Proceedings of the 1st International Conference on Natural Resources Engineering & Technology 2006
24-25th July 2006; Putrajaya, Malaysia, 186-194

The Effect of Alcohol on the Performance of Lipase-Immobilized Enzymatic Membrane Reactor for Esterification of (R,S)-Ketoprofen

Ai Lien Ong, Kian Chuan Fu, Azlina Harun @ Kamaruddin*, Subhash Bhatia,

School of Chemical Engineering, Engineering Campus, Universiti Sains Malaysia, Seri Ampangan, 14300 Nibong Tebal, Penang, Malaysia.

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 antartica*, 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

1.0 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.

* *Corresponding author. Tel.: +604-5996417; fax: +604-5941013 E-mail address: chazlina@eng.usm.my*

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.

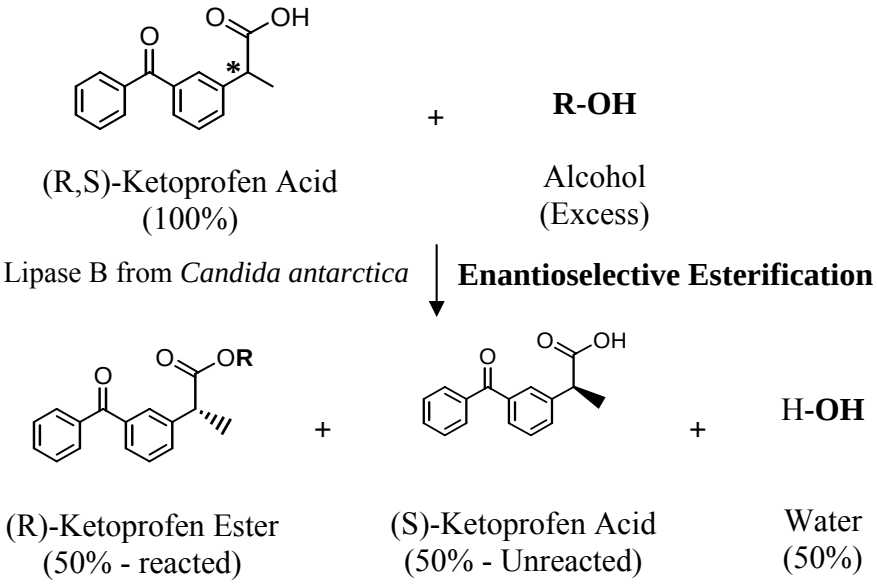

Fig. 1 Enantioselective esterification of (R,S)-ketoprofen.

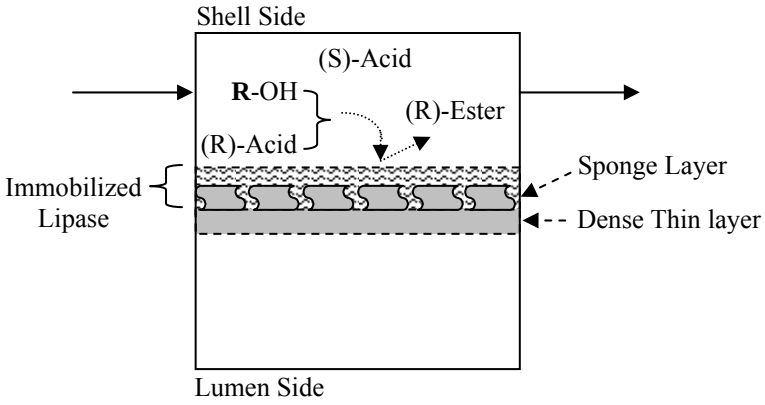

Fig. 2 Enantioselective esterification of (R,S)-ketoprofen in EMR.

2.0 Materials & Methods

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 ($Na_2HPO_4.2H_2O$) 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 $Na_2HPO_4.2H_2O$ 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^{o}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^{o}C. 60µl of the sample was injected. An example of HPLC chromatogram of pure (R,S)-ketoprofen is illustrated by Fig. 3.

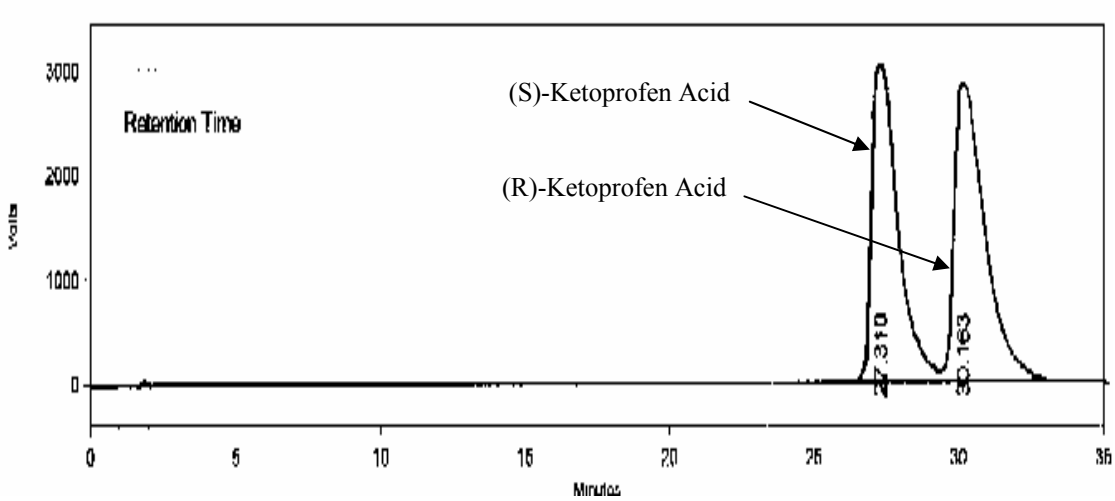

Fig. 3 HPLC chromatogram of pure (R,S)-ketoprofen.

The conversion of ketoprofen was calculated using the following equations:

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

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

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:

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

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

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

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 1μmol of (R)-ketoprofen acid per hour.

$$Lipase\ Activity = \frac{[X_R]\ x\ [C_0]}{[t]\ x\ [mg\ \ protein]} = \frac{\mu mol}{h.mg} = \frac{U}{mg} \tag{6}$$

3.0 Results and Discussion

3.1 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^{o}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.

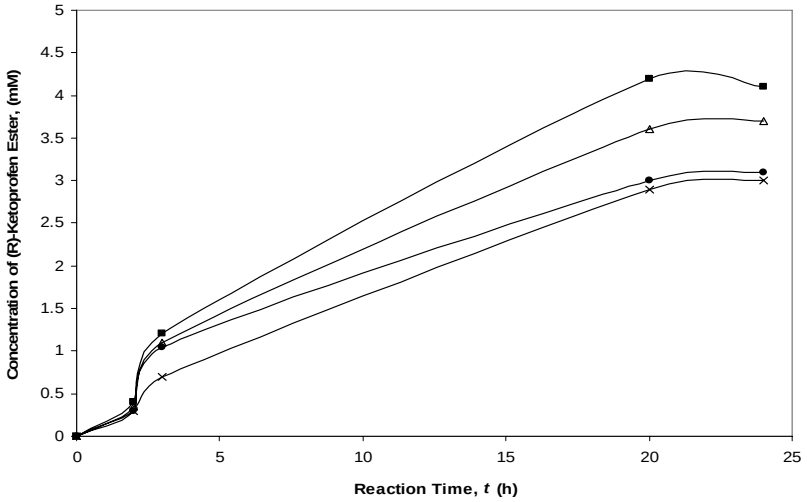

Fig. 4 Effect of different alcohols on the production of (*R*)-ketoprofen ester. Reaction conditions:10mM of (R,S)-ketoprofen, 2:1 molar ratio of *n*-butanol to (R,S)-ketoprofen, 24h, 1,2-dichloropropane:hexane (20:80, %v/v) as solvent, 158mg immobilized CALB, lipase solution pH of 7.0, 15ml/min. Symbols: (-■-) ethanol, (-Δ) butanol, (-●-) 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.

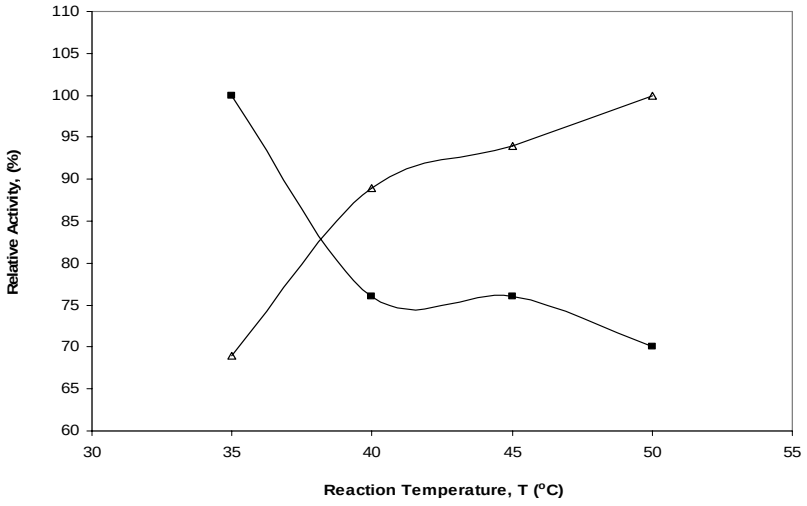

Fig. 5 Effect of different alcohols on the relative activity of immobilized lipase in EMR. Reaction conditions: 10mM of (R,S)-ketoprofen, 2:1 molar ratio of *n*-butanol to (R,S)-ketoprofen, 24h, 1,2-dichloropropane:hexane (20:80, %v/v) as solvent, 158mg immobilized CALB, lipase solution pH of 7.0, 15ml/min.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

3.2 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.

4.0 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.

Acknowledgments

The authors acknowledge Malaysian Ministry of Science, Technology and Innovation (MOSTI) for providing the long-term IRPA research grant no: 6012601 and National Science Fellowship (NSF) Scholarship that have resulted in this article.

References

[1] Harris, R.H. and I. Vavra. 1985. *Anti-Inflammatory and Anti-Rheumatic Drugs.* Boca Rotan: CRC Press.
[2] Haas, R.T. and E.S. Induyk. 2004. Ketopofen Compositions and Methods of Making Them. *USP 20,040,116,528*.

[3] Dorgan, J. 2003. *Ketoprofen Extended-Release Capsules.* Lauderdale: Andrx Pharmaceutical Inc.
[4] Stroble, M., P. Soderlund and J.W. Ryan. 2002. Ketoprofen Powder for Oral Use. *USP 20,020,169,212*.
[5] Ossipov, M.H., T.P. Jerussi, K. Ren, H. Sun and F. Porreca. 2000. Differential Effects of Spinal (R)-Ketoprofen and (S)-Ketoprofen Against Signs of Neuropathic Pain and Tonic Nociception: Evidence for a Novel Mechanism of Action of (R)-Ketoprofen Against Tactile Allodynia. *Pain* 87: 193-199.
[6] Diaz-Reval, M.I., R. Ventura-Martinez, M. Deciga-Campos, J.A. Terron, F. Cabre and F.J. Munoz. 2004. Evidence for a Central Mechanism of Action of S-(+)-Ketoprofen. *Eur. J. Pharmacol*. 483: 241-248.
[7] Kim, M.G., E.G. Lee and B.H. Chung. 2000. Improved Enantioselectivity of *Candida rugosa* Lipase Towards Ketoprofen Ethyl Ester by a Simple Two-Step Treatment. *Proc. Biochem.* 35: 977-982.
[8] Choi, G.S., J.Y. Kim, J.H. Kim, Y.W. Ryu and G.J. Kim. 2003. Construction and Characterization of a Recombinant Esterase with High Activity and Enantioselectivity to (S)-Ketoprofen Ethyl Ester. *Protein Expres. Purif.* 29: 85-93.
[9] Jin, J.N., S.H. Lee and S.B. Lee. 2003. Enzymatic Production of Enantiopure Ketoprofen in a Solvent-Free Two-Phase System. *J. Mol. Catal. B: Enzym*. 26: 209-216.
[10] Romero, M.D., L. Calva, C. Alba, A. Daneshfae and H.S. Ghaziaskar. 2005. Enzymatic Synthesis of Isoamyl Acetate with Immobilized *Candida antarctica* Lipase in n-Hexane. *Enzyme Microb. Technol*. 37: 42-48.
[11] McCabe, R.W. and A. Taylor. 2004. An Investigation of the Acryl-Binding Site of *Candida antarctica* Lipase B. *Enzyme Microb. Technol*. 35: 393-398.
[12] Rotticci, D. 2000. *Understanding and Engineering the Enantioselectivity of Candida antarctica Lipase B Toward Sec-Alcohols*. PhD Thesis. Royal Institute of Technology.
[13] Antona, N.D., P. Lombardi, G. Nicolosi and G. Salvo. 2002. Large Scale Preparation of Enantiopure (S)-Ketoprofen by Biocatalysed Kinetic Resolution. *Proc. Biochem*. 38: 373-377.
[14] Park, H.J., W.J. Choi, E.C. Huh, E.Y. Lee and C.Y. Choi. 1999. Production of Optically Active Ketoprofen by Direct Enzymatic Esterification. *J. Biosci. Bioeng*. 87: 545-547.
[15] Xi, W.W. and J.H. Xu. 2005. Preparation of Enantiopure (S)-Ketoprofen by Immobilized *Candida rugosa* Lipase in Packed Bed Reactor. *Proc. Biochem*. 40: 2161-2166.
[16] Ong, A.L., A.H. Kamaruddin and S. Bhatia. 2005. Current Technologies for the Production of (S)-Ketoprofen: Process Perspective. *Proc. Biochem*. 40: 3526-3535.
[17] Long, W.S., A.H. Kamaruddin and S. Bhatia. 2002. Enzymatic Membrane Reactor for Chiral Drugs Synthesis. *J. Teknologi.* 32: 27-44.
[18] Rios, G.M., M.P. Belleville, D. Paolucci and J. Sanchez. 2005. Process in Enzymatic Membrane Reactors: a Review. *J. Membr. Sci*. 242: 189-196.
[19] Chen, C.S. and C.J. Sih. 1989. General Aspects and Optimization of Enantioselective Biocatalysis in Organic Solvent: The Use of Lipases. *Angew. Chem. Int. Ed. Engl*. 28: 695-707.
[20] Ong, A.L., A.H. Kamaruddin, S. Bhatia, W.S. Long, S.T. Lim and R. Kumari. 2006. Performance of Free *Candida antarctica* Lipase B in the Preparation of Enantiopure (S)-Ketoprofen. *Enzyme Microb. Technol*. In Press.
[21] Ghamgui, H., M. Karra-Chaabouni and Y. Gargouri. 2004. 1-Butyl Oleate Synthesis by Immobilized Lipase from *Rhizopus oryzae*: a Comparative Study Between n-Hexane and Solvent-Free System. *Enzyme Microb. Technol*. 35: 355-363.
[22] Zaidi, A., J.L. Gainer, G. Carta, A. Mrani, T. Kadiri, Y. Belarbi and A. Mir. 2002. Esterification of Fatty Acids using Nylon-Immobilized Lipase in n-Hexane: Kinetic Parameters and Chain-Length Effects. *J. Biotechnol*. 93: 209-216.