Available online at www.sciencedirect.com
SCIENCE DIRECT
Enzyme and Microbial Technology 38 (2006) 551–556
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

Effect of solvent and initial water content on (*R*, *S*)-1-phenylethanol resolution

Lee Suan Chua *, Mohamad Roji Sarmidi

Department of Bioprocess Engineering, Faculty of Chemical and Natural Resources Engineering, Universiti Teknologi Malaysia, 81310 UTM Skudai, Johor, Malaysia

Received 3 October 2004; received in revised form 27 June 2005; accepted 19 July 2005

Abstract

The enzymatic resolution of (*R*, *S*)-1-phenylethanol was carried out in various organic solvents. Two commercial immobilized lipases, such as ChiroCLEC-PC and Chirazyme L2, c.f., C3, lyo used in this study could exhibit the highest performance in isooctane. The effects of initial water content on the activity and enantioselectivity of the enzymes were also investigated. The method of direct contact pre-equilibrium, such as substrate pre-equilibrium and enzyme pre-equilibrium were used in this study. The resolution was favourable in the absence of additional water and water mimicking compound such as glycerol in the reaction system. The enantioselectivity of the enzymes was not affected by the organic solvents tested and the initial water content ranging from 0 to 0.5% (v/v). The enzymes were very selective toward the (*R*)-1-phenylethanol with the enantioselectivity value more than 200.

Keywords: (*R*, *S*)-1-phenylethanol; Immobilized lipase; Organic solvent and water pre-equilibrium

1. Introduction

The first lipase used in non-aqueous solvent was already described in 1900 [1]. Only in 1960's, the interest in this field was renewed. The application of lipases in organic media began in the late 19th century. Zaks and Klibanov were the first researchers who consider enzyme performance in organic environment [2].

After the realization of enzymes can remain active in a highly apolar organic solvents, a considerable attention has been focused on the novel application of enzyme in chemical synthesis. In addition to the enhanced thermostability, many enzymes can also alter substrate specificity and selectivity in organic solvents.

The increasing use of enzymes in organic reaction has motivated researches into solvent selection. Several attempts have been made to establish rules for solvent choosing, but still did not achieve conclusion. To date, there is no principle to guide the selection of organic solvent for any particular process. The single most important criteria is the solvent compatibility with the maintenance of enzyme activity and stability [3,4].

Generally, organic solvents have been characterized according to their polarity. The parameters used to classify organic solvents include aquaphilicity, Hildebrand solubility, three-dimensional solubility, denaturation capacity, partition coefficient (log *P*), dielectric constant, dipole moment hydrogen bonding, polarizability, water solubility and electron pair acceptance index [5]. Among these parameters, log *P* (partition coefficient of a given compound in the octanol and water two phase system) is reported as the best parameter in providing information in choosing organic solvents. This is because log *P* meets all the requirements as a good indicator [6]. First, log *P* is a direct measure of polarity. Second, log *P* values can easily be determined from standard method or calculated from hydrophobic fragmental constants. Third, log *P* is very sensitive for polarity differences in a quite broad range.

The high hydrophobic solvents with $\log P > 4$ are considered the most suitable solvent for biocatalysis [7,8]. The solvents with log *P* values between 2 and 4 are moderately effective [7,8]. Those polar solvents with $\log P < 2$ are often

* Corresponding author. Tel.: +60 7 5532 502; fax: +60 7 5569 706.
E-mail address: chualeesuan@hotmail.com (L.S. Chua).

0141-0229/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2005.07.027

ineffective [7,8]. Although log *P* value does not always correlate with the degree of enzymatic synthesis, it provides valuable information for solvent selection.

The content of water in organic solvent is usually very limited. However, the presence of the limited water content cannot be neglected. It plays an important role in controlling enzyme performance in organic media.

It is a fact that a small amount of water is required for catalytic action of enzyme. However, the quantity of water required is varied from cases to cases. An optimal amount of water required depends on several parameters, including type of solvent, polarity of enzyme active site, substrate, solid support, and reaction conditions.

Although the amount of water needed is still unclear, some residually bound water seems to be absolutely essential for maintaining the enzyme active conformational state. This monolayer of water should be maintained on the enzyme surface. Actually, it is not a true layer of water but just a few clusters of water around the charged groups of protein. As long as a trace amount of essential water is present, the enzyme should be in active conformation. Therefore, the hydration level of enzyme is strongly affected by its bound water but not by free water in bulk solution. The bulk water could be replaced by organic solvents with no adverse effect on enzyme activity [9].

In this study, the effects of organic solvent on the activity and enantioselectivity of immobilized lipases were investigated. Two commercial immobilized lipases such as ChiroCLEC-PC (cross-linked enzyme crystals of *Pseudomonas cepacia* lipase) and Chirazyme L2, c.f., C3, lyo (lyophilzed carrier-fixed lipase B from *Candida antarctica*) were used in the resolution of (*R*, *S*)-1-phenylethanol. The enzymes performance was also studied with the addition of initial water content and glycerol. Molecular sieve was used to improve the reaction performance by removing water from the reaction system.

2. Materials and methods

2.1. Chemicals and enzymes

High purity grade of substrates, (*R*, *S*)-1-phenylethanol and lauric acid were purchased from Fluka (Switzerland). Isooctane was purchased from Merck (Germany). Commercial immobilized lipases such as ChiroCLEC-PC and Chirazyme L2, c.f., C3, lyo were purchased from Altus Biologics (USA) and Roche Molecular Biochemicals (Germany), respectively.

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

Lauric acid (150 mM) and (*R*, *S*)-1-phenylethanol (50 mM) were dissolved in isooctane in a reaction vessel of batch stirred tank reactor. The reaction was initiated after added in the enzymes (ChiroCLEC-PC = 12.5 mg, Chirazyme L2, c.f., C3, lyo = 250 mg) at 35 °C. The solution (25 ml) was continuously stirred to ensure all the enzyme particles were homogeneously dispersed in the reaction medium. Samples (0.01 ml) were periodically withdrawn to analyze the time course of reaction by GC [10]. No reaction was detected in the absence of the enzymes. Specifications mentioned above were followed in all the subsequent experiments unless otherwise indicated.

Table 1
log *P* values of organic solvents

Solvent	log *P*
Tert-butyl methyl ether	1.4
Toluene	2.5
Cyclohexane	3.2
Hexane	3.5
Heptane	4.0
Isooctane	4.5

2.3. Effect of organic solvent

The solvent effect was investigated by carrying out the reaction in the commonly used organic solvents. The organic solvents used were isooctane, heptane, hexane, cyclohexane, *tert*-butyl methyl ether and toluene. The organic solvents and their log *P* values are tabulated in Table 1. Triplicate data were collected for each set of experiments.

2.4. Effect of water content

The effects of water on the resolution and on the enzymes were investigated. The former study was carried out by pre-equilibrating the reaction solution with water (0–0.5%, v/v) for 16 h [11]. The reaction solution consisted of lauric acid (150 mM) and (*R*, *S*)-1-phenylethanol (50 mM) in isooctane (25 ml). The reaction was initiated after added in the enzymes. This method was called substrates pre-equilibrium method. The latter study was the pre-equilibration of lauric acid solution and enzyme with water (0–0.5%, v/v) in the absence of (*R*, *S*)-1-phenylethanol. The reaction was initiated following the addition of (*R*, *S*)-1-phenylethanol. This method was called enzyme pre-equilibrium method.

2.5. Effect of glycerol and molecular sieve

A water-mimicking solvent such as glycerol (0.1%, v/v) was added and stirred for 16 h in the reaction solution at room temperature. In the next study, a considerable amount of molecular sieve 3 Å, $K_{12}[(AlO_2)_{12}(SiO_2)_{12}]\cdot xH_2O$ (10%, w/v) was added to remove moisture from the reaction system. Two pre-equilibration methods as described in Section 2.4 were used in this study.

3. Results and discussion

3.1. Effect of organic solvent

The effect of solvent on the enzymatic reaction has been investigated by several researchers [12–17]. A good

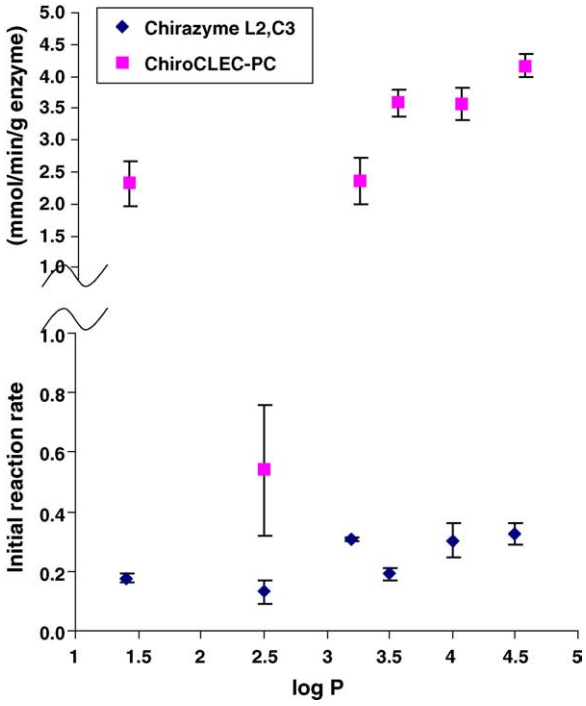

Fig. 1. Initial velocities of reactions in different log *P* values of solvents.

correlation has been achieved between the result and the polarity of solvent. However, the relationship is reverse in some cases. In this study, the solvents with log *P* values from 1.4 to 4.5 were used in order to investigate the effect of solvent polarity on the enzymatic resolution.

The relationship between the initial reaction rate and the hydrophobicity of organic solvents is presented in Fig. 1. The hydrophilic solvent may deactivate the enzyme in the way of distrupting the functional structure of enzyme or stripping off the essential water from the enzyme [17]. This is because water has higher affinity in hydrophilic solvent rather than bound to the enzyme. As a consequence, the catalytic activity of enzyme was decreased due to the lack of bound water to preserve the enzyme conformation flexibility. This structural mobility is necessary for its catalytic action. For example, the reaction in *tert*-butyl methyl ether exhibited lower reaction rate because the solvent is more hydrophilic than the other hydrocarbon solvents.

In addition to solvent hydrophobicity, the molecular structure of solvent may also play a role in affecting enzyme performance. The performance of the enzyme was decreased significantly in toluene. (*R*, *S*)-1-phenylethanol was more favourable to stay in the bulk phase of solvent containing benzene ring such as toluene instead of bound to the enzyme complex for the deacylation step. The π–π electron interaction on the benzene ring of toluene was more likely to attract the phenyl group of alcohol. The interaction might be due to the structural resemblance of alcohol and toluene.

Chirazyme L2, c.f., C3, lyo behaved the same in the solvent of toluene. There was a minor tendency that the catalytic activity of the enzyme in toluene was lower than the other solvents. Overall, no significant trend was noticed in the influence of solvent polarity on the reaction rate of Chirazyme L2, c.f., C3, lyo catalyzed reactions.

According to the hypothesis of Secundo et al. [18], some molecules of solvent may interact with the binding site of enzyme to form solvent–enzyme complex. The complex would behave differently depending on the nature of solvent. Therefore, the effect of solvent on the enzyme performance is greatly influenced by the bulkiness and hydrophobicity of solvent.

The factor of bulkiness is actually similar to the finding of Kamiya et al. [19]. They reported that the activity of enzymatic reaction is very sensitive to the structure of solvent. A small change in solvent structure would cause a great difference in the result of reaction. This fact is true, especially for highly enantioselective catalyst. For example, the difference between hexane and heptane is only one atom carbon. However, the activity of the enzymes was greatly different in both solvents.

Although the nature of solvent greatly affects the catalytic activity, it seems not influent the enantioselectivity of enzyme ($E > 200$). The enantioselectivity of the enzymes seems to be independent of organic solvent with log *P* values from 1.4 to 4.5. The enzymes are very selective in the aliphatic, branch, cyclic and aromatic solvents. This result is not in good agreement with the finding of previous researches [13]. They reported that the enantioselectivity of *Pseudomonas cepacia* lipase was influenced by organic solvent. Isooctane was used in the following study as it exhibited the highest initial reaction rate among the commonly used organic solvents.

3.2. Effect of water content

It is desirable to determine the optimum level of water content in the resolution. However, this is hard to predict in term of water content by weight or by volume. This is because the reaction mixture contains at least two distinct phases, where water was distributed between them. The water content in organic solvent may present as free water in bulk phase and as bound water on the surface of enzyme particles. The volume ratio of these phases is also changing during the reaction. Therefore, it is enough to study the effect of water added on the enzyme kinetics.

The effects of water content on the enzyme activity as well as enantioselectivity were investigated by using two different direct contact pre-equilibration methods [20]. The method of pre-equilibrating the reaction solution with water was carried out in order to investigate the enzyme performance in the reaction medium with different initial water content. The enzymes were initially not affected by the water content in this method.

The study of pre-equilibrating enzyme with different initial water content is to investigate the water effect on the enzyme itself. This study is essential as water affects the conformation of lipases, which is required for enzyme catalytic action.

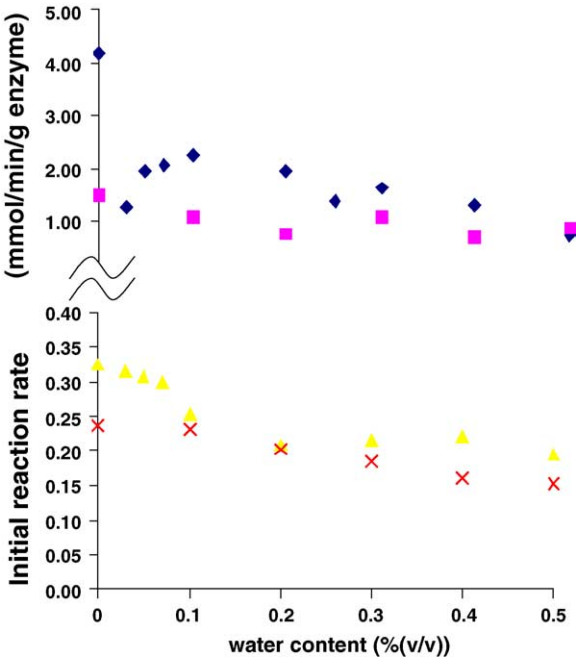

Fig. 2. Initial velocities of reactions at different initial water contents. ChiroCLEC-PC: (♦) substrates pre-equilibrium, (□) enzyme pre-equilibrium. Chirazyme L2, c.f., C3, lyo: (▲) substrates pre-equilibrium, (×) enzyme pre-equilibrium.

The activity of the enzymes was greatly influenced by the initial water content in the reaction mixture. Although the data points in Fig. 2 were scattered, the results seem following a decreasing trend when the initial percentage of water content was increased from 0.1 to 0.5% (v/v). Beyond the value of 0.5% (v/v), the suspended enzyme particles tend to aggregate together. This phenomenon would lead to diffusional limitation, which would also make the study of water effect more complicated. A sharp decrease in the reaction rate could be observed when the initial water content was increased up to 2% (v/v). For example, the initial reaction rate was decreased from 4.17 to 0.65 mmol/min/g enzyme at 2% (v/v) of initial water content catalyzed by ChiroCLEC-PC (data not shown).

As the water content increased, the amount of free water in the bulk phase of solution was also increased. Thus, the reaction is favourable toward reverse direction since this esterification reaction is reversible. Furthermore, bound water is more difficult to expel from the enzyme for the reaction to take place. The solution was already saturated with water; it is difficult for the repulsion of water molecules into the bulk phase. This explanation described the decrease in the reaction rate when the water content was increased.

Theoretically, 0.18% (v/v) of water was produced if 25 mM of (*R*)-1-phenylethanol reacted. However, only 0.015–0.025% (v/v) of water detected after reaction. The rest of water may be adhered to the wall of the reaction vessel because of surface tension. Some of the water molecules may also be bound to the enzyme particles. The reason is given based on the decrease in the performance of recycled enzymes. Most probably, the optimum level of water to preserve the catalytically active conformation of the enzyme was changed in the reused enzymes.

The degree of water effect was greater for the method of pre-equilibrating enzyme with water. The pre-equilibrated enzymes lost their catalytic activity more significantly. For the enzyme base comparison, the water effect was also more significant on the performance of ChiroCLEC-PC. The activity of the enzyme decreased significantly as the initial water content increased (Fig. 2).

As found with monomeric protein catalysts, cross-linked enzyme crystals require a small amount of water for activity when used in neat organics [21]. The amount of water required for high activity in non-polar organic solvents such as isooctane is typically low. However, the complete absence of water will result in the loss of enzyme selectivity and activity. A completely dehydrated enzyme is not catalytically active. When pre-equilibrating with additional water, the three-dimensional structure of the enzyme was altered since the enzyme itself contains 2–3% of bound water [21]. The bound water was the essential water that retained the enzymes in an ionization state where the enzymes could perform at maximum activity.

Chirazyme L2, c.f., C3, lyo itself contains less than 5% of water according to the specification given [22]. This water acts as water buffer, so that Chirazyme L2, c.f., C3, lyo is not sensitive to the water added in the experiments. Malcata et al. [23] found that the essential water is tightly bound to the *Porcine pancreatic* lipases but loosely bound to the yeast lipases. This observation is also agree with the result reported that water activity did not influent the activity and enantioselectivity of *Candida antarctica* lipase B as severely as most other lipases [24].

Therefore, both enzymes, ChiroCLEC-PC and Chirazyme L2, c.f., C3, lyo could perform at maximum activity without the addition of water in both pre-equilibrium methods. Letgeb and Knez [25] reported that the optimum amount of added water was very dependent on the temperature of esterification. They reported that immobilized lipases from *Mucor miehei* (Lipozyme) exhibited the highest activity in the synthesis of *n*-butyl oleate without the addition of water at 50 °C.

ChiroCLEC-PC and Chirazyme L2, c.f., C3, lyo used in this study are absolutely selective as their enantioselectivity value ($E > 200$) are not affected by the water content. As reported by Hogberg et al. [26], the enantioselectivity value of *Pseudomonas* lipase was independent of water activity. Edlund et al. [27] also reported that the enantioselectivity of immobilized enzyme was much less sensitive to water activity than crude enzyme. Water would only participate in the enantioselective step of reaction when the acyl part of the substrate is chiral [28]. This is because water is a competitive nucleophile for the acyl enzyme intermediate.

3.3. Effect of glycerol

Since the effect of water content was not favorable, a water mimic compound such as glycerol was introduced in the

Table 2
Comparison of initial reaction rates in the presence of water and glycerol

Enzyme (method)	Initial reaction rate (mmol/min/g enzyme)[a]		
	No additive	Water (0.1%, v/v)	Glycerol (0.1%, v/v)
ChiroCLEC-PC (substrates pre-equilibrium)	4.17 ± 0.67	2.25 ± 0.81	1.19 ± 0.64
ChiroCLEC-PC (enzyme pre-equilibrium)	1.50 ± 0.33	1.08 ± 0.43	0.47 ± 0.21
Chirazyme L2, c.f., C3, lyo (substrates pre-equilibrium)	0.32 ± 0.08	0.25 ± 0.09	0.37 ± 0.11
Chirazyme L2, c.f., C3, lyo (enzyme pre-equilibrium)	0.24 ± 0.06	0.23 ± 0.08	0.26 ± 0.10

[a] $X \pm Y$: X denotes for mean of triplicate data and Y denotes for standard deviation from triplicate data.

Table 3
Comparison of enzyme performance in the presence of molecular sieve

Enzyme (method)	Initial reaction rate (mmol/min/g enzyme)[a]		Final conversion (%)[a]		Equlibrium time (min)[a]	
	Blank	Mol. sieve	Blank	Mol. sieve	Blank	Mol. sieve
ChiroCLEC-PC (substrates pre-equilibrium)	4.17 ± 0.67	4.26 ± 0.55	46.9 ± 0.4	49.4 ± 0.2	320 ± 30	200 ± 40
ChiroCLEC-PC (enzyme pre-equilibrium)	1.50 ± 0.33	1.97 ± 0.43	45.8 ± 0.5	49.1 ± 0.2	250 ± 18	200 ± 25
Chirazyme L2, c.f., C3, lyo (substrates pre-equilibrium)	0.33 ± 0.08	0.32 ± 0.06	49.1 ± 0.4	49.5 ± 0.3	260 ± 10	35 ± 5
Chirazyme L2, c.f., C3, lyo (enzyme pre-equilibrium)	0.24 ± 0.06	0.41 ± 0.03	46.5 ± 0.2	49.3 ± 0.4	230 ± 21	35 ± 8

[a] $X \pm Y$: X denotes for mean of triplicate data and Y denotes for standard deviation from triplicate data.

reaction system. The study of glycerol effect was carried out by using the previous mentioned pre-equilibrium methods.

The role of glycerol was similar to water which acts as a hydrogen bond forming additive with the functional group of enzyme molecules [29]. It is a polyol with a higher propensity for forming hydrogen bonds compared to ethylene glycol and formamide.

Many studies used ethylene glycol and formamide as water mimicking compounds [30–32]. They found that the activity and enantioselectivity of lipases could be enhanced by the addition of these compounds. Gutman et al. [33] explained that water plays two distinct functions in the enzyme catalytic reactions. Only part of the water can be replaced by water-mimicking compound. The replacement not only increase the enzyme activity, but also the enantioselectivity of the lipases. Gubicza and Szakacs-Schmidt [30] reported that the reaction rate increased by approximately 30% when half of optimum water content was replaced by ethylene glycol in the enantioselectivity esterification of 2-substituted propionic acid. While the unreplacement portion of the water is presumeably the monomolecular water layer covering the enzyme.

The presence of glycerol (0.1%) in the resolution decreased the reaction rate of ChiroCLEC-PC catalyzed resolution. The degree of glycerol effect was more significant in reducing the reaction rate compared to the same percentage of additional water added into the reaction system (Table 2).

However, Chirazyme L2, c.f., C3, lyo was found to be more resistant toward the effect of either water or glycerol in 0.1% (v/v). The performance of the enzyme slightly increased when 0.1% (v/v) of glycerol was added but slightly decreased when 0.1% (v/v) of water was added into the reaction system. However, the increase or decrease of initial reaction rates was not significant. Overall, the addition of glycerol was not favourable in both enzymes catalyzed resolution.

3.4. Effect of molecular sieve

The addition of 10% (w/v) of molecular sieve 3 Å, $K_{12}[(AlO_2)_{12}(SiO_2)_{12}]\cdot xH_2O$ into the reaction solution would increase the enzyme performance in both pre-equilibrium methods. The increase of enzyme performance in term of initial reaction rate was not significant (Table 3). It was likely that the amount of water present in the reaction system was negligible initially. The water content increased gradually as the reaction proceeded.

The final conversion value was increased in the presence of molecular sieve. Water produced during the reaction was removed by molecular sieve. The removal of water had been reduced the problem of water inhibition. Therefore, the reaction could achieve higher final conversion value in shorter reaction time, especially for Chirazyme L2, c.f., C3, lyo catalyzed resolution.

In term of method comparison, the pre-equilibrium of substrates with molecular sieve has higher initial reaction rate, which is significantly shown by ChiroCLEC-PC catalyzed reactions. However, the decrease of initial reaction rate in the enzyme pre-equilibrium method was not mainly due to the effect of molecular sieve on the enzyme. ChiroCLEC-PC might less resistant towards the stirring effect over a long period of time. The reason is given because the initial reaction rate of the enzyme pre-equilibrium method was also decreased in the blank experiment.

4. Conclusion

The chiral resolution of (*R*, *S*)-1-phenylethanol catalyzed by immobilized lipases such as ChiroCLEC-PC and Chirazyme L2, c.f., C3, lyo was more favourable in the organic solvent with higher log *P* value. The activity of the enzymes

was significantly decreased in toluene. The reason for this observation was mainly due to the π–π electron interaction between the aromatic rings of toluene and substrate. Therefore, (*R*, *S*)-1-phenylethanol was more favourable to stay in the bulk phase of solvent instead of bound to the enzymes for reactions. The observation was obvious especially for ChiroCLEC-PC catalyzed resolutions.

The addition of initial water content into the reaction mixture could not increase the enzymes performance in both pre-equilibrium methods. The initial reaction rates of the resolution were decreased when 0.1–0.5% (v/v) of water added into the reaction mixture. The effect of glycerol as water mimicking additive was also unfavourable in this resolution. However, the reaction performance was increased when 10% (w/v) of molecular sieve 3 Å was added into the reaction mixture. In addition to initial reaction rate and final conversion value, the reaction time required for the resolution was reduced significantly.

References

[1] Berglund P. Controlling lipase enantioselectivity for organic synthesis. Biomol Eng 2001;18:13–22.

[2] Kamiya N, Goto M. How is enzymatic selectively of menthol esterification catalyzed by surfactant-coated lipase determined in organic media? Biotechnol Prog 1997;13:488–92.

[3] Janssen AEM, van der Padt A, van Sonsbeck HM, van't Riet K. The effect of organic solvents on the equilibrium position of enzymatic acylglycerol synthesis. Biotechnol Bioeng 1993;41:95–103.

[4] Carta G, Gainer JL, Gibson ME. Synthesis of esters using a nylon immobilized lipase in batch and continuous reactors. Enz Microb Technol 1992;14:904–10.

[5] Butler LG. Enzymes in non-aqueous solvents. Enz Microb Technol 1979;1:253–9.

[6] Cotton FA, Wilkinson G. Advanced inorganic chemistry: a comprehensive text. 4th ed. New York: John Wiley and Sons; 1980.

[7] Chen CS, Sih CJ. General aspects and optimization of enantioselective biocatalysis in organic solvents: the use of lipases. Angew Chem Int Ed Engl 1989;28:695–707.

[8] Kuo SJ, Parkin KL. Solvent polarity influences product selectivity of lipase-mediated esterification reactions in microaqueous media. J Am Oil Chem Soc 1996;73(11):1427–33.

[9] Klibanov AM. Enzymatic catalysis in anhydrous organic solvents. Trends Biochem Sci 1989;14:141–4.

[10] Chua LS, Sarmidi MR. Immobilized lipase-catalyzed resolution of (*R*, *S*)-1-phenylethanol in recirculated packed bed reactor. J Mol Catal B: Enz 2004;28:111–9.

[11] Persson M, Costes D, Wehtje E, Adlercreutz P. Effects of Solvent, water activity and temperature on lipase and hydroxynitrile lyase enantioselectivity. Enz Microb Technol 2002;30:916–23.

[12] Wu WH, Akoh CC, Philips RS. Lipase-catalyzed stereoselective esterification of D,L-menthol in organic solvent using acid anhydrides as acylating agents. Enz Microb Technol 1996;18:536–9.

[13] Wehtje E, Costes D, Adlercreutz P. Enantioselectivity of lipases: effects of water activity. J Mol Catal B: Enz 1997;3:221–30.

[14] Garros SB, Razafindramboa N, Pavia AA. Synthesis of (*z*)-3-hexen-1-yl butyrate in hexane and solvent free medium using *Mucor miehei* and *Candida antarctica* lipases. J Am Oil Chem Soc 1997;74(11):1471–5.

[15] Wu WH, Akoh CC, Philips RS. Stereoselective acylation of D.L-menthol in organic solvents by an immobilized lipase from *Pseudomonas cepacia* with vinyl propionate. J Am Oil Chem Soc 1997;74(4):435–9.

[16] Wu WH, Akoh CC, Philips RS. Lipase-catalyzed stereoselective esterification of D.L-menthol in organic solvent using acid anhydrides as acylating agents. Enz Microb Technol 1996;18:536–9.

[17] Perraud R, Laboret F. Optimization of methyl propionate production catalyzed by *Mucor miehei* lipase. Appl Microbiol Biotechnol 1995;44:321–6.

[18] Secundo F, Riva S, Carrea G. Effects of medium and reaction conditions on the enantioselectivity of lipase in organic solvents and possible rationales. Tetrahedron: Asymmetry 1992;3(2):267–80.

[19] Kamiya W, Goto M, Wakashio F. Surfactant-coated lipase suitable for the enzymatic resolution of menthol as a biocatalyst in organic media. Biotechnol Prog 1995;11:270–5.

[20] Kvittingen L. Some aspects of biocatalysis in organic solvents. Tetrahedron 1994;50:8253–74.

[21] Altus Biologics, Inc. ChiroCLEC-P.C. Cambridge: Information Booklet. Version: 2.0, 1999.

[22] Roche Diagnostics GmbH. Chirazyme L2, c.f., C3, lyo. Mannheim: Cat. No. 11835807103, 2001.

[23] Malcata FX, Reyes HR, Garcia HS, Hill Jr CG, Amundson CH. Kinetics and mechanisms of reactions catalyzed by immobilized lipases. Enz Microb Technol 1992;14:426–46.

[24] Rotticci D. Understanding and engineering the enantioselectivity of Candida antarctica lipase B towards sec-alcohols. Swedish: Royal Institute of Technology; 2000.

[25] Leitgeb M, Knez Z. The influence of water on the synthesis of *n*-butyl oleate by immobilized *Mucor miehei* lipase. J Am Oil Chem Soc 1990;67(11):775–8.

[26] Hogberg HE, Edlund H, Berglund P, Hedenstrom E. Water activity influences enantioslelectivity in a lipase-catalyzed resolution by esterification in an organic solvent. Tetrahedron: Asymmetry 1993; 4(10):2123–6.

[27] Edlund H, Berglund P, Jensen M, Hedenstrom E, Hogberg HE. Resolution of 2-methylalkanoic acids catalyzed by *Candida rugosa* lipase. Acta Chem Scandavica 1996;50:666–71.

[28] Janssen AEM, van der Padt A, van Sonsbeck HM, van't Riet K. The effect of organic solvents on the equilibrium position of enzymatic acylglycerol synthesis. Biotechnol Bioeng 1993;41:95–103.

[29] Zaks A, Klibanov AM. The effect of water on enzyme action in organic media. J Biol Chem 1988;263:8017–21.

[30] Gubicza L, Szakacs-Schmidt. Effects of the reaction conditions on the enantioselective esterification using *Candida cynlindracea* lipase. Biocatalysis 1994;9:131–43.

[31] Kitaguchi H, Itoh I, Ono M. Effects of water and water-mimicking solvents on the lipase-catalyzed esterification in an apolar solvent. Chem Lett 1990:1203–6.

[32] Gubicza L, Kelemen-Horvath I. Effect of water-mimicking addtives on the synthesis activity and enantioselectivity in organic solvents of a *Candida cynlindracea* lipase. J Mol Catal 1993;84:L27–32.

[33] Gutman AL, Brenner D, Boltanski A. Convenient practical resolution of racemic alkyl–aryl alcohols via enzymatic acylation with succinic anhydride in organic solvents. Tetrahedron: Asymmetry 1993;4(5):839–44.