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High yield and optical purity in biocatalysed acylation of *trans*-2-phenyl-1-cyclohexanol with *Candida rugosa* lipase in non-conventional media

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Abstract

Candida rugosa lipase (CRL) shows high enantioselectivity toward (1*R*,2*S*)-(−)-*trans*-2-phenyl-1-cyclohexanol enantiomer in acetylation reaction employing vinyl acetate as acyl donor. Attempts to improve reaction yields have pointed out that supercritical CO_2 is the best reaction medium in the studied biocatalytic process. In these conditions an immobilised lipase from *Candida rugosa* is able to quantitatively resolve racemate with e.e.$_p$ 100%.

Keywords: Lipase; Transesterification; *trans*-2-Phenyl-1-cyclohexanol; Resolution; Organic media; Supercritical fluids

1. Introduction

The synthesis of organic compounds with one or several stereogenic centres is one of the most challenging tasks in modern organic synthesis. Among the possibilities to achieve asymmetric induction or to carry out kinetic resolution, biocatalytical processes have been established as unrenounceable methods in contemporary organic synthesis, especially in non-conventional media such as organic solvents and supercritical fluids. Basic studies on these reaction systems have led to an understanding of the enzyme mechanism of activity, enzyme specificity, stability and structure, and there is currently much interest in using 'solvent engineering' to try to modulate enzyme activity and specificity in non-conventional media by changing the physicochemical properties of the solvent [1].

Very recently the use of enzymes in supercritical fluids has been proposed as a means of improving the activity and the utility of such enzymes in anhydrous environments [2]. As is well known, the use of supercritical fluids (SCFs) in chemical separation processes [3] has been of considerable research interest for the past decade, but one very interesting and, yet,

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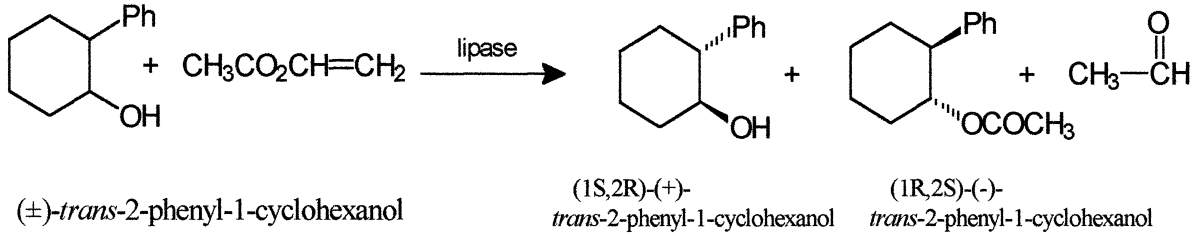


Fig. 1. Scheme of the transesterification reaction of (±)-*trans*-2-phenyl-1-cyclohexanol catalysed by lipase.

not fully tested offshoot of SCFs extraction technology is the use of a SCF solvent as a reaction medium in which an SCF either actively participates in the reaction or functions only as the solvent medium for reactants, or catalysts and/or products [4]. By exploiting the unique solvent properties of SCFs it could be possible to enhance reaction rates while maintaining or improving selectivity [5]. Also, separation of products from reactants can be greatly facilitated by the ease with which the solvent power of the SCF can be adjusted.

Among the biocatalysts used in non-conventional media, microbial lipases are the most frequently used. These enzymes are very easy to handle, many of them are stable, and they are able to accept a broad range of substrates due to their ability to change their conformation depending on the substrate structure (induced fit enzyme) [6]. This type of biocatalysts can be used to perform enantioselective hydrolytic reactions and formation of ester and amide bonds [7–12].

Vinyl acetate is one of the most widely used acyl donors in transesterification reactions because the unstable enol released during the course of the reaction immediately tautomerises to form the corresponding aldehyde, which shifts the reaction out of the equilibrium [11].

Among the cyclohexyl-based chiral auxiliaries, *trans*-2-phenyl-1-cyclohexanol plays an important role in asymmetric synthesis. Enantiomerically pure *trans*-2-phenyl-1-cyclohexanol has been used in a large number of reaction types [13] and to prepare optically pure pharmaceutical intermediates [14,15].

In this work we have studied lipase-catalysed transesterification of *trans*-2-phenyl-1-cyclohexanol with vinyl acetate as on acyl donor in order to separate both enantiomers of *trans*-2-phenyl-1-cyclohexanol (Fig. 1). Reaction parameters such as enzyme load, substrate concentration, vinyl acetate concentration and reaction media were investigated. We also examined several strategies to optimise the kinetic resolution of *trans*-2-phenyl-1-cyclohexanol, such as the employment of different lipases, immobilisation of biocatalysts, and the use of supercritical CO_2 as a reaction medium.

2. Experimental

2.1. Reagents

Lipase Type VII from *Candida rugosa* (L-1754), lipase Type VII-A from *C. rugosa* immobilised enzyme on macroporous acrylic beads (L-1150) and porcine pancreatic lipase (PPL) were obtained from Sigma (St. Louis, MO, USA). Lipase F-AP15 from *Rhyzopus Oryzae* were supplied by Amano Pharmaceutical (Nagoya, Japan), Lipase from *Rhyzomucor miehei* (RML) was obtained from Fluka Chemical (Buchs, Switzerland). (±)-*trans*-2-phenyl-1-cyclohexanol was purchased from Sigma (St. Louis, MO, USA). Vinyl acetate (99%) was obtained from Merck (Darmstadt, Germany). Non-commercial lipase from *C. rugosa* was kindly provided by Dr. Francisco Valero from Universitat Autonoma de Barcelona (UAB). All solvents were purchased from Carlo Erba

(Milano, Italy). Molecular sieves were purchased from Sigma (St. Louis, MO, USA). Carbon dioxide (99.998%) was purchased from SIAD (Bergamo, Italy).

2.2. Enzyme assay

Lipolytic activity was assayed by alkalimetric final titration. The assay mixture, containing 2.5 ml of buffer (phosphate buffer 10 mM, pH 7.4), 0.5 ml of tributyrin and 100 μl of the enzyme solution (10 mg/ml in buffer), was shaken for 30 s and incubated at 37°C under magnetic stirring for 30 min. An immobilised enzyme assay was performed by suspending 1 mg of solid in 2.5 ml of buffer solution. Then the reaction was stopped with 2.5 ml of ethanol/acetone 1:1 (v/v). The reaction mixture was titrated with 0.05 M NaOH in the presence of phenolphthalein using an automatic burette (Metrohm 645 multidosimat).

2.3. Transesterification procedure in organic solvent

The transesterification reactions were carried out in duplicate in screw capped vessels in which all reagents were added in appropriate ratios: racemic *trans*-2-phenyl-1-cyclohexanol was added to 2 ml of solvent followed by vinyl acetate and then by the enzyme. The sample was incubated in a thermostatic bath at 40°C under magnetic stirring at 600 rpm, along with its respective control (sample with no enzyme). No reaction took place in the absence of the enzyme.

2.4. Transesterification procedure in supercritical CO_2

The reaction was run in supercritical CO_2 at 40°C, 20 MPa. The apparatus (SFE 30 Carlo Erba Instruments) was specially designed to investigate various enzymatic reactions in supercritical carbon dioxide in a tank-like reactor with a reaction cell volume of 4 ml. The SFE (Supercritical Fluids Extractor) reactor can be used for operations at pressures of up to 500 atm in the temperature range 40–150°C. The pressure of CO_2 was controlled by a syringe pump that ensured a rapid pressure ramp-up. A precise amount of both enzyme and substrates was introduced within the reactor. After sealing, pressurisation was achieved by pumping liquid carbon dioxide to the desired final pressure (20 MPa, 40°C). During the reaction a six-way HPLC valve (Rheodyne 7125) allowed withdrawal of a sample for analysis without depressurisation. At the end of the reaction, depressurisation was achieved by opening a needle valve above the reaction cell. No reaction took place in the absence of the enzyme.

2.5. Experimental analysis

The enantiomeric excess (e.e.) of chiral esters was determined by enantioselective HRGC (High Resolution Gas Chromatography). A Carlo Erba Instrument Model 5300 (Milan, Italy) gas chromatograph equipped with a flame ionisation detector was used for all GC evaluations and separations. The injection port and detector temperature were set at 250°C. Helium was used as the carrier gas (0.75 kg/cm^2). Separations were performed on a 25 mt × 0.25 mm ID fused-silica enantioselective capillary column coated with Megadex 5 (30% 2,3-*O*-dimethyl-6-*O*-pentyl-β-cyclodextrin in OV 1701). The GC oven was operated at various temperatures for isothermal elution experiments between 140°C and 155°C, in 5°C increments. The injection volumes were 0.5 μl. The elution time of an unretained peak, used in calculating the capacity factors, was the mean of multiple injections of dichloromethane at the temperature used for chromatography.

Chromatographic data were collected and processed using the Millennium 2010 Chromatography Manager (Waters Chromatography, Division of Millipore, Milford, MA, USA) software.

In order to achieve the necessary overall selectivity, for both alcohol and ester, tandem arrangements of chiral (enantio- and diastereoselective) and achiral (diastereoselective) columns were performed, connecting in series the above-mentioned chiral column with a 5 mt × 0.20 mm ID fused-silica achiral capillary column coated with cross-linked methyl silicone HP1. In these experiments, the carrier gas pressure was increased to 1.40 kg/cm^2.

Synthetic yield of *trans*-2-phenyl-1-cyclohexylacetate was determined by the amount of product formed, using peak area integration by on-line software.

Mixtures of alcohol and ester enantiomers were baseline resolved by the aid of a multi-column approach in four peaks with the following retention times:

(1*S*,2*R*)-(+)-*trans*-2-phenyl-1-cyclohexanol (t_R = 16.77 min)
(1*R*,2*S*)-(−)-*trans*-2-phenyl-1-cyclohexanol (t_R = 17.48 min)
(1*S*,2*R*)-*trans*-2-phenyl-1-cyclohexylacetate (t_R = 17.95 min)
(1*R*,2*S*)-*trans*-2-phenyl-1-cyclohexylacetate (t_R = 18.82 min)

3. Results and discussion

3.1. Optimisation of the reaction conditions

Five commercial microbial and mammalian lipases were tested in acylation of *trans*-2-phenyl-1-cyclohexanol with vinyl acetate. The screened lipases showed a high enantioselectivity toward the (1*R*,2*S*)-(−)-*trans*-2-phenyl-1-cyclohexanol enantiomer. The *C. rugosa* lipase gave the best yield whereas other lipases performed poorly under the same reaction conditions (Table 1).

The transesterification of *trans*-2-phenyl-1-cyclohexanol with vinyl acetate was carried out at different vinyl acetate and alcohol concentrations. The best yield was obtained at a *trans*-2-phenyl-1-cyclohexanol/vinyl acetate molar ratio equal to 1:5 and at 0.0625 M *trans*-2-phenyl-1-cyclohexanol concentration.

Table 1
Lipase screening in vinyl acetate transesterification with (±)-*trans*-2-phenyl-1-cyclohexanol catalysed by different lipases

Lipases	Conversion[a] (%)	
	Reaction time 24 h	Reaction time 72 h
CRL (Sigma)	4.2[b]	8.7[c]
CRL immobilised (Sigma)	–[b]	1.4[c]
F-AP15	1.2[b]	3.1[c]
RML		–[d]
PPL		–[d]

[a] reaction medium: hexane; T = 40°C; substrate/vinyl acetate 1:1;
[b] enzyme: 40 mg; alcohol concentration: 0.5 M.
[c] enzyme: 400 mg; alcohol concentration: 0.5 M.
[d] enzyme: 100 mg; alcohol concentration: 0.125 M.

The amount of *C. rugosa* lipase in the reaction system was varied from 20 mg to 200 mg at 0.0625 M *trans*-2-phenyl-1-cyclohexanol concentration and at a 1:5 *trans*-2-phenyl-1-cyclohexanol/vinyl acetate molar ratio. Yields of 25% were observed just using 100 mg of lipase.

The reaction increased in yield up to 168 h when equilibrium was reached.

It has been proven that activity and enantioselectivity of lipases can be tuned by a reaction medium [16,17]. The reaction was run in a broad range of organic solvents with different physicochemical properties. Highest yields were performed in hexane, whereas no reaction took place in DMF and triethylamine. In the latter solvents, a denaturation effect was detected. The enzyme was incubated in the different solvents and the residual lipolytic activity was tested according to standard assay procedures. In all solvents the enzyme showed a stereo-preference to (1*R*,2*S*)-(−)-*trans*-2-phenyl-1-cyclohexanol. The data are shown in Table 2.

3.2. Improvement of trans-2-phenyl-1-cyclohexanol resolution by lipase-catalysed acetylation

At first, in order to increase the conversion of *trans*-2-phenyl-1-cyclohexanol in the corre-

Table 2
Effect of reaction medium on vinyl acetate transesterification with (±)-*trans*-2-phenyl-1-cyclohexanol catalysed by CRL (Sigma)

Solvent	Conversion[a] (%)	e.e.$_p$ (%)
DMF	0	–
NEt_3	0	–
$CHCl_3$	1.5	100
Dioxane	2.0	100
Cyclohexanone	2.7	100
CH_2Cl_2	3.2	100
CH_3CN	3.2	100
THF	4.2	100
Acetone	5.2	100
Benzene	6.3	100
CCl_4	6.8	100
*t*BuOMe	14.2	100
Decane	20.5	100
Et_2O	23.2	100
Hexane	24.4	100

[a]Alcohol concentration 0.0625 M; Substrate/vinyl acetate 1:5; CRL 100 mg; $T = 40°C$; reaction time 168 h.

sponding acetate, lipase from *C. rugosa* (UAB-CRL) produced in a pilot plant was used [18]. This enzyme has revealed some important differences in performance (qualitatively and quantitatively) compared to the commercial preparation (Sigma).

Time course was used to compare the activity of lipase from CRL (Sigma) to UAB-CRL. Using the latter preparation, reaction yields were 20% and the equilibrium was reached after 24 h. The acylation was highly enantioselective (e.e.$_p$ 100%).

Immobilisation of enzymes is an attractive worldwide technique to obtain a biocatalyst that is more stable and easier to handle than its free counterpart, and immobilised biocatalysts are being used extensively today [19]. Two different supporting materials were used to immobilise lipase from *C. rugosa*.

Both CRL Sigma and UAB-CRL were immobilised onto functionalised silica gel according to an already published procedure [20]. The immobilisation ratio enzyme/solid support was equal to 1:5 (w/w). Lipolytic activity of both lipases increased by immobilisation (Table 3). In particular, the activity of immobilised UAB-CRL was higher than that of immobilised CRL Sigma. Using immobilised UAB-CRL, the yield of the transesterification reaction reached 39.7% after 24 h, whereas in the presence of immobilised CRL (Sigma), conversion was very low, about 5.8% after 168 h.

Moreover, both lipases were immobilised onto CL-PVA (Cross-Linked Polyvinyl Alcohol) oleate polymer [21,22]. The immobilisation ratio enzyme/solid support was equal to 1:5 (w/w). Lipolytic activity of both immobilised lipases was lower than the respective free preparation (Table 3). Using UAB-CRL in the reaction of transesterification of *trans*-2-phenyl-1-cyclohexanol, the yield reaches 26% and e.e.$_p$ 100% after 24 h. The yield reaches 8% after 168 h, using immobilised CRL (Sigma).

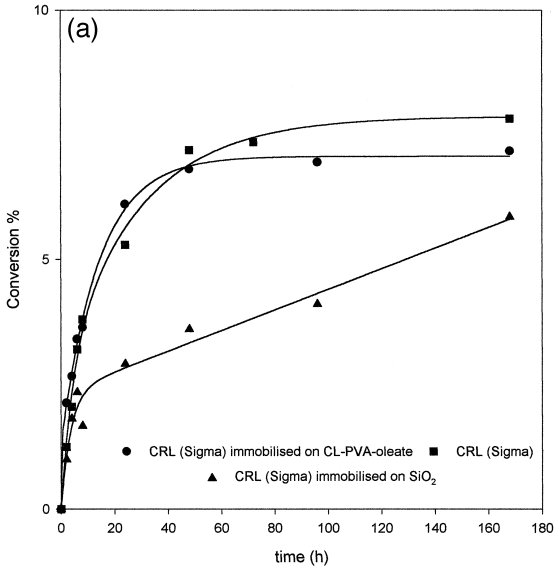
In order to evaluate the effect of immobilisation on enzyme activity, all data were compared to those obtained using 20 mg of free enzyme preparation (Fig. 2).

Results showed that immobilisation increases activity of UAB-CRL towards transesterification reaction of *trans*-2-phenyl-1-cyclohexanol. Otherwise, performance of CRL (Sigma) is not influenced by immobilisation on both functionalised silica gel and CL-PVA oleate polymer.

As is well known, the yeast *C. rugosa* produces different extracellular lipase isoforms [23]. The characterisation of catalytic activity of CRL has often given ambiguous results because experiments have generally been carried out with raw or partially purified enzyme preparations. CRL (Sigma) preparation includes the predomi-

Table 3
Lipolytic activity of *C. rugosa* lipase from Sigma and UAB

		Lipolytic activity (μeq/min mg)
CRL Sigma	free enzyme	3.0
	immobilised on silica gel	4.5
	immobilised on CL-PVA oleate	0.1
UAB-CRL	free enzyme	7.3
	immobilised on silica gel	15.7
	immobilised on CL-PVA oleate	0.1



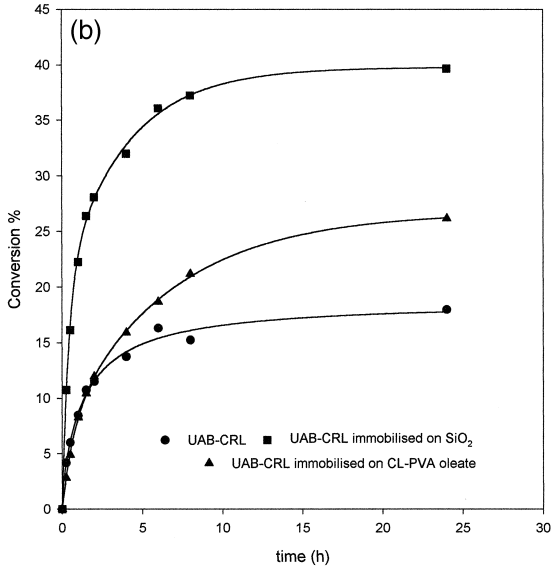


Fig. 2. (A) Vinyl acetate transesterification with (±)-*trans*-2-phenyl-1-cyclohexanol catalysed by free and immobilised CRL (Sigma). Alcohol concentration 0.0625 M; alcohol/vinyl acetate 1:5; CRL (Sigma) immobilised 100 mg; CRL (Sigma) free 20 mg; $T = 40$°C; solvent: hexane. (B) Vinyl acetate transesterification with (±)-*trans*-2-phenyl-1-cyclohexanol catalysed by free and immobilised UAB-CRL. Alcohol concentration 0.0625 M; alcohol/vinyl acetate 1:5; UAB-CRL immobilised 100 mg; UAB-CRL free 20 mg; $T = 40$°C; solvent: hexane.

nant isoform, Lip1 [24]. Based on recent communication [25], UAB-CRL's preparation shows the presence of two lipase isoforms Lip2 and Lip3 as prevalent hydrolytic enzymes. The different performance of two preparations may be due to a different isoform composition. This may also explain the different behaviour towards immobilisation. Activity of UAB-CRL is improved by immobilisation, whether it is due to hydrogen bonding (immobilisation on silica gel) or hydrophobic interaction (immobilisation on CL-PVA oleate). Immobilisation of CRL (Sigma) on both supporting materials doesn't involve any increase in the reaction yield. A first attempt to purify CRL (Sigma) showed that the isoforms have similar amino acid content, N-terminal sequence and molecular weight, but they differ on the neutral sugar content, hydrophobicity, stability to pH and temperature. They also show differences in substrate specificity [26].

Acetaldehyde is known to act as an alkylating agent on enzymes by forming Schiff bases in a Maillard-type reaction [27], in particular on N-amino residues of lysine [28]. A positive charge is removed from the enzyme surface during the course of this reaction, possibly leading to deactivation. Lipase from *C. rugosa* is extremely sensitive towards acetaldehyde and, as a consequence, it can lose up to 80% of activity in a typical acyl transfer reaction when vinyl acetate is employed as an acyl donor [29]. This drawback seems be overcome by immobilising the enzyme [30].

To test the stability of CRL towards acetaldehyde, transesterification was carried out by adding the same acetaldehyde as that produced after 72 h of reaction (12.5 mM).

CRL (Sigma) seems to be sensitive towards acetaldehyde at high conversion values, and after 72 h a decrease in yield is appreciable ($\approx 5\%$). Performance of UAB-CRL is not influenced by the presence of acetaldehyde. It was noted that immobilisation does not involve any change in enzyme stability towards acetaldehyde. Thus, the lower activity of CRL (Sigma) compared to that of UAB-CRL cannot be completely due to an acetaldehyde denaturation effect.

Moreover, we tried to investigate the effect of solid matrices on enzyme performance. 4 Å molecular sieves were employed either as beads or as powder. The reaction yield catalysed by CRL (Sigma) in the presence of molecular sieves as beads reached 33.0% after 168 h, whereas 16.4% of conversion was achieved under the same conditions using molecular sieves as powder (Fig. 3A).

The same behaviour was observed using UAB-CRL. In the presence of molecular sieves as beads, the yield reached 32.9% after 24 h (Fig. 3B).

Experimental data may suggest that molecular sieves can act not only as dehydration agents but also as a means of dispersing enzyme particles in the reaction system. In fact, in the heterogeneous system, beads seem to be more efficient than powder to increase the contact superficial area of biocatalysts.

Yields observed using CRL (Sigma) in the presence of molecular sieves as beads reach about 33% both in hexane and in Et_2O, but enantioselectivity observed is 87.7% in the former, 100% in the latter after 168 h. At high conversion a solvent effect on enantioselectivity of transesterification reaction seems to be apparent. Otherwise, using UAB-CRL in hexane, the $e.e._p$ observed was equal to 100%.

The above-mentioned supercritical fluids were employed to improve the activity of enzymes in anhydrous media. In particular a lipase-catalysed reaction was performed in supercritical fluids and its effect on activity and enantioselectivity was studied [31]. The physical properties of supercritical CO_2, such as the dielectric constant and the density, can be predictably manipulated by changing pressure. Thus, fine tuning of the physical properties of the reaction medium can be achieved by a small change in pressure. Our previous works have pointed out 40°C and 20 MPa as the optimal conditions for a lipase-catalysed acetylation reaction [32]. Under these conditions, transesterification of *trans*-2-phenyl-1-cyclohexanol with vinyl acetate was carried out in supercritical

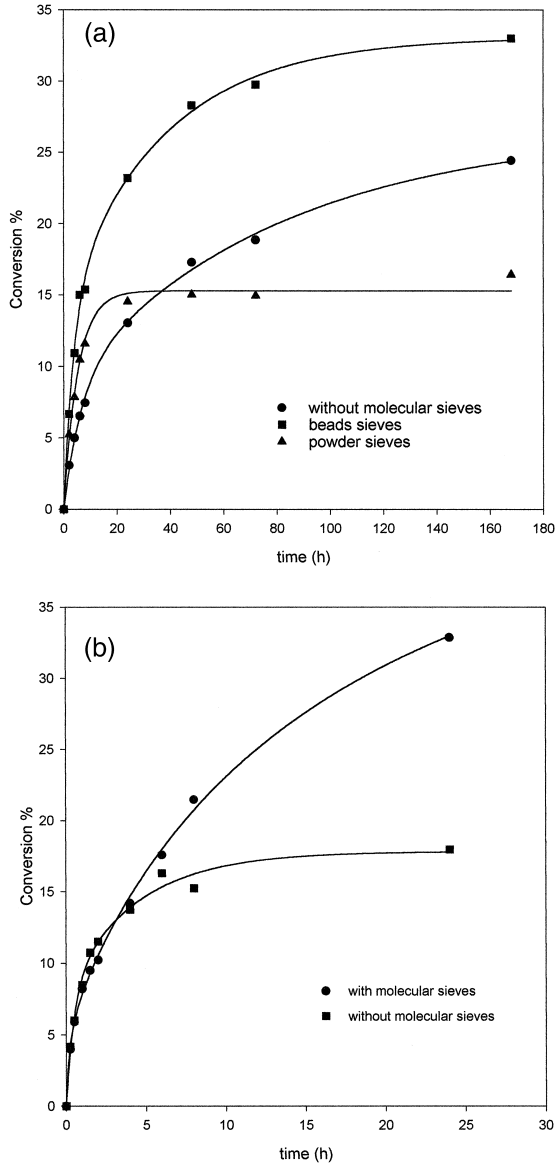


Fig. 3. (A) Vinyl acetate transesterification with (±)-*trans*-2-phenyl-1-cyclohexanol catalysed by CRL (Sigma) in the presence of molecular sieves. Alcohol concentration 0.0625 M; alcohol/vinyl acetate 1:5; CRL (Sigma) 100 mg; molecular sieves 200 mg; $T = 40$°C; solvent: hexane. (B) Vinyl acetate transesterification with (±)-*trans*-2-phenyl-1-cyclohexanol catalysed by UAB-CRL in the presence of molecular sieves. Alcohol concentration 0.0625 M; alcohol/vinyl acetate 1:5; UAB-CRL 20 mg; molecular sieves 40 mg; $T = 40$°C; solvent: hexane.

CO_2 using UAB-CRL immobilised on functionalised silica gel (400 mg). Equilibrium was achieved after 24 h and the initial rate was higher than that observed in hexane. The yield

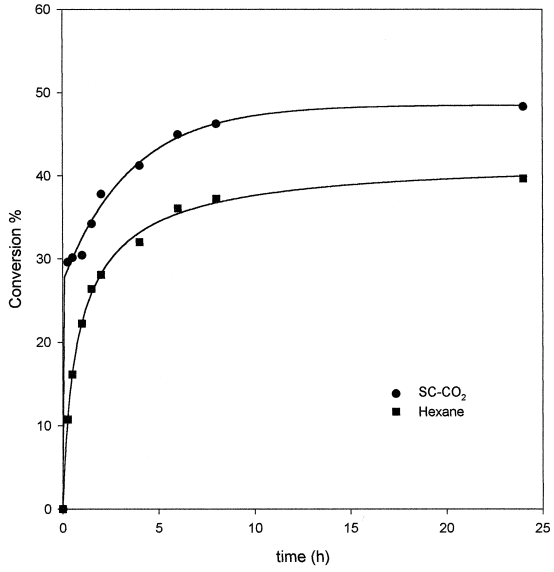


Fig. 4. Vinyl acetate transesterification with (±)-*trans*-2-phenyl-1-cyclohexanol catalysed by UAB-CRL immobilised on silica gel in hexane and supercritical CO_2 (40 MPa). Alcohol concentration 0.0625 M; alcohol/vinyl acetate 1:5; $T = 40°C$.

achieved 48% after 24 h and e.e.$_p$ observed was 100%, showing a kinetic resolution of *trans*-2-phenyl-1-cyclohexanol that was quite quantitative (Fig. 4). These results could be attributed to the specific properties of the medium in terms of low viscosity and surface pressure values and high diffusivity of substrates in the supercritical phase.

4. Conclusion

Lipase from *C. rugosa* is able to resolve a racemic mixture of (±)-*trans*-2-phenyl-1-cyclohexanol by a stereoselective acylation with vinyl acetate. CRL shows high enantioselectivity toward the (1*R*,2*S*)-(−)-*trans*-2-phenyl-1-cyclohexanol enantiomer.

Different organic solvents, biocatalysts from different sources, immobilisation procedures and solid matrixes in the reaction system were used to improve reaction yield. The resolution of (±)-*trans*-2-phenyl-1-cyclohexanol is finally achieved (100% e.e.$_p$) in supercritical CO_2 with quantitative yields. Supercritical CO_2 has been seen to be useful replacement for organic solvents. Moreover CO_2 is a natural product, more environmentally acceptable than organic solvents, it is also non-flammable, essentially non-toxic and it permits an easy product recovery, without any traces of solvent.

Further investigations are in progress to understand the role of supercritical CO_2 as a reaction medium in the acylation reaction of (±)-*trans*-2-phenyl-1-cyclohexanol. Determination of activation volume $\Delta V^{\#}$, valuation of solubility of reagents and effects of supercritical CO_2 on enzyme structure may clarify the effect of supercritical CO_2 on the studied reaction.

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