ELSEVIER

Journal of Molecular Catalysis A: Chemical 105 (1996) 79–86

JOURNAL OF MOLECULAR CATALYSIS A: CHEMICAL

# Different aspects of 'solvent engineering' in lipase biocatalysed esterifications

E. Catoni, E. Cernia *, C. Palocci

*Department of Chemistry, University of Rome 'La Sapienza', P.le A. Moro 5, 00185 Rome, Italy*

Received 4 April 1995; accepted 5 July 1995

**Abstract**

A microbial lipase from *Pseudomonas* sp., immobilized on an ACR–silica gel, has been used as catalyst for esterification reactions in organic solvents which were largely different in some fundamental physico-chemical characteristics. The results obtained were compared with the same reactions carried out in supercritical carbon dioxide. Significant variations in conversion values as function of log $P_{ow}$ of the solvent (dielectric constants and Hildebrand solubility parameters not providing consistent results) seem to demonstrate the importance of the enzyme–water content on the mechanism of action as in terms of conformations arrangements as well as in terms of substrates partition equilibrium with the polar matrix. Differently, the conversions and enantioselectivity of the reactions in the supercritical fluid, always higher than in organic solvents, could be attributed to the specific properties of the medium in terms of low viscosity and surface pressure values and higher diffusivity of substrates in the supercritical phase.

*Keywords:* Solvent engineering; Lipase; Esterification reactions

## 1. Introduction

The interest of the current research for the biocatalysed reactions in non-aqueous media is continuously increasing: the ability to synthesize chiral molecules through lipase catalysed esterification reactions is very promising for the so called 'racemic switches', i.e. the re-development of products now marketed as racemate, to enantiomers.

Being the catalytic performance of enzymes (particularly in terms of conversion and enantioselectivity) strictly dependant on the characteristics of the media, many attempts have been made to obtain empirical rules for the optimization of the whole system of reaction (substrate–enzyme–solvent) without reaching, up the date, general and univocal relationships.

Regarding the so called 'solvent effect' on enzymatic activity, many papers have appeared describing the effect of different organic solvents on biocatalytic activity and much attention has been paid to the influence of the solvent physico-chemical properties on enzymatic activity [1,2].

In our work a microbial lipase, immobilized on ACR–silica gel matrix, was employed to catalyse the esterifications of aromatic chiral alcohols with different substitution moieties using as reaction media traditional organic sol-

* Corresponding author.


*SSDI* 1381-1169(95)00153-0

vents. We tried to interpret the enzyme activity and selectivity using a direct correlation with the dielectric constant, Hildebrand solubility parameter and log $P_{ow}$ of the solvents employed. In addition we confirm some our preliminary results [3] connected with the utilization of supercritical carbon dioxide, enlarging the activity to the use of different aromatic compounds and obtaining anyhow high enantioselectivity and conversion values.

## 2. Experimental

### 2.1. Enzyme

The lipase from *Pseudomonas* sp. used in this work, was provided by Amano P, and immobilized on an ACR–silica gel (see our previous work [3]).

### 2.2. Substrates

As substrates were employed acetic anhydride as acylating agent and secondary racemic alcohols as 1-phenylethanol, 1-(4-fluorophenyl)-ethanol, 1-(4-chlorophenyl)-ethanol, 1-(4-bromophenyl)-ethanol, 1-(4-methoxyphenyl)-ethanol, 1-(2-methoxyphenyl)-ethanol, 1-(2-bromophenyl)-ethanol, 1-(2,4-dichlorophenyl)-ethanol, 1-(4-*tert*-butylphenyl)-ethanol, all purchased from Aldrich Chemical.

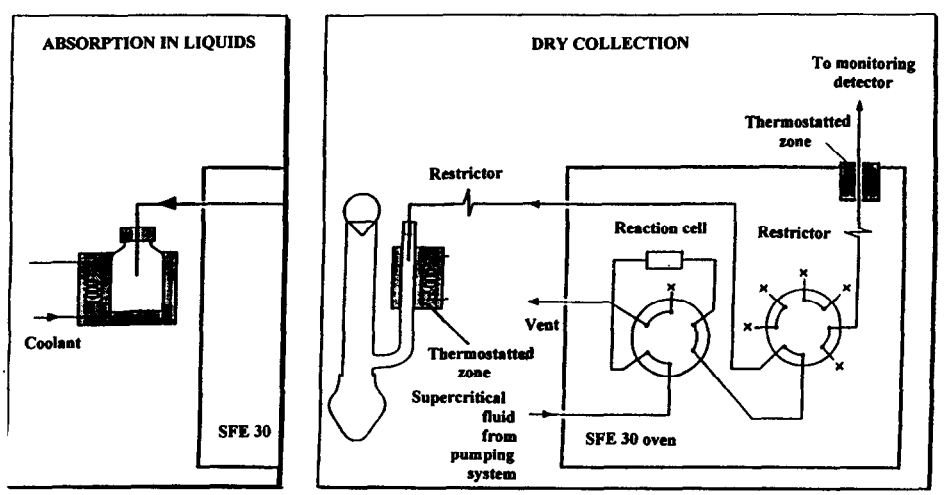
### 2.3. Solvents

All solvents (n-hexane, benzene, toluene, dichloromethane, chloroform, carbon tetrachloride, triethylammine, tetrahydrofuran, acetonitrile, dioxane, acetone, *t*-butylalcohol, dimethylformamide, cyclohexane, cyclohexanone), from Carlo Erba Analyticals, have a maximum water content of 0.02%.

## 3. Methods

### 3.1. Reactions in organic solvents

Esterifications were carried out in 1.75 ml of organic solvent containing 0.9 mmol of each alcohol, 0.9 mmol of acetic anhydride with an enzyme/substrates ratio equals to 33.2 U/mmol (where 1 U will hydrolyse 1 microequivalent of fatty acid from triacetin, in 30 min at pH 7.4 at 30°C). The mixture was incubated at 40°C for 6 h, under magnetic stirring at 600 r.p.m. Samples were taken for analysis at regular intervals, after 30 minutes and later every hour.




Scheme 1. Schematic diagram of the SFC reactor.

### 3.2. Reactions in supercritical carbon dioxide

The apparatus (SFE 30 Fisons Instruments), described schematically in Scheme 1, has been specially designed to investigate various enzymatic reactions in supercritical carbon dioxide in a reactor with a reaction cell volume of 0.7 ml. The pressure of $CO_2$ was controlled by a syringe pump that ensured a rapid target pressure achievement. 0.35 mmol of alcohol and anhydride and 10 mg of immobilized enzyme are introduced within the reactor. After sealing, pressurization is achieved by pumping liquid carbon dioxide to the desired final pressure (20 MPa), and the reactor is thermostated at 40°C.

During the reaction time a 6-way HPLC valve (Rheodyne 7125) permits to withdraw samples for analysis without depressurization. At the end of the reaction, depressurization is achieved by opening a needle valve above the reaction cell.

### 3.3. Chromatographic analysis

Progress of the reaction, for ester production, was followed by gas chromatography (Carlo Erba 5300) using a capillary column of polyphenyl-methylsiloxane (Mega: 25 m × 0.53 mm ID) and a flame ionization detector, with nitrogen as carrier gas, at a pressure of 0.8 bar, 473 K for the detector, 423 K for the injector.

The enantiomeric resolution was obtained employing two serial capillary column: the first one achiral (OV 1: 10 m × 0.53 mm ID or Carbowax: 20 m × 0.25 mm ID) the other one chiral (Cp-cyclodextrin-B-2, 3,6-M-19: 25 m × 0.25 mm ID).

Table 1
Esterification values, after 360 min of reaction time, of 1-phenylethanol with acetic anhydride in different organic solvents, employing an immobilized lipase from *Pseudomonas* sp.

| Reaction medium | % of conversion | % enantiomeric excess |
|---|---|---|
| $CHCl_3$ | 36 | 93 |
| Cyclohexane | 32 | 70 |
| Hexane | 32 | 58 |
| $CCl_4$ | 28 | 88 |
| Toluene | 28 | 88 |
| Benzene | 21 | 87 |
| Triethylamine | 20 | 67 |
| $CH_2Cl_2$ | 14 | 85 |
| Acetone | 9 | 93 |
| THF | 8 | 87 |
| Acetonitrile | 7 | 91 |
| 1,4-Dioxane | 5 | 91 |
| Cyclohexanone | 5 | 89 |
| *t*-Butylalcohol | 3 | 80 |
| DMF | 2 | 50 |

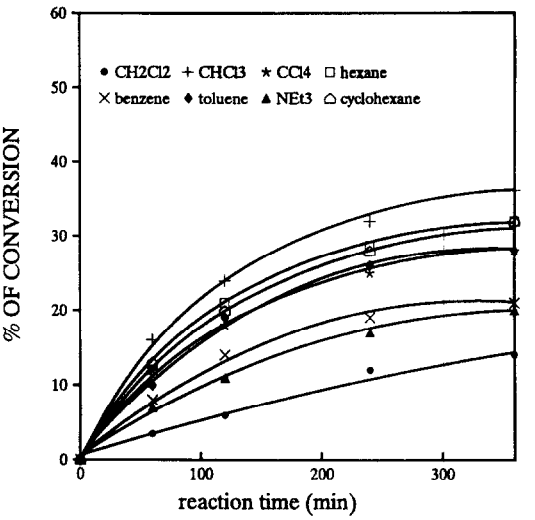



Fig. 1. Esterifications of 1-phenylethanol with acetic anhydride carried out in dichloromethane, chloroform, carbon tetrachloride, hexane, benzene, toluene, triethylamine and cyclohexane catalyzed by an immobilized lipase from *Pseudomonas* sp.

## 4. Results and discussion

Table 1 and Figs. 1 and 2 show the results of kinetic investigation of lipase catalysed esterification of 1-phenylethanol with acetic anhydride in fifteen different organic solvents. As can be clearly seen, the percentage of conversion and the enantioselectivity of the reaction dramatically depends on the solvent.

In order to explain this dependence we attempted to correlate the enzymatic activity and enantioselectivity, with basic physico-chemical characteristics of the solvent (see Table 2).

We have taken in consideration the dielectric constant ($\epsilon$), the Hildebrand solubility parame-

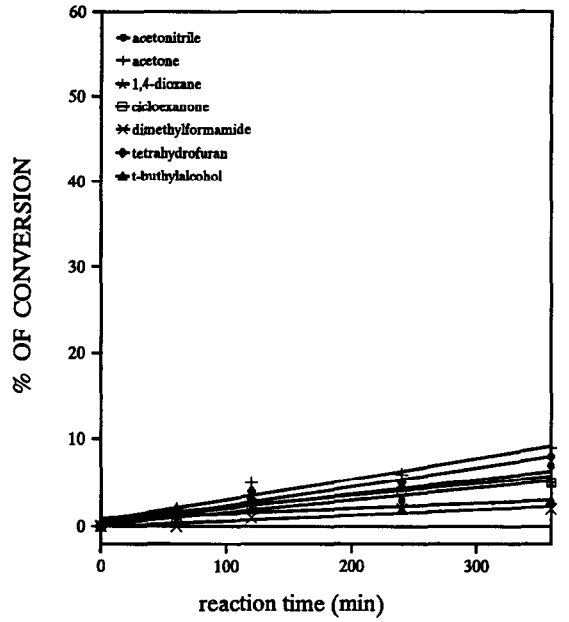



Fig. 2. Esterifications of 1-phenylethanol with acetic anhydride ($T = 40°C$) carried out in acetonitrile, acetone, 1,4-dioxane, cyclohexanone, dimethylformamide, tetrahydrofuran and *t*-butylalcohol catalyzed by an immobilized lipase from *Pseudomonas* sp.

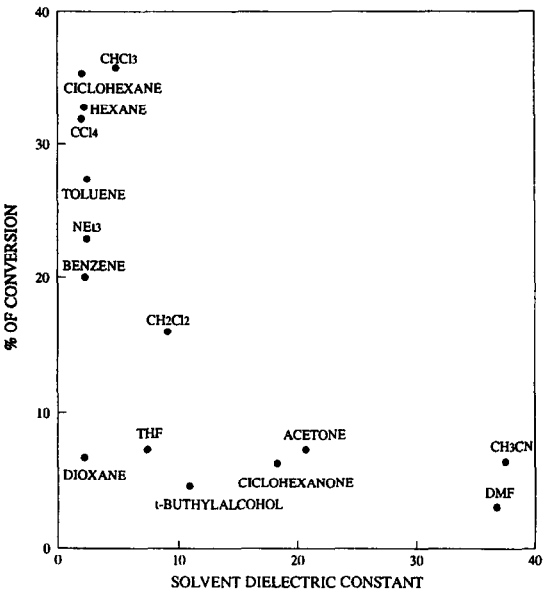



Fig. 3. Esterifications values of 1-phenylethanol with acetic anhydride ($T = 40°C$) carried out in different organic solvents vs. dielectric constant catalysed by an immobilized lipase from *Pseudomonas* sp.

ter ($\delta$) [4] and the solvent hydrophobicity, expressed as log $P_{ow}$ [5], which is the logarithm of the partition coefficient of a given compound in the standard octanol–water two-phase system.

The enantiomeric excess values plotted against $\epsilon$, $\delta$ or log $P_{ow}$ do not furnish significant correlation. In Fig. 3 is shown the correlation of the percentage of conversion with the dielectric constant of the solvents used. We can see that, in the conditions employed, no significant correlation exists between $\epsilon$ and enzymatic activity and selectivity.

Using the Hildebrand solubility parameter [4]

Table 2
Physico-chemical properties of different organic solvents

| Solvent | Dielectric constant (25°C, 1 atm) | Hildebrand solubility parameter ($Pa^{0.5}$) (25°C, 1 atm) | log $P_{ow}$ (25°C, 1 atm) |
|---|---|---|---|
| $CHCl_3$ | 4.81 | 9.3 | 2.0 |
| Cyclohexane | 2.02 | 8.2 | 3.2 |
| Hexane | 1.89 | 7.3 | 3.5 |
| $CCl_4$ | 2.24 | 8.6 | 3.0 |
| Toluene | 2.44 | 8.9 | 2.5 |
| Benzene | 2.28 | 9.2 | 2.0 |
| Triethylamine | 2.42 | – | 1.6 |
| $CH_2Cl_2$ | 9.08 | 9.7 | 0.93 |
| Acetone | 20.7 | 9.8 | −0.23 |
| THF | 7.4 | 9.1 | 0.49 |
| Acetonitrile | 37.5 | 11.9 | −0.33 |
| 1,4-Dioxane | 2.21 | 10.00 | −1.1 |
| Cyclohexanone | 18.3 | 9.9 | 0.96 |
| *t*-Butylalcohol | 10.9 | 10.22 | – |
| DMF | 36.7 | – | 1.0 |

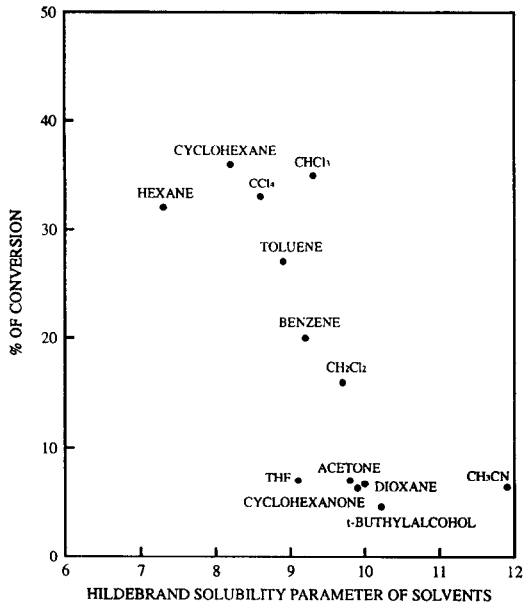



Fig. 4. Esterifications of 1-phenylethanol with acetic anhydride ($T$ = 40°C) carried out in different organic solvents vs. Hildebrand solubility parameter catalyzed by an immobilized lipase from *Pseudomonas* sp.

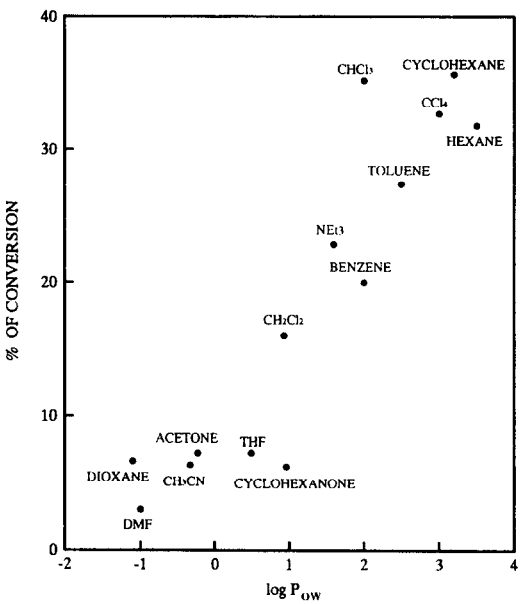



Fig. 5. Esterifications of 1-phenylethanol with acetic anhydride ($T$ = 40°C) carried out in different organic solvents vs. solvent log $P_{ow}$ catalyzed by an immobilized lipase from *Pseudomonas* sp.

just a weak correlation was observed. As well known $\delta$ is a rather poor solvent polarity indicator, especially for relatively apolar solvents. As shown in Fig. 4, in which is illustrated the percentage of conversion as function of the solubility parameter, approximately all solvents tested have a value within the quite narrow range of 7 to 11 and, moreover, the value of very apolar solvents do not differ significantly from each other.

Another more direct parameter reflecting the polarity of solvents is log $P_{ow}$. Log $P_{ow}$ values can easily be found out experimentally [6], or calculated from hydrophobic fragmental constants according to Rekker [7]. In Fig. 5 these values are plotted against the percentage of enzymatic conversion. The results clearly prove that there is a substantial correlation between activity and solvent polarity when log $P_{ow}$ is used as a polarity measure, and the results obtained show that the conversion increases with increasing log $P_{ow}$ values.

To quantify the correlation existing between the percentage of conversion and, log $P_{ow}$, we have found out the correlation coefficients [8] from the following expression:

$$r = \frac{N\sum_{1}^{N} x_i y_i \sum_{1}^{N} x_i \sum_{1}^{N} y_i}{\sqrt{N\sum_{1}^{N} x^2 - \left(\sum_{1}^{N} x\right)^2}\sqrt{N\sum_{1}^{N} y^2 - \left(\sum_{1}^{N} y\right)^2}}$$

where $r$ in a significant correlation has to be closed to 1. In Table 3 are reported $r$ values obtained.

The mathematical treatment of the experimental data, regarding enzymatic esterification

Table 3
Correlation coefficients $r$ between percentage of conversion and the different physico-chemical properties of solvents

| Solvent physico-chemical properties | $r$ | $P$ (%) [a] |
|---|---|---|
| log $P_{ow}$ | 0.90 | 0.1 |
| $\delta$ | 0.72 | 1 |
| $\epsilon$ | −0.63 | 2–5 |

[a] $P$ = probability to obtain an $r$ value higher than the $r$ obtained with our experimental data, with measurements conducted on non-correlated variables.

Table 4
Percentage of esterification, after 360 min of reaction time, between different secondary alcohols and acetic anhydride in organic solvents and in supercritical carbon dioxide ($T = 40°C$, $P_{CO_2}$ MPa) employing *Pseudomonas* sp. immobilized lipase

| Substrate | SC–$CO_2$ | Hexane | $CCl_4$ | Toluene | $CHCl_3$ | Benzene | $NEt_3$ | $CH_2Cl_2$ |
|---|---|---|---|---|---|---|---|---|
| 1-phenyl-ethanol | 46 | 32 | 30 | 27 | 33 | 20 | 20 | 15 |
| 1-(4-fluorophenyl)-ethanol | 57 | 48 | 34 | 42 | 28 | 28 | 25 | 16 |
| 1-(4-chlorophenyl)-ethanol | 52 | 28 | 27 | 23 | 28 | 18 | 25 | 18 |
| 1-(4-bromophenyl)-ethanol | 55 | 21 | 25 | 24 | 27 | 20 | 18 | 11 |
| 1-(4-methoxyphenyl)-ethanol | 28 | 18 | 28 | 26 | 29 | 25 | 19 | 18 |
| 1-(4-*tert*-butylphenyl)-ethanol | 50 | 32 | 28 | 24 | 24 | 16 | 20 | 10 |
| 1-(2-bromophenyl)-ethanol | 2 | 5 | 3 | 2 | 1 | 2 | 5 | 1 |
| 1-(2,4-dichlorophenyl)-ethanol | 12 | 9 | 4 | 8 | 2 | 7 | 9 | 1 |
| 1-(2-methoxyphenyl)-ethanol | 7 | 4 | 8 | 1 | 3 | 2 | 8 | 2 |

of 1-phenylethanol with acetic anhydride, confirms that log $P_{ow}$ is the physico-chemical property that better correlates with enzymatic activity.

The enzymatic esterifications were also carried out employing 1-phenylethanol derivatives as 1-(4-fluorophenyl)-ethanol, 1-(4-chlorophenyl)-ethanol, 1-(4-bromophenyl)-ethanol, 1-(4-methoxyphenyl)-ethanol, 1-(2-methoxyphenyl)-ethanol, 1-(2-bromophenyl)-ethanol, 1-(2,4-dichlorophenyl)-ethanol, 1-(4-*tert*-butylphenyl)-ethanol, to investigate on the influence of the nature of substrate on enzymatic activity.

These reactions were carried out in n-hexane, benzene, toluene, dichloromethane, chloroform, carbon tetrachloride, triethylamine, solvents in which we obtained high values of conversion using 1-phenylethanol as substrate.

In Table 4 are reported the conversion values, after 360 minutes of reaction time, as function of substrate structure. Also now $r$ data calculated (Table 5) confirm that enzymatic activity depends mainly on solvent hydrophobicity, compared to the other physico-chemical characteristics.

The percentage of esterification obtained shows that alogen atoms in *para* position generally increase the reaction rate and in particular with the fluoro derivative we obtained high conversion values. The presence, in *para* position in the aromatic ring of 1-phenylethanol, of a *tert*-butyl group, does not influence the enzymatic activity, providing conversion values close to those achieved with 1-phenylethanol. The 4-methoxy derivative of 1-phenylethanol usually induces a decrease of activity. The effect of *orto* substitutions in the aromatic ring of 1-phenylethanol, induces a steric hindrance in the complex enzyme/substrate determining a drastic decrease in activity.

As reported in our previous work [3], super-

Table 5
Correlation coefficients $r$ between percentage of conversion of esterification reactions, with different substrates, and the physico-chemical properties of solvents

| Substrate | Dielectric constant ($\epsilon$) | | Hildebrand solubility parameter ($\delta$) | | log $P_{ow}$ | |
|---|---|---|---|---|---|---|
| | $r$ | $P$ (%) [a] | $r$ | $P$ (%) [a] | $r$ | $P$ (%) [a] |
| 1-(4-fluorophenyl)-ethanol | −0.72 | 5 | 0.87 | 2 | 0.92 | 0.1 |
| 1-(4-chlorophenyl)-ethanol | −0.45 | – | 0.62 | 10 | 0.65 | 10 |
| 1-(4-bromophenyl)-ethanol | −0.62 | 10 | 0.29 | – | 0.64 | 10 |
| 1-(4-methoxyphenyl)-ethanol | −0.26 | – | 0.29 | – | 0.21 | – |
| 1-(4-*t*-butylphenyl)-ethanol | −0.70 | 8 | 0.84 | 5 | 0.93 | 0.1 |

[a] $P$ = probability to obtain an $r$ value higher than the $r$ obtained with our experimental data, with measurements conducted on non-correlated variables.

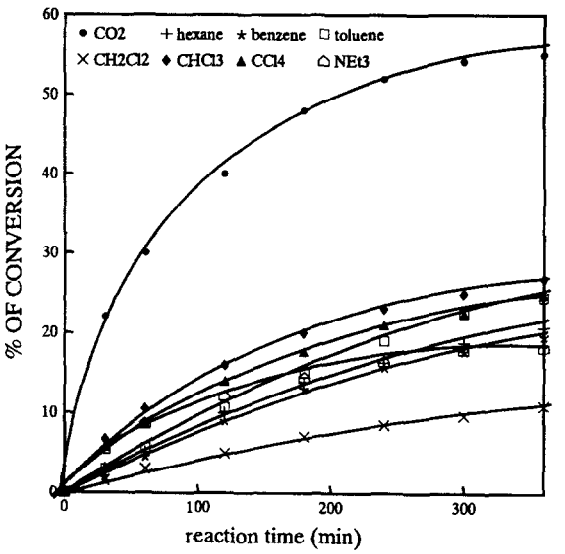



Fig. 6. Esterification rate of 1-(4-bromophenyl)-ethanol with acetic anhydride in supercritical carbon dioxide (20 MPa) in comparison with organic solvents like hexane, benzene, toluene, dichloromethane, chloroform and carbon tetrachloride at $T = 40°C$.

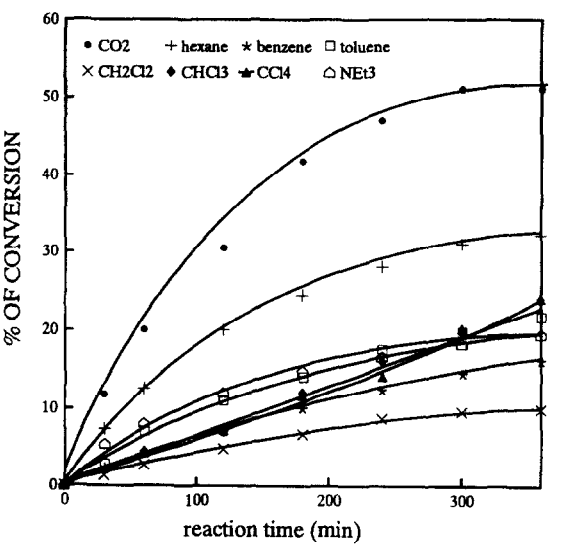



Fig. 7. Esterification rate of 1-(4-*tert*-butylphenyl)-ethanol with acetic anhydride in supercritical carbon dioxide (20 MPa) in comparison with organic solvents like hexane, benzene, toluene, dichloromethane, chloroform and carbon tetrachloride at $T = 40°C$.

critical fluids, in particular $SCCO_2$, could represent suitable media to carry out biocatalysed reactions. The aim of this work is to study different esterification reactions, employing 1-phenylethanol derivatives as substrates, using as catalyst an immobilized lipase from *Pseudomonas* sp. and to compare the results obtained in supercritical $CO_2$ to those achieved in organic solvents.

In Figs. 6–8 the kinetic curves obtained employing as substrates 1-(4-bromophenyl)-ethanol, 1-(4-*tert*-butylphenyl)-ethanol and 1-(4-methoxyphenyl)-ethanol and acetic anhydride as acylating agent, are reported, as an example, related to the reactions in organic solvents and compared to SC–$CO_2$. The results suggest that the reaction rates in supercritical $CO_2$ is significantly higher than in any conventional solvent tested. Although the lack of data regarding the log $P_{ow}$ values for supercritical $CO_2$, the solvent hydrophobicity doesn't seem to play an important role in reaction rate enhancement, while the higher extent of conversion in $CO_2$ compared to that in organic solvents can be explained as in terms of enhanced transport properties, high diffusivity of solute in supercritical medium as in terms of high stability of enzyme in supercritical phase. As well known the solutes diffusivity in the reaction medium has beneficial implications for enzymatic reac-

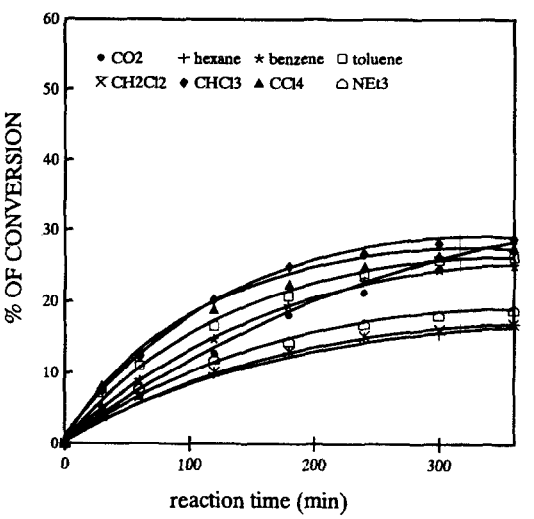



Fig. 8. Esterification rate of 1-(4-methoxyphenyl)-ethanol with acetic anhydride in supercritical carbon dioxide (20 MPa) in comparison with organic solvents like hexane, benzene, toluene, dichloromethane, chloroform and carbon tetrachloride at $T = 40°C$.

Table 6
Percentage of enantiomeric excess, after 360 min of reaction time, between different secondary alcohols and acetic anhydride in organic solvents and in supercritical carbon dioxide ($T = 40°C$, $P_{CO_2} = 20$ MPa) employing *Pseudomonas* sp. immobilized lipase

| 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*-butylphenyl)-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 |

tions when mass transfer in the immobilized matrix is the rate limiting step.

Table 6 shows that enantiomeric excess values obtained in $SC-CO_2$ are generally higher than those obtained in organic media.

## 5. Conclusions

In our work, using a microbial lipase immobilized on an ACR silica–gel matrix, we synthesized different esters of chiral alcohols, confirming the strong influence of the structure of substrate on the conversion and enantioselectivity of the reaction. The physico-chemical characteristics of media, as well as solubility Hildebrand parameters and dielectric constant of different organic solvents, do not provide reliable results, while the log $P_{ow}$ values give self-consistent relationships. Moreover, as reported by other authors [5], log $P_{ow}$/activity correlation is a general phenomenon that can be explained by differences in the ability of organic solvents to distort the 'essential water layer' around the enzyme.

Possible explanations for the results reported above could be referred to as regards to substrates behaviour, to the steric hindrance phenomena and for organic solvents activities to the superimposition of various parameters (presence of different phases in solution, association equilibrium of enzyme subunities) that can generate different and not reproducible performance of the enzyme, the hydrophobicity of the solvents (log $P_{ow}$), allowing the biocatalyst to reach in presence of the inner water the active conformation and the most suitable partition equilibrium with the polar matrix.

In addition we confirm some of our preliminary results relevant to the utilization, as a medium, of supercritical carbon dioxide, obtaining anyhow high enantioselectivity and conversion values. In this media the solvent hydrophobicity does not play a fundamental role in the biocatalytic reaction. The performance of enzyme in supercritical carbon dioxide, where predominant factors are low viscosity and surface tension values, could be explained in terms of enhanced mass transport properties and higher enzyme stability in comparison to organic solvents.

## References

[1] A. Zaks and A.M. Klibanov, J. Am. Chem. Soc., 108 (1986) 2767.
[2] T. Sakurai, A. Russeland A.M. Klibanov, J. Am. Chem. Soc., 110 (1988) 7236.
[3] E. Cernia, C. Palocci, F. Gasparini, D. Misiti and N. Fagnano, J. Mol. Catal., 89 (1994) L11–L18.
[4] J.H. Hildebrand, J.M. Prausnitz and R.L. Scott, Regular and Related Solutions, Van Nostrand Reinhold, New York, 1970.
[5] C. Laane, S. Boeren and K. Vos, Trends Biotechnol., 3 (1985) 10.
[6] M. Harnisch, H.J. Mockel and G. Schulze, J. Chromatogr., 282 (1983) 315.
[7] R.F. Rekker, The Hydrophobic Fragmental Constant, Elsevier, Amsterdam, 1977.
[8] H. Taylor, Elaborazione Statistica dei Dati Sperimentali, Veschi, Rome, 1988.