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Resolution of (*RS*)-2-phenylpropanoic acid by enantioselective esterification with dry microbial cells in organic solvent

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Abstract—The microbial direct esterification of racemic 2-phenyl-1-propanoic acid with ethanol by lyophilized mycelium of *Aspergillus oryzae* MIM and *Rhizopus oryzae* CBS 112.07 in organic solvents has been investigated. Dry cells of *Rhizopus oryzae* CBS 112.07 gave the (*R*)-ethyl ester with high enantiomeric excess (>97%) when the biotransformation was carried out in heptane or pentadecane. Dry mycelium of *Aspergillus oryzae* MIM furnished the ethyl ester of (*S*)-(+)-2-phenylpropanoic acid with 90% enantiomeric excess when used in toluene in a temperature range of 20–40°C.

1. Introduction

The resolution of racemic 2-arylpropionic acids by enzymatic esterification in organic solvents[1–5] is an easy way to obtain enantiomerically pure compounds which can be used as biologically active compounds.[6] Cell-bound esterases and lipases can be used directly as dry microbial cells in organic solvents for promoting ester synthesis.[7–10] They may offer economical and technical benefits, such as improved stability to the inhibitory effects of substrate/product/solvent and access to 'new' enzymes with interesting enantioselectivities, while avoiding costly and time-consuming purifications. Whole cells have been used to perform the resolution of racemic mixtures of different 2-alkanols by using dry mycelium of *Rhizopus oryzae* CBS 112.07 in heptane.[11] This approach seemed to be very promising so as a result the enantioselective esterification of (*RS*)-2-phenylpropanoic acid in organic solvent was studied using dry microbial cells.

2. Results and discussion

Dry mycelia of *Aspergillus oryzae* MIM and *Rhizopus oryzae* CBS 112.07 were employed These microorganisms had previously shown to be suited for catalyzing direct esterification of various substrates in organic solvents.[7,10,12] The microbial-catalyzed esterification of racemic 2-phenylpropanoic acid was carried out in heptane using different alcohols (Table 1).

Table 1. Esterification of 2-phenylpropanoic acid with different alcohols catalyzed by dry mycelium (30 g L^{-1}) of *Aspergillus oryzae* MIM and *Rhizopus oryzae* CBS 112.07 in heptane at 50°C. Molar conversion and e.e. of the ester formed after 3 days

Alcohol	*Aspergillus oryzae* MIM			*Rhizopus oryzae* CBS 112.07		
	Molar conv. (%)	E.e. (%)	*E*	Molar conv. (%)	E.e. (%)	*E*
Ethanol	15	90 *S*	22	12	72 *R*	6.8
1-Propanol	20	70 *S*	6.7	24	68 *R*	6.5
1-Butanol	31	60 *S*	5.2	26	60 *S*	4.9
1-Pentanol	24	52 *S*	3.7	27	52 *S*	3.8
1-Hexanol	30	39 *S*	2.7	29	39 *S*	2.7
1-Methyl-3-butanol	29	30 *S*	2.1	17	30 *S*	2.0

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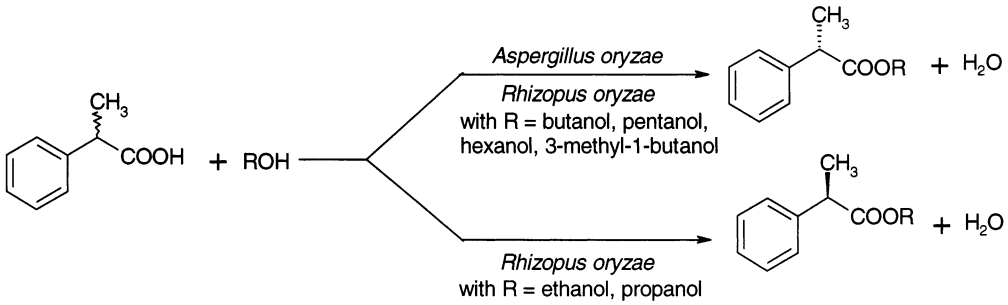

Scheme 1.

Table 2. Esterification of 2-phenylpropanoic acid with ethanol catalyzed by dry mycelium (30 g L^{-1}) of *Aspergillus oryzae* MIM and *Rhizopus oryzae* CBS 112.07 in different solvents. Molar conversion and e.e. of the ester formed after 6 days

Solvent	Log P	*Aspergillus oryzae* MIM			*Rhizopus oryzae* CBS 112.07		
		Molar conv. (%)	E.e. (%)	*E*	Molar conv. (%)	E.e. (%)	*E*
Diethyl ether	0.85	5	91	22	5	44	2.6
Dipropyl ether	1.9	5	73	6.7	5	94	33
Benzene	2.0	18	86	15	13	78	9.1
Toluene	2.5	25	89	22	11	70	6.2
Pentane	3.1	18	38	2.4	18	67	5.8
Heptane	4.0	47	90	46	30	70	7.6
Isooctane		45	84	23	21	66	5.8
Pentadecane	7.5	28	36	2.4	19	90	23

Aspergillus oryzae showed preference for the formation of the (*S*)-ester, the highest enantioselectivity being obtained with ethanol. The stereobias observed for *Rhizopus oryzae* was also strongly dependent upon the alcohol employed: esterification of 2-phenylpropanoic acid with ethanol and propanol gave the (*R*)-ester, while alcohols with longer chain lengths furnished predominantly the (*S*)-enantiomer (Scheme 1).

The highest enantiomeric ratios were obtained with ethanol, which was, therefore, employed in further experiments aimed at improving the enantioselectivity.

The polarity of the solvent often shows great influence on the enzymatic activity.[12] Solvents with different polarity were thus examined (dimethylsulfoxide, dioxane, acetonitrile, tetrahydrofuran, pyridine, diethyl ether, dipropyl ether, benzene, toluene, pentane, isooctane, pentadecane). Enzymatic activity could be observed only with solvents having higher hydrophobicity (expressed as log P)[13] as shown in Table 2. Reactions were performed at 50°C or at the boiling temperature for solvents having lower boiling points.

The highest enantioselectivity was observed with heptane and toluene for transformations catalyzed by *Aspergillus oryzae*, while heptane and pentadecane were the best solvents in the case of *Rhizopus oryzae*. The use of dipropyl ether also furnished good enantioselectivity, but with very low yields.

Experiments at different temperatures were carried out with *Aspergillus oryzae* in heptane and toluene (Table 3) and with *Rhizopus oryzae* in heptane and pentadecane (Table 4).

Table 3. Esterification of 2-phenylpropanoic acid with ethanol catalyzed by dry mycelium of *Aspergillus oryzae* MIM at different temperatures. Molar conversion and e.e. of the (*S*)-ester after 6 days

Solvent	Temperature (°C)	Molar conversion (%)	E.e. (%)	E
Toluene	20	8	84 *S*	12
	30	12	86 *S*	14
	40	21	89 *S*	21
Heptane	20	12	73 *S*	7.1
	30	21	75 *S*	8.5
	40	30	82 *S*	14

Table 4. Esterification of 2-phenylpropanoic acid with ethanol catalyzed by dry mycelium of *Rhizopus oryzae* CBS 112.07 at different temperatures. Molar conversion and e.e. of the (*R*)-ester after 6 days

Solvent	Temperature (°C)	Molar conversion	E.e. (%)	*E*
Heptane	20	9	>98 *R*	>100
	30	16	>98 *R*	>100
	40	24	95 *R*	52
Pentadecane	20	8	>98 *R*	>100
	30	15	>98 *R*	>100
	40	18	97 *R*	80

Temperature did not exert a strong influence on the stereoselectivity of *Aspergillus oryzae*, while almost complete enantioselectivity was obtained with *Rhizopus oryzae* at lower temperatures. It is likely that more than one enzyme of the mycelium can catalyze the esterification of 2-phenylpropanoic acid, but only the highly enantioselective ones are really active at lower temperatures.

It can be concluded that dry mycelium of *Aspergillus oryzae* MIM and *Rhizopus oryzae* CBS 112-07 can be used as enantioselective biocatalysts for the production of enantiomerically enriched ethyl (*R*)- or (*S*)-2-phenylpropanoate in organic solvents. Appropriate choice of solvent and temperature allowed for the formation of the (*R*)-ester as the only enantiomer using *Rhizopus oryzae*.

3. Experimental

3.1. Microorganisms, growth and biotransformation conditions

Aspergillus oryzae MIM (Microbiologia Industriale Milano) and *Rhizopus oryzae* CBS (Centraal Bureau voor Schimmelcultures, Baarn, Holland) 112.07 were used throughout this study and routinely maintained on malt extract (8 g L^{-1}, agar 15 g L^{-1}, pH 5.5). The microorganisms were cultured in 500 mL Erlenmeyer flasks containing 100 mL of medium and incubated for 48 h at 28°C on a reciprocal shaker (100 spm). The liquid media contained a basic medium (**BM**: Difco yeast extract 1 g L^{-1}, $(NH_4)_2SO_4$, 5 g L^{-1}, K_2HPO_4, 1 g L^{-1}, $MgSO_4 \cdot 7H_2O$, 0.2 g L^{-1}, pH 5.8) supplemented with Tween 80 (0.5%). Suspensions of spores (1.6×10^4) were used as inoculum. The microorganisms were also cultured in a 10 L stirred tank reactor containing 2 L of medium at 28°C, 200 rpm and aeration 1 vvm. Cells grown for 48 h in submerged cultures were harvested by filtration at 4°C, washed with phosphate buffer (pH 7.0, 0.1 M) and lyophilized. Ester synthesis was carried out in 10 mL screw capped test tubes by suspending lyophilized mycelium in organic solvent (5 mL) and then adding the alcohol and the acid. The reaction mixtures were magnetically stirred at different temperatures.

The work-up of the microbial-catalyzed esterification with ethanol is reported as an example: the mycelium was removed by centrifugation and the organic extracts were dried over Na_2SO_4. Evaporation of the solvent gave the crude product, which was purified by flash chromatography (hexane:ethyl acetate, 7:3) to give ethyl 2-phenylpropanoate.

3.2. Analytical methods

Samples (0.5 mL) were taken at intervals and centrifuged; 200 μL of the supernatant was added to an equal volume of a $CHCl_3$ solution containing an internal standard (2-phenyl-1-propanol). Molar conversions were determined by GLC carried out on a Carlo Erba Fractovap G1 gas chromatograph equipped with a hydrogen flame ionization detector; the column temperature was kept at 180°C. The column (3×2000 mm) was packed with Carbowax 20 M (10% 100/120 mesh, Supelcoport). The absolute configuration of the obtained esters was determined by comparison with the specific rotation of authentic samples of the pure enantiomers obtained by esterification of enantiomerically pure (*S*)-2-phenylpropanoic acid with different alcohols using conventional esterification procedures.[5] The enantiomeric composition was routinely determined by gas chromatographic analysis of the esters using a chiral capillary column (diameter 0.25 mm, length 25 m, thickness 0.25 μm, DMePeBeta-CDX-PS086, MEGA, Legnano, Italia). For ethyl, propyl and butyl 2-phenylpropanoates the column temperature was kept at 90°C for 15 min and then 1°C/min; retention times: (*R*)-ethyl ester 26.8; (*S*)-ethyl ester 27.5; (*R*)-propyl ester 35.5; (*S*)-propyl ester 36.2; (*R*)-butyl ester 35.8; (*S*)-butyl ester 36.5 min. For 2-phenylpropanoic pentyl and hexyl esters the column temperature was kept at 90°C for 10 min and then 1°C/min; retention times: (*R*)-pentyl ester 30.7; (*S*)-pentyl ester 31.4; (*R*)-hexyl ester 34.9; (*S*)-hexyl ester 35.6 min. For isoamyl 2-phenylpropanoate the column temperature was kept at 80°C for 15 min and then 1°C/min; retention times: (*R*)-isoamyl ester 44.1; (*S*)-isoamyl ester 27.5 min. The stereochemical outcome of the transformations was expressed as enantiomeric excess (e.e.) of the major enantiomer or as the enantiomeric ratio (*E*).[6,7]

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