the enantioselective esterification of (*R*)-ketoprofen

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Abstract

The performance of free *Candida antarctica* lipase B (CALB) in a mixed solvent system for enantioselective esterification of (*R*)-ketoprofen, leaving the target product (*S*)-ketoprofen in unreacted form was investigated. The effects of enzyme loading, substrate concentration, alcohol to acid molar ratio, type of solvents and reaction temperature were studied. The optimum reaction conditions obtained were 200 mg/ml of CALB and racemic ketoprofen concentration of 30 mM, with 20% (v/v) 1,2-dichloropropane–80% (v/v) *n*-hexane as solvent, alcohol to acid molar ratio of 1:1 at 40 °C. The conversion of (*R*)-ketoprofen, X_R obtained was 81% with enantioselectivity (*E*) value of 10, enantiomeric excess of product (ee_p) of 66.4% and enantiomeric excess of substrate (ee_s) of 59.4% for the reaction time of 24 h.

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Keywords: *Candida antarctica* lipase B; Ketoprofen; Enzymatic esterification; Enantiomer; Enantiopure

1. Introduction

The lipase obtained from *Candida antarctica* fraction B (CALB) is one of the most commonly used lipases [1]. It is commercially available in both free and immobilized form. CALB is a globular protein with an alpha/beta hydrolase fold. It consists of 317 amino acids and its molecular weight is 33 kDa [2]. The lipase originates from the basidiomyceteous yeast *Candida antarctica* [2]. The enantioselectivity of CALB towards *R*-enantiomer is well known and has been proven in several reports [3–5].

Ketoprofen [2-(3-benzoylphenyl) propionic acid] is a non-steroidal anti-inflammatory drugs (NSAID) with analgesic, anti-inflammatory and antipyretic properties, and is effective for the treatment of rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis [6,7]. Its anti-inflammatory effect is approximately 160 times the anti-inflammatory potency of aspirin on a unit weight basis [8]. It is found that these healing properties are mainly exerted by the (*S*)-enantiomer [7,9–13], while the (*R*)-enantiomer is effective as a therapeutic agent for toothache [3,14]. There is always a danger that one enantiomer of a drug

may possess the desired activity and the other, although inactive in producing the desired activity, may possess extraneous and even harmful pharmacologic properties [15]. However, still a major reason for the use of enantiomers mixtures is that the cost of separation of the enantiomers exceeds the potential advantage of a possible increase in the activity. Therefore, it has been a key issue in pharmaceutical industry to obtain the pure, active form of ketoprofen. An alternative enzymatic route in the preparation of enantiopure (*S*)-ketoprofen using lipase as biocatalyst is gaining importance because of their remarkable properties (e.g. regio, stereo and substrate specificity), milder reaction conditions reduction in the energy requirements and the production cost [16].

Although enzymatic preparation of enantiopure (*S*)-ketoprofen through various pathways has been well documented [3–6,10–14,17–19] and has been highlighted in previous study [20], none of them were studied using free CALB. Indeed, understanding of the basic performance of free lipase is very important before it can lead to any improvement of enzyme properties. The aim of the present study is to investigate the behaviour of free CALB for enantioselective esterification of racemic ketoprofen in the organic medium. The effect of enzyme loading, substrate concentration, alcohol to acid molar ratio, type of reaction medium and reaction temperature are reported.

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2. Materials and experimental methods

2.1. Materials

The lipase from *Candida Antarctica* fraction B (Novozym CALB L) was obtained from Novo Nordisk (Bagsvaerd, Denmark). The racemic ketoprofen was purchased from Shanghai Huaqu Imp. & Exp. Co. (Pudong, Shanghai, China). All other chemicals used in this work were of analytical grade and were supplied by Fisher Scientific (Nepean, Ont., Canada) and used without further purification.

2.2. Enzymatic preparation of (S)-ketoprofen

The enzymatic preparation of (S)-ketoprofen through enantioselective esterification reaction in an organic media was conducted in a 250 ml erlenmeyer flask. The racemic ketoprofen was added into 25 ml of selected organic solvent containing appropriate amount of *n*-butanol and enzyme (CALB). The mixture was incubated at 37 °C and shaken continuously at 250 rpm in an incubator shaker. Periodically, 1 ml aliquot was withdrawn from the reaction medium for HPLC analysis.

The effect of enzyme loading on the esterification reaction was studied by varying the amount of enzyme between 60 and 200 mg/ml keeping all other parameters constant. A similar mixture without enzyme was prepared under the same conditions and used as a control reaction.

To determine the effect of substrate concentration, the concentration of racemic ketoprofen was varied from 10 to 100 mM at a fixed alcohol to acid molar ratio while other parameters were held constant. In order to investigate the effect of alcohol to acid molar ratio on the reaction, the ratio of alcohol to acid was increased from 0:1 to 5:1.

The solvent effect was investigated by carrying out the reaction in different organic solvents. The solvents used were varied in dichloromethane–hexane fraction and 1,2-dichloropropane–hexane fraction.

The influence of temperature on the reaction was investigated by varying the temperature in the range of 30–60 °C.

2.3. Analytical methods

The concentration of ketoprofen enantiomers was determined using a HPLC (Shimadzu, Kyoto, Japan) equipped with a chiral stationary phase column: (R,R)-Whelk-O1 chiral column (Regis Technologies, Morton Grove, USA) and a UV detector (254 nm). A mixture of dichloromethane, *n*-hexane and ethanol (0.25 M ammonium acetate) (47:47:6, v/v/v) was used as an eluent. The flow rate was maintained at 1.5 ml/min and the column temperature at 25 °C. Sixty microliters of the sample was injected. The respective retention times of (R)-ketoprofen ester, (R)-ketoprofen acid and (S)-ketoprofen acid were 1.95, 16 and 17.9 min.

The conversion of ketoprofen ester was calculated using the following equations:

$$X(\%) = \frac{C_0 - C_i}{C_0} \times 100 \quad (1)$$

$$X_R(\%) = \frac{C_{R0} - C_{Ri}}{C_{R0}} \times 100 \quad (2)$$

where X is the overall conversion (%), X_R the conversion (%) of (R)-ketoprofen (fast reacting enantiomer), C_0 the initial amount of racemic ketoprofen (mM), C_i the amount of racemic ketoprofen (mM) at reaction time i , C_{R0} the initial amount of (R)-ketoprofen and C_{Ri} is the amount of (R)-ketoprofen (mM) at reaction time i .

Enantiomeric excess of substrate (ee_s) and product (ee_p) and enantiomeric ratio or enantioselectivity (E) were calculated using the following equations [3]:

$$ee_s(\%) = \frac{[S]_{acid} - [R]_{acid}}{[S]_{acid} + [R]_{acid}} \times 100 \quad (3)$$

$$ee_p(\%) = \frac{[R]_{ester} - [S]_{ester}}{[R]_{ester} + [S]_{ester}} \times 100 \quad (4)$$

$$E = \frac{\ln[1 - (X/100)(1 + (ee_p/100))]}{\ln[1 - (X/100)(1 - (ee_p/100))]} \quad (5)$$

where $[R]_{acid}$ and $[S]_{acid}$ represent the concentration of the (R) and (S) enantiomers of ketoprofen acid, while $[R]_{ester}$ and $[S]_{ester}$ represent the concentration of the (R) and (S) enantiomers of ketoprofen ester.

3. Results and discussion

3.1. Effect of enzyme loading

Enzymes have regioselectivity to simplify reaction steps and work under mild conditions, which make biocatalytic processes more competitive than chemical processes. The optimum amount of enzyme is important for shorter reaction time as well as higher yield to make the process more cost attractive. The effect of enzyme loading on the enantioselective esterification of racemic ketoprofen is shown in Fig. 1. The conversion increases dramatically with increasing amount of enzyme for the range of enzyme loading studied. The highest conversion of 57% was obtained for the enzyme loading of 200 mg/ml. No ester formation was detected for the reaction system in the absence of enzyme (data not shown). The higher enzyme concentration provided more active sites for substrate binding to enhance the reaction. Further experiments were carried out with CALB at 200 mg/ml to study the effect of other parameters.

3.2. Effect of substrate concentration

The substrate concentrations were varied from 10 to 100 mM, keeping the alcohol to acid molar ratio constant at 4:1. The effect of substrate concentration on the conversion is shown in Fig. 2. The conversion was first increased with increasing the racemic ketoprofen concentration up to 30 mM and then decreased at higher substrate concentration. The highest conversion of 93.4% was obtained at 30 mM of racemic ketoprofen for the reaction time of 96 h. However, it dropped with a further increase in substrate concentration. The loss of effectiveness of the enzyme measured in terms of conversion at higher substrate concentra-

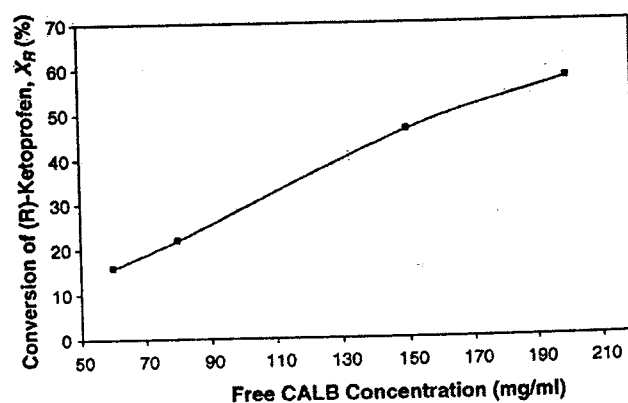


Fig. 1. Effect of enzyme loading on the conversion of (R)-ketoprofen. Reaction conditions: 50 mM of racemic ketoprofen, 4:1 molar ratio of *n*-butanol to racemic ketoprofen, reaction time = 48 h, hexane:dichloromethane (80:20, %v/v) as solvent, $T = 37$ °C, shaken at 250 rpm.

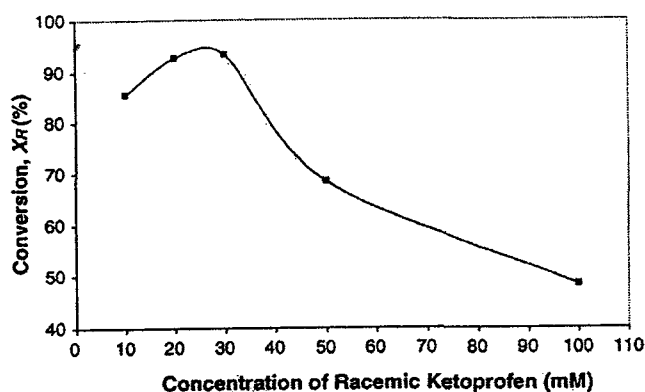


Fig. 2. Effect of substrate concentration (racemic ketoprofen) on the conversion of (*R*)-ketoprofen. Reaction conditions: 4:1 molar ratio of *n*-butanol to racemic ketoprofen, reaction time = 96 h, hexane:dichloromethane (80:20, %v/v) as solvent, 200 mg/ml of free CALB, $T = 37^\circ\text{C}$, shaken at 250 rpm.

tion may result from several factors including: (i) the properties of the medium (e.g. polarity) were modified at higher acid concentration [16]; (ii) the unreactive carboxyl group ($-\text{COOH}$) of substrate occupying the active site unproductively was significant at higher substrate concentration [1]; (iii) more water released at higher substrate concentration during esterification and favoured the hydrolysis reaction [21]; (iv) the amount of enzyme became the limiting substance at higher substrate concentration. Maugaid et al. [22] also reported that the catalytic site of CALB contained acid or basic residues which are able to take or to give a proton during catalytic reaction. The concentration of these ionisable groups being very high in the catalytic site and are very efficient acid/base catalysts. This is called the charge-relay system and originates from the alignment of Asp, His and Ser, linked by hydrogen bonds [22]. A small variation of pH (variation of acidic substrate concentration in the reaction medium) could influence the enzymatic activity, modifying the protonation state of an ionisable group of catalytic site, and so modify the reaction. The racemic ketoprofen concentration up to 30 mM was observed to be the optimum substrate concentration for the reaction.

3.3. Effect of substrate molar ratio

In order to achieve the highest product yield with the minimum side product, the molar ratio of substrates was optimized. The concentration of racemic ketoprofen was held constant, whereas the quantity of alcohol was varied to show the effect of the amount of nucleophilic agent (alcohol) on the reaction

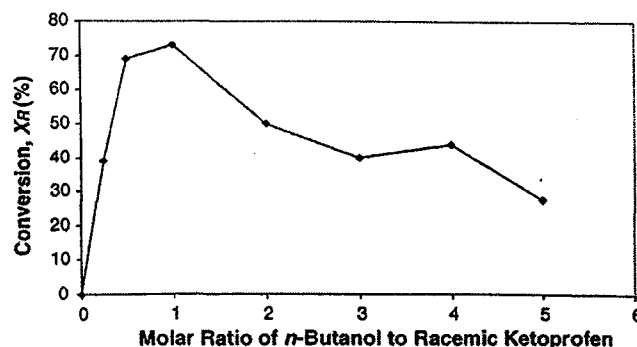


Fig. 3. Effect of *n*-butanol to racemic ketoprofen molar ratio on the conversion of (*R*)-ketoprofen. Reaction conditions: 30 mM of racemic ketoprofen, reaction time = 72 h, hexane:dichloromethane (80:20, %v/v) as solvent, 200 mg/ml of free CALB, $T = 37^\circ\text{C}$, shaken at 250 rpm.

performance. Fig. 3 shows the effect of molar ratio of *n*-butanol to racemic ketoprofen over the conversion. The highest conversion was obtained when the molar ratio of *n*-butanol to racemic ketoprofen was equivalent to 1:1. As the molar ratio increased from 1:1 to 5:1, the conversion was dropped from 72.9 to 28.3% for the reaction time of 72 h. The effect of substrate inhibition by the alcohol became notable and caused the conversion to fall. Duan et al. [23] reported that the reaction rate decreased with the increase in alcohol concentration. Alcohol was a dead end inhibitor of the enzyme that binds to an enzyme species and forms another species that can no longer participate in the esterification reaction (Bi Bi Ping Pong mechanism) [23]. Chua and Sarmidi [24] also reported that the decrease in final conversion value at high alcohol concentration was due to the enzyme deactivation by the alcohol because of the log P value of the alcohol was relatively low (less hydrophobic) where log P is defined as the logarithm of the partition coefficient of a given component in the octanol–water two-phase system [25]. The enzymes exhibited optimum activity in organic solvents with the higher log P values (more hydrophobic) [24].

In the literature, the most efficient alcohol to substrate molar ratio was found to be 3 propanol and 4 ethanol equivalents. Table 1 compiles the results of the present work to those reported in literature. Inspection of the data in Table 1 indicates that the higher carbon chain length of the alcohol, the lower the molar ratio of alcohol to acid was required for the esterification reaction. The chain length of alcohol was found to significantly affect the esterification reaction. Duan and Ching [26] reported that the increase in the carbon chain length of the alcohols, the activity of the enzyme decreased was observed in their work.

Table 1
Optimum alcohol to acid molar ratio in the present work and the literatures

Author [Ref.]	Racemic ketoprofen concentration (mM)	Enzyme	Solvent	Alcohol (molar ratio)	T ($^\circ\text{C}$)	X_R (%)
Park et al. [5]	5×10^{-6}	Novozym@435 (50 mg/ml)	CCl_4 , EDC	Ethanol (4:1)	37	76 (76 h)
Antona et al. [4]	4×10^{-5}	Novozym@435 (10 mg)	DIPE	Propanol (3:1)	45	60 (24 h)
Present work	30	Free CALB (200 mg/ml)	CH_2Cl_2 , hexane	<i>n</i> -Butanol (1:1)	37	73 (72 h)

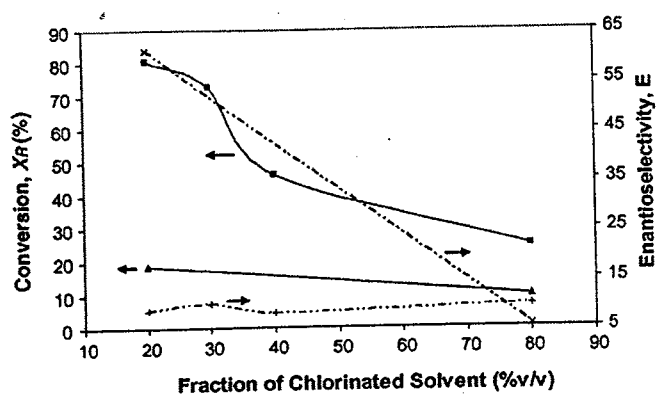


Fig. 4. Effect of chlorinated solvent fraction in *n*-hexane on the enantioselective esterification of racemic ketoprofen. Reaction conditions: 30 mM of racemic ketoprofen, 1:1 molar ratio of *n*-butanol to racemic ketoprofen, reaction time = 24 h, 200 mg/ml of free CALB, $T = 37^\circ\text{C}$, shake at 250 rpm. (■) Conversion for 1,2-dichloropropane, (▲) conversion for dichloromethane, (+) enantioselectivity for 1,2-dichloropropane and (x) enantioselectivity for dichloromethane.

3.4. Effect of reaction medium

Enzymatic catalysis in organic solvents significantly broadens conventional aqueous-based biocatalysis. Ghanem and Aboul-Enein [27] stated that water is a poor solvent for nearly all applications in industrial chemistry since most organic compounds of commercial interest are sparingly soluble and are sometimes unstable in aqueous solutions. The removal of water is tedious and expensive due to its high boiling point and high heat of vaporization. In contrast, the advantages of using organic solvents are: (i) the use of low boiling point organic solvents facilitates the recovery of the solvent and product with better overall yield; (ii) non-polar substrates are converted at a faster rate due to their increased solubility in the organic solvent; (iii) deactivation and/or substrate or product inhibition is minimized; (iv) side reactions are largely suppressed; (v) immobilization of enzyme is not required; (vi) denaturation of enzymes is minimized; (vii) shifting thermodynamic equilibria to favour synthesis over hydrolysis [27]. Xu et al. [28] reported that to favour the enzymatic catalysis, a reaction medium should satisfy the following two conditions: (i) it should have high log P value and (ii) it should readily dissolve the substrate. In order to improve the enantioselectivity of enzyme and the solubility of racemic ketoprofen in *n*-hexane, fraction of chlorinated solvent was used in the present study. The effect of chlorinated solvents fraction in *n*-hexane on the enzymatic behaviour were studied and illustrated in Fig. 4. The conversion decreased when the fraction of both chlorinated solvents in *n*-hexane were increased. Low conversion (19%) was obtained for the solvent composing of 20% (v/v) of dichloromethane/hexane. The solvent composed of 20% (v/v) of 1,2-dichloropropane/hexane showed the highest conversion of 81%. However, dichloromethane/hexane at the fraction of 20% (v/v) exhibited higher enantioselectivity (Fig. 4). The enantioselectivity decreased as the fraction of dichloromethane was increased. The enantioselectivity of enzyme was not influenced by the fraction of 1,2-chloropropane

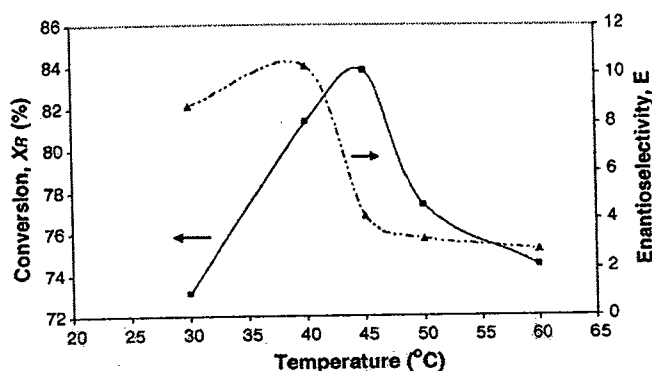


Fig. 5. Effect of reaction temperature on the enantioselective esterification of racemic ketoprofen. Reaction conditions: 30 mM of racemic ketoprofen, 1:1 molar ratio of *n*-butanol to racemic ketoprofen, reaction time = 24 h, hexane:1,2-dichloropropane (80:20, %v/v) as solvent 200 mg/ml of free CALB, shake at 250 rpm. (■) Conversion and (▲) enantioselectivity.

in *n*-hexane. Although the polarity of solvent greatly affected the catalytic activity, but it does not influence the enzyme enantioselectivity [24]. Due to the solubility limitation of ketoprofen in the fraction of chlorinated solvent less than 20%, the chlorinated solvent composition below 20% was not studied in the present work. Thus, the best fraction of dichloromethane and 1,2-dichloropropane in *n*-hexane was found at 20% (v/v) and gave the best results in terms of the enantioselectivity and conversion. The active site pocket of the CALB is accessible to external solvent and substrates through a narrow deep hydrophobic channel. The substrate accessibility to the active site through the narrow deep hydrophobic channel become difficult as the fraction of chlorinated solvent (less hydrophobic solvent) was increased. Thus, at higher chlorinated solvents fraction the conversion decreased. The result obtained was in agreement with the work of Park et al. [5] and Antona et al. [4] that using the same enzyme, CALB. Park et al. [5] reported that the highest conversion was obtained in *n*-hexane (but low in enantioselectivity), the highest enantioselectivity was observed in chlorinated solvent (1,2-dichloroethane; but low in conversion) and the highest in terms of both conversion and enantioselectivity were obtained with the solvent containing 20% (v/v) 1,2-dichloroethane-80% (v/v) *n*-hexane. Antona et al. [4] reported that the highest enantioselectivity was obtained using chlorinated solvent such as 1,2-dichloropropane and 1,2-dichloroethane. Besides, Liu et al. [29] quoted that the activity of lipase from *Candida rugosa* in esterification of ketoprofen was the highest when using cyclohexane or *iso*-octane as main solvents and toluene as co-solvent.

3.5. Effect of reaction temperature

The reaction temperature has a great influence on enzymatic reaction. The effect of temperature on performance of CALB in enzymatic esterification of racemic ketoprofen is shown in Fig. 5. The conversion increased when the reaction temperature was increased. The conversion reached maximum with the value of 84% at 45°C . At temperature higher than 45°C , the conversion decreased dramatically. The enantioselectivity increased

with increasing reaction temperature up to 40 °C. It decreased rapidly when the temperature rose to 45 °C. At higher temperature, the enantioselectivity decreased gradually. Reaction temperature of 40 °C was selected as an optimum temperature for the reaction as it exhibited highest enantioselectivity and relatively high conversion of 81% with the ee_p of 66.4% and ee_s of 59.4% (data not showed) at the reaction time of 24 h. The present work was in good agreement with the work of Chua and Sarmidi [24]. The CALB was unable to exhibit its highest activity at low temperature. At high temperature, the enzymes were partially deactivated. The elevated temperature is one of the most common causes for the enzyme deactivation, which results in the loss of the catalytically competent conformation [30]. Besides, alcohols are reported to be terminal inhibitors of lipases and their effects could be increased at high temperatures due to the increase of alcohol solubility in the reaction time [21].

4. Conclusions

The effects of various reaction parameters on the performance of CALB in the preparation of enantiopure (*S*)-ketoprofen were studied. The results showed that:

- (i) The conversion increased dramatically with increasing of enzyme loading. The highest conversion of 57% was obtained for the enzyme loading of 200 mg/ml.
- (ii) The highest conversion of 93.4% was obtained at 30 mM of racemic ketoprofen for the reaction time of 96 h. The conversion dropped at the higher racemic ketoprofen concentration.
- (iii) The optimum alcohol to acid molar ratio was 0.5:1. The longer carbon chain length of the alcohol may require lower molar ratio.
- (iv) The performance of CALB was found to be optimum when the solvent contained 20% (v/v) of 1,2-dichloropropane in *n*-hexane.
- (v) The reaction temperature of 40 °C gave the highest enantioselectivity (10) and relatively high conversion (81%) of (*R*)-enantiomer with the ee_p of 66.4% and ee_s of 59.4% at the reaction time of 24 h.

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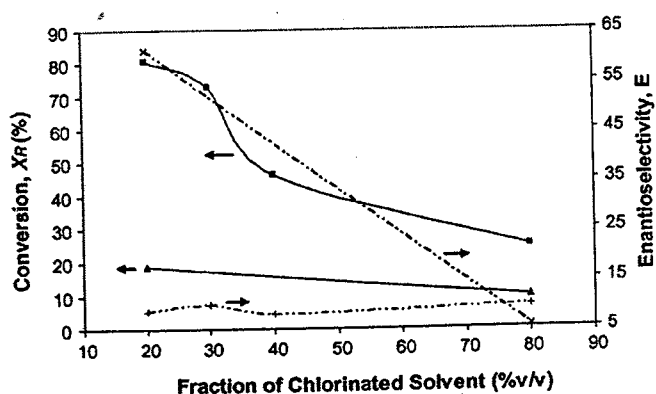


Fig. 4. Effect of chlorinated solvent fraction in *n*-hexane on the enantioselective esterification of racemic ketoprofen. Reaction conditions: 30 mM of racemic ketoprofen, 1:1 molar ratio of *n*-butanol to racemic ketoprofen, reaction time = 24 h, 200 mg/ml of free CALB, $T = 37^\circ\text{C}$, shake at 250 rpm. (■) Conversion for 1,2-dichloropropane, (▲) conversion for dichloromethane, (+) enantioselectivity for 1,2-dichloropropane and (x) enantioselectivity for dichloromethane.

3.4. Effect of reaction medium

Enzymatic catalysis in organic solvents significantly broadens conventional aqueous-based biocatalysis. Ghanem and Aboul-Enein [27] stated that water is a poor solvent for nearly all applications in industrial chemistry since most organic compounds of commercial interest are sparingly soluble and are sometimes unstable in aqueous solutions. The removal of water is tedious and expensive due to its high boiling point and high heat of vaporization. In contrast, the advantages of using organic solvents are: (i) the use of low boiling point organic solvents facilitates the recovery of the solvent and product with better overall yield; (ii) non-polar substrates are converted at a faster rate due to their increased solubility in the organic solvent; (iii) deactivation and/or substrate or product inhibition is minimized; (iv) side reactions are largely suppressed; (v) immobilization of enzyme is not required; (vi) denaturation of enzymes is minimized; (vii) shifting thermodynamic equilibria to favour synthesis over hydrolysis [27]. Xu et al. [28] reported that to favour the enzymatic catalysis, a reaction medium should satisfy the following two conditions: (i) it should have high log P value and (ii) it should readily dissolve the substrate. In order to improve the enantioselectivity of enzyme and the solubility of racemic ketoprofen in *n*-hexane, fraction of chlorinated solvent was used in the present study. The effect of chlorinated solvents fraction in *n*-hexane on the enzymatic behaviour were studied and illustrated in Fig. 4. The conversion decreased when the fraction of both chlorinated solvents in *n*-hexane were increased. Low conversion (19%) was obtained for the solvent composing of 20% (v/v) of dichloromethane/hexane. The solvent composed of 20% (v/v) of 1,2-dichloropropane/hexane showed the highest conversion of 81%. However, dichloromethane/hexane at the fraction of 20% (v/v) exhibited higher enantioselectivity (Fig. 4). The enantioselectivity decreased as the fraction of dichloromethane was increased. The enantioselectivity of enzyme was not influenced by the fraction of 1,2-chloropropane

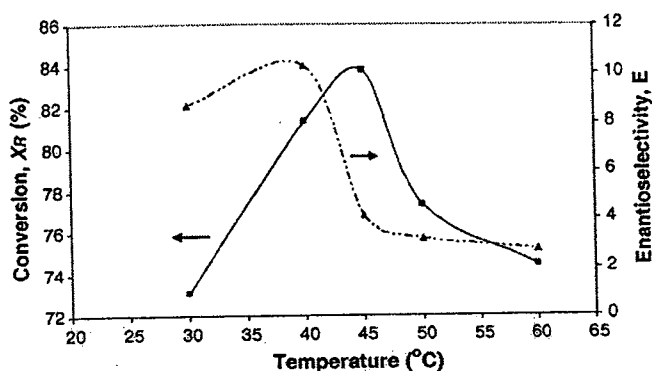


Fig. 5. Effect of reaction temperature on the enantioselective esterification of racemic ketoprofen. Reaction conditions: 30 mM of racemic ketoprofen, 1:1 molar ratio of *n*-butanol to racemic ketoprofen, reaction time = 24 h, hexane:1,2-dichloropropane (80:20, %v/v) as solvent 200 mg/ml of free CALB, shake at 250 rpm. (■) Conversion and (▲) enantioselectivity.

in *n*-hexane. Although the polarity of solvent greatly affected the catalytic activity, but it does not influence the enzyme enantioselectivity [24]. Due to the solubility limitation of ketoprofen in the fraction of chlorinated solvent less than 20%, the chlorinated solvent composition below 20% was not studied in the present work. Thus, the best fraction of dichloromethane and 1,2-dichloropropane in *n*-hexane was found at 20% (v/v) and gave the best results in terms of the enantioselectivity and conversion. The active site pocket of the CALB is accessible to external solvent and substrates through a narrow deep hydrophobic channel. The substrate accessibility to the active site through the narrow deep hydrophobic channel become difficult as the fraction of chlorinated solvent (less hydrophobic solvent) was increased. Thus, at higher chlorinated solvents fraction the conversion decreased. The result obtained was in agreement with the work of Park et al. [5] and Antona et al. [4] that using the same enzyme, CALB. Park et al. [5] reported that the highest conversion was obtained in *n*-hexane (but low in enantioselectivity), the highest enantioselectivity was observed in chlorinated solvent (1,2-dichloroethane; but low in conversion) and the highest in terms of both conversion and enantioselectivity were obtained with the solvent containing 20% (v/v) 1,2-dichloroethane-80% (v/v) *n*-hexane. Antona et al. [4] reported that the highest enantioselectivity was obtained using chlorinated solvent such as 1,2-dichloropropane and 1,2-dichloroethane. Besides, Liu et al. [29] quoted that the activity of lipase from *Candida rugosa* in esterification of ketoprofen was the highest when using cyclohexane or *iso*-octane as main solvents and toluene as co-solvent.

3.5. Effect of reaction temperature

The reaction temperature has a great influence on enzymatic reaction. The effect of temperature on performance of CALB in enzymatic esterification of racemic ketoprofen is shown in Fig. 5. The conversion increased when the reaction temperature was increased. The conversion reached maximum with the value of 84% at 45°C . At temperature higher than 45°C , the conversion decreased dramatically. The enantioselectivity increased

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Studies of reaction parameters on synthesis of Citronellyl laurate ester via immobilized *Candida rugosa* lipase in organic media

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Abstract

Immobilized *Candida rugosa* lipase was used for the synthesis of citronellyl laurate from citronellol and lauric acid. Screening of different types of support (Amberlite MB-1 and Celite) for immobilization of lipase and solvent (*n*-hexane, *n*-heptane, and *iso*-octane) and optimization of reaction conditions such as catalyst loading, effect of substrates molar ratio and temperature have been studied. The maximum enzyme activity was obtained at 310K. The immobilized *Candida rugosa* lipase onto Amberlite MB-1 support was found to be the best support with conversion of 89% of citronellyl laurate ester in *iso*-octane compared to Celite 545. Deactivation of *Candida rugosa* lipase at 313K, 318K and 323K were observed. Ordered bi bi mechanism with dead end complex of lauric acid was found to fit the initial rate data and the kinetic parameters were obtained by non-linear regression analysis.

Keywords: Lipase, *Candida rugosa*, Esterification, Amberlite MB-1, ordered bi bi mechanism.

Introduction

Esters are one of the most important natural fragrances. The traditional extractions from plant materials and direct biosynthesis by fermentation are the two generally used methods for flavor and fragrance production [1]. Natural flavor esters extracted from plant materials are often either too scarce or expensive for commercial use. For industrial use, flavors are usually produced by chemical synthesis and are not considered as natural products. Thus, their market value is less than esters from natural sources. Such esters however may be considered natural when produced by lipase-mediated synthesis [2].

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and industrial potential. Lipases are conventionally defined as enzymes which catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. Enzymes such as lipases accept a broad range of substrates and they are readily available at low cost and require no cofactor for reaction [3]. Both native and immobilized lipases are available commercially. Crude lipases are used in laundry detergents and many other applications; however, an increasing number of specialty applications of lipases in synthesis and biotransformation demand the use of immobilized biocatalyst for production efficiency and process economics.

Economically, crude lipases can be easily found in the market today but they are costly. The possibility of regenerating and reusing the enzyme would be an attractive field in biocatalysis. Immobilization is believed to stabilize the enzyme molecules and supporting materials [4]. Immobilization helps to keep the dispersion of enzyme in organic solvents homogenous, which is essential for efficient enzymatic reactions [5]. Additionally, when enzymes are present in solution they will leave the reactor with end

products and this means separation stage must be performed to separate end products from the enzyme and new enzymes will be used in the next process. Conversely, using an immobilized enzyme will simplify product recovery in which the enzymes can be reused and placed near other enzymes and by this way a catalytic sequence is produced [6].

Moreover, immobilization will enhance the stability of lipase due to the ability of the support material chosen to retain just the right amount of water for the enzyme to remain active. Selecting the right support material is crucial in esterification process using immobilized lipase. The best support material should allow the effective utilization of the enzyme by having the enzyme molecules accessible to the substrates [4]. This study was carried out by immobilizing the *Candida rugosa* lipase using adsorption method on two types of support which were Amberlite MB-1 and Celite.

Esters of cyclic terpene alcohol citronellol with long-chain fatty acid have attracted researchers recently in investigating their aroma qualities. Many publications of terpene esters mostly reported on the use of short-chain fatty acid. Shintre *et al.* (2002) carried out esterification of lauric acid with various fatty alcohols using Lipolase 100 L lipase. Yadav *et al.* (2004) have synthesized lauric acid with citronellol with various lipases and they reported Novozym SP 435 (*Candida antartica*) lipase was found to be the best catalyst with 53% conversion at 303K.

It is known that the lipase-catalyzed esterification of fatty acid with fatty alcohol follows the reversible Ping Pong Bi-Bi type mechanism [7]. Many researchers have also proposed kinetic models for lipase catalyzed esterification and transesterification reactions in organic media based on this mechanism [1, 8-10]. Yadav and Lathi (2004) had proposed for the lipase catalyzed reactions for the synthesis of citronellyl laurate was

an ordered bi bi mechanism with dead-end complex of lauric acid. In spite of the fact that ping pong bi bi and ordered bi bi mechanism were proposed by researchers, kinetic study for both mechanisms has been carried out. The present article also discusses on the reaction parameters affecting lipase-catalyzed synthesis of citronellyl laurate using immobilized *Candida rugosa* lipase on various types of support material, various types of solvent, pH, effect of substrate concentration and reaction temperature.

Enzyme Kinetics

There are two general types of kinetic mechanism recognized for bi bi reaction, *i.e.* ping pong (non-sequential) or ternary complex (sequential) mechanism [11, 12]. In ping pong mechanism, only one substrate is bound to the enzyme at any time to form the acyl-enzyme complex. As soon as one product is formed and released, the other substrate binds to the modified enzyme (substituted form of the free enzyme) to form the second product. While in the ternary complex mechanism, two substrates [A] and [B] are bound to the enzyme [E] in either a specific or random order to form an [ABE] complex, which then reacts to form the products [P] and [Q] [13, 14].

Currently, many researchers accepted ping pong mechanism for two-substrate two-product (bi bi) reactions for lipase-catalyzed esterification and transesterification reaction in organic media [9, 15-17]. Therefore, it is necessary to consider the enzyme kinetics using ping pong mechanism model. The reaction mechanism for the ping pong bi bi mechanism with dead end complex of lauric acid can be illustrated by the Cleland convention diagram as shown in Figure 1:

According to it, the lipase (E) may react with lauric acid [B] to yield a dead end complex (EB) or it may bind to A (DL-Citronellol) site to give EA. EA is the enzyme citronellol complex. F is the modified formed of enzyme which will react with P (citronellyl laurate) forming FP, the modified enzyme citronellyl laurate as intermediate complexes before P was released. Similarly, F and lauric acid [B] then binds together to form modified enzyme lauric acid complex (FB) which is an ineffective complex. At the same time, EQ, the enzyme water complex was also formed before second product Q (water) was released.

The resultant equation for the ping pong bi bi reaction with dead end complex of lauric acid sequence is expressed by eq. (1) [18].

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

where, v is the initial rate of reaction ($\text{mol L}^{-1} \text{min}^{-1} \text{g}^{-1}$ of enzyme); $V_{\max}$ is maximum rate of reaction ($\text{mol L}^{-1} \text{min}^{-1} \text{g}^{-1}$ of enzyme); $[A]$ is initial concentration of citronellol ($\text{mol L}^{-1} \text{g}^{-1}$ of enzyme), $[B]$ is the initial concentration of lauric acid ($\text{mol L}^{-1} \text{g}^{-1}$ of enzyme); K_B is Michealis Menten constant for lauric acid ($\text{mol L}^{-1} \text{g}^{-1}$ of enzyme); K_A is the Michealis Menten constant for citronellol ($\text{mol L}^{-1} \text{g}^{-1}$ of enzyme); K_i is inhibition constant for lauric acid ($\text{mol L}^{-1} \text{g}^{-1}$ of enzyme).

The reaction sequence for ordered bi bi mechanism with dead end complex inhibition is shown in Figure 2 where A is citronellol; B is lauric acid; EA is enzyme citronellol complex; BE is dead end enzyme lauric acid complex; BEB is dead end enzyme lauric acid complex; EB is effective enzyme lauric acid complex; EAB is effective enzyme citronellol lauric acid complex; P is citronellol laurate and Q is water. It is assumed that the binding of B to the A site does not prevent B from binding to its usual site so that dead end complexes form, BE and BEB [18].

In the case of lipase-catalyzed reactions, it has been established that the lipase first forms an acyl enzyme complex with the acyl donor, ruling out the random mechanism [1]. Thus, it was suggested by Yadav and Lathi (2004) that lipase catalyzed reactions was an ordered bi bi mechanism with dead-end complex of lauric acid. The resultant equation for ordered bi bi reaction sequence is expressed by eq (2) with its symbol and coefficients being identically defined as those in eq. (1) with additional terms of $\alpha=1$ and $\beta=1$ (dimensionless) [18].

$$\frac{v}{V_{\max}} = \frac{[A]}{\alpha K_A \left(1 + \frac{K_B}{[B]} + \frac{K_B}{K_i} + \frac{[B]}{\beta K_i} \right) + [A] \left(1 + \frac{\alpha K_B}{[B]} \right)} \quad (2)$$

Material and methods

3.1 Materials

Lauric acid was purchased from Fluka (Switzerland) with 98% purity and DL-Citronellol (95% pure) from Sigma Aldrich (USA). Lipase (EC 3.1.1.3) from *Candida rugosa* (Type VII) was purchased from Sigma-Aldrich (Japan), support Amberlite MB-1 from Sigma-Aldrich (USA), and Celite from Fluka (Switzerland). *n*-heptane and *n*-hexane are supplied by Merck (Germany) and *iso*-octane (99.84% assay) by Fischer Chemicals (UK). All chemicals are of analytical grade and used without further purification.

Lipase Immobilization

Lipase from *Candida rugosa* (E.C.3.1.1.3) with nominal specific lipolytic activity of 910 unit/mg was immobilized by adsorption onto Amberlite MB-1 and Celite 545 supports. During immobilization, the enzyme (20 mg) was dissolved in 5 ml sodium phosphate buffer (pH 7.5) and mixed with 2 g of dried support and stirred at 200 rpm for 24 hr at 310K. The immobilized lipase was filtered and washed thoroughly with deionized water and rinsed with sodium phosphate buffer solution of the same pH. The resultant immobilized lipase was then dried in a vacuum oven at room temperature.

Esterification method

Esterification was carried out in 100 ml screw-capped bottle. The reaction mixture consisted of 30 mM of DL-Citronellol and 20 mM of lauric acid diluted to 10 ml with

iso-octane (otherwise specified) as solvent. The reaction mixture was added with 60 mg of immobilized lipase and agitated at 310K at a speed of 200 rpm in an orbital shaker (Certomat, B. Braun). *Iso*-octane was the solvent used in all the experiments except in the solvent screening studies. Aliquots of the reaction mixture were taken for analysis after 24 hr of reaction time and analyzed by gas chromatography. All other reaction parameters were kept constant unless otherwise specified.

Sample Analysis

The concentration of the reactants and products were determined by gas chromatography (Perkin Elmer Autosystem XL, USA) equipped with a flame ionization detector (FID). The column used was NukolTM fused silica capillary column (Supelco, USA) with dimension of 0.53 mm i.d. x 15 m x 0.50 μ m film thicknesses. Injector and detector temperatures were set at 473K and 523K, respectively. After injection of sample, the temperature of the column oven was kept constant for 1 min (323K), linearly increased to 463K (10K/min). The carrier gas used was helium. Conversion of the lauric acid in the reaction was calculated by comparing the initial and final acid concentration.

Results and discussion

Effect of hydrophilic/hydrophobic supports for lipase immobilization

After immobilization, the *Candida rugosa* lipase was used to synthesize citronellyl laurate with varying loading of immobilized lipase to determine the best support material to be used in subsequent experiments. The results obtained in the immobilization studies of *Candida rugosa* lipase on two different types of support are

shown in Figure 3. As observed, the catalytic conversion shown by Amberlite MB-1 was much better compared to Celite 545 as support material for immobilization of *Candida rugosa* lipase in the synthesis of citronellyl laurate.

Lower enzymatic activity is observed for Celite 545 for the synthesis of citronellyl laurate. Conversely, Amberlite-MB1 showed an increase in conversion value with increase amount of immobilized lipase. The result clearly shows that Celite 545 support did not work well for *Candida rugosa* lipase. Amberlite MB-1 are hydrophobic beads with both strongly acidic and basic resin [19]. Other common hydrophobic supports include polyethylene, polypropylene, styrene, and acrylic polymers. Celite 545 belongs to the hydrophilic supports among the others include Duolite, Celite, silica gel, activated carbon, clay and Sepharose. Hydrophilic supports are generally characterized by high losses of lipase activity upon immobilization. These losses in lipase activity have been attributed to (i) a change in the conformation of the lipase upon adsorption to a form which has reduced activity; (ii) a situation in which only small amount of lipase is immobilized; (iii) a decrease in the ability of hydrophobic substrates to reach the active site of the lipase; or (iv) the existence of steric hindrance imposed by the carrier matrix which constrains the flexibility of the lipase molecule [5]. Use of microporous supports with a hydrophobic character (e.g., made from standard and high density polypropylene, or high density polyethylene) leads to very good performance for the immobilized lipase [5]. Dahlan (2004) also found Amberlite MB-1 to be the best support in the synthesis of citronellyl butyrate by direct esterification using continuous packed bed reactor.

Effect of different solvents

High hydrophobic solvents with $\log P > 4$ are considered the most suitable solvent for biocatalysis. The solvents with $\log P$ values between 2 and 4 are moderately effective. Those polar solvents with $\log P < 2$ are often ineffective. Although $\log P$ value does not always correlate with the degree of enzymatic synthesis, it provides valuable information for solvent selection [20]. Three different types of solvent had been chosen to investigate the effect of reaction media. Experiments were conducted with *n*-hexane ($\log P = 3.5$), *n*-heptane ($\log P = 4.0$) and *iso*-octane ($\log P = 4.5$).

An acid conversion of 75.9% was achieved with *n*-hexane, 75.8% with *n*-heptane and 89.4% acid conversion with *iso*-octane when 60 g/l of immobilized lipase was used in the esterification process (Figure 4). At 80 g/L of enzyme loading, 94.3% conversion was observed for *iso*-octane. Enzyme loading at 100 g/L does not exhibit higher conversion value but the same as that at 80 g/L loading. A similar trend was also observed for *n*-hexane and *n*-heptane. This can be explained by the leveling off behavior of esterification at higher enzyme concentration where the excess active sites of enzyme molecules would not be exposed to the substrates and remain inside in the bulk of enzyme particles without contributing significantly to the reaction [21]. It can be further explained as all the available substrate is forming the enzyme/substrate complex, and so the extra enzyme molecules (active sites) cannot further increase the reaction rate [22].

Reaction in *iso*-octane with $\log P$ of 4.5 gave the highest acid conversion followed by *n*-heptane with $\log P$ of 4.0 and *n*-hexane with $\log P$ of 3.5 with slightly lower acid conversion than that of *n*-heptane. The $\log P$ value of the solvent is usually used as the parameter to characterize their possible effect on enzyme activity and

performance. Log P is the logarithm of the partition coefficient of the organic liquid between n -octanol and water. The higher the log P , the more non-polar (hydrophobic) is the solvent. Enzyme requires water layer around the support to maintain its activity and hydrophobic solvents such as n -heptane and iso -octane (log $P > 4.0$) to preserve the catalytic activity without stripping the water from the enzyme. Wu et al. (2002) reported an esterification reaction catalyzed by surfactant-coated *Candida rugosa* lipase in iso -octane to be successful. Other literature also reported that the use of iso -octane as the media for esterification or transesterification reaction was successful when *Candida rugosa* lipase was employed [3, 22-25]. All subsequent experiments to be discussed in the successive sections were conducted using 60 g/L of immobilized lipase in iso -octane as solvent.

Effect of substrate molar ratio

Two sets of experiment were conducted by fixing lauric acid concentration at 20mM and varying DL-citronellol concentration (20-60mM) in iso -octane and vice versa. The percentage of acid conversion increased from 42.3 to 89.7% when the concentration of alcohol increased (Figure 5). However, an increase in acid concentration causes the percentage of acid conversion to drop tremendously from 42.3 to 7.6%. This showed that excess of alcohol leads to a higher citronellyl laurate production yields. Conversely, excessive amount of acid decreases the reaction rate as this may be due to enzyme inhibition and the possibility of the substrate being strongly adsorbed on the enzyme active site as a result of excessive amount of substrate [26]. Above 1.5 of alcohol to acid molar ratio, a slight decrease of acid conversion was observed from 89.7 to 88.0%. Higher substrate ratios will raise the reaction equilibrium and increase the theoretical

maximum incorporation and product yields. On the other hand, higher acid content will increase the inhibition effect and lead to longer reaction time to reach equilibrium; thus lower conversion [27]. Yadav and Lathi (2004) found similar findings on the synthesis of citronellol laurate using lauric acid as acyl donor. Therefore, 1.5 of alcohol to acid molar ratio was used in the subsequent experiments.

Effect of temperature

The conversion of citronellyl laurate ester by immobilized *Candida rugosa* lipase was also studied as a function of temperature (Figure 6). Temperature was varied from 303K to 328K to observe the product formation. Temperature may affect the esterification efficiency in a positive way or vice versa. A rise in temperature will increase the reaction rate as explained by the transition state theory. However, at a higher reaction temperature, enzyme tertiary structure may also disrupt causing it to denature [22]. Further study was carried out at 310K because no significant changes in acid conversion value were observed at 308K and 313K. Consequently, it is interesting to note that at 310K the acid conversion increases up to 89.7%. However, at temperature above 313K, the conversion profile changed appreciably with low conversion values. This may be due to the enzyme denaturation phenomenon, which is a physical mechanism [28].

Kinetic model based on initial rate measurement

Initial rates of ester synthesis were determined for various concentrations of acid and alcohol. Two sets of experiments were conducted using 60 mg of immobilized *Candida rugosa* lipase with appropriate quantities of lauric acid and DL-citronellol and the total volume was made up to 10ml with *iso*-octane. In one set of experiment, DL-

citronellol amount was varied from 20mM to 100mM at a fixed quantity of lauric acid. In another set of experiment, the lauric acid concentration was varied from 20mM to 100mM at a fixed quantity of DL-citronellol. The mechanism of enzymatic reactions can be obtained from the Lineweaver-Burk double reciprocal plots (Figure 7).

It was observed that the initial reaction rate increased as the concentration of citronellol (A) was increased (Figure 8). When lauric acid (B) concentration increased, the reaction rate dropped. This is possibly due to the effect of substrate inhibition. The Lineweaver-Burk double inversion plot ($1/r$ versus $1/[A]$) in Figure 7 shows the slope and intercepts decreases when the concentration of lauric acid increases. It was also observed that the lines do not cross at the same point. Thus the result suggested that the system was affected by a noncompetitive inhibition. The inhibition may due to the formation of a dead end complex of lauric acid [26, 28].

The initial reaction rates obtained at various acid and alcohol concentrations were fitted to both types of mechanism by a non-linear regression analysis to obtain kinetic constant using polymath software. The accuracy of experimental data was computed by analyzing the initial reaction rate using equation 1 and 2 and presented in Table 1.

The theoretical (simulated) initial rate were calculated by using the parameters in Table 1 for the ordered bi bi with dead-end complex of lauric acid model and is compared against the experimental data for different alcohol concentration (Figure 8). There is a match between theory and experiment at $R^2 = 0.9927$, proving the validity of this ordered

bi bi model. In the other case, the estimated parameter was found to be negative and unrealistic although the determination coefficient value is approximately one. It is thus concluded that the reaction sequence follows the ordered bi bi with dead-end complex of lauric acid mechanism.

Table 1 also presents the kinetic value for the synthesis of citronellyl laurate using immobilized Novozym SP 435 from *Candida Antarctica* lipase studied by Yadav and Lathi (2004). A similar trend can be observed from the values obtained. The kinetic values obtained from the studies were notably different from the reported values by Yadav and Lathi (2004). The use of different type of enzyme, different operating temperature, and type of organic media operating can affect the reaction rate. Table 2 presented the differences of operating conditions between the present study and the research done by Yadav and Lathi (2004) in the synthesis of citronellyl laurate.

Conclusion

In the present work, it can be concluded that *Candida rugosa* lipase has a high catalyzing ability in citronellyl laurate production. The data obtained from the esterification analysis on various parameters showed that the optimum production of citronellyl laurate (89% of acid conversion) was achieved at 310K using 60g/l of catalyst loading, synthesized in *iso*-octane for 24 hr of reaction period. Amberlite MB-1 was found to be the best support for immobilization of *Candida rugosa* lipase. The analysis of the initial rates showed that the esterification of citronellol and lauric acid catalyzed by immobilized *Candida rugosa* lipase follows the ternary mechanism (ordered bi bi

mechanism) with dead-end complex inhibition of lauric acid from the two proposed mechanism.

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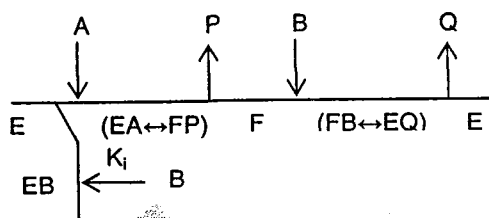


Figure 1: Cleland convention diagram for ping pong bi bi mechanism with dead complex inhibition.

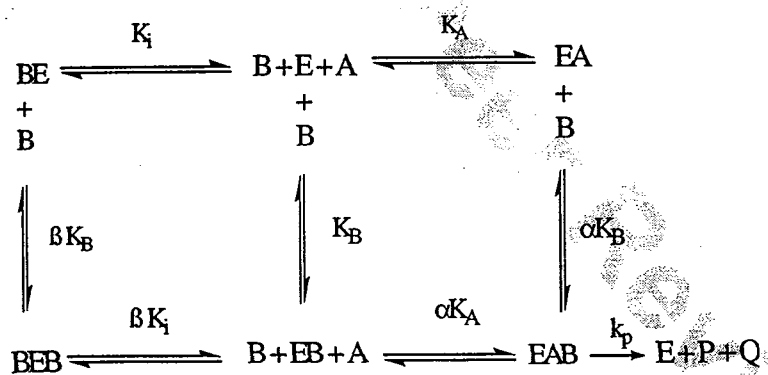


Figure 2: Reaction sequence for ordered bi bi mechanism with dead end complex inhibition.

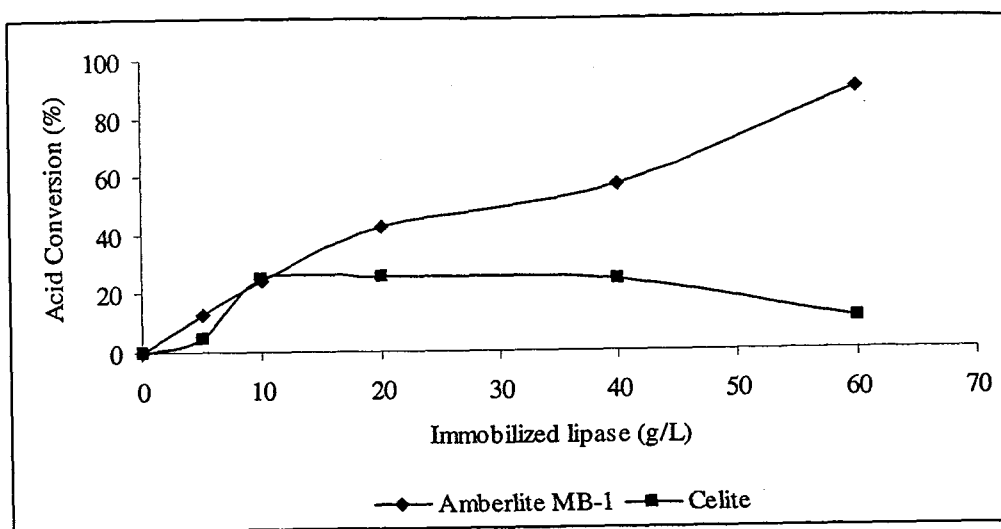


Figure 3: Effect of different types of support for lipase immobilization. Reaction condition; substrates concentration 0.02 M lauric acid; 0.03 M citronellol; agitation speed 200 rpm; temperature 310K; incubation period 24 hr.

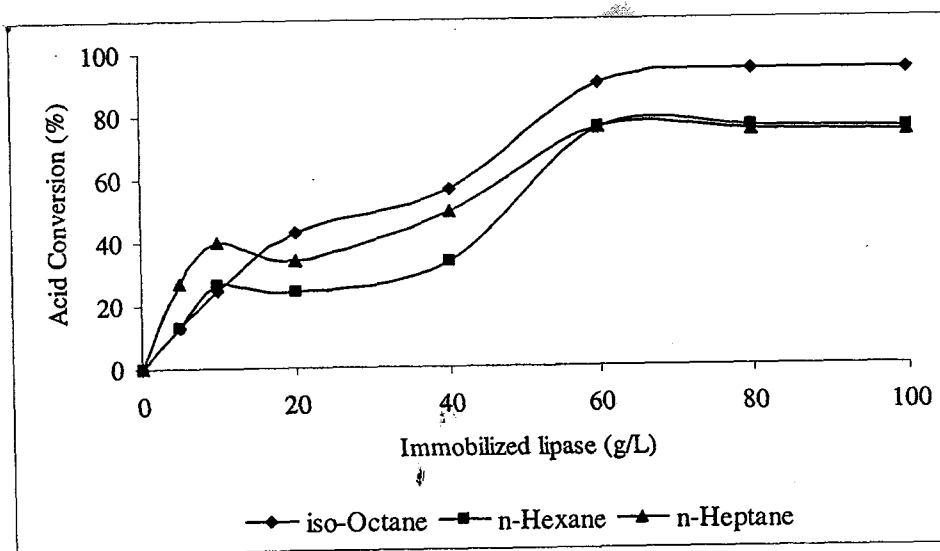


Figure 4: Effect of different solvents. Reaction condition; substrates concentration 0.02 M lauric acid; 0.03 M citronellol; agitation speed 200 rpm; temperature 310K; incubation period 24 hr.

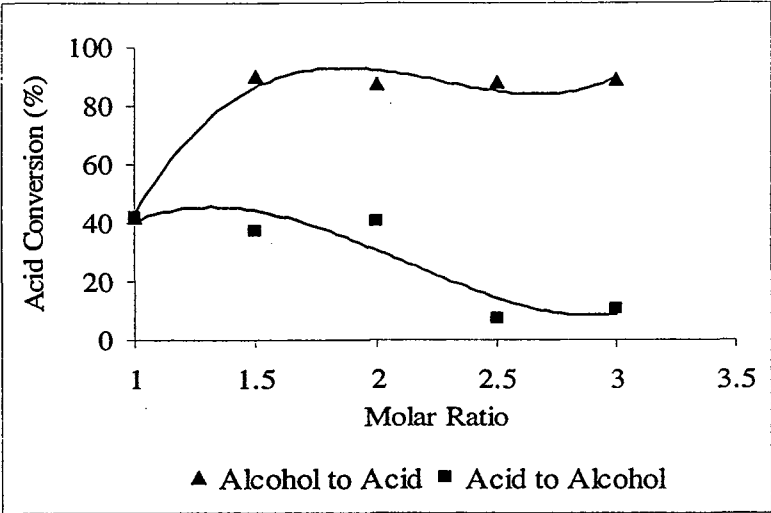


Figure 5: Effect of substrate molar ratio on the synthesis of citronellyl laurate. Reaction condition; catalyst load (immobilized lipase) 60 g/L; agitation speed 200 rpm; temperature 310K; incubation period 24 hr.

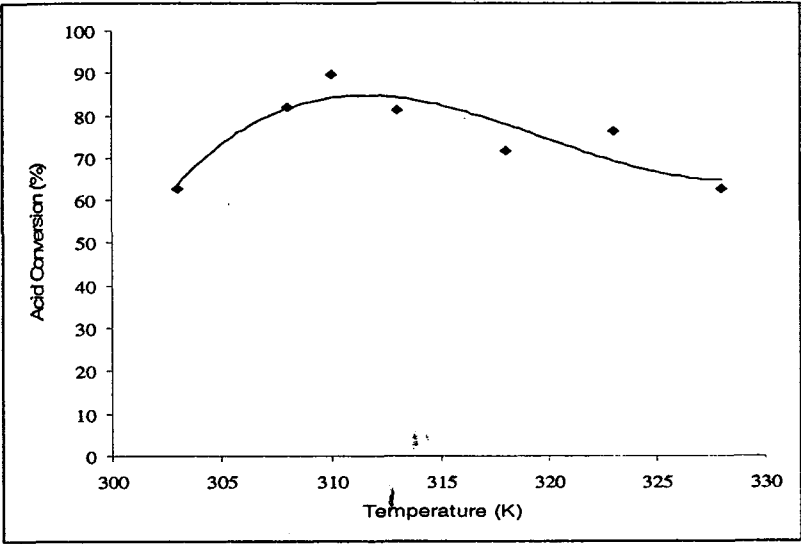


Figure 6: Effect of temperature on the synthesis of citronellyl laurate in *iso*-octane. Reaction condition; 0.02 M lauric acid; 0.03 M alcohol; agitation speed 200 rpm; temperature 303K-328K; incubation period 24 hr.

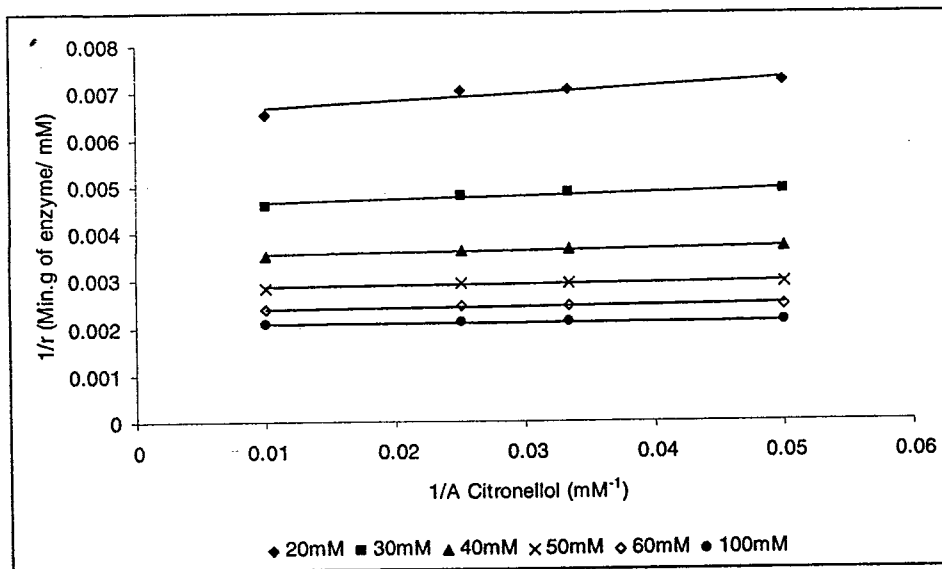


Figure 7: Double reciprocal of Linear-Burk plot for different concentration of lauric acid (B).

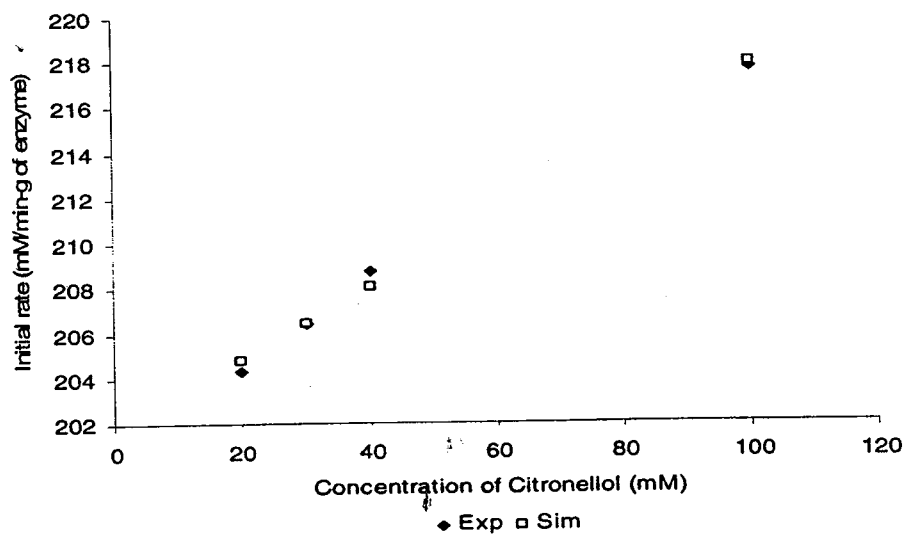


Figure 8: Comparison of experimental and simulated (theoretical) rates as a function of citronellol concentration; ($\blacklozenge$) $v_{\text{experimental}}$, ($\square$) $v_{\text{simulated}}$

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Table 1

Kinetic constants obtained for esterification of citronellyl laurate catalyzed by immobilized *Candida rugosa* lipase.

Parameter	Ping Pong bi bi with dead end complex of lauric acid ($\times 10^{-3}$)	Ordered bi bi with dead end complex of lauric acid ($\times 10^{-3}$)	Ordered bi bi with dead end complex of lauric acid (Yadav and Lathi, 2004)
$V_{\max}$ (mol L ⁻¹ min ⁻¹ g ⁻¹ enzyme)	0.5509	0.0698	10.5531
$K_{M,acid}$ (mol L ⁻¹ g ⁻¹ enzyme)	-50.4998	0.3486	25.3566
$K_{M,alcohol}$ (mol L ⁻¹ g ⁻¹ enzyme)	1.0172	0.7471	38.9845
K_i (mol L ⁻¹ g ⁻¹ enzyme)	0.0834	0.0078	1.6641
R^2	0.9926	0.9927	NA

NA = not available

Table 2

Comparison between the present study and Yadav and Lathi (2004) on the synthesis of citronellyl laurate.

Subject	Present study	Yadav and Lathi (2004)
1) Catalyst	<i>Candida rugosa</i> (self-immobilized by adsorption method onto Amberlite MB-1 resin)	Novozym SP 435 (<i>Candida Antarctica</i>) immobilized on macroporous polyacrylic resin.
2) Operating temperature	37°C	30°C
3) Solvent	<i>Iso</i> -octane	<i>n</i> -heptane
4) Agitation speed	200 rpm	500 rpm

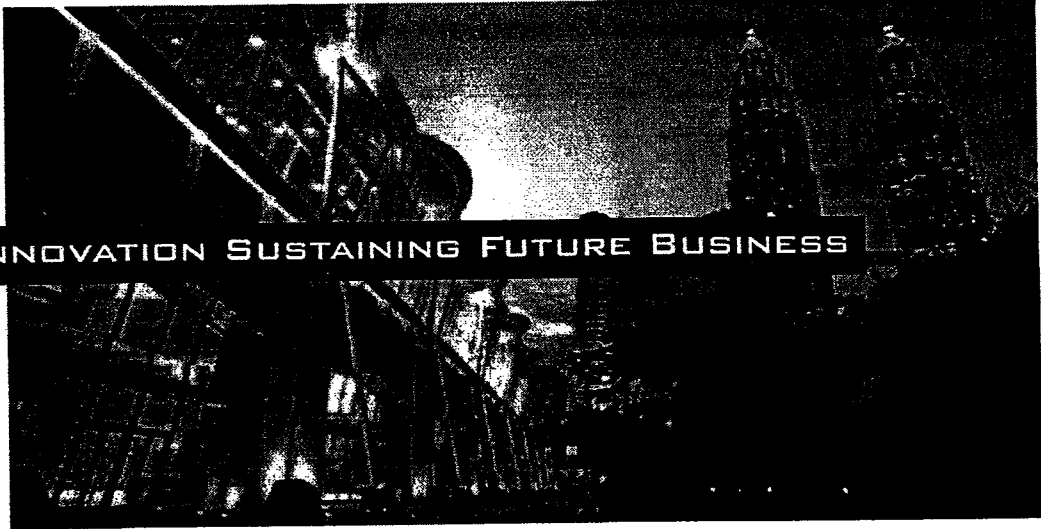


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KINETICS OF FREE *CANDIDA ANTARCTICA* LIPASE B IN THE ESTERIFICATION OF (R,S)-KETOPROFEN: MODEL AND EXPERIMENTAL STUDIES

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ABSTRACT

Kinetics of the esterification reaction of (R,S)-ketoprofen acid and *n*-butanol has been studied using free *Candida antarctica* lipase B for the production of enantiopure (S)-ketoprofen acid. The experimental data indicated that the reaction follows a Michaelis–Menten kinetics and described by the Ping Pong Bi Bi mechanism. The kinetic parameters of the proposed model for the esterification reaction were determined. The kinetic results showed that the inhibition effect of *n*-butanol was higher than the inhibition effect of (R,S)-ketoprofen acid. The simulated data obtained from the kinetic model with (R,S)-ketoprofen acid and *n*-butanol inhibition agreed well with the experimental data at the acid concentration lower than 50mM.

Keywords: *Candida antarctica* lipase B; Ketoprofen; Enzymatic esterification; Kinetics; Ping Pong Bi Bi Mechanism.

1 INTRODUCTION

Lipases are the most widely investigated of all enzymes due to their remarkable properties (e.g. regio, stereo, and substrate specificity), milder reaction conditions, reduction in the energy requirements and the production cost have been observed (Yadav and Devi, 2004; Romera et al., 2005). The lipase obtained from *Candida antarctica* fraction B (CALB) is one of the most commonly used lipase [McCabe and Taylor, 2004] because of its high level of activity and stability (Lozano et al., 2002). It is commercially available in both free and immobilized form. CALB is a globular protein with an alpha/beta hydrolase fold. It consists of 317 amino acids and its molecular weight is 33 kDa (Rotticci, 2000). The lipase originates from the basidiomyceteous yeast *Candida antarctica* (Rotticci, 2000). The enantioselectivity of CALB towards R-enantiomer is well known and has been proven in several reports (Jin et al., 2003; Antona et al., 2002; Park et al., 1999).

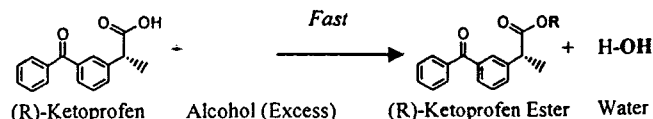
Ketoprofen [2-(3-benzoylphenyl) propionic acid] is a non-steroidal anti-inflammatory drug (NSAID) with analgesic, anti-inflammatory and antipyretic properties, and is effective for the treatment of rheumatoid arthritis, osteoarthritis and ankylosing spondylitis (Xi and Xu, 2004; Diaz-Reval, et al., 2004). It was reported that these healing properties are mainly exerted by an (S)-enantiomer (Diaz-Reval, et al.,

2004; Zhu et al., 2005), while the (R)-enantiomer is effective as a therapeutic agent for toothache (Jin et al., 2003; Liu et al., 2000). There is always a danger that one enantiomer of a drug may possess the desired activity and the other, although inactive, may possess extraneous and even harmful pharmacologic properties (Rossi et al., 1993). An alternative enzymatic route in the preparation of enantiopure (S)-ketoprofen using lipase as a biocatalyst has gained attention and was highlighted intensively in the previous study (Ong et al., 2005).

Although enzymatic preparation of enantiopure (S)-ketoprofen through various pathways has been well documented (Ong et al., 2005), none of them were studied using free CALB. Understanding the performance of free lipase is very useful in the work of improvements of enzyme properties. As it can be the basic margin to enable researcher measuring the improvement extent that they have been workout. In order to identify the behaviour of free CALB in the ester synthesis, it is useful to know the reaction kinetics and the binding constants that describe the kinetic behaviour. In this paper, the kinetics of the (R,S)-ketoprofen acid-butanol esterification reaction catalysed by free CALB is reported. The schematic diagram of the enantioselective esterification reaction of (R,S)-ketoprofen acid using free CALB was shown by Fig. 1. Thus, R-enantiomer as the fast-reacting enantiomer and S-enantiomer as the slow-reacting enantiomer. The kinetic parameters were estimated and the simulated data obtained from the kinetic model were compared with the experimental data.

ENANTIOSELECTIVE ESTERIFICATION

Fast Reacting Enantiomer:



Slow Reacting Enantiomer:

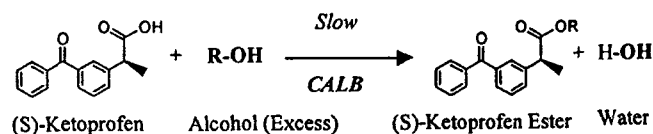
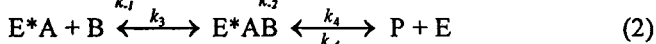
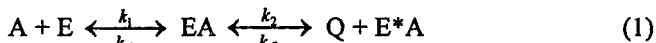


Fig. 1. Schematic diagram of the enantioselective esterification reaction of (R,S)-ketoprofen acid by using free CALB.

2 KINETIC MODEL

Most of the earlier kinetic studies on lipase-catalysed esterifications reactions are based on a model incorporating a Ping Pong Bi Bi mechanism, which seem to be valid for most simple enzymatic esterification reactions (Yadav and Devi, 2004; Maury et al., 2005; Duan et al., 1997). In the Ping Pong Bi Bi mechanism, "Ping Pong" describes a mechanism in which one product must be released before the addition of second substrate while "Bi Bi" represent a reaction in which is bimolecular in reactants and bimolecular in products (Piszkiewics, 1977). The mechanism for esterification reaction can be expressed as:



Where A, B, Q, P, I, E, E*A, EI and E*AI represent acid, alcohol, water, ester, inhibitor, enzyme, acyl-enzyme complex, dead-end inhibition complexes of enzyme with inhibitor, respectively.

When there is no inhibition, the initial reaction rate equation can be expressed as (Piszkiewics, 1977):

$$\frac{v_i}{V_{max}} = \frac{[S_{acid}][S_{alcohol}]}{K_{M_{acid}}[S_{alcohol}] + K_{M_{alcohol}}[S_{acid}] + [S_{acid}][S_{alcohol}]} \quad (5)$$

If there is inhibition by alcohol, the equation is:

$$\frac{v_i}{V_{max}} = \frac{[S_{acid}][S_{alcohol}]}{K_{M_{acid}}[S_{alcohol}](1 + \frac{[S_{alcohol}]}{K_{I_{alcohol}}}) + K_{M_{alcohol}}[S_{acid}] + [S_{acid}][S_{alcohol}]} \quad (6)$$

If there is inhibition by acid, the equation is:

$$\frac{v_i}{V_{max}} = \frac{[S_{acid}][S_{alcohol}]}{K_{M_{acid}}[S_{alcohol}] + K_{M_{alcohol}}[S_{acid}](1 + \frac{[S_{acid}]}{K_{I_{acid}}}) + [S_{acid}][S_{alcohol}]} \quad (7)$$

If there is inhibition by both acid and alcohol, the equation is:

$$\frac{v_i}{V_{max}} = \frac{[S_{acid}][S_{alcohol}]}{K_{M_{acid}}[S_{alcohol}](1 + \frac{[S_{alcohol}]}{K_{I_{alcohol}}}) + K_{M_{alcohol}}[S_{acid}](1 + \frac{[S_{acid}]}{K_{I_{acid}}}) + [S_{acid}][S_{alcohol}]} \quad (8)$$

Where v_i is the initial reaction rate, V_{max} is the maximum reaction rate, $[S_{acid}]$ is the acid concentration, $[S_{alcohol}]$ is the alcohol concentration, $K_{M_{acid}}$ is the binding constants for acid, $K_{M_{alcohol}}$ is the binding constants for alcohol, $K_{I_{acid}}$ is the inhibition constants for acid and $K_{I_{alcohol}}$ is the inhibition constants for alcohol.

3 MATERIALS AND METHODS

3.1 MATERIALS

The lipase from *Candida antarctica* fraction B solution (CALB Novozym 525) was obtained from Novo Nordisk (Bagsvaerd, Denmark) with 1-10% protein content [18]. (R,S)-ketoprofen acid was purchased from Shanghai Huaqu Imp. & Exp. Co. (Pudong, Shanghai, China). All other chemicals used in this work were of analytical grade and supplied by Fisher Scientific (Nepean, Ontario, Canada) and used without further purification.

3.2 ENZYMATIC PREPARATION OF (S)-KETOPROFEN

The enzymatic preparation of (S)-ketoprofen through enantioselective esterification reaction in an organic media was conducted in 250ml erlenmeyer flask. The (R,S)-ketoprofen acid was added into 25ml of organic solvent (20% 1,2-dichloropropane-80% *n*-hexane) containing appropriate amount of *n*-butanol and 0.17ml of free enzyme (CALB). The mixture was incubated at 40°C and shaken continuously at 250rpm in an incubator shaker. Periodically, 1ml aliquot was withdrawn from the reaction medium for HPLC analysis.

3.3 ANALYTICAL METHODS

The concentration of ketoprofen enantiomers was determined using a HPLC system (Shimadzu, Kyoto, Japan) equipped with a chiral stationary phase column: (R,R)-Whelk-O1 chiral column (Regis Technologies, Morton Grove, USA) and a UV detector (254nm). A mixture of dichloromethane, *n*-hexane and ethanol (0.25M ammonium acetate) (47:47:6, v/v/v) was used as an eluent. The flow rate was maintained at 1.5ml/min and the column temperature at 25°C. 60μl of the sample was injected.

3.4 DETERMINATION OF INITIAL RATES

The effect of substrates concentration on the initial rate of reaction was investigated. In order to determine the initial rates, two sets of experiments were performed. In the first set of experiments, the (R,S)-ketoprofen acid concentration was varied in the range of 20mM to 50mM at fixed concentration of *n*-butanol while other variables were held constant. In another set of experiments, the concentration of *n*-butanol was varied in the range of 20mM to 120mM at fixed concentration of (R,S)-ketoprofen acid while other parameters were held constant. The initial reaction rates were determined from the initial slope of the tangent to the curve of product concentration versus time.

3.5 DETERMINATION OF KINETIC PARAMETERS

Based on the initial reaction rate experiments, the value of kinetic parameters can be determined from Lineweaver-Burk plots (Piszkiewics, 1977). Equation (5) can be lineared in double-reciprocal form. When (R,S)-ketoprofen acid

concentration $[S_{acid}]$ is varied at constant n -butanol concentration $[S_{alcohol}]$, the equation is:

$$\frac{1}{v_i} = \left(\frac{K_{M\ acid}}{V_{max}} \right) \left(\frac{1}{[S_{acid}]} \right) + \left(\frac{K_{M\ alcohol}}{[S_{alcohol}]} + 1 \right) \left(\frac{1}{V_{max}} \right) \quad (9)$$

When n -butanol concentration $[S_{alcohol}]$ is varied at constant (R,S)-ketoprofen acid concentration $[S_{acid}]$, the equation is:

$$\frac{1}{v_i} = \left(\frac{K_{M\ alcohol}}{V_{max}} \right) \left(\frac{1}{[S_{alcohol}]} \right) + \left(\frac{K_{M\ acid}}{[S_{acid}]} + 1 \right) \left(\frac{1}{V_{max}} \right) \quad (10)$$

A primary plot of $(1/v_i)$ versus $(1/[S_{acid}])$ for equation (9) yields a linear line with a slope of $(K_{M\ acid}/V_{max})$ and intercept as:

$$\text{Intercept} = \frac{K_{M\ alcohol}}{V_{max} [S_{alcohol}]} + \frac{1}{V_{max}} \quad (11)$$

Similar plot was drawn for equation (10) to yield a slope of $(K_{M\ alcohol}/V_{max})$ and intercept of:

$$\text{Intercept} = \frac{K_{M\ acid}}{V_{max} [S_{acid}]} + \frac{1}{V_{max}} \quad (12)$$

Those primary plots are useful in indicating the operation of a Ping Pong mechanism. Thus, parallel lines will be obtained if the reaction obeyed Ping Pong mechanism. Since both slopes and intercepts are complex constants containing two unknowns, secondary plot was employed. A plot of the y-axis intercept values of primary plot versus $(1/[S_{alcohol}])$ for equation (11) yields straight line with a slope of $(K_{M\ alcohol}/V_{max})$ and y-axis intercept of $(1/V_{max})$. Thus, the value of V_{max} , $K_{M\ acid}$ and $K_{M\ alcohol}$ were obtained. Similar plots were used for equation (12).

3.6 INHIBITION EFFECT OF SUBSTRATES

Substrates inhibition study involved consideration of competitive, noncompetitive, uncompetitive and mixed inhibition models (Piszkiewics, 1977). The initial reaction rates obtained from experimental data were used for the primary plots (Lineweaver-Burk plot) and plotting secondary plot of slope (for competitive and noncompetitive inhibition model) or intercept (for uncompetitive and noncompetitive inhibition model) of primary plot versus inhibitor concentration gave estimates of K_i in equation (8) by dividing the y-intercept and the slope of secondary plots.

3.7 SIGMAPLOT SOLUTION

For better accuracy, non-linear fitting program embedded in *SigmaPlot 2000* software was used to determine all the kinetic parameters. The initial reaction rates determined experimentally were fitted to the kinetic models using the software. Initial parameters values were obtained from the Lineweaver-Burk plot (graphical method), a linear

transformation of Michaelis-Menten equation which was previously mentioned in section 3.5.

4 RESULTS AND DISCUSSION

The kinetics of free CALB-catalysed esterification reaction of (R,S)-ketoprofen acid with n -butanol was investigated by studying the effect of both substrates concentration on the initial reaction rate. The reaction involved two reactants that give rise to two products as shown in Fig. 1; therefore the kinetics was termed as Bi Bi. The data from the initial reaction rates measurement were used for the estimation of kinetic parameters using graphical methods (Lineweaver-Burk plot) as well as *SigmaPlot* software.

Through graphical method, the exact mechanism of enzymatic reactions can be identified from the Lineweaver-Burk plot. Fig. 2 shows the Lineweaver-Burk double reciprocal plot of esterification reaction for variation in (R,S)-ketoprofen acid concentration at five fixed concentrations of n -butanol. As the concentration of n -butanol is increased from 20mM to 120mM, both slope and intercept are affected. Thus, mixed inhibition (competitive and non-competitive) by n -butanol was observed from the types of lines obtained (Fig. 2). According to mixed inhibition model, both free enzyme and acyl-enzyme complex were inhibited with the presence of inhibitor, where reaction equations (3) and (4) were obeyed. The trend of parallel lines obtained at low n -butanol concentration is in agreement with Ping Pong Bi Bi mechanism. According to Ping Pong Bi Bi mechanism (Piszkiewics, 1977), the enzyme releases a product in between the addition of substrates to the enzyme as shown by the reaction equations (1) and (2). By referring to reaction (1), after (R,S)-ketoprofen acid binds to the enzyme, water as first product is released and acyl-enzyme complex is formed. It is followed by binding of n -butanol to the acyl-enzyme complex and the second product, the ester is then released as shown in equation (2). At low concentration of (R,S)-ketoprofen acid, reaction (1) was the rate limiting (Yadav and Lathi, 2005). Increasing the concentration of n -butanol converted more dead-end complex (E^*B) and thereby decreased the concentration of enzyme to form acyl-enzyme (E^*A). At extremely high n -butanol concentration, excess amount of n -butanol for reaction (2) and (3) exhibited reaction (4) with the existing acyl-enzyme complex to form (E^*AB) unproductively. Duan et al. (1997) reported that the esterification reaction of (R,S)-ketoprofen acid with n -propanol catalysed by immobilized CALB also showed inhibited by the alcohol.

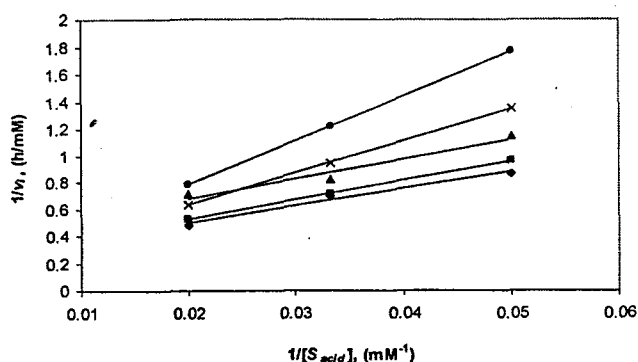


Fig. 2. Lineweaver-Burk double reciprocal plot of esterification reaction for variation in (R,S)-ketoprofen acid concentration at five fixed concentration of *n*-butanol. Reaction conditions: (1,2)-dichloropropane-hexane (20:80, %v/v) as solvent, 0.17ml of free CALB, T = 40°C, shaken at 250rpm. Symbols: (♦) 20mM, (■) 40mM, (▲) 60mM, (×) 80mM, (●) 120mM of *n*-butanol.

Fig. 3 shows the Lineweaver-Burk double reciprocal plot of esterification reaction for variation in *n*-butanol concentration at three fixed concentrations of (R,S)-ketoprofen acid. Thus, the shape of the graph suggested that the same Ping Pong Bi Bi kinetic mechanism is followed. The lines are parallel and concave upward suggesting uncompetitive inhibition and substrate inhibition by (R,S)-ketoprofen acid (Fig. 3). According to these inhibition models, the acyl-enzyme complex was inhibited by the inhibitor before reacted with the second substrate (Piszkiwics, 1977) as shown by equation (4). Effect of inhibition by (R,S)-ketoprofen acid can be explained by the hypothesis that (R,S)-ketoprofen acid (A) may react with acyl-enzyme complex (E*A) to yield a dead-end complex (E*AA) that cannot transfer acyl moiety to *n*-butanol (Segal, 1975) as shown by the reaction equation (4). Thus, excess amount of (R,S)-ketoprofen acid for reaction (1) will exhibit more reaction (4) to form (E*AA).

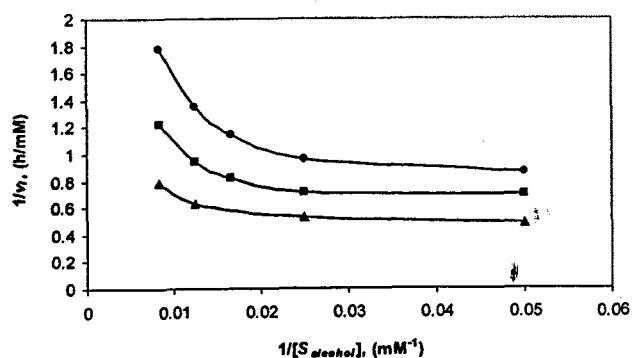


Fig. 3. Lineweaver-Burk double reciprocal plot of esterification reaction for variation in *n*-butanol concentration at three fixed concentration of (R,S)-ketoprofen acid. Reaction conditions: (1,2)-dichloropropane-hexane (20:80, %v/v) as solvent, 0.17ml of free CALB, T = 40°C, shaken at 250rpm. Symbols: (●) 20mM, (■) 30mM, (▲) 50mM of (R,S)-ketoprofen acid.

The kinetic parameters determined using nonlinear regression embedded in *SigmaPlot* software are given in Table 1. The Ping Pong Bi Bi mechanism with both substrates inhibition (equation (8)) fitted well to the

experimental data with the standard error less than 1.0 and the parameters coefficient of variation, CV less than 1.0% compared to when using other kinetic models (e.g. no inhibition model-equation (5), with only alcohol inhibition model-equation (6) or with only acid inhibition model-equation (7)). The value of inhibition constant for (R,S)-ketoprofen acid ($K_{I \text{ acid}}$) is 3.5-times larger than that of *n*-butanol. Thus, the lack of significant of inhibition by (R,S)-ketoprofen acid is not surprising as the $K_{M \text{ acid}}$ was high and the interaction with the lipase was weak if compared to *n*-butanol.

Table 1. Kinetic parameters for free CALB-catalysed esterification of (R,S)-ketoprofen acid based on Ping Pong Bi Bi mechanism using *SigmaPlot 2000*

Kinetic parameter	Value	Standard Error	CV, (%)
V_{max} (mM/h)	7.45	1.44×10^{-07}	1.94×10^{-06}
$K_{M \text{ acid}}$ (mM)	39.60	1.15×10^{-06}	2.90×10^{-06}
$K_{M \text{ alcohol}}$ (mM)	28.87	3.39×10^{-06}	1.18×10^{-05}
$K_{I \text{ acid}}$ (mM)	83.80	5.11×10^{-05}	6.10×10^{-05}
$K_{I \text{ alcohol}}$ (mM)	23.92	2.61×10^{-07}	1.09×10^{-06}

The comparisons between simulated and experimental data are illustrated in Fig. 4. Fig. 4 shows that the initial reaction rates increased with *n*-butanol concentration up to 20mM and decreased with further increase of concentration. The initial reaction rates were proportional to acid concentration in the study range of 20mM to 50mM with respect to (R,S)-ketoprofen acid. Thus, higher *n*-butanol concentrations appear to inhibit the catalytic activity of free CALB. The kinetic model with acid-alcohol inhibition fitted well to the experimental data up to 30mM of (R,S)-ketoprofen acid concentration in this study. However, further increase in (R,S)-ketoprofen acid concentration led to some variation between the simulated and experimental values. This may be due to the significant formation of water in the reaction medium at higher substrate concentration that affects the experimental values which the effect has been neglected by the classical kinetic model.

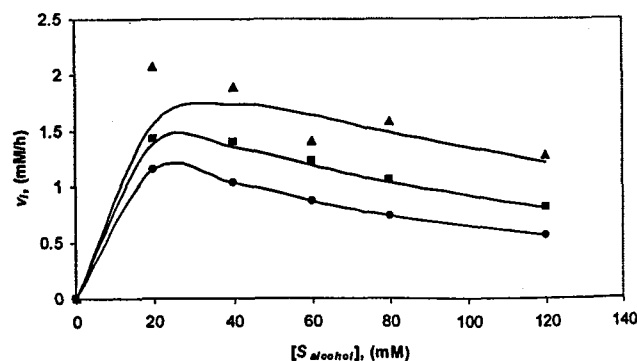


Fig. 4. Initial reaction rate data for variation in *n*-butanol concentration at three fixed concentration of (R,S)-ketoprofen acid. Reaction conditions: (1,2)-dichloropropane-hexane (20:80, %v/v) as solvent, 0.17ml of free CALB, T = 40°C, shaken at 250rpm. Symbols: (●) 20mM, (■) 30mM, (▲) 50mM of (R,S)-ketoprofen acid. Simulated values: solid lines, Experimental values: symbols.

5 CONCLUSIONS

Production of (S)-ketoprofen acid via direct esterification reaction was catalysed by free CALB. Initial reaction rates and progress curve data were used to develop a suitable model. The kinetic parameters were computed as: $V_{max} = 7.45$ mM/h; $K_{M\text{ acid}} = 39.60$ mM; $K_{M\text{ alcohol}} = 28.87$ mM; $K_i\text{ acid} = 83.80$ mM; and $K_i\text{ alcohol} = 23.92$ mM. The proposed kinetic model was used to simulate the initial reaction rates data which were in agreement with experimental values with the standard error less than 1.0 and the CV less than 1%. The results show that the Ping Pong Bi Bi mechanism with inhibition by both (R,S)-ketoprofen acid and *n*-butanol substrates was obeyed. The effect of *n*-butanol inhibition was found to be more significant as compared to the (R,S)-ketoprofen acid inhibition.

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THE EFFECT OF ALCOHOL ON THE PERFORMANCE OF LIPASE-IMMOBILIZED ENZYMATIC MEMBRANE REACTOR FOR ESTERIFICATION OF (R,S)-KETOPROFEN

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Abstract

The effect of alcohols on the performance of lipase-immobilized enzymatic membrane reactor (EMR) for enantioselective esterification of (R,S)-ketoprofen has been studied. In this work, mixed solvent medium was used and the (R)-ketoprofen was reacted with the different alcohols in the presence of immobilized lipase B from *Candida antarctica*, leaving the target product (S)-ketoprofen in its unreacted form. The alcohols involved in the reactions were ethanol, butanol, heptanol and 2-ethoxyethanol. The chain length of alcohol was found to significantly affect the performance of the esterification in EMR. Through the alcohols screening, the esterification reaction using ethanol in EMR showed higher initial rate, conversion, enzyme enantioselectivity, enantiomeric excess of substrate (ee_s) and enantiomeric excess of product (ee_p). However the results with butanol showed less inhibition effect on a wider range of temperature

Keywords: Effect of alcohol; Enzymatic membrane reactor, *Candida antarctica* lipase B; Ketoprofen; Esterification

Abstrak

Kesan alkohol ke atas prestasi reaktor membran berenzim (EMR) yang mengandungi lipase tersekatgerak telah dikaji untuk tindak balas pengesteran secara memilih bagi (R,S)-Ketoprofen. Di dalam kajian ini, pelarut bercampur telah digunakan dan (R)-ketoprofen telah bertindak balas dengan pelbagai alkohol di dalam kehadiran lipase B daripada *Candida antarctica* tersekatgerak. Hasil tindak balas ini adalah produk sasaran (S)-ketoprofen dalam bentuk asal. Alkohol yang terlibat dalam kajian ini adalah etanol, butanol, heptanol and 2-etoksietanol. Panjang rantai alkohol didapati mempengaruhi prestasi EMR. Daripada kajian saringan ke atas semua alkohol, tindak balas pengesteran di dalam EMR yang mengandungi ethanol menunjukkan halaju awal, kadar pertukaran, enantio-kememilihan enzim, lebihan substrat (ee_s) dan lebihan produk (ee_p) yang lebih tinggi. Manakala keputusan bagi butanol pula menunjukkan kurangnya kesan rencatan pada julat suhu yang lebih besar.

Kata kunci: Kesan alkohol; Reaktor membran berenzim, *Candida antarctica* lipase B; Ketoprofen; Pengesteran

1. INTRODUCTION

Ketoprofen [2-(3-benzoylphenyl)-propionic acid], is a non-steroidal anti-inflammatory drug (NSAID) with analgesic and antipyretic properties that has long been recognized as being useful in the treatment of pain, inflammation, and fever [1,2]. Its anti-inflammatory effect is approximately 160 times the anti-inflammatory potency of aspirin on a unit weight basis [3]. Therefore, it has received attention since the past two decades. Although (R,S)-ketoprofen can cause gastrointestinal side effects in patients, these are usually less severe than with aspirin. However, aspirin is less expensive than most of the propionic derivatives for those who can tolerate it [4].

Commercially, ketoprofen is still marketed and administered as racemic mixture of (R) and (S) enantiomers, which are equivalent on a unit weight basis. However, (S)-ketoprofen and (R)-ketoprofen display significantly different pharmacologic activities and benefits [5]. (S)-ketoprofen has long been recognized for its therapeutic activities of reducing inflammation, relieving pains and fever [6-8] while the (R)-enantiomer is effective as a therapeutic agent for toothache [9]. Thus, the production of single enantiomer is essential to avoid the consumption of unwanted enantiomers. However, still a major reason for the use of enantiomers mixtures is that the cost of separation of the enantiomers exceeds the potential advantage of a possible increase in the activity. Therefore, it has been a key issue in pharmaceutical industry to obtain the pure, active form of ketoprofen.

An alternative enzymatic route in the preparation of enantiopure (S)-ketoprofen using lipase as biocatalyst is gaining importance because of their remarkable properties (e.g. regio, stereo, and substrate specificity), milder reaction conditions reduction in the energy requirements and the production cost [10]. The lipase obtained from *Candida antarctica* fraction B (CALB) is one of the most commonly used lipases [11]. It is commercially available in both free and immobilized form. CALB is a globular protein with an alpha/beta hydrolase fold. It consists of 317 amino acids and its molecular weight is 33 kDa [12]. The lipase originates from the basidiomyceteous yeast *Candida antarctica* [12]. The enantioselectivity of CALB towards R-enantiomer is well known and has been proven in several reports [9,13,14].

Although enzymatic preparation of enantiopure (S)-ketoprofen has been well documented [9,13-15] and has been highlighted in the previous study [16], none of them were studied using CALB immobilized on enzymatic membrane reactor (EMR). The EMR technology offers higher efficiency, easy to scale-up, applicable in continuous and steady state mode, easier retention and reuse of enzyme, reduction in substrate/product inhibition, free enzyme end-product, flexibility of its system configuration, enhance the stability of enzyme and resistance towards dilution by solution. The membrane used can be made from either hydrophobic or hydrophilic polymeric material [17]. The use of EMR is to ensure the complete rejection of the enzyme in order to maintain the full activity inside the reacting volume [18] by controlling the MWCO of the membrane. The aim of the present study is to investigate on the effect of alcohols on the performance of lipase-immobilized EMR for enantioseparation of (R,S)-ketoprofen. The enantioseparation occurred by enantioselective esterification of (R)-ketoprofen, leaving the target product (S)-ketoprofen in unreacted form as shown in Fig. 1. The enantioselective esterification reaction of (R,S)-ketoprofen occurs at the distinct layer of lipase immobilization on the membrane surface in EMR as illustrated in Fig. 2.

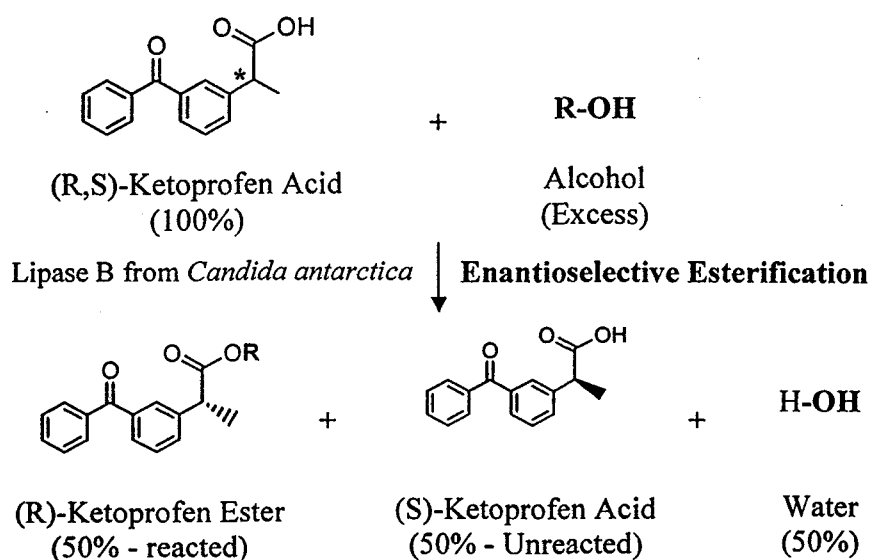


Fig. 1. Enantioselective esterification of (R,S)-ketoprofen.

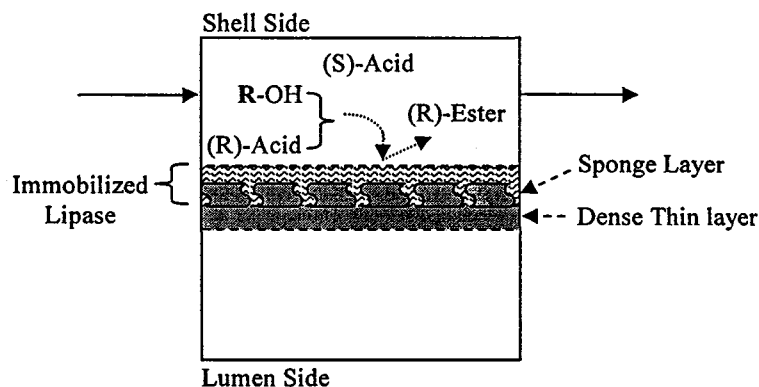


Fig. 2. Enantioselective esterification of (R,S)-ketoprofen in EMR.

2. EXPERIMENTAL

The lipase from *Candida antarctica* fraction B solution (CALB Novozym 525) having 5285 Units per g protein was obtained from Novo Nordisk (Bagsvaerd, Denmark). This lipase was used without further purification. BCA protein assay was supplied by Pierce (Illinois). (R,S)-ketoprofen acid 99% was purchased from Shanghai Huaqu Imp. & Exp. Co. (Pudong, Shanghai, China). All other chemicals used in this work were of HPLC grade and supplied by Fisher Scientific (Nepean, Ontario, Canada) and used without further purification.

2.1. Experimental Setup

The experimental rig of the EMR consists of a commercially available hollow fiber membrane module (Pall, Malaysia) which was constructed from hydrophobic polysulfone (PS) membrane with a nominal cut-off of 10,000Dalton and a specific membrane area of 0.015m^2 in polysulfone housing. A custom-made incubator (H x W x L: 12" x 13" x 26") with built-in temperature controller (Watlow Series 93, a 1/16 DIN microprocessor-based auto-tuning) was used to keep the reaction temperature constant. Two distinctive compartments of EMR, shell side stream and lumen side stream are circulated in countercurrent mode using dosing pumps (capacity: 15 – 280ml/min; Cole Parmer). Two back-pressure regulators (capacity: 0 – 150PSI; Tescom Back Pressure Model 44-2363-24-025) were fitted at each outlet streams of the EMR for trans-membrane pressure adjustment by changing the flow-rate. Pressure gauges (capacity: 0 – 100PSI; Swagelok and Millipore) were placed at each inlet and outlet streams of EMR for trans-membrane pressure quantification. All valves and fittings were connected with 6mm ID stainless steel tubing.

2.2. Preparation of Phosphate Buffer Solution

Phosphate buffer solution was prepared by mixing 50mM disodium hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) with 50mM potassium dihydrogen phosphate (KH_2PO_4) at an appropriate ratio to give the desired pH. The desired pH value was adjusted by adding KH_2PO_4 to decrease the pH or by adding $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ to increase the pH.

2.3. Preparation of Lipase Concentration

CALB solution was prepared by mixing CALB with 1L of 50mM phosphate buffer solution at the desired pH value and the solution was gently stirred by magnetic stirrer for 2h. The solution was centrifuged at 3000rpm for 5min to remove the insoluble substances. Some amount of the solution was stored in the refrigerator for the measurement of initial protein concentration.

2.4. Enzyme Immobilization via Physical Adsorption Technique

1L of CALB solution was prepared using phosphate buffer at desired pH value. Fresh phosphate buffer solution at desired pH value was circulated in the shell side and lumen side streams for pre-conditioning of membrane. The residual of fresh phosphate buffer solution in shell side and lumen side were then completely purged with nitrogen gas. The lipase solution of 1L was circulated in the shell side of the hollow fiber membrane module at the pressure of 0.25bar and flow velocity of 40ml/min at room temperature. Thus, lipase was immobilized on the membrane sponge layer by cross-flow filtration of the lipase solution. When the permeate volume reached more than 80%, the circulation was stopped. The residual of lipase solution in shell side and lumen side were completely purged with nitrogen gas. The retentate and permeate were collected and the volume was recorded. Fresh organic solvent of 250ml was pumped and circulated through the shell side and lumen side of the reactor at 15ml/min for 2h to create the reaction microenvironment of lipase. The residual of fresh organic solvent in shell side and lumen side were then completely purged with nitrogen gas. The protein concentrations of the initial lipase solution, the permeate and the retentate were quantified by BCA test kit (Pierce,

Rockford, IL). The amount of the protein retained on the membrane was calculated from the protein mass balance of these solutions.

2.5. Experimental Procedure

Enantioselective esterification of (R,S)-ketoprofen in organic medium was conducted in EMR. The (R,S)-ketoprofen was added into 250ml of organic solvent (20% 1,2-dichloropropane - 80% hexane). The solution was heated until all the substrate was fully dissolved. After the solution was cooled to room temperature, appropriate amount of alcohol was added. Subsequently, the solution was stirred to homogenize the reaction mixture. The reaction mixture of 250ml was then circulated in the shell side at the flow-rate of 15ml/min where the lipase was immobilized. The reaction temperature was increased to 40°C to start the esterification reaction. Periodically, 1ml aliquot was withdrawn from the reaction medium for HPLC analysis.

2.6. Analytical Methods

The concentration of ketoprofen enantiomers was determined using a HPLC system (Shimadzu, Kyoto, Japan) equipped with a chiral stationary phase column: (R,R)-Whelk-O1 chiral column (Regis Technologies, Morton Grove, USA) and a UV detector (254nm). A mixture of dichloromethane, *n*-hexane and ethanol (0.25M ammonium acetate) (47:47:6, v/v/v) was used as an eluent. The flow rate was maintained at 1.5ml/min and the column temperature at 25°C. 60μl of the sample was injected. An example of HPLC chromatogram of pure (R,S)-ketoprofen is illustrated by Fig. 3.

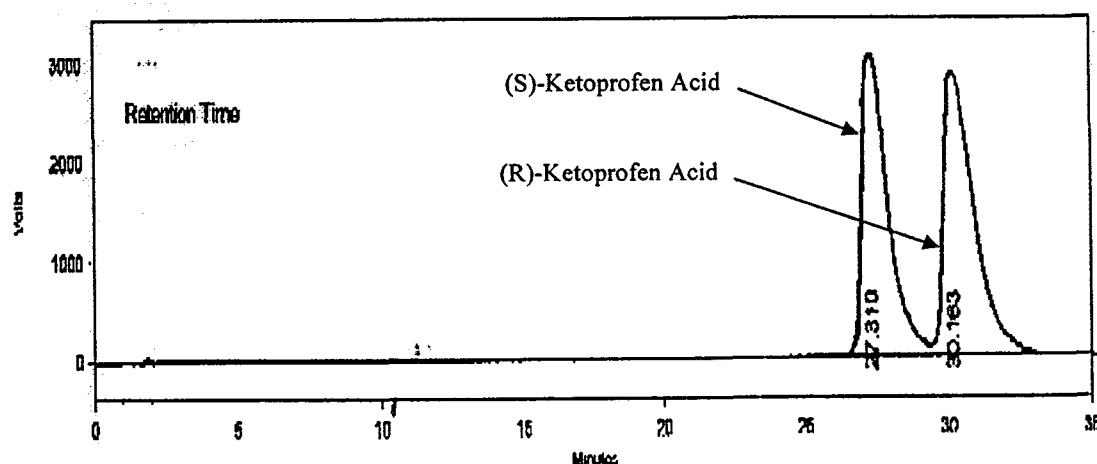


Fig. 3. HPLC chromatogram of pure (R,S)-ketoprofen.

The conversion of ketoprofen was calculated using the following equations:

$$X (\%) = \frac{C_0 - C_t}{C_0} \times 100 \quad (1)$$

$$X_R (\%) = \frac{C_{R0} - C_{Rt}}{C_{R0}} \times 100 \quad (2)$$

Where X is the overall conversion (%), X_R the conversion (%) of (R)-ketoprofen (fast reacting enantiomer), C_0 the initial amount of racemic ketoprofen (mM), C_t the amount of racemic ketoprofen (mM) at reaction time t , C_{R0} the initial amount of (R)-ketoprofen and C_{Rt} the amount of (R)-ketoprofen (mM) at reaction time t .

Enantiomeric excess of substrate (ee_s) and product (ee_p) [12] and enantiomeric ratio or enantioselectivity (E) [18] were calculated using the following equations:

$$ee_s (\%) = \frac{([S]_{acid} - [R]_{acid})}{([S]_{acid} + [R]_{acid})} X 100 \quad (3)$$

$$ee_p (\%) = \frac{([R]_{ester} - [S]_{ester})}{([R]_{ester} + [S]_{ester})} X 100 \quad (4)$$

$$E = \frac{\ln [1 - X(1 + ee_p)]}{\ln [1 - X(1 - ee_p)]} \quad (5)$$

Where $[R]_{acid}$ and $[S]_{acid}$ represent the concentration of the (R) and (S) enantiomers of ketoprofen acid while $[R]_{ester}$ and $[S]_{ester}$ represent the concentration of the (R) and (S) enantiomers of ketoprofen ester.

The enzymatic activity was defined as μmol of substrate consumed per hour per milligram of immobilized protein as illustrated by the following relation (3.11) where 1U of lipase was defined as the amount of lipase necessary to consume $1\mu\text{mol}$ of (R)-ketoprofen acid per hour.

$$\text{Lipase Activity} = \frac{[X_R] \times [C_0]}{[t] \times [\text{mg protein}]} = \frac{\mu\text{mol}}{\text{h.mg}} = \frac{U}{\text{mg}} \quad (6)$$

3. RESULTS

It is often believed that only primary alcohol are effective for esterification reactions catalysed by lipases in organic media due to the steric hindrance of the secondary and tertiary alcohols [19]. Thus, the primary alcohol was tested for the effect of alcohols in this study. The effect of alcohols on the performance of lipase-immobilized EMR for enantioseparation of (R,S)-ketoprofen was studied under constant condition (15ml/min, 40°C and pH 7.0). The alcohols screened were ethanol, butanol, heptanol, and 2-ethoxyethanol. Fig. 4 shows the effect of alcohols on the production of (R)-ketoprofen ester. As shown in the Fig. 4, the performance of lipase immobilized in EMR for enantioselective esterification of (R,S)-ketoprofen was proportional to the increase of the primary alcohol chain length. The enantioselective esterification of (R,S)-ketoprofen, with ethanol in lipase immobilized EMR resulted in the highest reaction rate among the tested alcohols followed by butanol, heptanol and 2-ethoxyethanol.

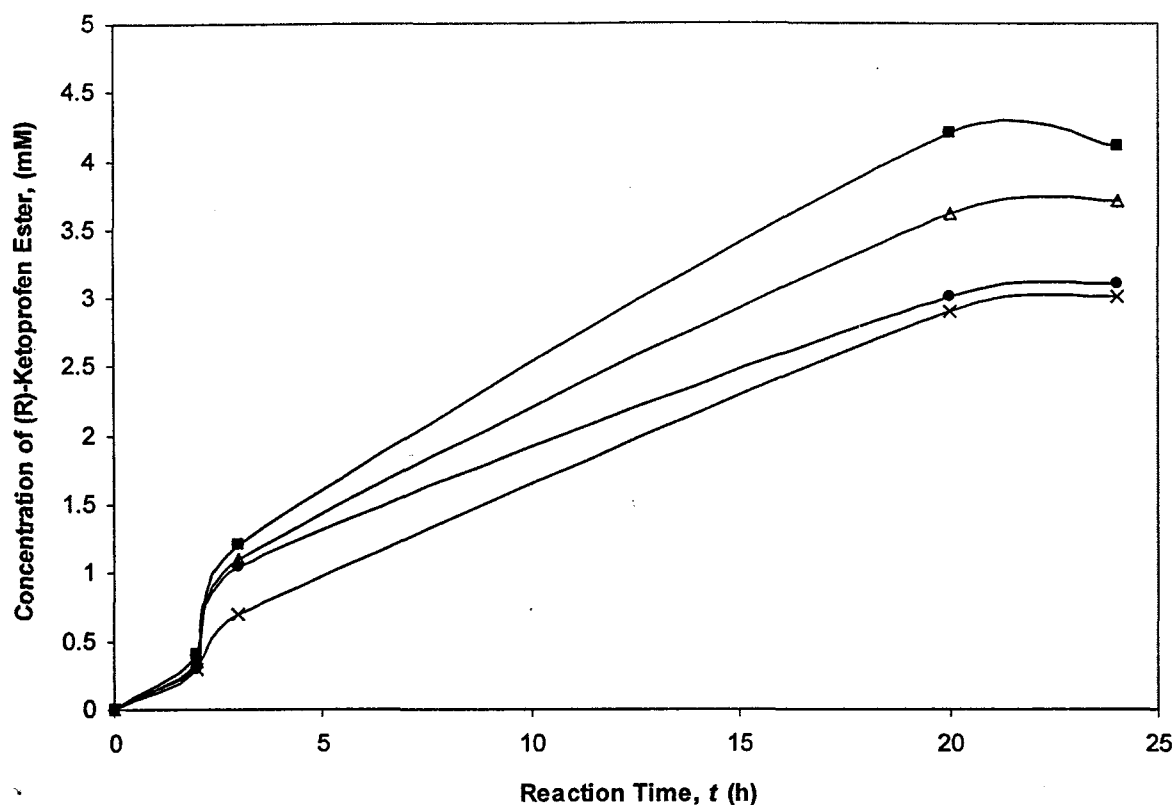


Fig. 4. Effect of different alcohols on the production of (*R*)-ketoprofen ester. Reaction conditions: 10mM of (*R,S*)-ketoprofen, 2:1 molar ratio of *n*-butanol to (*R,S*)-ketoprofen, (1,2)-dichloropropane:hexane (20:80, %v/v) as solvent, 158mg immobilized CALB, lipase solution pH of 7.0, 40°C, 15ml/min. Symbols: (■) ethanol, (Δ) butanol, (●) heptanol, (x) 2-ethoxyethanol.

The effect of alcohols on the enantioselective esterification of (*R,S*)-ketoprofen in lipase immobilized EMR in terms of enantiomeric excess of substrate (ee_s), enantiomeric excess of product (ee_p), conversion of (*R*)-ketoprofen (X_R) and lipase enantioselectivity (E) at the reaction time of 24h were summarized in Table 1. As shown in Table 1, the enantioselective esterification of (*R,S*)-ketoprofen in lipase immobilized EMR containing ethanol showed higher conversion, enzyme enantioselectivity, enantiomeric excess of substrate (ee_s) and enantiomeric excess of product (ee_p). With the increase of the carbon chain length of the alcohols, the ee_p and E decreased while the relationship between ee_s and conversion of (*R*)-ketoprofen with the increase of carbon chain length of alcohols remained unclear. Since the enantioselective esterification of (*R,S*)-ketoprofen with ethanol and butanol gave competitive performance in lipase immobilized EMR, these alcohols were selected for the following study.

Table 1. Effect of alcohols on the enantioselective esterification of (R,S)-ketoprofen after 24h reaction

Alcohols	ee_S (%)	ee_P (%)	X_R (%)	E
Ethanol	25	26	28	2.3
Butanol	8	25	27	1.7
Heptanol	11	18	16	1.6
2-Ethoxyethanol	10	5	26	1.2

The utilization of enzymes in processes often encounters the problem of thermal inactivation of enzyme. The dependence of the enzymatic esterification on reaction temperature in the presence of ethanol and butanol respectively were investigated by varying the reaction temperature in the range of 35–50°C holding all other parameters constant. The effect of alcohols on relative activity (the highest enzyme activity achieved was defined as 100% activity) of immobilized lipase in EMR for enantioselective esterification of (R,S)-ketoprofen at various reaction temperature are shown in Fig. 5. Thus, Fig. 5 shows that by increasing the reaction temperature from 35°C to 50°C, the relative activity of immobilized lipase in EMR significantly improved from 69% to 100% for the case of butanol as the alcohol for the esterification of (R,S)-ketoprofen. In contrast, the relative activity of immobilized lipase in EMR decreased from 100% to 76% when the ethanol was used as the alcohol for the reaction. The highest lipase relative activity of 100% at 50°C was obtained for the case of butanol while the highest lipase relative activity of 100% for the case of ethanol falls at 35°C.

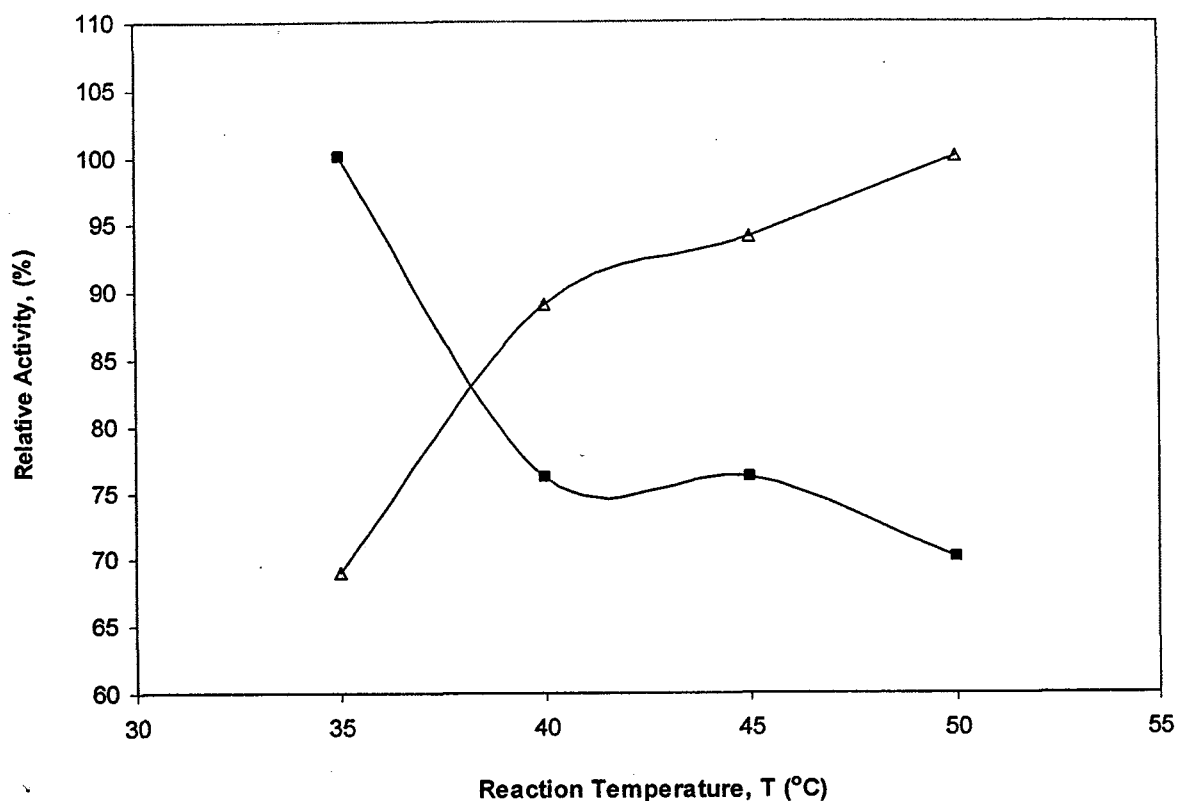


Fig. 5. Effect of different alcohols on the relative activity of immobilized lipase in EMR. Reaction conditions: 10mM of (R,S)-ketoprofen, 2:1 molar ratio of *n*-butanol to (R,S)-ketoprofen, 24h, 1,2-dichloropropane:hexane (20:80, %v/v) as solvent, 158mg immobilized CALB, lipase solution pH of 7.0, 15ml/min. Symbols: (-■-) ethanol, (-Δ-) butanol.

4. DISCUSSION

The carbon chain length of alcohol was shown to affect the free lipase catalysed esterification reaction of (R,S)-ketoprofen in a previous study [20]. The alcohols with shorter chain were likely to enhance the reaction rate and gave better lipase catalytic activity than those with long chain [14]. In the present work, the results obtained may be explained by a slower diffusion rate of the long chain alcohols into the lipase immobilized support [21] and the nucleophilicity of the alcohols [22]. In esterification reaction, ester formation and enzyme regeneration occurred after the nucleophilic attack by the alcohol on the acid at the active site of the lipase. It is expected that the more nucleophilic an alcohol is, the faster its reaction rate [22]. In the case of ethanol, it is more electron-releasing which have resulted to stronger nucleophilicity. However, this effect decreases with the increasing distance of the carbon chain length to its reacting group meaning that the alcohol nucleophilicity decreases with increasing the number of carbon atoms, resulting in decreasing reactivity. Besides, the nucleophilic attack by the hydroxyl group of alcohol becomes increasingly difficult as the size of the alcohol moiety becomes larger; the steric effect becomes stronger and engaged the acylation process between enzyme and substrate [22]. Thus,

the ethanol with shorter carbon chain length resulted in higher initial reaction rate, conversion of (R)-ketoprofen, ee_s and ee_p in the present study. However, the polarity of alcohol decreases with increasing the carbon chain length and reduces its acidity. This indicated that there will be stronger inhibition effect by the smaller alcohol molecule with shorter carbon chain length [22]. It is also known that, an increase in operating temperature increases the solubility of the substrate thus assisted in the diffusion of the substrate to the enzyme immobilized on the membrane where the reaction took place. Figure 5 reflected this behaviour and showed that since butanol which is less inhibitory to the ketoprofen acid, gave an increased enzyme activity compared to the ethanol in the temperature ranged studied.

5. CONCLUSION

(R,S)-ketoprofen can be successfully resolved by an esterification reaction catalysed by an immobilized lipase, *Candida antarctica* lipase B in EMR. The effects of alcohols on the performance of lipase immobilized EMR in terms of initial reaction rate, conversion of (R)-ketoprofen, ee_s , ee_p and thermal stability of the immobilized lipase have been studied. The immobilized lipase in EMR gave the highest initial reaction rate, conversion of (R)-ketoprofen, ee_s and ee_p for reaction containing ethanol while with butanol showed less inhibitory effect in the EMR.

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Dynamic kinetic resolution: alternative approach in optimizing S-ibuprofen production

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Abstract A lipase catalysed enantioselective hydrolysis process under in situ racemization of the remaining (R)-ibuprofen ester substrate with sodium hydroxide as the catalyst was developed for the production of S-ibuprofen from (R,S)-ibuprofen ester in isooctane. Detailed investigations on parameters study indicated that 0.5 M NaOH, addition of 20% (v/v) co-solvent (dimethyl sulphoxide), operating temperature of 45 °C, and 40 mmol/L substrate gave 86% conversion and 99.4% optical purity of S-ibuprofen in dynamic kinetic resolution. Meanwhile, in common enzymatic kinetic resolution process, only 42% conversion of the racemate and 93% enantiomeric excess of the product was obtained which are of lower values as compared to dynamic kinetic resolution. The S-ibuprofen produced during each process was evaluated and approximately 50% increment in concentration of S-acid product was produced when dynamic kinetic resolution was applied into the process.

Keywords Dynamic kinetic resolution · Lipase · Sodium hydroxide · Hydrolysis · Ibuprofen ester

Abbreviations NaOH: Sodium hydroxide · DKR: Dynamic kinetic resolution · DMSO: Dimethyl sulphoxide · NSAIDs: Non-steroidal anti-inflammatory drugs · HPLC: High pressure liquid chromatography · E_e : Enantiomeric excess of the product

Introduction

Racemic compounds can be resolved chemically, enzymatically, and by asymmetric synthesis. Of all these

methods, enzymatic resolution of racemate is increasingly used because of high enantioselectivity of the enzymes [1–3]. However, the disadvantage of this method is that a maximum product yield of 50% in the kinetic resolution procedures is obtained, based on the racemic starting substrate [4]. In order to overcome the limitation, dynamic kinetic resolution has proved to be a potentially efficient process in which the standard kinetic resolution is coupled with continuous in situ racemization of the unreacted starting substrate [4, 5]. The process can theoretically result in a single product enantiomer with 100% yield [6]. Although such process has industrial practicality, only fairly specific examples on the resolution of amino acids, carboxylic acids, alcohols, cyanohydrin, and hemithioacetal esters have been reported [7]. 2-(4-Isobutylphenyl) propionic acid or commonly known as ibuprofen acid is one of the important member of non-steroidal anti-inflammatory drugs (NSAIDs) that belong to the family of propionic acid. It is widely used to treat rheumatoid arthritis, headache, muscular strain, cephalalgia, etc. [8]. Common ibuprofen is marketed in the racemic form, which contain an equal amount of S and R enantiomer. This is because such racemates are easily obtainable through chemical synthesis. However, in many chemical and pharmaceutical reactions, chirality of each enantiomer does play an important role in defining the properties of the product, especially in the pharmaceutical industry when safety is of utmost importance. For example, ibuprofen itself, the physiological activity was mainly from S-enantiomer which showed 100 times more potential in therapeutics effects [1, 9]. Meanwhile, the presence of R-ibuprofen will cause serious side effect to the human body such as gastrointestinal pain. The pharmacology behaviours of each enantiomer in the racemate ibuprofen have been reported by Evans [10] on the pharmacology studies of acidic NSAIDs. Therefore, the two enantiomers of ibuprofen can be regarded as two different drugs because of difference in their pharmacological properties [11, 12]. Considerable efforts have been made to obtain (S)-profen from enzymatic

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resolution processes [1, 9, 13, 14]. This included also the use of membrane reactor in enzymatic resolution as reported by Long et al. [14]. Recently, a facile dynamic kinetic resolution process was further reported, in which S-naproxen, S-suprofen, and S-fenoprofen were obtained in high yield and optical purity by using *Candida rugosa* lipase and organic base as catalysts [4, 5, 15–18]. As this methodology is promising, it is the purpose of the present study to produce S-ibuprofen from (R,S)-ibuprofen ethoxyethyl ester according to Fig. 1. Detailed investigations on the effects of sodium hydroxide concentration, addition of co-solvent, temperature, and substrate concentration on racemization and enzymatic resolution were conducted. Moreover, a comparison between kinetic resolution and dynamic kinetic resolution performance was made for the production of the desired S-ibuprofen enantiomer.

Materials and methods

Materials

Racemic ibuprofen was purchased from Shasun Drugs Company, India. Meanwhile the free enzyme, lipase from *C. rugosa* (EC 3.1.1.3, type VII, 724 units/mg solid) were purchased from Sigma. Amberlite XAD 7 was purchased from Sigma-Aldrich companies. Hexane, 2-propanol and acetic acid were of high pressure liquid chromatography (HPLC) grade. 2-Ethoxyethanol, sodium hydroxide pellets, isooctane and other solvents as well as reagents used were of analytical grade, and were obtained commercially. Racemic ibuprofen acid and racemic ethoxyethyl ibuprofen ester were resolved on a Regis Whelk-01 Chiral Column (15 cm×4.6 mm i.d., 5 µm particle size) with *n*-hexane, isopropanol and acetic acid in 98/2/0.5% (v/v/v) as a mobile phase. An amount of 50 mM phosphate buffer solution was used as buffer solution. The pH required in the process was adjusted by dosing the solution of potassium dihydrogen phosphate

buffer (Fisher). A constant mobile phase with flow rate of 1.0 mL/min was used at the wavelength of 254 nm with Shimadzu's UV detector.

Synthesis of (R,S)-ibuprofen ester

Esterification of racemic ibuprofen was carried out in the batch reaction using 96.94 mmol racemic mixture with 224 mmol 2-ethoxyethanol. The solution was dissolved in 180 mL isooctane with *p*-toluenesulphonic acid (0.44 g, 2.44 mmol) as a catalyst in the reaction. The solution was refluxed for 8 h. The reaction mixture was cooled at room temperature and washed with 5% sodium hydroxide and water. The organic layer was dried over anhydrous sodium sulphate to remove the water and evaporated to dryness yielding up to 87.25 mmol (R,S)-ibuprofen ester.

Lipase immobilization on Amberlite XAD 7

The immobilization of lipase was prepared by adsorption of the lipase on Amberlite XAD 7. The enzyme solution (50 mL) was prepared by dissolving 2.2 g crude lipase in the 50 mL phosphate buffer solution, pH 7. The solution was brought in contact with 2.0 g of support washed with phosphate buffer (pH 7, 50 mM) in succession for 1.5 h. The lipase-support was mildly shaken at room temperature for 24 h. The supports were separated from the solution by filtration which was subsequently dried and stored at 4 °C for further use.

Kinetic resolution of (R,S)-ibuprofen ester

Twenty-five millilitres of isooctane was added with 0.2 g of the immobilized lipase and different (R,S)-ibuprofen ester concentration (range from 20 to 50 mM). The solution was agitated in agitation shaker at 45 °C,

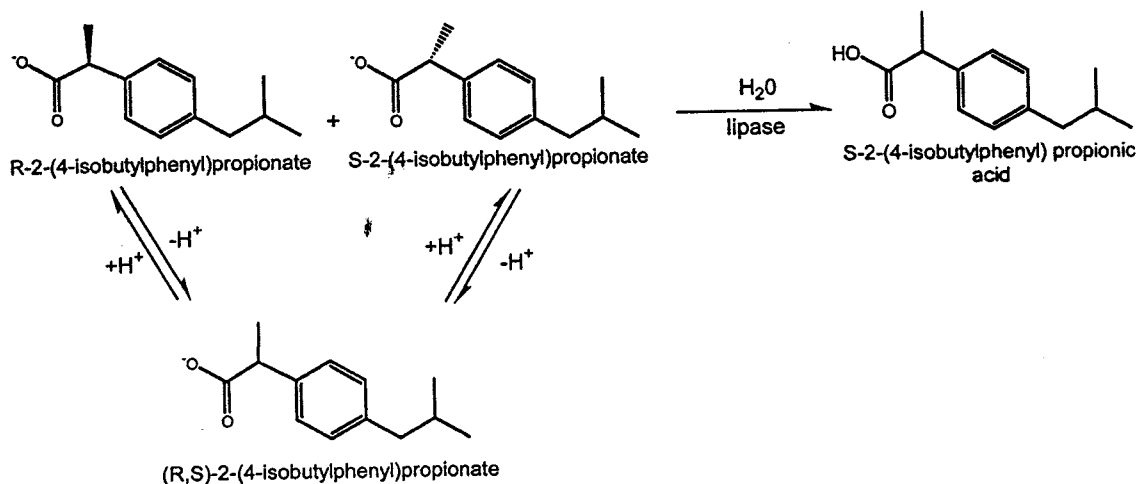


Fig. 1 Overall mechanism for S-ibuprofen synthesis via dynamic kinetic resolution process

200 rpm. Sample was removed and injected onto HPLC system at different time intervals for analysis. The conversion and enantiomeric excess of the product were calculated from equation of Chen et al. [19] as given below:

$$ee_p = \frac{[P] - [Q]}{[P] + [Q]} = \text{enantiomeric excess of product P}$$

$$X = 1 - \frac{[A] + [B]}{[A_0] + [B_0]} = \text{extent of conversion}$$

where the symbol $[P]$ and $[Q]$ represent S-ibuprofen concentration and R-ibuprofen concentration, respectively. Meanwhile, the symbol $[A]$ and $[B]$ for S-ibuprofen and R-ibuprofen ester concentration, $[A]_0$ and $[B]_0$, for S-ibuprofen and R-ibuprofen ester concentration at $t=0$. Then, the X symbol represents the conversion of the process.

Dynamic kinetic resolution of (R,S)-ibuprofen ester

To 25 mL of isooctane was added 40 mM of (R,S)-ibuprofen ester, 0.5 M NaOH, and 0.2 g immobilized lipase. The reaction medium consisted of two layers of solution; organic ibuprofen ester and an aqueous NaOH where the reaction only occurred at the contact surface between these two layers. Then, the resultant solution was agitated in orbital shaker at constant temperature of 45 °C and 200 rpm. Sample from organic phase was withdrawn and injected onto the HPLC systems at different time intervals for analysis. Then, the time course conversion for enantiomers, X_R and X_S , the racemate conversion, X_t and enantiomeric excess of the product were calculated.

Analysis

The hydrolysis of (R,S)-ibuprofen ester and racemization of R-ibuprofen ester in aqueous-isooctane was monitored using HPLC with chiral column from Regis [(R,R)-WHELK-01: Morton Grove, IL, USA] capable of separating the internal standard of (R)- and (S)-ibuprofen, (R)- and (S)-ibuprofen ester with typical retention time of 5.467, 5.617, 5.267, and 5.367 min, respectively. The mobile phase was a mixture of *n*-hexane:isopropanol:acetic acid with the ratio of 98:2:0.5 (v/v). The flow rate was set to 1.0 mL/min with the UV detection of 254 nm.

Results and discussion

Kinetic resolution versus dynamic kinetic resolution

The performance between dynamic kinetic resolution and conventional enzymatic kinetic resolution is illustrated in Fig. 2. Dynamic kinetic resolution showed the

potential in enhancing the production of S-ibuprofen. The S-ibuprofen produced in dynamic kinetic resolution after 120 h reaction time was 27.7 mmol/L. Approximately 50% increment in the concentration of S-ibuprofen was observed in the dynamic kinetic resolution process as compared to conventional kinetic resolution. This trend showed the potential of base to racemize unreacted R-enantiomer substrate into its antipode and thus, a continuous S-ibuprofen ester was supplied into the resolution process. Meanwhile, enzymatic kinetic resolution with immobilized lipase gave only 16.8 mmol/L of S-ibuprofen after 120 h reaction hour as shown in the result. It can be noted that the dynamic kinetic resolution (DKR) process is a suitable process in obtaining pure enantiomer with 100% theoretical yield. However, the performance in the DKR process will be improved by varying the parameters that gave profound effects on the process.

Effect of NaOH loading

The addition of NaOH into the reaction medium showed a profound effect on improving the hydrolysis rate and enhancing the formation of S-ibuprofen. Thus, further study was carried out by varying the concentration of NaOH from 0.3 to 1.0 M. The observed results are shown in Fig. 3. Lower conversion and concentration of the product were observed at 0.3 M NaOH. The conversion achieved was 56% and resulted in 22.3 mmol/L of product. A 0.5 M NaOH gave the highest formation of S-ibuprofen which was 27.7 mmol/L at 69.3% conversion. However, further increase of NaOH concentration to 0.7 and 1.0 M resulted in a decrease of product concentration. About 66.8% conversion was achieved after 120 h reaction time using 0.7 M NaOH and produced 26.7 mmol/L of the S-ibuprofen. Then, at 1.0 M NaOH concentration only 50% of conversion was achieved and gave approximately

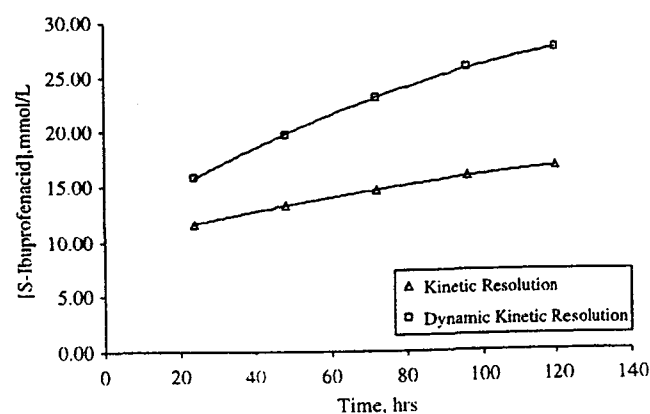


Fig. 2 The comparison between dynamic kinetic resolution and kinetic resolution process in the production of S-ibuprofen acid using similar reaction conditions

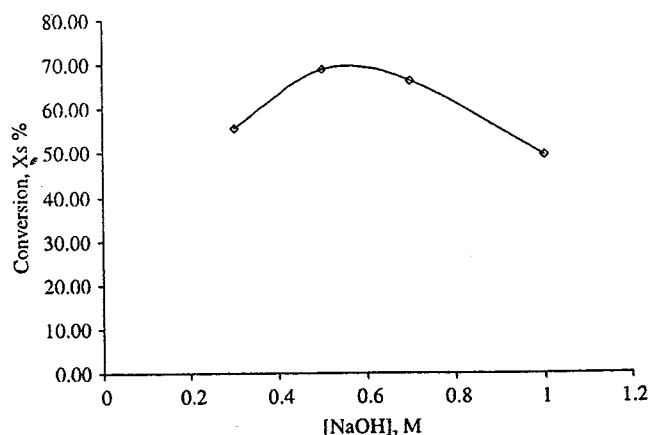


Fig. 3 Effect of addition of various NaOH concentrations on the conversion of (R,S)-ibuprofen ester in dynamic kinetic resolution process. Reaction conditions: 20 mmol/L (R,S)-ibuprofen ester, 0.2 g immobilized lipase, isooctane as a solvent and agitation speed of 200 rpm. The reaction takes place for 120 h

19.9 mmol/L of the product. This indicated that the optimum racemization process occurred at lower NaOH concentration of 0.5 M. In addition, the optical purity of the product was increased to 98.4% as shown by enantiomeric excess value. By comparing the product concentration, the highest base concentration (1.0 M) resulted in 30% reduction of product concentration as compared to 0.5 M NaOH. The optimum performance obtained with lower base concentration of 0.5 M may be due to the ability of only small amount or concentration of OH required at physiological pH (pH 7.0–7.3). Generally, the proton is transferred to the base in the transition state of the slow step [12]. Since this process deals with different reaction and different catalyst simultaneously in one pot reaction, the extreme condition should be avoided to obtain the best performance in both reactions involved. Generally, high concentration of base was favoured for racemization process but it caused a rapid decrease in enzymatic catalyst reaction since lipase activity was interfered with the presence of base [7]. The racemization process with addition of NaOH at 0.5 M concentration was also reported by Xin et al. [4] using naproxen methyl ester as a substrate.

Effect of temperature

The effect of temperature became one of the crucial factors in the enzymatic resolution since the enzyme required a mild temperature condition in order to obtain the optimum catalytic activity. Thus, when dealing with a combination of chemical catalyst and biocatalyst, temperature is the factor that needs to be considered in the experiment. The effect of temperature was studied in the range of 30–50 °C. According to Fig. 4, the highest conversion of S-ibuprofen in the DKR process was achieved at 45 °C. About 79% conversion was obtained at the reaction temperature of 45 °C. Generally, with

increasing reaction temperature, the conversion of S-ibuprofen increases. As shown in Fig. 4, at 30 °C only 67.3% conversion was achieved. However, when the reaction temperature was increased to 45 °C, about 15% increment in conversion was observed. Meanwhile, at high temperature of 50 °C, the conversion obtained was 76% which is 3.7% lower than the optimum result. The reaction temperature of 45 °C gave a maximum production of S-ibuprofen of 31.6 mmol/L at 120 h reaction time. Approximately 46.8% increment in S-ibuprofen concentration was observed when base catalysed racemization was added into the kinetic resolution at the same reaction condition. This indicated that at temperature 45 °C, large amount of R-ibuprofen ester was simultaneously converted into (R,S)-ibuprofen ester and hence, encouraged the formation of S-ibuprofen through the hydrolysis process. For the racemization process to be effectively performed, the higher temperature was required in order to invert R-enantiomer into the racemic form [20]. Thus, temperature at 45 °C was selected as a suitable temperature for both reaction conditions (kinetic and dynamic kinetic resolution). The immobilized enzyme was also effective at 45 °C before denaturation process occurs. The combination process between racemization by base catalyst and enzymatic catalysis process has been studied previously in the temperature range of 40–60 °C by Li et al. [21] on formation of mandelonitrile acetate. Meanwhile, at high temperature of 50 °C, a small decrease in S-ibuprofen concentration was observed but it was still higher compared to the value obtained in conventional kinetic resolution, that is, 12.40 mmol/L. At 50 °C, slow hydrolysis process occurred due to the decrease in enzyme activity at high temperature and this resulted in a lower racemization rate as well as S-ibuprofen formation. Thus, it gave a lower concentration of S-ibuprofen when compared with the result obtained at 45 °C in the dynamic kinetic resolution process. However, at 50 °C

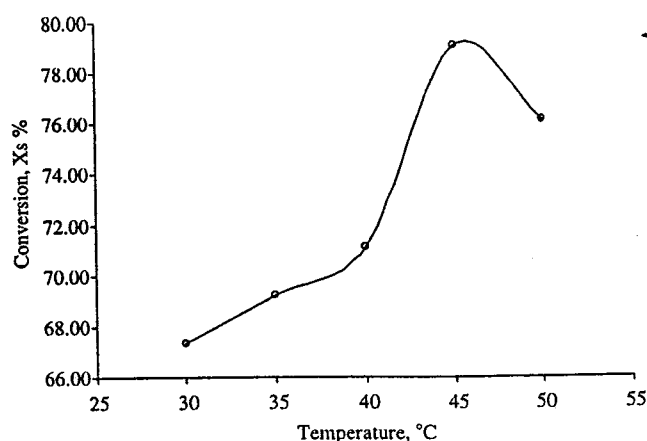


Fig. 4 Effect of various temperatures on the conversion of (R,S)-ibuprofen ester. Reaction conditions: 0.5 M NaOH, 20 mmol/L (R,S)-ibuprofen ester, 0.2 g immobilized lipase, isooctane as a solvent and agitation speed of 200 rpm. The reaction takes place for 120 h

there was no significant change observed for the enantiomeric excess of the product. The highest optical purity of the product was observed at 45 °C which gave 98.6% of enantiomeric excess.

Effect of substrate concentration

Different substrate concentrations were introduced in the racemization process in order to indicate the performance of the racemization; assuming that at high concentration of the racemic ester, more unreacted R-ibuprofen ester is expected to be present. Hence, this increased the possibility of the racemization process to occur. The range of substrate concentration used in the study was between 20 and 50 mmol/L. From Fig. 5, the highest conversion was obtained using 40 mM (R,S)-ibuprofen ester as a substrate. By increasing the substrate concentration, the conversion of the reaction was also increased which indicated that large amount of the R-ibuprofen ester was present for the racemization process. However, the conversion of S-ibuprofen was reduced when higher substrate concentration of 50 mM was introduced into the system. The conversion observed at 50 mmol/L of (R,S)-ibuprofen ester was 66.8% which was 19% lower than the conversion observed at 40 mmol/L substrate. At higher substrate concentration, high amount of ethoxyethanol (side product) will also be produced which may result in low enzyme activities. During this condition, the probability of converting the substrate into S-product decreased. The product inhibition at high concentration of substrate has previously been observed by Madhav and Ching [1]. Therefore, the overall conversion in DKR process was reduced when high substrate concentration was applied in this reaction. Similar to the conversion, the formation of the product also showed a reduction at high concentration of the substrate. By increasing the substrate concentration from 20 to 40 mmol/L, the increment in product

conversion was observed. The highest concentration of the product obtained was 68.6 mmol/L at 40 mmol/L of substrate. A 28.6% increment in product concentration from total substrate concentration used indicated that around 50% racemization of R-ibuprofen ester had occurred. However, at 50 mmol/L substrate concentration, 66.8 mmol/L S-ibuprofen was produced after 120 h reaction time which is slightly lower than at substrate concentration of 40 mmol/L. Only 3% difference in the S-acid produced at 40 mmol/L substrate and 50 mmol/L substrate was observed even though a large difference in conversion was obtained. This is because at 50 mmol/L substrate, the quantity of substrate is about 10% extra as compared to 40 mmol/L. So, the S-ibuprofen produced will still be in high concentration even when a low conversion was obtained. Therefore, this parameter study should be optimized for better performance in both processes.

Effect of co-solvent

The effect of the addition of co-solvent into the reaction medium is shown in Fig. 6. The effect of co-solvent on conversion and concentration of the product was studied in the range of 20–80% (v/v). From the observation, the conversion was increased with the addition of the dimethyl sulphoxide (DMSO) solvent. However, at higher amount of DMSO added (>20%), a reduction of conversion was observed. The optimum percentage of DMSO required in the reaction medium was 20% which gave the conversion of the (R,S)-ethoxyethyl ibuprofen ester of 87.7%. Nevertheless, reaction condition without addition of co-solvent gave better performance and more stability for the whole experimental time interval. The optimum conversion obtained was with the addition of 20% DMSO and gave 35.7 mmol/L S-ibuprofen. Meanwhile, without addition of DMSO into the reac-

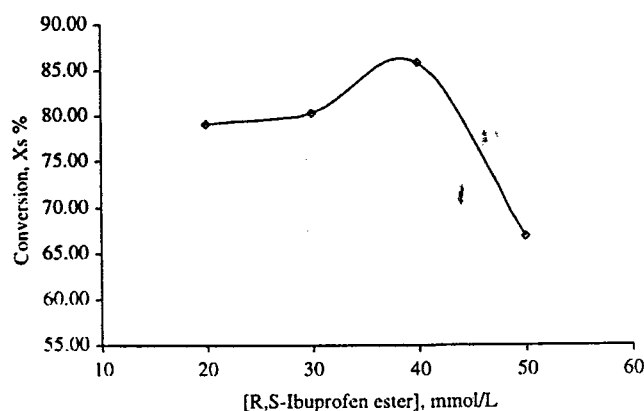


Fig. 5 Effect of addition of various substrate concentrations on the conversion of (R,S)-ibuprofen ester. Reaction conditions: 0.5 M NaOH, 0.2 g immobilized lipase, isooctane as a solvent and agitation speed of 200 rpm. The reaction takes place for 120 h

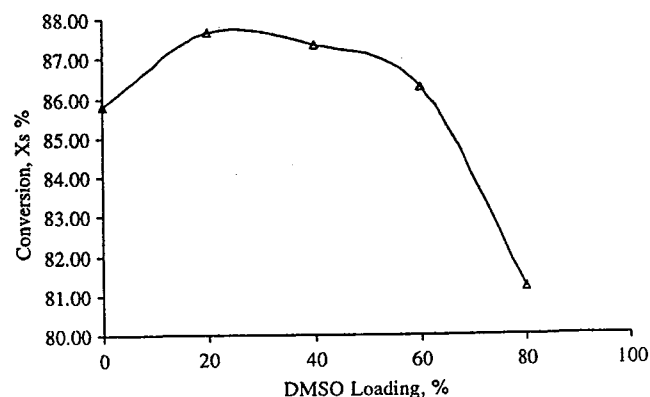


Fig. 6 Effect of addition of DMSO to the conversion of (R,S)-ibuprofen ester in dynamic kinetic resolution process. Reaction conditions: 0.5 M NaOH, 0.2 g immobilized lipase, 45 °C, isooctane as a solvent and agitation speed of 200 rpm. The reaction takes place for 120 h

tion, the S-ibuprofen produced after 120 h reaction time was 34.3 mmol/L. Only a small increment in total S-acid concentration was observed in the reaction. Generally, addition of DMSO–water content to the reaction medium increased the basic condition that is required in the racemization process. However, with higher DMSO content, lower NaOH concentration was allowed to form a homogeneous phase. Only a small amount of DMSO is required in basic medium to accelerate the racemization rate of ibuprofen enantiomer. On the other hand, the enantiomer is highly stable in pure DMSO and DMSO–water mixed phase. The same percentage of DMSO was observed to enhance the conversion as well as the racemization process [22, 23]. The optical purity of the product in terms of enantiomeric excess of the product decreased as the amount of DMSO was increased in the reaction medium. The maximum enantiomeric excess of the product produced was at 20% DMSO and gave about 98.5% enantiomeric excess of the product. Meanwhile, the highest enantiomeric excess of the product was also shown at 40 mM of (R,S)-ibuprofen ester and gave the value of 99.4%. High value of enantiomeric excess of the product suggested that racemization and kinetic resolution effectively occurred at this condition. This condition was proved by the value of interconversion constant which is $3.85 \times 10^{-2} [B]$ where $[B]$ represents the concentration of base catalyst. It was found that the interconversion constant obtained in this work was approximately in the same order of magnitude as that reported in the literature even when different substrates were used. The higher interconversion constant obtained implies that the α -substituents of ibuprofen ester has contributed greater inductive and resonance polar effects on the acidity of the α -hydrogen, and hence, enhances the racemization rate [15]. The enantiomeric excess of the product against conversion is illustrated in Fig. 7 to determine the effect of optimum condition with different substrate concentration in the

racemization process. From the figure, the enantiomeric excess of the product showed almost a consistent value with the increment of conversion. The highest enantiomeric excess of the product was achieved in the reaction with 40 mmol/L of substrate (R,S)-ibuprofen ester. The high enantiomeric excess value explained that the ability of NaOH to racemize the unreacted R-ibuprofen ester into the (R,S)-ibuprofen ester form thus produced the S-ester substrate for continuous kinetic resolution process.

Conclusion

It is feasible to combine racemization into the kinetic resolution process in the resolution of (R,S)-ibuprofen ethoxyethyl ester. Through this method, the enantiopure S-ibuprofen can be obtained at 99.4% and the conversion higher than 85% can be achieved. It was demonstrated that the synthesis of S-ibuprofen via dynamic kinetic resolution process is highly dependent on the suitability of the reaction medium between enzymatic resolution and racemization process. The best performance for both process occurred at sodium hydroxide concentration of 0.5 M, temperature of 45 °C, and addition of only 20% (v/v) DMSO. Meanwhile, in order to avoid inhibition in enzymatic kinetic resolution, 40 mM of (R,S)-ibuprofen ethoxyethyl ester was selected as the best substrate concentration for dynamic kinetic resolution process. The highest S-ibuprofen produced with these conditions was 68.6 mmol/L. These improvements have made the dynamic kinetic resolution in S-ibuprofen synthesis truly competitive to other S-ibuprofen synthesis.

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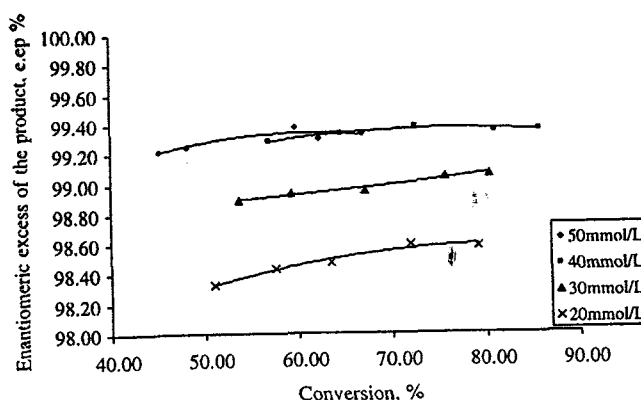
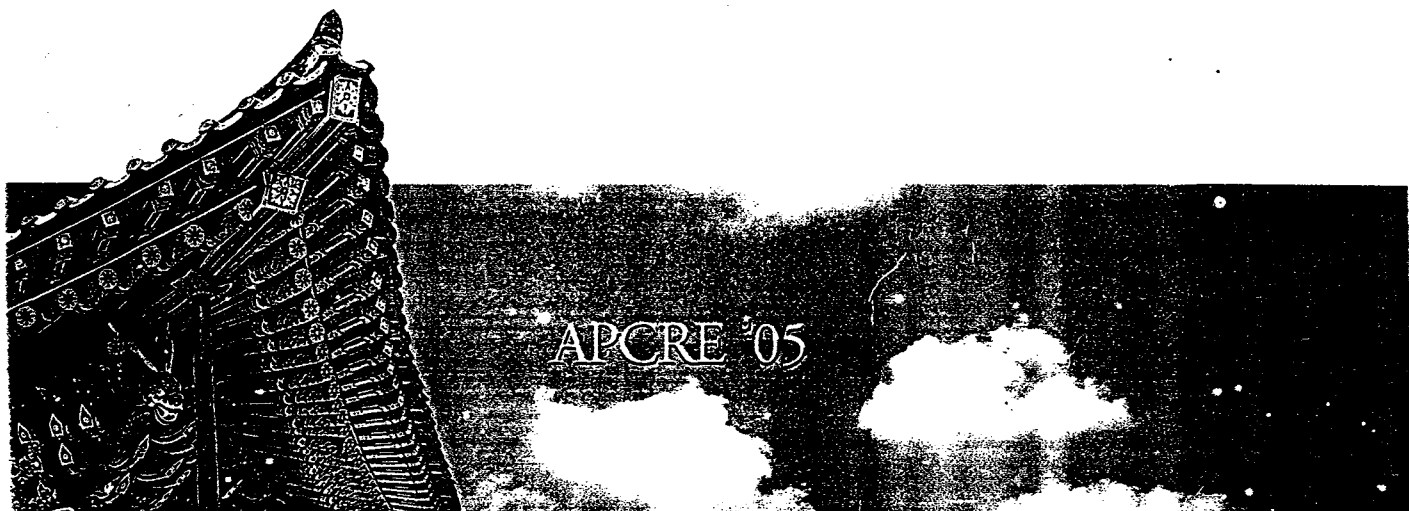


Fig. 7 Effect of different substrate concentration to the racemization condition and production of S-ibuprofen acid in terms of enantiomeric excess of product and conversion. Reaction conditions: 0.5 M NaOH, 0.2 g immobilized lipase, 45 °C, isooctane and agitation speed of 200 rpm

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Kinetics and enantioselectivity of lipase in the kinetic resolution of racemic ibuprofen ester

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Introduction

The kinetic studies of immobilized lipase in a hollow fiber membrane reactor system for kinetic resolution of racemic ibuprofen esters are reported. Ibuprofen is a nonsteroidal anti-inflammatory drug (NSAID) effective in treating fever, pain, and inflammation in the body and is being sold as racemic compound (a racemic is a mixture of R- & S-enantiomers). Only the (S)-enantiomer of ibuprofen provides the desired therapeutic (healing) effect, while the other enantiomer is inactive and causes side effects [Adams et al., 1976]. The production of an optically pure single enantiomer from its racemic mixture may be achieved by chiral resolutions, which can be initiated either by a prochiral intermediate, precursor or natural occurring racemate. In the present study, kinetic resolution is applied based on the use of an enantioselective lipase capable of preferentially catalyzing the (S)-ibuprofen ester rather than its (R) counterpart, thus producing the enantio-enriched (S)-ibuprofen acid. Lipase from *Candida rugosa* was employed in the hydrolysis reaction both in free form in a batch system and in immobilized form in an enzymatic membrane reactor (EMR). Figure 1 shows the hydrolysis reaction in the kinetic resolution. The kinetic resolution was studied at two conditions: (i) lipase was immobilized on the spongy layer, with organic phase at the shell side and aqueous in the fiber lumen; (ii) lipase was immobilized on inner lumen, with organic phase in the lumen circuit and aqueous in the shell circuit. The immobilized lipase on the spongy layer exhibited higher operational stability. The optimum operating conditions for the lipase-catalyzed hydrolysis reaction in an EMR system were pH 8, 40 °C and at 80 ml/min organic phase flow rate with lipase immobilized on the spongy layer.

Summary

The present kinetic study is aimed at identifying the kinetic model for the resolution undertaken and enabling a reaction rate (velocity) equation to be written for a specific kinetic model. The kinetic analysis covers various factors affecting the rates of enzymatic hydrolysis reactions. The initial reaction rate (v_0) obtained from each substrate concentration was fitted to Michaelis-Menten kinetics using *Enzyme Kinetics! Pro* (EKP) Software (ChemSW product, Singapore) to obtain estimates of V_{max} and K_m for free lipase reaction and $V_{max,app}$ and $K_{m,app}$ for immobilized lipase reaction. Michaelis constant ($K_{m,app}$) and maximum reaction rate ($V_{max,app}$) for immobilized lipase were 36.47 mmol L⁻¹ and 3.27 mmol L⁻¹ h⁻¹, respectively; and that for free lipase were 63.43 mmol L⁻¹ and 2.83 mmol L⁻¹ h⁻¹, respectively. Excessive substrate was found to inhibit the reaction as an uncompetitive inhibitor. The by-product 2-ethoxyethanol was found to be inhibitory to the hydrolysis reaction (non-competitive) when it was present in an excess. Product inhibition constant (K_{IP}) was larger than the substrate inhibition constant (K_{IS}) by an order of magnitude, i.e. K_{IP} value was 337.94 mmol L⁻¹ for batch system and 354.20 mmol L⁻¹ for EMR while K_{IS} value was 43.07 mmol L⁻¹ for batch system and 49.52 mmol L⁻¹ for EMR. The enzyme reaction kinetics was modeled on the basis of rapid equilibrium assumption. Figure 2 portrays the kinetic mechanism computed from the rapid equilibrium condition. The overall mechanism was developed based on the combined inhibition effects by 2-ethoxyethanol product as a non-competitive inhibitor and (S)-ibuprofen ester as an uncompetitive inhibition. Equation 1 shows the resultant lipase-catalyzed hydrolysis reaction rate. It is desirable to use an enzyme that is able to display high catalytic activity as well as high enantioselectivity for a chosen reaction. There was good agreement between the kinetic models and experimental data with maximum error of $\pm 5.94\%$.

Keywords: Lipase; Enzyme kinetics; Inhibition; Membrane reactor; Enantioselectivity; Optical purity; Enantiomer

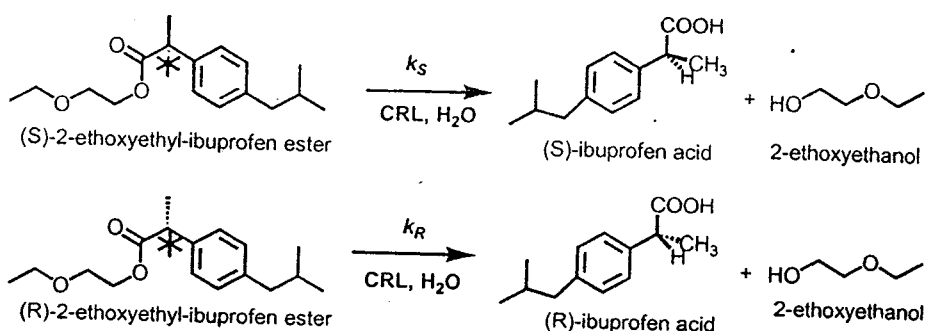


Figure 1. Lipase-catalyzed hydrolysis of racemic ibuprofen ester. CRL: *Candida rugosa* lipase

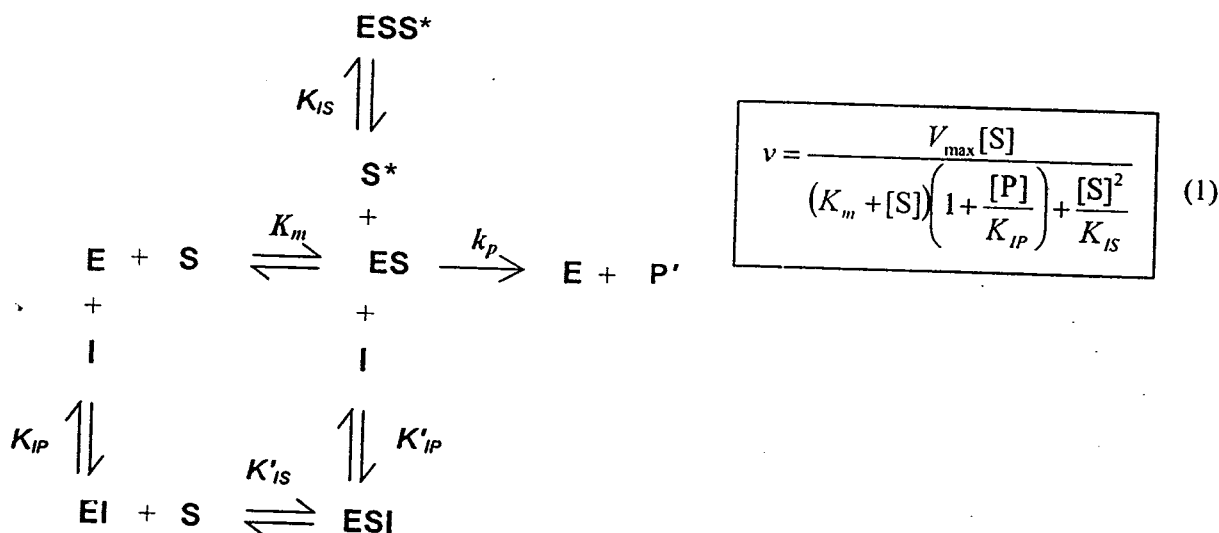


Figure 2. Kinetic mechanism of enzymatic hydrolysis of racemic ibuprofen ester with uncompetitive substrate inhibition and non-competitive product inhibition. S: substrate, S*: excessive substrate inhibitor, P': desired product, E: enzyme, I: by-product inhibitor, k_p : dissociation constant, K_m : Michaelis constant, K_{IP} : product inhibition constant, K_{IS} : substrate inhibition constant.

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Modelling and Simulation of Enzymatic Membrane Reactor for Hydrolysis of Ibuprofen Ester: Separation of Products

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*The enzymatic hydrolysis of racemic ibuprofen ester by lipase from *Candida rugosa* using a hollow-fiber membrane reactor was studied. The modeling and simulation of an enzymatic membrane reactor was based on the diffusion of (S)-ibuprofen acid product which leaves the reactor from the lumen side of the capillary fibers. The mathematical model containing a set of nonlinear partial differential equations was solved using orthogonal collocation technique. Orthogonal collocation technique is a weighted residual method based on Jacobi polynomials incorporating orthogonal trial functions. This paper presents a numerical technique of high efficiency, based on the orthogonal collocation of weighted residuals in solving the nonlinear partial differential equations of product diffusion in the capillary fibers. The modelling simulation study provided a better understanding of the process dynamics and product separation in the integrated enzymatic membrane reactor. The simulation study shows that noticeable product diffusion and transport occurred throughout the region of the fiber length, with 63.0% of product concentration (solute concentration) obtained at the exit of the fiber. The input parameters investigated were Peclet number and weighing function parameters, α and β . The best simulation results were achieved at the optimal 10 collocation points.*

Keywords: *Enzymatic membrane reactor, orthogonal collocation, racemic ibuprofen ester, (S)-ibuprofen acid.*

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Introduction

Orthogonal collocation is one of the widely used collocation methods for solving many chemical engineering problems (Rice and Do, 1995; Tayakout et al., 1995; Mehdizadeh and Dickson, 1990). Collocation methods have been found to be a powerful numerical problem-solving method of weighted residuals for engineering and science applications. These methods are distinguished by the choice of the test functions, used in the minimization of the residuals. In orthogonal collocation method, the dependent variable is expanded in a series of orthogonal polynomials, and the differential equation is evaluated at certain collocation points (Villadsen and Stewart, 1967). The collocation points are the roots to an orthogonal polynomial, as first used by Lanczos (1956). A major improvement was proposed by Villadsen and Stewart (1967) whereby the entire solution process is done in terms of solution at the collocation points rather than the coefficients in the expansion. These collocation methods differ from other weighted residual methods in that the residual is not directly orthogonalized, but is matched to an orthogonal function at its zeros, which eliminates the need to integrate the residual and consequently simplify calculations (Rice and Do, 1995). This method is especially useful for reaction-diffusion problems that frequently arise when modeling chemical reactors (Do, 1998).

In this paper, the modeling and simulation study is reported for the diffusion of (S)-ibuprofen acid, which leaves the reactor from the lumen side of the capillary fibers. Theoretical model equations which simulate the diffusion profiles were developed, and transformed into approximate differential equations by orthogonal collocation approximation formula. The resulting system of non-linear algebraic equations was solved using collocation method.

Process Description

The reaction under study was the enzymatic hydrolysis of racemic ethoxyethyl-ibuprofen ester. The hydrolysis reaction involves two substrates, i.e. water and racemic ethoxyethanol ibuprofen ester. Excess of water is present in the hydrolysis reaction to make it a single substrate mechanism. The Michaelis-Menten model is employed here as it is appropriate in the case of a 'one substrate-one product' system. The (R)-ester is not active in the above reaction (Giorno, 1997; Madhav and Ching, 2001). Hence, the reaction mechanism was simplified as shown in Figure 1.

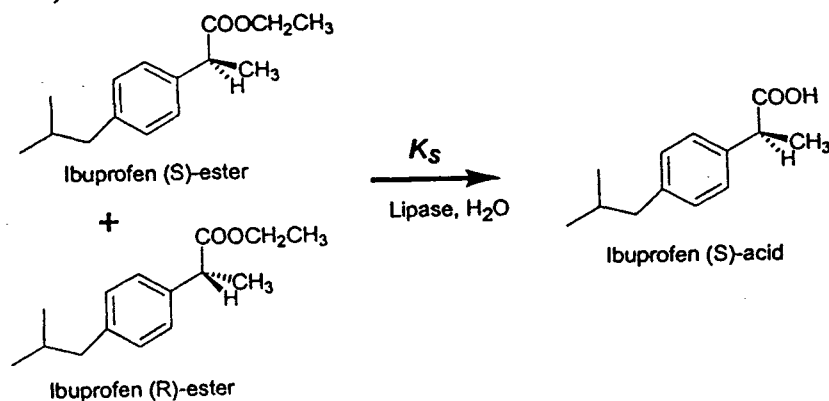


Figure 1. Hydrolysis reaction of racemic ibuprofen ester.

The hollow-fiber membrane module comprised of 65 asymmetric polymeric fibers in a tube-and-shell arrangement. The external surface of the asymmetric hollow fiber membrane is spongy while the internal surface is a dense thin layer. The hydrophobic substrate, i.e. racemic ibuprofen ester in organic medium, flows in the shell side. The aqueous phase flows in the fiber lumen. Both the organic substrate and aqueous phase come in contact only at the enzyme-membrane interface. Due to the use of a hydrophilic membrane, the water-soluble (S)-ibuprofen acid produced from the reaction diffuses simultaneously into the aqueous stream. For simplification, the mass transfer equation for the reaction-diffusion phenomenon in the membrane reactor was formulated based on a single hollow fiber. The development of model equations was carried out based on the following assumptions:

1. isothermal process;
2. the reaction-diffusion regime are identical in each hollow fiber;
3. diffusion of the water-soluble product across the membrane skin is at a faster rate than diffusion through the organic phase, due to the nature of the hydrophilic membrane.

Operation of Hollow Fiber Membrane Reactor

In this work, the product diffusion in the fiber lumen was considered without taking into account the reaction at the enzyme-membrane active layer. The effects of pressure drop along the hollow fiber and osmotic pressure on the effective transmembrane pressure are of fundamental importance. The operating conditions used as the input parameters in the simulation were a pressure difference of 25 kPa across the hollow fiber, permeation velocity of 10^{-3} cm/s, and axial velocity inside the fibers about 5 cm/s. Laminar flow was assumed (Yuan et al., 1956) and the angular effect was ignored. The physical properties of the solution (i.e. density, viscosity and solute diffusivity) were assumed to be unchanged throughout the process.

Model Description

The modelling and simulation was based on the diffusion of (S)-ibuprofen acid into the aqueous stream in the lumen side of the membrane fibers. The diffusion process was studied for both the axial and radial coordinates of the fiber lumen. Partial differential equations (PDE) describing the dimensionless concentration of (S)-ibuprofen acid and its velocity profiles inside the hollow fiber region have been derived. The mathematical model proposed has nonlinear partial differential equations and was solved using the orthogonal collocation technique in order to simulate the model reaction behavior. Upon transforming the PDE to first and second derivative matrices of the interpolation polynomial using approximation formula, the roots of the n -th order Jacobi polynomial were calculated based on the method of Do (1998). The model equation is a second-order partial differential equation (PDE) as a function of dimensionless radial coordinate R and axial coordinate y . In cylindrical geometry, the dimensionless equation of continuity combined with the convective-diffusive flux equation for the acid is given as:

$$\frac{\partial C}{\partial \tau} = \frac{1}{Pe \cdot R} \frac{\partial}{\partial R} \left(R \frac{\partial C}{\partial R} \right) - U \frac{\partial C}{\partial y} \quad (1)$$

with the following boundary conditions:

$$C(\tau, R, 0) = 1 \quad (2)$$

$$\left. \frac{\partial C}{\partial R} \right|_{R=\infty} = 0 \quad (3)$$

$$\left. \frac{\partial C}{\partial R} \right|_{R=1} = V_m(y)C(\tau, 1, y)Pe \quad (4)$$

and initial condition:

$$C(0, R, y) = 1 \quad (5)$$

In the above equations R denotes the dimensionless radial coordinate; y is dimensionless axial coordinate; and C is dimensionless concentration of (S)-ibuprofen acid. Symmetry properties were employed here as the problem is symmetrical in x at $x = 0$ as well as in y at $y = 1$. The transformations were made before the application of orthogonal collocation:

$$x = R^2 \quad (6)$$

$$\frac{\partial x}{\partial R} = 2R \quad (7)$$

$$s = (1 - y)^2 \quad (8)$$

$$\frac{\partial s}{\partial y} = 2(y - 1) \quad (9)$$

With this transformation, Equation (1) becomes:

$$\frac{4x}{Pe} \frac{\partial^2 C}{\partial x^2} + \frac{4}{Pe} \frac{\partial C}{\partial x} + 2\sqrt{s}U(x, y) \frac{\partial C}{\partial s} = 0 \quad (10)$$

The boundary condition at $R = 0$ and $y = 1$ are not needed, due to the transformation. The boundary conditions for Equations (4) and (5) rewritten in terms of x and s are:

$$x = 1; \quad \frac{\partial C}{\partial x} = \frac{Pe}{2} V_m C \quad (11)$$

$$s = 1; \quad C = 1 \quad (12)$$

Steady-state axial velocity profiles for laminar flow through a porous fiber with small permeation velocity and fiber diameter are given by Equation (13), which is based on the Navier Stokes equation reported by Yuan and co-workers (1956) after being reduced to dimensionless form:

$$U = 2(1 - 2V_m y)(1 - x^2) \quad (13)$$

where V_m is the dimensionless membrane permeation velocity.

The first step in the orthogonal collocation method was the appropriate selection of interpolation points. Two spatial domains, x and s , are involved in the model formulation. N interior collocation points were chosen for the x domains and M points in the s domain. Thus, the total number of interpolation points in the x domain was $N+1$, including the point at $x = 1$, and in the s domain was $M+1$, including the point at $s = 1$. Study of the optimum number of points was also carried out in this work. The algorithms of orthogonal collocation were written in MATLAB software. The resulting systems of linear algebraic equations were solved using MATLAB.

Results and Discussion

The simulation work presented here focuses only on the diffusion of (S)-ibuprofen acid in the fiber lumen. The simulation was studied for a variety of input parameters, including weight functions parameters α , β and collocation points (n). Simulations were investigated at different collocation points of 5, 10, 15, 17, 20, with α and β values varied from 0 to 2. Figure 2 shows the dimensionless concentration profiles as a function of reactor length along the capillary fiber represented by y coordinates, and across the fiber radius represented by R coordinates. When comparing results of different α and β values, the simulated results show that the approximation fluctuates significantly for β values other than zero. Simulations at α value of 2 approximated the concentration profile most appropriately (figure not included). The best results were obtained with $\alpha = 2$, $\beta = 0$ at $n = 10$, as shown in Figure 2(a). Figure 3 shows the concentration profiles in three-dimensional form.

Figure 2(a) shows that the acid concentration diffused from a maximum value (most concentrated) at the membrane surface, and the (S)-acid produced leaves the enzyme-membrane interface to the center of the fiber. Initially there was no substrate in the bulk exit concentration. Along the axial coordinates, the concentration profile approached a maximum level at the fiber exit. This explains how axial velocity influences the axial diffusion of the acid. In the aqueous phase of fiber lumen, the concentration of the (S)-acid product was observed to rise rapidly along the fiber length. The membrane flux was sufficiently large with no concentration polarization by the acid at the membrane surface, mainly because of solubility of (S)-ibuprofen acid in aqueous phase causing immediate diffusion into the fiber lumen. The concentration profiles become elevated when approaching the fiber edge, as shown in Figure 3. This phenomena was probably due to radial diffusion and axial convection due to continuous production of (S)-ibuprofen acid, and its rapid diffusion into the aqueous phase (from reaction layer to fiber lumen) until steady-state was achieved.

The use of different numbers of collocation points (n) showed significant influence on the simulated results. The selection of collocation points more profoundly influenced the simulations in the mesh of the radial direction than that for the fiber length. For collocation points of odd numbers ($N = 5$, $N = 15$) as indicated in Figures 4(a) and 4(c), the methods were poorly approximated when approaching the exit of the fiber and the membrane surface. The value $N = 5$ results in significant inaccuracy of the computed concentration profiles, due to insufficient points for evaluation of the PDE solution along the entire length of the fiber. However, a large number of collocation points ($N = 20$) makes calculations complicated because of the larger dimension of matrices involved in the formulation. The model geometry was a

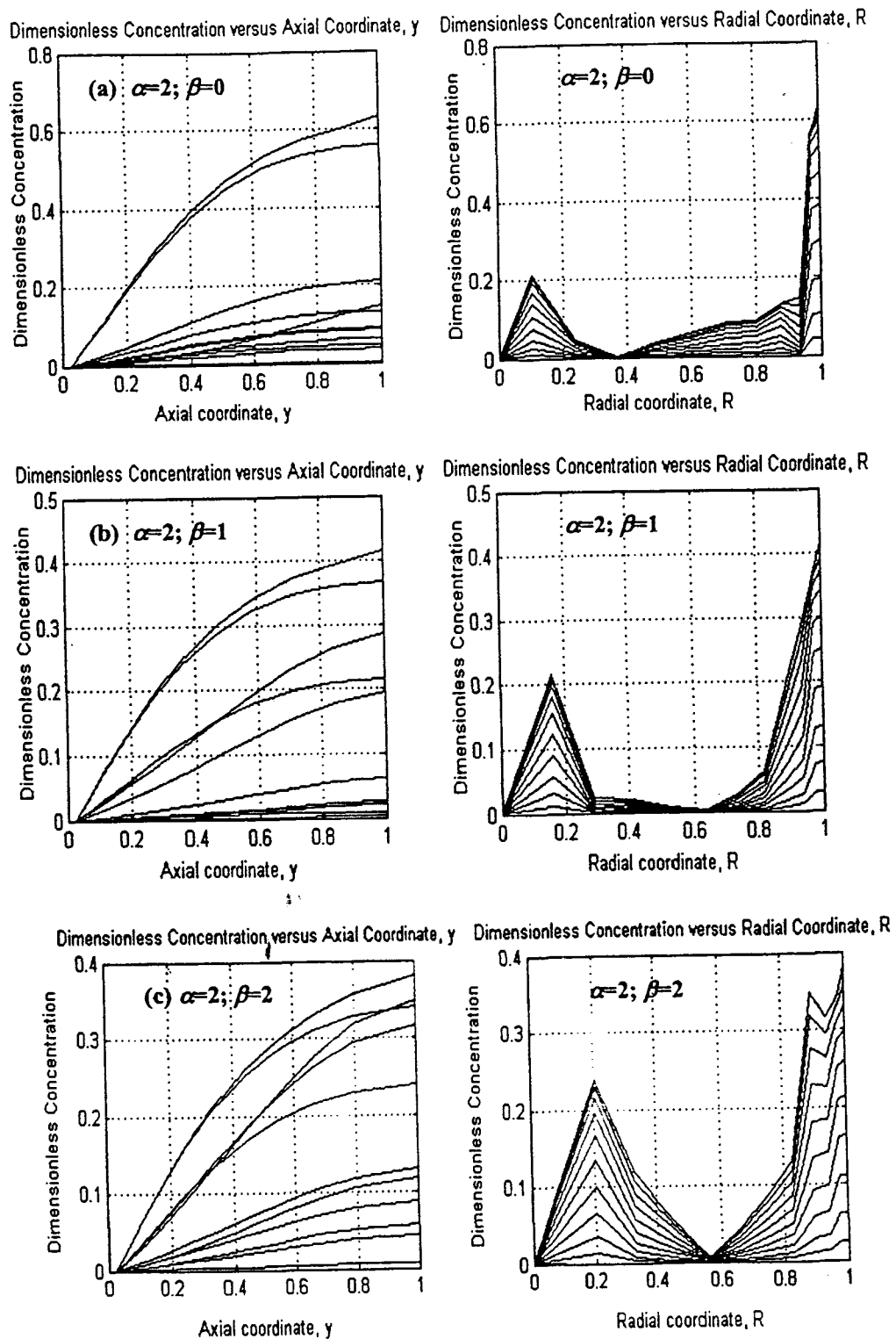


Figure 2. Dimensional concentration profiles at $\alpha=2$ and $\beta=0, 1, 2$; (a) $\alpha=2, \beta=0$; (b) $\alpha=2, \beta=1$; and (c) $\alpha=2, \beta=2$.

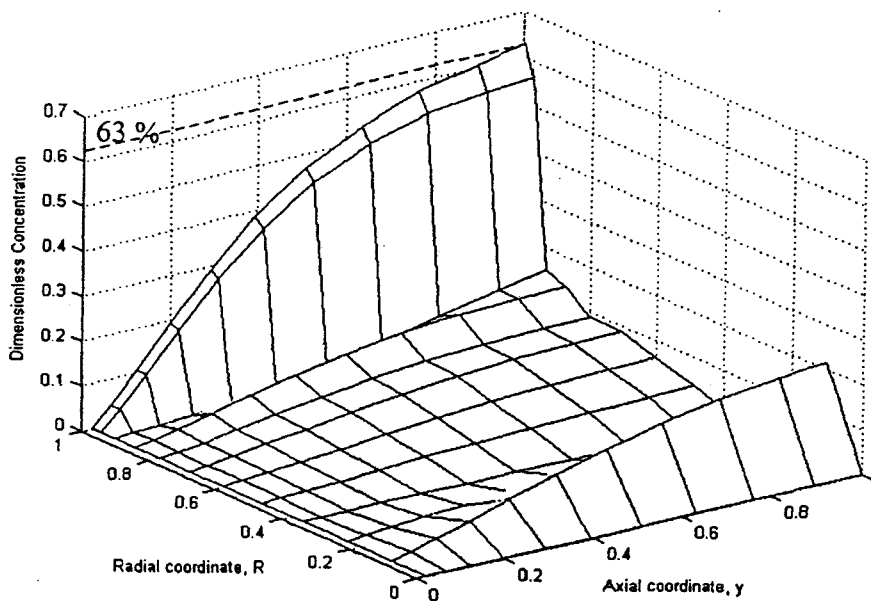


Figure 3. Three dimensional simulated results $N = 10$ at $\alpha = 2$ and $\beta = 0$.

symmetrical system. The selection of even functions used for the trial functions was essential because a selection of odd functions might lead to a zero value for the multiplicative coefficient (Tayakout et al., 1995). The number of 10 collocation points was found to be sufficient in this work. The precise selection of the collocation points is the most crucial part in determining the efficiency of the collocation method. It was decided to use fixed but larger collocation points for the weight function parameters for the whole extension of the fiber.

From the results in Figure 4(a-d), the concentration profiles were comprised of multiple but not evenly distributed lines. Each line represents different collocation point evaluated from the roots of a Jacobian polynomial, and was constructed simultaneously from a large number of mesh points. The simulated results showed that product separation using a biphasic hollow-fiber membrane reactor was sufficiently effective throughout the region of the fiber length, with 63.0% degree of conversion of product concentration achieved at the exit of the fiber. This value of product concentration was the bulk (S)-acid concentration taken at the aqueous phase in the fiber lumen. The polar product in the aqueous phase was absolutely free from substrate molecules due to the distinctive range of pore size distribution of the ultrafiltration membrane. The molecular weight of the substrate was too high to penetrate the micro-pores, thus ensuring the substrate molecules were enclosed in the organic phase in the shell side. This type of biphasic enzymatic membrane reactor configuration has the added advantage of working as an integrated reactor and separator. The unreactive nonpolar substrate can be recovered from the system by the continuous organic stream, while the polar product can be removed by the aqueous sweeper stream.

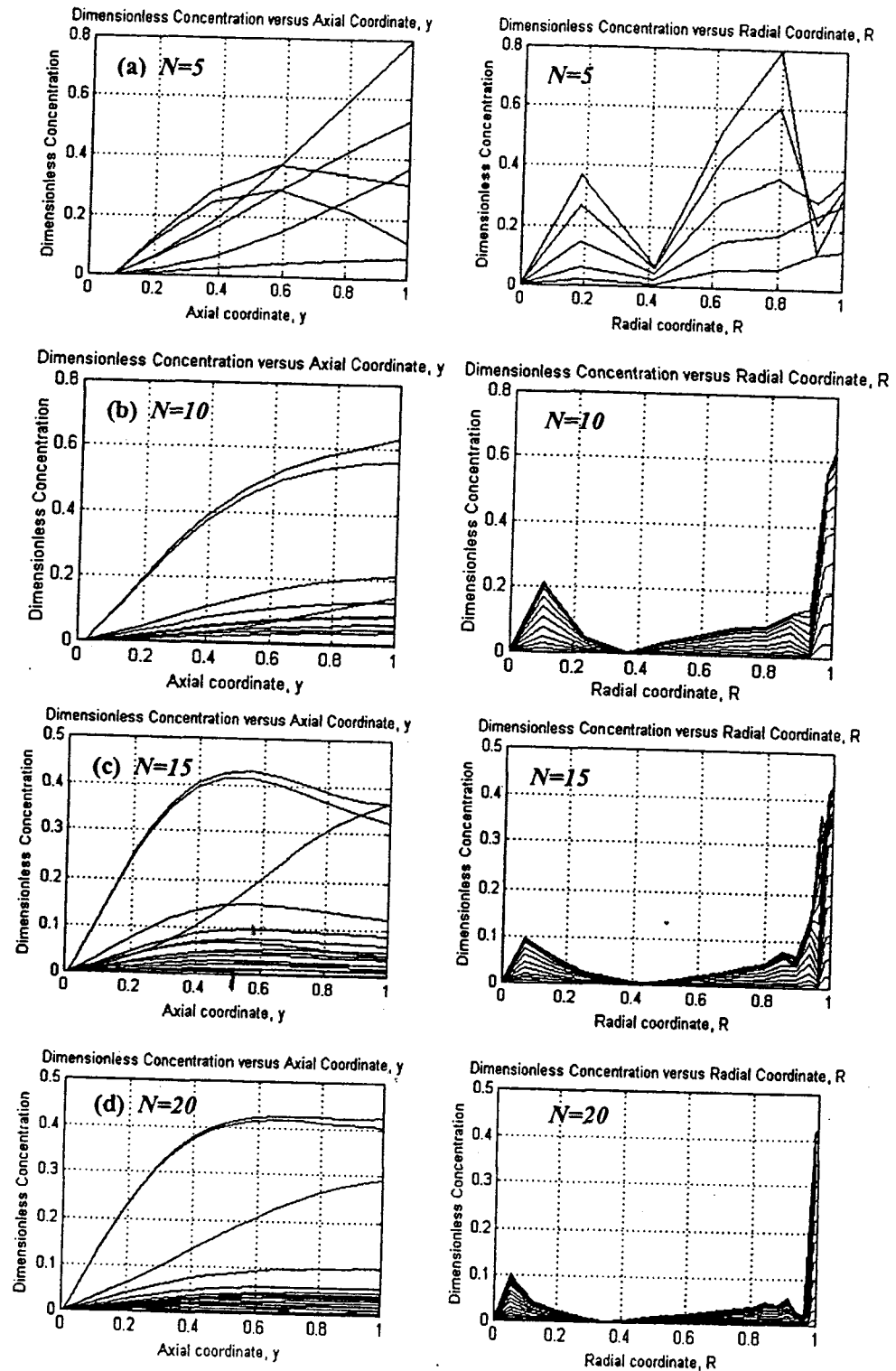


Figure 4. Simulation results at various collocation points at $\alpha = 2$ and $\beta = 0$; (a) $N = 5$; (b) $N = 10$; (c) $N = 15$; and (d) $N = 20$.

Conclusions

The orthogonal collocation technique was found to be applicable for modelling the diffusion behaviour in a hollow-fiber membrane reactor. The methodology used to select the weight functions was shown to be very effective in determining an adequate polynomial approximation with reduced order for nonlinear PDEs. The method provides rapid and precise parameters estimation, which is an additional benefit especially when parameters prediction is essential during the preliminary stage of the experimental work.

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Nomenclature

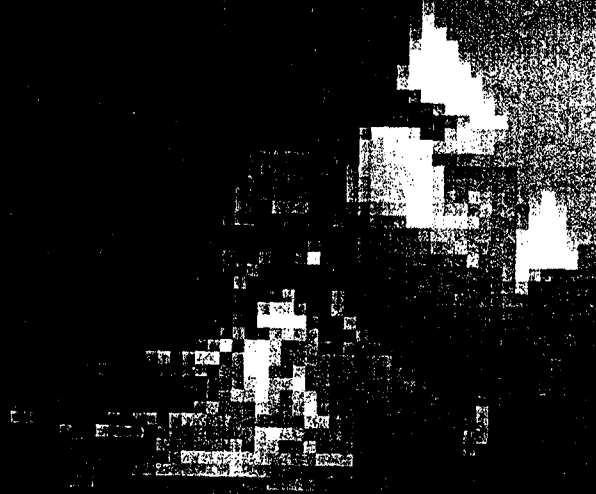
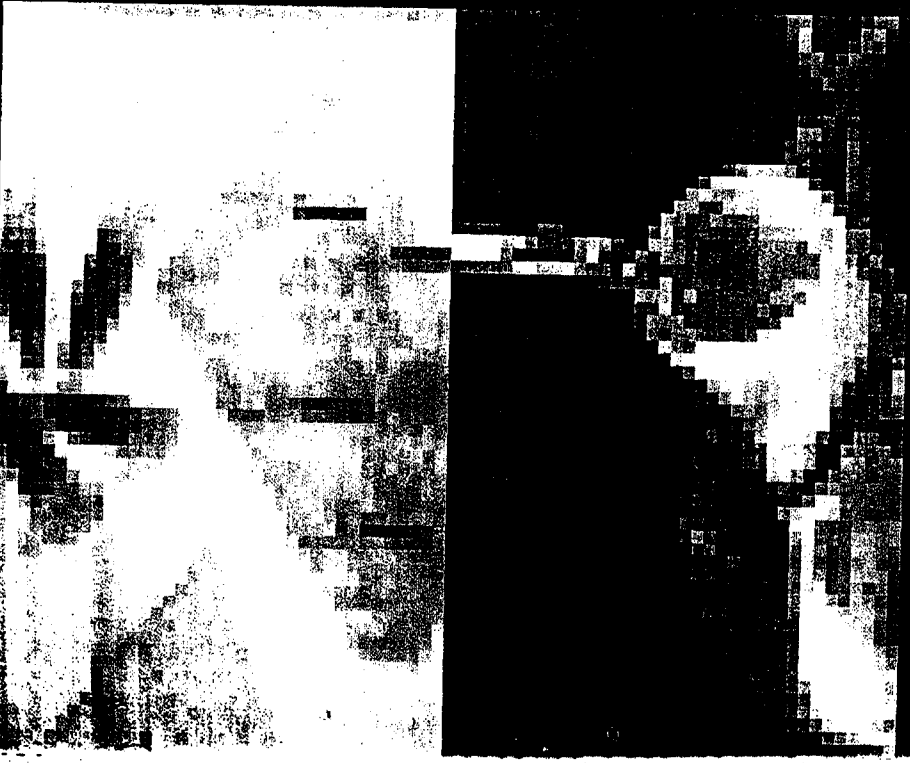
α	Weight function parameter
β	Weight function parameter
C	Dimensionless product concentration of (S)-ibuprofen acid
L	Fiber length (cm)
r	Radial coordinate (cm)
U	Dimensionless axial velocity
V_m	Dimensionless radial velocity at membrane
z	Axial coordinate (cm)
$Pe = \frac{V_{mi} r}{D}$	Peclet number
$R = \frac{r}{r_m}$	Dimensionless radial coordinate
$s = (1-y^2)$	Axial coordinate after transformation
$V_m = \frac{V_i}{V_m}$	Dimensionless membrane permeation velocity
$W(x) = x^\beta (1-x)^\alpha$	Weight function
$y = \frac{V_{mi}}{u_o r_m} z$	Dimensionless axial coordinate
$x = R^2$	Radial coordinate after transformation
$Z = \frac{z}{L}$	Dimensionless axial coordinate

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PROJECTS



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Current technologies for the production of (*S*)-ketoprofen: Process perspective

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Abstract

Ketoprofen (2-(3-benzoylphenyl)-propionic acid), belongs to the family of 2-arylpropionic acids (profens), is one of the nonsteroidal anti-inflammatory drugs. The production of single enantiomer of ketoprofen is expected to grow due to its enantiomer displaying better pharmacologic activities and benefits. Kinetic resolution is the most widely used method in the production of enantiomer pure ketoprofen. However, the process has a number of limitations, which are yet to be overcome. These limitations call for an innovative approach where two processes (enantioselective esterification and dynamic kinetic resolution) can be combined and operated under a single operating enzymatic membrane reactor system. The technology is more economical, more enantiomerically benign and has a potential maximum yield more than 50%. This paper will highlight the current technology in the production of biologically active (*S*)-ketoprofen, the limitations associated with the production and solutions to overcome these limitations.

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Keywords: Ketoprofen; Enantiomer; Kinetic resolution; Lipase; Enzymatic membrane reactor

1. Introduction

Ketoprofen (2-(3-benzoylphenyl)-propionic acid), one of the most useful NSAIDs, has received attention since the past two decades and its anti-inflammatory effect is approximately 160 times the anti-inflammatory potency of aspirin on a unit weight basis [1,2]. Commercially, ketoprofen is formulated as a racemic mixture of “*R*” and “*S*” enantiomers, which are equivalent on a unit weight basis. A major reason for the use of mixtures of enantiomers remains the cost of separation of the enantiomers which exceeds the potential advantage of a possible increase in the activity. However, (*S*)-ketoprofen and (*R*)-ketoprofen display significantly different pharmacologic activities and benefits [3]. (*S*)-enantiomer has the therapeutic activity of reducing inflammation and relieving pains while the (*R*)-enantiomer can be used as a toothpaste additive to prevent periodontal disease [4]. Thus, the effort of producing single

enantiomer of ketoprofen is important and expected to grow due to the pharmacological benefits of both enantiomers.

This paper will highlight the current technology in the production of biologically active (*S*)-ketoprofen, the limitations of current methods of production and strategies in overcoming the limitations associated with its production.

2. Current technology in the production of (*S*)-ketoprofen and the limitations

Generally, there are two ways of obtaining enantiomeric forms through enantiomeric technology. The first is to start with a racemic form, and separate out one of the enantiomers. The second is to synthesize the enantiomeric form directly. The primary methods of separation are crystallization [5,6], chromatography [7,8] and kinetic resolution [9–13]. Other methods of separation include capillary electrophoresis [14,15], solvent extraction [16] and receptor-based resolution [17]. Synthesis usually involves the use of chiral intermediates, asymmetric chemical

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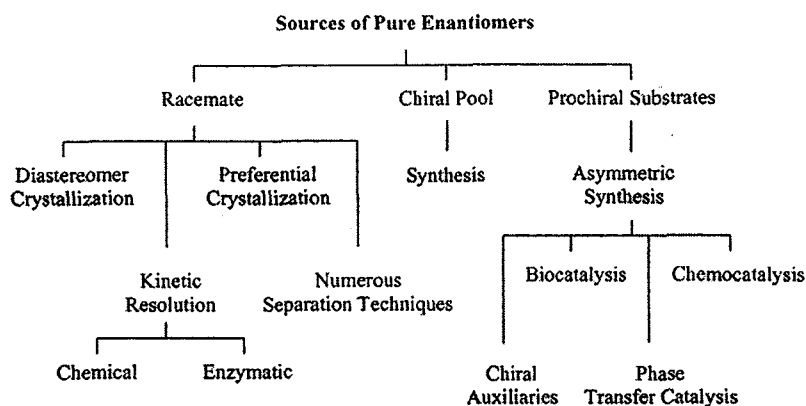


Fig. 1. Sources of pure enantiomers.

catalysts or enzymes [18]. Fig. 1 presents the sources of pure enantiomers.

Various approaches such as diastereomer crystallization [19,20], kinetic resolution [21–36], asymmetric synthesis [37–42], chromatography separation [15,43,44] and preferential crystallization [45,46] were reported for the production of (*S*)-enantiomer of ketoprofen (Fig. 2). However, the major limitation of (*S*)-ketoprofen production is that the maximum yield is limited to 50% of the desired enantiomer based on the racemic starting substrate, which consists of equimolar of (*R*)-enantiomer and (*S*)-enantiomer. The use of enantiomeric technology is only in the separation of the 50% of the desired (*S*)-enantiomer, while the other 50% of (*R*)-enantiomer remained as byproduct. The effort of recovering the 50% of the byproduct is important in order to achieve the maximum yield of more than 50%.

Application of organic solvent and additional reactant in the reaction medium especially in the batch system requires additional steps for downstream processes to obtain the purity of the final product. Downstream processing include filtering the enzyme or mass of microbial from the reaction medium, evaporating the organic solvent together with the excess volatile reactant, separation of the residues using fresh solvent and extraction of the unreacted substrates.

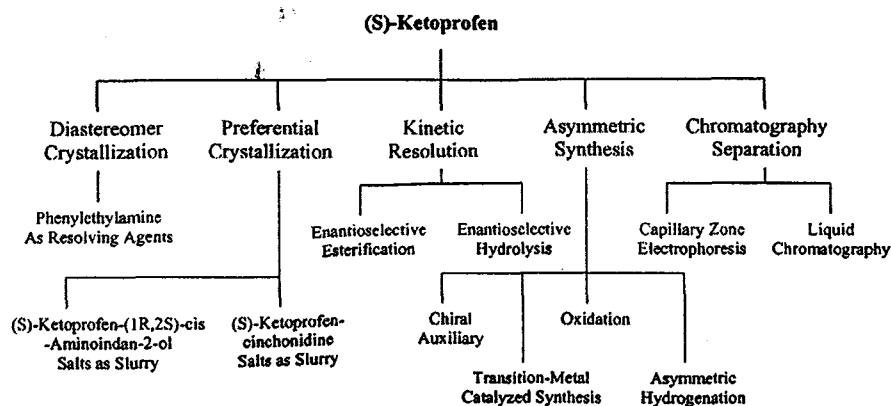
These processes do not only involve extra steps but are also time consuming particularly in the batch processes. These purification processes for each batch reaction affect the productivity of the desired product and enzyme recovery capability, which in turn influences, the profitability of the process.

The approaches and its limitations in the production of optically pure (*S*)-ketoprofen are reviewed in the following sections. These limitations must be overcome in order to obtain yield more than 50% and higher purity of the desired enantiomer.

2.1. Diastereomer and preferential crystallization

Diastereomer crystallization is a method, which utilizes an enantiomerically pure acid or base (the resolving agent), which forms diastereomeric salts with the racemate. Pure enantiomers are obtained by selective crystallization of the diastereomeric salts [47].

Yoneyoshi et al. [19] reported that 43.1% yield of (*S*)-ketoprofen was obtained through diastereomer crystallization with the product enantiomeric excess (ee_p) of 79.1%. In their work, equimolar of racemic ketoprofen and (*S*)-*N*-(*p*-hydroxybenzyl)-1-phenylethylamine were added

Fig. 2. (*S*)-Ketoprofen production technology.

to methanol, and heated to dissolve these reactants in the methanol. The reaction mixture was cooled and kept over night. The material was crystallized out and subsequently separated by filtration. The crystalline material was decomposed with 1% hydrochloric acid, and followed by an extraction operation carried out twice with toluene. The organic layer was concentrated under reduced pressure to give (*S*)-ketoprofen.

In another (*S*)-ketoprofen production system using the same approach, 40% yield was reported by Lukas et al. [20]. They reported that the racemic ketoprofen was dissolved in 2-propanol in a heated mixing chamber and phenylethylamine (chiralic base) was added to the mixture. Once the addition was completed, the mixture was stirred at its boiling point and subsequently cooled. The precipitated diastereoisomer was pressed out and washed twice with ice-cold 2-propanol.

Preferential crystallization is a method where a super-saturated racemic solution is seeded by the crystals of one enantiomer which then crystallizes out preferentially. It is technically feasible only with racemates which are conglomerates [47,48].

Eikeren et al. [45] reported that ee_p of 97.2% of (*S*)-ketoprofen was obtained through preferential crystallization of racemic ketoprofen. In their work, pure enantiomer of 1-aminoindan-2-ol was used as resolving agent to exhibit the resolution of racemic ketoprofen. (*R,S*)-ketoprofen was combined with an acetonitrile/water mixture followed by heating and stirring until the mixture was dissolved. The solution was treated with (1*R*,2*S*)-*cis*-1-aminoindan-2-ol, mixed and seeded with a slurry containing (*S*)-ketoprofen-(1*R*,2*S*)-*cis*-1-aminoindan-2-ol diastereomer salt. The solids that formed were collected by filtration, washed twice with acetonitrile and dried under vacuum to yield 66.4 g of (*S*)-ketoprofen (1*R*,2*S*)-*cis*-1-aminoindan-2-ol diastereomer. A portion of the salt was treated to release the acid. The enantiomeric excess of the released acid was measured by chiral HPLC and found to be 97.2% (*S*)-ketoprofen.

(*S*)-Ketoprofen production using cinchonidine was reported by Manimaran et al. [46]. In their research, cinchonidine was added to a solution of racemic ketoprofen and ethyl acetate under vigorous stirring and heating conditions. The mixture was diluted with methanol. After cooling, the solution was seeded with 98% enantiomerically pure (*S*)-ketoprofen-cinchonidine salts to induce crystallization. The precipitated diastereomeric salt was filtered under vacuum, washed three times with ethyl acetate and three times with ether, and then dried under vacuum for few hours. Yield of 31% and ee_p of 97% were obtained after recrystallization.

However, these processes are not being used for various reasons. Due to several recrystallization operations which are necessary, these processes require longer time but are also uneconomical, as they only lead for example to low yield. Moreover, the bases used in diastereomer crystallization are expensive and chemically unstable; they are only

reusable in a pure state with losses [49]. The solubility of racemic ketoprofen is influenced by its two aromatic rings. Thus, external heat is required in order to resolve the starting material. Yoneyoshi et al. [19] reported that a temperature up to 60 °C was required. The operating temperature is relatively high if compared to the enzymic reaction in which the temperature is in the range of 25–45 °C. Other than the requirement of external heating sources, external cooling facilities are also required. Manimaran et al. [46] reported that a temperature as low as 5 °C was needed to enhance salts formation.

In addition, the process requires a considerable volume of solvent during a salts washing step [45,46] and extraction operation [19]. Despite this, the yield of the final product is still not high. The application of pressure was also necessary during operation because certain portions of the process must be carried out under pressure before yielding the (*S*)-ketoprofen. The process is energy intensive as well as time consuming.

Furthermore, it has a drawback that reuse of unnecessary diastereomer is not economical. These resolution methods are, however, cumbersome and expensive. Generally, the chemical resolution methods entail the selective stoichiometric crystallization of a diastereomeric salt by the use of an expensive amine such as cinchonidine or dehydroabietylamine acetate or the use of a water-soluble amine such as glucamine, which is difficult to recover [50].

The rate of reaction of the chiral reagent with individual enantiomers may be different thus leading to a ratio of diastereomers which does not reflect the initial ratio of analyte enantiomers. Further, these procedures require scrupulous maintenance of the enantiomeric purity of the chiral reagent and avoidance of partial racemization of the analyte and chiral reagent during the course of the derivatization procedure. Lastly, the diastereomeric product ultimately obtained may give non-identical detection results, thus requiring additional validation steps [51].

2.2. Kinetic resolution

Kinetic resolution is still the most widely used approach for the production of optically active fine chemical and pharmaceutical especially (*S*)-ketoprofen. The preparation of optically active (*S*)-ketoprofen could be achieved by enzymic resolution [4,52]. The enzymic reaction process in the pure enantiomer synthesis is a much preferred route because of its high activity at room temperature. The enzymic process assists in avoiding the formation of by-products and minimizing the problems of separation [4]. The catalytic behaviour of enzymes is highly selective as compared to chemical catalysts, as these enzymes demonstrate a higher reaction rate, at milder condition, and greater stereo-specificity [53].

Until recently, two methods of enzymatic kinetic resolution have been applied in the production of optically

pure enantiomer of ketoprofen. These are enantioselective esterification and enantioselective hydrolysis and are generally operated in the batch mode of production.

2.2.1. Enantioselective esterification

In the process of enantioselective esterification, racemic ketoprofen acid will react with alcohol (e.g. methanol, ethanol, etc.) in the presence of enantioselective enzyme to yield enantiomer ester (*R*)-ketoprofen ester or (*S*)-ketoprofen ester depending on the type of enantioselective enzyme and water. The enantiomeric acid of ketoprofen was further separated from ester either through neutralization and precipitation of the enantiomer acid [21] or extraction using saturated NaHCO_3 [22].

Among the most commonly used enzymes, lipase is reported to be most suitable for organic synthesis because of its stability, availability, acceptance of a broad range of substrates and have no coenzyme requirement for the catalysis [23]. The lipase of *Candida antarctica* has been widely used in this esterification reaction of ketoprofen with the enantiospecific toward (*R*)-enantiomer. Park et al. [22] reported using immobilized lipase from *C. antarctica* in the esterification of racemic ketoprofen. They observed that conversion as high as 59% and enantiomeric excess (ee_p) of 75% could be obtained. The same lipase was applied by Crescenzo et al. [24] in their enantioselective esterification of racemic ketoprofen. A comparison between the lipase of *C. antarctica*, *Mucor miehei*, *Pseudomonas cepacia*, *Candida cylindracea*, *Aspergillus niger*, *Rhizopus javanicus*, *Mucor javanicus*, Porcine pancreas and wheat germ was reported by Antona et al. [21]. The lipase from *C. antarctica*

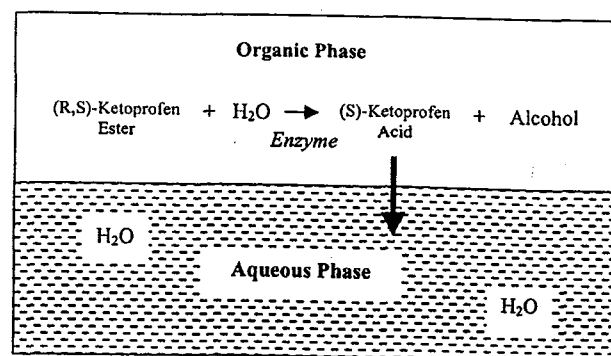


Fig. 3. Hydrolysis of racemic ketoprofen in two-phase system.

gave the highest conversion and enantiomeric excess of the substrate in the esterification process.

2.2.2. Enantioselective hydrolysis

For an enantioselective hydrolysis reaction racemic ketoprofen ester is the starting material. In the presence of enantioselective enzyme, one of the enantiomers is hydrolyzed to yield enantiomer acid ((*R*)-ketoprofen acid or (*S*)-ketoprofen acid depending on the type of enantioselective enzyme) and alcohol. The reaction medium could be a single phase (organic solution) or two phases (organic-aqueous solution) system. Additional steps for the separation of excess water and targeted enantiomer from the reaction medium are required for the single phase system. The excess amount of water and targeted enantiomer acid were extracted simultaneously into aqueous phase during the reaction for two phase system (Fig. 3) [25].

Table 1
Summary of enantioselective hydrolysis of racemic ketoprofen ester

Starting material	Enantioselective enzyme	Yield/ ee_p (%)	Ref.
Ketoprofen trifluoroethyl ester	Lipase from <i>Mucor javanicus</i> , $E = 50$	Yield = 53 $ee_p = 86$	[25]
Ketoprofen butyl ester	Lipase from <i>Candida Antarctica</i> , $E = 3.9$	Yield = 45 $ee_p = -$	[26]
Ketoprofen ethyl ester	Lipase from <i>Candida rugosa</i> , $E = 100$	Yield = - $ee_p = 98$	[29]
2-Chloroethyl ketoprofen ester	Lipase from <i>Candida rugosa</i> , $E = 100$	Yield = - $ee_p = 96$	[30]
Ketoprofen ethyl ester	Esterase of <i>Trichosporon brassicae</i> , $E = 51$	Yield = - $ee_p = -$	[31]
Ketoprofen ethyl ester	Esterase of <i>Pseudomonas</i> sp. S34, $E = -$	Yield = 47 $ee_p = 99$	[32]
Ketoprofen ethyl ester	Recombinant esterase, $E = -$	Yield = - $ee_p = 99$	[33]
Ketoprofen ethyl ester	Esterase of <i>Bacillus stearothermophilus</i> JY144, $E = -$	Yield = - $ee_p = 99$	[34]
2-Chloroethyl ketoprofen ester	Lipase from <i>Candida rugosa</i> , $E = -$	Yield = 30 $ee_p = 99$	[35]
2-Chloroethyl ketoprofen ester	Lipase from <i>Candida rugosa</i> , $E = 50$	Yield = 22 $ee_p = 94$	[36]

There are two common enzymes used in this reaction: lipase and esterase. Lipase from *M. javanicus* and *C. antarctica* shows enantioselectivity towards (*R*)-ketoprofen as reported by Kato et al. [25] and Jin et al. [26] in the enzymic hydrolysis reaction of ketoprofen ester while lipase from *Candida rugosa* was enantiospecific for (*S*)-enantiomer [28,29,54]. However, the enantioselectivity (or enantiomeric ratio is the ratio of the amounts of the two enantiomers in a particular sample of a chiral compound, *E*) of this enzyme towards (*S*)-ketoprofen was far from satisfactory [22,54]. Kim et al. [29] and Liu et al. [30] emphasized that the enantioselectivity of this lipase for ketoprofen could be improved through pre-treatment before being used in the hydrolysis reaction. Table 1 summarizes the enantioselective hydrolysis of racemic ketoprofen ester reported in the literature.

2.2.3. Limitations of kinetic resolution

The alkyl esters of ketoprofen are insoluble in water and are generally poor substrates for commercial enzymic hydrolysis because multiphase/heterogeneous reaction systems suffer from a number of drawbacks on the industrial scale. For example, scale-up and reliability problems are frequently associated with the processing of dispersions and emulsions, and continuous operation and pH control (especially in hydrolytic reactions) are difficult to achieve. Additionally, the phases must be separated before product can be recovered and excessive inter-phase mass transfer resistances are often encountered. These are associated with diffusion of the poorly soluble substrate in the aqueous phase. Many of these disadvantages associated with the enzymic resolution of water insoluble esters of chiral carboxylic esters in heterogeneous reaction systems could be minimized or eliminated if the water-solubility of the ester derivative could be substantially increased [55].

The proper control of water amount or concentration is important in the process for ensuring the efficiency of the enzyme activity and reaction. Thus, an excess amount of water content in the reaction medium lead to the reverse reaction, loss of enzyme activity is one of the drawbacks of the resolution reaction. In order to achieve the yield of pure enantiomer at more than 50% through kinetic resolution, another batch process can be followed in series. In the first reactor, racemic esterification is conducted to produce racemic ester. This intermediate product is subjected to the enantioselective hydrolysis in the presence of enantioselective enzyme to yield pure enantiomer in another reactor. The undesired (unreacted) enantiomer is recovered by dynamic kinetic resolution where this enantiomer is further racemized and reintroduced in enzymic resolution to enrich the desired enantiomer. These subsequent processes cause limitations due to the deactivation and contamination of the intermediate products between the processes.

Application of microorganism as a source of hydrolyses in kinetic resolution created several limitations such as required additional biomass treatment, proper nutrient supplement and

productivity for each batch of reaction are strongly dependent on the batch of microorganism used. The limitations totally influenced the priority of this technology that needs to be adapted in the industrial scale production.

2.3. Asymmetric synthesis

Asymmetric synthesis is referred as enantioselective conversion of a prochiral substrate to an optical active product using a chiral addend via asymmetric step [47].

(*S*)-ketoprofen was produced by various pathways under asymmetric synthesis (Fig. 2) [37–42]. Invention relates to asymmetric syntheses in which a prochiral or chiral compound is reacted in the presence of an optically active metal-ligand complex catalyst to produce an optically active (*S*)-ketoprofen was reported by Babin et al. [37]. In their work, 3-ethenylphenylphenyl ketone was used as a starting material and oxidation of an optically active *S*-(2)-(3-benzoylphenyl)-propionaldehyde prepared by an asymmetric hydroformylation to give the pure (*S*)-ketoprofen was disclosed.

Process oxidation and process asymmetric hydrogenation were applied by Laue et al. [38] to produce optically pure (*S*)-ketoprofen in the presence of chemical catalyst. In their work, various intermediates products were being prepared and multiple-step reactions were involved to yield the (*S*)-ketoprofen with the *ee*_p higher than 80%.

However, efficient asymmetric synthesis required the ability to control both regioselectivity and stereoselectivity [37]. Failures of regioselectivity and stereoselectivity control affect the productivity. Multiple reaction steps in the asymmetric synthesis are certainly unsuitable for operation on an industrial level owing to the extremely high labour cost and space consuming. Furthermore, significant amount of solvent is required during downstream processes for each step of intermediate products extraction and purification.

Conventional chemical catalysts are basic needs in asymmetric synthesis, such as $\text{PdCl}_2(\text{PPh}_3)_2$, copper(I) iodide, $\text{PdCl}_2(\text{PhCN})_2$, $[\text{Ni}(\text{MeCN})_6][\text{BF}_4]_2$ and $\text{Pd}(\text{dba})_2$ in order to facilitate the overall reaction [39]. However, most of the catalysts are generally highly toxic, pyrophoric and “environmentally unfriendly,” often resulting in harmful byproducts. In addition, the chiral catalysts to be used can be prepared only with extreme difficulty. It requires an expensive catalyst as well as a complicated reaction.

In the preparation of various types of arylacetic acid derivatives such as ketoprofen, one of the problems associated with the present day processes is the use of a catalyst directly in an organic solvent to convert the 3-vinylbenzophenone via carbonylation to ketoprofen [39]. This requires, after reaction, separation of the desired end product (i.e. ketoprofen) from the catalyst in the overall reaction mass and recycling of the catalyst. There are substantial losses in catalyst as well as decreased efficiencies such as difficulties in the separation of the catalyst and end product.

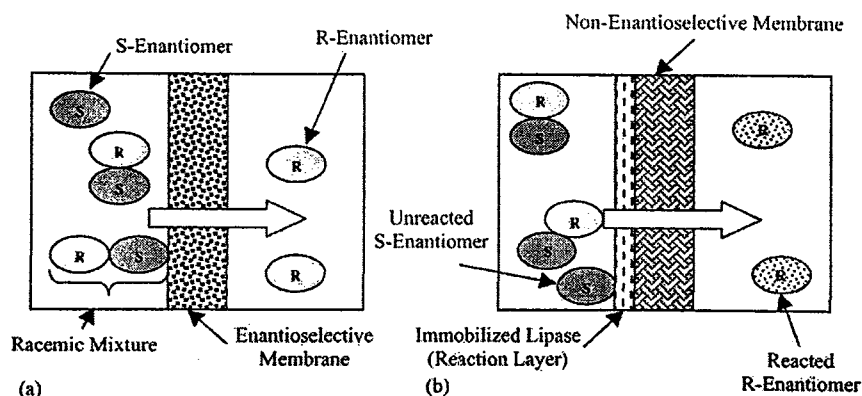


Fig. 4. Two directions of membrane technology in assisting kinetic resolution: (a) enantioselective membrane in direct separation of a racemic mixture and (b) non-enantioselective membrane in assisting an enantioselective process.

2.4. Chromatographic separation

The chromatographic enantiospecific resolution of racemic NSAIDs is generally accomplished by two methods. One is pre-column derivatization of the enantiomers with a chiral reagent followed by separation of the resulting diastereoisomers on an achiral phase. Second is the temporary formation of diastereoisomers: (A) on a chemically bonded chiral stationary phases (CSP) with an achiral mobile phase and (B) by addition of a chiral mobile phase complexing agent and an achiral stationary phase [43].

Boisvert et al. [44] used high performance liquid chromatography (HPLC) for the quantification and separation of ketoprofen enantiomers using a chiral column [Chirex 3005; (*R*)-1-naphthylglycine 3,5-dinitrobenzoic acid] with the mobile phase, 0.02 M ammonium acetate in methanol, set at a flow-rate of 1.2 ml/min. In their work, baseline separation of ketoprofen enantiomers and byproducts, free from interferences, were achieved in less than 20 min.

A method for resolving the enantiomers of ketoprofen by capillary zone electrophoresis (CZE) using a background electrolyte (BGE) containing a cyclodextrin as chiral

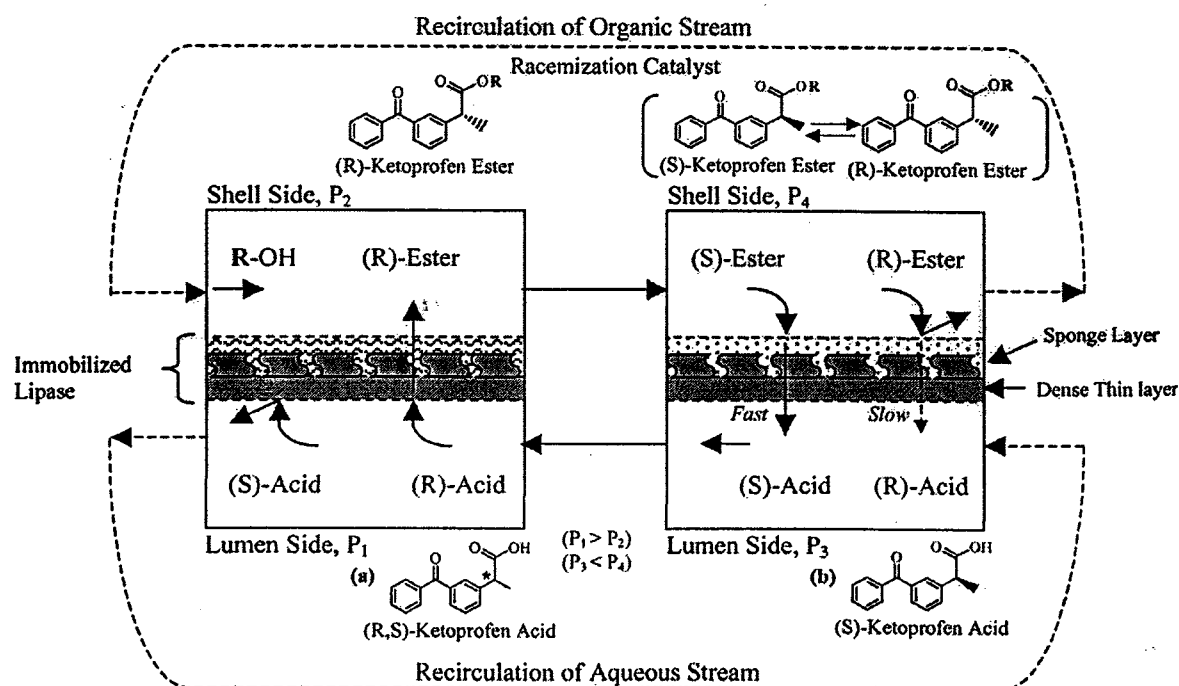


Fig. 5. New configuration of EMR in production of (*S*)-ketoprofen: (a) direct enzymatic esterification of (*R,S*)-ketoprofen acid and (b) dynamic kinetic resolution of racemized (*R,S*)-ketoprofen ester.

selector was proposed by Blanco et al. [15]. The resolution with a high efficiency in a short time (less than 20 min) with no capillary treatment was claimed.

The disadvantage of chromatographic separation is time-consuming, and the yield is low due to the resolution of racemic mixtures lead to yield of a maximum 50% of one enantiomer, and 50% of the other enantiomer. In addition, this process while useful as an analytical tool is not typically capable of producing large amounts of materials for commercial utility. High sensitization of HPLC in sample detection required extremely pure substrate, every single contamination of starting material affects the results accuracy and the lifetime of the technology.

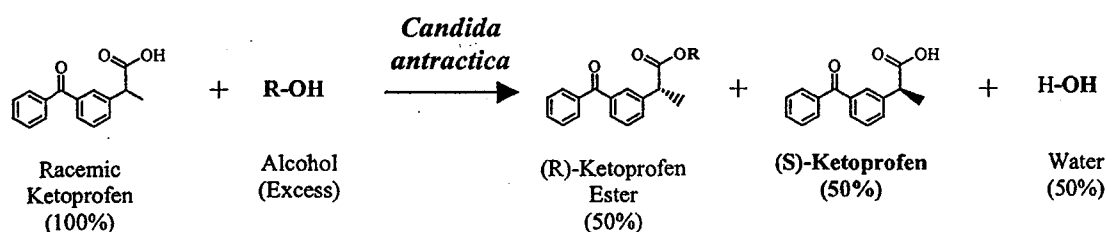
The high-performance liquid chromatographic method known as a method of analysis directed to optical purity may be utilized for optical resolution. It is not suitable for mass production because it is an expensive technology even on the laboratory scale. The considerable amount of mobile phase used as substrate carrier in the process causes environmental problem if the technology is being scaled up to the industrial production.

3. Strategies to overcome

Enzymic membrane reactors (EMR) have been used in the study of many important enzymic reactions. However, in the production of enantiomeric pure ketoprofen, EMR has not yet been applied in a two-phase system. Due to the flexibility and ability of EMR to work in a single phase, two phases and multiple phases media, it would be possible to employ EMR in carrying out enzymic kinetic resolution in the wider region of operation.

Challener [56] reported EMR technology applied to assist kinetic resolution in two directions. In the first approach, an enantioselective membrane is utilized to enable direct separation of a racemic mixture. Fig. 4(a) shows that one of the enantiomers will be enantioselectively permeate and the other enantiomer will be the retentate. In the second method, a non-enantioselective membrane is employed to assist in an enantioselective process. The enantioselective reaction will convert one of the enantiomers into a new compound which will permeate across the non-enantioselective membrane

ENANTIOSELECTIVE ESTERIFICATION



DYNAMIC KINETIC RESOLUTION

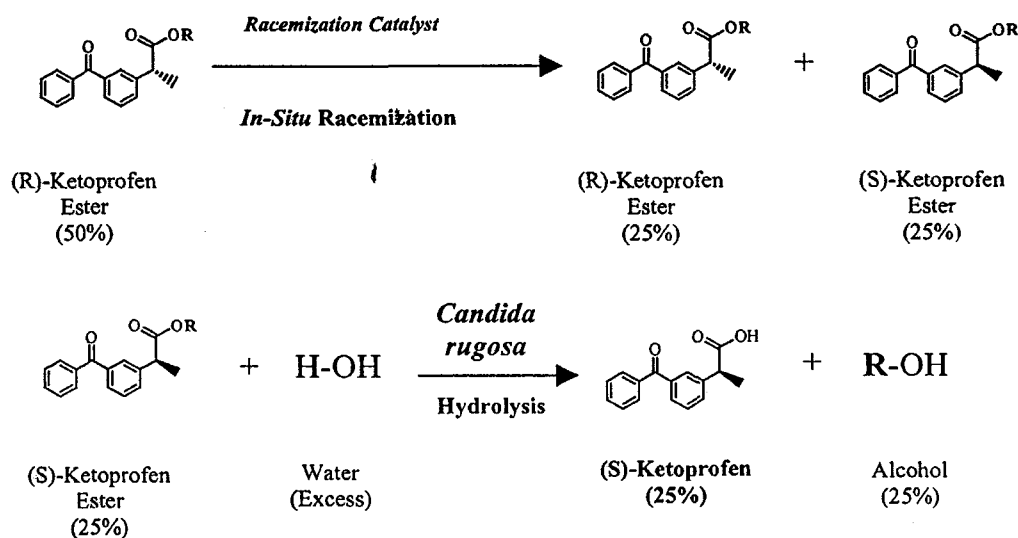


Fig. 6. Synthetic route of (S)-ketoprofen.

Table 2
Strategies to overcome existing limitations

Current limitations	Strategies to overcome
Maximum yield less than or equal to 50%	Higher yield by combining dynamic kinetic resolution into the process to recover the byproduct Simplify the processes into a single operating system
Multiple processes/steps involved to obtain high yield of desired enantiomer	
Downstream processing	In situ separation are possible by using enzymatic membrane reactor
Multiple steps involved: time consuming and not economical	Overcome by introducing a single operating enzymatic membrane reactor system
Low productivity as batch processes involved	Applying enzymatic membrane reactor where continuous operation are possible
Low enzyme recovery	Higher recovery through immobilization of the enzyme
Low enzyme stability	Higher stability through immobilization of the enzyme
Difficulties in water content management	Using biphasic enzymatic membrane reactor where water will remain in aqueous phase from the reaction region
Intermediate products deactivation and contamination as multiple batch processes involved	Lesser by applying a single operating system where the processes are combined

and the remaining unreacted enantiomer will remain in the retentate (Fig. 4(b)).

Thus, EMR technology can be applied in the production of optically active enantiomer of ketoprofen either by enantioselective esterification using monophasic/biphasic EMR, enantioselective hydrolysis using biphasic EMR, racemization using monophasic EMR, combined processes using single operating system of EMR or other configuration to overcome the current limitations.

One of the most novel methods in the production of (*S*)-ketoprofen acid is through the employment of biphasic (two phases) EMR system. In this type of EMR, enantioselective esterification and dynamic kinetic resolution are combined and operated under a single operating EMR system as shown in Fig. 5. Thus, in situ separation of the enantiomers, higher productivity, higher enzyme recovery, proper water content and intermediate products purity control are possible in achieving the production yield of more than 50% of the desired pure enantiomer. This approach is expected to reduce the capital and production cost.

The process first starts with the enantioselective esterification reaction where the carboxylic acid of (*R,S*)-ketoprofen acid reacts enantioselectively with alcohol in the presence of lipase to yield (*R*)-ester as shown in Fig. 6. The reaction occurs at the distinct layer of lipase immobilization on the membrane where the interface to the organic phase and aqueous phase are present (Fig. 5(a)). The main target of this reaction is to separate the (*S*)-enantiomer from the (*R*)-enantiomer by forming a water-insoluble (*R*)-ketoprofen ester through the esterification reaction. This ester is recovered in the organic phase while the unreacted substrate, (*S*)-ketoprofen acid is recovered in the aqueous phase. Transmembrane pressure (TMP) is applied to enhance the filtration rate. The recirculation of both streams of the membrane is able to enhance the productivity of the process. A different solubility behaviour of the products is able to increase the in situ separation of (*R*)-enantiomer ester and (*S*)-enantiomer acid in EMR.

Dynamic kinetic resolution (defined as combination reactions of in situ racemization and hydrolysis) is introduced to the reaction system by applying another module of biphasic EMR with lipase immobilized on the membrane in order to recover the undesired enantiomer (*R*)-enantiomer. The in situ racemization reaction occurred in the organic stream while the hydrolysis reaction occurred at the interface of organic phase and immobilized lipase in the EMR (Fig. 5(b)). The (*R*)-ketoprofen ester racemized to (*R,S*)-ketoprofen ester in the presence of racemization catalyst. The (*S*)-ketoprofen ester from (*R,S*)-ketoprofen ester is hydrolyzed in the presence of enantioselective lipase to form (*S*)-ketoprofen acid and alcohol as shown in Fig. 6. Transmembrane pressure (TMP) is applied to enhance the diffusion rate. The main target of this process is to recover the undesired (*R*)-enantiomer ester in order to enrich the desired (*S*)-enantiomer.

The process is a more economical and more enantiomerically benign process for the synthesis of enantiomerically pure compound which is expected to reach maximum theoretical yield of 100% using the dynamic kinetic resolution process [10,57–60]. Table 2 summarizes the different strategies in overcoming the existing limitations in production of (*S*)-ketoprofen.

In the EMR process, the reaction rate and optical purity depend on the type of solvent, type of enzyme, the amount of water present and chiral or achiral substrate molar ratio [13,24]. In order to appreciate fully the potential of EMR technology in ketoprofen production, further studies on the type of membrane material, direction of transmembrane pressure, choice of substrate stream, types of solvent, choice of catalysts for racemization, diffusion rate, reaction rate, type of efficient aqueous solution for product extraction and related kinetics would be useful.

4. Conclusions

The potential applications of enzymic membrane reactor technology over the more conventional approaches are

numerous, especially in the production of optically active (*S*)-ketoprofen acid. This is due to its higher efficiency, reduced costs through simultaneous reaction and separation process, easy to scale-up, applicable in continuous and steady state mode, enhancement of the stability of immobilized lipase and flexibility of its system configuration. Lipase is becoming increasingly important in high-value applications for the production of fine chemicals. The ability of lipase to be regioselective and stereoselective, allows resolution of racemic mixtures and biotransformation. Lipase-based and membrane-based processing has a promising future; however, there are several limitations to overcome. These include a relatively high cost, lack of optimal range of catalytic or membrane specificities and properties required for a particular application. The market for industrial enzyme and membrane is expected to grow steadily. The reason lies in the improved production efficiency resulting from the availability of selective enzymes and membrane in addition to the new applications. Enzymes and membrane technology however, should not be considered alone but rather as a combination technology. Combining enzyme and membrane technology in enzymic membrane reactor is a practical approach that should be encouraged and this combination can be followed in other fields of application.

Acknowledgements

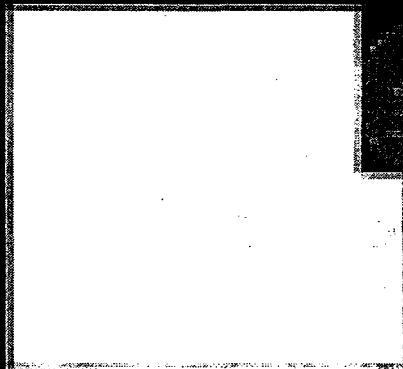
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CHEMICAL ENGINEERING SCIENCE





Enzyme kinetics of kinetic resolution of racemic ibuprofen ester using enzymatic membrane reactor

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Abstract

An ultrafiltration hollow fiber enzymatic membrane reactor was employed to study the kinetics of lipase-catalyzed kinetic resolution of racemic ibuprofen ester. Lipase from *Candida rugosa* was employed in the hydrolysis reaction both in free form in a batch system and in immobilized form in an enzymatic membrane reactor (EMR). The half life ($t_{1/2}$) of immobilized lipase on spongy layer was 105 h at reaction temperature of 40 °C and 62 h at 45 °C. This value was 94 h for lipase immobilized on the inner lumen and 45 h for free lipase in batch system at 40 °C. Excessive substrate was found to inhibit the reaction as an uncompetitive inhibitor. The by-product 2-ethoxyethanol was found to be non-competitive inhibitor to the reaction when it was present in an excess. Michaelis constant (K_m) and maximum reaction rate (V_{max}) for immobilized lipase were 36.47 mmol L⁻¹ and 3.27 mmol L⁻¹ h⁻¹, respectively; and that for free lipase were 63.43 mmol L⁻¹ and 2.83 mmol L⁻¹ h⁻¹, respectively.
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1. Introduction

(S)-ibuprofen acid was reported 160 times more reactive in their analgesic effect than the (R)-acid (Adams et al., 1976). There is increasing demand for economical and highly efficient methods for commercial synthesis of pure enantiomers which mainly focuses on chiral drugs production. Most of the modern medicines comprise of only single pure enantiomer. The demand for these optically pure compounds is becoming more crucial due to its more target-specific therapeutic effect than that possessed by racemic mixtures. Many of the conventional technologies produce a mixture of both enantiomers either to be consumed as racemate (1:1 molar ratio) or at different molar ratios. Chiral resolutions can be initiated either by a prochiral intermediate, precursor or natural occurring racemate. The cost of

substrates influences the choice of method as well as the possibility of recycling the catalyst or the resolving agent. Kinetic resolution utilizes natural occurring racemic compounds (racemate) as its starting material and hence is beneficial in terms of process economics. Being a subject of recent investigation, kinetic resolution is a method in which the residual substrate fraction can be obtained in high enantiomeric purity. Ideally one enantiomer reacts faster than the other with a chiral entity (Sheldon, 1993). A number of literature reported on enzymatic resolution of naproxen and ibuprofen acids and esters by lipases in which high enantioselectivity was observed for the (S)-form of the racemate (Battistel et al., 1991; Sheldon, 1993; Arroyo and Sinisterra, 1994; Roure et al., 1997; Lopez-Belmonte et al., 1997; Sanchez et al., 2000).

Of all the lipases available, lipase from *Candida rugosa* is given particular attention as an ideal biocatalyst for the resolution of racemic esters and alcohols (Kirchner et al., 1985; Holmberg et al., 1991; Parida and Dordick, 1991; Holmberg and Hult, 1992; Kim and Lee, 1996; Sanchez

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ét al., 1996; Roure et al., 1997; Cardenas et al., 2001), as it acts enantioselectively and prefers to catalyze the synthesis of one of the enantiomers through hydrolysis with higher preference. Enzymes have started to be utilized by the fine chemicals manufacturers. For example, Gist-Brocades found that addition of the methyl ester of racemic ibuprofen to growing cultures of *Pseudomonas*, *Mucor*, *Arthrobacter* or *Bacillus* species produced (S)-ibuprofen at 74–98% ee (Stahly and Starrett, 1997). Gist-Brocades uses a growing microorganism, Sepracor uses isolated enzyme preparations and Rhône-Poulenc uses highly purified enzyme called isozymes (Stahly and Starrett, 1997). An advantage of many enzymatic kinetic resolutions employing lipases and esterases in organic media, is that they can be performed at high substrate concentration. These features possessed by enzymes have made kinetic resolutions of racemic mixtures practical for industrial applications. On the other hand, catalytic asymmetric syntheses often require high dilutions in order to achieve acceptable enantioselectivity (Sheldon, 1996). Furthermore, hydrolytic enzymes are sufficiently inexpensive.

Ultrafiltration is an energy-efficient method used in product separations at a high degree of purity. Overall production costs can be reduced by the use of high-surface-to-volume catalytic hollow fiber membrane. The use of polyacrylonitrile (PAN) UF membrane for kinetic resolution of racemate has been reported by Matsumae et al. (1994) for the production of optically active 3-phenylglycidic acid ester. PAN hollow fiber membrane was also utilized for other non-enantiomeric resolution processes, such as hydrolysis of triacetin by immobilized lipase from *Candida rugosa* was reported by Guit et al. (1991). The use of enzymatic membrane reactor (EMR) allows easy replacement of enzyme (i.e., by removal of immobilized enzyme and repeated enzyme immobilization) and secondly EMR can also be used for biphasic reactions. Models that predict the performance of hollow fiber enzymatic membrane reactor on kinetic resolution of racemic ibuprofen ester had been reported in previous work (Long et al., 2003), where mathematical formulations were developed to simulate hydrolysis reaction in the membrane matrix support and product diffusion in the fiber lumen.

2. Process description

In the present study, the biphasic organic/aqueous membrane reactors are separated with the two liquid phases, the membrane is in contact with the organic phase on one side and with the aqueous phase on the other. As the organic/aqueous interface is located at the membrane level, the hollow fiber membrane provides high exchange surface per unit volume. The schematic diagram of the EMR system employed for the kinetic resolution is shown in Fig. 1. The racemic 2-ethoxyethyl-ibuprofen ester in organic solvent (isooctane) was circulated along the shell side and

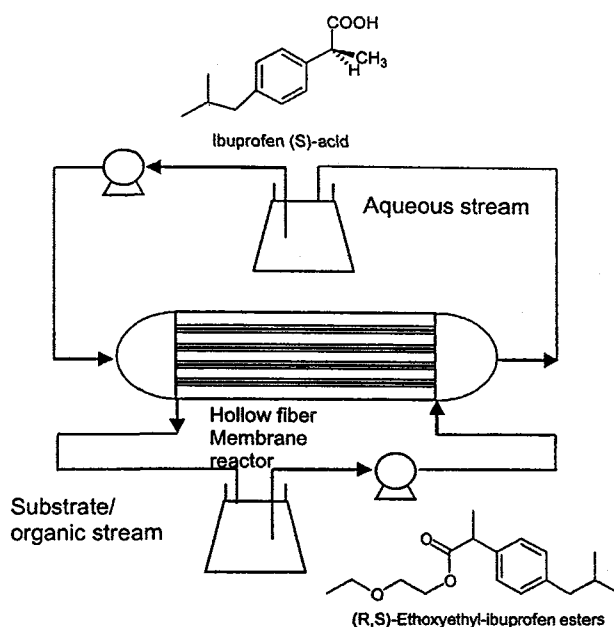


Fig. 1. Hollow fiber enzymatic membrane reactor for kinetic resolution of racemic 2-ethoxyethyl-ibuprofen ester.

aqueous buffer in the fiber lumen, in a counter-current mode.

In the hydrolysis reaction, the reaction layer is located at the aqueous–organic interface in the porous matrix. The use of circulating aqueous stream neglects the presence of water as a reactant; the *Candida rugosa* lipase catalyzes preferentially the (S)-ibuprofen ester, the (R)-ibuprofen ester is far less reactive, thus reducing the kinetics to one substrate–one product, as shown in Fig. 2. The substrate is in intimate contact with the immobilized enzyme at the membrane by means of a trans-membrane pressure (TMP) with excess pressure (40 kPa) from the shell side to avoid dispersion of aqueous phase through the hydrophilic membrane into the organic phase.

The enzyme reaction kinetics in this work was modeled on the basis of rapid equilibrium assumption. Rapid equilibrium condition (also known as quasi-equilibrium) assumes that only the early components of the reaction are at equilibrium (Segel, 1976). In rapid equilibrium condition, the E, S and ES equilibrate rapidly compared to the dissociation rate of ES into E and P. The product 2-ethoxyethanol was found to be a non-competitive inhibitor and (S)-ibuprofen showed uncompetitive inhibition. The proposed mechanism is given in Fig. 3. The overall enzyme reaction kinetics modified for uncompetitive substrate inhibitor and non-competitive product inhibitor was given in Eq. (1), which is in agreement with that obtained by Madhav and Ching (2001):

$$v = \frac{C_S V_{\max}}{(K_m + C_S)(1 + P/K_{IP}) + C_S^2/K_{IS}} \quad (1)$$

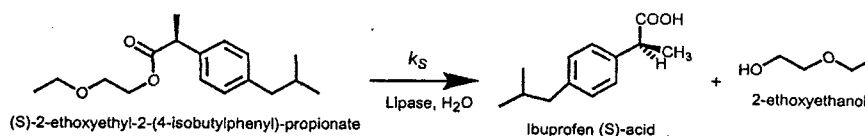


Fig. 2. Kinetic resolution of racemic 2-ethoxyethyl-ibuprofen ester, where lipase catalyzed the more reactive enantiomer, i.e., the (S)-ester viz. the (S)-ester into the corresponding (S)-acid and 2-ethoxyethanol as the by-product.

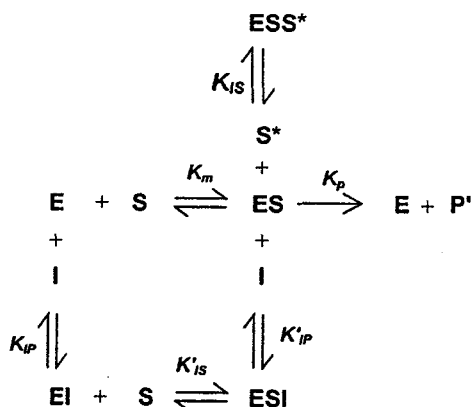


Fig. 3. Kinetics mechanism of enzymatic hydrolysis of racemic ibuprofen ester with uncompetitive substrate inhibition and non-competitive product inhibition.

v is the hydrolysis reaction rate; C_S is the substrate concentration of (S)-2-ethoxyethyl-ibuprofen ester; P is the concentration of by-product, 2-ethoxyethanol; K_{IS} and K_{IP} are the substrate and the product inhibition constants, respectively.

The objective of the present work were firstly to model reaction kinetics with reference to Michaelis–Menten rate equation taking into account possible substrate and product inhibitions, and secondly to study the performance of the EMR in terms of activity of immobilized lipase and operational stability of EMR.

3. Materials and methods

3.1. Enzymes and chemicals

Lipase from *Candida rugosa* EC 3.1.1.3 (Type VII, 724 units per mg solids) was supplied by Sigma-Aldrich (MI). The lipase was used as purchased without further purification. BCA protein assay was obtained from Pierce (IL). (R,S)-ibuprofen acid 99% and (S)-ibuprofen acid 99% were purchased from Acros (Belgium). Isooctane 99%, Sodium dihydrogen phosphate (KH_2PO_4 , MW 136.09), disodium hydrogen phosphate dehydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, MW 177.99) were supplied by Fisher Chemicals (UK). Other chemicals and reagents used were of analytical grade.

3.2. Chemical syntheses of ibuprofen ester

Racemic 2-ethoxyethyl-ibuprofen ester was chemically synthesized in our laboratory from the racemic ibuprofen acid, and 2-ethoxyethanol using isooctane as co-solvent and *p*-toluenesulfonic acid as catalyst. The esterification was carried out using a reflux setup in a Dean and Stark apparatus for 8 h. After cooling at room temperature, the organic mixture was washed with 5% sodium hydroxide and water. The organic layer was dried using sodium sulfate and evaporated by a rotary evaporator (Buchi Model R-114, Switzerland). The residual was further purified by micro-distillation to remove impurities. The ester structure was determined by H-NMR (Bruker Model Advance 400, Swiss) and FTIR (Perkin-Elmer Model System 2000, Shelton). For synthesis of (S)-ibuprofen ester, (S)-ibuprofen acid was used in the reflux, other conditions remain the same.

3.3. Enzyme immobilization on membrane reactor

2 g/L of *Candida rugosa* lipase solution was prepared using phosphate buffer at desired pH value. Enzyme immobilization on the membrane sponge layer was carried out by ultrafiltration of one liter lipase solution from shell side to fiber lumen with TMP of 35 kPa and flow velocity 1.5×10^{-2} m/s, at room temperature. The volume of the buffer collected from the permeate and retentate were recorded. After filtration of lipase solution, ultrafiltration was continued using 1 L of fresh buffer for washing. Residual water in shell side was completely filtered by purging with nitrogen gas. The washing solution in the permeate and retentate were collected for protein measurement. For lipase immobilized on the inner lumen, the same procedure was used except that the direction of ultrafiltration was from lumen to shell. The amount of protein (lipase) adsorbed onto the fibers of the hollow fiber membrane reactor was measured as the difference between the protein content of the lipase buffer solutions before and after the immobilization procedure. The protein concentration was quantified based on BCA standard test tube protocol (Pierce, Rockford, IL).

3.4. EMR operation and activity of immobilized lipase

Koch ultrafiltration membrane model HF1.0-45-XM50 (Koch Membrane Inc, USA) was made of asymmetric hydrophilic PAN material with MWCO 50 kDa. The

membrane module has 65 fibers with fiber inner diameter 1.14 mm and effective surface area 0.093 m². The membrane module was installed in a custom-made incubator. Its shell circuit was connected to an organic tank and lumen circuit to an aqueous tank. The organic phase composed of known concentration of racemic ibuprofen ester in isooctane and the aqueous phase was 50 mmol L⁻¹ phosphate buffer solution at pH 8. The flow of the organic and aqueous loops was in countercurrent mode in order to achieve higher interfacial contact of reactants. The operation of EMR was started by circulating organic substrate solution in the shell side. TMP of 40 kPa from shell to lumen was set prior to circulation of phosphate buffer solution in the lumen loop. The organic flow velocity at shell side was fixed at 4.0×10^{-3} m/s and aqueous flow velocity in the lumen was fixed at 6.5×10^{-2} m/s. The hydrolysis reaction was carried out for 8–10 h. Samples were collected from both organic and aqueous tanks.

3.5. Membrane cleaning and regeneration

After each experiment, the membrane was regenerated by backflushing with 50% v/v 0.1 M sodium hydroxide in isopropanol solution from lumen to shell (for cases where lipase was immobilized on the spongy layer) and from shell to lumen (for cases where lipase was immobilized on inner lumen) to remove all entrapped enzyme. This was followed by rinsing with deionized water.

3.6. Activity of free lipase in batch reaction

The activity of free lipase in aqueous/organic emulsion was performed in batch runs (emulsion) at 40 °C using (R,S)-ibuprofen ester as the substrate for a period of 48 h. The emulsion system consisted of an aqueous phase with 0.5–5.0 g/L of lipase solution (500 µg protein ml⁻¹) dissolved in 100 ml of phosphate buffer at pH 8.0 and an organic phase with 25 mmol L⁻¹ racemic ibuprofen ester in 100 ml isooctane. Orbital stirring of 150 rpm was fixed for batch reaction. Samples were withdrawn from aqueous/organic emulsion at regular time intervals and analyzed by HPLC.

3.7. Inhibition analysis in free lipase and immobilized lipase systems

The analysis of substrate and product inhibition was examined differently in two distinguished cases, and each case was studied for both batch runs and EMR. In this study, the enzyme kinetics has one substrate (C_S), one product (P) and one inhibitor, I (either excessive substrate or product).

4. Analytical procedures

4.1. Sample analysis using HPLC

The concentrations of the respective (R)- and (S)-ibuprofen ester and its acid form was analyzed by HPLC (Shidmazu Model 10VP, Japan) using (R,R)-Whelk-O1 Chiral Column (Regis-Pirkle, USA) and a UV detector. The mobile phase composed of hexane/isopropanol/acetic acid with the ratio 98:2:0.5. The organic sample (substrate in isooctane) was directly injected to HPLC without prior sample preparation. The aqueous sample (product in phosphate buffer) required liquid–liquid extraction step based on a method reported by Sakaki et al. (2001). The amount of (S)-ibuprofen acid produced was calculated based on the following formula:

$$m_i = C_{S,i} V_{\text{buffer}} + \sum_{j=0}^{i-1} C_{S,j} V_{\text{out}}, \quad (2)$$

m_i is the mass production of (S)-ibuprofen acid at i th sampling; $C_{S,i}$ and $C_{S,j}$ are concentrations of (S)-ibuprofen acid at different concentrations at different sampling time; V_{buffer} is the volume of buffer solution in the reactor system; V_{out} is the volume of sample taken from aqueous buffer.

4.2. Evaluation of kinetic parameters and inhibition constants

The reaction initial rates for kinetic resolution of racemic ibuprofen ester in both the EMR and batch systems were determined by plotting a tangent at the initial part of the progress curve. Data of initial reaction rates from various runs performed at different concentrations were fitted using *Enzyme Kinetics! Pro* (EKP) Software (ChemSW product, BioFrontier Technology, Singapore). The data were fitted to Michaelis–Menten kinetics using non-linear regression to obtain the constants K_m and V_{max} for free lipase system as well as $K_{m \text{ app}}$ and $V_{\text{max app}}$ for immobilized lipase system. These calculations were based on the classical Hanes–Wolf transformations. Substrate and product inhibitions analyses involved considerations of competitive, non-competitive, non-competitive and mixed inhibition models. The initial reaction rate obtained from experimental data was used in the primary plots (Hanes–Wolf plot) and plotting of secondary plots ($1/V_{\text{max}}$ vs. I) gave estimates of K_{IS} and K_{IP} . The initial reaction velocity (v_0) obtained from each substrate concentration was used to fit Michaelis–Menten equation (the inhibition model) thus obtaining estimates of $V_{\text{max app}}$ and $K_{m \text{ app}}$.

5. Results and discussion

Experiments were carried out with substrate concentration in the range of 5–50 mmol L⁻¹ at various operating

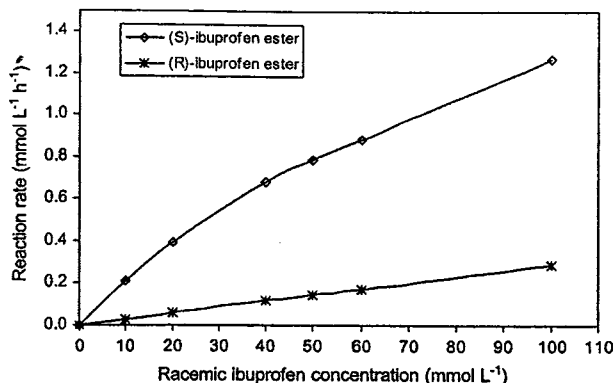


Fig. 4. Reaction rates for hydrolysis of (S)-ibuprofen ester and (R)-ibuprofen ester as a function of racemic ibuprofen ester concentration.

temperature and pH. This range was selected due to the fact that substrate concentration should fall in the range of 0.5–10 K_m in order to obtain accurate values of K_m and V_{max} (Bowden, 1981). The amount of lipase immobilized on the sponge layer was found to be in the range of 1.8 ± 0.2 g/m² and on the inner lumen was in the range of 1.3 ± 0.2 g/m² when fixed 2 g/L lipase solution was used. The type of lipase immobilization to the PAN membrane was physical adsorption. Guit et al. (1991) reported that enzyme half life and activity of the immobilized lipase from *C. cylindracea* (currently known as *C. rugosa*) was not significantly influenced by the type of membrane material and thus the binding of the lipase to the membrane was not very specific and the lipase was generally bound via surface adsorption. Fig. 4 shows the results of typical reaction rates for hydrolysis of (S)- and (R)-ibuprofen ester. The reaction rate of the lipase-catalyzed hydrolysis of (S)-ibuprofen ester was about 10 times higher than that of the (R)-ibuprofen ester. The enantiomeric ratio (E value) of the enzyme in the kinetic resolution can be calculated from the ratio of the two reaction rates (hydrolysis reaction rate of the (S)-ester over the (R)-ester), which in this case the E value was about 10. Since the (S)-ibuprofen ester was found to be less reactive in the kinetic resolution, the assumption of one-substrate and one-product was applied in the enzymatic reaction as depicted in Fig. 2.

5.1. Half life of Lipase in EMR and Batch[†] System

The half life ($t_{1/2}$) of lipase activity for both immobilized and free lipase was studied at initial 35 mmol L⁻¹ substrate concentration and 50 mmol L⁻¹ phosphate buffer solution (pH 8) at 40 °C. Enzyme activity measurements were made from multiple sampling of product (from the aqueous buffer) and substrate (sample). The relative activity is expressed as

$$\text{Relative activity (\%)} = \frac{\text{Lipase activity at any time}(A_t)}{\text{Initial lipase activity}(A_0)} \times 100. \quad (3)$$

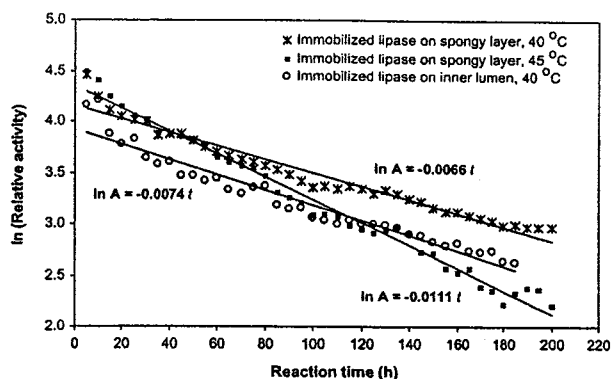


Fig. 5. Relative activity of immobilized lipase as a function of reaction time at three different reaction temperature and immobilization conditions.

The lipase activity was expressed as a function of initial activity, inactivation constant (k_d) and reaction time as defined by Pronk et al. (1992):

$$A_t = A_0 e^{-k_d t}. \quad (4)$$

The half life ($t_{1/2}$) of the lipase activity was defined as the reaction time when the lipase activity reduced to half of its initial activity, which is expressed as

$$\text{Half life of lipase activity, } t_{1/2} = \frac{0.693}{K_d}. \quad (5)$$

Fig. 5 shows the linearized relative activity of immobilized lipase as a function of reaction time. The amount of immobilized lipase on spongy layer was 1.79 g/m² for hydrolysis reaction carried out at 40 °C and 1.81 g/m² for reaction at 45 °C, and that for the inner lumen was 1.32 g/m² at 40 °C. The deactivation constant (k_d) for immobilized lipase on spongy layer at 40 °C reaction temperature was 0.0066 h⁻¹ and that for 45 °C was 0.0074 h⁻¹, whereas for the inner lumen was 0.0111 h⁻¹. Therefore, the half life calculated from Eq. (5) for the immobilized lipase on spongy layer was 105 h at 40 °C reaction temperature and 62 h at 45 °C. The half life for immobilized lipase on inner lumen was 94 h.

The half life of free lipase in the batch system at 40 °C determined from the same method was 45 h ($k_d = 0.0153$). The immobilized lipase on the spongy layer had a longer half life ($t_{1/2} = 105$ h) than that exhibited by both the free lipase and immobilized lipase on the inner lumen ($t_{1/2} = 94$ h) at reaction temperature of 40 °C. This observation might be due to the fact that the spongy region has a definite volume on which lipase molecules were stably retained in the three-dimensional space as compared to a fairly thin two-dimensional membrane skin which might be a less stable support for enzyme immobilization. In addition, in the latter case the enzyme molecules were subjected to shear stress induced by flowing organic stream along the fiber lumen. Unlike the former case where its structure was being well preserved in the spongy region, and thus it was protected from friction with the flowing substrate stream. The half life

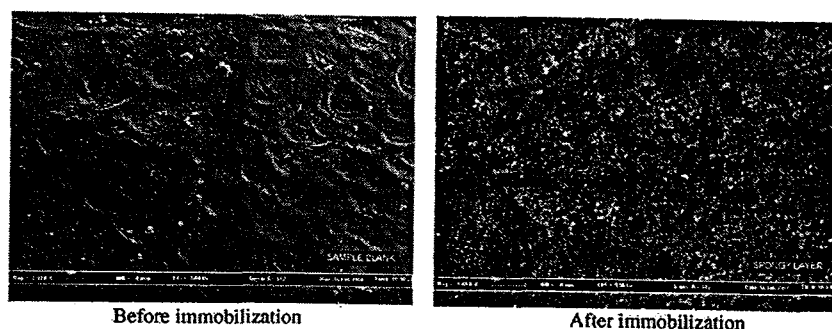


Fig. 6. SEM photographs of the spongy layer before and after immobilization.

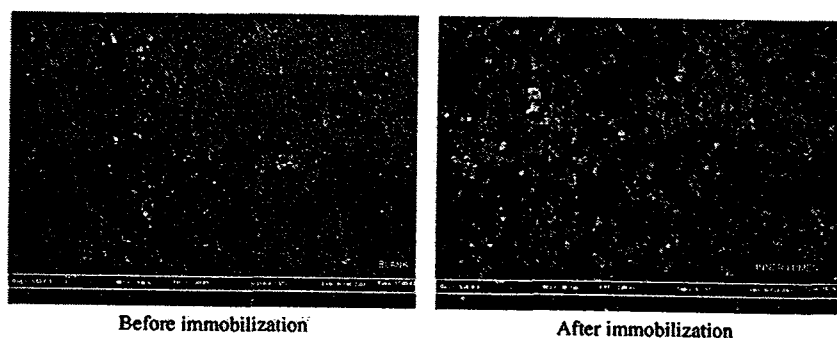


Fig. 7. SEM photographs of the inner lumen before and after immobilization.

of immobilized lipase dropped from 105 h (40 °C) to 62 h at elevated temperature (45 °C).

Figs. 6 and 7 show the SEM photographs of the spongy layer and inner lumen before and after lipase immobilization, respectively. The photographs were taken at 5000 magnification. It was observed that the lipase distribution on the spongy layer was more uniform and higher in its adsorption amount compared to that on the inner lumen. Thus, the higher enzyme productivity exhibited by immobilized lipase on the spongy layer might be due to proper binding or interaction of lipase functional groups with the spongy region which maintained the open-structure of lipase molecules for more stereospecific substrate–enzyme binding.³ On the other hand, this was not always possible for lipase immobilized on the dense inner lumen skin, as can be seen in Fig. 7. The latter case might be associated with random conformational change on membrane adsorption surface which only adsorbed lipase preferentially. This resulted in a loss of adsorption efficiency and consequently low activity.

5.2. Operational stability of immobilized lipase in EMR

Experiment performed in batch system was compared to the performance of EMR in order to justify the advantages of the EMR process over the batch system. Lipase stability was measured in terms of its half-life ($t_{1/2}$). Fig. 8 shows the free lipase activity for a period of 150 h operation. The amount of protein in the free lipase solution was 0.17 g (2 g/L lipase

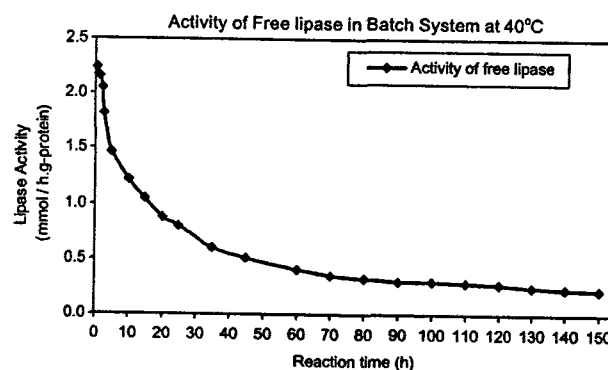


Fig. 8. Activity of free lipase in batch system for hydrolysis reaction taking place at 40 °C, pH 8 and 0.17 g protein.

solution) at 50 mmol L⁻¹ phosphate buffer solution (pH 8) and reaction temperature 40 °C. The E value of free lipase was in the range of 5.2–6.4. Fig. 9 shows the lipase activity in the EMR as a function of time which was examined at two different modes: firstly, the lipase was immobilized on spongy layer; and secondly, the lipase was immobilized on inner lumen. The amount of protein immobilized on the spongy layer was 1.79 g/m² and that on the inner lumen was 1.32 g/m². The E value of immobilized lipase on the spongy layer fell in the range 11.2–15.3 and that on the inner lumen was 9.5–13.1, which was independent of sides of immobilization on the membrane.

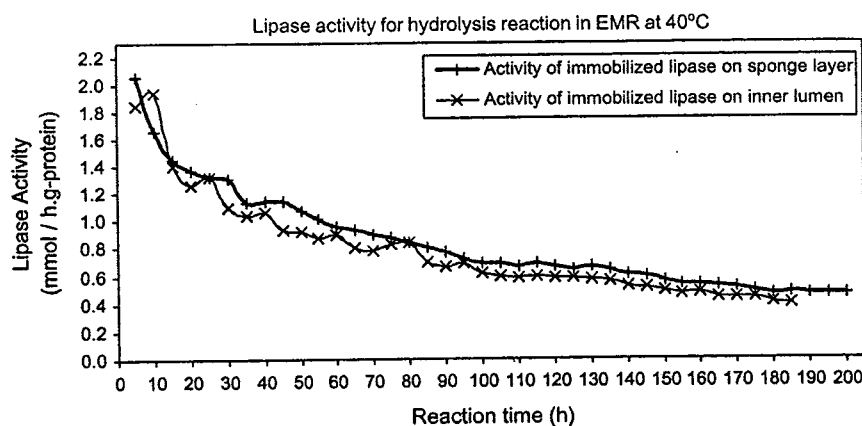


Fig. 9. Lipase activity for enzymatic hydrolysis in the EMR at 40°C, pH 8 with lipase immobilized at two different sides of membrane.

The initial lipase activity in batch system was 2.25 mmol/h g-protein while that for EMR was 2.05 mmol/h g-protein for lipase immobilized on spongy layer and 1.85 mmol/h g-protein for lipase immobilized on inner lumen. Although the batch system exhibited relatively higher initial activity than that in the EMR, there was a fast decrease in the lipase activity after 10-hour reaction. The free lipase activity dropped to 1.24 mmol/h g-protein at 10th hour. The EMR operation showed a better stability than the free lipase. This might be due to lower mobility of immobilized lipase in the membrane and thus its conformation was maintained for a prolonged operation as compared to the free lipase. The result was in agreement with literature that immobilized lipase exhibited greater stability in its activity than that exhibited by free lipase (Giorno et al., 1995; Ceynowa and Rauchfleisch, 2003).

The overall lipase activity exhibited by the immobilized lipase was higher than the free lipase. This revealed the different specific interactions between the enzyme active site and the substrate. During the adsorption (immobilization) process, the protein molecules (which are initially folded and able to contact the surface at only one side) begin to slightly unfold following contact, thus creating multiple binding sites (Bohnert and Horbett, 1986). This resulted in much greater activity than that achieved by a fixed amount of enzyme in batch reaction. The accessibility of lipase by the substrate molecules was much greater at the organic-aqueous membrane interface than that in the batch system, as a result of larger volumetric surface area (interfacial contact area) in the immobilized system. This resulted in much greater activity than that achieved by the free lipase in the batch reaction. Pugazhenthil and Kumar (2004) reported that the activity of lipase upon immobilization increased by 39%.

Lipase immobilized on ultrafiltration membranes might be subjected to leakage and losses as a result of prolonged operation normally associated with EMR. The aqueous phase was regularly refreshed to avoid buildup of the (S)-acid

concentration in the aqueous loop due to long operation time. BCA test was carried out for aqueous sample in the lumen side to check if there was leakage or breakage of lipase from the membrane spongy matrix. No protein was detected in the sample, thus it was concluded that no loss of lipase were observed during the long-term EMR operation.

5.3. Temperature stability of immobilized lipase in EMR

The effect of temperature on the stability of immobilized lipase for hydrolysis reaction in the membrane reactor was examined at two different temperatures (40 and 45 °C) and fixed pH 8 at TMP 40 kPa with recirculation of both phases for a period of 200 h. The temperature stability was carried out for two different immobilization conditions: (i) lipase immobilized on spongy layer; and (ii) lipase immobilized on inner lumen. The organic and aqueous flow velocities were 4.0×10^{-3} and 6.5×10^{-2} m/s, respectively, for the first condition. In the second condition, the organic and aqueous flow velocities were 1.3×10^{-2} and 1.5×10^{-2} m/s, respectively.

Hanes–Woolf method was employed for the evaluation of kinetic constant due to its strength in errors handling. Immobilized lipase showed higher maximum reaction rate with $V_{\max \text{ app}} = 3.27 \text{ mmol L}^{-1} \text{ h}^{-1}$ and greater enzyme–substrate affinity with $K_m \text{ app} = 36.47 \text{ mmol L}^{-1}$, as compared to free lipase where $V_{\max} = 2.83 \text{ mmol L}^{-1} \text{ h}^{-1}$ and $K_m = 63.43 \text{ mmol L}^{-1}$. The Michaelis constants of lipases were found to be altered by immobilization. K_m indicates the extent of binding between the enzyme and its substrate for a given substrate concentration. In the present work, the $K_m \text{ app}$ value of immobilized lipase showed a lower value, indicating a greater extent of binding. Kinetic parameters reported by other investigators for kinetic resolution of racemic ibuprofen ester are given in Table 1. It was found that the kinetic data reported in this work was in the same order of magnitude as that reported in the literature.

Table 1
Hydrolysis of racemic ibuprofen ester by *C. cylindracea* (or *C. rugosa*)

Substrate	<i>T</i> (°C)	pH	<i>K_m</i> or <i>K_{m app}</i>	<i>V_{max}</i> or <i>V_{max app}</i>	Reactor configuration	Solvent	Stability	Ref.
Cyanomethyl- ibuprofen ester	25 & 40	8	47 ± 3 ^a mmol L ⁻¹	4 ± 1.2 ^a mmol L ⁻¹ h ⁻¹	Polyamide 50kD membrane reactor with immobilized lipase	Toluene	No deactivation up to 16 days	Giomo et al. (1995)
Methyl- ibuprofen ester	37	7	25 μmol L ⁻¹	3.2 μmol L ⁻¹ h ⁻¹	Shake flask	Isooctane	—	Madhav and ching (2001)
2-ethoxyethyl- ibuprofen ester	40	8	36.47 mmol L ⁻¹	3.27 mmol L ⁻¹ h ⁻¹	PAN membrane 50kD reactor with immobilized lipase	Isooctane	EMR show stability after 180 h	Present study
	35	8	63.43 mmol L ⁻¹	2.83 mmol L ⁻¹ h ⁻¹	Batch system	Isooctane	Stable up to 150 h	

^a Evaluated from free lipase in solution in an stirred tank reactor.

5.4. Substrate inhibition study

Substrate inhibition was observed by investigating the kinetics of the reaction system. Fig. 10 shows the kinetics plot for substrate inhibition in the hydrolysis reaction performed in batch system at $[S^*] > 100 \text{ mmol L}^{-1}$. $[S^*]$ is the (S)-ibuprofen ester substrate inhibitor concentration, and v_0 is the initial reaction rate for (S)-ester conversion. The substrate inhibition was also examined for the hydrolysis reaction at inhibitor concentration (substrate) below 75 mmol L^{-1} . Fig. 11 shows the kinetics plot for substrate inhibition for the EMR system at $[S^*] > 100 \text{ mmol L}^{-1}$. It was observed from both the Hanes–Wolf plots in Figs. 10 and 11 that both batch and EMR systems show similar trend of substrate inhibition. The K_{IS} value was calculated by the EKP program. The K_{IS} value was calculated by the EKP program and is presented in Table 2. The inhibition constant (K_{IS}) in EMR was $49.52 \text{ mmol L}^{-1}$, higher than value obtained in batch system ($43.07 \text{ mmol L}^{-1}$). The inhibitor constant (K_{IS} or K_{IP}) is a measure of enzyme–inhibitor affinity. It is the dissociation constant of the enzyme–inhibitor complex.

Tables 3 and 4 present the intrinsic kinetic parameters (K_m and V_{max}) for free lipase in the batch system and apparent kinetic parameters ($K_{m app}$ and $V_{max app}$) for immobilized lipase in the EMR. K_m indicates the extent of binding between the enzyme and its substrate for a given substrate concentration. The Michaelis constants of lipases were found to be altered by immobilization. The $K_{m app}$ value of immobilized lipase showed a lower value, indicating a greater extent of binding. A similar trend was seen from the two system presented in Tables 3 and 4 that there were reductions in both $K_{m app}$ and $V_{max app}$ values when (S)-ibuprofen ester concentration above 100 mmol L^{-1} was used. Maximum reductions of 28.5% in K_m and 78.1% in V_{max} were observed for batch system when substrate concentration above 100 mmol L^{-1} was used, and that for EMR were 23.2% in $K_{m app}$ and 75.8% in $V_{max app}$. A possible substrate inhibition

in the system was uncompetitive inhibition, as determined from the kinetic study using EKP software.

The reduction of $K_{m app}$ was caused by a possible uncompetitive inhibition because excessive substrate binds to the ES complex forming the ternary ESS* complex, as depicted in Fig. 3. This phenomenon showed an apparent increase in the affinity of enzyme against the substrate as a result of more substrate being bound to the enzyme, i.e., the ESS* formation. An increase in substrate concentration increased the enzymatic reaction rate. However, once substrate concentration was increased ($> 1.5 K_m$), enzyme concentration became limiting and the enzyme–substrate complex reached saturation. At saturating substrate concentration, the substrate inhibition was profound. Since the ESS* complex would not be converted into product, thus $V_{max app}$ was decreased. This uncompetitive inhibition effect was in agreement with the literature that substrate inhibition in enzyme-catalyzed reaction decreased the reaction rate for Michaelis–Menten kinetics, resulting in the drop of racemate conversion (Madhav and Ching, 2001). Matson and López (1990), Xiu and Jiang (2000) and Xiu et al. (2001) found in their theoretical analysis that when substrate inhibition took place, the conversion dropped and the enantiomeric reaction rate decreased, causing the optimal time for terminating the reaction for a racemic resolution became longer. However, no significant change in the K_m and V_{max} was observed for inhibitor concentration below 75 mmol L^{-1} for both the batch system (maximum of 1.3% change in K_m and 3.2% change in V_{max}) and EMR (0.4% change in $K_{m app}$ and 0.3% change in $V_{max app}$). This shows that K_m (or $K_{m app}$) value was consistent when substrate was not inhibitory to the system.

The uncompetitive inhibitor increased the amount of substrate which bound to the enzyme, giving an apparent increase in enzyme–substrate affinity and a decrease in the value of $K_{m app}$. Since this inhibition was severe at high substrate concentration and therefore it decreased $V_{max app}$. This could happen if the inhibitor bound to the enzyme only

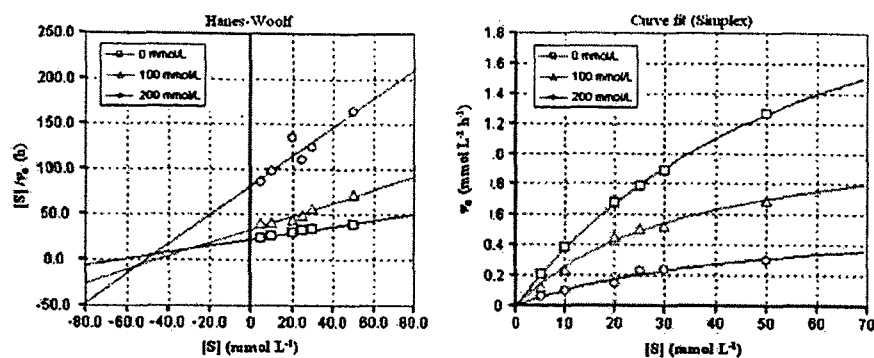
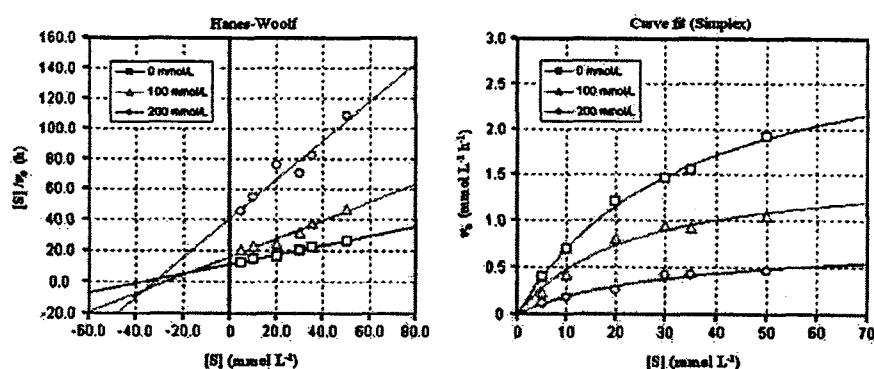
Fig. 10. Substrate inhibition plots at $[S^*] > 100 \text{ mmol L}^{-1}$ for batch system using EKP software.Fig. 11. Substrate inhibition plots at $[S^*] > 100 \text{ mmol L}^{-1}$ for EMR system using EKP software.

Table 2

Substrate and product inhibitions parameters for batch system and EMR based on Hanes–Woolf method obtained at reaction temperature of 40°C and buffer solution of pH 8

Batch				EMR			
$V_{\max}$ $\text{mmol L}^{-1} \text{ h}^{-1}$	K_m mmol L^{-1}	K_{IP}^a mmol L^{-1}	K_{IS}^b mmol L^{-1}	$V_{\max \text{ app}}$ $\text{mmol L}^{-1} \text{ h}^{-1}$	$K_{m \text{ app}}$ mmol L^{-1}	K_{IP}^a mmol L^{-1}	K_{IS}^b mmol L^{-1}
2.83	63.43	337.94	43.07	3.27	36.47	354.20	49.52

^aNon-competitive product inhibition.

^bUn-competitive substrate inhibition.

Table 3

Substrate inhibition: kinetic parameters for free lipase in the batch system

$[S^*]^a$ (mmol L^{-1})		K_m (mmol L^{-1})		$V_{\max}$ ($\text{mmol L}^{-1} \text{ h}^{-1}$)	
		Batch system	% Change with respect to $[S^*] = 0$	Batch system	% Change with respect to $[S^*] = 0$
Blank	0	63.43	—	2.83	—
> 100	100	45.34	28.5	1.36	51.9
	200	50.60	20.2	0.62	78.1
≤ 75	15	62.58	1.3	2.74	3.2
	75	64.09	1.0	2.84	0.4

^a $[S^*]$ is the (S)-ibuprofen ester substrate inhibitor concentration.

Table 4

Substrate inhibition: apparent kinetic parameters for immobilized lipase in the EMR

[S*] ^a (mmol L ⁻¹)		$K_{m \text{ app}}$ (mmol L ⁻¹)		$V_{\text{max app}}$ (mmol L ⁻¹ h ⁻¹)	
		EMR	% Change with respect to [S*] = 0	EMR	% Change with respect to [S*] = 0
Blank	0	36.47 (K_m)	—	3.27 (V_{max})	—
> 100	100	27.63	23.2	1.70	48.0
	200	32.39	11.2	0.79	75.8
≤ 75	15	36.61	0.4	3.28	0.3
	75	36.60	0.4	3.28	0.3

^a[S*] is the (S)-ibuprofen ester substrate inhibitor concentration.

after the substrate making the ternary complex ESS* unreactive. The inhibitor and substrate bound to the enzyme independently of each other. The consequence was a decrease in apparent limiting rate $V_{\text{max app}}$ by the same extent (Segel, 1976).

Excessive substrate acted as an uncompetitive inhibitor by binding to the enzyme–substrate complex to form the inactive ternary enzyme–substrate–inhibitor complex. Substrates should have fairly low aqueous solubility in order to maintain the conformation of enzyme activity. It was observed that under the experimental conditions employed, the reaction rate was independent of substrate concentrations in the range of $[S] < 1.5 K_m$. In such situation when the substrate concentration was low (dilute form, $[S] \ll K_m$), the enzyme kinetics could be assumed as a first-order reaction and inhibition effects were absent.

5.5. Product inhibition study

In hydrolysis reaction as shown in Fig. 2, the 2-ethoxyethanol was a by-product and its rate of formation was the same as the conversion rate of the (S)- or (R)-ibuprofen ester: one mole of 2-ethoxyethanol would be formed when one mole of ester was catalyzed. Some known concentration of 2-ethoxyethanol was added in the organic phase prior to the start of the reaction for product inhibition study. The product inhibition study was carried out at 2-ethoxyethanol concentration above 90 mmol L⁻¹ and below 50 mmol L⁻¹ with substrate concentration in the range of 5–50 mmol L⁻¹. The kinetics plot for batch system at $[I] > 90 \text{ mmol L}^{-1}$ are presented in Fig. 12 and that for EMR system is shown in Fig. 13. The K_{IP} value was 337.94 mmol L⁻¹ for batch system and 354.20 mmol L⁻¹ for EMR system. The product inhibition constants (K_{IP}) for the two systems are given in Table 2. Tables 5 and 6 show the kinetic parameters (K_m and V_{max}) for free lipase in the batch system and immobilized lipase in the EMR.

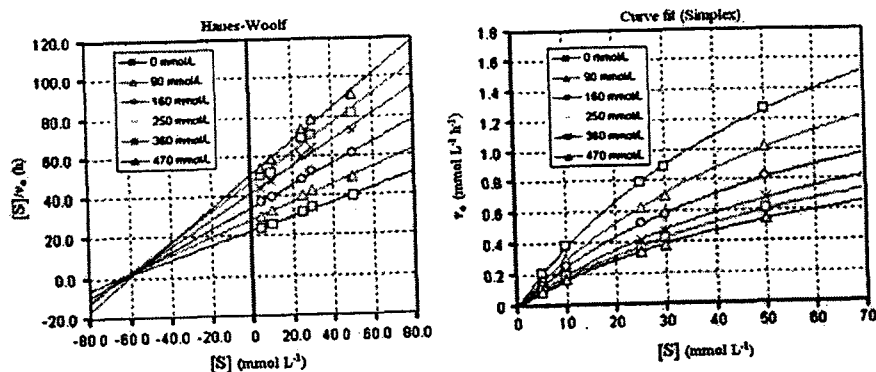
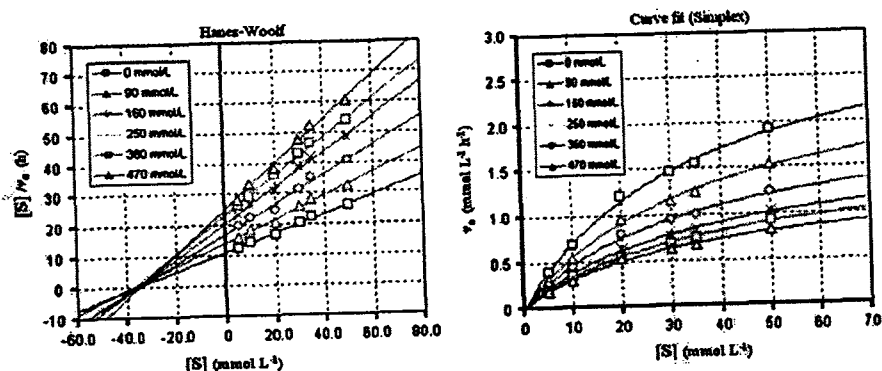
It was observed in both tables that the K_m and $K_{m \text{ app}}$ values were fairly consistent while V_{max} and $V_{\text{max app}}$ values were seen decreasing at inhibitor concentration $[I]$ above 90 mmol L⁻¹. A maximum of 58.0% reduction in V_{max} value

was observed in batch system and that for EMR system was 57.8% in $V_{\text{max app}}$ value. The 2-ethoxyethanol was found to be a non-competitive inhibitor to the substrate as determined by the EKP software.

In non-competitive inhibition, the substrate (S) and inhibitor (I) have equal potential to bind to both the free enzyme (E). The inhibitor will form a ternary complex with enzyme–substrate (ES) whereas the substrate will form another ternary complex with enzyme–inhibitor (EI). Since the non-competitive inhibitor had no effects on the binding of substrate to the enzyme, the enzyme–substrate affinity ($K_{m \text{ app}}$) remained consistent (or unchanged). There are two different ways for the formation of ESI ternary complex and this complex would not form the product and therefore $V_{\text{max app}}$ was decreased.

As can be seen from Tables 5 and 6, the product inhibition was only significant at $[I] > 90 \text{ mmol L}^{-1}$. A possible reason of product inhibition was due to the nature of 2-ethoxyethanol which was miscible in both aqueous buffer and organic solvent. This could have stripped out the water molecules bound on the lipase and caused the lipase inactive. In addition, this inhibition effect was severe in long EMR operation (about 10 hours) when the buffer solution was saturated with the desired polar (S)-acid product and 2-ethoxyethanol. There was continuous build up of 2-ethoxyethanol in the reactor after a long operation, thus inhibiting the enzymatic hydrolysis reaction. If this was the case, the phosphate buffer solution should be changed on a periodic basis to avoid product inhibition in the EMR system. K_{IP} value was larger than the K_{IS} value by an order of magnitude, i.e., K_{IP} value was 337.94 mmol L⁻¹ for batch system and 354.20 mmol L⁻¹ for EMR while K_{IS} value was 43.07 mmol L⁻¹ for batch system and 49.52 mmol L⁻¹ for EMR, as shown in Table 2.

The combined inhibition effects by 2-ethoxyethanol product as a non-competitive inhibitor and (S)-ibuprofen ester as an uncompetitive inhibition resulted in an overall mechanism shown in Fig. 3. Kinetic parameters reported by other investigators for kinetic resolution of racemic ibuprofen ester are given in Table 1. It was found that the kinetic parameters reported in this work were in the same order of magnitude as that reported in the literature, except for the case

Fig. 12. Product inhibition plots at $[I] > 90 \text{ mmol L}^{-1}$ for free lipase in batch system using EKP software.Fig. 13. Product inhibition plots at $[I] > 90 \text{ mmol L}^{-1}$ for immobilized lipase in EMR using EKP software.Table 5
Product inhibition: kinetic parameters for free lipase in the batch system

[I] ^a (mmol L ⁻¹)		K_m (mmol L ⁻¹)		V_{max} (mmol L ⁻¹ h ⁻¹)	
		Batch system	% Change with respect to [I] = 0	Batch system	% Change with respect to [I] = 0
Blank > 90	0	63.43	—	2.83	—
	90	66.12	4.2	2.32	18.0
	160	63.31	0.2	1.83	35.3
	250	62.59	1.3	1.51	46.6
	360	63.47	0.1	1.35	52.2
	470	60.44	4.7	1.19	58.0
≤ 50	5	63.55	0.2	2.88	1.8
	10	65.65	3.5	2.94	3.89
	20	63.31	0.2	2.88	1.8
	30	63.88	0.71	2.89	2.1
	50	65.60	3.42	2.94	3.89

^a[I] is the 2-ethoxyethanol inhibitor concentration.

of Madhav and Ching (2001) as substrate concentration was in the unit of $\mu\text{mol L}^{-1}$.

Increasing the reaction rate by in situ separation of the inhibiting product was verified by Liese et al. (2000), in which a hydrophilic membrane was used to separate the product present in the aqueous phase (containing product as inhibitor) from the organic phase (which was retained in the reactor). It was also reported that continuous removal

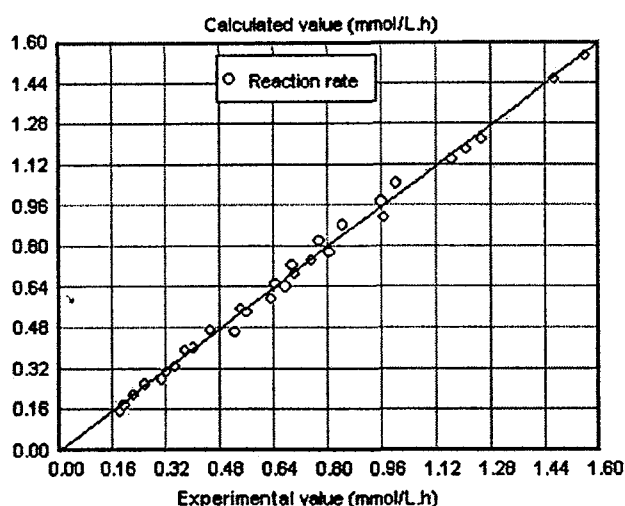
of products and retention of reactants could improve the reaction conversion for the equilibrium-limited reversible reactions (Paturzo et al., 2002).

The verification of the enzyme kinetic mechanism was made by plotting calculated value from the rate equation given by Eq. (1) against true experimental values using Polymath 5.1. The experimental data were taken from different reaction conditions and different experimental runs. The

Table 6

Product inhibition: apparent kinetic parameters for immobilized lipase in the EMR

[I] ^a (mmol L ⁻¹)		$K_{m \text{ app}}$ (mmol L ⁻¹)		$V_{\text{max app}}$ (mmol L ⁻¹ h ⁻¹)	
		EMR	% Change with respect to [I] = 0	EMR	% Change with respect to [I] = 0
Blank	0	36.47 (K_m)	—	3.27 (V_{max})	—
> 90	90	37.61	3.1	2.65	19.0
	160	35.56	2.5	2.09	36.1
	250	35.90	1.6	1.74	46.8
	360	37.43	2.6	1.60	51.1
	470	34.92	4.3	1.38	57.8
≤ 50	5	37.03	1.5	3.34	2.1
	10	37.13	1.8	3.35	2.4
	20	36.98	1.4	3.34	2.1
	30	37.05	1.6	3.34	2.1
	50	37.01	1.5	3.34	2.1

^a[I] is the 2-ethoxyethanol inhibitor concentration.Fig. 14. Plot of experimental values in comparison with calculated value from reaction rate equation (Eq. (1)) with $R^2 = 0.9949$.

results are given in Fig. 14. The experimental reaction rate fits the calculated rate equation with $R^2 = 0.9949$.

5.6. Comparison of experimental data and simulated results

Since the kinetic parameters have been estimated, the time-course conversion of the hydrolysis reaction was simulated using the following equation:

$$t = - \int_{C_{S_0}}^{C_S} \frac{dC_S}{-v}, \quad (6)$$

t is the time-course conversion of the hydrolysis reaction in the EMR; v is the reaction rate given in Eq. (1) with the kinetic parameters presented in Table 2. Fig. 15 shows the

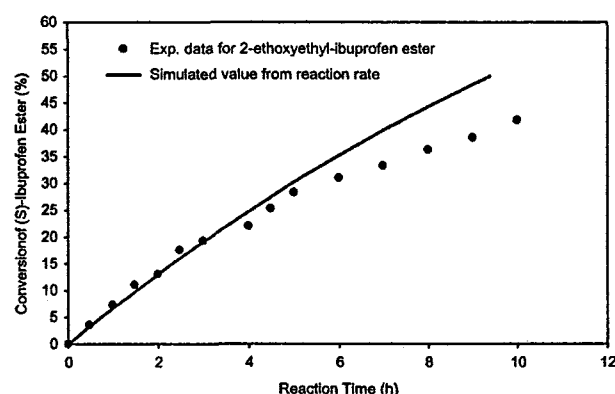


Fig. 15. Time-course conversion of hydrolysis reaction in EMR.

plot of the time-course conversion of (S)-ester, X_S which is calculated from Eq. (7).

$$X_S = \frac{C_{S_0} - C_S}{C_{S_0}}, \quad (7)$$

C_{S_0} is the initial concentration of (S)-ibuprofen ester. The experimental data were in good agreement with the values simulated from the time-course conversion (Eq. (6)) for the first 5 hours of reaction and started to diverge thereafter. The deviation was most probably caused by enzyme deactivation.

6. Conclusions

Immobilization of lipase from *C. rugosa* on enzymatic membrane reactor showed satisfactory hydrolysis activity with kinetic parameters $K_{m \text{ app}} = 36.47 \text{ mmol L}^{-1}$ and $V_{\text{max app}} = 3.27 \text{ mmol L}^{-1} \text{ h}^{-1}$. Results showed that the studied hydrolysis reaction obeyed Michaelis–Menten kinetics. Substrate and product inhibitions in the hydrolysis

reaction can be eliminated when low substrate concentrations were used. The selective removal of products from the reaction site increases the conversion of product-inhibited reactions. The substrate diffusion analysis showed the enzymatic hydrolysis reaction in the EMR was kinetically controlled. This makes the enzymatic membrane reactor potential for the production of single enantiomers at the highest possible product purity. Membrane application is very much beneficial for most enzymatic reaction and separation as most organic membrane could sustain low operating temperature while these reactions require low operating temperatures. By applying this EMR, the reaction time was decreased and longer operational stability was maintained.

Acknowledgements

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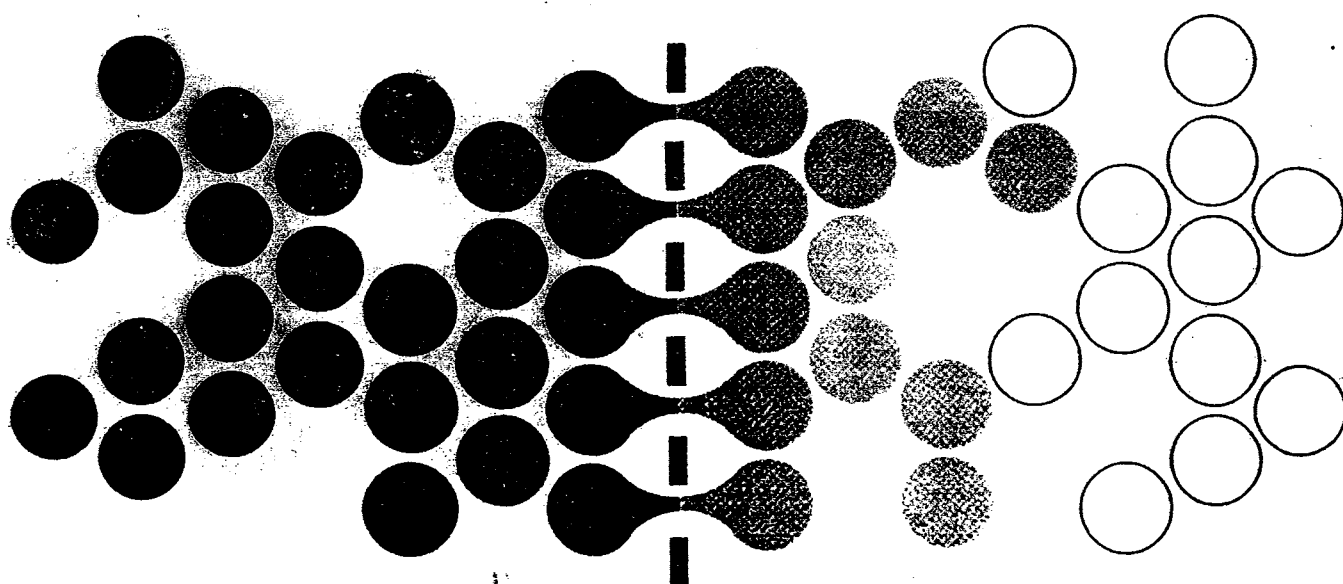
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Chiral resolution of racemic ibuprofen ester in an enzymatic membrane reactor

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Abstract

In the present study, the production of (*S*)-ibuprofen acid is reported using lipase-catalyzed hydrolysis reaction in a hollow fiber membrane reactor, as an efficient and cleaner route to obtain single-enantiomer compound. The performance of an enzymatic membrane reactor (EMR) was evaluated by varying operating temperature, organic phase flow rate, aqueous phase flow rate and enzyme loadings. The optimal operating conditions: temperature of 40 °C, pH 8 at EMR residence time (τ_{org}) of 10.87 h (organic inlet flow rate of 80 mL/min) with enzyme loading of 1.78 g/m² on the spongy layer of membrane were obtained. The kinetic resolution yielded an *E* value of 13, 31% *ee_S* and 85% *ee_P*. Both substrate and product purity were achieved by recovering the less reactive substrate in the organic stream while enhancing the optical purity of the desired product in the aqueous stream. The EMR system was modeled by incorporating Michaelis–Menten kinetics and treating the system as a continuous flow stirred tank reactor (CSTR). The simulated results agreed well with the experimental data.
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Keywords: Enzymatic membrane reactor; Kinetic resolution; Ibuprofen; Optical purity; CSTR

1. Introduction

Chiral drugs are major contributors to the global pharmaceutical market. Single-isomer drugs accounted for 30% of the \$335 billion total drug sales worldwide in 1998, raised up to 32% of the \$360 billion market in 1999 [1]. This fact has led to great efforts to obtain optically pure compounds, among which, non-steroid anti-inflammatory drugs (NSAIDs) are rated as the top chiral drugs. Ibuprofen and naproxen are commonly used NSAIDs for the treatment of arthritis and body pains in which the higher activity is exhibited by the (*S*)-configuration at the chiral center. The (*S*)-enantiomer of ibuprofen has the desired therapeutic effect, while the (*R*)-ibuprofen is inactive and can cause side effects [2]. There are number of industrial routes for the production of optically active (*S*)-ibuprofen [3–5]. Albermarle, BASF and Boots are

currently the three major suppliers of bulk active racemate. Ibuprofen was first introduced in the UK market as a racemic mixture in 1969 and in the USA in 1974 [6]. The Boots Company first patented the synthesis of ibuprofen in the 1960's (U.S. Patent 3,385,886). Boot's synthesis of racemic ibuprofen is a six-step synthesis. It was later that a new company, called BHC was formed by both Hoechst Celanese Corporation and Boots Company to develop a new 'green' synthesis of ibuprofen from the same starting material, i.e. isobutylbenzene and marketed ibuprofen. The catalyst used by the BHC's synthesis was hydrogen fluoride (HF) which the company claimed that no waste was generated by the catalyst as it was recovered and reused in their synthesis.

Ultrafiltration membrane process is an energy-efficient method used in product separations at a high degree of purity. The usage of hollow fiber and flat membrane reactors for biochemical reactions with immobilized lipases have been extensively reported [7–10]. The most common being the aqueous/organic biphasic system with immobilized enzyme or liquid membrane system with soluble enzyme. Several

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configurations of enzymatic reactor have been reported for the hydrolysis reaction involving chiral centers, e.g. a packed bed column with immobilized *Candida cylindracea* lipase has been used for the resolution of naproxen by hydrolysis [11], and for other hydrolysis reactions using hollow fiber membrane reactors in which high enantioselectivity for the (*S*)-enantiomer of the racemate was achievable [12–17]. Lipases are commonly used as chiral catalysts in organic synthesis [18]. One characteristic feature of lipase reactivity is that it reacts rapidly with insoluble rather than with soluble ester substrates in aqueous solutions. *C. cylindracea*-catalyzed (*S*)-specific hydrolysis of ibuprofen esters in an enzymatic membrane reactor and its 'state of the art' of the membrane reactor have been described by Sepracor [4] with an ee value of 96% achievable by the application of membrane reactor operated in batch mode [19].

Enzymatic membrane reactor (EMR) has great potential towards the development of chirotechnology. As the result of the role played by membrane in separation of two immiscible fluids, EMRs are normally employed in biphasic reactions in which reaction and separation occurs simultaneously. Models that predict the performance of EMR on kinetic resolution of racemic mixtures have been described [20–24]. The continuous operation of a membrane reactor can be modeled as a recycle reactor by high recirculation of both the substrate and product streams [20,25–27]. The volume of the ultrafiltration module is generally small relative to the total volume, and the recirculation flow rate is much larger than the flow rate leaving the reactor. This recycle reactor system is accomplished only when the conversion per pass is limited to less than 5%, to achieve satisfactory substrate resolution [20]. Early attempts to incorporate a membrane separation device to a continuous stirred tank reactor (CSTR) was motivated to continuously remove an inhibitory or desired product during fermentation process [7]. Microporous hollow fiber membrane reactors (HFMR) are geometrically suitable to be operated in a continuous mode by means of their mass transfer characteristics, i.e. the diffusion (concentration difference) and convection (by TMP) of reactants and products across the fiber wall. The main transport mechanism through which substrate is converted include diffusion of substrate from the bulk liquid phase to the membrane; diffusion of substrate

within membrane pores; and diffusion of substrate to the enzyme.

Lipases hydrolyze esters that are insoluble in water. Most of the immobilized enzymes are active in hydrophilic membranes and there is intertwining between both phases inside the membrane [9]. Upon immobilization, lipases require a minimum amount of water molecule surrounding and enclosing its active site which serves as a catalytic interfacial area. The hydrophilic membrane favors the aqueous buffer flowing at the lumen side and enables the water molecule to pass through the membrane pore. The organic phase containing the substrate flows in the shell side of the membrane. Hence, the immiscible organic–aqueous interface will be formed in the spongy region of the membrane in which the interfacial area, coupled with the immobilized lipase, provides a reaction layer or catalytic surface for the hydrolysis reaction. The control of TMP from shell to lumen is critical in order to stabilize the two phases within the membrane as well as to avoid dispersion of organic phase into the aqueous phase [28].

In the present study, an ultrafiltration membrane reactor is employed for the resolution of racemic mixtures of ibuprofen esters. The objective is to examine the performance of EMR in kinetic resolution of racemic compounds to achieve the highest product purity and to optimize the recovery quality of the less reactive enantiomers. The EMR was operated as a continuous stirred tank reactor. The experimental data were compared with the simulated results obtained from a model treating EMR as CSTR.

2. Enzymatic reaction in the membrane porous support

In the enzymatic kinetic resolution, the (*S*)-ester is the fast reacting enantiomer and it is catalyzed by lipase in the hydrolysis reaction to form the corresponding (*S*)-acid, as shown in Fig. 1. 2-Ethoxyethanol was chosen as the alkyl group for the (*R,S*)-ibuprofen ester as the ester contains an electron-withdrawing group which increases the rate of *Candida rugosa* lipase catalyzed hydrolysis [29,30]. In EMR operation, membrane containing the enzyme acts as a catalytic interface; it is in contact with the organic phase on one side

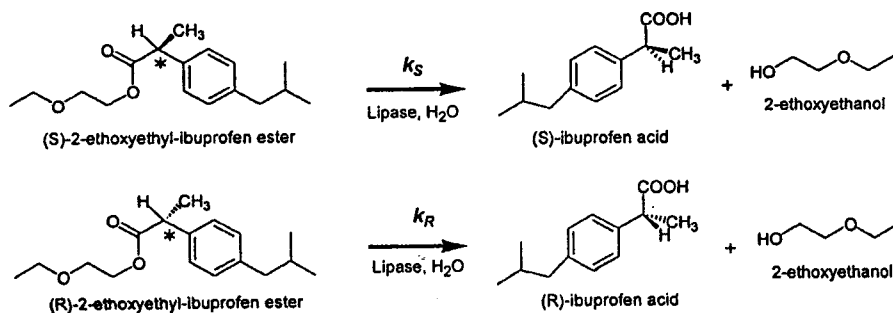


Fig. 1. Kinetic resolution of racemic 2-ethoxyethyl-ibuprofen ester by *C. rugosa* lipase-catalyzed hydrolysis.

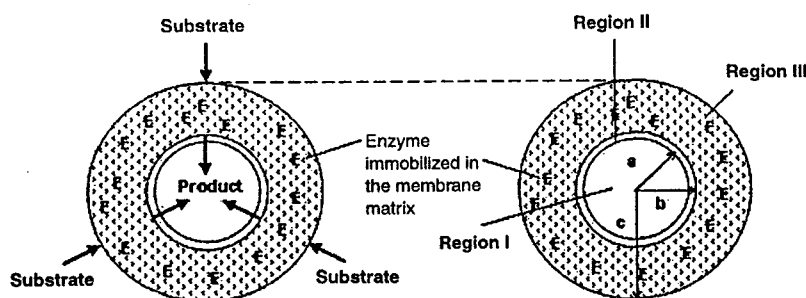


Fig. 2. Mass transfer of substrate and product in a single hollow fiber membrane where membrane acts as a semipermeable barrier with a selective enzyme. a , Fiber internal radius; c , fiber external radius; $c - b$, thickness of porous matrix; $b - a$, thickness of dense skin.

and with the aqueous phase on the other side. With the hydrophilic membrane, the aqueous phase was in close contact with the immobilized lipase while the membrane serves as a barrier to separate the immiscible phases due to its hydrophilic nature.

The enzymatic hydrolysis of racemic ibuprofen ester takes place in the membrane porous matrix, at which the substrate flows in from the shell side, as shown in Fig. 2. The driving force (convection) was primarily induced by transmembrane pressure (TMP) from the shell to lumen side of the membrane module [26]. A detailed model development of the enzymatic reaction in the membrane porous matrix has been reported [24]. In the present EMR system, an identical configuration is applied where the enzyme was immobilized in the membrane porous matrix and substrate diffuses across the spongy matrix in order to react with loaded enzyme at the enzyme-membrane reaction layer.

3. Experimental studies and apparatus

3.1. Chemical synthesis of racemic ibuprofen ester and (*S*)-ibuprofen ester

Thirty grams or 0.15 mol (*R,S*)-ibuprofen 99% (Acros, Belgium), 0.6 g *p*-toluenesulfonic acid (Acros, Belgium) and 30 g or 0.33 mol 2-ethoxyethanol (Acros, Belgium) were dissolved in 265 mL of isooctane. A two-fold molar excess of alcohol was used because the non-chiral compound should be present in a molar excess over the chiral compound to ensure complete conversion. The solution was refluxed for 8 h using a Dean and Stark apparatus. The reaction mixture was cooled at room temperature and washed with 5% sodium hydroxide followed by washing with water. The organic layer was dried with sodium sulfate and evaporated in the rotary evaporator (Buchi Model R-114, Switzerland). The residual from the heating flask was further purified by micro-distillation to remove impurities giving 12.5 g of (*R,S*)-ethoxyethyl-ibuprofen esters. All components were analyzed by ^1H NMR (Bruker Model Advance 400, Swiss) and FTIR spectroscopy (Perkin-Elmer Model System 2000, Shelton).

3.2. Preparation of phosphate buffer solutions

Sodium dihydrogen phosphate (KH_2PO_4) and disodium hydrogen phosphate dehydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) were supplied by Fisher Chemicals (UK). Phosphate buffer was prepared from sodium dihydrogen phosphate and disodium hydrogen phosphate. This buffer solution was further used for the preparation of lipase solution.

3.3. Preparation of lipase solution

Lipase from *C. rugosa* EC 3.1.1.3 (Type VII, 724 units per mg protein) was supplied by Sigma-Aldrich (MI). Two gram per liter of *C. rugosa* lipase solution was prepared using 50 mmol/L phosphate buffer at pH 8.0 and the solution was gently stirred by magnetic stirrer for 2 h.

3.4. Enzyme immobilization

One liter of 2 g/L *C. rugosa* lipase solution (0.0005×10^6 to 1.492×10^6 units) was circulated in the shell loop of the membrane reactor by cross-flow filtration at flow rate of 300 mL/min at room temperature and transmembrane pressure of 35 kPa. The volume of the buffer collected from the permeate and retentate were recorded. After filtration of lipase solution, ultrafiltration was proceeded with 1 L buffer solution. Residual water in permeate and retentate was collected. Five hundred milliliters of fresh isooctane in the shell loop and 500 mL of phosphate buffer (of the same pH) in the lumen loop were circulated for 15 min at TMP of 35 kPa from shell to lumen to introduce a consistent microenvironment to the enzyme for the hydrolysis reaction. For lipase immobilized on the inner lumen (the internal side of the fiber lumen), the same procedure was used except that the direction of ultrafiltration was from lumen to shell. The commercial lipase normally contains 12–20% protein. In this work, the amount of immobilized lipase onto the membrane was calculated from the difference of protein concentration before and after immobilization. The protein concentration was determined based on BCA Standard Test Tube Protocol (Pierce, Rockford, IL) using bovine serum albumin (BSA) as a stan-

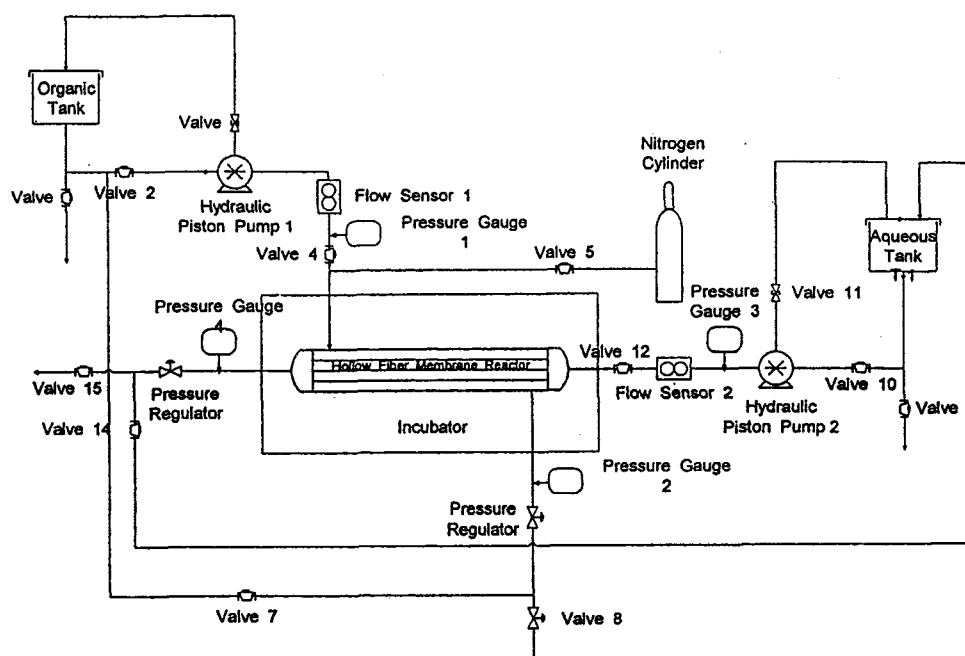


Fig. 3. Schematic diagram of enzymatic membrane reactor setup.

dard. Since the lipase was immobilized by physical method, it can be removed by backflushing using sodium hydroxide solution from lumen to shell. The membrane could be reused for immobilization of fresh enzyme.

3.5. Enzymatic membrane reactor configuration and operation

The hollow fiber membrane module employed was Koch ultrafiltration membrane HF1.0-45-XM50 (Koch Membrane Inc., USA) constructed of hydrophilic polyacrylonitrile material (PAN) with molecular weight cut off (MWCO) of 50 kDa, 65 fibers with fiber i.d. 1.14 mm and effective surface area of 0.093 m². The PAN membrane is asymmetrical having a tight skin layer on the lumen side and a loose sponge layer on the shell side with thickness of 0.56 mm. The schematic diagram of enzymatic membrane reactor setup is shown in Fig. 3. The organic and aqueous phases were immiscible and brought together at the enzyme-membrane interface to catalyze the reaction. The interfacial contact area for the enzymatic hydrolysis reaction was on the membrane interface. The flow of both phases was kept in counter-current mode in order to achieve higher mass transfer rate of reactants and products.

Isooctane was used as the organic solvent. The hydrophobic substrate was dissolved in the organic stream and channeled to the system at feed concentration of 25–100 mmol/L in the shell side and aqueous buffer in the fiber lumen. The substrate partitioned from the organic phase into the hydrophilic membrane by means of transmembrane pressure from shell to lumen. The operation of EMR was started by channeling the organic substrate solution in the shell side. A

TMP of 40 kPa from shell to lumen was set prior to circulation of phosphate buffer solution in the lumen loop. This is to avoid penetration of aqueous solution into the organic phase.

The EMR was operated continuously for a period of 10 h. In experiments with varying temperature, aqueous buffer of fixed pH 8.0 was used. In standard experimental runs, lipase solution of 2 g/L in 50 mM phosphate buffer pH 8 was used in the immobilization prior to enzymatic membrane reactions (the pH of aqueous buffer was identical to the pH value used for immobilization), the reaction temperature was 40 °C, the aqueous phase flow rate recirculating in the lumen circuit was 250 mL/min, the organic phase to the reactor consisted of both fresh and recycle substrate. The outlet stream consisted of two flows, i.e. returning to the reactor inlet and leaving the system as shown in Fig. 4. The EMR was running at constant volume with $F_{Sr} = F_{S0}$. The TMP was set at 40 kPa unless otherwise stated.

4. Analytical procedures

4.1. HPLC analysis

The concentration of the optically pure (*R*)- and (*S*)-enantiomer of ibuprofen ester and ibuprofen acid from the hydrolysis reaction was obtained by HPLC analysis (Shimadzu Model 10VP, Japan) using a reversed phase (*R,R*)-Whelk-Ol Chiral Column (Regis Pirkle, USA) with a UV detector. The mobile phase used was hexane/isopropanol/acetic acid (98:2:0.5). (*S*)-ibuprofen acid was eluted faster than the (*R*)-acid. The organic sample (ibuprofen ester in isooctane) was directly injected into HPLC without prior sample prepara-

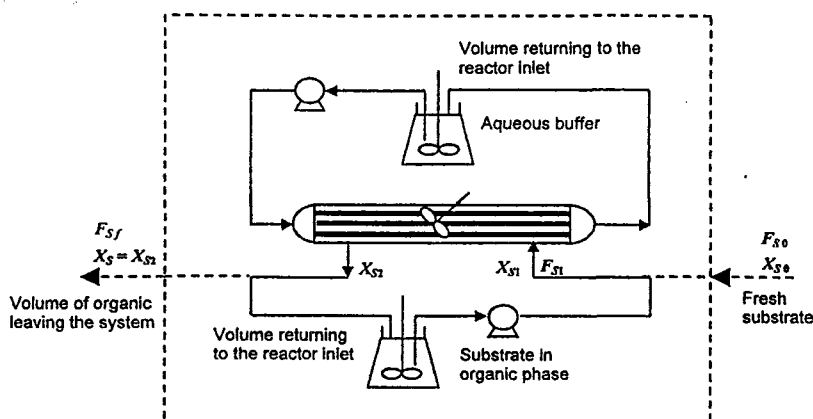


Fig. 4. EMR operated in a recycle mode modeled as a CSTR. F_{S0} , molar flow rate of substrate at the outlet stream. F_{Sf} , molar flow rate of substrate at the feed stream.

tion. The aqueous sample (ibuprofen acid in buffer) could not be directly measured and required liquid–liquid extraction step following the method suggested by Sakaki et al. [16]. The cumulative amount of (*S*)-ibuprofen acid produced was calculated from the aqueous phase.

4.2. Calculation of racemate optical purity and lipase enantiomeric ratio

The efficiency of the kinetic resolution was evaluated in terms of enantiomeric excess of the product (*ee_p*) and enantiomeric excess of the remaining less reactive substrate (*ee_s*). The optical purity of the (*S*)-ibuprofen acid is measured by *ee_p* while the optical purity of the remaining less reactive substrate is quantified by *ee_s*. A simple method to determine the enantiomeric ratio in enantioselective biocatalyst was reported by Rakels et al. [31]. The *ee_s* and *ee_p* are calculated from the molar fraction of each enantiomer as follows:

$$ee_s = \frac{C_R - C_S}{C_S + C_R} \quad (1)$$

$$ee_p = \frac{P_S - P_R}{P_S + P_R} \quad (2)$$

where *C* and *P* denote the substrate and product concentrations respectively with subscript 'R' and 'S' designate the respective (*R*)- and (*S*)-enantiomer. The enantiomeric ratio (*E*) characterizes the enantioselectivity of a particular enzyme in a reaction and is expressed as [32]:

$$E = \frac{\ln[1 - X(1 + ee_p)]}{\ln[1 - X(1 - ee_p)]} \quad (3)$$

An *E* value of 1 means no enantioselectivity. *E* value greater than 20 portrays a sufficiently good enantioselectivity. *X* is the overall conversion for the racemic substrate calculated from Eq. (4):

$$X = \frac{C_0 - C_S}{C_0} \quad (4)$$

where C_0 is the initial concentration of (*R,S*)-2-ethoxyethyl-ibuprofen ester and C_S is the concentration of (*S*)-2-ethoxyethyl-ibuprofen ester.

4.3. Enzyme kinetics

The initial reaction rates (v_0) were calculated from each substrate concentration. The rate was fitted to Michaelis–Menten kinetics using *Enzyme Kinetics! Pro* (EKP) software (ChemSW product, Singapore) to obtain estimates of Michaelis–Menten parameters, V_{max} and K_m for immobilized lipase reaction. The enzyme kinetics of the hydrolysis reaction incorporating substrate and product inhibitions analyses was acquired from biphasic membrane reactor process. The enzyme kinetic study shows a possible uncompetitive inhibition when excessive substrate was used (data not shown); and the 2-ethoxyethanol (byproduct from the hydrolysis reaction) was found to be a non-competitive inhibitor when it was present in an excess (data not shown). In the present work, the kinetic model modified for substrate and product inhibitions based on rapid equilibrium assumption is given by Eq. (5):

$$v = \frac{V_{max} C_S}{(1 + (C_b/K_{IP}))(C_S + K_m) + (C_S^2/K_{IS})} \quad (5)$$

where *v* is the hydrolysis reaction rate; C_b , is the concentration of 2-ethoxyethanol (byproduct), K_{IS} is substrate inhibition constant; K_{IP} is the product inhibition constant. Table 1 shows the corresponding kinetic parameters and inhibition constants obtained. The same inhibiting phenomena by different substrate and alcohol in a batch system were reported in the literature [33] and are also presented in Table 1. Alcohol is known to disrupt the three-dimensional architecture of the lipase [10]. Substrate concentration should always be greater than K_m so as to imitate a first-order reaction. It was found in this work that when $[C_S] \leq 1.5 K_m$ (data not shown), the enzyme kinetics was reduced to simple Michaelis–Menten

Table 1

Kinetic parameters and inhibition constants from present study and literature

System	Substrate	$V_{\max}$	K_m	K_{IS} (uncompetitive)	K_{IP} (non-competitive)	Reference
EMR	2-Ethoxyethanol-ibuprofen ester	3.27 mmol/L h	36.47 mmol/L	49.52 mmol/L	354.20 mmol/L	Present work
Batch system	Methyl-ibuprofen ester	3.2 μ mol/L h	25 μ mol/L	83 μ mol/L	2390 μ mol/L	[33]

model given by Eq. (6) in which inhibition was absent:

$$v = \frac{V_{\max} C_S}{C_S + K_m} \quad (6)$$

5. EMR modeled as CSTR

The kinetic resolution of racemic ibuprofen ester taking place in the EMR was treated as a CSTR operation. Previous study on modeling and simulation of EMR showed that the reactor behaved like a well-mixed tank (CSTR) as indicated by flat concentration profiles in the porous support when operated with a wide range of substrate flow rates [24]. This conceptual model was accomplished by continuous recirculation of organic and aqueous streams which were in the recycle loops as depicted in Fig. 4. Membrane reactor could be considered as a CSTR when the recycle ratio value is very high (approaches infinity), in which the recycle ratio as defined by Levenspiel [34] is:

Recycle ratio (R)

$$= \frac{\text{volume of fluid return to the reactor inlet}}{\text{volume leaving the system}} \quad (7)$$

The experimental conversion was compared with the predicted conversion values calculated from the CSTR model. The CSTR model was developed on the basis of steady-state mixed flow reactor using the following equation [26,34]:

$$F_{S1} X_{S1} = (v) V_{\text{matrix}} \quad (8)$$

where

$$F_{S1} = FC_{S1} \quad (9)$$

As seen in Fig. 4, X_S is the conversion measured at the stream leaving the reactor (exit stream), X_{S1} is the conversion at the inlet stream and X_{S2} is the conversion at the outlet stream of the reactor. X_{S1} is related to X_S by Eq. (10):

$$X_{S1} = \left(\frac{R}{R+1} \right) X_S \approx X_S \quad (10)$$

Since the recycle ratio is substantially large ($R = \infty$), attempts were made by considering that X_{S1} and X_S are identical. Thus, in this case, X_S is calculated from Eq. (11):

$$X_S = \frac{C_{S0} - C_S}{C_{S0}} \quad (11)$$

The reaction rate (v) is given by Eq. (5). F_{S1} is the total inlet molar flow rate of (S)-ester (includes both fresh feed and recycle stream); F is the total inlet volumetric flow rate

of substrate (fresh feed and recycle stream); C_{S1} is the inlet (S)-ester concentration to the reactor; V_{matrix} is volume of porous support calculated from the following equation:

$$V_{\text{matrix}} = \alpha_p 2\pi cL (c-b)N \quad (12)$$

where α_p is the membrane porosity ($\alpha_p = 0.8$); L is the fiber length ($L = 43.2$ cm); $c-b$ is the thickness of porous matrix as denoted in Fig. 2; N is the total number of fibers ($N = 65$). C_{S1} can be determined from Eq. (13) given as follows [34]:

$$C_{S1} = C_{S0} \left(\frac{1+R-RX_S}{1+R} \right) \quad (13)$$

where C_{S0} is the initial concentration of (S)-2-ethoxyethyl-ibuprofen ester. The recycle ratio (R) was kept high in order to avoid mass transfer limitation and to avoid the buildup of a concentration gradient in the loop. Substituting Eq. (9) into (8) and after rearranging, the following third-order polynomial equation was obtained:

$$\begin{aligned} \frac{F}{V_{\max} V_{\text{matrix}}} \frac{C_{S1}^2}{K_{IS}} X_S^3 - \frac{F}{V_{\max} V_{\text{matrix}}} \left[C_{S1} \left(1 + \frac{P}{K_{IP}} \right) \right. \\ \left. + \frac{2C_{S1}^2}{K_{IS}} \right] X_S^2 + \left[\frac{F}{V_{\max} V_{\text{matrix}}} (K_m + C_{S1}) \left(1 + \frac{P}{K_{IP}} \right) \right. \\ \left. + \frac{F}{V_{\max} V_{\text{matrix}}} \frac{C_{S1}^2}{K_{IS}} + 1 \right] X_S - 1 = 0 \end{aligned} \quad (14)$$

The solution of the third-order polynomial would have three roots (X_S), among which only the positive root was considered.

6. Results and discussion

The important process variables affecting the performance of EMR in the kinetic resolution were studied. The main parameters were organic phase flow rate (50–300 mL/min), aqueous phase flow rate (50–450 mL/min), reaction temperature (25–45 °C) and enzyme loading (0.5–5 g/L). Additional assessments were also studied for the EMR system, which include diffusion analysis of the substrate in the membrane matrix, EMR operation treated as a BSTR and as a CSTR respectively.

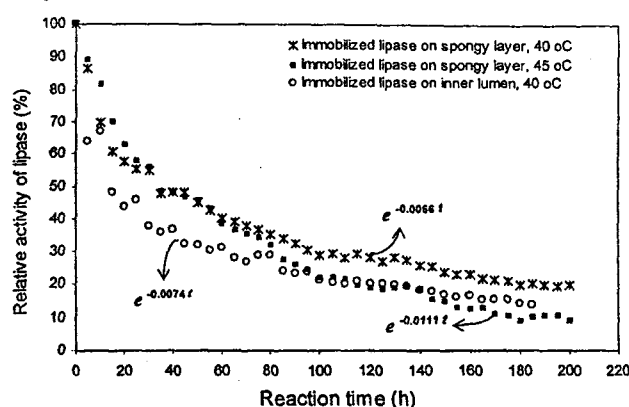


Fig. 5. Relative activity of immobilized lipase as a function of reaction time.

6.1. Lipase activity in EMR

The lipase activity is presented in Eq. (15) as defined by Liese et al. [35]:

$$\begin{aligned} \text{Activity} &= \frac{[\text{hydroxyester}] \times \text{conversion}}{\text{time} \times [\text{lipase}]} \\ &= \frac{\text{mM}}{\text{min}(\text{g-protein/L})} \times \frac{60 \text{ min}}{\text{h}} \\ &= \frac{60 \text{ mmol}}{\text{h} \times \text{g-protein}} \end{aligned} \quad (15)$$

The amount of immobilized lipase on spongy layer was 0.16 g-protein for hydrolysis reaction carried out at 40 °C and 0.17 g-protein for reaction at 45 °C, and that for the inner lumen was 0.13 g-protein at 40 °C. All reactions were carried out at pH 8. In this work, the relative activity is expressed as:

$$\begin{aligned} \text{Relative activity (\%)} &= \frac{\text{Lipase activity at any time}}{\text{Initial lipase activity}} \times 100 \end{aligned} \quad (16)$$

Fig. 5 shows the relative activity of immobilized lipase as a function of reaction time in the EMR in which enzymatic hydrolysis was occurring. It was observed from Fig. 5 that the exponential decay of enzyme activity followed first order kinetics. The half time ($t_{1/2}$) of the lipase activity is the reaction time when the lipase activity reduced to half of its initial activity.

The deactivation constant (k_d) for immobilized lipase on spongy layer at 40 °C reaction temperature was 0.0066 h^{-1} and that for 45 °C was 0.0111 h^{-1} , whereas for the inner lumen was 0.0074 h^{-1} as shown in Fig. 5. Therefore, the half time calculated for the immobilized lipase on spongy layer was 105 h at 40 °C reaction temperature and 62 h at 45 °C. The half time for immobilized lipase on inner lumen was 94 h. It is believed that substrate depletion was not the cause of the enzyme deactivation. As the reaction proceeded, substrate depletion caused the substrate concentration gradient across

the membrane decreased, hence decreased the transport rate of substrate to the reaction layer. In this case, continuous feed of fresh substrate could avoid the substrate depletion problem.

6.2. Diffusion of substrate in the membrane matrix

Membrane reaction–separation process is based on mass transfer mechanism between each substance in the shell and lumen sides. In this work, the film transport mechanism involves: (i) substrate transport through external film and membrane matrix, (ii) substrate diffusion to enzyme, and (iii) product diffusion to bulk aqueous, as depicted in Fig. 6. The substrate transports from the bulk organic phase via convection/diffusion into the reaction layer, viz. organic–aqueous membrane interface with loaded enzyme. The resultant product (i.e. (S)-ibuprofen acid) diffuses/extracts through the hydrophilic membrane into the aqueous phase circulating in the lumen circuit. The substrate molecule has to diffuse all these resistances to reach the enzyme active site. The substrate and product exchange surface was at the membrane for aqueous/organic interfacial reaction. It was reported that neither the skin nor the spongy matrix offer much diffusional resistance, and hence an enzymatic reaction carried out in a hollow fiber membrane reactor will usually be limited either by diffusion across the membrane fiber or reaction kinetics on the sponge-side [36].

The diffusion analysis for transport of substrate molecule from the bulk organic phase to the reaction layer was studied on the basis of effective diffusion coefficient (D_{eff}), characteristic length or molecular path in the spongy matrix (L_m), initial concentration of limiting substrate (C_{S0}), maximum reaction rate (V_{max}) and deactivation constant (k_d). The effective diffusion coefficient (D_{eff}) of substrate and product in the hollow fiber membrane was calculated based on a method given by Wu et al. [21]. The expressions of L_m^2/D_{eff} , C_{S0}/V_{max} and $1/k_d$ indicated good estimates of time scales associated with mass transfer of substrate. Table 2 presents the parameters obtained from experimental data used for the substrate diffusion study. L_m^2/D_{eff} gave the time a substrate molecule takes to travel across the porous matrix, which was in the range of 1.1–312 min. These values indicate the length of the transport time of the substrate molecule across the region was not sufficient for it to diffuse throughout. Hence, no significant substrate concentration profiles should build up in the radial direction. The range of substrate concentration used in this work, i.e. 25–100 mmol/L gave the effective diffusion coefficient in the range of 1.004×10^{-5} to $2.843 \times 10^{-3} \text{ cm}^2/\text{min}$, which is in the order of magnitude as that reported by researchers working on biocatalytic membrane reactor using other types of UF membrane [9,21,26]. A process was kinetically controlled, if $L_m^2/D_{\text{eff}} \leq C_{S0} \leq V_{\text{max}} \leq 1/k_d$; it is diffusion controlled if $C_{S0}/V_{\text{max}} \leq L_m^2/D_{\text{eff}} \leq 1/k_d$ [8]. The value of L_m^2/D_{eff} was between 1.1 and 312 min, C_{S0}/V_{max} was in the range of 527–1587 min and $1/k_d$ varied from 8108 to 9091 min. Thus, the enzymatic hydrolysis reaction studied

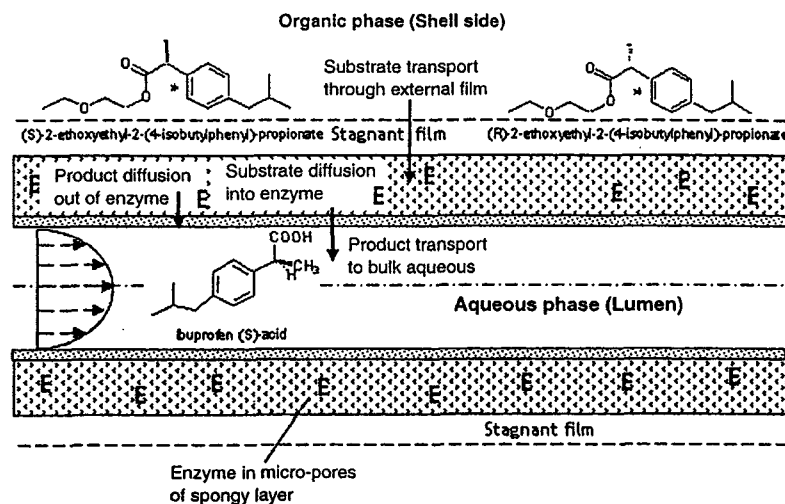


Fig. 6. Diffusion mechanism of substrate and product at the membrane interface.

in the EMR was kinetically controlled as it was in the order of $L_m^2/D_{eff} \leq C_{S0}/V_{max} \leq 1/k_d$ and diffusion rate was faster as compared to the reaction rate.

6.3. Effect of aqueous phase flow rate

The volumetric flow rate of aqueous phase was an important operating parameter which influenced the performance of EMR. The effect of aqueous flow rate was studied for lipase immobilized on the sponge layer. Four hundred milliliters of aqueous phase (50 mmol/L phosphate buffer, pH 8.0) at flow rates of 50 mL/min ($\tau_{aq} = 0.55$ min), 250 mL/min ($\tau_{aq} = 0.11$ min) and 450 mL/min ($\tau_{aq} = 0.06$ min) was carried out at fixed 80 mL/min ($\tau_{org} = 10.87$ h) of organic flow rate. The residence time of the aqueous phase in the fiber lumen (τ_{aq}) was calculated from Eq. (17):

$$\tau_{aq} = \frac{V_{lumen}(mL)}{F_{aq}(mL/min)} \quad (17)$$

where

$$V_{lumen} = \pi a^2 L N \quad (18)$$

Fig. 7 shows the effect of aqueous residence time on the (S)-acid production rate with lipase immobilized on spongy layer. It was observed that at the lower residence time of fiber

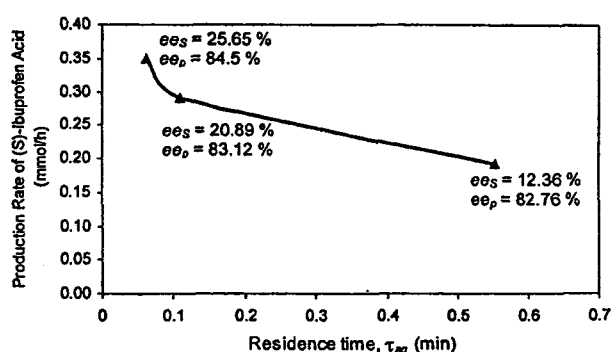


Fig. 7. Effect of aqueous residence time (τ_{aq}) on (S)-acid production rate with lipase immobilized on spongy layer. Total EMR operation time was 10 h. The ee_S and ee_P values were determined at the end of hydrolysis reaction.

lumen (τ_{aq}), the production rate of (S)-ibuprofen acid was high up to 0.35 mmol/h at $\tau_{aq} = 0.06$ min ($F_{aq} = 450$ mL/min). In this hydrolysis reaction of racemic ibuprofen ester, both the (R)- and (S)-ester were non-polar substances whereas the product (S)-acid was polar and diffused into the flowing aqueous phase. The selection of membrane materials and types for a particular process was dependent not only on pore size and structure but also on its hydrophilicity. The less reactive

Table 2
Diffusion analysis for substrate molecule in the boundary layer of organic phase and the membrane porous matrix

Parameter	Description	Unit	Porous matrix
L_m	Characteristic length	cm	0.056
D_{eff}	Effective diffusion coefficient	cm ² /min	1.004×10^{-5} to 2.843×10^{-3}
L_m^2/D_{eff}	–	min	1.1–312
C_{S0}	Initial substrate concentration	mmol/L	50
V_{max}	Apparent maximum reaction rate	mmol/L h	1.89–5.69
C_{S0}/V_{max}	–	min	527–1587
k_d	Deactivation constant	h ⁻¹	0.0066–0.0074
$1/k_d$	–	min	8108–9091

(*R*)-ester was retained in the organic stream at the shell side. The enantiomeric excess of the remaining substrate (*ees*) and product (*eep*) were greatly influenced by the residence time of the fiber lumen of EMR as shown in Fig. 7. An *ees* value of 25.65% associated with *eep* of 84.50% was obtained at $\tau_{aq} = 0.06$ min. It is believed that higher aqueous phase flow rate (thus lower τ_{aq}) facilitated the diffusion of polar product, and thus the flux of (*S*)-acid increased especially when hydrophilic membrane was employed. An increase in aqueous phase flow rate increases the diffusion rate thus giving rise to an increase in the (*S*)-acid diffusion due to reduced laminar film. However, *C. rugosa* lipase has been reported to lose activity as a function of shearing time and shear rate [10]. Hence, a fixed aqueous phase flow rate of 250 mL/min ($\tau_{aq} = 0.11$ min) was chosen for other EMR operations so as to cope with operational stability yet maintaining satisfactory productivity.

6.4. Effect of organic phase flow rate

The performance of the EMR operation was investigated as a function of organic phase flow rate in two immobilization conditions: (i) lipase immobilized on the spongy layer; (ii) lipase immobilized on inner lumen. The organic phase (substrate) flowed in the circuit in contact with the lipase and phosphate buffer solution at the other side. The organic phase flow rate (fresh and recycle) was in the range of 50 mL/min ($\tau_{org} = 14.41$ h), 80 mL/min ($\tau_{org} = 10.87$ h) and 200 mL/min ($\tau_{org} = 5.7$ h) at fixed aqueous phase flow rate of 250 mL/min in the lumen circuit for lipase immobilized on the spongy layer; and 25 mL/min ($\tau_{org} = 7.87$ h), 50 mL/min ($\tau_{org} = 5$ h), 130 mL/min ($\tau_{org} = 3$ h) at fixed aqueous phase flow rate of 300 mL/min in the shell loop ($\tau_{aq} = 0.2$ min) for lipase immobilized on inner lumen. The expression for the residence time of the organic phase (τ_{org}) for this type of recycle reactor (CSTR) is presented by Eq. (19) [34]:

$$\tau_{org} = \frac{C_{S0} V_{matrix}}{F_{S0}} = -(R+1) \int_{(C_{S0}+RC_S)/R+1}^{C_S} \frac{dC_S}{(-v)} \quad (19)$$

where V_{matrix} is the volume of porous membrane matrix given in Eq. (12) and v is the enzymatic reaction rate given in Eq. (5).

6.4.1. Enzyme immobilized on spongy layer

The effect of organic flow rate was contrary to that observed in the effect of aqueous flow rates. The τ_{org} parameter was related with reactor retention time whereas τ_{aq} addressed about the mass transport of product diffusion. Fig. 8 shows the performance of EMR for hydrolysis reaction in terms of (*S*)-ibuprofen acid production rate at different values of τ_{org} . A general trend was observed that lower organic flow rates yielded higher production rate. Higher *ees* and *eep* values were associated with higher production rate at lower flow rates. EMR operated at 50 mL/min ($\tau_{org} = 14.41$ h) gave 30.91% *ees* and 84.74% *eep* but operation at 200 mL/min

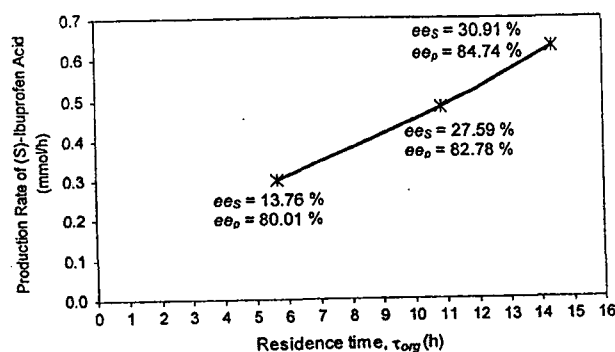


Fig. 8. Effect of organic residence time (τ_{org}) on (*S*)-acid production rate with lipase immobilized on spongy layer. The *ees* and *eep* values were determined at the end of hydrolysis reaction.

($\tau_{org} = 5.7$ h) gave only 13.76% of *ees* and 80.01% *eep*. The residence time of EMR shell side, i.e. organic volumetric flow rate showed great influence on the reactor performance. This was due to sufficient contact time between the substrate and enzyme at the membrane interface when diffusion of substrate molecule to the spongy region was not limiting, which was only possible at lower organic flow rates. The organic flow rate was kept low, i.e. 80 mL/min (equivalent to $\tau_{org} = 10.87$ h) so as to achieve more promising (*S*)-acid production rate.

6.4.2. Enzyme immobilized on inner lumen

Similarly, longer organic residence time yielded higher production rate, as shown in Fig. 9. The *ees* and *eep* values were independent of different immobilization conditions. Instead, it was governed by (*S*)-acid production rate. 28.21% *ees* and 81.34% *eep* were obtained at (*S*)-acid production rate of 0.35 mmol/h at $\tau_{org} = 7.87$ h ($F_{org} = 25$ mL/min), while 9.16% of *ees* and 67.32% *eep* were obtained at 0.15 mmol/h of (*S*)-acid production rate at $\tau_{org} = 3$ h ($F_{org} = 130$ mL/min). Lower (*S*)-acid production was obtained at lower τ_{org} (higher inlet organic flow rates) due to higher cross-flow velocities, because part of the enzyme may be extracted in the organic phase causing decrease in enzyme activity [13]. Fig. 10 com-

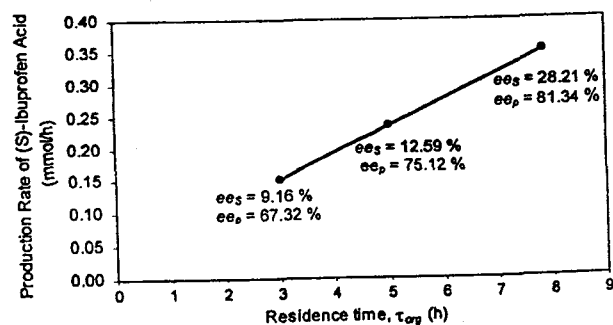


Fig. 9. Effect of organic residence time (τ_{org}) on (*S*)-ibuprofen acid production rate with lipase immobilized on inner lumen. The *ees* and *eep* values were determined at the end of hydrolysis reaction.

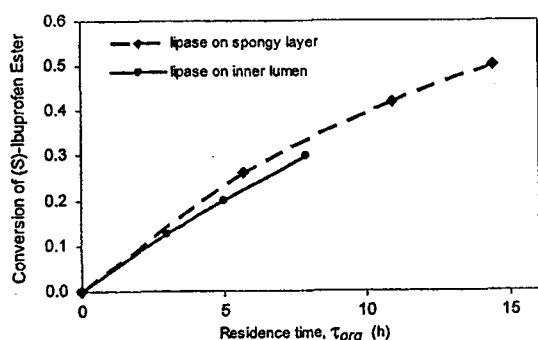


Fig. 10. Conversion of (S)-ibuprofen ester by lipase immobilized on spongy layer and inner lumen against different organic residence time (τ_{org}).

compares the conversion of (S)-ibuprofen ester between lipase immobilized on spongy layer and inner lumen against the residence time of EMR. The e_{es} and e_{ep} values were determined at the end of hydrolysis reaction. It was shown that lipase immobilized on the spongy layer exhibited greater catalytic activity with higher (S)-ester conversion as compared to lipase immobilized on the inner lumen.

6.5. Effect of temperature on activity

The operating temperature applied for the hydrolysis reaction in the EMR was varied from 25 to 45 °C. The plot of the initial reaction rate at different temperatures is shown in Fig. 11. The initial reaction rate increased once the temperature increased from 25 to 40 °C but dropped at 45 °C. Higher temperature resulted in higher substrate mobility and thus increasing reaction rate. The optimal reaction temperature was found to be 40 °C with initial reaction rate 1.85 mmol/L h. Relatively high operating temperatures may reduce the viscosity of the substrate phase and thus assist the substrate diffusion [10]. The drop in reaction rate with further increase in temperature up to 45 °C could be due to enzyme deactivation. Enzyme molecules are complex protein structure that arranges specific amino acid R-groups to form the stereospecific substrate binding site with the enzyme catalytic site. When enzyme molecules absorb too much energy, its pro-

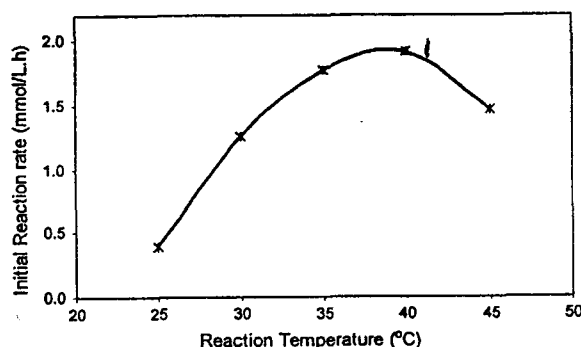


Fig. 11. Initial reaction rate at different operating temperature in EMR at $\tau_{org} = 4.4$ min and $\tau_{aq} = 1.6$ min.

tein structure could disrupt and the enzyme could undergo irreversible denaturation and lost its activity [37,38].

6.6. Effect of temperature on stability

The optimum temperature depends on the assay time chosen. The true "optimum temperature" for an assay is the maximum temperature at which the enzyme exhibits a constant activity over a time period as long as the assay time [37]. The effect of temperature on the stability of immobilized lipase for hydrolysis reaction in the membrane reactor was examined at two different temperatures (40 and 45 °C) and fixed pH 8 at TMP 40 kPa with recirculation of both phases for a period of 200 h. The temperature stability was carried out for lipase immobilized on the spongy layer. Fig. 12 shows that the lipase gave higher hydrolysis rate when operated at 45 °C at the beginning as compared to that of 40 °C, but its activity started to drop rapidly after 60 h of operation.

When the protein was exposed for longer period at the elevated temperature, partial deactivation could take place due to protein denaturation by heating. The expected increase in reaction rate at relatively high temperatures was offset by the increasing rate of denaturation. This result agreed with the literature that a higher reaction temperature increased the initial activity of the enzyme but decreased its stability [38]. Therefore, relatively high operating temperature was beneficial only when increased rate of reaction was essential. This was the case for EMR which was normally operated until the activity reduced to a minimum allowable limit.

Fig. 13 shows the e_{es} and e_{ep} values at various temperatures (25–45 °C) at pH 8.0. The e_{ep} values were between 82 and 85% which were consistent and independent of reaction temperature. The e_{es} value varied with reaction temperature, with optimum value of 31% obtained at 40 °C. The e_{es} and e_{ep} were slightly lower than the target values of $e_{es} \geq 50\%$ and $e_{ep} \geq 90\%$. This value could be further improved by in situ extraction of (R)-ester by a chiral resin or ion exchange membrane in order to prevent the catalysis of (R)-ester by lipase.

When operating the membrane reactor system in a batch stirred tank reactor (BSTR) mode with both organic and aqueous circuit recirculating in their respective loop, data of batch system can be obtained. The EMR with high recycle ratio of the organic phase and recirculation of the buffer solution in the lumen circuit by taking into account the system with solid lines is the representative of BSTR (Fig. 4). The time of reaction for a batch system is calculated as [34]:

$$t = - \int_{C_{S0}}^{C_S} \frac{dC_S}{(-v)} \quad (20)$$

Fig. 14 shows the plot of (S)-ester conversion (X_S) against the time of reaction using BSTR model given in Eq. (20) at different effectiveness factor ($0.1 \leq \eta \leq 1$). The experimental data were found to be consistent with the values predicted

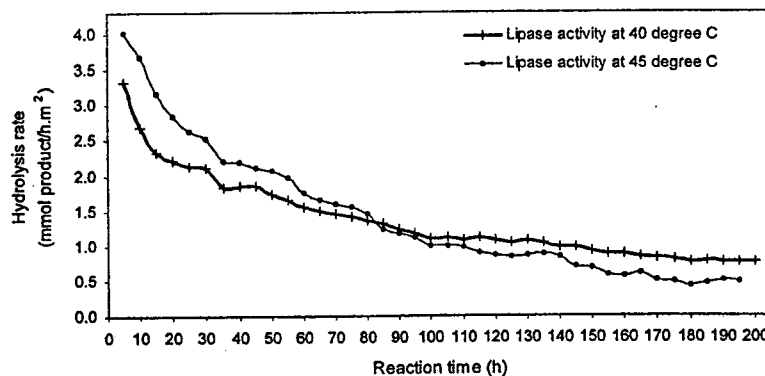
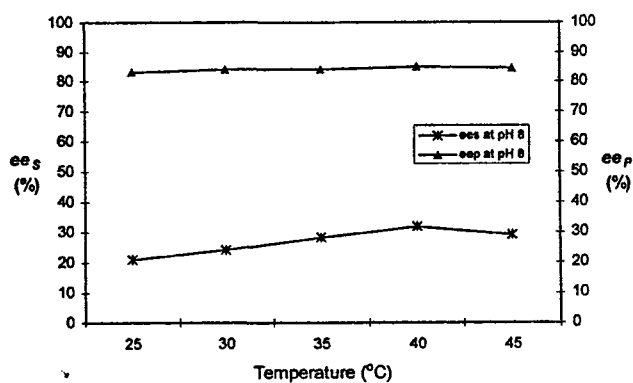
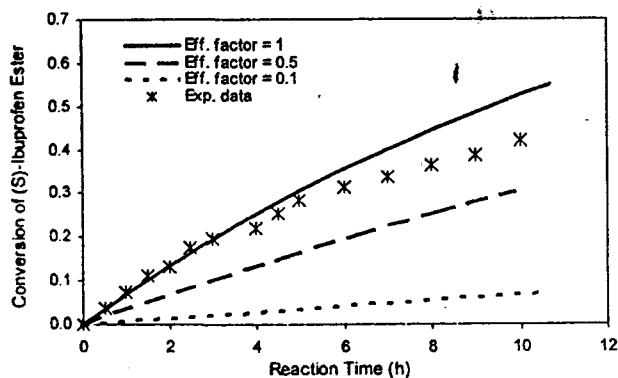


Fig. 12. Temperature stability of the immobilized lipase on the membrane spongy layer.

Fig. 13. e_{es} and e_{ep} values at various temperatures (fixed pH 8.0).

from BSTR model for the first 3 h of reaction with $\eta = 1$. The data starts to deviate from the line ($\eta = 1$) after 4 h and approaches effectiveness factor of about 0.75 (by estimation). This shows that reaction rates with diffusion limitation were comparable to reaction without diffusion limitation during the first few hours of reaction where enzyme was greatly available for the substrate and byproduct concentration was too low to inhibit the reaction.

Fig. 14. BSTR model, experimental data from reactor operated in BSTR mode (inner reactor system) and experimental data from inner reactor system equivalent to a BSTR. Eff. factor is the short form of effectiveness factor (η).

6.7. Effect of enzyme loading

Enzyme may be more effective when present as an aqueous solution (when lipase is in its monolayer form) in the membrane pores than when gelified on the membrane surface. Therefore, the range of enzyme loading in which the specific activity remains constant should be known, the stage when all the loaded protein is distributed as a monolayer (i.e. until all the enzyme can be reached by the substrate) in order for the system to work under reaction-limited condition. In this study, the lipase was uniformly distributed in the interstices of asymmetric hydrophilic membrane. The lipase was immobilized in the spongy matrix and the membrane shell side was in contact with the organic solvent. The low distribution coefficient of lipase prevented it from partitioning into the organic phase.

The protein binding capacity was affected by the electrostatic charge distribution and the number of potential coupling sites in the lipase molecules [8]. The amount of protein adsorbed was also dependent on pH, ionic strength, surface area, porosity, and the physical characteristics of the enzyme and the support [37,39]. Table 3 shows that immobilization capacity on the spongy layer was different from that on the inner lumen although the same concentration of lipase solution was used. The e_{es} value was higher at lower enzyme loading while e_{ep} value was independent of the amount of loading. This showed that the lipase enantioselectivity was influenced by the amount of loading. Immobilized protein on the spongy layer was in the range of 0.14–2.85 g/m² on the basis of outer surface area of the membrane. Enzyme loading in these experiments was in the range of 0.09–2.36 g/m² for lipase immobilized on the inner dense layer (lumen side) based on the inner surface area of membrane. The protein loading was within the same order of magnitude with an enzyme loading of 1.0–1.5 g/m² using PAN membrane reported by Guit [40]. Sakaki et al. reported a range of 200–6400 mg-protein/m² with the use of polyamide membrane [16].

The spongy region adsorbed more protein compared to the dense inner skin of the membrane. This might be due to dissimilarity in the membrane surface polarity of the spongy

Table 3
ee_S and ee_P values for different enzyme loadings

Lipase <i>C. rugosa</i> with molecular weight of 58500 g mol ⁻¹ dissolved in 50 mM phosphate buffer						
Lipase solution (g/L)	Immobilized on shell side			Immobilized on inner lumen		
	g-protein/m ²	ee _S (%)	ee _P (%)	g-protein/m ²	ee _S (%)	ee _P (%)
0.5	0.14	25.98	82.76	0.09	22.94	79.84
1.0	0.92	23.39	82.48	0.71	16.47	80.16
2.0	1.78	17.06	81.90	1.43	14.36	78.62
5.0	2.85	11.60	83.94	2.36	10.58	77.39

layer and inner lumen and different charges in the distribution of lipase as a function of loading which resulted in different hydrophobic–hydrophilic balance between lipase and the two membrane surfaces, thus showing different loading capacity.

6.7.1. Comparison between immobilized lipase on spongy layer and inner lumen

Fig. 15 shows the enzyme productivity for two different immobilization conditions as a function of immobilized protein. The enzyme productivity for lipase immobilized on spongy layer was about two-fold higher than that immobilized on the inner lumen. The enzyme productivity was about 5.5 g-product/h g-protein for reaction carried out at 1.78 g-protein immobilized per m² external spongy area while only 2.8 g-product/h g-protein was obtained at 1.43 g-protein immobilized per m² inner lumen. The higher enzyme productivity exhibited by immobilized lipase on the spongy layer might be due to proper binding or interaction of lipase functional groups with the spongy region which maintained the open-structure of lipase molecules for more stereospecific substrate–enzyme binding. On the other hand, this was not always possible for lipase immobilized on the dense inner lumen skin. The latter case was associated with random conformational change on membrane adsorption surface which only adsorbed lipase preferentially. This results in a loss of adsorption efficiency and consequently low enzyme productivity. Calabró et al. [41] reported that the reactor productivity reaches a maximum either when the enzyme is equally distributed in the membrane regions or when it is mostly entrapped in the fiber spongy layer. As opposed to lipase

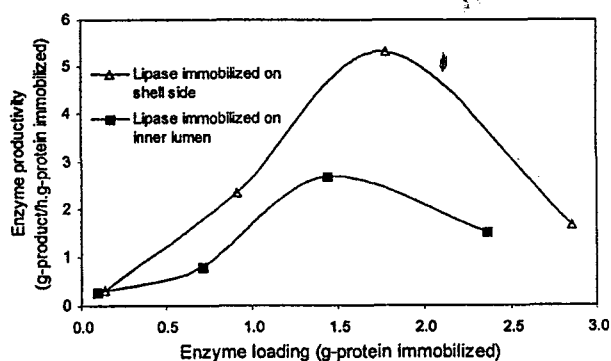


Fig. 15. Comparison of enzyme productivity for different immobilization conditions.

immobilization on spongy layer, the lipase immobilized on the inner lumen stayed near the organic–aqueous interface to catalyze the reaction as its enzyme–membrane reaction layer was aligned with the organic–aqueous interface [16].

Bouwer et al. [9] pointed out that in lipase immobilized membrane reactor, the lipase does not only stay on the two-dimensional surface but also spreads out in the membrane. In cases where enzyme immobilized on the spongy layer (with thickness of 0.56 mm), the amount of immobilized protein should be expressed in terms of mg-protein/m³ and mg-protein/m² for that immobilized on the inner lumen. The volume of the spongy region was 8.512×10^{-7} m³. However, for the sake of unit consistency, a standard unit of g-protein/m² was used in this work for both immobilization cases. For reaction to occur in a kinetically controlled regime, enzyme loading should be optimized based on an empirical correlation, i.e. Thiele modulus expressed as:

$$\phi^2 = \frac{(c-b)^2 \alpha_p V_{\max}}{K_m D_{\text{eff}}} \quad (21)$$

where $c-b$ is the thickness of the spongy layer (porous matrix). At low loading, most enzyme adsorbed on the layer far from membrane external surface (i.e. the furthest of spongy region which is near the membrane skin). As a result, the reaction in the bulk phase becomes dominant compared to the reaction at organic–aqueous interface. Hence, the enantioselectivity decreased because not all lipase contribute to the hydrolysis reaction [16].

6.7.2. Effect of enzyme loading on specific activity

In the present study, specific activity defined as the production rate per gram of immobilized protein (mmol/h g-protein). The specific activity of lipase was reported for optimal operation condition, i.e. at initial 35 mmol/L substrate concentration, 50 mmol/L phosphate buffer solution (pH 8), 40 kPa TMP and 40 °C reaction temperature. Fig. 16 compares the effect of enzyme loading specific activity between immobilized lipase on the spongy layer and inner lumen.

The specific activity was found to decrease with an increase in lipase loading on the membrane, from 26.5 mmol/h g-protein at 0.14 g/m² lipase loading to 2.0 mmol/h g-protein at 2.85 g/m² lipase loading for lipase immobilized on spongy layer. The specific activity dropped from 13.0 mmol/h g-protein at 0.09 g/m² lipase loading to 1.5 mmol/h g-protein at 2.36 g/m² lipase loading for lipase

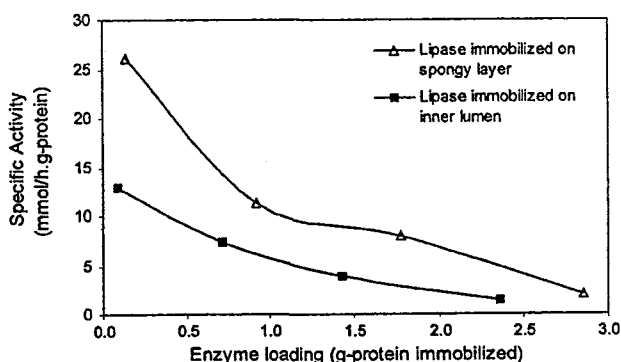


Fig. 16. Specific activity of immobilized lipase for hydrolysis reaction at different enzyme loading.

immobilized on inner lumen. As loading was increased, a great amount of lipase was aggregated throughout the fixed membrane space as a result of limited surface area for it to adsorb. Lipase in its aggregated form was less stable as its 'open-structure' was no longer available. These results agreed with predictions and observations reported in the literature [20,21,32]. Severe mass transfer limitations were observed at very high lipase loading as the specific activity dropped to a great extent. Mass transfer limitation might occur at high lipase loading due to lower D_{eff} (data not shown), causing the drop in specific activity to a greater extent. There might be pores resistance that decreased the mass transfer rate of the substrate molecules through the membrane when high enzyme concentration was used [14].

6.7.3. Comparison between simulated and experimental results

Enzyme loading is an important factor controlling the optical purity of (S)-acid and (R)-ester. A parameter that measures the enzyme loading is the Thiele modulus (Φ^2) presented in Eq. (21), which is a function of membrane porosity (α_p), K_m , V_{max} , effective diffusivity of substrate (D_{eff}) and thickness of spongy layer ($c-b$). V_{max} is an indicative of the amount of enzyme loading in the immobilization. The effective diffusion of substrate and product in the hollow fiber membrane was calculated based on methods outlined by Treybal [42] and Wu et al. [21]. Figs. 17 and 18 show the influence of (S)-ester conversion on ee_s and ee_p at different enzyme loading. The optical purity of less reactive (R)-ester increased with conversion whereas the optical purity of the desired (S)-acid decreased with conversion at fixed enzyme loading. The substrate conversion was proportional to Thiele modulus (Φ^2).

The conversion increased with increasing D_{eff} (lower Φ^2), due to the fact that the rate of product formation was diffusion-controlled for small value of D_{eff} and kinetics-controlled for large D_{eff} values. However, the overall ee_s values were declined with an increase in the value of Φ^2 . The same qualitative trend was also observed in the ee_p - Φ^2 relations. The drop in enantiomeric purity of the product (ee_p) might be due to mass transport limitations in the porous matrix. The

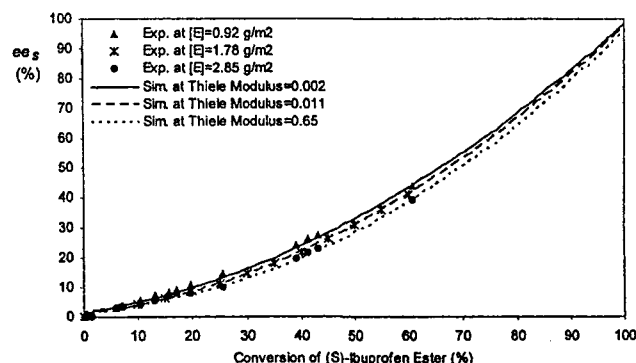


Fig. 17. Comparison between experimental and simulated results on the effect of Φ^2 on (S)-ester conversion and optical purity of less reactive (R)-ester.

product purity was consistent throughout the whole range of (S)-ester conversion. The figures show that the predicted ee_s and ee_p values (curves shown by Φ^2) were of the same order of magnitude as that obtained from the experiments (curves shown by enzyme loadings). The effect of enzyme loading on optical purity of racemate was found to be consistent with that reported in the literature [21,23,24].

The conversion played an important role in determining the optical purity of less reactive (R)-ibuprofen ester. Although substrate conversion increased with increasing enzyme loading but it was offset by reduced ee_s and ee_p values. In addition, the enzyme effectiveness factor dropped when enzyme loading was too high [41]. Both Figs. 17 and 18 could be used as a guide to select an appropriate enzyme loading so as to enhance ee_s and ee_p values while achieving satisfactory conversions.

6.7.4. Optical purity in relation to substrate and product resolutions

In this work, the enzyme shows enantioselectivity in catalyzing the more reactive (S)-ibuprofen ester to form the corresponding (S)-acid was produced. The substrate (racemic 2-ethoxyethyl-ibuprofen ester) was insoluble in water. The byproduct, 2-ethoxyethanol was miscible in both organic and

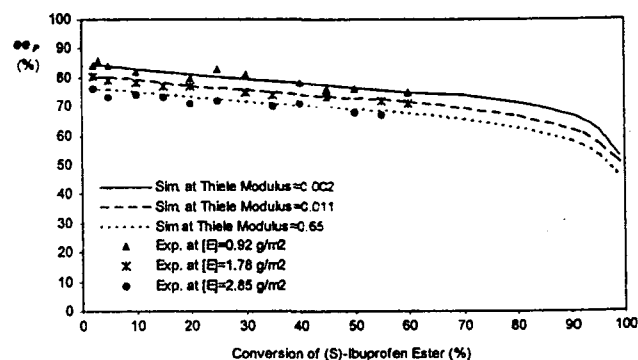


Fig. 18. Comparison between experimental and simulated results on the effect of Φ^2 on (S)-ester conversion and optical purity of (S)-acid product.

Table 4
Kinetic resolution of racemic naproxen and ibuprofen by *C. cylindracea* (or *C. rugosa*)

Type of drugs	Reaction	<i>T</i> (°C)	Conversion (%)	ee (%)	Reactor configuration	Immobilization method	Solvent/medium	Stability	Reference
Naproxen	Hydrolysis	–	25	ee _S > 95	Closed-loop column packed with Amberlite XAD-7, XAD-8 and Duolite ES762	Adsorption	2-Ethoxyethanol	1200 h operation at 35% with 20% loss of activity	[29]
Ibuprofen	Esterification	–	17–40	16 < ee _S < 75.3	Lipase immobilized on agarose, silica and alumina	Covalent binding	Isooctane	144–192 h operation	[43]
Ibuprofen	Esterification; hydrolysis	Room temperature	32; 41	ee _P = 100	AOT/isooctane microemulsion system	–	Sodium bis(2-ethylhexyl) sulfosuccinate (AOT)/isooctane	–	[44]
Naproxen	Esterification	37	48.3	ee _P = 74.4	Reaction in organic solvent media	–	Anhydrous isooctane	–	[45]
Ibuprofen; free fatty acid	Hydrolysis	25 and 40	–	–	Biphasic hollow fiber membrane reactor	Covalent binding	Toluene	No deactivation after 16 days	[13]
Ibuprofen	Esterification	–	1.6–46.5	–	Micro-aqueous organic medium	–	Ethyl ether, benzene, 2-heptanone, anisole, chloroform, toluene, butyl ether, carbon tetrachloride, <i>n</i> -hexane, cyclohexane, <i>n</i> -heptane, <i>n</i> -octane	Deactivation was not observed up to 200 h	[46]
Naproxen	Hydrolysis	30	–	ee _S = 92	Polyamide UF hollow fiber membrane reactor	–	Isooctane	Immobilized lipase showed stable activity after 200 h	[16]
Ibuprofen	Hydrolysis	40	31	ee _S = 31; ee _P = 85	PAN UF hollow fiber membrane reactor	–	Isooctane	EMR show stability after 180 h	Present study
Ibuprofen	Hydrolysis	20	–	ee _S = 96	Multiphase membrane reactor in batch mode	–	–	–	[19]

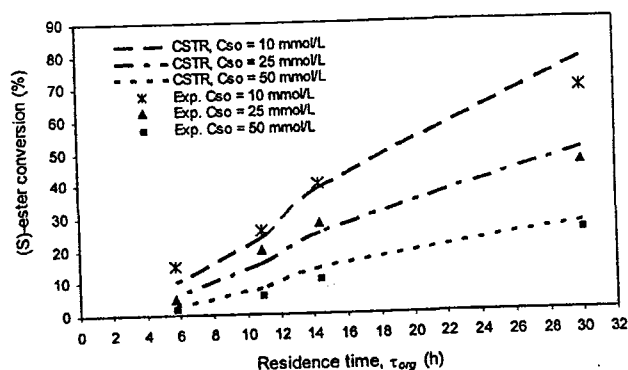


Fig. 19. Comparison between CSTR model equation and experimental data from reactor operated in CSTR mode (outer reactor system). All data were taken after 10 h of EMR operation.

aqueous phases. The optical purity of the (*S*)-acid product (*eep*) was achieved in the product recovery stream in the aqueous phase and the optical purity of the remaining less reactive (*R*)-ester (*ees*) was enhanced in the substrate recovery stream in organic phase. An *E* value of 13 was calculated from Eq. (3).

A high *ees* value is always associated with a low *eep* value [31]. It is desirable to choose a set of operating conditions in order to obtain a significant purity of desired product and the remaining substrate in order to make the kinetic resolution a success. The work reported by other investigators on the kinetic resolution of racemic ibuprofen esters and acids are presented in Table 4 for comparison with the present study. Table 4 summarizes optimum temperature, operational stability, optical purity values and conversion. It is clear from the table that the (*S*)-ester conversion and *ee* values reported in the present study were very promising for EMR process in kinetic resolution of racemic esters.

6.8. Comparison between EMR operation and CSTR model

The EMR system in the present work was operated as CSTR by continuous feeding of organic and aqueous streams. The substrate stream was continuously pumped into the reactor at the same time as the product stream was removed. The internal circuit of hollow fiber membrane reactor could be considered as a mixed flow reactor because the recirculation time through the fibers was more than an order of magnitude lower than the characteristic time for the hydrolysis in the reactor.

The performance of EMR in terms of (*S*)-ester conversion is plotted in Fig. 19 as a function of organic feed flow rate (25–200 mL/min) and (*S*)-ester concentration in the range of 10–50 mmol/L at 40 °C, pH 8, TMP 40 kPa and 250 mL/min aqueous flow rate treated as a CSTR. The dots depict the EMR experimental (*S*)-ester conversion and the lines represent conversion obtained from CSTR model equation in Eq. (19). The experimental conversions were consistent with that calcu-

lated by CSTR model equation with maximum difference of 10.3%. Higher (*S*)-ester conversion was achieved for reaction carried out at lower flow rate and (*S*)-ester concentration. A fairly low (*S*)-ester conversion was observed at 200 mL/min organic phase flow rate. The CSTR system was appropriate for enzymatic reaction involving soluble substrate or product, or cases where the product was inhibitory to the enzymatic reaction [7].

7. Conclusions

The kinetic resolution of racemic ibuprofen esters was carried out in a biphasic EMR system. *C. rugosa* exhibited higher enantioselectivity for (*S*)-ester while (*R*)-ester was less reactive. Enzyme immobilized on spongy layer exhibited better stability and yielded higher productivity. Satisfactory results were achieved in the kinetic resolution with an *E* value of 13, 31% *ees* and 85% *eep*. The maximum production rate of (*S*)-ibuprofen acid was 0.62 mmol/h at $\tau_{org} = 14.41$ h (50 mL/min) and that at $\tau_{aq} = 0.06$ min (450 mL/min) was 0.35 mmol/h. A working temperature of 40 °C was selected as a compromise between maximum productivity and stability. The diffusion of substrate and product to and from the enzyme–membrane interface (where the reaction took place) was rate-limiting.

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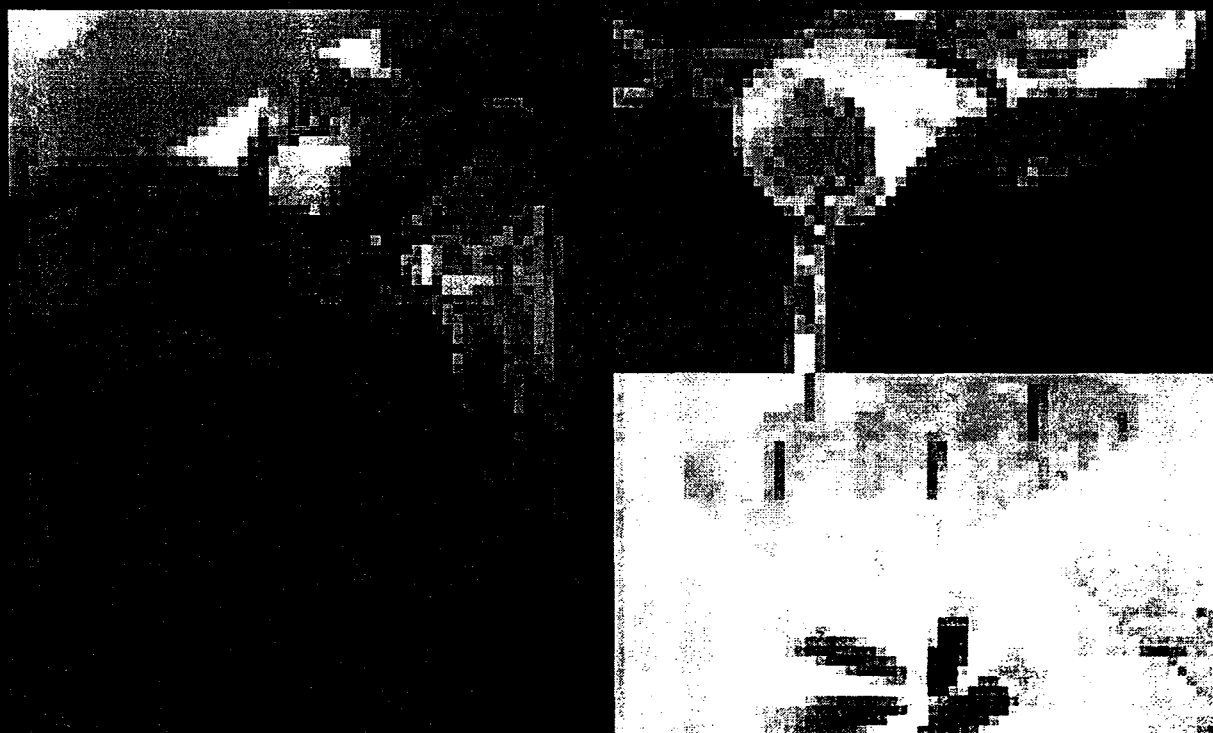
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Comparison of kinetic resolution between two racemic ibuprofen esters in an enzymic membrane reactor

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Abstract

The enantioselective hydrolysis of racemic esters and simultaneous separation of the corresponding optically pure (*S*)-acid as pure isomer is of considerable interest to the pharmaceutical industry as a route to non-steroidal anti-inflammatory drugs. In the present study, an enzymic membrane reactor (EMR) was employed for the optical resolution of racemic ibuprofen ester. EMR consisted of a lipase immobilized polymeric membrane, an organic phase dissolving ester and an aqueous phase to recover the reaction products. The catalytic behaviour of lipase immobilized in a polymeric hollow fibre membrane was investigated using 1-heptyl-ibuprofen ester and 2-ethoxyethyl-ibuprofen ester. The two ester substrates differ in the nature of the alkyl group of the alcohol moiety. The performance of the immobilized enzyme was studied as a function of temperature, pH of phosphate buffer solution and substrate flow rate. The operational conditions that favoured the enzyme selectivity for the (*S*)-ibuprofen esters, in order to obtain the corresponding (*S*)-ibuprofen acid as an optically pure single enantiomer, were identified. Lipase from *Candida rugosa* was used in the hydrolysis of racemic ibuprofen ester. (*R*)-Ibuprofen ester was found to be less reactive in the reaction. Highest enantioselectivity of enzyme was obtained with phosphate buffer solution of pH 8.0 at temperature of 40 °C and at lower substrate flow rate for both racemic 1-heptyl-ibuprofen and 2-ethoxyethyl-ibuprofen esters. The hydrolysis of racemic 1-heptyl-ibuprofen ester gave 4% *ee_S*, 90% *ee_P* and *E* value of 1–4, while the hydrolysis of 2-ethoxyethyl-ibuprofen ester resulted in 31% *ee_S*, 85% *ee_P* and *E* value of 9.5–13 by running the experiment under optimum operating condition in an enzymatic membrane reactor.

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Keywords: Membrane reactor; Ibuprofen; *Candida rugosa*; Hydrolysis; Enantioselectivity; 1-Heptyl-ibuprofen ester; 2-Ethoxyethyl-ibuprofen ester

1. Introduction

Ibuprofen, 2-(4-isobutylphenyl) propionic acid, constitutes the largest single group of drugs used in the treatment of rheumatoid arthritis and as general analgesics, which is a widely used for headache and minor pains [1]. Resolution of racemic ibuprofen by *Candida rugosa* lipase can be achieved through stereospecific esterification in non-aqueous media, while in aqueous media this approach is extended to stereospecific hydrolysis of the corresponding esters. The two enantiomers of racemic ibuprofen show physiologically different behaviour, the (*S*)-enantiomer form exhibiting an anti-inflammatory property [2]. This drug is a chiral

molecule and was originally marketed as a racemate. The development and administration of (*S*)-ibuprofen was later in progress when the (*S*)-enantiomer of ibuprofen proved to be 100 times medically more active than the (*R*)-form [3]. The pharmacological activity of this chiral drug is primarily shown by the corresponding (*S*)-enantiomer. The (*R*)-enantiomer is the unwanted enantiomer as it is often the cause of side effects or toxicity. Pharmacological studies have indicated that gastrointestinal problems are the most frequent side effects associated with profens consumption [4]. Much emphasis has been therefore laid on the production of the useful (*S*)-enantiomer in pure form.

Kinetic resolution using enantioselective catalysts (enzymes) has been reported in the literature [5–10]. This process is a practical method for producing single enantiomers of chiral drugs because less energy is needed

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and is a comparatively cheaper process. In the production of chiral drugs by enzymic hydrolysis reaction, the racemic mixture undergoes a kinetic resolution in the presence of an enantioselective enzyme, i.e. lipase from *C. rugosa*. The kinetic resolution in this work is based on substrate selectivity since the lipase is enantioselective for only one of the two substrates (i.e. (*R*)- and (*S*)-enantiomers) present in a racemic mixture. The two enantiomers of a racemate react at different rates with the chiral catalyst (enzyme), in which the enzyme shows a greater stereopreference towards one of the two enantiomers. An example is the kinetic resolution of racemic ibuprofen ester via enzymic hydrolysis reaction as shown in Fig. 1.

Kinetic resolution occurs when k_R is not equivalent to k_S . In this work, the (*S*)-enantiomer of ibuprofen acid has a higher reaction rate than the (*R*)-enantiomer ($k_S \gg k_R$). The pure product (*S*)-ibuprofen acid is transported through the membrane pores into the aqueous phase because the product (*S*)-ibuprofen acid has higher water solubility than (*R*)-ibuprofen acid.

The application of membrane technology in the fine chemical and pharmaceutical industry is of worldwide research interest [11,12]. Enzymic membrane reactors (EMR) comprise a combination of enzymic reaction, substrate recovery and product separation in one unit. The enzymic membrane reactor system consists of a polymeric hollow fibre membrane and the immobilized enzyme as catalyst. The membrane is used as separation medium and immobilization support to retain the enzyme. The hollow fibre configuration is widely used with enzyme immobilized on the membrane [11–15]. Immobilized enzymes are confined in a well-defined region, e.g. the spongy region. This configuration is more advantageous because of its longer contact time of reactants with the enzymes due to its high surface to volume ratio that permits high enzyme density in a small reaction volume. The reaction media in the enzymic membrane reactor can be non-aqueous (organic), two-liquid phases (organic-aqueous) or multiphase (enzymic reaction in an aqueous-organic medium with the enzyme present as the solid form). An added advantage of membrane reactor application is the reusability of the membrane by removal of inactivated enzymes and replacement with new enzyme.

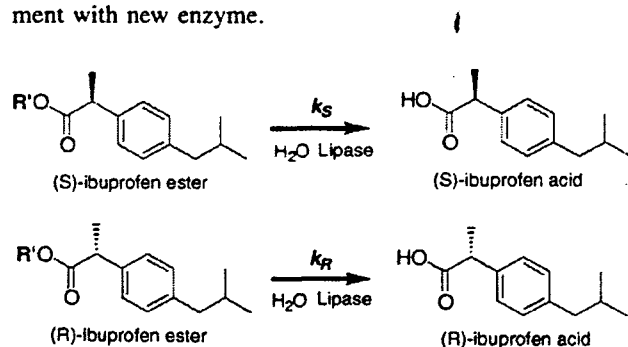


Fig. 1. Kinetic resolution of racemic ibuprofen ester, via lipase catalyzed hydrolysis reaction, R' designates an alkyl group.

The objectives of the present work are to compare the kinetic resolution of two different ibuprofen esters, viz. 1-heptyl-ibuprofen ester and 2-ethoxyethyl-ibuprofen ester and to identify the optimum operating conditions favouring the enzyme stereoselectivity to obtain the optically pure single enantiomer, i.e. the (*S*)-ibuprofen acid.

2. Biphasic organic/aqueous catalytic membrane reactor

The biphasic catalytic membrane reactor is a two-separated phase membrane reactor. The membrane separates the two immiscible phases with the two phases flowing along two separate circuits of the membrane module. The organic phase was recirculated in the shell side of the membrane, while buffer solution flowed in the lumen side. Fig. 2 shows the interaction of the enzyme–substrate (organic)-aqueous in the membrane spongy region with enzymic hydrolysis reaction taking place in the membrane. Substrate or racemic mixture in the organic phase partitioned from the shell side and reacted with immobilized enzyme in the membrane where the hydrolysis reaction took place and the product (*S*)-ibuprofen acid was extracted into aqueous phase as shown in Fig. 3. Bioconversion and separation of chiral compounds occur simultaneously, with the enantioselective enzyme as a chiral system and the membrane as a barrier that separates the converted enantiomer. The organic substrate could not pass through the membrane due to non-polarity and insolubility of the substrate as well as hydrophilicity of the membrane. The membrane is wetted by the aqueous phase because the membrane material is hydrophilic. The (*S*)-ibuprofen acid product is water-soluble and freely passed through the membrane into the aqueous phase in the lumen.

3. Experimental procedures

3.1. Materials and chemicals

Lipase from *C. rugosa* EC 3.1.1.3 (Type VII, 724 units per mg solids using olive oil as substrate) was supplied by Sigma–Aldrich (MI). (*R,S*)-ibuprofen acid 99% and (*S*)-ibuprofen acid 99% were purchased from Acros (Belgium). Other chemicals and reagents used were of analytical grade. 2-ethoxyethanol 99% and isooctane 99% were supplied by Acros (Belgium). 1-Heptanol 99 % was supplied from Merck (Germany).

3.2. Chemical synthesis of racemic ibuprofen ester

(*R,S*)-Ibuprofen acid (10 g, 0.048 mol), *p*-toluenesulphonic acid (0.2 g, 1.11 mmol) and 1-heptanol (5.57 g, 0.096 mol, 13.5 ml) were dissolved in 88.5 ml of isooctane. The solution was refluxed for 8 h using a Dean and Stark

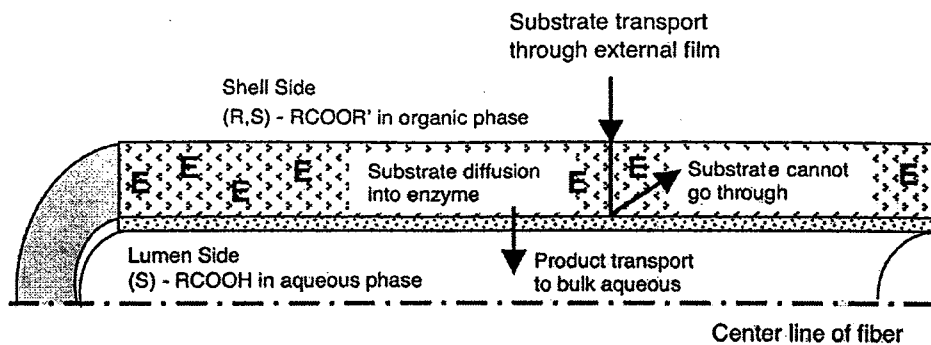


Fig. 2. Cross-section of hollow fiber membrane portraying the diffusion of substrate and product at the vicinity of organic-aqueous membrane interface.

apparatus. The reaction mixture was cooled at room temperature and washed with 5% sodium hydroxide to dissolve *p*-toluenesulfonic acid and followed by washing with water for removing impurities. The organic layer was dried using sodium sulfate. The residual from the heating flask was further purified by micro-distillation to remove impurities, giving (R,S)-1-heptyl-ibuprofen ester. The chemical synthesis for the esterification of 2-ethoxyethyl-ibuprofen ester was carried out using the same method with 2-ethoxyethanol as alcohol.

3.3. Preparation of phosphate buffer solution

Phosphate buffer solution was prepared by mixing 50 mM sodium dihydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) with 50 mM disodium hydrogen phosphate (KH_2PO_4) at an appropriate ratio to give the desired pH. The pH was adjusted by adding KH_2PO_4 to decrease the pH or by adding $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ to increase the pH.

3.4. Preparation of lipase solution

2 g/L of *C. rugosa* lipase solution was prepared using 2 g of lipase powder dissolved in 1 L of 50 mM phosphate buffer solution at desired pH value and the solution was gently stirred by magnetic stirrer for 2 h. The solution was centrifuged at 3000 rpm for 5 min to remove the insoluble substances.

3.5. Enzyme immobilization

The lipase was immobilized into the spongy layer of the membrane by cross flow filtration of the lipase solution across the membranes from the shell side to the fiber lumen at constant flow rate of 300 ml/min at transmembrane pressure of 35 kPa. After immobilization, the capillary membranes were washed by circulation of 50 mM phosphate buffer at desired pH value from the shell side to the fiber lumen for 20 min. In such a way, the lipase was physically

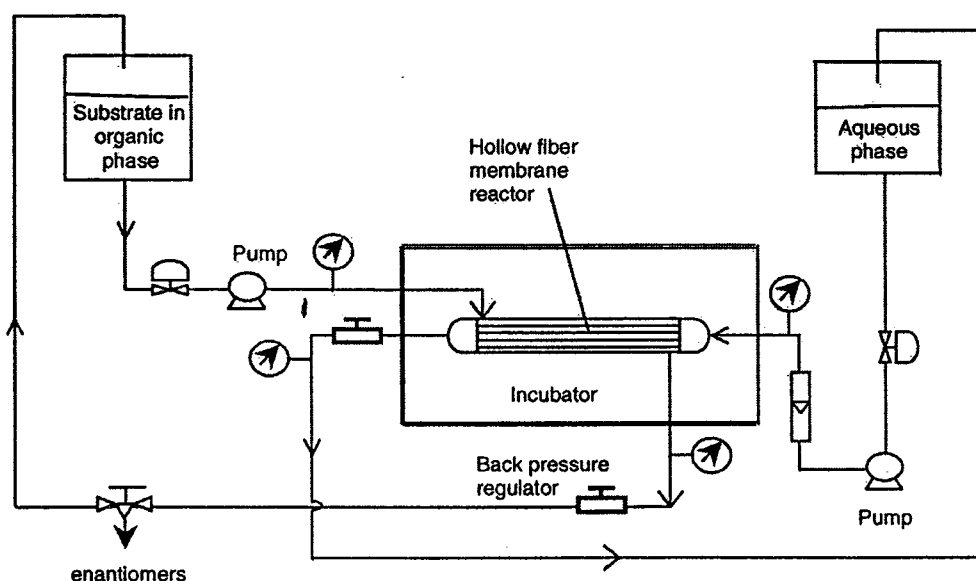


Fig. 3. Schematic diagram of EMR set up with hollow fiber membrane module kept in an incubator, the shell side loop was connected to an organic tank and lumen loop connected to an aqueous tank.

immobilized on the spongy layer of the shell side. The protein concentrations were measured by BCA Protein Assay Reagent Kit. The amount of protein immobilized on the membrane was calculated from the difference of protein concentration before and after immobilization.

3.6. Enzymic membrane reactor operation

A Koch ultrafiltration membrane model HF1.0-45-XM50 (MWCO 50 kDa, Koch Membrane Inc., USA) made of polyacrylonitrile material (PAN) was employed. The membrane is asymmetrical and hydrophilic. In the EMR operation, 350 ml of 25 mM (*R,S*)-ibuprofen ester in isooctane was circulated in the shell side and 400 ml of 50 mM phosphate buffer was circulated in lumen side. Flow rates in both loops (shell and lumen) were maintained at 80 ml/min and 250 ml/min, respectively. Transmembrane pressure (TMP) from organic to aqueous phase was maintained at 40 kPa to avoid the filtration of aqueous solution into the organic phase. The pH of phosphate buffer solution used in aqueous phase was identical to the pH value used for the immobilization. A small amount of sample was collected at appropriate interval, and the concentrations of products were measured. Fig. 3 shows the schematic diagram for the enzymatic hydrolysis reaction taking place in the EMR system.

3.7. Analysis of ibuprofen ester concentration in the organic phase

Chiral analysis of both enantiomers and the optical purity of ibuprofen were conducted by chiral HPLC analysis using a (*R,R*)-Whelk-01 chiral column (Regis Pirkle, USA) with the mobile phase comprised of hexane:2-propanol:trifluoroacetic acid (98:2:0.5 v/v) at a flow rate of 1.3 ml/min. UV detector with wavelength set at 254 nm was used and injection volume was 10 μ l. The mode of HPLC separation is normal phase.

3.8. Analytical procedures

The concentrations of racemic ibuprofen ester and acid were analyzed by HPLC (Shimadzu Model 10VP, Japan). The (*S*)-enantiomer was eluted faster than the (*R*)-enantiomer on this column. A calibration curve of chromatographic peak area versus ibuprofen concentration was obtained from the data of standard solutions. The conversion of the substrate in the enzymic reaction was measured from the decrease of the instantaneous peak area in comparison to the starting peak area.

4. Calculation of optical purity and enantiomeric ratio

The efficiency of kinetic resolution was evaluated based on the optical purity of the desired product, expressed in

terms of enantiomeric excess of the product (*ee_P*) and on the optical purity of the remaining substrate, expressed in terms of enantiomeric excess of the remaining less reactive substrate (*ee_S*), referred to the (*R*)-form. Racemate optical purity can be calculated from the molar fraction of each enantiomer using the following equations:

$$ee_S = \frac{C_R - C_S}{C_R + C_S} \quad (1)$$

$$ee_P = \frac{P_S - P_R}{P_S + P_R} \quad (2)$$

C and *P* indicate the concentration of substrate and product, respectively. The subscripts *R* and *S* represent the corresponding (*R*)- and (*S*)-enantiomer in the mixture, respectively.

The enantiomeric ratio (*E*) is also a measure of the efficiency of a kinetic resolution. It characterizes the enantioselectivity of a particular enzyme and is defined as the ratio of pseudo first order rate constants for the two enantiomers [16]: that is, for a reaction in which the (*S*)-enantiomer selectively reacts $E_S = k_S/k_R$. The enantiomeric ratio (*E*) of a racemic mixture was calculated from the degree of conversion (*X*) and the enantiomeric excess of the remaining substrate or product (*ee*) by the following equations:

$$E = \frac{\ln[(1-X)(1-ee_S)]}{\ln[(1-X)(1+ee_S)]} \quad (3)$$

$$E = \frac{\ln[1-X(1+ee_P)]}{\ln[1-X(1-ee_P)]} \quad (4)$$

X is the overall conversion for the racemic substrate calculated from Eq. (5):

$$X = \frac{C_0 - C_S}{C_0} \quad (5)$$

C₀ is the initial concentration of (*R,S*)-2-ethoxyethyl-ibuprofen ester and *C_S* is the concentration of (*S*)-2-ethoxyethyl-ibuprofen ester. In cases where one-substrate and one-product was applied in the enzymatic reaction, only the more reactive (*S*)-ibuprofen ester was considered and its conversion, *X_S* is calculated from Eq. (6).

$$X_S = \frac{C_{S0} - C_S}{C_{S0}} \quad (6)$$

C_{S0} is the initial concentration of (*S*)-ibuprofen ester. When the degree of conversion is low, the enantiomeric ratio is approximated as [17]:

$$E = \frac{[1 + ee_P]}{[1 - ee_P]} = \frac{P_S}{P_R} \quad (7)$$

P_S and *P_R* are the concentrations of (*S*)-isomer and (*R*)-isomer in the product, respectively.

Table 1
Kinetics parameters for different substrate (reaction occurred at 40 °C and pH 8)

Substrate	$V_{\max}$ (mmol/L h)	K_m (mmol/L)
2-Ethoxyethyl-ibuprofen ester	3.27	36.47
1-Heptyl-ibuprofen ester	0.7	85.29

5. Result and discussion

5.1. Enzyme kinetic parameters

$V_{\max}$ is the maximal reaction rate possible if every enzyme molecule present is saturated with substrate and is a property of the particular enzyme. The Michaelis constant (K_m) is defined as the substrate concentration for which the observed reaction rate is half of $V_{\max}$. This K_m value characterizes the affinity between substrate and enzyme. Table 1 shows the kinetic parameters for the hydrolysis reactions of 1-heptyl-ibuprofen ester and 2-ethoxyethyl-ibuprofen ester. Both the esters of ibuprofen are hydrolyzed stereoselectively by *C. rugosa* lipase. The kinetic parameters for the EMR process with 2-ethoxyethyl-ibuprofen ester as substrate are $V_{\max} = 3.27$ mmol/L-h and $K_m = 36.47$ mmol/L at reaction temperature of 40 °C and buffer solution of pH 8 with 1.78 g/m of lipase immobilized at the spongy layer of the PAN membrane. The kinetic values for 1-heptyl-ibuprofen ester as substrate are $V_{\max} = 0.7$ mmol/L h and $K_m = 85.29$ mmol/L. Enzyme-substrate molecular recognition is a critical step in enzyme-catalyzed reactions. The efficiency of the molecular recognition is defined by the absolute configuration at the chiral center of the substrate [18]. The 2-ethoxyethyl-ibuprofen ester was seen to be a

better substrate for the kinetic resolution process. A possible reason for this is the use of activated ester (2-ethoxyethyl is more electronegative than the long-chain 1-heptyl and thus it is more reactive) might increase the lipase stereoselectivity and hence its affinity towards the chiral substrate. The *C. rugosa* lipase exhibited a relatively higher stereoselectivity towards 2-ethoxyethyl-ibuprofen ester in the kinetic resolution with the E value in the range of 9.5–13, while for the case of racemic 1-heptyl-ibuprofen ester as the substrate, the E value ranged from 1 to 4. The respective chemical structures of the two esters are given in Fig. 4. The use of 2-ethoxyethyl as the acyl group of naproxen has also been cited in the literature in which the water insoluble 2-ethoxyethanol-naproxen ester was found to be readily and selectively hydrolyzed by *C. cylindracea* lipase [19].

The kinetic study of the hydrolysis reaction was carried out in a preliminary study which showed the enzyme kinetics follow the Michaelis-Menten model given by Eq. (8) when inhibition was absent, which was validated when $[C_S] \leq 1.5 K_m$ (data not shown):

$$v = \frac{V_{\max} C_S}{C_S + K_m} \quad (8)$$

v is the hydrolysis reaction rate with the unit of mmol/L h.

5.2. Effect of temperature

The effect of temperature on kinetic resolution of ibuprofen ester was studied under the same flow rate, constant amount of immobilized lipase and phosphate buffer solution fixed at pH 8. The different temperatures studied were 30, 35, 40 and 45 °C. Fig. 5 shows the effect of reaction

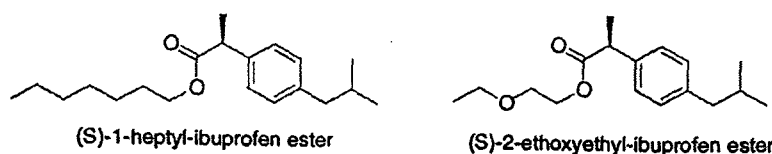


Fig. 4. Chemical structures of (S)-1-heptyl-ibuprofen ester and (S)-2-ethoxyethyl-ibuprofen ester.

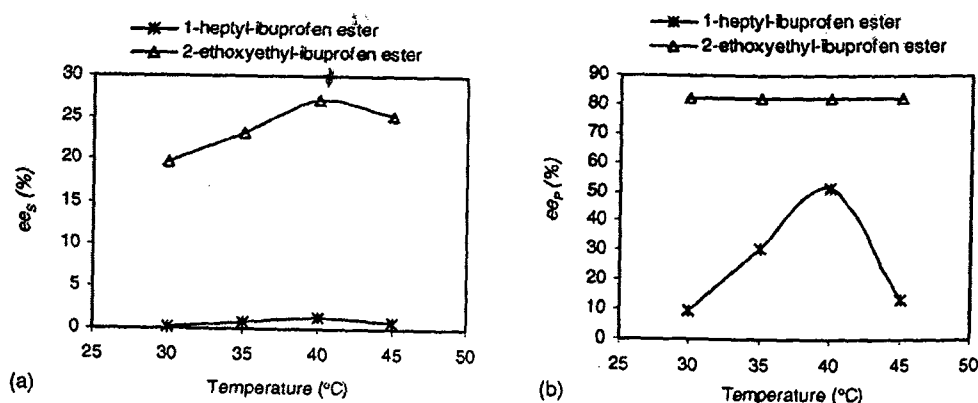


Fig. 5. Effect of operating temperature for hydrolysis reaction of: (a) 1-heptyl-ibuprofen ester and (b) 2-ethoxyethyl-ibuprofen ester carried out at 250 ml/min aqueous phase flow rate, 80 ml/min organic phase flow rate and pH 8.

temperature on enantiomeric excess of the remaining substrate (ee_S) and the pure product (ee_P). The ee_S value for the hydrolysis of 2-ethoxyethyl-ibuprofen ester increased from 19 to 27% with increasing temperature from 30 to 40 °C but it dropped at 45 °C. The ee_S values for the kinetic resolution of (*R,S*)-1-heptyl ibuprofen ester were fairly low, which were $\leq 3\%$ ee_S . In fact, lipases are capable of catalyzing the hydrolysis of both of the optical antipodes (enantiomers) of their substrates, but at different rates [20]. The difference in the stereoselectivity and ee_S value between the two esters reflects the individual susceptibility of the two substrates to undergo different conformational changes and interfacial binding efficiency with *C. rugosa* lipase [18]. Fig. 5(b) shows the effect of reaction temperature on the ee_P values for the kinetic resolution of the two esters. The hydrolysis product of racemic 1-heptyl-ibuprofen ester was highest in its ee_P (55% ee_P) at 40 °C but much lower at other temperatures. On the other hand, the ee_P of the hydrolysis product of racemic 2-ethoxyethyl-ibuprofen ester was observed to be consistent with about 82% ee_P obtainable at all the temperatures under study. The (*S*)-form of 2-ethoxyethyl-ibuprofen ester was seen to be better recognized by *C. rugosa* lipase at its molecular level as compared to the (*S*)-form of 1-heptyl-ibuprofen ester. The ester group of 2-ethoxyethyl-ibuprofen ester and its acyl group (2-ethoxyethyl) in position 2 (chiral carbon) may fit a more specific pocket within the molecular recognition site than that possessed by 1-heptyl ibuprofen ester. Fig. 6 shows the membrane productivity as a function of reaction temperature. The membrane productivity for 2-ethoxyethyl-ibuprofen ester as substrate falls in the range of 0.7 and 0.82 g-product/h m². The hydrolysis of 1-heptyl-ibuprofen ester gave relatively low membrane productivity which ranges from 0.05 to 1.5 g-product/h m², compared to that exhibited by 2-ethoxyethyl-ibuprofen ester. An increase in operating temperature increased the solubility of substrate and thus assisted the diffusion of substrate to enzyme immobilized on membrane where the reaction took place. However, further increase in temperature up to 45 °C

resulted in a drop in membrane productivity. This could be due to enzyme deactivation which caused a poorer resolution. There is a certain temperature range for lipase at which the conformation of the enzyme is optimal. The optimal temperature of the membrane reactor should be determined carefully since it has a dualistic effect. Enzyme is inactive and shows low reaction rate at low temperature. With increasing temperature, the mobility of substrate molecule to the lipase active site increased, the lipase activity increased proportionally.

5.3. Effect of pH

Enzymes are polyelectrolytes containing both positive and negative ionizations of amino acid at the surface, which may be protonated or unprotonated depending on the pH of the surrounding medium. The effect of pH in the water phase localized around the enzyme is an important study. However, the effect of pH on the enantioselectivity of enzymes has been rarely reported. In this work, the phosphate buffer solution served two functions: (1) providing a medium surrounding the lipase as to regulate its charge distribution; (2) assisting the mass transport or flux of the polar (*S*)-acid from the enzyme–substrate–membrane reaction layer into the aqueous stream. The pH value of phosphate buffer solution used for hydrolysis reaction in the membrane reactor was the same as that used for lipase immobilization in order to remain the identical microenvironment at the vicinity of the lipase. There is a pH range for lipase at which the conformation of the enzyme as catalyst is optimal, while extreme pH conditions will cause an irreversible inactivation of the enzyme. Three different pH values studied at constant temperature 40 °C were 6, 7 and 8.

As indicated in Fig. 7, the ee_S value increased with increasing pH for both substrates. The ee_P value for 2-ethoxyethyl-ibuprofen ester seems to be independent of pH as a constant value of 82% was observed throughout the pH range (6.0–8.0). This study has shown that pH greatly influenced the enantioselectivity of lipase, which probably resulted from conformational changes of lipase against pH variation.

Buffer solution with pH 8 results in highest optical purity (both ee_S and ee_P) and enantioselectivity of lipase enzyme. This is due to the fact that ibuprofen acid was dissociated and dissolved more easily in aqueous solution at alkaline pH. Phosphate buffer solution with higher pH assists the mass transport of the (*S*)-acid from the enzyme–substrate reaction place into the aqueous stream more easily. Fig. 8 compares the results of membrane productivity shown by the two esters. The more basic the phosphate buffer solution, the higher the productivity exhibited by 2-ethoxyethyl-ibuprofen ester, with the highest membrane productivity value obtained at pH 8.0, i.e. 1.6 g-product/h m². However, these values were too low for the case of 1-heptyl-ibuprofen ester.

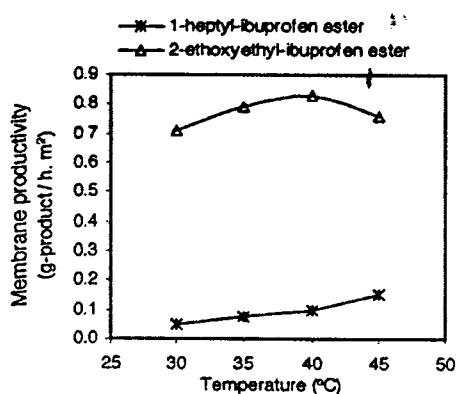


Fig. 6. Comparison of membrane productivity for two different acyl groups of ibuprofen ester as a function of reaction temperature.

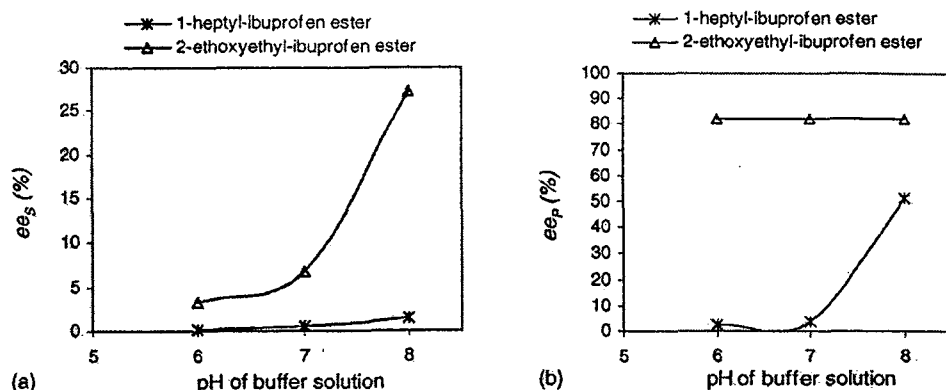


Fig. 7. Effect of operating pH on optical purity for hydrolysis reaction of: (a) 1-heptyl-ibuprofen ester and (b) 2-ethoxyethyl-ibuprofen ester carried out at 250 ml/min aqueous phase flow rate, 80 ml/min organic phase flow rate and 40 °C.

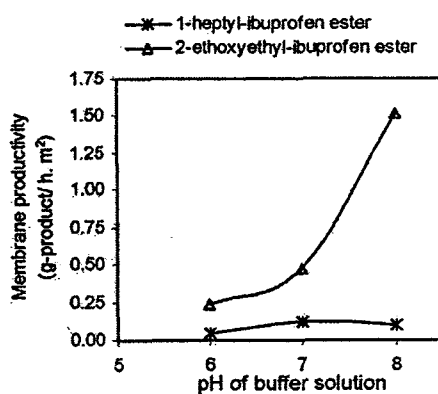


Fig. 8. Effect of pH on membrane productivity for two different acyl groups of ibuprofen ester.

5.4. Effect of organic phase flow rate

The performance of the EMR operation was investigated as a function of organic phase flow rate in hydrolysis reaction for 1-heptyl-ibuprofen ester and 2-ethoxyethanol-ibuprofen ester. Enzyme-loaded membrane separates the two immiscible phases by recirculating the two phases along the two separate circuits of the membrane module. The organic stream (substrate) was recirculated in the circuit in contact with the lipase and phosphate buffer solution flowed on the other side. The organic flow rate was in the range of 100, 180, 250 ml/min for hydrolysis reaction using 1-heptyl ibuprofen ester; and 50, 80 and 200 ml/min for

2-ethoxyethyl-ibuprofen ester at fixed aqueous flow rate of 250 ml/min. Experiments were carried out at 40 °C and pH 8.0 phosphate buffer solution. Table 2 shows the performance of EMR for hydrolysis reaction in terms of membrane productivity, ee_S and ee_P at different organic phase flow rate.

It was observed that lower organic flow rates yielded higher membrane productivity for both the esters, with 4% ee_S and 90% ee_P shown by 1-heptyl-ibuprofen ester while 31% ee_S and 85% ee_P given by 2-ethoxyethyl-ibuprofen ester at 100 ml/min organic phase flow rate. Lower flow rate caused longer retention time of the substrate molecule at the enzyme microenvironment (immobilized enzyme on membrane surface). This would also lengthen the enzyme–substrate contacting time and create higher hydrolysis activity. Higher ee_S and ee_P values were associated with higher productivity at lower flow rates. This was probably due to diffusive transport limitation of the substrate molecule in the direction tangential to the membrane at increased organic flow rates [21], which resulted in poor resolution. Generally, 2-ethoxyethyl-ibuprofen ester showed higher optical purity in terms of the desired pure product (ee_P) and the remaining less reactive substrate (ee_S) that that exhibited by 1-heptyl-ibuprofen ester. It is reported in the literature that alkyl esters containing an electron-withdrawing group such as chloroethyl, carboethoxyl and cyanomethyl are hydrolyzed more readily than those are not substituted [19].

The EMR with high recirculation of buffer solution and organic phase in the lumen and shell loop respectively, can be treated as a batch-stirred tank reactor (BSTR) as shown

Table 2
Effect of substrate phase flow rate on membrane productivity, ee_S and ee_P values for hydrolysis reaction of 1-heptyl-ibuprofen ester and 2-ethoxyethyl-ibuprofen ester

1-heptyl-ibuprofen ester				2-ethoxyethyl-ibuprofen ester			
Organic phase flow rate	Membrane productivity	ee_S (%)	ee_P (%)	Substrate phase flow rate	Membrane productivity	ee_S (%)	ee_P (%)
100	0.91	4	90	50	4.31	31	85
180	0.47	1.4	52	80	3.55	25	83
250	0.03	0.07	46	200	2.26	14	80

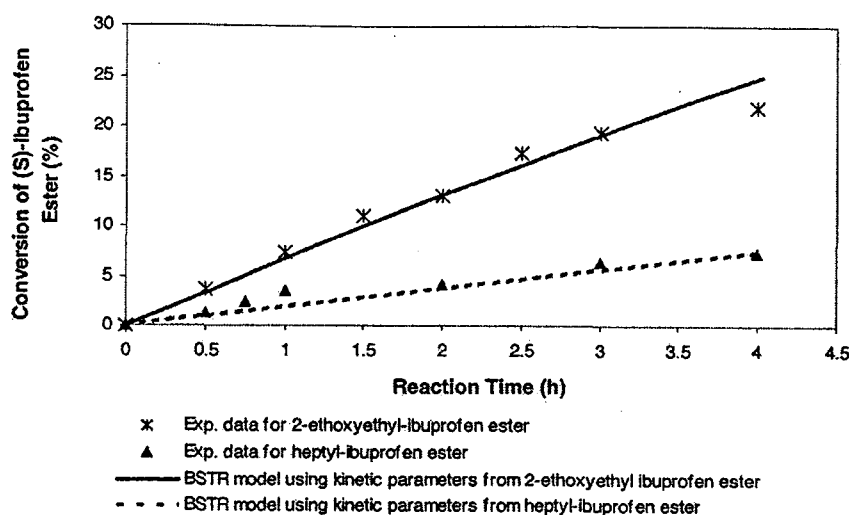


Fig. 9. Time-course conversion of hydrolysis reaction for two different esters: experimental data and BSTR model.

from the schematic diagram given in Fig. 3. The time-course conversion of a batch reaction can be calculated using the expression as follows [22]:

$$t = - \int_{C_{S0}}^{C_S} \frac{dC_S}{-v} \quad (9)$$

t is the time-course conversion of the hydrolysis reaction in the EMR; v is the enzymatic reaction given in Eq. (8) in which the respective kinetic parameters for the two esters substrate are presented in Table 1. Fig. 9 shows the plot of the time-course conversion of (S)-ester (X_S) with points designating the experimental data and lines showing the predictions calculated from the BSTR model given in Eq. (9). The experimental data for both the esters were found to be in agreement with the values predicted from BSTR model.

6. Conclusions

An experimental analysis of optical resolution of racemic 1-heptyl-ibuprofen ester and 2-ethoxyethyl-ibuprofen ester in lipase immobilized membrane reactor in biphasic configuration at various conditions has been carried out. It was found that lipase from *C. rugosa* has a high selectivity for (S)-ibuprofen ester in the hydrolysis of racemic ibuprofen ester. The performance of the immobilized enzyme was studied as a function of temperature, pH of phosphate buffer solution and substrate flow rate. The present work aimed at identifying the conditions that favored the enzyme selectivity for the (S)-ibuprofen ester, in order to obtain the corresponding (S)-ibuprofen acid as a pure isomer. It was observed that highest enantioselectivity of enzyme was obtained with phosphate buffer solution of pH 8.0 at temperature of 40 °C and at lower substrate flow

rate for both racemic 1-heptyl-ibuprofen ester (i.e. 100 ml/min) and 2-ethoxyethyl-ibuprofen ester (i.e. 50 ml/min). 2-ethoxyethyl-ibuprofen ester shows higher activity and enantioselectivity than 1-heptyl-ibuprofen ester due to the fact that selectivity decreased with the increasing aliphatic chain of the alcohol.

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BPE-39

Enzymatic synthesis of citronellyl laurate with immobilized *Candida rugosa* lipase in organic media

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Abstract

Terpene esters of fatty acid can be used in many applications especially in food, pharmaceutical and cosmetic industries as flavor and fragrance compounds. The *Candida rugosa* lipase immobilized on Amberlite MB-1 resin was investigated for its use in the synthesis of citronellyl laurate by direct esterification in organic media. The effect of various reaction parameters such as effect of catalyst loading, temperature effect and type of solvent on the progress of the esterification reaction have been studied. The optimal conversion >90% of citronellyl laurate was obtained from direct esterification between lauric acid and citronellol at reaction temperature of 37°C and iso-octane as the reaction medium.

Keywords: Terpene ester, *Candida rugosa*, Flavors and Fragrances.

Introduction

Most of the flavor compounds are currently provided by traditional methods such as chemical synthesis or extraction from natural sources. Due to the great demand for natural products, this factor has pushed the flavor industries to seek new methods to obtain flavor compounds naturally. The use of biotechnology through direct biosynthesis by fermentation has been described as potential source of ester [1].

However, the esters concentration and productivities obtained using fermentation are rather low. A new possibility to produce natural esters was opened by enzyme catalysis carried out in non-aqueous organic solvents or in free-solvent reaction medium [2]. Direct esterification and transesterification reactions have been performed using lipases to produce, tailored triglycerides and numerous flavor esters [3].

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and industrial potential. Lipases catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. Enzymes such as lipases accept a broad range of substrates and they are readily available at low cost and require no cofactor for reaction. [4]. Both native and immobilized lipases are available commercially. Crude lipases are used in laundry detergents and many other applications; however, an increasing number of specialty applications of lipases in synthesis and biotransformation demand an immobilized biocatalyst for efficiency of use.

Beside that, immobilization improves recyclability of expensive lipases. Also, immobilization can enhance enzyme stability and activity [5]. Additionally, immobilization of lipase has many advantages: when enzymes are present in solution they will leave the reactor with end products and this means that we will have to separate from end products and new enzymes used in the next process: immobilized enzyme can be placed near other enzymes and this way, a catalytic sequence is produced [6].

Yadav and Lathi, 2004 have reported that the synthesis of citronellyl laurate from citronellol and lauric acid using various immobilized lipases. The results showed that Novozym SP 435 was found to be the best catalyst with 53% conversion and 100% selectivity in 2 hour at 30°C. Furthermore, it was found to be an ordered bi-bi mechanism with dead-end complex of lauric acid to represent the kinetic data. The experiment was conducted using fully baffled mechanically agitated glass reactor of 50 ml capacity, which was immersed in a thermostatic water bath.

This paper will elaborate on the esterification of citronellyl laurate as seen in Figure 1 in batch mode using immobilized *Candida rugosa* lipase. Several parameters will be discussed briefly that includes effects of catalyst loading, effect of types of solvent and effect of temperature.

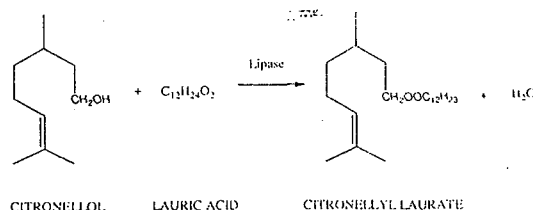


Figure 1: Schematic diagram: esterification of citronellyl laurate

Approach and Methods

Materials

Lauric acid was purchased from Fluka (Switzerland) with the purities of 98% and DL-Citronellol (95% pure) from Sigma Aldrich (St. Louis MO USA), lipase (EC 3.1.1.3) from *Candida rugosa* (Type VII) was purchased from Sigma Aldrich (Japan), Isooctane (99.84% assay) from Fischer Chemicals (UK). All chemicals are analytical grade.

Lipase Immobilization

Lipase from *Candida rugosa* (E.C.3.1.1.3) was immobilized by adsorption onto Amberlite MB-1 support. In aqueous environment, the enzyme (20 mg) was dissolved in 5 ml sodium phosphate buffer (pH 7.5) and mixed with 2 gram of the support and stirred at 200 rpm for 24 hr at 37°C. Then, the immobilized lipase preparation was filtered and washed thoroughly with deionized water and rinsed with sodium phosphate buffer solution. The resultant immobilized lipase was then dried in a vacuum oven at room temperature.

Esterification method

Esterification was carried out in 10 ml screw-capped bottle. 30mM of DL-Citronellol were added to 20mM of lauric acid followed by different amounts of immobilized lipase. The mixture was stirred in an orbital shaker (200 rpm) at 37°C and sample was taken for analysis after 24 hr of reaction time. Samples were analyzed by gas chromatography.

Analysis

The concentrations of the reactants and products were determined on a gas chromatography (Perkin Elmer Autosystem XL, USA) equipped with a flame ionization detector (FID). The column was a Nukol™ fused silica capillary column with dimension 0.53 mm i.d. x 15 m x 0.50 µm film thicknesses (Supelco, USA). Consumption of the acid in the reaction was calculated by comparing the gas chromatography analysis of the sample obtained with the one at the commencement of the reaction.

Results and Discussion

Effect of catalyst loading

The catalyst loading effect was studied by varying catalyst loading from 5 g/L to 100 g/L and keeping all other parameters constant. The experiment were carried out in iso-octane at a fixed substrate concentration of 20 mM lauric acid and 30 mM citronellol, at a temperature of 37°C with shaking rate of 200 rpm for 24 hours. It was found that the percentage of citronellyl laurate produced increased with an increase of catalyst loading from 5 g/L to 40 g/L with conversion 3% to 56%. However, an increase in catalyst loading from 60 g/L to 100 g/L did not have any significant effect on conversion (Figure 2). This behavior of leveling-off can be explained by considering the active site of the enzyme molecules present in excess would not be exposed to the substrates and remain inside the bulk of enzymes particles without contributing significantly to the reaction [7]. In the work of Yadav and Lathi (2005) it was suggested this behavior is due to the increase in the concentration of catalyst above the substrate concentration, which might bring in external mass transfer resistances. All further experiments were carried out using 60 g/L of immobilized lipase.

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Figure 2: Effect of catalyst loading

Effect of solvent

Choosing the appropriate solvent are crucial for the enzymatic reaction because many organic solvents are known to inactivate and denature lipase. In general, enzymes are more stable when suspended in non-polar solvents that have low solubility for water than in polar solvent [8]. On the other hand, the enzyme requires water layer around the support to maintain its activity and the solvents which strip it from the enzyme are the least effective [9]. Log P value are also essential information in determining the suitable reaction media. Solvents having log P > 4 do not distort the essential water coat around the support, thereby leaving the biocatalyst in active site [8]. The acid conversion was high for iso-octane and almost the same for n-hexane and heptane as shown in Figure 3.

The results are summarized in Table 1 and it has shown the correlation between the values of log P and the acid conversion in the study. Wu et al. (2002) reported that surfactant-coated *Candida rugosa* lipase activity in iso-octane was much higher than that in octane, although the two solvents are of the same log P. This might suggest that the solvent with a branched structure is more suitable for the enzymatic reaction compared with the solvent with a straight chain. All further experiments were carried out using iso-octane as solvent.

Table 1: Log P values of the organic solvent used and the optimum acid conversion in synthesis of citronellyl laurate.

Solvent	Log P	Optimum Acid Conversion (%)
<i>n</i> -Hexane	3.5	75.9
<i>n</i> -Heptane	4.0	75.8
<i>iso</i> -Octane	4.5	94.3

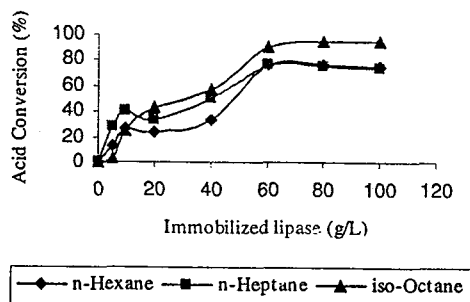


Figure 3: Effect of various type of solvent on acid conversion

Effect of temperature

The optimal temperature for enzyme activity was determined by incubating the reaction mixture at 30°C, 35°C, 37°C, 40°C, 50°C or 55°C. With an increase in temperature from 30°C to 37°C, the rate of reaction and acid conversion increased from 62% to 89% in 24 hr of incubation time. At higher temperatures than 40°C thermal deactivation of enzyme occurs. From the Figure 4, it clearly shows the percentages of acid conversion dropped and tend to increase slowly but not higher than 80% of acid conversion. At low temperature, the rate of a lipase-catalyzed reaction increases with temperatures according to the Arrhenius model (with a low activation energy). However, at elevated temperature this model breaks down due to extensive irreversible denaturation of lipase. This explained the enzyme behavior at higher temperature processes, almost all enzymes become deactivated resulting in the loss of activity as well as their selectivity. All further experiments were carried out at 37°C.

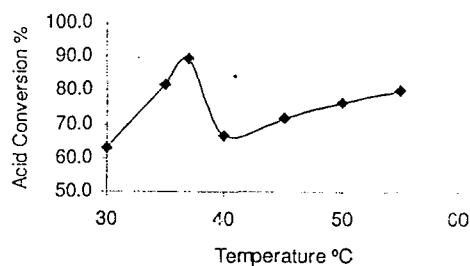


Figure 4: Effect of temperature

Conclusion

The aim of this work was to optimize the synthesis of citronellyl laurate esters using immobilized lipase from *Candida rugosa*. Esterification of lauric acid was carried out with citronellol in various solvent. 60 g/L of catalyst loading gives the maximum acid conversion for the esterification reaction. It was also found that *iso*-octane was the most suitable solvent which gives the maximum conversion of acid. At 37°C of reaction temperature, the yield obtained was the highest with conversion of acid 89%.

Further work on other parameter such as substrate molar ratio, pH for immobilization effect, time course effect, water effect and effect of support for immobilization will be carried out during the research.

Acknowledgements

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CITRONELLYL BUTYRATE SYNTHESIS IN NON-CONVENTIONAL MEDIA USING PACKED-BED IMMOBILIZED *CANDIDA RUGOSA* LIPASE REACTOR

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Abstract The synthesis of citronellyl butyrate by direct esterification reaction catalyzed by immobilized lipase from *Candida rugosa* was studied in a continuous packed bed reactor using *n*-hexane as organic solvent. Parameters such as residence time, temperature, and pH were examined. The optimum conversion was obtained at a flow rate of 1 ml/min (residence time 8 min), temperature of 50 °C, and pH 7.5. At high temperature, biocatalyst was rapidly deactivated with respect to time. The immobilized lipase was stable at pH range of 6 to 9. The deactivation of biocatalyst was increased at pH of immobilized lipase 4 and 10 in comparison with pH range from 5 to 9. The esterification reaction in packed-bed reactor was modeled by ping pong model and ping pong with product inhibition model. The observed kinetics behaviour of esterification reaction was found to follow a ping pong with product inhibition model. In this study, the conversion of butyric acid and stability of immobilized lipase was higher in the system with molecular-sieve than in the system without molecular-sieve.

Keywords Citronellyl Butyrate, Flavor, Esterification, Immobilized Lipase, Non-Conventional Media, Packed-Bed Reactor, Ping Pong Model

چکیده سنتز سیترونیل بوتیرات با واکنش استری شدن به کمک آنزیم لیپاز تثبیت شده ناشی از میکروارگانیسم *Candida rugosa* در راکتور بستر فشرده مورد مطالعه قرار گرفت. پارامترهای مورد مطالعه شامل زمان ماند، دمای واکنش و pH بودند. ضریب تبدیل بهینه یا دبی جریان ۱ میلی لیتر بر دقیقه (زمان ماند ۸ دقیقه) در دمای ۵۰ درجه سانتیگراد و pH برابر ۷/۵ به دست آمد. واکنش در دمای بالا موجب غیر فعال شدن سریع بیوکاتالیست شد. لیپاز ثبت شده در محدوده pH برابر ۶ تا ۹ پایدار بود. این بیوکاتالیست در pH برابر ۴ تا ۱۰ غیرفعال تر از دامنه pH برابر ۵ تا ۹ بود. واکنش استری شدن در راکتور با بستر فشرده به دو روش پینگ پنگ و پینگ پنگ با بازدارنده مورد آنالیز قرار گرفت. نتایج نشان داد که داده های تجربی از مدل بازدارنده پینگ پنگ تبعیت می کند. در این تحقیق تبدیل اسید بوتیریک و پایداری آنزیم لیپاز ثبت شده در سیستم غربال مولکولی به میزان قابل ملاحظه ای بیشتر از بدون غربال مولکولی بود.

1. INTRODUCTION

Citronellyl esters (in particular citronellyl butyrate) are flavor ester of terpene alcohols of short-chain fatty acids. Citronellyl butyrate has a strong "sweet fruity floral like green citrus linalool" flavor, and hence has been used mainly in baked goods, frozen dairy, candy, gelatins and puddings, non-alcoholic

beverages, etc. Formerly, these esters are obtained by various methods such as chemical synthesis, extraction from natural products and by fermentation [1-3]. With the high demand for natural products, flavor industry is more interested in the use of biotechnology to produce natural flavors.

Biotechnology has been an area of major research

activity in the past decade or so for the production of flavor and fragrance compounds by using enzyme-catalyzed reaction and esters produced, however, may be considered natural. Many microbial lipase have been immobilized and a number of studies dealing with advantages of the use of immobilized lipase as biocatalyst in non-conventional media have been reported, for example, the increased in solubility of hydrophobic compounds [4], such as terpene alcohol. However, study of continuous process, especially on lipase-catalyzed synthesis of terpene esters, has received relatively little attention. Although lipase catalyzed reaction has significant benefits, the industrial application of the technology has been slow. Due to their high efficiency, low cost and ease of construction, operation and maintenance, packed-bed reactor have been reported as being the most suitable kind of reactors in industrial scale applications for ester synthesis. The ratio between substrate and enzyme is much lower in a packed bed reactor than in conventional batch reactors, and this result in shorter reaction times [5].

The objectives of this research paper are: (1) to study the reaction parameters of citronellyl butyrate synthesis using an immobilized lipase as catalyst and *n*-hexane as organic (non-conventional) media in a continuous packed-bed reactor, (2) to investigate lipase deactivation mechanism, (3) to determine the kinetic parameters based on ping pong mechanism, and (4) to study the performance of different experimental reactor for the synthesis of citronellyl butyrate, *i.e.* single packed-bed immobilized lipase reactor; packed-bed reactor with bed mixture of immobilized lipase and molecular sieve; and packed-bed immobilized lipase reactor with a separate molecular sieve column.

2. MATERIALS AND METHODS

2.1 Materials

Powdered lipase (EC-3.1.1.3) Type VII from *Candida rugosa* (901 U/mg), DL-Citronellol (95% pure), Amberlite XAD-1180 and molecular sieve were obtained from Sigma-Aldrich (M) Sdn.Bhd. Butyric acid (99% pure) was purchased from Across Organic (NJ, USA). Celite 545

diatomaceous earth, *n*-hexane, phenolphthalein, acetone, and ethanol were supplied by Fisher Scientific (M) Sdn. Bhd. Amberlite MB-1 was obtained from Organo Co. (Japan), and sodium hydroxide was supplied by Merck Co. (Darmstadt, Germany).

2.2 Immobilization Procedure

Immobilization of lipase by adsorption method was carried out by modification according to Omar and Suguna [6]. Two grams of support was washed three times with deionized water and dried in an oven at 80 °C. A 20 mg of lipase was dissolved in 5 ml phosphate buffer solution of pH 7.5 (unless otherwise specified). The dried support was added to the lipase solution. After shaking at 200 rpm for 24 hours at room temperature, then the immobilized lipase preparation was filtered and washed thoroughly with deionized water and rinsed with *n*-hexane. The resultant immobilized lipase was then dried in a vacuum dry desiccator at room temperature and stored in glass vials in refrigerator until further use. The amount of lipase adsorbed to the support was determined by using Pierce BCA (bicinchoninic acid) protein assay with BSA (bovine serum albumin) as standard.

2.3 Reactor Operation

Tubular packed-bed reactor (1.2 cm ID, 24 cm length) was used (Figure 1) for continuous operation which consisted of a jacketed glass tube. The bed was packed with 21 g of immobilized lipase directly into the column. There was a perforated plate at the bottom of the reactor to make uniform flow pattern and to avoid any flow channeling. Density of biocatalyst was experimentally determined given a value of 1.10 g/ml. At zero time, the feed solution (with equimolar concentration of 50 mM) was introduced into the reactor in an up-flow configuration at a flow rate of about 1 ml/min (linear flow rate 3 cm/min) at 37 °C (unless otherwise specified). In immobilized enzyme bioreactor, the external mass transfer is eliminated with high mass flow rate [7]. In the present operating immobilized lipase system, the external mass transfer with the linear flow rate greater than 3×10^{-5} m/s (0.18 cm/min) was omitted. Duplicate

samples of the effluent were periodically taken and acid values were monitored by titration method.

Duplicate samples of the effluent were also periodically taken to monitor the reactor activities.

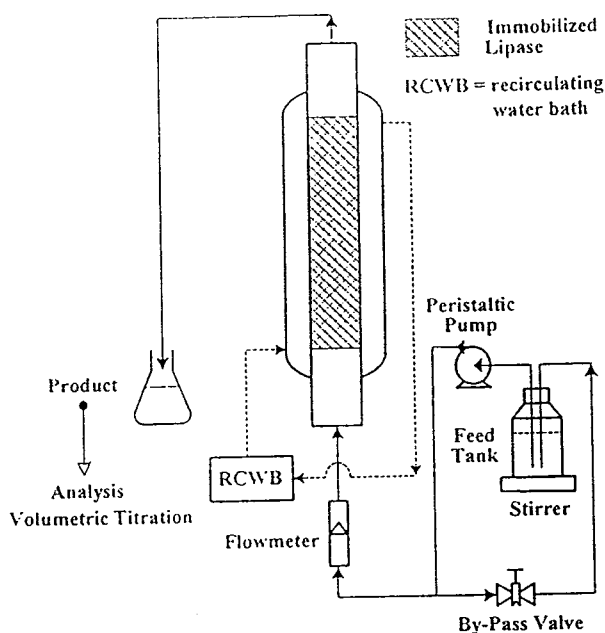


Figure 1. Schematic diagram for experimental set up with single packed bed immobilized lipase reactor.

The water generated through the lipase-catalyzed esterification reactions is considered to be the most important parameter. Adding adsorbents such as molecular sieve to the enzymatic reaction system is the simplest way to remove the produced water. In this case, another two experimental set up were also studied. In the *first case*, two glass reactors with the same size were used. Each reactor was packed with 3.5 g of immobilized lipase directly into the column (4 cm in height), followed by 3.17 g of molecular sieve (4 cm in height) until each reactor is filled with immobilized lipase and molecular sieve. In the *second case*, two glass reactors with the same size were also used. The first reactor was packed with 21 g of immobilized lipase directly into the column. While the second reactor was packed with 19 g of molecular sieve particles (density 1.63 g/ml). The purpose of having two different immobilized lipase reactors set up was to observe and compare the degree of conversion and stability of immobilized lipase.

2.4 Analytical methods

Samples from the inlet and outlet streams of the reactor were withdrawn periodically for analysis. Acid consumption analysis were conducted and monitored by titration method using Digitrate™ digital burette (Jencons, UK) with NaOH solution, phenolphthalein as the indicator, and ethanol-acetone solution as quenching agent. The difference in the results between duplicates was less than $\pm 5\%$. The average values of replicate data were used in calculation. The mole of acid reacted was calculated from the values obtained for the blank and test samples. The ester produced was determined as being equivalent to the acid consumed. Then the acid conversion was calculated.

3. RESULTS AND DISCUSSION

3.1 Effect of residence time

One of the most crucial parameter that was responsible for the higher yields in the packed bed bioreactor was residence time. Residence time directly relates to the volumetric flow rate when the packed bed used is the same. As a consequence, flow rate is one of the important parameters for the packed bed bioreactor. In this study, the residence time dependence of the immobilized lipase activity in a packed-bed reactor was investigated in the range of 1.3 to 8 min by adjusting volumetric flow rate from 1 to 6 ml/min (with linear flow rate from 3 to 18 cm/min). The effect of residence time on the synthesis of citronellyl butyrate in the packed bed reactor at steady state condition are shown in Figure 2. Experimental results revealed that by increasing residence time (decreasing volumetric flow rate) the conversion of butyric acid to produce citronellyl butyrate was also increased and reached to the highest acid conversion of 95 % at the residence time of 8 min (flow rate of 1 ml/min). This is because, the longer residence time (smaller flow rate) would lengthen the enzyme substrate contact time and create higher esterification activity. High substrate flow rate will reduce mass

transfer of substrate into the supported-enzyme interface, thus less distribution and short contact time of substrate and enzyme active site, yielded lower conversion of substrate.

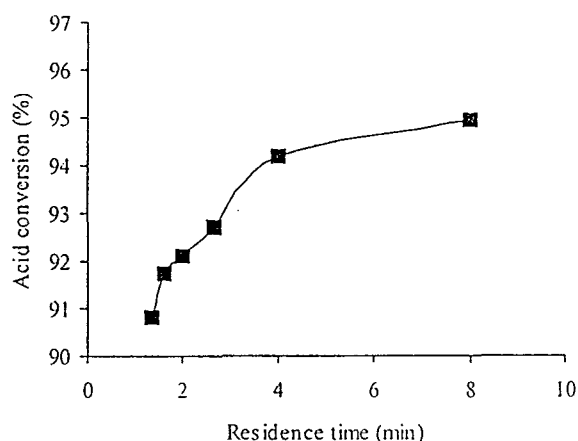


Figure 2. The effect of residence time on citronellyl butyrate synthesis in a continuous packed-bed immobilized lipase reactor at steady-state condition

3.2 Effect of temperature

The temperature dependence of the immobilized lipase activity in a packed-bed reactor was investigated in the range of 20 to 60 °C. The effect of various temperatures on enzymatic synthesis of citronellyl butyrate at steady state condition are shown in Figure 3. Thermal stability of *Candida rugosa* lipase used in a continuous packed-bed reactor was improved and stabilized by immobilization, where the highest conversion of 97 % was obtained at 50 °C. However, as reported that lipase stability was also influenced by temperature, where higher temperature will greatly reduce the lipase stability. As the temperature increase up to 60 °C, lipase activity decreased as reflected in the decrease on acid conversion to 90 %. This results show that any change of temperature causes change in the rate of reaction and degree of conversion.

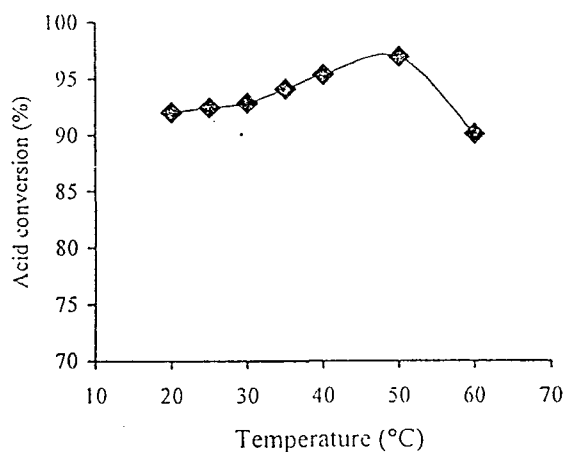


Figure 3. The effect of temperature on citronellyl butyrate synthesis in a continuous packed-bed immobilized lipase reactor at steady-state condition.

It was also found from the experimental data that the enzyme decayed rapidly with time for a period of time. The thermal denaturation has a profound effect on deactivation of the enzyme. It is assumed that thermal denaturation of an enzyme may be modelled by the following serial deactivation scheme [8-9],



the simple first order deactivation expression results

$$a = \exp(-k_d t) \quad (2)$$

where k_d is deactivation rate constant, and can be found from packed-bed equation of first-order reaction and first-order deactivation rate constant given by the following equation [10]:

$$\ln \ln \left(\frac{1}{1-X_a} \right) = \ln(k\tau) - k_d t \quad (3)$$

From equation (3), the evaluation of the deactivation rate constant with the data at various temperature studied are shown in Figure 4. The deactivation rate constants, k_d , was found to increase (0.61 to 0.92 h⁻¹) with increasing

operating temperature (30 to 60 °C). This means, the conversion of acid decreases more rapidly with increasing temperature. In other words, from the graph of activity of immobilized lipase, *i.e.* according to equation (2), versus time at various

temperatures studied, it was found that deactivation of biocatalyst is more rapid with increasing temperature as reflected in the decrease of biocatalyst activity shown in Figure 5.

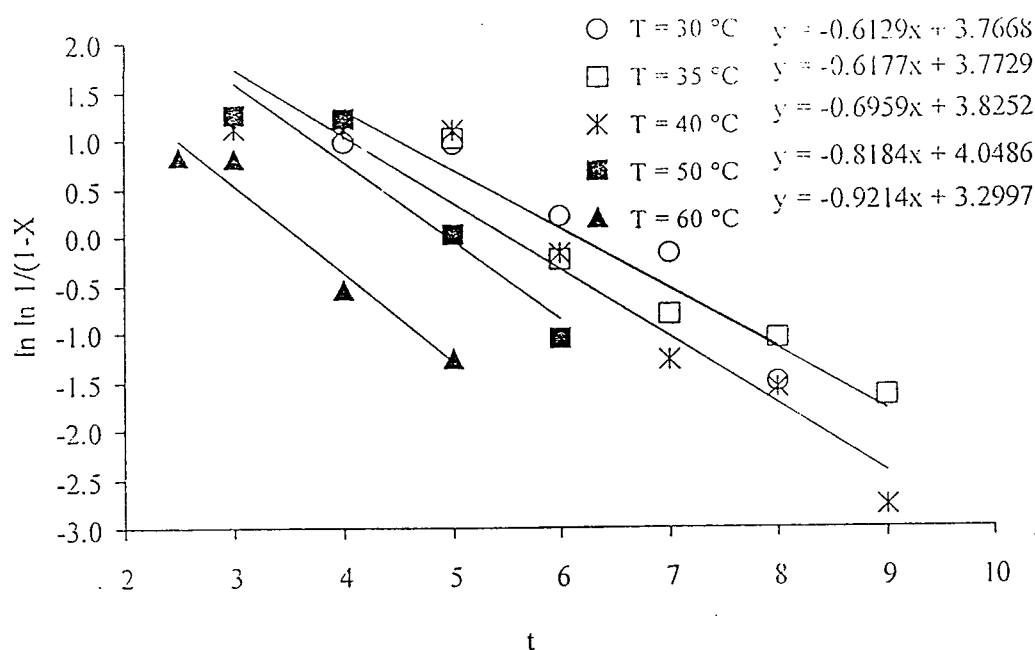


Figure 4. Deactivation kinetics of immobilized lipase in a continuous packed-bed reactor on citronellyl butyrate synthesis at various temperatures studied

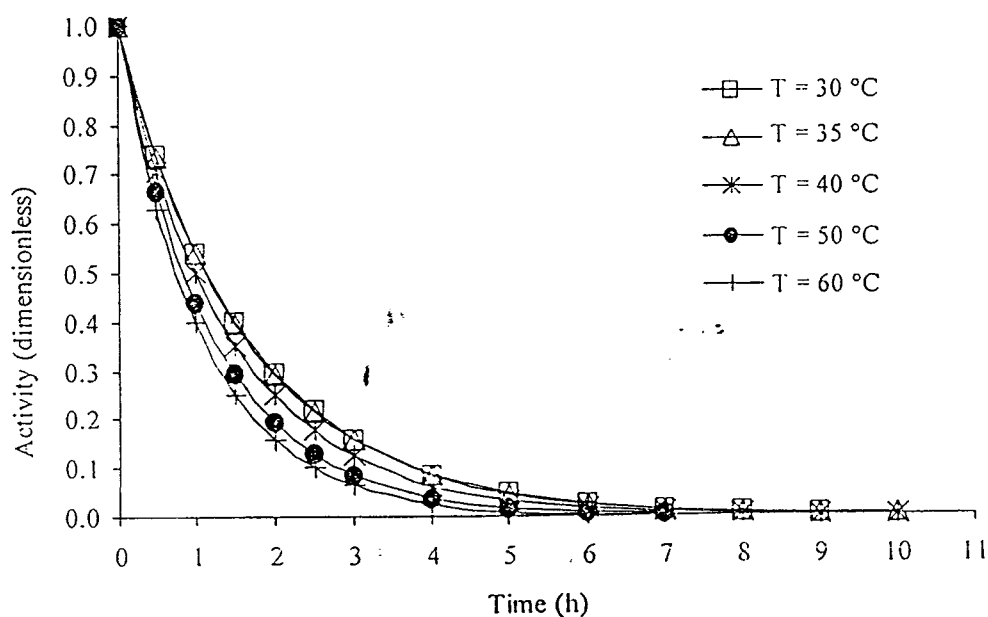


Figure 5. Activity of immobilized lipase in packed-bed reactor in catalyzing citronellyl butyrate as a function of time at various temperatures studied.

3.3 Effect of PH

The effect of pH on the synthesis of citronellyl butyrate was investigated in a continuous packed-bed immobilized lipase reactor in the pH range of 4 to 10. Figure 6 shows the relationship between lipase adsorbed and the acid conversion by immobilized *Candida rugosa* lipase at different pH values in a packed-bed reactor at steady-state condition. About 92 – 95 % of lipases were adsorbed to the support in the range of pH studied. In the pH range from 6 to 10, immobilized lipase were stable, where acid conversion of 88 - 95 % were observed. At pH 4 and 5, the immobilized lipase were more sensitive to pH, where conversion of 66 and 70 %, respectively, were obtained. In this study, the optimal pH was 7.5 with 95 % conversion.

Enzyme deactivation analysis was also carried out for pH dependency using equation (3). Figure 7 shows the evaluation of the deactivation rate constant with the data at various pH studied. The deactivation rate constants (k_d) were found greater at immobilized lipase pH of 4 and 10, i.e. 1.5 and 1.07 h^{-1} , respectively. This shows that deactivation of biocatalyst at this lowest and highest pH studied are more rapid in comparison with pH range of 5 to 9. In other words, from the graph of activity of immobilized lipase versus time, it was found that deactivation of biocatalyst was more rapid with extreme pH of immobilized lipase at 4 and 10, respectively, as presented in Figure 8.

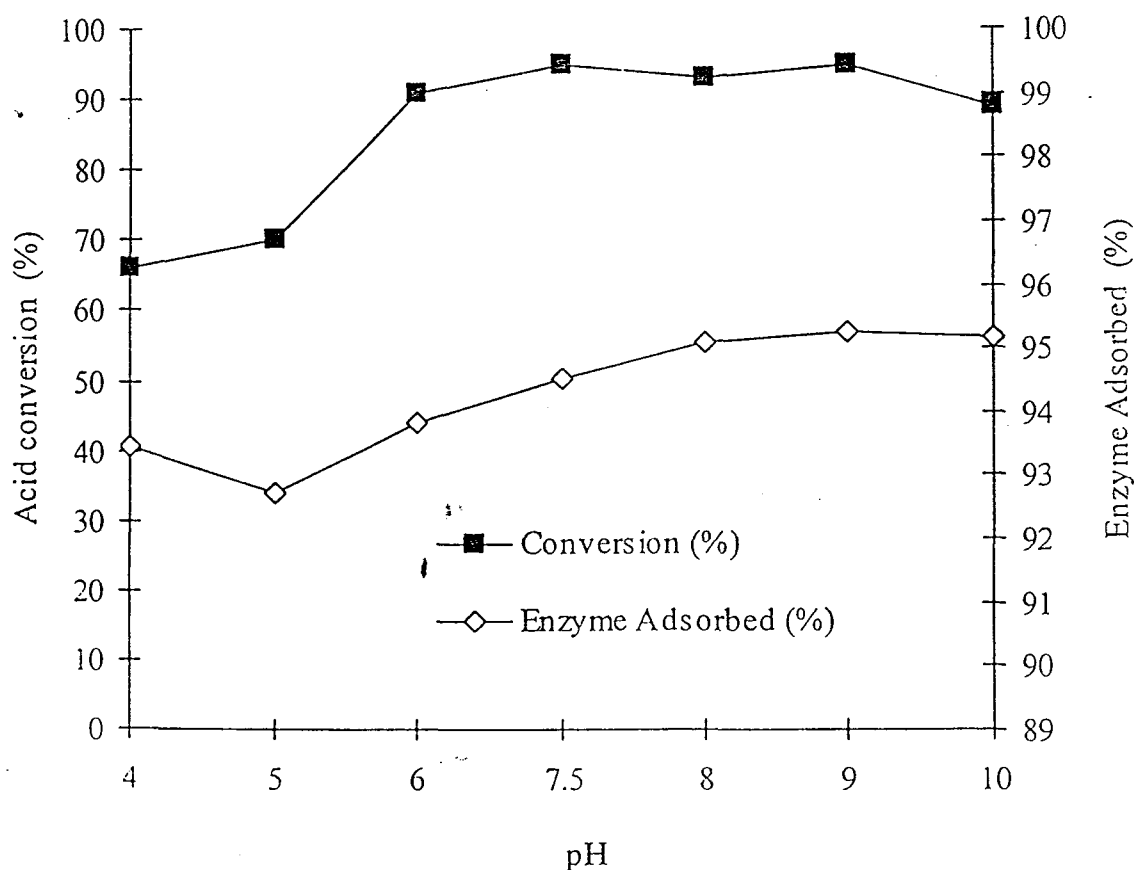


Figure 6. Effect of pH of immobilized lipase during the synthesis of citronellyl butyrate in a continuous packed-bed reactor at steady-state condition.

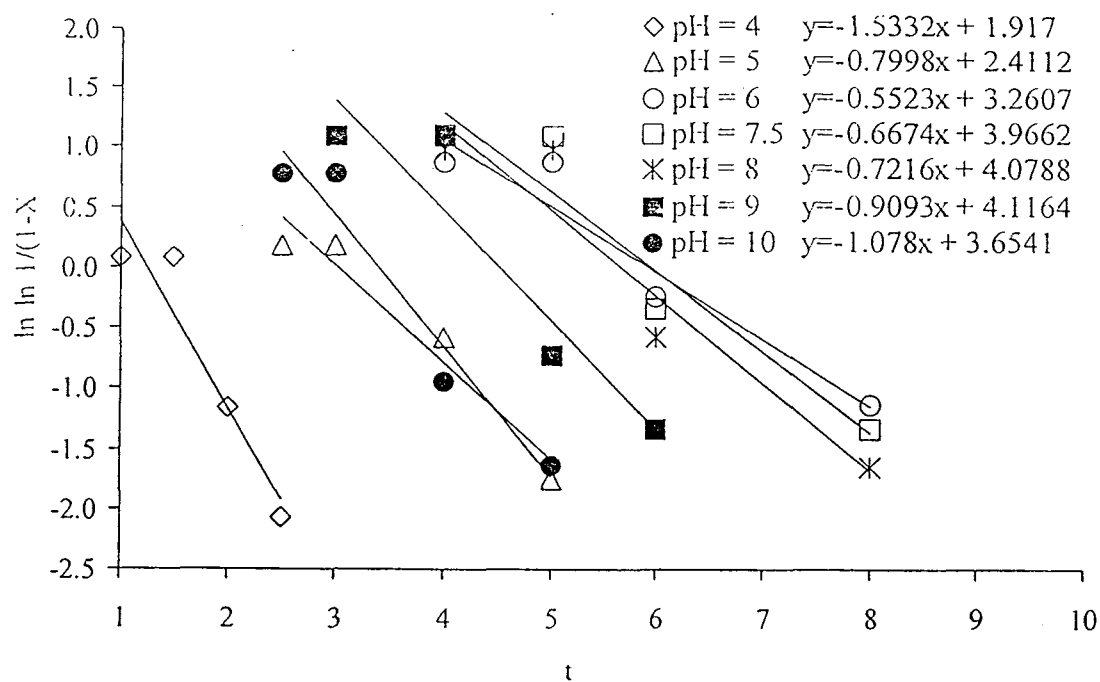


Figure 7. Deactivation kinetics of immobilized lipase in a continuous packed-bed reactor on citronellyl butyrate synthesis at various pH studied.

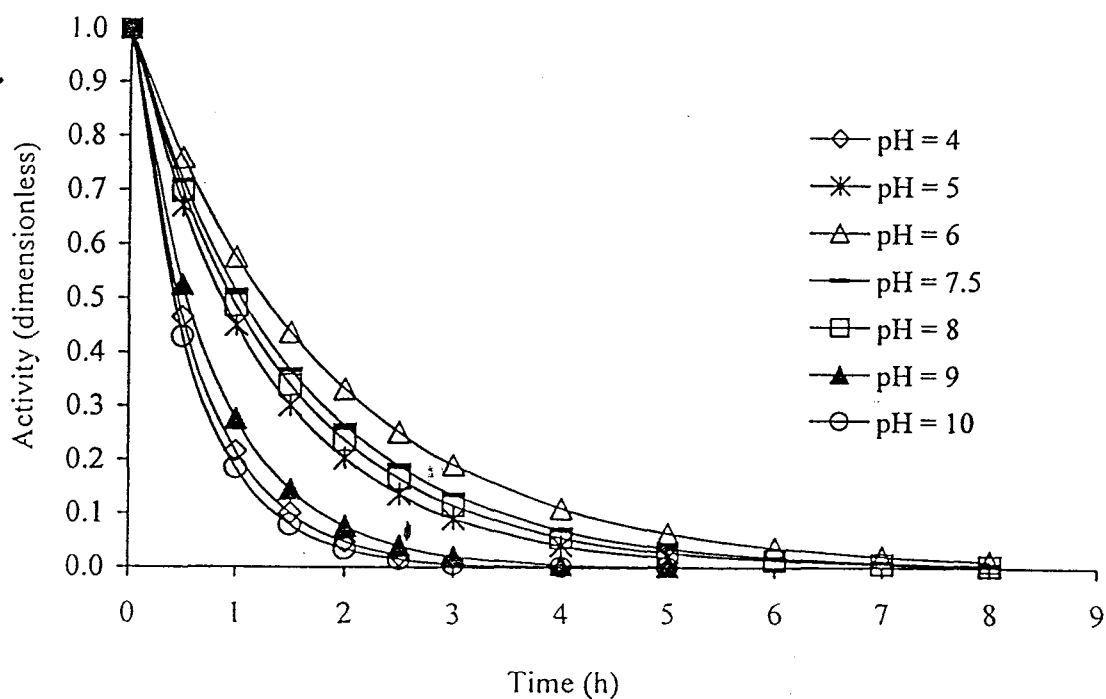


Figure 8. Activity of immobilized lipase in packed-bed reactor in catalyzing citronellyl butyrate as a function of time at various pH studied

3.4 Mathematical models

Various kinetics mechanisms have been proposed for the description of lipase-mediated reactions [11-12]. For two-substrate two-product (bi-bi) reactions, the most generally accepted mechanism for lipase-catalyzed reactions is ping-pong mechanism. This mechanism was first established by Chulalakasananukul *et.al* [13] for the kinetics of esterification of oleic acid with ethanol using immobilized *R. miehei* lipase as biocatalyst and *n*-hexane as organic solvent. After that, many researchers have also proposed kinetic models for lipase-catalyzed esterification and transesterification reactions in organic media based on this mechanism [14-22]. In this study, ping pong and ping pong with product inhibition models, were tested with the experimental data. The experiments were carried out in *n*-hexane with equimolar substrate concentrations of 0.1, 0.05 and 0.02 M (initial butyric acid concentration of 9.35, 4.50 and 1.84 g/l, respectively) at fixed flow rate of 1 ml/min and 37 °C.

Based on a single displacement reaction of ping pong and ping pong with product inhibition mechanisms, the rates of decrease of butyric acid [A] may be given by [16],

$$-\frac{d[A]}{dt} = \frac{V_m'}{1 + \frac{K_3 + K_4}{[A]}} \quad (4)$$

$$\text{and} \quad -\frac{d[A]}{dt} = \frac{V_m'}{1 + \frac{(K_3 + K_4)}{[A]} + \frac{K_3 K_i ([A]_0 - [A])}{[A]}} \quad (5)$$

The decrease of butyric acid concentration as a function of time at various initial concentrations were fitted with these two models by non-linear regression using Polymath software (version 5.1). Figures 9 – 11 shows the comparison of the experimental data and the simulation results for two models tested. The parameters estimated for kinetic constants are given in Table 1. In all the initial butyric acid concentration tested, the ping pong model with product inhibition, *i.e.* equation (5), predicted the observed behaviour more accurately than the ping pong without product inhibition model, *i.e.* equation (4). The estimated value for the second model at various initial butyric acid concentrations has a correlation coefficients (R^2) greater than 0.99 and the sum of squares of errors (SSE) were much less than the first model.

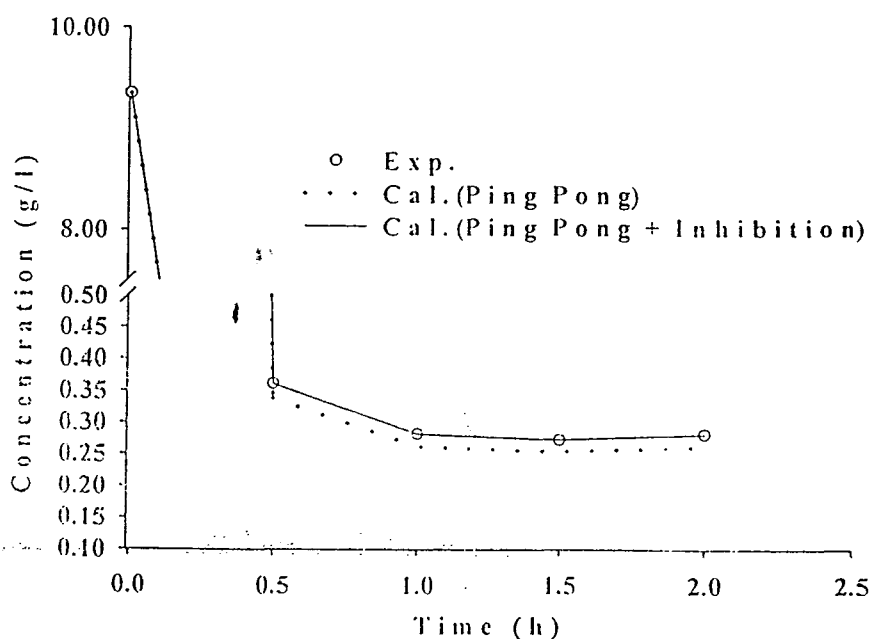


Figure 9. Comparison of experimental data with model predictions of esterification of citronellyl butyrate at initial butyric acid concentration of 0.1 M (9.35 g/l).

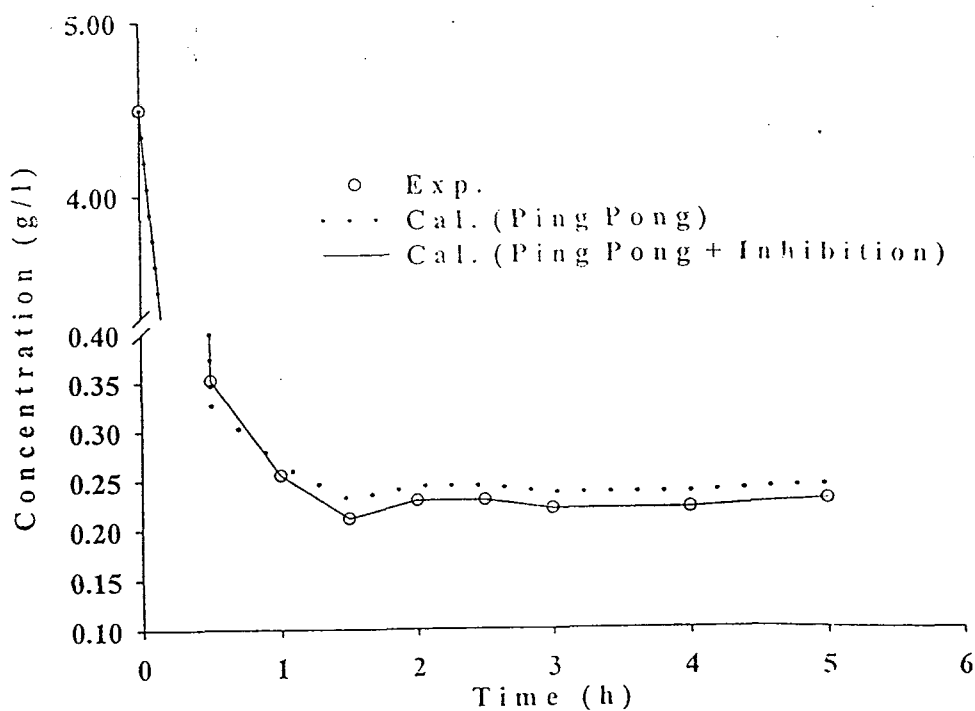


Figure 10. Comparison of experimental data with model predictions of esterification of citronellyl butyrate at initial butyric acid concentration of 0.05 M (4.50 g/l).

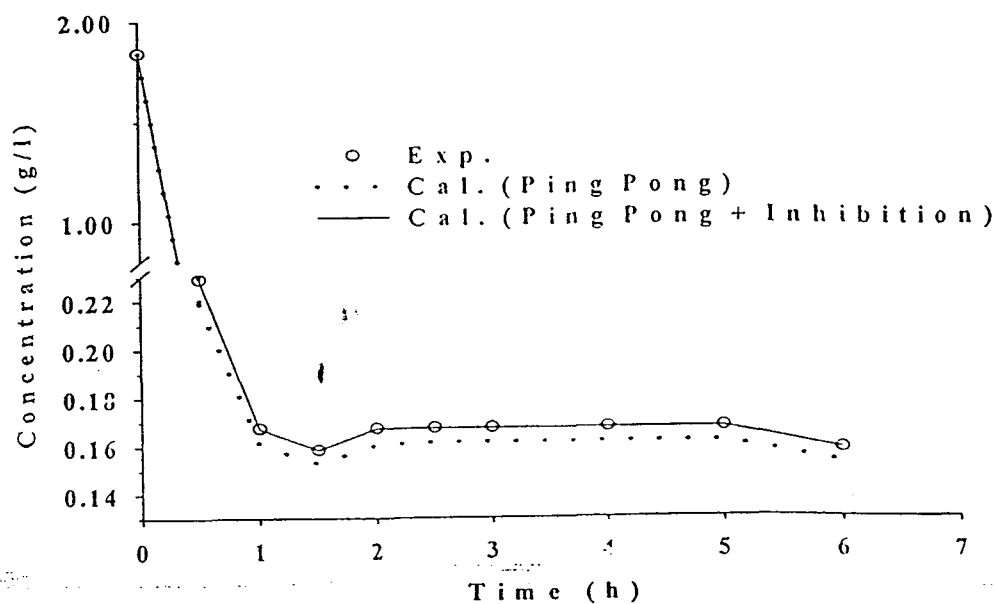


Figure 11. Comparison of experimental data with model predictions of esterification of citronellyl butyrate at initial butyric acid concentration of 0.02 M (1.84 g/l).

Table 1. Estimated kinetic constants for esterification of citronellyl butyrate

Butyric acid concentration	Models	Parameters				
		V_m (g/l.min)	$K_3 + K_4$ (g/l)	K_3K_i (g/l)	R^2	SSE ($\times 10^9$)
0.1 M	Ping pong	0.1758	1.1010		0.9428	4320.0
	Ping pong with product inhibition	1.1010	1.3679	0.7894	0.9997	5.8
0.05 M	Ping pong	0.1580	1.0101		0.9409	13200.0
	Ping pong with product inhibition	0.6091	0.3395	1.0101	0.9999	2.0
0.02 M	Ping pong	0.1481	1.0101		0.9690	1820.0
	Ping pong with product inhibition	0.2366	0.0577	0.9973	0.9999	4.4

3.5. Performance of packed-bed immobilized lipase reactor with molecular-sieve

In this study, citronellyl butyrate synthesis was conducted in the packed-bed immobilized lipase reactor with mixed and separated molecular-sieve particle. The comparison in the synthesis of citronellyl butyrate by using different experimental reactor set up are shown in Figure 12. As observed, the reactions were quite fast and reached a steady state within 1 to 1.5 hours for all experimental reactor set up studied. A conversion of about 92 - 93 % was maintained over a period of 15 hours at steady state condition for the system with molecular-sieve. For the system without molecular-sieve, only 90 % of conversion is maintained over a period of 10 hours. Based on this experiment on the performance of different reactor set up, the conversion of butyric acid to produce ester and the stability of immobilized *Candida rugosa* lipase increased more in the system with molecular-sieve than in the system without molecular-sieve. Although higher conversion of 93 % was obtained by using packed-bed reactor with a bed mixture of immobilized lipase and molecular-sieve, however, this

experimental set up was not practical and extensive effort was required for regeneration. Hence, packed-bed immobilized lipase reactor with a separate molecular sieve column was preferred in carrying out this study. The main advantage of this system was that regeneration of the molecular-sieve column could be independently carried out upon saturation without disrupting the immobilized enzyme reactor.

4. CONCLUSIONS

Continuous esterification of citronellyl butyrate by immobilized *Candida rugosa* lipase in a packed-bed reactor with and without molecular-sieve particle has been carried out in non-conventional media (*n*-hexane). The esterification reaction was simulated by two models namely, ping pong model and ping pong with product inhibition model. The second model was found to predict the observed behaviour accurately. In order to improve the efficiency, packed-bed immobilized lipase reactor with a separate molecular sieve column was preferred than packed-bed reactor with a bed mixture of immobilized lipase and molecular sieve.

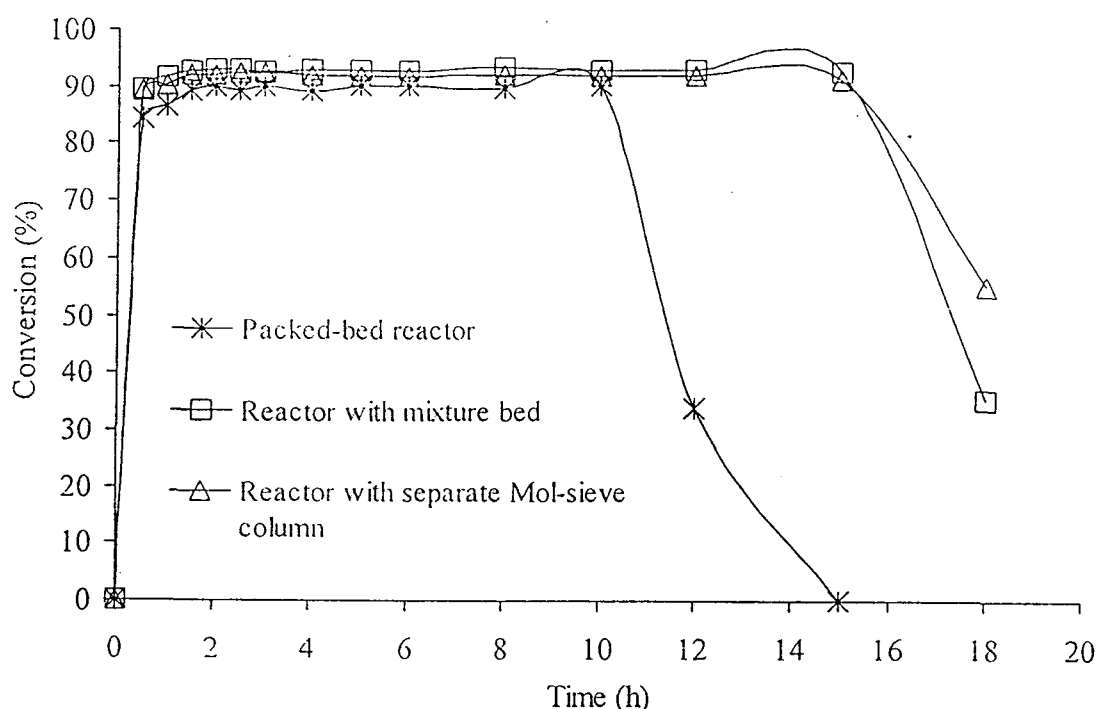


Figure 12. Comparison of experimental reactor set up in the synthesis of citronellyl butyrate.

5. ACKNOWLEDGEMENTS

The authors acknowledge the research grant provided by Universiti Sains Malaysia that has resulted the present paper.

NOTATION	
a	enzyme activity
[A]	concentration of butyric acid (mole/l)
[A] ₀	initial concentration of butyric acid (mole/l)
E, E _i , E _d	active, intermediates and deactivated enzyme (g/l)
k	rate constants (mole/l.h)
K ₃ , K ₄	constants related to reaction rate constants
K _i	inhibition constant (mole/l)
k _d , k _{d1} , k _{d2}	first-order deactivation rate constants (h ⁻¹)
t	time (h)
V _m	constant related to maximum velocity (mole/l.h)
X _A	conversion of butyric acid

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