Novel Hydrolases from Thermophilic Filamentous Fungi for Enantiomerically and Enantiotopically Selective Biotransformations

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Abstract: Fourteen thermophilic filamentous fungi were cultivated on two shake flask media and the supernatants were assayed for lipase/carboxylesterase activities using olive-oil, *p*-nitrophenyl palmitate and *p*-nitrophenyl butyrate as substrates. The crude enzyme powders (acetone precipitated supernatants) were tested as biocatalysts in organic solvents. Kinetic resolution of racemic 1-phenylethanol (*rac*-**1**) and desymmetrisation of 2-acyloxypropan-1,3-diols (**3a, b**) by acetylation with vinyl acetate were chosen for testing the biocatalytic abilities of these preparations. The tested biocatalysts proved to be comparable to the commercially available enzymes with respect to the degree of enantiomer selectivity, whereas they exhibited a wider range of enantiotopic selectivity than the most common commercial enzymes.

Keywords: asymmetric catalysis; enzyme catalysis; hydrolases; kinetic resolution; thermophilic filamentous fungi

Introduction

The development of novel biocatalytic methods is a continuously growing area of chemistry, microbiology and genetic engineering – due to the fact that biocatalysts are selective, easy-to-handle and environmentally friendly.[1] Industrial applications are widespread as biocatalytic steps are already being used to manufacture a wide range of products, including drugs, agricultural chemicals, organics, fine chemicals and plastics.[2] Because the demand for the enantiopure form of the chiral compounds has been increasing rapidly, novel microorganisms and/or their enzymes are the subjects of screening to produce such chemicals.[1,2]

Enzyme preparations from fungi has been studied since the early 1950's.[3] Several fungal biocatalysts are commercially available and they are valuable tools for the synthetic chemists.[4] Thermophilic fungi are the only representatives of eukaryotic organisms that can grow at temperatures above 45 °C with a maximum temperature of growth extending up to 60 to 62 °C.[5] Their extracellular enzymes display activity maxima that are close to or above the optimum temperature for the growth of the organisms and, in general, are more heat stable than those of the mesophilic fungi. Therefore, thermophilic fungi are potential sources of thermotolerant enzymes of scientific and commercial interest. Some extracellular enzymes from thermophilic fungi are being produced commercially, and a few others have commercial prospects.[5]

Lipases represent the most important group of biocatalysts for biotechnological applications.[6] Although several lipases were isolated from thermophilic fungi, only few, e.g., *Thermomyces lanuginosus* (formerly *Humicola lanuginosa*) or *Rhizomucor miehei* have been commercialised[7] and investigated as synthetic biocatalysts.[8,9] Genes encoding these enzymes have also been cloned into recombinant hosts.[7] Immobilisation,[10,11] genetic modifications by site directed mutagenesis,[12] gene shuffling[13] or directed evolution[14] of these recombinant enzymes have been used to modify/enhance their practical applicability.

Because some lipases from thermophilic fungi are highly valuable biocatalysts, less studied thermophilic fungi for organic synthesis have been chosen to investigate their extracellular lipase/carboxylesterase pro-

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duction. In order to obtain additional data for such thermotolerant hydrolases, these fungal enzymes were also evaluated for enantiomer and enantiotopic selective biotransformations.

Results and Discussion

Among the thermophilic fungal strains selected for our lipase/carboxylesterase study, *Chaetomium thermophilum*,[15,16] *Humicola grisea*,[17] *Humicola insolens*,[11] *Paecilomyces* sp.,[13,18,19] *Sporotrichum thermophile*,[20] *Talaromyces emersonii*,[21,22,23] *Talaromyces thermophilus*,[17,21] *Thermoascus aurantiacus*,[15,16] *Thermoascus thermophilus*[15,16,17,24] and *Thermomyces lanuginosus*[11],[16],[21] have been reported for such enzyme production. However, only a few of the above fungi have been used for the industrial production of lipase or have had their enzymes tested thoroughly for organic synthesis. It is noteworthy that extremely stable microenzymes (1.6 to 4.1 kDa) with esterase activity were found in *Talaromyces emersonii* and *Emericella nidulans*.[23] According to our best knowledge, lipase/carboxy ester hydrolase activity of *Myceliophthora thermophila* and *Thermomucor indicae-seudaticae* has not yet been published.

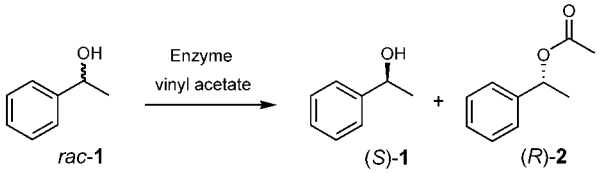
In a previous screen of thermophilic fungi, lipase activity was detected in the culture filtrates of fungi within 48 h of incubation at 45 °C.[17] The presence of lipids in the growth medium enhanced the production of extracellular lipases but the lack of lipids in the growth medium did not prevent lipase synthesis.[17] In our study, fourteen different thermophilic fungal strains were cultured in two different media which contained olive-oil to induce the lipase production (Table 1). The lipase/carboxylesterase activities in the supernatants obtained from the cultures after incubating at 45 °C for 72 h were determined by three different activity tests. The lipase activity was characterised by a titrimetric method using olive oil as substrate and by a spectrophotometric test using *p*-nitrophenyl palmitate as substrate. The general carboxylesterase activity was determined by spectrophotometry using *p*-nitrophenyl butyrate as substrate. In line with previous observations made during lipase activity screening,[25] in several instances practically no activity with olive oil was determined for preparations exhibiting activity on other substrates. The results listed in Table 1 strongly support that most of the fungal strains produced more than one extracellular enzyme exhibiting carboxy ester hydrolase activity. It is clearly seen, e.g., for *Talaromyces emersonii* NRRL-3221 or *Talaromyces thermophilus* NRRL-2155, that the esterase *vs.* lipase activity pattern was dependent on the culturing conditions. In many cases growth in LIP1 medium favoured the esterase production, whereas culturing in LIP2 medium increased the lipase-like activity.

Although hydrolases (lipases/esterases) were detected in numerous thermophilic fungi strains, only a few have been evaluated as synthetic biocatalysts as yet.[8,9] Therefore, we thought it worthwhile to characterise the biocatalytic abilities of enzymes from thermophilic fungi by two typical enantioselective processes. Enantiomer selectivity was evaluated by kinetic resolution of racemic 1-phenylethanol (**1**), and the enantiotopic selectivity by desymmetrisation of 2-acyloxypropane-1,3-diols (**3a, b**), both by enzymatic acetylation with vinyl acetate.

The enantiomers of 1-phenylethanol (**1**) are of interest since they are used as chiral reagents for the determination of enantiomeric purity and for resolution of acids[26] or for asymmetric opening of cyclic anhydrides and of epoxides.[27] The enzymatic kinetic resolution of racemic 1-phenylethanol (**1**) or its esters is well documented.[1] Therefore, acetylation of *rac*-**1** with vinyl acetate in hexane was chosen for testing the enantiomer selectivity of our enzyme preparations (Scheme 1, Table 2). The degree of enantiomer selectivity (E) was precisely calculated[28] from the conversion-enantiomeric composition data obtained by GC on a chiral stationary phase. For comparison and for unambiguous assignment of the absolute configurations of the produced acetate and alcohol enantiomers, acylation of *rac*-**1** with vinyl acetate in hexane by Lipozyme TL IM (*Thermomyces lanuginosus* lipase immobilised on silica by granulation) has also been performed on both small and preparative scales.

Our data (Table 2; only the reactions exceeding 5% conversion after 168 h are listed) indicate that many of the isolates were capable of performing enantiomer-selective acetylation of the test substrate *rac*-**1** with (*R*)-enantiomer preference exhibiting enantiomer selectivities from $E = 1.1$ up to 231. Although the observed enantiomer selectivity for Lipozyme TL IM under these non-optimised conditions was quite high ($E = 102$), several of our enzyme preparations exhibited even higher selectivities [e.g., *Myceliophthora thermophila* TUB-F-39 (LIP2): $E = 163$; *Humicola insolens* CBS-147.64 (LIP2): $E = 171$; *Chaetomium thermophilum* TUB-F-69 (LIP2): $E = 231$]. In addition, not only the selectivity but also the productivity (50% conversion within 72 h) of the enzyme preparation from *Chaetomium thermophilum* TUB-F-69 (LIP2) proved to be appropriate for synthetic purposes.

2-Substituted-propane-1,3-diols are important chiral building blocks of numerous biologically active mole-



Scheme 1. Enzymatic acetylation of racemic 1-phenylethanol (*rac*-**1**).

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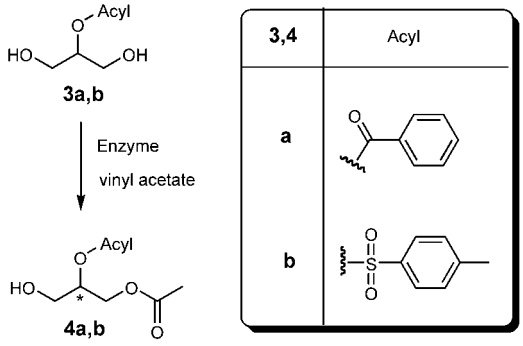
Table 1. Lipase and carboxylesterase activities of thermophilic filamentous fungi.

Fungal strain (Medium)	Precipitate [mg/mL][a]	Hydrolase activity[b] [mU/mL]		
		Olive oil	*p*-NPP	*p*-NPB
Chaetomium thermophilum TUB-F-69 (LIP1)	4.5	0	97.3	109.6
Chaetomium thermophilum TUB-F-69 (LIP2)	1.0	0	15.3	73.3
Humicola grisea var. *thermoidea* CBS-183.64 (LIP1)	2.8	0	429.0	210.0
Humicola grisea var thermoidea CBS-183.64 (LIP2)	5.0	0	188.0	526.0
Humicola insolens CBS-147.64 (LIP1)	1.4	0	55.2	96.3
Humicola insolens CBS-147.64 (LIP2)	3.4	0	22.4	309.0
Myceliophthora thermophila TUB-F-39 (LIP1)	7.8	0	84.5	191.3
Myceliophthora thermophila TUB-F-39 (LIP2)	3.9	0	100.2	320.4
Paecilomyces sp. TUB-F-70 (LIP1)	16.0	0	0.35	1.4
Paecilomyces sp. TUB-F-70 (LIP2)	13.5	0	9.5	6.4
Sporotrichum thermophile ATCC-36.347 (LIP1)	1.3	0	30.0	13.8
Sporotrichum thermophile ATCC-36.347 (LIP2)	2.3	9.3	94.7	63.5
Sporotrichum thermophile WFPL-264 A (LIP1)	0.3	0.2	80.1	68.4
Sporotrichum thermophile WFPL-264 A (LIP2)	0.2	0	76.4	66.8
Talaromyces emersonii NRRL-3221 (LIP1)	3.3	0	5.1	15.3
Talaromyces emersonii NRRL-3221 (LIP2)	2.4	2.7	4.9	5.8
Talaromyces thermophilus NRRL-2155 (LIP1)	1.6	0	7.3	6.7
Talaromyces thermophilus NRRL-2155 (LIP2)	13.5	0	28.8	6.0
Thermoascus aurantiacus TUB-F-43 (LIP1)	1.3	0	5.9	23.5
Thermoascus aurantiacus TUB-F-43 (LIP2)	1.0	0	7.8	18.0
Thermoascus thermophilus NRRL-5208 (LIP1)	2.3	1.8	34.5	30.0
Thermoascus thermophilus NRRL-5208 (LIP2)	1.9	3.1	3.9	0
Thermomucor indicae-seudaticae NRRL-6429 (LIP1)	1.1	0	104.0	42.5
Thermomucor indicae-seudaticae NRRL-6429 (LIP2)	4.6	0	1.7	0.6
Thermomyces lanuginosus ATCC-38.905 (LIP1)	2.4	20.3	4.6	20.7
Thermomyces lanuginosus ATCC-38.905 (LIP2)	1.1	17.9	26.0	3.1
Thermomyces lanuginosus CBS-224.63 (LIP1)	1.6	0	0	16.9
Thermomyces lanuginosus CBS-224.63 (LIP2)	2.6	12.8	31.5	16.9

[a] Mass of dried precipitate from 1 mL of supernatant of the corresponding culture.
[b] For activity tests, see Experimental Section. Activities below 0.1 mU/mL are reported as 0.



Scheme 2. Enzymatic acetylation of 2-acyloxypropane-1,3-diols (**3a, b**).

cules, such as phospholipids,[29] PAF (platelet-activating factor)[30] or β-blockers.[31] Our group has recently published results on enantiotopic selective enzymatic acetylation of prochiral 2-acyloxypropan-1,3-diols[32] which proved to be useful for the preparation of such chiral building blocks in optically active form. Therefore, acylation reactions of 2-benzoyloxypropane-1,3-diol (**3a**) and 2-(*p*-toluenesulfonyl)oxypropane-1,3-diol (**3b**) with vinyl acetate (Scheme 2, Tables 3 and 4) have been chosen for testing the enantiotopic selectivities of the novel biocatalysts from the thermophilic fungi as well.

Because the configuration of (*R*)-**4a** was previously determined by correlation with compounds of ambiguous low optical rotation data,[32a] the absolute configuration of (*R*)-**4a** was unambiguously re-determined by chemical correlation starting from (*R*)-(**5**)[33] *via* benzoylation and subsequent catalytic hydrogenation (Scheme 3) before performing the enzymatic reactions with **3a**. Afterwards, asymmetric acylation reactions of the prochiral diol (**3a**) were performed (Scheme 2, Table 3) to test the enantiotopic selectivity of the thermophilic fungal enzymes. The results of these reactions proved also to be quite promising. The prochiral diol (**3a**) was accepted as substrate by many preparations. The asymmetric acylation of **3a** with *Talaromyces emersonii* NRRL-3221 (LIP2) proceeded with the same enantiotopic preference and almost as selectively as the reaction with the best commercial enzyme PPL[32a] (94% ee *vs.* 96% ee of (*R*)-**4a**, respec-

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Table 2. Acetylation of racemic 1-phenylethanol (*rac*-**1**) with thermophilic fungal enzymes.

Enzyme Fungal strain (Medium)	*t* [h]	*c* [%][a]	ee [%][a]	*E*[b]
Chaetomium thermophilum TUB-F-69 (LIP2)	72	50	96.4	231
Humicola insolens CBS-147.64 (LIP2)	24	11	98.7	171
Myceliophthora thermophila TUB-F-39 (LIP2)	96	5	98.7	163
Chaetomium thermophilum TUB-F-69 (LIP1)	72	50	94.3	123
Lipozyme TL IM	20	47	95.0	102
Humicola grisea var. *thermoidea* CBS-183.64 (LIP2)	96	14	97.7	101
Thermomyces lanuginosus CBS-224.63 (LIP1)	168	6	97.1	72
Thermomyces lanuginosus CBS-224.63 (LIP2)	168	10	95.6	50
Thermomyces lanuginosus ATCC-38.905 (LIP1)	168	5	91.5	25
Talaromyces emersonii NRRL-3221 (LIP2)	168	14	82.1	12
Sporotrichum thermophile ATCC-36.347 (LIP2)	72	24	76.1	7.5
Sporotrichum thermophile WFPL-264 A (LIP1)	72	26	41.7	2.8
Thermoascus aurantiacus TUB-F-43 (LIP2)	168	5	10.3	1.3
Sporotrichum thermophile WFPL-264 A (LIP2)	72	31	0.9	1.0

[a] Conversion and enantiomeric composition of the produced acetate [(*R*)-**2**] were determined by GC on chiral stationery phase, for details see Experimental Section.

[b] The degree of enantiomer selectivity (*E*) was calculated by using the equation $E = \ln[1 - c(1 + ee(P))]/\ln[1 - c(1 - ee(P))]$ as defined for the irreversible enantiomer selective transformations.[28]

Table 3. Acetylation of 2-benzoyloxypropane-1,3-diol (**3a**) with thermophilic fungal enzymes.

Enzyme Fungal strain (Medium)	*t* [h] *m* [mg]	Yield [%]	**4a** Config.	ee [%]
PPL: lipase from porcine pancreas[32a]	300 1	63	*R*	96
Talaromyces emersonii NRRL-3221 (LIP2)	48 48	87	*R*	94
Talaromyces emersonii NRRL-3221 (LIP1)	101 120	86	*R*	67
Sporotrichum thermophile ATCC-36.347 (LIP1)	73 168	14	*R*	23
Myceliophthora thermophila TUB-F-39 (LIP2)	42 168	12	*R*	15
Sporotrichum thermophile ATCC-36.347 (LIP2)	126 168	27	-	0
Chaetomium thermophilum TUB-F-69 (LIP2)	101 168	12	*S*	5
Humicola grisea var. *thermoidea* CBS-183.64 (LIP2)	202 168	14	*S*	12
Thermomucor indicae-seudaticae NRRL-6429 (LIP2)	78 168	12	*S*	16
Thermomyces lanuginosus CBS-224.63 (LIP1)	65 168	12	*S*	29
Paecilomyces sp. TUB-F-70 (LIP2)	103 72	47	*S*	53
Thermoascus thermophilus NRRL-5208 (LIP2)	101 168	30	*S*	53
Thermomyces lanuginosus ATCC-38.905 (LIP2)	96 15	51	*S*	59
Talaromyces thermophilus NRRL-2155 (LIP1)	70 21	47	*S*	71

tively). In addition, enzymes preferring the formation of the opposite enantiomer (*S*)-**4a** – up to 71% ee with *Talaromyces thermophilus* NRRL-2155 (LIP1) – were also found. The results indicate that these thermophilic fungi produce various hydrolases with different selectivity patterns – not only the degree but also the sense of selectivity can differ – which may enrich the selection of the synthetically useful biocatalysts. In some instances, data observed with the two different isolates from the same strain [e.g., *Talaromyces emersonii* NRRL-3221: (LIP2), 94% ee *vs.* (LIP1), 67% ee of (*R*)-**4a** or *Thermoascus thermophilus* NRRL-5208: (LIP2), 53% ee *vs.* (LIP1), 71% ee of (*S*)-**4a**] clearly indicate that thermophilic fungi produce different extracellular hydrolases with remarkably different selectivities. Thus, for a highly selective optimised biotransformation either a fully optimised enzyme production process or production of the enzyme in question by recombinant organisms overexpressing its gene might be necessary. It is also shown that enzymes from closely related strains *Talaromyces emersonii* NRRL-3221 *vs. Talaromyces thermophilus* NRRL-2155 can behave significantly differently.

Because a wide range of enantiotopic selectivity was observed in the asymmetric acetylation of **3a**, it seemed worthwhile to investigate the acylation reaction of **3b** as well. The prochiral **3b** has also been tested with a selection of commercially available enzymes for asymmetric acylation,[32b] but only moderate enantiotopic selectivities towards the formation of (*S*)-**4b** were found.

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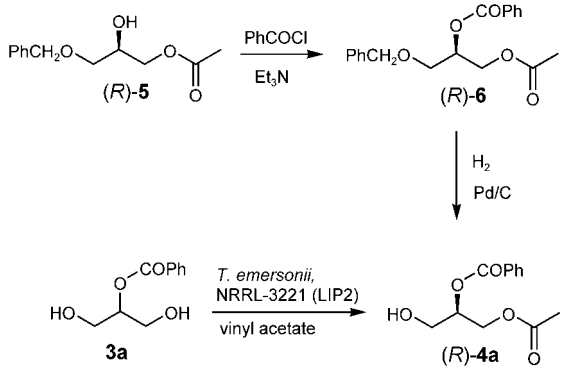
Table 4. Acetylation of 2-(4′-toluenesulfonyl)oxypropane-1,3-diol (**3b**) with thermophilic fungal enzymes.

Enzyme Strain, Collection No (Medium)	*m* [mg]	*t* [h]	**4b** Yield [%]	Config.	ee [%]
Talaromyces emersonii NRRL-3221 (LIP2)	103	168	44	*R*	4
Sporotrichum thermophile ATCC-36.347 (LIP1)	248	168	61	*S*	2
Thermomyces lanuginosus CBS-224.63 (LIP2)	86	178	76	*S*	8
Sporotrichum thermophile ATCC-36.347 (LIP2)	245	168	29	*S*	13
Talaromyces emersonii NRRL-3221 (LIP1)	100	72	52	*S*	16
Myceliophthora thermophila TUB-F-39 (LIP1)	232	168	12	*S*	17
Thermomyces lanuginosus CBS-224.63 (LIP1)	69	168	8	*S*	21
Talaromyces thermophilus NRRL-2155 (LIP2)	61	168	45	*S*	23
Humicola grisea var. *thermoidea* CBS-183.64 (LIP2)	196	168	16	*S*	32
Sporotrichum thermophile ATCC-36.347 (LIP2)	157	4	23	*S*	41
Lipozyme IM [32b]	50	0.5	82	*S*	42
Thermoascus thermophilus NRRL-5208 (LIP1)	52	168	5	*S*	45
Thermomyces lanuginosus ATCC-38.905 (LIP2)	100	10	24	*S*	50
Thermoascus thermophilus NRRL-5208 (LIP2)	174	168	31	*S*	51
Paecilomyces sp. TUB-F-70 (LIP2)	78	21	28	*S*	52
Talaromyces thermophilus NRRL-2155 (LIP1)	74	10	45	*S*	53



Scheme 3. Determination of the absolute configuration of (*R*)-**4a** by chemical correlation with (*R*)-**5**.

The best result with commercially available enzymes was achieved with Lipozyme IM (immobilised *Rhizomucor miehei* lipase),[32b] thus this reaction providing (*S*)-**4b** with 41% ee is also listed in Table 4 for comparison. Although a wider selectivity range than with the previously tested commercial lipases[32b] was observed [from (*R*)-**4b** of 4% ee with *Talaromyces emersonii* NRRL-3221 (LIP2) to (*S*)-**4b** of 53% ee with *Talaromyces thermophilus* NRRL-2155 (LIP1)], these selectivities are still unsatisfactory for practical purposes.

Conclusions

Our present study indicates that thermophilic filamentous fungi are a promising and not yet fully exploited source of hydrolases with valuable biocatalytic properties. The results from three stereoselective test reactions – acylation of *rac*-**1**, **3a** and **3b** in organic media with vinyl acetate catalysed by a series of acetone-dried extracellular enzyme preparations – indicated that these enzymes might be superior in synthetic biotransformations over the commercialised thermophilic fungal lipases with respect to the degree of enantiomer selectivity or direction/degree of enantiotopic selectivity.

Experimental Section

Materials and Methods

The NMR spectra were recorded in $CDCl_3$ on a Bruker DRX-500 spectrometer (at 500 MHz for ^1H and 125 MHz for ^{13}C spectra); chemical shifts are expressed in ppm (δ scale). IR spectra (film) were taken on a Specord 2000 Series spectrophotometer; bands are listed in cm^{-1} (ν). GC analyses were carried out on HP 5890 or Agilent 4890D instruments equipped with FID detector and HP Chiral column (30 m × 0.32 mm, 0.25 μm film of 20% permethylated β-cyclodextrin; HP Part No.: 190916-B213) using H_2 carrier gas (oven: 100 °C, injector: 250 °C, detector: 250 °C, head pressure: 10 psi, 50:1 split ratio). Optical rotations were determined on a Perkin Elmer 241 polarimeter. TLC was carried out on Kieselgel 60 F_{254} (Merck) sheets. Spots were visualised by treatment with 5% ethanolic phosphomolybdic acid solution and heating of the dried plates. All solvents were freshly distilled prior their use.

Racemic 1-phenylethanol (*rac*-**1**) and vinyl acetate used in enantiomer selective acylations were products of Aldrich. 2-Benzoyloxypropane-1,3-diol (**3a**) and 2-(4′-toluenesulfonyl)-oxypropane-1,3-diol (**3b**) as test substrates for the enantiotopic selective biotransformations were prepared by our established methods.[32] Lipozyme TL IM was a product of Novozymes, Denmark. The inorganic salts and materials for biomass production were the corresponding products of Sigma, Aldrich or Fluka.

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Microorganisms

Thermophilic fungi have been purchased from the following culture collections: ATCC (American Type Culture Collection, Manassas, Virginia); CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; NRRL (Northern Regional Research Center, USDA, Peoria, Illinois); TUB (Technical University of Budapest, Hungary); WFPL (Western Forest Products Laboratory, Vancouver, Canada). The TUB strains have the following accession numbers at the ATCC collection: *Chaetomium thermophilum* TUB F-69 (=ATCC 58136), *Myceliophthora thermophila* TUB F-39 (=ATCC 58152), *Paecilomyces* sp. TUB F-70 (=ATCC 58155) and *Thermoascus aurantiacus* TUB F-43 (=ATCC 58156).

The fungi were grown and maintained on potato dextrose agar (PDA) slants at 45 °C until appropriate sporulation then stored at room temperature. Fully sporulated Petri plate PDA cultures were harvested by washing with 0.1% Tween-80 containing water and the dense spore suspension was used for shake flask inoculation. Viable spore content was determined by serial dilution and colony forming unit (CFU) counting on PDA at 45 °C.

Shake Flask Fermentation

For shake flask fermentation, 150 mL medium was distributed into cotton-plugged 500-mL Erlenmeyer flasks, and sterilised at 121 °C for 30 min. The flasks were inoculated to a final concentration of 1×10^6 spores of the respective fungus/mL, and incubated on a rotary shaker at 45 °C and 300 rev/min for 3 days. Fourteen thermophilic filamentous fungal strains were screened, each in two different shake flask media (LIP1 and LIP2). The compositions were as follows (in g/L):

LIP1: olive oil, 10; sucrose, 10; defatted soybean meal, 2.5; KH_2PO_4, 2; $CaCO_3$, 1.5; $(NH_4)_2HPO_4$, 1; Tween-80, 0.5; $MgSO_4 \cdot 7\,H_2O$, 0.5; NaCl, 0.5; $NaNO_3$, 0.5; trace element solution, 0.5.

LIP2: olive oil, 10; corn meal, 15; KH_2PO_4, 2; $CaCO_3$, 2; Tween-80, 2; $(NH_4)_2HPO_4$, 1.5; corn steep liquor, 1; $MgSO_4 \cdot 7\,H_2O$, 0.5; NaCl, 0.5; KNO_3, 0.5; trace element solution, 0.5.

Trace element solution (g/L): $MnSO_4$, 1.6; $ZnSO_4 \cdot 7\,H_2O$, 3.4; $CoCl_2 \cdot 6\,H_2O$, 2; $FeSO_4 \cdot 7\,H_2O$, 5.

Lipase/Carboxylesterase Activity Tests from Supernatants

The whole fermentation broths of the resulting 28 cultures were centrifuged (3000 rpm, 10 min at 20 °C) and aliquots from the supernatants were used for enzyme activity measurements. The hydrolase activities were determined by three different, well established and properly adopted activity tests:

Method A: titrimetric method using olive oil as substrate;[34]

Method B: spectrophotometric method using *p*-nitrophenyl palmitate as substrate;[25]

Method C: spectrophotometric method using *p*-nitrophenyl butyrate as substrate.[25]

Preparation of the Biocatalysts

The main portion of the supernatants was treated with acetone (2–3 v/v). The forming precipitate was filtered and treated with acetone (2–3 v/v). The residual solvent from the precipitate was evaporated in vacuum and the resulting dry powder was stored at 4 °C in sealed tubes. Many of the preparations maintained almost unaltered biocatalytic activity over a year.

Enantiomer-Selective Acetylations of Racemic 1-Phenylethanol (*rac*-1)

Suspensions of the enzyme preparations (20 mg) with a solution of *rac*-**1** (20 mg) in hexane (2 mL) and vinyl acetate (0.5 mL) were shaken at 1000 rpm in sealed glass vials at room temperature for the time indicated in Table 2. The conversions were checked by TLC (hexane-acetone, 10:4, v/v). At the reaction time indicated in Tables 2, the enzyme was filtered off and the enantiomeric compositions and the ratios of the product and residual substrate fractions were analysed by GC on a chiral stationary phase (Table 2). GC retention times: 7.64 min, (*S*)-**2**; 8.04 min, (*R*)-**1**; 8.23 min, (*R*)-**2**; 8.65 min, (*S*)-**1**; GC molar response factor of acetate/alcohol (*rac*-**2**/*rac*-**1**): 1.18 [used for calculations of *c* and *E* throughout in Table 2]. Table 2 lists only those reactions which exhibited more than 5% conversion after 168 h.

A preparative scale conversion of *rac*-**1** (200 mg) catalysed by Lipozyme TL IM (200 mg) was also performed in hexane (20 mL) and vinyl acetate (5 mL). After shaking the reaction mixture at 1000 rpm, room temperature for 5 h, the enzyme was removed by filtration. The solvent was distilled off from the filtrate by rotary evaporation and the residue was separated by vacuum chromatography (silica gel, hexane-acetone, 10:1, v/v) to give alcohol (*S*)-**1** (yield: 156 mg 78%; 23% ee by GC); $[\alpha]_D^{22}$: −11.1 (*c* 1.0, $CHCl_3$) {lit.: $[\alpha]_D$: −45.3 ($CHCl_3$);[35] $[\alpha]_D$: −53.5 (*c* 1.13, $CHCl_3$);[36] $[\alpha]_D^{25}$: −55.1 (*c* 1.63, $CHCl_3$)[37]}; IR: ν = 3352, 3008, 2976, 2952, 1466, 1430, 1368, 1204, 1080, 1008, 900, 760, 690 cm^{-1}; ^1H NMR: δ = 1.513 (d, *J* = 6.9 Hz, 3H, 2-CH_3), 3.38 (br s, 1H, OH), 4.861 (q, *J* = 6.3 Hz, 1H, 1-CH), 7.40 (mc, 5H, Ar H); ^{13}C NMR: δ = 25.034, 69.843, 125.071, 126.862, 127.970, 145.525 and acetate (*R*)-**2** (yield: 43 mg, 16%; 97% ee by GC); $[\alpha]_D^{22}$: 99.0 (*c* 1.0, $CHCl_3$) {lit.: $[\alpha]_D^{20}$: 103.5 (*c* 1.0, $CHCl_3$);[38] $[\alpha]_D$: 105.1 (*c* 1.3, $CHCl_3$)[39]}; IR: ν = 1740, 1456, 1376, 1244, 1208, 1064, 944, 760, 700 cm^{-1}; ^1H NMR: δ = 1.574 (d, *J* = 7.4 Hz, 2-CH_3), 21.103 (s, 3H, Ac CH_3), 5.926 (q, *J* = 6.9 Hz, 1H, 1-CH), 7.30–7.40 (m, 5H, Ar H); ^{13}C NMR: δ = 21.416, 22.283, 72.241, 125.860, 127.632, 128.258, 141.422, 169.946; both as colourless oils. IR and NMR data agreed with the reported spectra of (*S*)-**1**[40] and (*R*)-**2**.[41]

Enantiomeric Excess Determination of 4a and 4b

The racemic or optically active 3-acetoxy-2-acyloxypropan-1-ols (**4a** or **4b**, 9–12 mg), pyridine (25 μL) and 4-(*N*,*N*-dimethylamino)pyridine (2 mg) were added to a solution of 5% (*R*)-MTPA-Cl in CCl_4 (350 μL) and the mixture was heated in a sealed ampoule at 50 °C for 3 h. The resulting mixture was successively washed with 5% HCl solution (1 mL), saturated Na_2CO_3 solution (1 mL) and brine (1 mL). The organic phase was dried over Na_2SO_4 and the solvent was evaporated. The

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diastereomeric excess (de) values of the forming MTPA esters (MTPA-**4a** and MTPA-**4b**) were determined from their ^{1}H NMR spectra (500 MHz, $CDCl_3$). The following ^{1}H NMR signals were used for de determinations: MTPA-**4a**: 3.52 (s) and 3.54 (s); MTPA-**4b**: 1.93 (s); 1.96 (s).

Asymmetric Acetylation of the Prochiral Diols 3a and 3b

To the solution of prochiral diol **3a** or **3b** (200 mg) and vinyl acetate (0.8 mL) in THF (2 mL) and hexane (2 mL) fungal enzyme preparation was added (for quantities, see Tables 3 and 4) and the mixture was stirred at room temperature (for reaction times, see Tables 3 and 4). The conversion was checked by TLC (hexane-acetone, 10:4, v/v). At the reaction time indicated in Tables 3 and 4, the enzyme was filtered off, the solvent was removed from the filtrate in vacuum and the residue was purified by vacuum chromatography (silica gel, hexane-acetone, 10:1, v/v) to give **4a** or **4b** (yield, configuration and enantiomeric excess data are listed in Tables 3 and 4; data are shown only for those reactions which exhibited more than 10% conversion after 168 h).

Yield, configuration and enantiomeric excess data for **4a** are listed in Table 3. As an example, data for (*R*)-**4a** prepared by *Talaromyces emersonii* NRRL-3221 (LIP2) enzyme are given here. Yield: 211 mg (87%); 94% ee by ^{1}H NMR of MTPA-**4a**; $[\alpha]_D^{22}$: −26.9 (*c* 1.0, EtOH), {lit.: $[\alpha]_D$: −27.4 (*c* 1, EtOH), 96% ee[32a]}; IR: ν = 3390, 2956, 2908, 2848, 1732, 1720, 1586, 1476, 1438, 1352, 1274, 1108, 1016, 948, 708 cm^{-1}; ^{1}H NMR: δ = 2.09 (s, 3H, CH_3), 3.30 (br s, 1H, OH), 3.89 (m, 2H, 1-CH_2), 4.43–4.44 (m, 2H, 3-CH_2), 5.32–5.35 (m, 1H, 2-CH), 7.45 (t, 2H, *J* = 7.5 Hz, 3′-Ar-H), 7.58 (t, 1H, *J* = 7.5 Hz, 4′-Ar-H), 8.07 (d, 2H, *J* = 7.5 Hz, 2′-Ar-H). The IR and ^{1}H NMR data agreed with the reported spectra of (*R*)-**4a**.[32a]

Yield, configuration and enantiomeric excess data for **4b** are listed in Table 4. As an example, data for (*S*)-**4b** prepared by *Talaromyces thermophilus* NRRL-2155 (LIP1) enzyme are given here. Yield: 105 mg (45%); 53% ee by ^{1}H NMR of MTPA-**4b**; $[\alpha]_D^{22}$: 15.7 (*c* 1.0, MeOH), {lit.: $[\alpha]_D$: 12.5 (*c* 1, MeOH), 42% ee[32b]}; IR: ν = 3507, 2959, 2887, 1731, 1698, 1602, 1584, 1453, 1372, 1265, 1124, 1034, 725 cm^{-1}; ^{1}H NMR: δ = 1.92 (s, 3H, CH_3), 2.45 (s, 3H, 4′-CH_3), 3.75 (m, 2H, 1-CH_2), 4.14–4.26 (m, 2H, 3-CH_2), 4.70 (m, 1H, 2-CH), 7.34 (d, 2H, 3′-Ar-H), 7.82 (d, 2H, 2′-Ar-H). The IR and ^{1}H NMR spectra agreed with the reported data of *rac*-**4b**.[32b]

(*R*)-1-Acetoxy-2-benzoyloxy-3-benzyloxypropane (*R*)-6

(*R*)-**5**, with $[\alpha]_D^{20}$: −3.7 (*c* 1, $CHCl_3$), (2.24 g, 10 mmol), benzoyl chloride (1.28 mL, 11 mmol), Et_3N (1.7 mL, 12 mmol) and 4-(*N*,*N*-dimethylamino)pyridine (40 mg) were dissolved in CH_2Cl_2 (20 mL) and heated under reflux for 3 h. Afterward, the resulting mixture was washed with 5% HCl (2 × 5 mL), 10% Na_2CO_3 (5 mL), 10% $NaHCO_3$ (5 mL) and brine (5 mL) and the solvent was removed in vacuum. The residue was purified by vacuum chromatography (silica gel, hexane-acetone, 10:0.5, v/v) to give (*R*)-**6** as a colourless oil, yield: 2.46 g (75%); $[\alpha]_D^{20}$: −12.1 (*c* 1.0, MeOH); IR: ν = 1744, 1721, 1452, 1367, 1315, 1274, 1232, 1110, 712 cm^{-1}; ^{1}H NMR: δ = 2.03 (s, 3H, CH_3), 3.73 (m, 2H, 3-CH_2), 4.37–4.47 (dd, 2H, 1-CH_2), 4.57 (dd, 2H, CH_2-Ph), 5.48 (m, 1H, 2-CH), 7.26–8.06 (m, 10H, 2 × Ar).

(*R*)-3-Acetoxy-2-benzoyloxypropan-1-ol (*R*)-4a

To a solution of (*R*)-**6** (1.64 g, 5.0 mmol) in *i*-PrOH (16 mL) 10% Pd-C catalyst (100 mg) was added and the resulting mixture was hydrogenated at room temperature under atmospheric pressure for 1 h. After the catalyst was removed by filtration, the solvent was evaporated off from the filtrate in vacuum and the residue was purified by vacuum chromatography (silica gel, hexane-acetone, 10:1, v/v) to give (*R*)-**4a** as a colourless oil, yield: 1.09 g (92%); $[\alpha]_D^{20}$: −27.2 (*c* 1.0, MeOH); the IR and ^{1}H NMR spectra were indistinguishable from those of product (*R*)-**4a** prepared with *Talaromyces emersonii*, NRRL-3221 (LIP2).

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