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Kinetic Resolution of Both 1-Phenylethanol Enantiomers Produced by Hydrolysis of 1-Phenylethyl Acetate with *Candida antarctica* Lipase B in Different Solvent Systems[1]

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Abstract—The enzymatic resolution of (*R*, *S*)-1-phenylethanol produced by hydrolysis of (*R*, *S*)-1-phenylethyl acetate catalyzed by immobilized *Candida antarctica* lipase B (CALB) was successfully carried out in different solvent systems. A systematic screening and optimization of the reaction parameters such as enzyme amount, the nature and the content of organic solvent, pressure and temperature in supercritical carbon dioxide (SC-CO_2) and phosphate buffer, with respect to the conversion rate, were performed. CALB exhibits high enantioselectivity in both *tert*-butanol with 0.025 mol/l phosphate buffer (pH 7.5) and SC-CO_2 with 0.025 mol/l phosphate buffer (pH 7.5) systems. The conversion rate was 41.2% higher in SC-CO_2 with 0.025 mol/l phosphate buffer (pH 7.5) then in *tert*-butanol with 0.025 mol/l phosphate buffer (pH 7.5) and the reaction time decreased from 8 h to 90 min.

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Chiral alcohols are important intermediates for the synthesis of many chiral medicines, pesticides, flavors, fragrances, liquid crystals and chiral auxiliaries which can be prepared by chemical or biological methods. Biological approaches, including biocatalysis and bioconversion, have drawn broad attention for its high efficiency, mild reaction conditions, outstanding stereospecificity, benign to the environment, and so on [1–5]. These methods normally refer to kinetic resolution of racemic alcohols or asymmetric reduction of prochiral ketones.

The optically active 1-phenylethanol, especially (*R*)-1-phenylethanol, is used as chiral building block and synthetic intermediate in fine chemical, pharmaceutical and agrochemical industries [6]. In pharmaceutical industry, (*R*)-1-phenylethanol is used as ophthalmic preservative and may also inhibit cholesterol intestinal adsorption and thus decrease the cholesterol level [6]. The other application area of the enantiomers is in the chemical analysis. Both (*R*)- and (*S*)-1-phenylethanol are used as chiral reagent for the determination of enantiomeric purity and for the asymmetric opening of cyclic anhydrides and epoxides [7]. Lipases (triacyl glycerol hydrolases EC 3.1.1.3) are enzymes that catalyze a broad spectrum of reactions, such as hydrolysis of ester bonds and transesterification and ester synthesis at the interface between substrate and water or in non-aqueous organic solvents [8, 9]. They are used in a wide range of industrial processes including food, chemical, pharmaceutical and detergent production, because lipases possess wide substrate specificity, have an excellent ability to recognize chirality, and do not require labile cofactors.

Therefore, the kinetic resolution of (*R*, *S*)-1-phenylethanol by means of lipases has been investigated by many research groups and shown to be very efficient [10]. However, most kinetic resolutions of (*R*, *S*)-1-phenylethanol catalyzed by lipases are carried out through transesterification [11–14]. Only a few research groups tried to use the hydrolysis of esters to resolve the racemates. Kawashima and Hasegawa [14] were first who studied the enantioselective hydrolysis of (*R*, *S*)-1-phenylethanol alkyl carbonate by means of porcine pancreatic lipase using 0.7 mol/l borate buffer (pH 8.0) as the reaction medium with enantiomeric excess (***ee***) 53% and yield of (*R*)-1-phenylethanol 54%. Hydrolysis of (*R*, *S*)-1-phenylethyl acetate with *Candida rugosa* lipase in water was investigated by Bellezza et al. [15]. The rate of hydrolysis was very low and the enantioselectivity was disappointing.

In the present study, the kinetic resolutions of (*R*, *S*)-1-phenylethanol produced by hydrolysis of (*R*, *S*)-1-phenylethyl acetate with a commercial immobilized *Candida antarctica* lipase B (CALB, Novozym 435) in different solvent systems (see Scheme) were investigated. The conditions of hydrolysis were also optimized.

[1] The article is published in the original.

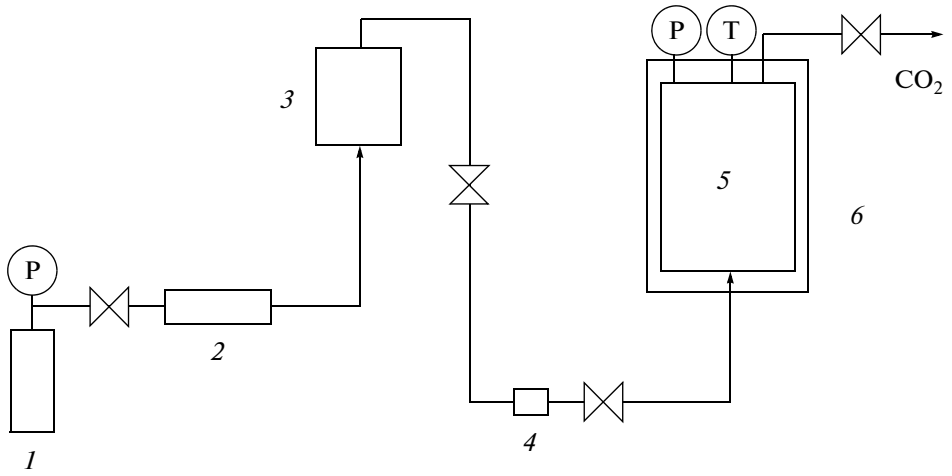
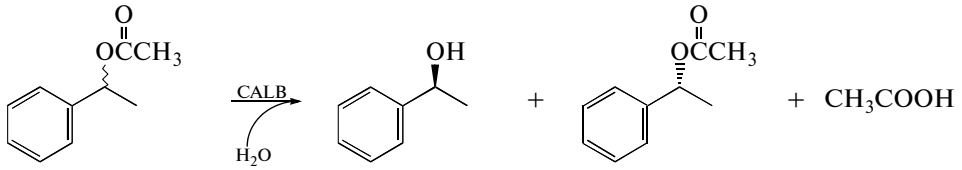


Fig. 1. Supercritical CO_2 treatment system: *1*—CO_2 cylinder, *2*—filter, *3*—cooling bath, *4*—high-pressure plunger-pump, *5*—reactor, *6*—constant temperature oven. The symbols T and P means temperature probe and pressure gauge, respectively.

Schematic representation of kinetic resolution of (*R*, *S*)-1-phenylethanol produced by hydrolysis of (*R*, *S*)-1-phenylethyl acetate by CALB



(*R*, *S*)-1-Phenylethanol acetate | (*S*)-1-Phenylethanol | (*R*)-1-Phenylethanol acetate | Acetate acid

Scheme.

MATERIALS AND METHODS

Materials

(*R*, *S*)-1-Phenylethyl acetate (≥98%), (*R*)-1-phenylethanol (≥97%) and (*S*)-1-phenylethanol (≥97%) were provided by Sigma. The commercially available enzyme preparation, CALB-Novozym 435, was purchased from Novozymes AS (Bagsvaerd, Denmark). The biocatalyst was used without any pretreatment. Other organic solvents and chemicals were all purchased from the local market with analytical grade.

Analytical Methods

The enantiomer contents during the reaction time course were monitored by a Runan RuiHong SP-6890 gas chromatograph equipped with a FID detector, using high-purity nitrogen as carrier gas and a capillary chiral column (CYCLODEX-B, 30 m × 0.25 mm × 0.25 μm, Agilent Technology), at following temperature program: 100°C (5 min) – 120°C (5°C/min, 12 min) – 200°C (10°C/min, 10 min). Detector and injector temperatures were both set at 250°C. It was splitless in the analysis.

Reaction Procedure

Enantiomerical hydrolysis of (*R*, *S*)-1-phenylethyl acetate to produce (*S*)-1-phenylethanol by CALB in different solvent systems was performed. Experiments under normal pressure were carried out in 50-ml batch conical flasks with lids in a rotating shaker. The rotating rate and temperature were set 150 rpm and 30°C, respectively. If organic solvent was used as the sole medium, 1% 0.025 phosphate buffer (pH 7.5) was added. The initial concentration of (*R*, *S*)-1-phenylethyl acetate was 1% (v/v).

Experiments under supercritical pressure were carried out batchwise in a 50-ml stainless steel extraction vessel as shown in Fig. 1. The reaction medium was 0.025 mol/l phosphate buffer/supercritical CO_2 (40/60, v/v). The experiments proceeded as follows: first, 20 ml 0.025 mol/l phosphate buffer, 0.5 ml (*R*, *S*)-1-phenylethyl acetate and 150 mg immobilized enzyme were loaded into the reactor with thermostat and the pipelines were linked immediately and tightly. Then CO_2 was pumped into the reactor with a high-pressure plunger-pump. The pressure and temperature were held for a given period of time after the system reached the set state. Afterwards, gas-valve was

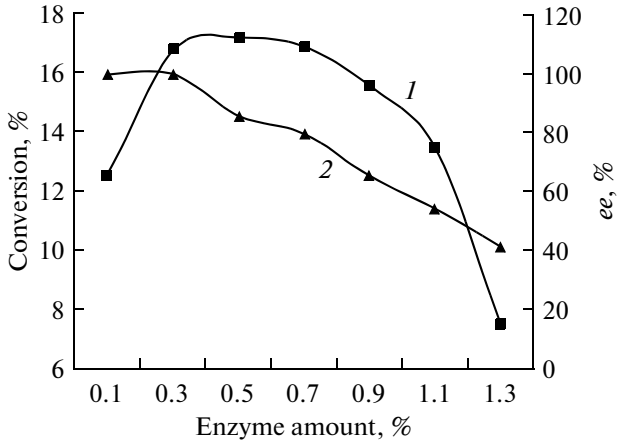


Fig. 2. Effect of enzyme amount on the conversion (*1*) and *ee* (*2*). Reaction conditions: rotating rate 150 rpm, temperature 30°C, pH 7.5, 0.025 mol/l phosphate buffer, initial concentration of (*R*, *S*)-1-phenylethyl acetate 1.0% (*v*/*v*).

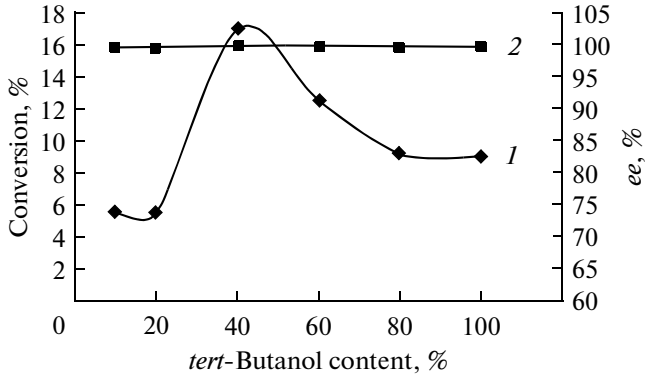


Fig. 3. Effect of *tert*-butanol content on the conversion (*1*) and *ee* (*2*). Reaction conditions: rotating rate 150 rpm, temperature 30°C, pH 7.5, solution of 0.025 mol/l phosphate buffer and *tert*-butanol, initial concentration of (*R*, *S*)-1-phenylethyl acetate 1.0% (*v*/*v*), CALB amount 0.3% (*w*/*v*).

opened to let CO_2 out until a complete discharge of pressure was attained. The samples from different runs were taken out from the reactor and were analyzed by GC in order to establish the product formation profile. The enantiomers of (*R*, *S*)-1-phenylethyl acetate and (*S*)-1-phenylethanol were baseline separated in the GC-analysis. The conversion rate and *ee* were calculated by applying the equation, which is valid for reactions:

$$\text{conversion rate} = (R_s - S_s)/2R_s \times 100\%,$$

$$ee = (S_p - R_p)/(S_p + R_p) \times 100\%,$$

where R_s and S_s are the concentrations of (*R*)- and (*S*)-1-phenylethyl acetate, R_p and S_p are the concentrations of (*R*)- and (*S*)-1-phenylethanol, respectively.

At least two experiments were run at each operative condition. The relative deviation was within ±1.5%).

RESULTS AND DISCUSSION

Effect of the Enzyme Concentration

The commercial immobilized form of CALB was assayed to catalyze the (*R*, *S*)-1-phenylethyl acetate hydrolysis in 0.025 mol/1 phosphate buffer. CALB concentration was varied in the range of 0.1–1.3% (*w*/*v*) (Fig. 2). At low CALB concentration (less than 0.3%), the hydrolysis of ester increased with increasing enzyme amount and high *ee* values were obtained in the experiments. But when CALB concentration reached more than 0.3%, *ee* began to decrease. Furthermore, the conversion rate also decreased when CALB concentration was beyond 0.7%. This might be caused by the adsorption of substrate by the particles of CALB. For this reason, 0.3% was selected for the following experiments, and no further experiments were performed at higher CALB amounts.

Screening of Reaction Media under Normal Pressure

A comparison of activity on CALB in various organic solvents is shown in table. CALB could efficiently catalyze the hydrolysis in hexane and heptane. But the enantioselectivity was poor. *ee* Value was the highest for *tert*-butanol, although the converstion rate was low. In literature [16, 17], *tert*-butanol was usually selected as the medium for lipase-catalyzed reaction. Therefore, in the following experiments *tert*-butanol was selected as medium.

In order to investigate the effect of the water content on the hydrolysis, different concentrations of *tert*-butanol in 0.025 mol/l phosphate buffer (pH 7.5) were used for the hydrolysis, from 10 to 100%. The results are shown in Fig. 3. The conversion rate grows with increasing *tert*-butanol content. At 40% of *tert*-butanol, the conversion rate reaches a maximum. At higher concentrations of *tert*-butanol (more than 40%), the conversion rate decreases with increasing *tert*-butanol content. The *ee* values in all the experiments are higher than 99%.

Comparison of conversion rate and enantiomeric excess in various organic solvents

Parameter	Solvent					
	tert-butanol	hexane	heptane	ethyl acetate	toluene	benzene
Conversion rate, %	3.62	49.77	40.42	6.57	17.02	9.65
ee	35.98	20.54	1.92	1.08	11.05	3.25

Time Course of Hydrolysis under Normal Pressure

Figure 4 shows the time course of reaction during enantioselective hydrolysis. The *ee* value in the reaction was more than 99% (data not shown). When the reaction began, the reaction substrate, (*R*, *S*)-1-phenylethyl acetate, was immediately consumed and (*S*)-1-phenylethanol was produced. After 8 h reaction, the conversion rate reached 24.5%.

Preliminary Optimization of Reaction Conditions under Supercritical CO_2

SC-CO_2 has benefits of an environmental benign nature, low toxicity, non-flammability, high availability and ambient critical temperature (T_c = 31.0°C) [18]. SC-CO_2, like other supercritical fluids, is different from ordinary solvents and characterized by a low gas-like viscosity, a high diffusivity and a liquid-like solubilizing power. Furthermore, these properties are tunable by manipulating the pressure and temperature. Small changes in pressure or temperature can lead to significant changes in density and density-dependant solvent properties, such as the dielectric coefficient. Since the first report on the lipase–catalyzed reactions in supercritical fluids in 1985 by Randolph et al. [19], use of supercritical fluids, especially SC-CO_2, as the reaction media in lipase-catalyzed reactions has been one of the important research points in recent decades. The benefits [20, 21] of using supercritical CO_2 for lipase-catalyzed reactions are high reaction rates, good control of selectivities [22–24], including regioselectivities and stereoselectivities, etc.

In order to improve the conversion rate and the efficiency of the hydrolysis by CALB, reactions were carried out under supercritical CO_2. The reaction conditions, such as pressure and temperature were preliminarily optimized. Taking into account the results obtained at normal pressure, 60% of supercritical CO_2 was chosen as the reaction medium throughout the experiments.

Effect of pressure on the hydrolysis. Since possible loss of enzyme activity caused by high pressure of SC-CO_2 may lead to undesirable poor reaction rates and reduction of desired product production, the effect of pressure was investigated first. The reaction pressure was varied from 8 to 25 MPa at 40°C. As seen from Fig. 5, the conversion rate reached a maximum at 10 MPa and then decreased. In the experiments, *ee* value was more than 99%. Perhaps because the reaction was performed in the multi-phase system, the hydrolysis rate was low at low pressures due to the low solubility of water. With increasing pressure, the solubility of water increased resulting in a deeper hydrolysis of the substrate. Upon further pressure increase, the hydrolysis decreased, because the water content in the immobilized enzyme diminished due to poorer solubility of water. So 10 MPa was the pressure that is most suitable for the lipase-catalyzed hydrolysis under SC-CO_2.

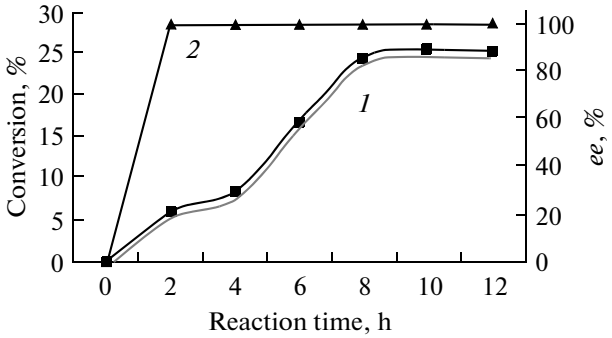


Fig. 4. Time course of hydrolysis under normal pressure: *1*—conversion, *2*—*ee*. Reaction conditions: rotating rate 150 rpm, temperature 30°C, pH 7.5, solution of 0.025 mol/l phosphate buffer and *tert*-butanol in ratio 60/40, initial concentration of (*R*, *S*)-1-phenylethyl acetate 1.0% (*v*/*v*), CALB amount 0.3% (*w*/*v*).

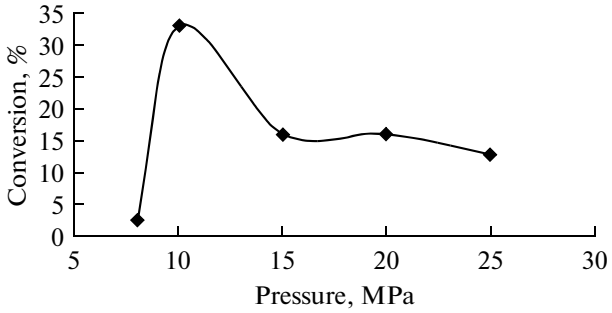


Fig. 5. Effect of pressure on the conversion. Reaction conditions: temperature 40°C, solution of 0.025 mol/l phosphate buffer and supercritical CO_2 in ratio 60/40, initial concentration of (*R*, *S*)-1-phenylethyl acetate 1.0% (*v*/*v*), CALB amount 0.3% (*w*/*v*).

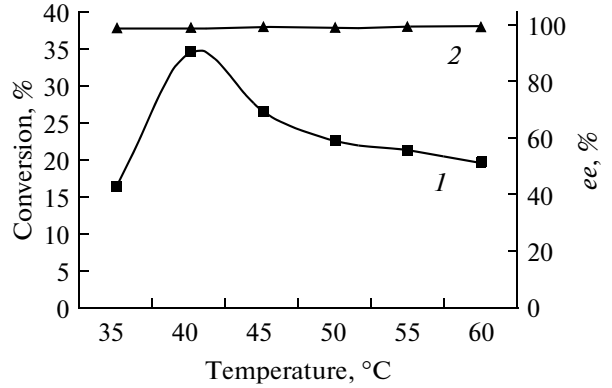


Fig. 6. Effect of temperature on the conversion (*1*) and *ee* (*2*). Reaction conditions: pressure 10 MPa, solution of 0.025 mol/l phosphate buffer/ and supercritical CO_2 in ratio 60/40, initial concentration of (*R*, *S*)-1-phenylethyl acetate 1.0% (*v*/*v*), CALB amount 0.3% (*w*/*v*).

Effect of temperature on the hydrolysis. The temperature was varied from 35 to 60°C at 10 MPa. The results are presented in Fig. 6. Below 40°C, the conversion rate rose with the increasing temperature, whereas above 40°C, the conversion rate decreased with increasing temperature. The possible explanation is similar to that for the pressure effect and relates to different solubility of

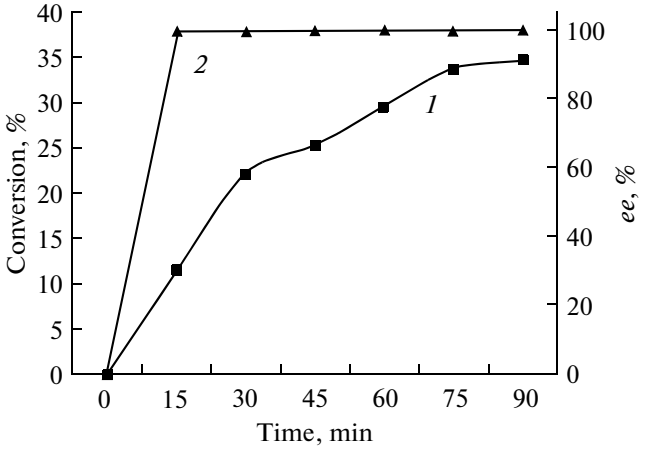


Fig. 7. Time course of hydrolysis in supercritical CO_2: *1*—conversion, *2*—*ee*. Reaction conditions: pressure 10 MPa, temperature 40°C, solution of 0.025 mol/l phosphate buffer and supercritical CO_2 in ratio 60/40, initial concentration of (*R*, *S*)-1-phenylethyl acetate 1% (*v*/*v*), CALB amount 0.3% (*w*/*v*).

water in the SC-CO_2. So 40°C was the best temperature for the enzymatic reaction in SC-CO_2. In the experiments, *ee* was more than 99%.

Time course of hydrolysis under supercritical pressure. Figure 7 shows the time course of the kinetic resolution of (*R*, *S*)-1-phenylethanol by chiral hydrolysis of (*R*, *S*)-1-phenylethyl acetate catalyzed by CALB in SC-CO_2 and 0.025 mol/l phosphate buffer (pH 7.5) at 10 MPa and 40°C. As can be seen, the substrate was hydrolyzed rapidly up to 30 min. After 30 min, the hydrolysis rate gradually decreased. At the end of hydrolysis, the conversion rate reached 34.6%. The hydrolysis time 90 min in SC-CO_2 and 0.025 mol/l phosphate buffer (pH 7.5) considerably decreased compared to 8 h in *tert*-butanol and 0.025 mol/l phosphate buffer (pH 7.5). In the experiments, *ee* was more than 99%.

So, the kinetic resolutions of (*R*, *S*)-1-phenylethanol by chiral hydrolysis of (*R*, *S*)-1-phenylethyl acetate catalyzed by a commercial CALB were carried out successfully in *tert*-butanol and 0.025 mol/l phosphate buffer (pH 7.5) or in SC-CO_2 and 0.025 mol/l phosphate buffer (pH 7.5). The high enantioselectivity was achieved with CALB for both media. The higher conversion rate (41.2%) was obtained in SC-CO_2 and 0.025 mol/l phosphate buffer (pH 7.5) compared to that in *tert*-butanol and 0.025 mol/l phosphate buffer (pH 7.5). In addition, the reaction time decreased from 8 h to 90 min. The study has highlighted that by selecting suitable solvent and reaction conditions, the hydrolysis of (*R*, *S*)-1-phenylethyl acetate by CALB is a successful approach in a shorter reaction time. The results presented here clearly demonstrated the potential of lipase for hydrolysis of (*R*, *S*)-1-phenylethyl acetate to resolve racemic 1-phenylethanol. Further tests are being conducted to investigate the enzymatic resolution of 1-phenylethanol racemates by hydrolysis of (*R*, *S*)-1-phenylethyl acetate in SC-CO_2/phosphate buffer biphasic systems in order to carry out an integral green biocatalytic process.

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