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Kinetic resolutions with novel, highly enantioselective fungal lipases produced by solid state fermentation

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Abstract

Thirty-eight filamentous fungi cultivated under solid state fermentation (SSF) conditions were screened for lipase activity and enantioselectivity in kinetic resolutions of racemic secondary alcohols (*rac*-**1a–c**) by acetylation with vinyl acetate performed in organic solvents. Many of the target fungi have not been studied previously for lipase/esterase activity and enantioselectivity. Without special enzyme isolation processes, the room temperature (25 °C) dried SSF cultures as such were tested in the enantiomer selective biotransformations. The majority of these SSF preparations proved to be effective as enantiomer selective biocatalysts exhibiting high but usual enantioselectivities according to the Kazlauskas rule. However, the SSF preparation of *Mucor hiemalis* origin acted as a selective anti-Kazlauskas catalyst. The best SSF products were successfully applied in preparative scale resolutions.

Keywords: Lipases; Solid state fermentation; Kinetic resolution; Enantioselectivity

1. Introduction

Synthetic application 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 [1–3]. Biocatalytic steps are already being used industrially to manufacture a wide range of products, including drugs, agricultural chemicals, organics, fine chemicals and plastics [4,5]. There is a rapidly increasing demand for the enantiopure form of chiral compounds, therefore novel microorganisms and/or their enzymes are subject of screening to produce such chemicals [1,4].

Screening of thermophilic filamentous fungi in our laboratory for production of lipase/esterase enzymes by submerged fermentations (SmF) resulted in preparations exhibiting high enantiomeric and enantiotopic selectivity in synthetic biotransformations [6]. Although SmF, which is widely used in the enzyme industry, has advantages in process control and good yields of extracellular enzymes, the products in fermentation beer are relatively dilute and therefore the downstream process results in high volumes of effluents (sewage). As an alternative, solid state fermentation (SSF) has been developed and proved to be an economical way to produce various enzymes including lipases and esterases [7–9]. Lipase production of various strains by SSF including *Penicillium simplicissimum* [10], *Penicillium restrictum* [11–13], *Penicillium candidum* [14,15], *Penicillium camembertii* [15], *Rhizopus* sp. [16], *Rhizopus oligosporous* [17], *Rhizopus rhizopodiformis* and *Rhizomucor pusillus* [18], *Rhizomucor miehei* [19,20], *Mucor miehei* [15], *Mucor racemosus* [21], *Aspergillus niger* [22–25], *Yarrowia lipolytica* [26] or *Candida rugosa* [27–30] have been reported. Economic analysis of lipase production by *P. restrictum* in solid state and submerged fermentations revealed that for a production scale of 100 m^3/year, SSF was more economical than SmF [31].

Several of these organisms, such as *A. niger* [24] which is considered as GRAS, can be used for large-scale production of enzymes even in the food industry. Agro-industrial residues such as olive oil cake [18], soy cake [10], coconut oil cake [27,28], babassu cake [11], gingelly oil cake [25], wheat bran [15,19,22,24], rice bran [30], almond meal [17], sugar cane bagasse [18], rice husks [22] or ricinus seed litters [23] can

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be used as substrates in the production of lipases by SSF. In some cases, the use of mixed solid substrate, such as coconut oil cake:wheat bran (1:1) [32], might be advantageous. By the aid of artificial solid supports, such as nylon sponge [26], liquid agricultural wastes can also be applied as substrates.

Supplementation of the SSF medium with lipase inducers strongly effected the lipase production [10,12,24]. It has been reported that the addition of olive oil led to the highest lipase activity [12].

Although numerous lipases were produced by SSF, only a few reports may be found on the applications of the lipases produced by SSF. For example, SSF lipases were applied for hydrolysis of dairy wastewater [11,12] or for enhancement of aroma in black tea [20]. The only SSF lipase used for synthetic purposes so far was reported from a *Rhizopus* sp. [16]. The lyophilized powder of the enzyme after extraction and partial purification was applied for synthesis of short chain citronellyl esters in hexane and neat.

The above precedents prompted us to cultivate a series of filamentous fungi by solid state fermentation and screen them for novel lipase activities. Our goal was to examine strains which were not or not frequently investigated as lipase producers. Since the gently dried SSF preparations do not require costly downstream processes, they can be considered as inexpensive, naturally immobilized biocatalysts. Therefore, these materials were tested for lipase activity and their catalytic activity in synthetic enantioselective reactions were also evaluated.

2. Experimental

2.1. Microorganisms

The fungi indicated in Tables 1–5 were obtained from American Type Culture Collection (ATCC), Manassas, VA; Botany School, University of Melbourne (BSUM), Parkville, Vic., Australia; Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands; Department of Agriculture, Eastern Cereal and Oilseed Research Centre (DAOM), Ottawa, Canada; Institute for Fermentation (IFO), Osaka, Japan; Food Research Laboratory (FRR), CSIRO, North Ryde, NSW, Australia; Hungarian Institute for Plant Protection (HIPP), Budapest, Hungary; Northern Regional Research Center (NRRL), USDA, Peoria, IL, USA; National Institute of Public Health (OKI), Budapest Hungary; Technical University of Budapest (TUB), Hungary; University of Alberta Mold Herbarium and Culture Collection (UAMH), Alberta, Canada; Russian Culture Collection of Microorganisms (VKM), Moscow, Russia; Western Forest Products Laboratory (WFPL), Vancouver, Canada. Strains were stored as freeze-dried cultures and revitalized on potato–dextrose–agar (PDA) medium in Petri plates at 30 °C.

2.2. Solid state fermentation

Solid state fermentation was carried out in cotton-plugged 500 ml Erlenmeyer flasks on solid wheat bran medium (10 g)

Table 1
Effect of substrate, moisture content, inducer and drying method of SSF preparations from *Gliocladium* strains on lipase activity and biotransformation of *rac*-1-phenylethanol, *rac*-**1a**

No.[a]	Strain	Substrate/ Inducer[b]	Moisture content (%)	SSF time (day)	Lipase activity (U/g)	Drying[c]	c[d] (%)	(*S*)-**1a** ee[d] (%)	(*R*)-**2a** ee[d] (%)	E[e]
9	*Gliocladium catenulatum* NRRL 1093	Wb/Oo	60	3	138	RT	49.1	98.5	98.4	>200
10	*Gliocladium catenulatum* NRRL 1093	Wb/Oo	60	3	138	A	45.5	85.4	97.5	>100
11	*Gliocladium catenulatum* NRRL 1093	Wb/Oo	70	3	134	RT	48.2	97.8	98.9	>200
12	*Gliocladium catenulatum* NRRL 1093	Wb/Oo	70	3	134	A	48.5	97.0	98.1	>200
13	*Gliocladium catenulatum* NRRL 1093	Rs/Oo	70	3	73	RT	–	–	–	–
14	*Gliocladium catenulatum* NRRL 1093	Rs/Oo	70	3	73	A	–	–	–	–
23	*Gliocladium roseum* NRRL 1085	Wb/Oo	60	4	1010	RT	36.8	58.6	97.7	>100
24	*Gliocladium roseum* NRRL 1085	Wb/Oo	60	4	1010	A	46.7	90.4	98.2	>200
25	*Gliocladium roseum* NRRL 1085	Wb/Oo	70	4	1230	RT	26.1	35.7	97.9	>100
26	*Gliocladium roseum* NRRL 1085	Wb/Oo	70	4	1230	A	29.4	41.3	97.3	>100
27	*Gliocladium roseum* NRRL 1085	Wb/Po	60	3	1370	RT	40.9	68.3	97.5	>100
28	*Gliocladium roseum* NRRL 1085	Wb/Po	60	3	1370	A	40.8	69.4	97.7	>100
29	*Gliocladium roseum* NRRL 1085	Wb/Po	70	3	890	RT	34.4	52.2	97.6	>100
30	*Gliocladium roseum* NRRL 1085	Wb/Po	70	3	890	A	32.2	47.0	94.8	58

[a] SSF batch numbers are shown as identifiers.
[b] Wb, wheat bran; Rs, rapeseed (as such); Oo, olive oil; Po, palm oil.
[c] RT, air drying at room temperature; A, drying with acetone.
[d] At 48 h reaction time. The ee values of (*R*)-**2a** and (*S*)-**1a** and c were determined by GC on Hydrodex β-PM column.
[e] Enantiomer selectivity (E) was calculated from c and ee_{2a} [49] (and confirmed by independent calculation from ee_{1a} and ee_{2a} [50]). Due to sensitivity to experimental errors, E values calculated in the 100–300 range are reported as >100, and E values calculated above 300 are given as >200.

Table 2
Kinetic resolution of racemic 1-phenylethanol *rac*-**1a** by air-dried SSF biocatalysts

No.[a]	Strain	Lipase activity (U/g)	Reaction time (h)	c[b] (%)	(*S*)-**1a** ee[b] (%)	(*R*)-**2a** ee[b] (%)	E[c]
101	*Chaetomium elatum* UAMH 2672	0.2	24	21.9	27.7	99.6	≫200
97	*Scopulariopsis brevicaulis* WFPL 248A	110	24	19.4	24.7	99.6	≫200
9	*Gliocladium catenulatum* NRRL 1093	138	48	49.1	98.5	98.4	>200
105	*Chaetomium funicola* UAMH 3034	135	24	48.4	95.8	97.2	>100
23	*Gliocladium roseum* NRRL 1085	1010	120	48.4	86.9	96.0	>100
109	*Chaetomium virescens* ATCC 32319	365	24	39.7	65.8	97.3	>100
65	*Chaetomium globosum* OKI 270	61	120	52.1	99.8	91.4	>100
55	*Thamnidium elegans* ATCC 18.191	138	120	50.7	79.8	90.2	65
121	*Chaetomium cochliodes* NRRL 2320	27	24	21.7	26.8	96.1	65
125	*Chaetomium globosum* NRRL 6296	12	24	23.6	30.1	95.6	59
15	*Gliocladium vermoesenii* NRRL 1752	161	120	52.2	99.5	87.2	55
59	*Tolypocladium geodes* CBS 723.70	129	120	50.0	83.3	88.7	49
85	*Trichoderma harzianum* TUB F-886	224	24	36.7	54.3	92.1	42
43	*Aspergillus terreus* FRR 2532	222	120	45.6	68.3	87.8	34
117	*Trichoderma harzianum* TUB F-946	1597	24	31.9	43.4	91.4	34
89	*Trichoderma harzianum* TUB F-947	995	24	34.1	48.2	90.5	32
49	*Fusarium oxysporum* f. sp. *dianthi* HIPP	20	120	11.2	12.2	90.9	24
57	*Thamnostylum pyriforme* ATCC 8686	138	120	45.2	63.0	82.6	21
47	*Myrothecium verrucaria* NRRL 2003	20	120	15.4	14.3	88.7	19
77	*Trichoderma effusum* TUB F-354	275	120	8.5	7.9	64.3	5
51	*Aspergillus niger* ATCC 10864	86	120	24.0	16.6	56.9	4
71	*Trichoderma harzianum* TUB F-791	76	120	9.9	5.7	39.0	2
73	*Paecilomyces varioti* IFO 4855	16	120	9.3	2.4	11.4	1
63	*Mucor hiemalis* NRRL 13.009	107	120	14.1	14.7[d]	85.6[d]	15

[a] SSF batch numbers are shown as identifiers.
[b] The ee values of (*R*)-**2a** and (*S*)-**1a** and c were determined by GC on Hydrodex β-PM column.
[c] Enantiomer selectivity (E) was calculated from c and ee_{2a} [49] (and confirmed by independent calculation from ee_{1a} and ee_{2a} [50]). Due to sensitivity to experimental errors, E values calculated in the 100–300 range are reported as >100, values in the 300–500 range are reported as >200 and values calculated above 500 are given as ≫200.
[d] The reaction proceeded with opposite enantiomer selectivity resulting (*R*)-**1a** and (*S*)-**2a**.

Table 3
Kinetic resolution of racemic 1-cyclohexylethanol *rac*-**1b** by air-dried SSF biocatalysts

No[a]	Strain	Lipase activity (U/g)	Reaction time (h)	c[b] (%)	(*S*)-**1b** ee[b] (%)	(*R*)-**2b** ee[b] (%)	E[c]
23	*Gliocladium roseum* NRRL 1085	1010	48	50.0	99.1	99.3	≫200
97	*Scopulariopsis brevicaulis* WFPL 248A	110	48	44.8	96.6	99.6	≫200
93	*Trichoderma harzianum* TUB F-963	1267	48	37.3	68.6	99.5	≫200
9	*Gliocladium catenulatum* NRRL 1093	138	48	49.8	99.6	98.5	≫200
105	*Chaetomium funicola* UAMH 3034	135	48	50.0	96.2	98.2	>200
101	*Chaetomium elatum* UAMH 2672	0.2	48	24.5	35.3	99.3	>200
125	*Chaetomium globosum* NRRL 6296	12	48	21.8	31.8	99.2	>200
49	*Fusarium oxysporum* f. sp. *dianthi* HIPP	20	72	19.6	33.6	99.1	>100
65	*Chaetomium globosum* OKI 270	61	72	50.5	99.5	96.2	>100
89	*Trichoderma harzianum* TUB F-947	995	48	15.6	31.2	98.9	>100
85	*Trichoderma harzianum* TUB F-886	224	48	15.5	38.3	98.9	>100
61	*Trichoderma harzianum* ATCC 56.678	5275	72	49.7	96.6	95.6	>100
15	*Gliocladium vermoesenii* NRRL 1752	161	48	51.1	99.5	92.8	>100
109	*Chaetomium virescens* ATCC 32319	365	48	8.5	12.9	98.1	>100
121	*Chaetomium cochliodes* NRRL 2320	27	48	27.5	38.7	96.3	76
59	*Tolypocladium geodes* CBS 723.70	129	72	39.2	67.8	93.5	55
43	*Aspergillus terreus* FRR 2532	222	72	36.6	58.1	92.9	46
57	*Thamnostylum piriforme* ATCC 8686	138	72	42.3	73.0	91.6	46
47	*Myrothecium verrucaria* NRRL 2003	20	72	31.1	37.4	92.1	37
55	*Thamnidium elegans* ATCC 18.191	138	72	44.1	76.7	88.4	34
117	*Trichoderma harzianum* TUB F-946	1597	48	25.6	34.2	92.0	33
63	*Mucor hiemalis* NRRL 13.009	107	48	4.0	3.0[d]	95.9[d]	50

[a] SSF batch numbers are shown as identifiers.
[b] The ee values of (*R*)-**2b** and (*S*)-**1b** and c were determined by GC on Hydrodex β-PM column.
[c] Enantiomer selectivity (E) was calculated from c and ee_{2b} [49] (and confirmed by independent calculation from ee_{1b} and ee_{2b} [50]). Due to sensitivity to experimental errors, E values calculated in the 100–300 range are reported as >100, values in the 300–500 range are reported as >200 and values calculated above 500 are given as ≫200.
[d] The reaction proceeded with opposite enantiomer selectivity resulting (*R*)-**1b** and (*S*)-**2b**.

Table 4
Kinetic resolution of racemic 1-(naphth-2-yl)ethanol *rac*-**1c** by air-dried SSF biocatalysts

No.[a]	Strain	Lipase activity (U/g)	Reaction time (h)	c[b] (%)	(*S*)-**1c** ee[b] (%)	E[c]
9	*Gliocladium catenulatum* NRRL 1093	138	120	49.8	97.9	≫200
23	*Gliocladium roseum* NRRL 1085	1010	120	37.8	60.2	>200
55	*Thamnidium elegans* ATCC 18.191	138	120	41.5	70.0	>200
65	*Chaetomium globosum* OKI 270	61	120	50.8	98.0	>100
71	*Trichoderma harzianum* TUB F-791	76	120	38.7	61.5	>100
15	*Gliocladium vermoesenii* NRRL 1752	161	120	52.2	99.5	>100
43	*Aspergillus terreus* FRR 2532	222	120	36.7	55.8	93
1	*Actinomucor elegans* ATCC 6476	110	72	8.4	8.1	18
63	*Mucor hiemalis* NRRL 13.009	107	120	14.7	14.8[d]	15

[a] SSF batch numbers are shown as identifiers.
[b] The ee value of (*S*)-**1c** and c were determined by GC on BetaDex 225 column.
[c] Enantiomer selectivity (E) was calculated from c and $ee_{\mathbf{1c}}$ [50]. Due to sensitivity to experimental errors, E values calculated in the 100–300 range are reported as >100, values in the 300–500 range are reported as >200 and values calculated above 500 are given as ≫200.
[d] The reaction proceeded with opposite enantiomer selectivity resulting in (*R*)-**1c**.

Table 5
Preparative scale kinetic resolution of the racemic secondary alcohols *rac*-**1a–c** by air-dried SSF biocatalysts

No.[a]	Strain	Compound **1**,**2**	Reaction time (h)	c[b] (%)	(*S*)-**1** Yield (%)	(*S*)-**1** ee[b] (%)	(*R*)-**2** Yield (%)	(*R*)-**2** ee[b] (%)	E[c]
9	*Gliocladium catenulatum* NRRL 1093	**a**	120	27	65	36	25	97	91
		b	120	32	46	53	28	98	>200
		c	120	23	71	29	16	99[c]	>200
65	*Chaetomium globosum* OKI 270	**a**	120	44	53	89	35	94	91
		b	120	44	40	76	34	97	>100
		c	120	43	53	66	35	96[d]	44

[a] SSF batch numbers are shown as identifiers.
[b] The ee values and c were determined by GC.
[c] Enantiomer selectivity (E) was calculated from c and $ee_{\mathbf{1a–c}}$ [50]. Due to sensitivity to experimental errors, E values calculated in the 100–300 range are reported as >100, values in the 300–500 range are reported as >200.
[d] The ee values for (*R*)-**2c** were calculated from the enantioselectivity (E) and ee of (*S*)-**1c** [50].

containing mixture of wheat bran (9 g) and olive oil (1 g), and wetted with a salt solution to a 60% or 70% moisture content. In a few experiments, crude palm oil (1 g) was used instead of olive oil (1 g) as an enzyme inducer. Composition of the salt solution (w/v) was 0.5% NH_4NO_3, 0.5% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% NaCl and 0.1% (v/v) trace element solution, pH 6.0. The composition of the trace element solution (w/v) was the following: 0.08% $MnSO_4$, 0.17% $ZnSO_4 \cdot 7H_2O$, 0.25% $FeSO_4 \cdot 7H_2O$. In some experiments performed by *Gliocladium* strains, rapeseed was also tested as a potential substrate for SSF. In these media, rapeseed (with natural oil content, 10 g seed as such per Erlenmeyer flask) was wetted with the previously described salt solution, to a 60% or 70% moisture content. The wet SSF media were routinely sterilized in autoclave, inoculated with dense spore suspension prepared in sterile water from fully sporulating Petri plate cultures (10^6 viable spores/g dry matter SSF medium) and incubated at 30 °C without shaking (static culture).

Lipase activity was determined from the culture extract of SSF samples. The content of each Erlenmeyer flask was mixed thoroughly with an appropriate volume (to make a total volume of 150 ml) of 0.1% Tween-80 solution. The SSF material was extracted at room temperature[1] for 2 h with occasional shaking, then centrifuged at 7000 × g for 10 min and the supernatant was used for the assay.

For the organic chemical tests dry SSF samples were used. The wet SSF material was dewatered by two different methods, namely (a) direct drying at room temperature for 24 h and/or (b) removing water with 3 (v/w) cold acetone and final drying at room temperature for 24 h.

2.3. Lipase activity test from SSF preparations

Lipase activity was determined according to Vorderwülbecke et al. [33] using *p*-nitrophenyl-palmitate (pNPP, SIGMA) as a substrate. Solution A contained 90 mg of pNPP dissolved in 30 ml of propane-2-ol. Solution B contained 2 g Triton X-100 and 0.5 g gum arabic (SIGMA) dissolved in 450 ml buffer (Tris–HCl, 50 mM, pH 8.0). The assay solution was prepared by adding 1 ml of solution A to 9 ml of solution B to obtain

[1] Room temperature refers to 25 ± 3 °C (summer season in Hungary) throughout this work.

an emulsion, which remained stable for 2 h. The assay mixture contained 2 ml of the emulsion and 0.5 ml of the appropriately diluted enzyme extract. After incubation at 45 °C for 30 min, the liberated *p*-nitrophenol (pNP) was measured at 410 nm.

2.4. 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) and are reported in ppm on the δ scale. IR spectra (film) were taken on a Specord 2000 Series spectrophotometer and the wavenumbers of the absorptions are reported in cm^{-1}. GC analyses were carried out on HP 5890 or Agilent 4890D instruments equipped with FID detector and Hydrodex-β-PM column (25 m × 0.25 mm, 0.25 μm film with permethylated β-cyclodextrin; Macherey&Nagel) or Betadex 225 fused silica capillary column (30 m × 0.25 mm × 0.25 μm film; Supelco) 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 visualized 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*-**1a**), 1-cyclohexylethanol (*rac*-**1b**), 1-(naphth-2-yl)ethanol (*rac*-**1c**) and vinyl acetate were products of Aldrich.

The inorganic salts and materials for biomass production were purchased from either Sigma, Aldrich or Fluka.

2.5. Enantiomer selective acetylations of racemic alcohols *rac*-*1a–c*

Suspensions of the enzyme preparations (20 mg) with a solution of *rac*-**1a–c** (20 mg) in a mixture of hexane (1 ml), tetrahydrofurane (0.5 ml) and vinyl acetate (0.5 ml) were shaken at 1000 rpm in sealed glass vials at room temperature for the time indicated in Tables 2–4. The conversions were checked by TLC (hexane–acetone 10:4, v/v). At the reaction time indicated in Tables 2–4, the enantiomeric compositions and the ratio of the product and residual substrate fractions were analyzed by GC on chiral stationary phase (Tables 2–4). Tables 2–4 list results only for those reactions which exhibited more than 5% conversion after 120 h.

2.5.1. GC retention times

R_t (Hydrodex β-PM column, 65–118 °C, 2.5 °C/min, 12 psi): 17.6 min, (*R*)-**2a**; 18.5 min, (*S*)-**2a**; 18.9 min, (*R*)-**1a**; 19.5 min, (*S*)-**1a**; GC molar response factor of acetate/alcohol (*rac*-**2a**/*rac*-**1a**): 1.23 [used for calculations of *c* and *E* in Tables 1 and 2].

R_t (Hydrodex β-PM column, 100–112 °C, 1 °C/min, 12 psi): 7.5 min, (*R*)-**1b**; 7.6 min, (*S*)-**2b**; 8.2 min, (*R*)-**2b**; 8.9 min, (*S*)-**1b**; GC molar response factor of acetate/alcohol (*rac*-**2b**/*rac*-**1b**): 1.25 [used for calculations of *c* and *E* in Table 3].

R_t (Beta-Dex 225 column, 130–185 °C, 2 °C/min, 12 psi): 23.1 min, (*R*)-**1c**; 23.4 min, (*S*)-**1c**; 24.1 min, **2c**; GC molar response factor of acetate/alcohol (*rac*-**2c**/*rac*-**1c**): 1.21 [used for calculations of *c* and *E* in Table 4].

Preparative scale conversions of *rac*-**1a–c** (200 mg each) catalyzed by SSF preparation #9, from *Gliocladium catenulatum* NRRL 1093 or by #65, from *Chaetomium globosum* OKI 270 (200 mg, each) were also performed in hexane (10 ml), tetrahydrofurane (5 ml) and vinyl acetate (5 ml). After shaking the reaction mixture at 1000 rpm, room temperature for 120 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 10:0.5, v/v) to give alcohol (*S*)-**1a–c** and acetate (*R*)-**2a–c**.

(*S*)-**1a**: colorless oil; SSF-9: [yield: 131 mg, 65%; ee: 36% by GC; $[\alpha]_D^{22} = -18.8$ (*c* 1.0, $CHCl_3$)]; SSF-65: [yield: 105 mg, 53%; ee: 89% by GC; $[\alpha]_D^{33} = -57.7$ (*c* 1.0, $CHCl_3$)] {lit.: $[\alpha]_D$: −45.3 ($CHCl_3$) [34]; $[\alpha]_D$: −53.5 (*c* 1.13, $CHCl_3$) [35]; $[\alpha]_D^{25}$: −55.1 (*c* 1.63, $CHCl_3$) [36]}; IR: 3352, 3008, 2976, 2952, 1466, 1430, 1368, 1204, 1080, 1008, 900, 760, 690; ^1H NMR: 1.51 (d, *J* = 6.9 Hz, 3H), 3.38 (br s, 1H), 4.86 (q, *J* = 6.9 Hz, 1H), 7.40 (m, 5H); ^{13}C NMR: 25.03, 69.84, 125.07, 126.86, 127.97, 145.53; IR and NMR data agreed with the reported spectra [37].

(*R*)-**2a**: colorless oil; SSF-9: [yield: 65.5 mg, 25%; ee: 97% by GC; $[\alpha]_D^{22} = +125$ (*c* 1.0, $CHCl_3$)]; SSF-65: [yield: 95 mg, 35%; ee: 94% by GC; $[\alpha]_D^{22} = +123$ (*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: 1740, 1456, 1376, 1244, 1208, 1064, 944, 760, 700; ^1H NMR: 1.57 (d, *J* = 6.9 Hz), 2.10 (s, 3H), 5.93 (q, *J* = 6.9 Hz, 1H), 7.30–7.40 (m, 5H); ^{13}C NMR: 21.42, 22.28, 72.24, 125.86, 127.63, 128.26, 141.42, 169.95; IR and NMR data agreed with the reported spectra [40].

(*S*)-**1b**: colorless oil; SSF-9: [yield: 120 mg, 46%; ee: 53% by GC; $[\alpha]_D^{22} = +1.6$ (*c* 1.0, $CHCl_3$)]; SSF-65: [yield: 105 mg, 40%; ee: 76% by GC; $[\alpha]_D^{22} = +2.1$ (*c* 1.0, $CHCl_3$)] {lit.: $[\alpha]_D^{20}$: +8.43 Et_2O [41]; $[\alpha]_D^{20}$: +3.51 (*c* 3.1, $CHCl_3$) [42]; IR and NMR data agreed with the reported spectra [43].

(*R*)-**2b**: colorless oil; SSF-9: [yield: 76 mg, 28%; ee: 98% by GC; $[\alpha]_D^{22} = +9.5$ (*c* 1.0, $CHCl_3$)]; SSF-65: [yield: 90 mg, 33.8%; ee: 97% by GC; $[\alpha]_D^{22} = +7.2$ (*c* 1.0, $CHCl_3$)] {lit.: $[\alpha]_D^{20}$: +6.6 (*c* 2.6, $CHCl_3$) [44]; IR: 2928, 1736, 1244; ^1H NMR: 1.09 (d, *J* = 6.4 Hz, 3H), 1.65 (m, 11H), 1.96 (s, 3H), 4.75 (q, *J* = 6.4 Hz, 1H); ^{13}C NMR: 17.05, 21.3, 26.04, 26.23, 28.5, 42.57, 74.56, 170.08.

(*S*)-**1c**: white powder; SSF-9: [yield: 142 mg, 71%; ee: 29% by GC; $[\alpha]_D^{22} = -15$ (*c* 1.0, $CHCl_3$); mp.: 71–72 °C {lit.: 70–72 °C (hexane) [45]}]; SSF-65: [yield: 106 mg, 53%; ee: 66% by GC; $[\alpha]_D^{22} = -34.8$ (*c* 1.0, $CHCl_3$)] {lit.: $[\alpha]_D^{20}$: −43.1 (*c* 2.6, EtOH) [46]; $[\alpha]_D$: −25 (*c* 2.2, MeOH) [47]; $[\alpha]_D^{25}$: −23.0 (*c* 4.55, MeOH) [44]; mp.: 70–71 °C}; IR: 3610, 3550–3250, 3060, 3020, 2980, 2930, 1450, 860; ^1H NMR: 1.58 (d, *J* = 6.9 Hz, 3H), 2.0 (br s, 1H), 5.06 (q, *J* = 6.9 Hz, 1H), 7.5 (mc, 3H), 7.8 (mc, 4H); ^{13}C NMR: 25.09, 70.48, 123.76, 123.77, 125.75, 126.11, 127.63, 127.90, 128.27, 132.88, 133.28, 143.15; IR and NMR data agreed with the reported spectra [43].

(*R*)-**2c**: both as colorless oil; SSF-9: [yield: 42 mg, 16%; ee: 99% by GC; $[\alpha]_D^{22} = +125.5$ (*c* 1.0, $CHCl_3$)]; SSF-65: [yield: 89 mg, 35%; ee: 96% by GC; $[\alpha]_D^{22} = +102.5$ (*c* 1.0, $CHCl_3$)]

{lit.: $[\alpha]_D^{20}$: +33 (*c* 1, $CHCl_3$) [47]; $[\alpha]_D$: +109 (*c* 1.83, $CHCl_3$) [44]}; IR: 1064, 1244, 1372, 1740, 2984; ^{1}H NMR: 1.61 (d, J = 6.9 Hz, 3H), 2.10 (s, 3H), 6.04 (q, J = 6.9 Hz, 1H), 7.5 (d, 3H), 7.8 (t, 4H); ^{13}C NMR: 21.37, 22.15, 72.43, 124.06, 124.99, 126.03, 126.20, 127.63, 127.99, 128.32, 133.00, 133.15, 138.97, 170.37; IR and NMR data agreed with the reported spectra [48].

3. Results and discussion

Because our previous lipase/esterase activity screening of some thermophilic filamentous fungi in submerged fermentation conditions resulted in biocatalysts exhibiting high enantioselectivity in synthetic biotransformations [6], we thought it worthwhile to investigate several poorly studied mesophilic fungi for similar activities as well. Since solid state fermentation is well known as an economical way to produce various enzymes including lipases and esterases [7–9], we also considered this method as a promising possibility to produce lipases of these mesophilic fungi. Moreover, considering the SSF matrix as a natural immobilization carrier, the dried SSF materials as cheap biocatalyst were tested without further downstream processing.

First, the effects of substrate matrices (wheat bran or whole rapeseed), the water content during SSF (60% or 70% moisture contents) and the drying method of the biomass (simple air drying at room temperature or washing with acetone) on the lipase productivity and on the conversion rate/enantiomer selectivity in a typical biotransformation of *rac*-**1a** were studied (Table 1).

The preliminary screening results with *G. catenulatum* NRRL 1093 samples indicated that wheat bran supplemented with vegetable oil as substrate (nos. 9–12 in Table 1) is superior over rapeseed (nos. 13, 14 in Table 1). Therefore, in the later tests only wheat bran medium was employed. Considering the best lipase activities (determined by *p*-nitrophenyl palmitate), the highest rate of conversion (*c*) and the highest degree of enantiomer selectivity (*E*) in acetylation of racemic 1-phenylethanol, *rac*-**1a** and the simplicity of the final treatment of crude biocatalyst, the SSF conditions using wheat bran having 60% moisture content as substrate, olive oil as inducer and air drying at RT after 3 days cultivation at 35 °C were chosen for further screening.

Next, air-dried SSF preparations as such were tested as biocatalysts in enantiomer-selective biotransformations. For this purpose, kinetic resolutions of various racemic secondary alcohols (*rac*-**1a–c**) by acetylation in organic solvents with vinyl acetate catalyzed by the air-dried SSF preparations were carried out (Fig. 1) and the conversion rates and enantioselectivities of the reactions were determined.

The biocatalytic abilities and enantiomer selectivities of our SSF biocatalysts has been tested first on acetylation of racemic 1-phenylethanol *rac*-**1a** as a typical racemic secondary alcohol with a rigid aromatic ring adjacent to the asymmetric carbon centre (Table 2). As expected, almost all enzyme preparations followed the so-called "Kazlauskas rule" [51]. In acylation of racemic 1-phenylethanol *rac*-**1a**, the best enantiomer selectivities ($E > 200$) has been observed with SSF preparations of strains *Chaetomium elatum* UAMH 2672 (sample #101), *G. catenulatum* NRRL 1093 (#9) and *Scopulariopsis brevicaulis* WFPL 248 A (#97).

Somewhat lower but still significant selectivities ($E > 100$) has been found for SSF samples of *Chaetomium funicola* UAMH 3034 (sample #105), *C. globosum* OKI 270 (#65), *Chaetomium virescens* ATCC 32319 (#109) and *Gliocladium roseum* NRRL 1085 (#23). One enzyme preparation, from *Mucor hiemalis* NRRL 13.009 strain (#63), revealed not enormous but significant anti-Kazlauskas selectivity in the acylation of *rac*-**1a**.

Racemic 1-cyclohexylethanol *rac*-**1b** in which a more flexible and relatively bulky cyclohexyl moiety is attached directly to the asymmetric centre has been selected as the second test substrate (Table 3). Due to the increased bulkiness of the cyclic substituent in this substrate (*rac*-**1b**), considerably more strains exhibited high enantiomer selectivity in its enzymatic acetylation. In addition to the *Chaetomium* (sample #65, 101, 105, 109), *Gliocladium* (#9, 23), and *Scopulariopsis* (#97) species which were highly selective on substrate *rac*-**1a**, other strains like *C. globosum* NRRL 6296 (#125), *C. virescens* ATCC 32319 (#109), *Gliocladium vermoesenii* NRRL 1752 (#15), *Trichoderma harzianum* ATCC 56.678 (#61), TUB F-963 (#93),

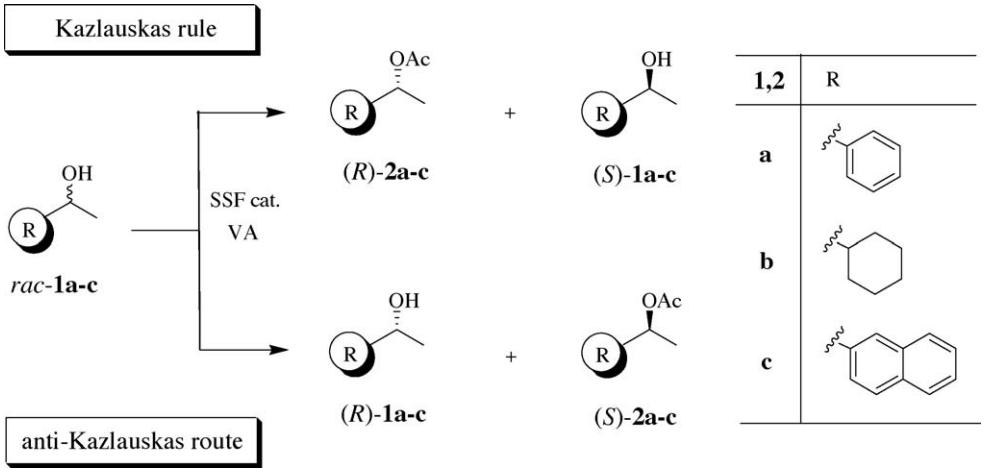

Fig. 1. Kinetic resolution of racemic secondary alcohols *rac*-**1a–c** by SSF biocatalysts.

TUB F-947 (#89) and TUB F-886 (#85) catalyzed the acylation reaction of *rac*-**1b** according to the Kazlauskas-rule with high enantiomer selectivity ($E > 100$). Interestingly, the enzyme preparation from *M. hiemalis* NRRL 13.009 (#63) retained the anti-Kazlauskas selectivity on this secondary alcohol *rac*-**1b** as well.

When the SSF biocatalysts were tested on the third test substrate, racemic 1-(naphth-2-yl)ethanol *rac*-**1c** containing a rigid and bulky 2-naphthyl moiety, significantly less strains than for *rac*-**1a** or *rac*-**1b** were found capable catalyzing the acetylation (Table 4). In addition to the SSF biocatalysts from *Chaetomium* (sample #65) and *Gliocladium* (#9, 15, 23) strains which also showed high selectivity towards the smaller substrates *rac*-**1a** or *rac*-**1b**; two strains, *Thamnidium elegans* ATCC 18.191 (#55) and *Trichoderma harzianum* TUB F-791 (#71) were found catalyzing only the bulky *rac*-**1b** substrate with high selectivity ($E > 100$). The anti-Kazlauskas nature of selectivity of the SSF preparation from *M. hiemalis* NRRL 13.009 (#63) remained consequent in the acylation of *rac*-**1c**.

To demonstrate the synthetic usefulness of the SSF biocatalysts, two of them, namely *G. catenulatum* NRRL 1093 (sample #9) and *C. globosum* OKI 270 (#65) were successfully applied in preparative scale enzymatic acetylation of the tested racemic secondary alcohols *rac*-**1a–c** (Table 5). These two SSF biocatalysts were among the most effective samples on all three substrates *rac*-**1a–c** screened. The selected SSF biocatalysts catalyzed the preparative scale (200 mg of substrate) reactions with high selectivities (Table 5) although the selectivities were somewhat lower as found for analytical scale reactions (Tables 2–4), especially in acetylation reactions of the aromatic substrates *rac*-**1a** and *rac*-**1c**. According to GC on chiral stationary phase, the isolated products (*R*)-**2a–c** were almost enantiomerically pure (Table 5).

In addition to precise determination of the enantiomeric composition, the products were fully characterized and their specific rotations were also determined. In some cases specific rotations determined in this work exceeded the previously determined values (c.f. Section 2.5; e.g. for (*R*)-**2a** of 97%ee by GC: $[\alpha]_D^{22} = +125$ (*c* 1.0, $CHCl_3$), lit.: $[\alpha]_D$: 105.1 (*c* 1.3, $CHCl_3$) [39]; for (*R*)-**2b** of 98%ee by GC: $[\alpha]_D^{22} = +9.5$ (*c* 1.0, $CHCl_3$), lit.: $[\alpha]_D^{20}$: +6.6 (*c* 2.6, $CHCl_3$) [44]; for (*R*)-**2c** of 99%ee by GC: $[\alpha]_D^{22} = +125.5$ (*c* 1.0, $CHCl_3$), lit.: $[\alpha]_D$: +109 (*c* 1.83, $CHCl_3$) [43]).

4. Conclusions

Solid state fermentation was evaluated for microbial production of lipase/esterase enzymes using wheat bran and olive oil as carbon source and enzyme inducer. Screening 38 of mesophilic fungi yielded good activities for many isolates. The whole SSF culture preparations were used without prior downstream processing for testing the raw biocatalysts in organic reactions.

The majority of these SSF preparations proved to be effective as enantiomer selective biocatalysts. In the enzyme-catalyzed acetylation of three selected racemic secondary alcohols (*rac*-**1a–c**), some of them exhibited high but usual enantioselectivities according to the Kazlauskas rule [51], whereas the *M. hiemalis* NRRL 13.009 SSF preparation proved to be an anti-Kazlauskas biocatalyst. Some of the best SSF preparations were successfully applied in preparative scale kinetic resolutions of *rac*-**1a–c**, indicating their usefulness as inexpensive, naturally immobilized biocatalysts.

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