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The role of the reaction medium in lipase-catalyzed esterifications and transesterifications

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Abstract

The nature of the reaction medium (different organic solvents and supercritical fluids) markedly influenced the microbial lipase activity in esterification and transesterification reactions employing as substrates natural and synthetic compounds in terms of reaction rates and lipase enantioselectivity. The experimental data obtained show that while there are no substantial correlations between enantioselectivity and some physicochemical characteristics of the solvent as hydrophobicity and dielectric constant, the solvent polarity and hydrophobicity is able to modulate greatly lipase activity. The possible effects of the solvent characteristics on the catalytic performance of the enzymes are discussed and a rationale is proposed to explain the results obtained.

Keywords: Lipase; Biocatalysis; Organic solvent; Supercritical fluids

1. Introduction

The use of organic media for enzymatic reactions is now well documented and several systems (biphasic systems, reverse micelles and monophasic systems) and enzymes (oxidoreductases, hydrolases and isomerases) have been successfully employed for synthetic purposes (Chen and Sih, 1989; Wescott and Klibanov, 1994). Moreover, the demand by health authorities for enantiomerically pure, pharmaceutical active compounds, has led to the increased use of biocatalysts and increased research in order to obtain enzymes with higher activity and selectivity (Roberts et al., 1993).

In this field the lipases are especially widespread as biocatalysts for selective acylation and deacylation of natural and synthetic compounds and for resolution of racemic alcohols and acids (Bjorkling et al., 1991).

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Besides the activity in the field of new and more economical lipolytic enzymes from different sources (microbial, mammalian and plant sources), as well as in the field of recombinant lipases preparation through protein and genetic engineering, many efforts have been devoted to optimize reaction conditions in order to improve lipase performance. A study is being carried out on the reaction medium in order to modulate the yield as well as the stereoselectivity and regioselectivity of the biocatalysed reactions (Van Tol et al., 1995).

Our research group has been involved in the study of microbial lipases (fungal and bacterial source) to be employed in biotransformations of natural and synthetic compounds. On this basis we have carried out studies on the different parameters that can be employed to modulate microbial lipases catalytic activity. Recently we have devoted a large part of our study to the investigation of the importance of reaction medium, employing organic solvents and fluids in supercritical conditions.

The use of microbial lipases in non conventional media such as supercritical fluids (SCFs), has been proposed as a means of improving the activity and utility of such enzymes in anhydrous environments (Cernia and Palocci, 1997). It is well known, that by exploiting the unique solvent properties of SCFs (Table 1) (viscosity wide variations are possible with small changes in pressure and/or temperature) it may be possible to enhance reactions rates while maintaining or improving selectivity (Catoni et al., 1996). Separation of products from reactants can be greatly facilitated by the ease with which the solvent power of the SCFs can be adjusted.

2. Materials and methods

2.1. Chemicals

The lipase from *Pseudomonas cepacea* (Pseudomonas SP) was provided by Amano P, and was immobilised on ACR-silica gel (Catoni et al., 1996).

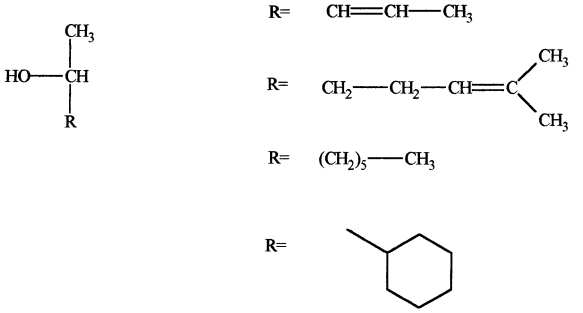
Table 1
Physical properties of supercritical fluids

	Gas	Supercritical fluid	Liquid
Density (g/cm^3)	10^{-3}	0.1–1	1
Diffusion (cm^2/s)	10^{-1}	10^{-3}–10^{-4}	$<10^{-5}$
Viscosity (g/cm*s)	10^{-4}	10^{-3}–10^{-4}	10^{-2}

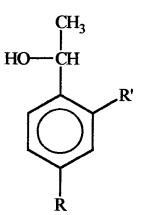
Gas like: High diffusivity (mass transport in complex matrices); low viscosity (favorable flow characteristics).
Liquid like: high solvatating power (dependent on density).

All phenyl ethanol derivatives, acylating agents, menthol and the primary alcohols (ethanol, butanol, hexanol, octanol, decyl alcohol, dodecanol, tetradenanol, 3 - methyl 2 - buten 1 - ol, crotylic alcohol) were purchased from Aldrich Chemical. (±) - *endo* - bicicyclo (2.2.1)hept - 5 - en - 2 - ol was

Aliphatic secondary alcohols



Aromatic secondary alcohols



R=H; R'=H
R=F; R'=H
R=Cl; R'=H
R=Br; R'=H
R=H; R'=Br
R=Cl; R'=Cl
R=OCH_3; R'=H
R=H; R'=OCH_3
R=*ter*-Bu; R'=H

Scheme 1. Secondary aliphatic and aromatic alcohols with different substitution moiety.

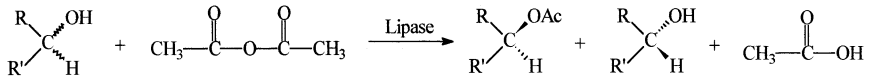
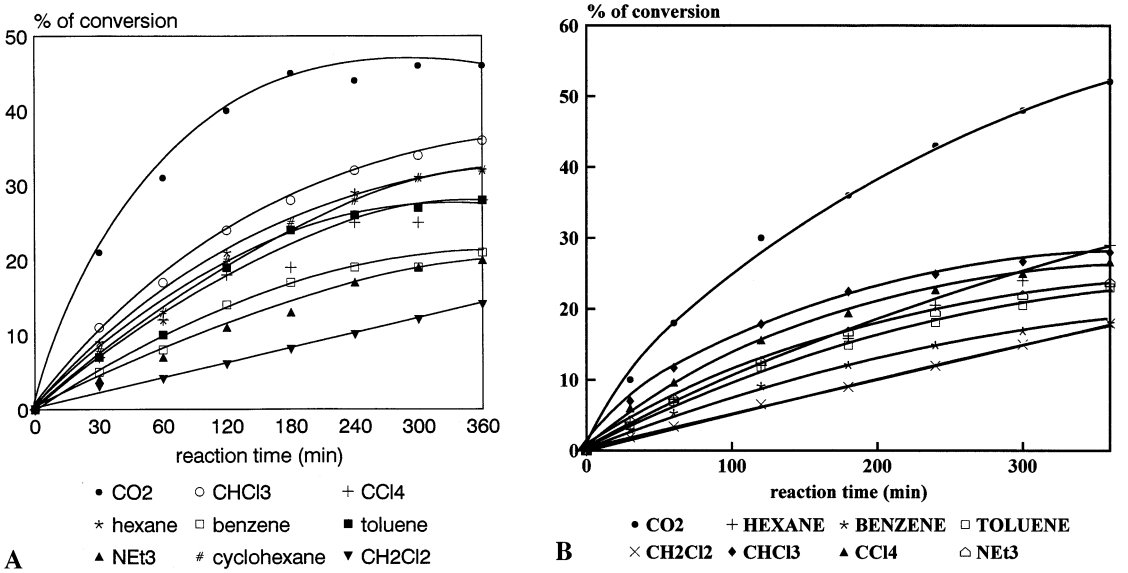


Fig. 1. General scheme of enzymatic esterification of secondary alcohols in different organic solvents and supercritical carbon dioxide.



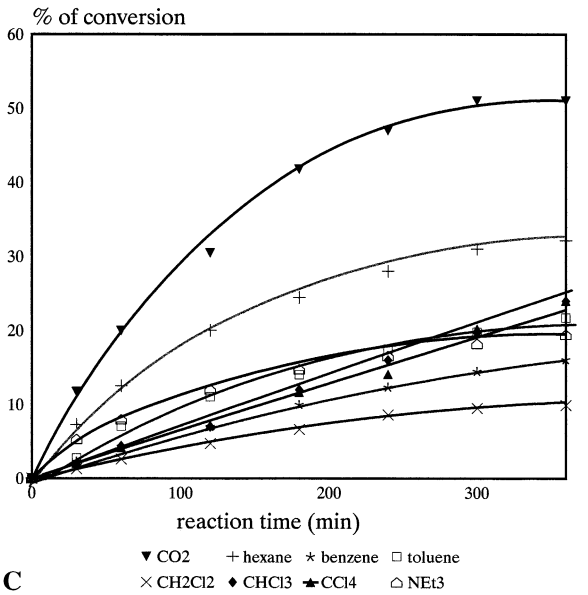


Fig. 2. Esterification reactions catalysed by *Pseudomonas cepacea* lipase in organic solvents and supercritical carbon dioxide. As substrates: acetic anhydride and: (A) 1-phenylethanol; (B) 1-(4-chlorophenyl)-ethanol; (C) 1-(4-*tert*-buthylphenyl)-ethanol. Conversion (%) versus reaction time.

Table 2
Enantiomeric excess (%) at 360 min and 40°C of esterification reaction of different secondary alcohols and acetic anhydride biocatalysed by immobilised *Pseudomonas cepacea* lipase in organic solvents and in supercritical CO_2

Substrate	SC–CO_2	Hexane	CCl_4	Toluene	Benzene	$CHCl_3$	Net_3	CH_2Cl_2
1-Phenyl-ethanol	97	42	88	88	87	93	67	85
1-(4-Fluorophenyl)-ethanol	96	69	76	80	82	85	7	75
1-(4-Chlorophenyl)-ethanol	80	58	69	70	67	90	12	85
1-(4-Bromophenyl)-ethanol	96	88	70	89	73	80	4	84
1-(4-Methoxyphenyl)-ethanol	100	60	49	88	86	55	20	39
1-(4-*tert*-Buthylphenyl)-ethanol	99	93	62	70	75	80	3	78
1-(2-Bromophenyl)-ethanol	100	71	13	100	100	100	4	31
(2,4-Dichlorophenyl)-ethanol	90	76	75	75	60	60	88	43
1-(2-Methoxyphenyl)-ethanol	95	10	13	54	14	7	1	16

synthesised according to Oberhauser (Oberhauser et al., 1987) and Honda (Honda et al., 1981). (2*R**,3*S**) Methyl *trans* 3-(4-methoxyphenyl) glycidate was a generous gift from Lusochimica (Italy). All solvents were from Carlo Erba Analyticals, with a maximum water content of 0.02%.

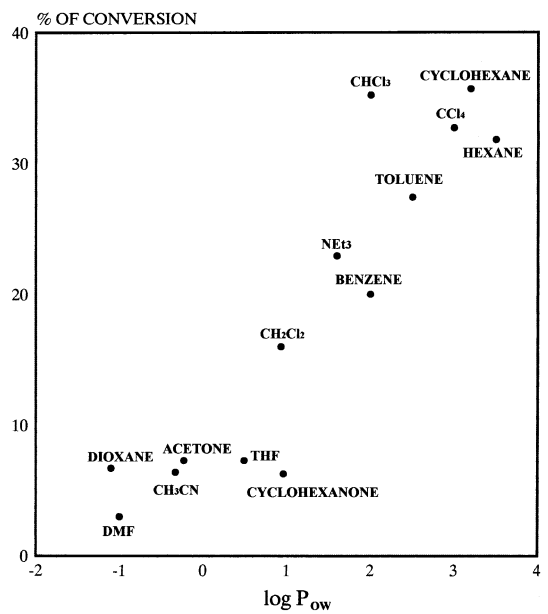


Fig. 3. Esterification reactions of 1-phenylethanol and acetic anhydride catalysed by *Pseudomonas cepacea* lipase in organic solvents. Conversion (%) versus solvent characteristics expressed as log P_{ow}.

2.2. Reactions in organic solvents

Esterification and transesterification reactions were carried out in 1.75 ml of organic solvent containing 0.9 mmol of each alcohol (phenyl ethanol derivatives menthol and (±)-*endo*-bicicyclo (2.2.1)hept-5-en-2-ol), 0.9 mmol of acylating agent and from 25 to 60 mg of free and immobilised *Pseudomonas cepacea* lipase. Transesterifications reactions were carried out in 3.2 ml of organic solvent containing 9 mmol of methyl *trans* 3-(4-methoxyphenyl) glycidate, 9 mmol of each alcohol and 53 mg of free *Pseudomonas cepacea* lipase.

All the reaction mixtures were incubated at 40°C under magnetic stirring at 600 rpm. Samples were taken for analysis at regular intervals.

2.3. Reaction in supercritical carbon dioxide

The apparatus employed to carry out the reactions in supercritical medium (SFE 30 Fison Instruments) were specially designed to investigate various enzymatic reactions in supercritical carbon dioxide in a reactor with a reaction cell volume of 0.9 ml. The pressure was controlled by a syringe pump that ensured a rapid target pressure achievement. A 0.35 mmol quantity of alcohol, 0.35 mmol of acylating agent and 10 mg of immobilised enzyme were introduced to the reactor. After sealing, pressurisation is achieved by pumping liquid carbon dioxide to the

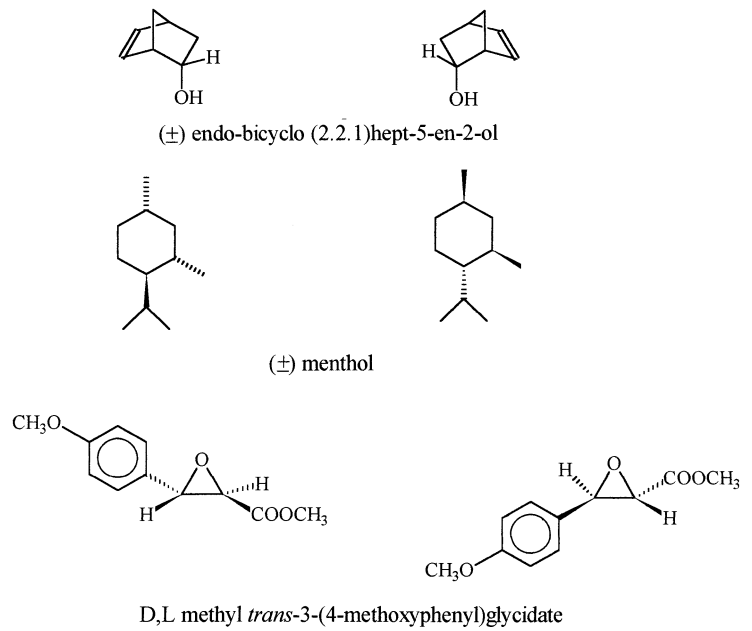


Fig. 4. Substrates employed in transesterification reactions catalysed by *Pseudomonas cepacea* lipase.

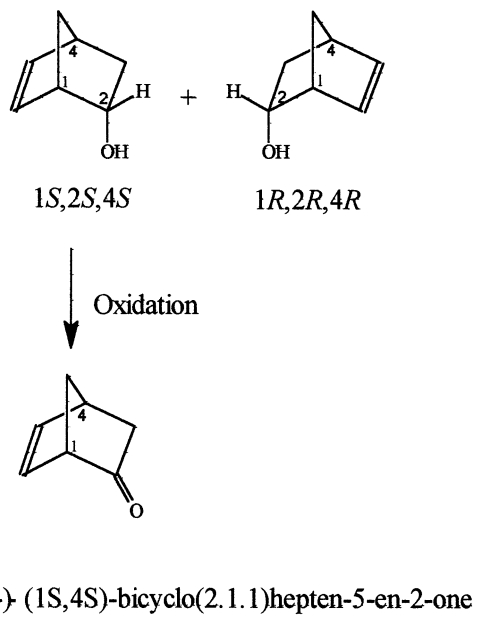


Fig. 5. Scheme of oxidation reaction to obtain (−)-(1*S*,4*S*)-bicyclo-(2.2.1)hept-5-en-2-one from (±)-endo-bicyclo-(2.2.1)hept-5-en-2-one.

Table 3
Conversion (%) and enantiomeric excess (%) at 48 h and 40°C of transesterification reaction of (±)-norbornenol and vinyl acetate biocatalysed by immobilised *Pseudomonas cepacea* lipase in organic solvents

Reaction media	Initial rate (μmol/min)	*C* (%)	e.e. (%)
n-Hexane	0.59	48	80
Cyclohexane	0.80	75	65
CCl_4	0.58	50	78
Toluene	0.32	49	79
Benzene	0.26	46	84
$CHCl_3$	0.12	18	89
NEt_3	0.40	52	70
Cyclohexanone	0.38	32	82
CH_2Cl_2	0.11	17	84
THF	0.19	32	87
Vinyl acetate	0.33	45	72
Aceton	0.17	28	83
CH_3CN	0.11	20	75
DMF	—	0	—
Dioxane	0.19	36	82

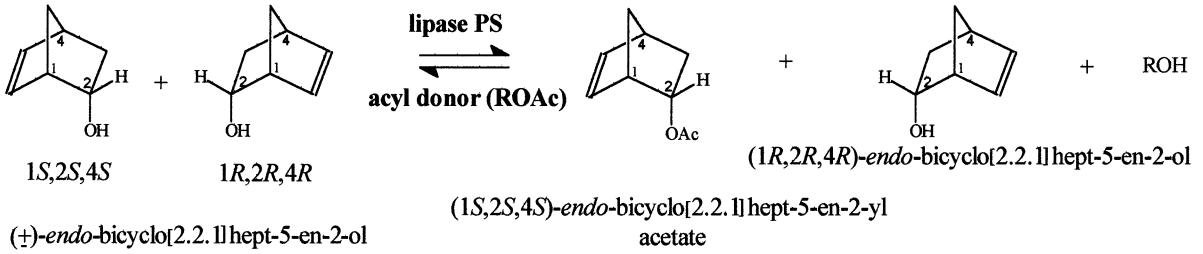


Fig. 6. Scheme of transesterification reaction of (±)-endo-bicyclo-(2.2.1)hept-5-en-2-one catalysed by Pseudomonas cepacea lipase in organic solvents.

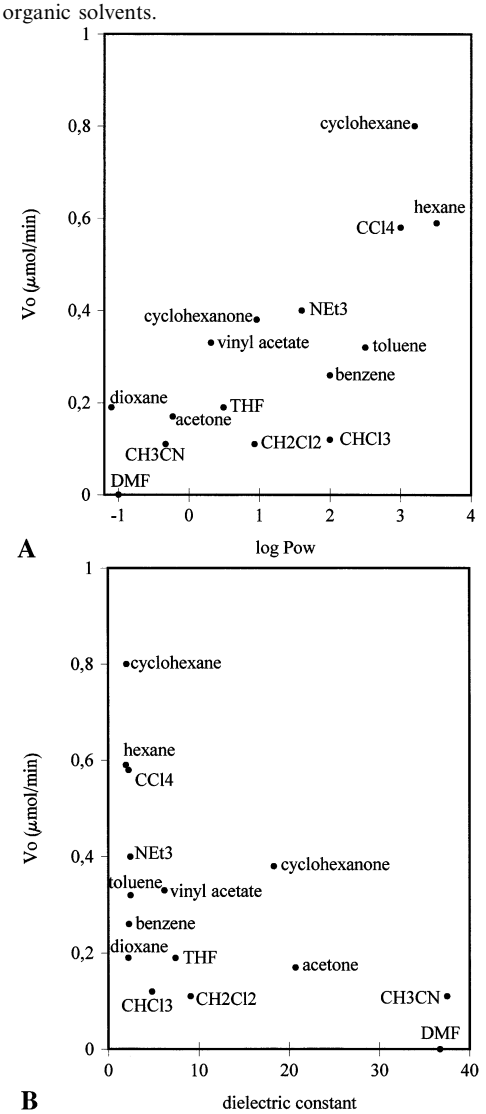


Fig. 7. Transesterification reactions of (±)-endo-bicyclo-(2.2.1)hept-5-en-2-one and vinyl acetate catalysed by *Pseudomonas cepacea* lipase in organic solvents. Conversion (%) versus solvent characteristics expressed as: (A) log P_{ow}; (B) dielectric constant.

desired final pressure (20 MPa), and the reactor is thermostated at 40°C. During the reaction time a 6-way valve (Rheodyne 7125) allows sample withdrawal for analysis without depressurisation. At the end of reaction, depressurisation is achieved by opening a needle valve above the reaction cell.

2.4. Chromatographic analysis

Esters production of phenyl ethanol derivatives, menthol and (±)-*endo*-bicicyclo (2.2.1)hept-5-en-2-ol, was followed by gas chromatography (Carlo Erba 5300) and flame ionisation detector, with nitrogen as carrier gas at a pressure of 1 bar using capillary columns. Phenyl ethanol derivatives were analysed using a polyphenyl-methylsiloxane column (OV1: 25 m × 0.53 mm ID); the enantiomeric resolution was obtained employing two serial capillary column: the first one achiral (OV1: 10 m × 0.53 mm ID or Carbowax: 20 m × 0.25 mm ID) the other one chiral (Cp-cyclodextri-B-2, 3,6-M-19: 25 m × 0.25 mm ID). Menthol was detected using a CDX-B column (Cp-cyclodextri-B-2, 3,6-M-19: 25 m × 0.25 mm ID). Progress of the reaction of -*endo*-bicicyclo (2.2.1)hept-5-en-2-ol was followed on OV-1 column (OV 1:10 m × 0.53 mm ID) while enantiomeric excess was detected on CDX-B column (Cp-cyclodextri-B-2, 3,6-M-19: 25 m × 0.25 mm ID). Progress of the reactions of methyl *trans* 3-(4-methoxyphenyl) glycidate and the enantiomeric excess (e.e.) (A. Gentile, 1992) were followed by HPLC (HPLC Perkin Elmer Series 10), using a chiral column (CHIRALCEL OD-H 25 × 0.46 cm I.D. from DAICEL) with a mixture of *n*-hexane/*iso*-propyl alcohol (90:10) as an eluent phase.

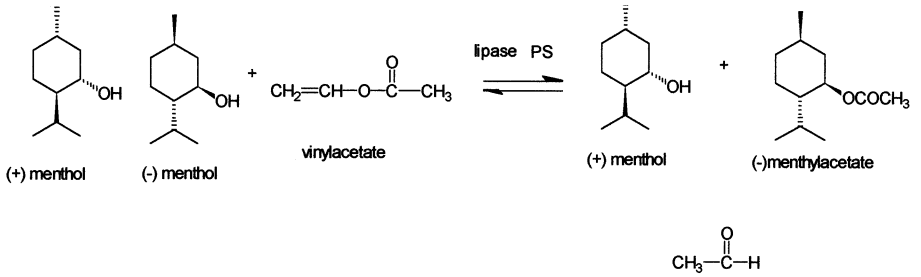


Fig. 8. Scheme of transesterification reactions of (±)menthol and vinyl acetate catalysed by *Pseudomonas cepacea* lipase in organic solvents.

3. Results and discussion

As model substrates, secondary aliphatic and aromatic alcohols with different substitution moieties (Scheme 1) were firstly employed in esterification reactions with acetic anhydride in some organic solvents and supercritical carbon dioxide using as catalyst an immobilized lipase from *Pseudomonas cepacia*.

Fig. 1 shows the reaction scheme of the esterification reactions of some of the above mentioned substrates with acetic anhydride in supercritical carbon dioxide and organic solvents. Fig. 2 shows the kinetic curves of some employed substrates and Table 2 summarizes the enantiomeric excess values obtained.

As can be clearly seen the percentage of conversion and the enantioselectivity of the studied reactions, dramatically depend on the employed solvent while the use of differently activated substrates do not substantially improve the reaction rates in the employed conditions.

In order to explain this dependence we attempted to correlate the enzymatic activity, with some physic-chemical properties of the solvent.

The enantiomeric excess values plotted against solvent dielectric constant (ε), the Hildebrand solubility parameter (δ) and $\log P_{ow}$ ($\mathrm{Log}\, P_{ow}$ is defined as the logarithm of the partition coefficient of a given compound in the standard octanol-water two-phase system) do not furnish significant results; otherwise a linear correlation between the percentage of conversion and $\log P_{ow}$ was found (Fig. 3). The results clearly prove that there is an increase of lipase activity with increasing solvent hydrophobicity expressed in terms of $\log P_{ow}$ values.

As far as the results obtained in supercritical fluids, we observed an higher extent of conversion in $SCCO_2$ compared to that in organic solvents. Although the lack of data regarding the dielectric constant and the $\log P_{ow}$ value for supercritical CO_2, the solvent hydrophobicity does not seem to play an important role in reaction rate enancements. Our results can be explained in terms of enhanced transport properties, high diffusivity of solute in supercritical medium as in terms of high enzyme stability in supercritical phases. Moreover the use of a reaction medium in a supecritical state determined a sharp increase of the reaction enantioselectivity with e.e. values ($\geq 98\%$) higher than those obtained in organic solvents.

Lipases from *Pseudomonas cepacia* were also used in the enantioselective transterification reactions using racemic alcohols ((±)-*endo*-bicyclo

Table 4
Conversion (%) and enantiomeric excess (%) at 48 h and 40°C of transesterification reaction of (±)-menthol and vinyl acetate biocatalysed by immobilised *Pseudomonas cepacea* lipase in organic solvents

Reaction media	*C* (%)	e.e. (%)
n-Hexane	39	95
Cyclohexane	35	96
CCl_4	26	98
Toluene	25	95
Benzene	27	94
$CHCl_3$	12	—
NEt_3	15	94
CH_2Cl_2	16	94
THF	19	94
Aceton	17	93
Dodecane	35	95
Decane	34	95
Dioxane	17	97

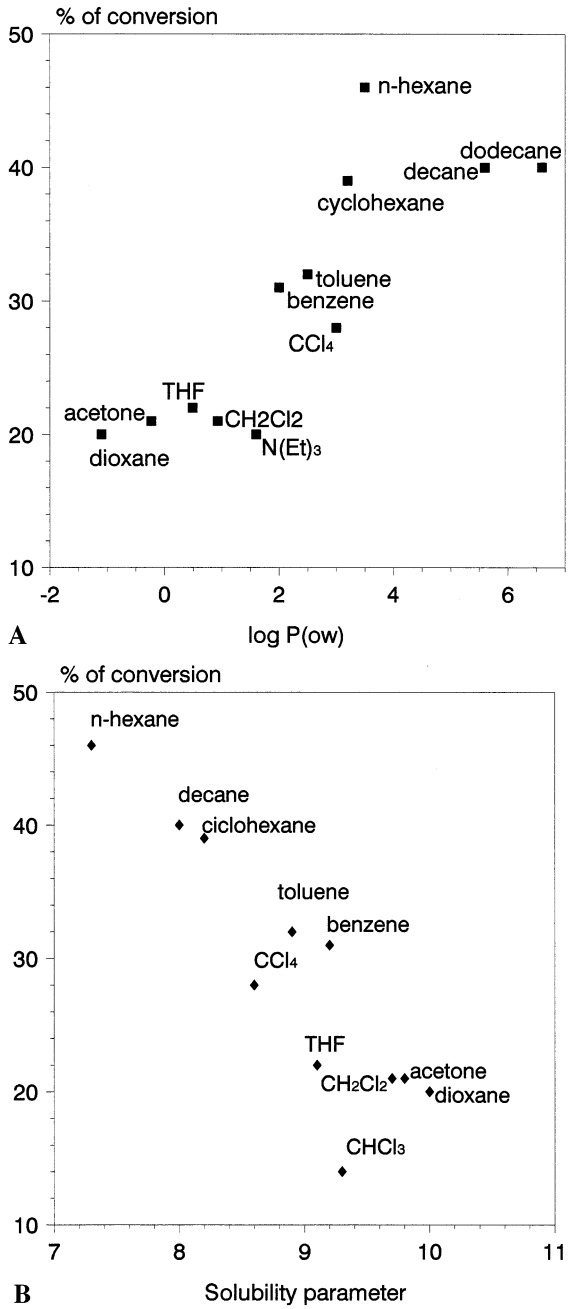


Fig. 9. Transesterification reactions of (±)menthol and vinyl acetate catalysed by *Pseudomonas cepacea* lipase in organic solvents. Conversion (%) versus solvent characteristics expressed as: (A) log P_{ow}; (B) Hildebrand solubility parameter.

(2.2.1)hept-5-en-2-ol, (±) menthol) and esters (2*R**,3*S**) methyl *trans* 3-(4-methoxyphenyl) glycidate) as substrates of pharmaceutical interest as reported in Fig. 4.

The chiral alcohol (±)-*endo*-bicyclo (2.2.1)hept-5-en-2-ol is a useful starting material for the stereospecific synthesis of cyclopentane systems such as methaneprostacyclins, nucleoside analogues and Brefeldin A. (Betina et al., 1965; Bartlett and Greene, 1978; Baudouy et al., 1977; Eichberger et al., 1986).

Brefeldin A is a fungal metabolite with a chemical structure containing a macrocyclic lactone ring which has a wide range of biological activities (Betina, 1969; Harri et al., 1971). One of the synthetic pathways to synthesize Brefeldin A employs a (1*S*,4*S*) bicyclopenthanone (Corey and Wollenberg, 1976; Berger and Faber, 1991).

Therefore we thought it would be interesting to investigate the lipase catalyzed resolution of the racemic mixture of (±)-norbornenol from which optically active chetone can be easily obtained (Fig. 5).

The transesterification reactions were carried out employing vinyl acetate as acyl donor in different reaction media (Fig. 6). Besides studies on the use of different acetyl donors, with different chemical structures (esters of saturated, insaturated alcohols and enol acetates), our interest was focused on the possibility of improving the reaction yield and the enantioselectivity of the considered reactions using different organic solvents with different physic-chemical properties. The percentage of conversion at fixed reaction time, depends greatly on the solvent employed but there is no effect on the enantioselectivity of the process (Table 3). Moreover the e.e. ($\geq 80\%$) values are substantially independent of the acetyl donor structure.

Fig. 7 shows the plot of the initial velocities in different reaction media against some physic-chemical properties of the chosen solvents.

Results pointed to the impossibility of finding a direct correlation with a single parameter.

Menthol, a secondary alcohol of terpenic structure obtained from *Mentha piperita* or *Mentha arvensis* plants by steam distillation (Eccles, 1994) has three asymmetric carbon atoms in its cyclohexane ring (Bauer et al., 1990; Eccles et al., 1990). Only the (−)menthol has an effect on the cold receptors of the human skin (interfere with calcium ions crossing through the cell membrane causing oscillation in the membrane potential).

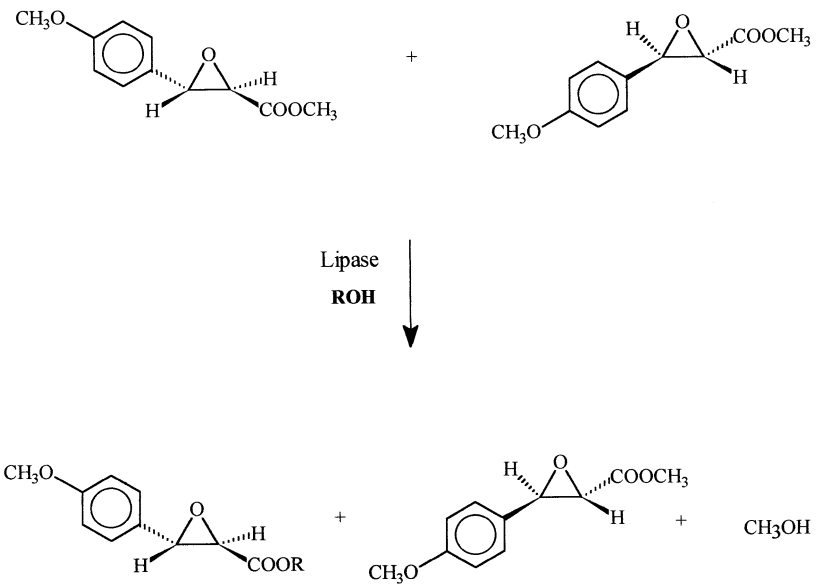


Fig. 10. Scheme of transesterification reactions of methyl *trans*-3-(4-methoxyphenyl)glycidate and buthanol catalysed by *Pseudomonas cepacea* lipase in organic solvents.

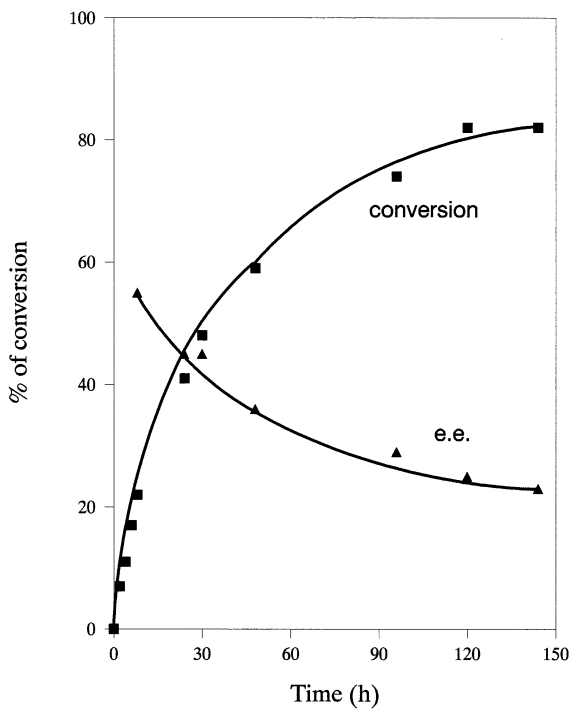


Fig. 11. Transesterification reaction of *trans*-3-(4-methoxyphenyl)glycidate and buthanol catalysed by *Pseudomonas cepacea* lipase in *n*-hexane. Conversion (%) and enantiomeric excess (%) versus reaction time.

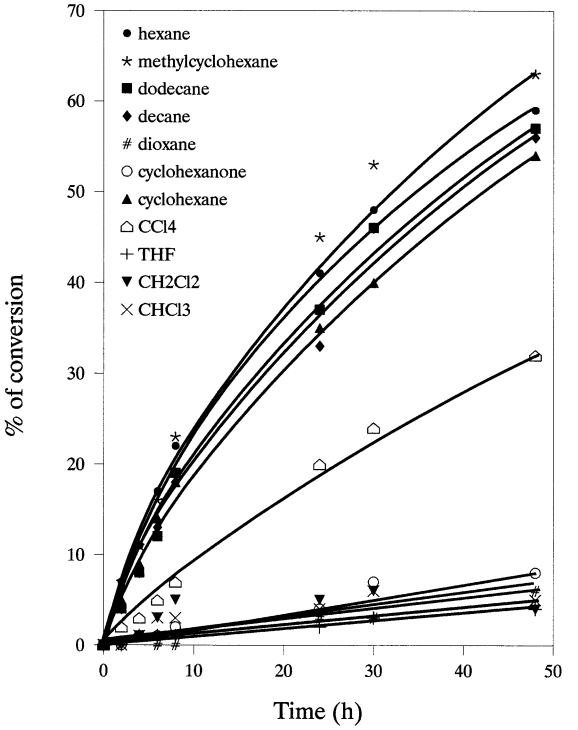


Fig. 12. Transesterification reactions of *trans*-3-(4-methoxyphenyl)glycidate and buthanol catalysed by *Pseudomonas cepacea* lipase in organic solvents. Conversion (%) versus reaction time.

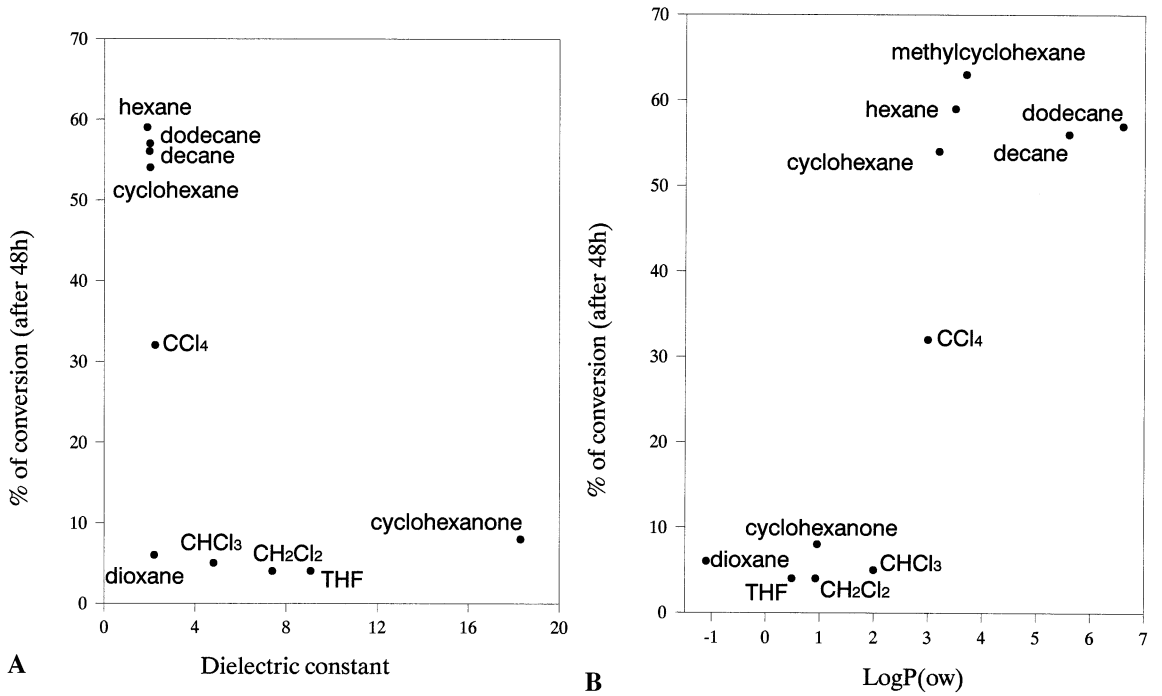


Fig. 13. Transesterification reactions of *trans*-3-(4-methoxyphenyl)glycidate and buthanol catalysed by *Pseudomonas cepacea* lipase in organic solvents. Conversion (%) versus solvent characteristics expressed as: (A) dielectric constant; (B) log P_{ow}.

We carried out a study on the possibility of separating menthol enantiomers using a transterification reaction catalyzed by lipase from *Pseudomonas cepacia* in organic solvents (Fig. 8). Besides the temperature optimization of the reaction conditions, the substrates molar ratio (menthol and vinyl acetate), the amount of the enzyme used, particular attention was devoted to the possibility of modulating enzyme activity and selectivity by varying the reaction medium.

The percent of conversion depends greatly on the solvent employed, the yield varying from 10 to 50% after 96 h reaction time. The e.e. values (Table 4) are very high ($\geq 90\%$) and independent from the solvent chosen.

The correlation between enzyme activity and some physic-chemical properties, such as the solvent dielectric constant, log P_{ow} and the Hyldebrand solubility parameter, gave us very interesting results. Only the solvent hydrophobicity and the Hyldebrand solubility parameter (Fig. 9) was an unequivocal correlation found, showing an enzymatic yield inversely dependent on solvent polarity.

Diltiazem (1), (+) *cis*-(2*S*,3*S*)-3-acetoxy-5-[2-(dimethylamino) ethyl] 2,3-dihydro-2-(4-methoxyphenyl) 1,5-benzothiazepin-4-(5*H*), is a very strong vasodilating agent discovered by Tanabe Seiyaku, which features calcium channel blocking activity (Schwartz et al., 1992). Among the several synthetic approaches that have been described (Watson et al., 1990; Miyata et al., 1991) one of the most important is based on the Tanabe synthesis (Tanabe, 1984) which begins with the Darzens' condensation of 4-methoxybenzaldehyde (a) with methyl chloroacetate. The subsequent reaction opens the oxirane ring of the methyl *trans* 3-(4-methoxyphenyl) glycidate (b) with 2-nitrothiophenol. Several subsequent steps (intramolecular ring closure, reduction of the NO_2 group, ester hydrolysis, acetylation and *N*-alkylation) complete the synthesis. Only the (2*R*,3*S*) methyl *trans* 3-(4-methoxyphenyl) glycidate is necessary in the synthesis of Diltiazem, however, after Darzens' condensation a racemic mixture is obtained.

Some authors (Kaverna and Sundholm, 1993)

studied the transesterification reaction of (2*R**,3*S**) methyl *trans* 3-(4-methoxyphenyl) glycidate with aliphatic alcohols catalyzed by *Candida cylindracea* lipase. They focused their attention on the water content of different organic solvents used as reaction media. As a matter of fact their results show a rough correlation between the yield of the reaction and the amount of the water stripped by the organic solvent.

On this basis we decided to study in depth the influence of the reaction medium in the transesterification reaction of (2*R**,3*S**) methyl *trans* 3-(4-methoxyphenyl) glycidate with aliphatic alcohols employing a lipase from *Pseudomonas cepacia* (Fig. 10).

As reported in Fig. 11 the enzymatic reaction proceeds very slowly and although the final conversion approaches 80%, the enantiomeric excess, calculated for the (2*R*,3*S*) enantiomer, decreases from 55 to 23%.

In the condition we employed, the best relationship between yield and enantiomeric excess was observed within only 48 h reaction times.

The kinetic curves of the transesterification reaction between butanol and methyl *trans* 3-(4-methoxyphenyl) glycidate in different organic solvents are shown in Fig. 12. As can be seen clearly the conversion greatly depends on the reaction medium employed.

In order to better understand these results trials were carried out to correlate the enzymatic activity with some physic-chemical characteristics of the organic solvents. The dielectric constant (ε), Hildebrand solubility parameter (δ), solvent density (ρ) and solvent hydrophobicity, expressed as $\log P_{ow}$, were taken into consideration.

In Fig. 13 the dielectric constant and the $\log P_{ow}$ values against the percentage of conversion (after 48 h) are plotted respectively. Solvents with high solubility parameter and relatively high dielectric constant values show a common feature in terms of solvent hydrophobicity showing low $\log P_{ow}$ values. The enzymatic reactions carried out in these solvents show very low conversion rates ($\leq 10\%$). In contrast hydrophobic solvents with low solubility parameter and dielectric constant values furnished yields greater than 30%.

4. Conclusions

The possibility of varying the activity and the selectivity of the lipase biocatalysed reactions, by changing the nature of the reaction medium, can be considered a useful tool to modulate the chemical interactions between enzyme, substrate and reaction media with different physico-chemical properties (Secundo et al., 1992). These studies not only improve the understanding of enzymes mechanism of action but even contribute to elaborate better strategies for industrial applications of lipolytic enzymes.

From the results obtained, carrying out esterification or transesterification reactions with model substrates in different organic solvent, *Pseudomonas cepacia* lipase activity shows a dependence on the physic-chemical properties of the reaction medium employed. In particular solvent hydrophobicity, expressed as $\log P_{ow}$, is the physicochemical characteristic best correlated with the enzymatic activity. As matter of fact apolar and hydrophobic solvent with low dielectric constant values increase the reaction rate, however, polar solvent or containing heteroatoms, carbonilyc or amidic groups decrease the reaction rate. The use of hydrophobic solvents in lipases biocatalysed reactions preserves enzyme conformational rigidity and stability (even in the presence of fluids in supercritical conditions). Moreover the motions in proteins are partially governed by electrostatic interactions (Affleck et al., 1992). In fact electrostatic forces are the dominant factor correlating protein structure and function. Attenuation of such forces by the solvent environment can be dramatic (use of solvents with high dielectric constant values) particularly in solvent-accessible regions of enzyme proteins.

On this basis, we could speculate that one or more molecules of solvents could interact with the enzyme in or near the substrate binding site and depending on their physicochemical properties they could interfere with the transformation of the substrate, thus affecting the reaction rate. Therefore according to our hypothesis it could be the solvent-enzyme complex that influences the activity. The formation of a such complex requires that

in organic media, as in aqueous media in which there is bulk water and water tightly bound to the enzyme, there is bulk solvent and bound solvent. However this model cannot be of predictive value because of the great number of possible solvent-enzyme complexes (equal to the number of the solvent used) and because each complex might behave different depending on the nature of the substrate used.

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