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Enzymatic esterification of DL-menthol with propionic acid by lipase from *Candida cylindracea*

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Abstract: This work deals with the resolution of DL-menthol with propionic acid by *Candida cylindracea* lipase (Ccl) in organic solvent reaction systems and a reverse micelles system of sodium 1,4-bis (2-ethylhexyl) sulfosuccinate (AOT). The activity and stability as well as enantioselectivity of the lipase in two systems were studied. The results indicate that the lipase showed higher stability in reverse micelles than in organic solvent, which proved that the reverse micelles system has potential application for maintaining the activity of the enzyme for a long time. This is because lipase molecules can be entrapped in water-containing micro-drops of reverse micelles, avoiding direct-contract with unfavorable organic medium. The enantioselectivity ($E > 30$, $ee_p = 92.5$) in the two systems is relatively high, although the conversion is moderate. The influence of the characteristic parameters of the two systems, such as pH, temperature, w_0 (molar ratio of water to AOT in reverse micelles systems) and water content (organic solvent) on the conversion of DL-menthol was also investigated.

Keywords: enzymatic; resolution; DL -menthol; reverse micelles

INTRODUCTION

In recent years, extensive studies have been carried out on enzyme-containing reverse micelles systems.[1–5] Reverse micelles are thermodynamically stable, nanometer-sized water droplets dispersed in an organic phase by means of surfactants. One of the most important properties of reverse micelles is their ability to entrap enzymes and other biomolecules into their water pools, which avoids direct contact with organic solvents that potentially denature the enzyme.[6] The very large aqueous/organic interface in reverse micelles is also of great technological interest because it leads to an increase in the number of substrate molecules available to react. Due to the above-mentioned properties, the activity and stability of lipase in reverse micelles will be enhanced as compared with organic solvent.

Nowadays there is an increased demand for optically pure enantiomers as building blocks for pharmaceutical and agrochemicals.[7] One such compound is L-menthol. Because of its cooling and refreshing effect, L-menthol is an important fragrance and flavor compound and is used widely in cosmetics, toothpaste, chewing gums, cigarettes, sweets and medicines. The esters of L-menthol can be expected to have interesting characteristics, such as fragrance which is emitted as the ester bond is hydrolyzed.[8] The chiral menthol esters have been used in asymmetric synthesis.[8,9]

Enantiomeric resolution of L-menthol and preparation of L-menthol esters from racemic DL-menthol are problems of interest to the flavor and fragrance industries. *Candida rugosa* lipase (Crl)-catalyzed esterification of racemic menthol in organic solvents and emulsions has been investigated extensively.[10–15] Orlich *et al*[10] have studied the Crl-catalyzed reactions in nonionic W/O-microemulsions with a technical surfactant, Marlipal O13-60. Wang *et al*[11] have studied the factors affecting the resolution of DL-menthol by immobilized lipase-catalyzed esterification in organic solvent. Kahlow *et al*[12] have studied a model of the pressure dependence of the enantioselectivity of Crl towards (+/−)-menthol. Babali *et al*[13] have studied the enzymatic esterification of (−)-menthol with fatty acids in solvent by a commercial lipase from *Candida rugosa*. They also studied the enzymatic esterification of (−)-menthol with lauric acid in isooctane by sorbitan monostearate-coated lipase from Crl. Shimada *et al*[14] have studied the enzymatic synthesis of L-menthyl esters in an organic solvent-free system. But to date, few studies have been carried out on the resolution of DL-menthol in reverse micelles systems.

In this work, we selected a less expensive *Candida cylindracea* lipase (Ccl) which has never been used in the resolution of DL-menthol. The surfactant

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we selected in our work was sodium 1,4-bis (2-ethylhexyl) sulfosuccinate (AOT), which is readily obtained and is relatively cheap. We investigated the esterification of racemic menthol with propionic acid in reverse micelles systems and in an *n*-hexane/water mixture using *Candida cylindracea* lipase (Ccl) as the biocatalyst. The aim of our work was to investigate the difference in the stability and activity of Ccl in these two systems and to prove the superiority of the reverse micelles system for resolution. The conversion of DL-menthol to its propionate was measured as a function of buffer pH, water content and temperature, and optimum conditions (ie highest yield) were identified. Our experiments proved that under optimum reaction conditions, the activity and stability of Ccl were found to be greater in reverse micelles than in *n*-hexane.

MATERIALS AND METHODS

Chemicals

Sodium 1,4-bis (2-ethylhexyl)sulfosuccinate(AOT, ~96%purity) was purchased from ACROS organics (New Jersey, USA); *n*-hexane (AR ≤95.0%) was purchased from Beijing Beihua Fine Chemicals Co Ltd; lipase from *Candida cylindracea* was purchased from Fluka; propionic acid (AR ≤99.5%) was purchased from Tianjin Chemical Reagent Co Inc; DL-menthol (99%) was purchased from Alfa Aesar.

Reaction in organic solvent system

The DL-menthol (50 mmol) and propionic acid (100 mmol) was added to the hexane (10 mL) in a 50 mL sealed flask, then a buffer solution of suitable concentration (pH = 6 − 9) was added and the final water content was adjusted by the addition of the required amount of the buffer solution. Finally the required amount of lipase was added to initiate the reaction. The reaction mixture was stirred continuously and kept at a constant temperature in the water bath at various temperatures. After some time, samples (200 μL) were withdrawn from the reaction medium, dissolved with ethanol to stop any enzymatic reaction then analyzed subsequently by gas chromatography (GC).

Reaction in reverse micelles system

Reverse micelles were typically prepared by the addition of a known volume of phosphate buffer at a known pH value into a hexane (10 mL) solution containing 100 mmol surfactant. The mixture was briefly shaken until an optically clear single-phase solution was formed. The DL-menthol (50 mmol) and propionic acid (100 mmol) was then added to 10 mL AOT/hexane reverse micelles in a 50 mL sealed flask, finally the desired amount of lipase was added. The reaction mixture was stirred in a constant temperature reactor at various temperatures. After some time, samples (200 μL) were withdrawn from the reaction medium, dissolved with ethanol to stop any enzymatic reaction then analyzed subsequently by GC.

Chromatographic Analysis

The progress of the reactions was monitored using a Agilent 6890 Gas Chromatograph (FID) equipped with an HP-chiral 30 m × 0.25 mm × 0.25 μm capillary column. The temperature program used begins at 65 °C with an isothermal hold at 65 °C for 1 min, then is increased to 170 °C at a gradient of 5 °C min^{-1}; the carrier gas is hydrogen and the velocity of the carrier gas:39cm s^{-1}.

RESULTS AND DISCUSSION

Comparation of the stability of lipase in organic media and AOT/hexane reverse micelles systems

The stability of the lipase in the two systems was indicated by the change in the conversion reaction over time. The progress curves are shown in Fig 1. Figure 1 shows that the conversion in reverse micelles increased faster than that in the organic system and it was higher than that in the organic system after 3 days. The conversion reaction in the organic system remained steady at about 15.7% after 7 days, whereas in the reverse micelles system, the conversion increased up to 31.8% in 20 days and remained steady after that. This indicates that the stability was enhanced in the reverse micelles systems as compared with the organic media. This may be due to the reverse micelles being useful as microreactors for enzymatic reactions.[16,17] The catalytic reaction occurs because of the large internal interface between water and the continuous oil or because of the exchange of water within the droplets, the aqueous interior of the reverse micelles serving as a host for the water-soluble enzyme[10] which can maintain the activity of the enzyme for a long time; in the organic solvent containing a lower water content, the reaction proceeded very quickly in the first 3 days, but after that the degree of conversion of the reaction was nearly unchanged, which demonstrates

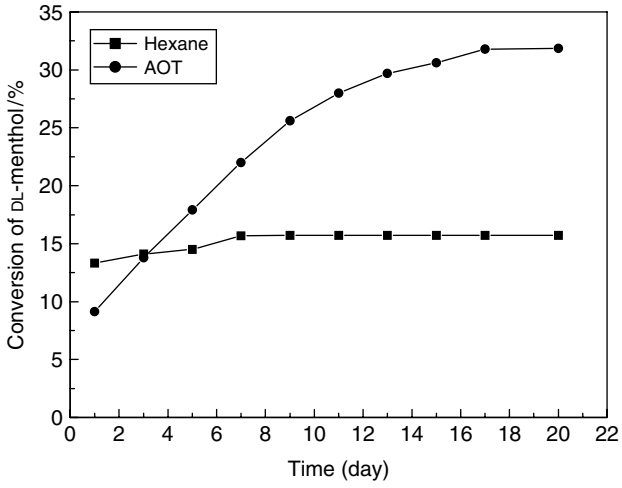

Figure 1. Progress curves of enzymatic esterification of DL-menthol. The reaction was performed in hexane (or reverse micelles)at 1:2 mole ratio of DL-menthol (50mmol) to propionic acid at 45 °C (solvent: water content 4uL, pH = 6.5; AOT/hexane reverse micelles: water content w_0 = 6, pH = 7.5).

that the reaction had been completed in a relatively short time, this is because lipase in this system keeps in direct touch with the organic solvent, which can deactivate the enzyme in a short time.

In the first 3 days the conversion in reverse micelles was lower than that in organic solvent. The inhibition against enzymatic activity that occurred in reverse micelles systems was observed in this work. Such inhibition has been caused by the strong electrostatic interaction that exists between the enzyme and polar head group of the ionic amphiphilic in the reaction system.[18]

Enantioselectivity of lipase in AOT/hexane reverse micelles systems and organic media

Enantiomeric excess is an index of optical purity and defined by eqn (1):

$$ee = \frac{|C_S - C_R|}{C_S + C_R} \tag{1}$$

where C_S and C_R are the concentrations of the (*S*)-isomer and the (*R*)-isomer in the mixture, respectively. The enantioselectivity of the enzyme can be expressed by the enantiomeric ratio E defined by eqn (2), and it is expressed by eqn (3) for irreversible biocatalytic systems:

$$E = \frac{\ln(C_S/C_{S0})}{\ln(C_R/C_{R0})} \tag{2}$$

$$E = \frac{\ln[1 - c(1 + ee_p)]}{\ln[1 - c(1 - ee_p)]} = \frac{\ln[(1 - c)(1 - ee_s)]}{\ln[(1 - c)(1 + ee_s)]} \tag{3}$$

where C_{S0} and C_{R0} are the initial concentration of the (*S*)-isomer and the (*R*)-isomer before the enzymatic reaction and c is the degree of reaction conversion, and ee_p and ee_s are enantiomeric excess of the product and the substrate, respectively.

The relationship between ee_p (and E) and reaction time is shown in Figs 2 and 3. Figures 2 and 3 show that the enantioselectivities (E and ee_p) for both AOT/hexane reverse micelles and hexane organic solvent are similar and the ee_p values in the two systems are relatively high. They also show that the ee_p and E decreased over time. This is because the concentration of substrate decreased over time and according to the formula $v = kc$ (v: the velocity of the reaction; k: the constant of the reaction velocity; c: the concentration of the substrate), the concentration of L-menthol, which reacted faster than D-menthol in this system, will decrease faster than D-menthol. So the enantioselectivity of system will decrease over time. But at the end both the ee_p and E are relatively high. So the resolution of DL-menthol with propionic acid by Ccl can perform successfully in two systems. *Candida cylindracea* lipase is an excellent catalyst for preparing optically pure L-menthol. The effects of pH, temperature, w_0 (for AOT/hexane reverse micelles), water content (for hexane solvent) on the E or ee_p were found to be very small. So, subsequently we only discussed the effect of pH, temperature, w_0 (molar ratio of water to AOT in reverse micelles systems; for AOT/hexane reverse micelles), water content (for hexane solvent) on the conversion reaction.

Effect of temperature on the esterification

The effects of different temperatures on the esterification are shown in Fig 4. The two curves followed rather bell-shaped profiles. For reactions in organic media and AOT/hexane reverse micelles systems, at the lower temperatures, the velocity of reaction increased with increasing temperature so the conversion was enhanced, which obeys the dynamic reaction law. In addition when the temperature is low, the number of active molecules in the reaction system is small, and the amount of conversion is low. However increasing the temperature above 45 °C resulted in a sharp decrease in the conversion. This could be attributed to the fact that it was difficult for the enzyme to keep its active structure when the temperature was high. In addition, for the reverse micelles

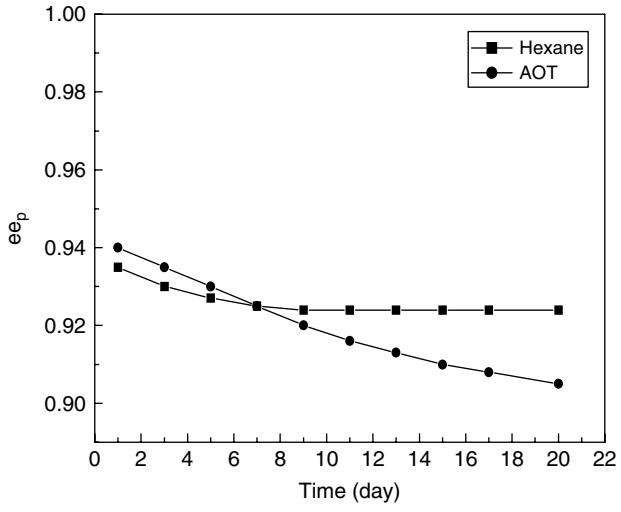

Figure 2. Relationship between ee_p and reaction time.

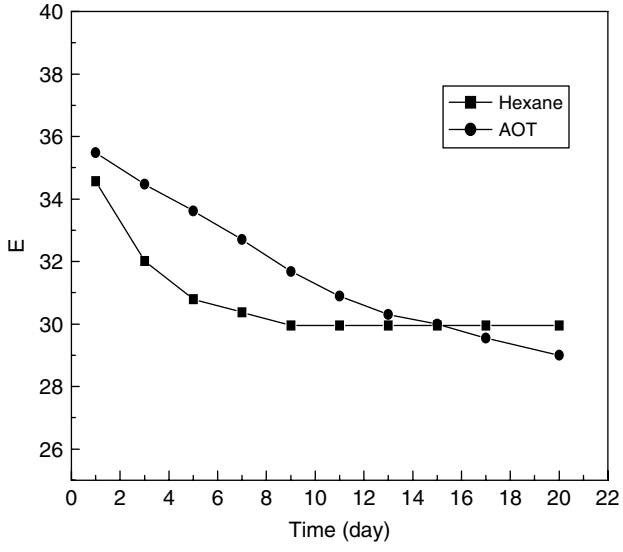

Figure 3. Relationship between E and reaction time.

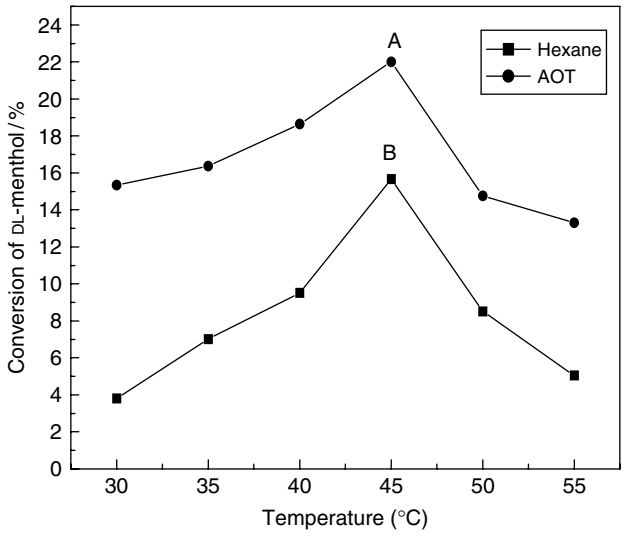

Figure 4. Effect of temperature on the esterification. The reaction was performed in hexane or reverse micelles at 1:2 mole ratio of DL-menthol (50mmol) to propionic acid for 7 days (organic solvent: pH = 6.5, water content: 4uL; AOT/hexane: pH = 7.5, $w_0 = 6$).

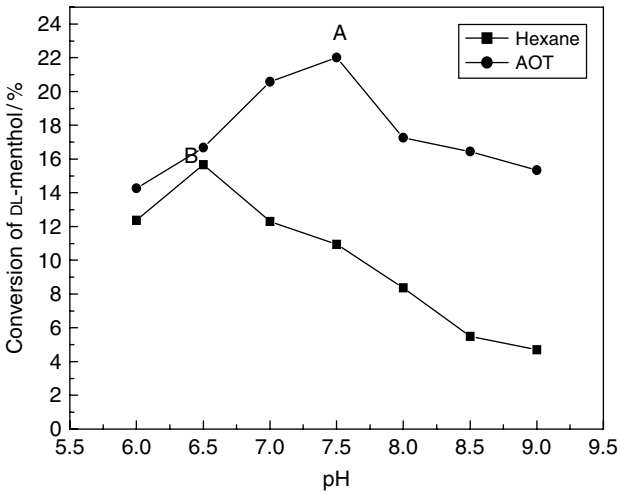

Figure 5. Effect of pH on the esterification. The reaction was performed in hexane or reverse micelles at 1:2 mole ratio of DL-menthol (50mmol) to propionic acid at 45 °C for 7 days (organic solvent: water content: 4uL; AOT/hexane: $w_0 = 6$) A: $ee_p = 0.925$ E = 32.7; B: $ee_p = 0.925$ E = 30.37.

system, because of molecular thermal movement, the reverse micelles system will become unstable with the temperature increase, then the activity of the enzyme in the unstable reverse micelles system will decrease. From Fig 4, it is evident the optimum temperature for keeping the active structure of the lipase in the two systems is 45 °C.

Effect of pH of buffer solution on the esterification

The dependence of conversion on pH is shown in Fig 5. The optimum pH for the lipase reaction has been determined in the different systems. For reactions in *n*-hexane organic media a sharp increase at pH 6.5 was observed. For reactions in AOT/hexane reverse micelles systems the optimum conversion was at pH 7.0–7.5. Lipase in these systems seems to have high activity in the neutral pH range. This is because the enzyme is a kind of protein; the neutral condition will thus suit the activity of the enzyme. However this reaction is reversible and acidic conditions will accelerate the production of ester. The curves are the result of the two functions working together.

Effect of water content on the conversion of esterification

The water content in reverse micelles has been reported to be an important parameter for enzymatic reactions.[6,19,20] In this paper the water content in reverse micelles is indicated by the molar ratio of water to surfactant in organic phase ($w_0 = [H_2O]/[\text{Surfactant}]$). In AOT/hexane reverse micelles systems, the reaction conversion was dependent on the water content and reached a maximum at a water content of around $w_0 = 6$ as shown in Fig 6. The curve is bell-like. For reaction in reverse micelles systems the initial amount of water is bound to the polar head of the surfactant molecule till it is fully saturated,[16,21]

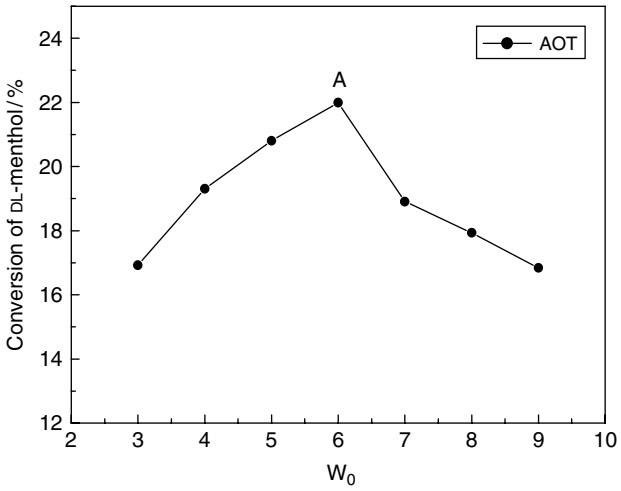

Figure 6. Effect of water content on the esterification. The reaction was performed in reverse micelles at 1:2 mole ratio of DL-menthol (50mmol) to propionic acid at 45 °C for 7 days (AOT/hexane: pH = 7.5) A: $ee_p = 0.925$ $E = 32.7$.

thus they can provide the appropriate hydrophilic environment for catalytic activity of esterification.[17] When the w_0 is small, the water content of the reaction system is low so a large amount or all of water is bound to the surfactant and the amount of free water available to maintain the activity of the enzyme is low, hence the activity of the enzyme is low. The amount of free water increases as the w_0 increases, so the activity of the enzyme also increases. But when the w_0 is too high, not only the internal interfaces decrease but also the structure of the enzyme is too loose. In addition, too much water will shift the equilibrium to the reverse direction.

The effect of water content on the conversion in organic media is shown in Fig 7. The curve is bell-shaped. It has been observed that the conversion increased with an increase in water content whereas the

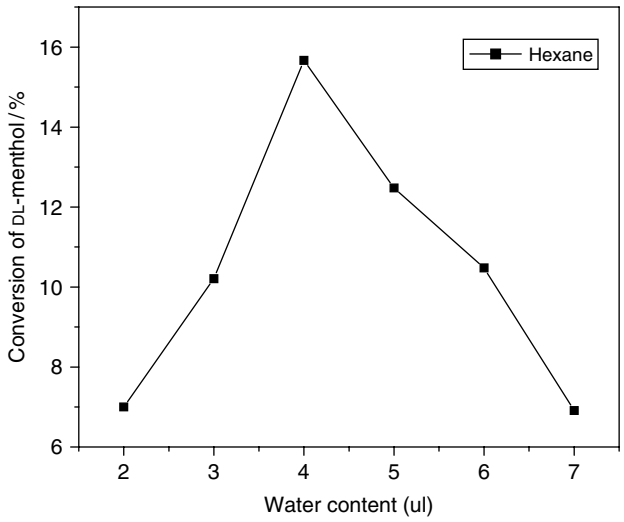

Figure 7. Effect of water content on the esterification. The reaction was performed in hexane at 1:2 mole ratio of DL-menthol (50mmol) to propionic acid at 45 °C (pH = 6.5) for 7 days; *B*: $ee_p = 0.925$ $E = 30.37$.

highest conversion was observed at around 0.04% and then decreased with an increase in water content. This is because water is necessary for the catalytic activity of Ccl because water is involved in the formation of oxyanion holes with the polar residues of the catalytic site. When the water content of system is low, there is not enough water to maintain the active structure of the enzyme, and the enzyme is too rigid to exert all its activity. As the water content increases, the activity of the enzyme increases; when the water content is too high, a large amount of enzyme is embedded by the water and cannot keep in touch with the substrate, which means that the enzyme cannot exert all its activity. Too high a water content will accelerate the decomposition of ester.

Both Figs 6 and 7 are bell-shaped, this shows that with a suitable water content of the two reaction systems the lipase will exert the optimal activity in the optimal water content.

CONCLUSIONS

In this work, we have studied the enantioselectivity, activity and stability of a lipase in reverse micelles and in organic solvent. The results demonstrated that both in reverse micelles and organic solvent media, relatively high enantioselectivities were obtained, which proved that *Candida cylindracea* lipase is an excellent catalyst for preparing optically pure L-menthol, but conversion is moderate. The results also showed the lipase showed higher stability in reverse micelles than in organic solvent, this should be attributed to the formation of reverse micelles. Because the aqueous interior of the reverse micelles can entrap water-soluble enzyme, this means that the enzyme cannot contact with unfavorable organic medium directly. This proved that the reverse micelles system has potential application for maintaining the activity of the enzyme for a long time. The reaction parameters (the temperature, pH of buffer and water content) that influenced the enzymatic resolution of DL-menthol in the two systems was also studied, these parameters were found to have profound effects on the conversion reaction. Based on the present study, we also confirmed the optimum reaction conditions for the resolution of DL-menthol in two systems, the maximum conversion (15.7%) was obtained at pH 6.5, water content 0.04%, temperature 45 °C, time 20days. For reactions in AOT/hexane reverse micelles systems the maximum conversion (31.87%) was obtained at pH 7.5, $w_0$6, temperature 45 °C, time 20days.

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REFERENCES

1 Klyachko NL and Levashov AV, Bioorganic synthesis in reverse micelles and related systems. *Curr Opin In Collo Int* **8**:179–186 (2003).
2 Nagayama K, Nishimura R, Doi T and Imai M, Enhanced recovery and catalytic activity of *Rhizopus delemar* lipase in an AOT microemulsion system with guanidine hydrochloride, *J Chem Technol Biotechnol* **74**:227–230 (1999).
3 Franueville E, Loutrari H, Mellou F, Stamatis H, Friboulet A and Kolisis FN, Reverse micelles, a system for antibody-catalysed reactions, *J Mol Catal B: Enzymatic* **21**:15–17 (2003).
4 Das D and Das PK, Improving the lipase activity profile in cationic water-in-oil microemulsions of hydroxylated surfactants, *Langmuir* **19**:9114–9119 (2003).
5 Muralidhar RV, Marchant R and Nigam P, Lipases in racemic resolutions, *J Chem Technol Biotechnol* **76**:3–8 (2001).
6 Hashimoto Y, Ono T, Goto M and Hatton TA, Protein refolding by reversed micelles utilizing solid-liquid extraction technique, *Biotechnol Bioeng* **57**:620–623 (1998).
7 Persson M, Costes D, Wehtje E and Adlercreutz P, Effects of solvent, water activity and temperature on lipase and hydroxynitrile lyase enantioselectivity, *Enzyme Microb Technol* **30**:916–923 (2002).
8 Athawale V, Manjrekar N and Athawale M, Enzymatic synthesis of chiral menthacrylate monomer by *pseudomonas cepacia* lipase catalyzed resolution of (±)-menthol, *J Mol Catal B: Enzymatic* **16**:169–173 (2001).
9 Impastat FJ, Barash L and Walborsky HM, Partial asymmetric synmmetric synthesis in the addition of diphenyldiazomethane to olefins, *J Am Chem Soc* **81**:1514–1515 (1959).
10 Orlich B and Schomäcker R, *Candida rugosa* lipase reactions in nonionic w/o-microemulsion with a technical surfactant. *Enzyme Microb Technol* **28**:42–48 (2001).
11 Wang DL, Nag A, Lee GC and Shaw JF, Factors affecting the resolution of DL-menthol by immobilized lipase-catalyzed esterification in organic solvent, *J Agr Food Chem* **50**:262–265 (2002).
12 Kahlow UHM, Schmid RD and Pleiss J, A model of the pressure dependence of the enantioselectivity of *Candida rugosa* lipase, *Protein Science* **10**:1942–1952 (2001).
13 Babali B, Aksoy HA, Turer M and Ustun G, Enzymatic esterification of (-)-menthol with fatty acids in solvent by a commercial lipase from *Candida rugosa*, *J Am Oil Chem Soc* **78**:53–56 (2001).
14 Shimada Y, Hirota Y, Baba T, Kato S, Sugihara A, Moriyama S, Tominaga Y and Terai T, Enzymatic synthesis of L-menthyl

esters in organic solvent-free system, *J Am Oil Chem Soc* **76**:1139–1142 (1999).
15 Babali B, Aksoy HA, Turer M and Ustun G, Enzymatic esterification of (-)-menthol with lauric acid in isooctane by sorbitan monostearate-coated lipase from *Candida rugosa*, *J Am Oil Chem Soc* **78**:173–175 (2001).
16 Luisi PL, Meier P, Wolf R, Properties of enzymes solubilized in hydrocarbon via reversed micelles, in: *Enzyme Engineering*, ed by Weetall HH and Royer GP. Plenum Publishing Co, New York, Vol5, pp 369–371 (1980).
17 Naoe K, Ohsa T, Kawagoe M and Imai M, Esterification by *Rhizopus delemar* lipase in organic solvent using sugar ester reverse micelles, *Biochem Eng J* **9**:67–72 (2001).
18 Kuboi R, Mori Y and Komasawa I, Determination and deactivation of *porcine pancreas* lipase in reverse micelle extraction (in Japanese), *Kagaku Kogaku Ronbunshu* **16**:1060–1066 (1990).
19 Sawada K and Ueda M, Characteristics of aqueous microenvironments in non-ionic surfactant reverse micelles and their use for enzyme reactions in non-aqueous media, *J Chem Technol Biotechnol* **79**:369–375 (2004).
20 Hayes D, Mechanism of protein extraction from the solid state by water-in-oil microemulsions, *Biotechnol Bioeng* **53**:583–593 (1997).
21 Hossain MJ, Takeyama T, Hayashi Y, Kawanishi T, Shimizu N and Nakamura R, Enzymatic activity of *Chromobacterium visosum* lipase in an AOT/Tween 85 mixed reverse micellar system, *J Chem Technol Biotechnol* **74**:423–428 (1999).