Journal of Molecular Catalysis B: Enzymatic 63 (2010) 87–92

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Microbial-catalysed resolution of sterically demanding cyanohydrins

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ARTICLE INFO

Article history:
Received 17 September 2009
Received in revised form 8 December 2009
Accepted 14 December 2009
Available online 29 December 2009

Keywords:
Enzyme catalysis
Mycelium
Hydrolases
Cyanohydrins
tert-Alcohols

ABSTRACT

Mycelia containing carboxyl-esterases, a novel source of enzymes, have been investigated in the hydrolytic kinetic resolution of one type of *tert*-alcohols, α,α-disubstituted cyanohydrins. This class of enzymes was found to be both active and selective towards these *tert*-alcohols, giving *E*-values as high as 42.

1. Introduction

Cyanohydrins are versatile building blocks in organic synthesis and fine chemical industry [1]. Their enantioselective, catalytic preparation has attracted much attention and both chemical and enzymatic approaches have been developed. In most cases the (bio)catalytic methods focus on aldehydes as starting materials since the stereo-differentiation of these prochiral molecules is relatively easy [2–4]. However, when starting from ketones the induction of chirality becomes considerably more difficult. In addition the reaction equilibrium is unfavourable since the product, an α,α-disubstituted cyanohydrin, is sterically congested [5]. Indeed, it is a *tert*-alcohol (Fig. 1). When viewing α,α-disubstituted cyanohydrins as alcohols, this also opens up a new avenue of approach towards their enantioselective synthesis, the kinetic resolutions of their esters.

Only few examples of the successful enzymatic hydrolysis of esters of *tert*-alcohols have been reported in the literature [6]. Enantioselective conversions are even fewer and to date only three reports on the enantioselective kinetic resolution of sterically congested cyanohydrin acetates, derived from ketones, have been published [7–10]. *Bacillus subtilis* esterase 2 (BS2), which displayed excellent enantioselectivity towards other *tert*-alcohols, was completely unselective in the hydrolysis of α,α-disubstituted cyanohydrin acetates [11]. Consequently, a great need for new enzymes to enantioselectively hydrolyse these α,α-disubstituted cyanohydrin acetates exists.

Fungal mycelia and cell-bound hydrolases are such a versatile source of new enzymes, that to date has not yet been fully explored [12–16]. Many microorganisms have cell-bound esterases and other hydrolases. These enzymes are thus firmly immobilized [17] to the whole cells and show several technical advantages compared to commercially available enzymes, such as easy scale-up since only the cells need to be grown and no enzyme purification needs to be performed. In addition, the enzymes display high stability in organic solvents. It has previously been shown that the catalytic activity for the esterification and ester hydrolysis of mycelia of different moulds from various species (*Rhizopus oryzae*, *Rhizopus javanicus*, *Rhizopus liquefaciens* and *Aspergillus oryzae*) is promising [13,16,18]. Similar results were obtained with cell-bound carboxyl-esterases from *Bacillus coagulans* [14,15] and *Kluyveromyces marxianus* [12]. All these hydrolases hence have the properties desirable for being catalysts in the enantioselective hydrolysis of α,α-disubstituted cyanohydrin acetates.

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doi:10.1016/j.molcatb.2009.12.014

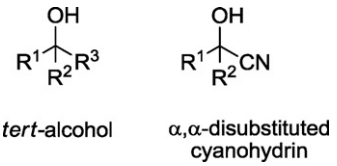


Fig. 1. α,α-Disubstituted cyanohydrins are *tert*-alcohols.

2. Experimental

CAUTION: All procedures involving hydrogen cyanide were performed in a well-ventilated fume hood equipped with a HCN detector. HCN-containing wastes were neutralised using commercial bleach and stored independently over a large excess of bleach for disposal.

2.1. Materials and methods

n-Dodecane (99+%, Sigma–Aldrich), 1,3,5-triisopropylbenzene (97%, Fluka), phosphate buffer (10 mM, pH 7.0), ethyl acetate (>99%, Acros Organics), *n*-heptane (99%, CHROMASOLV® for HPLC, Sigma–Aldrich), 2-propanol (99.9%, HPLC grade, Fisher Scientific), trifluoroacetic acid (99%, extra pure, Acros Organics), 2,4-di-*tert*-butylphenol (99%, Sigma–Aldrich), acetic acid (99.5%, Acros Organics) and (1*R*, 2*R*)-(-)-1,2-diaminocyclohexane (98%, Sigma–Aldrich) were used as received. Racemic α,α-disubstituted cyanohydrin acetates were prepared according to established procedures by cyanation, deprotection and acetylation [10]. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker Avance 400 (400 and 100 MHz, respectively) or a Varian Unity Inova 300 instrument (300 and 75 MHz, respectively). Chemical shifts are expressed in parts per million (δ) relative to tetramethylsilane. Coupling constants *J* are expressed in Hertz (Hz). Elemental analyses were performed by Dr. A. Verwey Analytical Laboratories Rotterdam; HRMS analyses were performed on a Bruker micrOTOF-Q instrument using Electrospray Ionisation (ESI) in positive ion mode (capillary potential of 4500 V). Mass spectra were obtained with a Shimadzu GC-2010 Gas Chromatograph coupled to a Shimadzu GCMS QP-2010S Gas Chromatographic Mass Spectrometer. Enantiomeric purity was determined by GC using an enantioselective β-cyclodextrin column (CP-Chirasil-Dex CB 25 m × 0.32 mm) and a Shimadzu Gas Chromatograph GC-17A equipped with an FID detector and a Shimadzu Auto-injector AOC-20i, using He with a linear gas velocity of 75 cm s^{-1} as the carrier gas. Alternatively, it was determined by HPLC using a Waters System (Waters 486 UV detector, Waters 515 pump and Waters 717+ injector) equipped with a Chiralpak AD-H column from Daicel (4.5 μm × 250 mm) with UV-detection at 215 nm and *n*-heptane:2-propanol 80:20 + 0.1% trifluoroacetic acid (TFA) as solvent (flow: 0.5 mL/min). Optical rotations were measured using a Perkin-Elmer 241 polarimeter.

2.2. Microorganisms and culture conditions

The following microbial strains have been employed:

Rhizopus oryzae CBS 112.07, CBS 391.34 (*javanicus*), CBS 260.38 (*liquefaciens*) (Central Bureau voor Schimmelcultures, Utrecht, The Netherlands).
Aspergillus oryzae MIM (Industrial Microbiology Section, DISTAM, University of Milan).
Bacillus coagulans NCIMB 9365 (National Collection of Industrial and Marine Bacteria, Aberdeen, UK).
Kluyveromyces marxianus CBS 1553 (Central Bureau voor Schimmelcultures, Utrecht, The Netherlands).

Rhizopus and *Aspergillus* strains 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 basal medium (Difco yeast extract 1 g/L, $(NH_4)_2SO_4$ 5 g/L, K_2HPO_4 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, pH 5.80) added with Tween 80 (0.5%). Suspensions of spores (1.6×10^4) were used as inoculum. Mycelium grown for 48 h in submerged cultures was harvested by filtration at 4 °C, washed with phosphate buffer (pH 7.0, 0.1 M) and lyophilized.
Kluyveromyces marxianus CBS 1553 was routinely maintained on malt extract (8 g/L, agar 15 g/L, pH 5.5) and cultured in 2.0 L Erlenmeyer flasks containing 200 mL of malt broth (pH 6.0) and incubated for 48 h at 28 °C on a reciprocal shaker (100 spm).
Bacillus coagulans NCIMB 9365 was routinely maintained on Difco nutrient broth (8 g/L, agar 15 g/L, pH 7.0) and cultured in 2.0 L Erlenmeyer flasks containing 200 mL of CYSP broth (casitone 15 g/L, yeast extract 5 g/L, soytone 3 g/L, peptone 2 g/L, $MgSO_4 \cdot 7H_2O$ 15 mg/L, $FeCl_3$ 115 mg/L, $MnCl_2$ 20 mg/L, pH 7.0) and incubated for 24 h at 45 °C on a reciprocal shaker (120 spm).

The dry weights were determined after centrifugation of 100 mL of cultures, cells were washed with distilled water and dried at 110 °C for 24 h.

Liquid mycelia preparations (grown on medium and stored in phosphate buffer at 4 °C, used within a short time after preparation): *Bacillus coagulans*, *Kluyveromyces marxianus*.

2.3. General procedure for the hydrolytic kinetic resolution

The reactions were run on a scale of 2 g/L (substrate concentration). Cells (40.0 mg) and substrate (4.0 mg) were mixed together in phosphate buffer (10 mM, pH 7.0, 2.0 mL) and stirred at 30 °C for 6 h. The substrate did not dissolve completely in the phosphate buffer and gave a turbid mixture. *n*-Dodecane or 1,3,5-triisopropylbenzene (0.05 or 0.1 mL of a solution with concentration 2 g/L in ethyl acetate) was added at the end of the reaction. *n*-Dodecane (0.1 mL) was used for substrates **1b**, **1c** and **1e**. 1,3,5-Triisopropylbenzene was used for substrates **1a** (0.1 mL), **1d** (0.05 mL), **1f** (0.05 mL), **1 h** (0.1 mL) and **1 g** (0.05 mL). The reaction mixture was extracted with ethyl acetate (2 × 2.5 mL), dried over Na_2SO_4, filtered and concentrated to a volume of 1 mL under N_2. Enantiomeric excess (*ee*) and conversion (c) was calculated from chiral GC or HPLC analysis. The experiments where no reaction occurred were considered as a blank reaction (Table 1). No autohydrolysis of the substrates was observed, even after long periods of time in phosphate buffer. This is in perfect agreement with a recent publication on these substrates [10].

2.4. Spectroscopic data for α,α-disubstituted cyanohydrin acetates

1a: R^1 = Ph, R^2 = Me: see Ref. [10].

1b: R^1 = 4-Cl–C_6H_4, R^2 = Me: ^{1}H NMR ($CDCl_3$, 300 MHz): 1.98 (s, 3H), 2.13 (s, 3H), 7.36–7.49 (m, 4H); ^{13}C NMR ($CDCl_3$, 300 MHz): 20.9, 29.6, 72.7, 117.8 (C≡N), 126.0 (2C), 129.2 (2C), 135.2, 136.8, 168.3 (C=O); MS: 225/223 (M^+), 208, 183/181, 164, 146, 139, 113, 101; GC: 160 °C: 3.7 min (*S*) and 4.0 min (*R*).

1c: R^1 = 4-OMe–C_6H_4, R^2 = Me: see Ref. [10].

1d: R^1 = 3,4-*di*-OMe–C_6H_3, R^2 = Me: ^{1}H NMR (400 MHz, $CDCl_3$): 2.00 (s, 3H), 2.13 (s, 3H), 3.89 (s, 3H), 3.92 (s, 3H), 6.89 (d, *J* = 8.2 Hz, 1H), 7.02 (s, 1H), 7.10 (d, *J* = 8.2 Hz, 1H); ^{13}C NMR (400 MHz, $CDCl_3$): 21.1, 31.0, 56.0, 56.1, 73.1, 108.1, 111.1, 117.3 (C≡N), 118.3, 130.5, 149.2, 149.7, 168.4 (C=O); elemental analysis calcd for $C_{13}H_{15}NO_4$: C, 62.6; H, 6.1; N, 5.6; found: C, 62.8; H, 6.2; N, 5.5; HRMS: *m*/*z* calcd for $C_{13}H_{15}NNaO_4$ $(M+Na)^+$ 272.0893, found 272.0887; MS: 249 (M^+), 232, 210, 207, 190, 165, 138, 119, 104, 89, 77, 63, 43, 40;

Table 1
Conversions, enantiomeric excesses and *E*-values for hydrolytic kinetic resolutions of α,α-disubstituted cyanohydrin acetates catalysed by mycelia.

Substrate	Mycelia strain	Conversion	*ee*	*E*
1a	*Rhizopus oryzae*	45%	1% (*R*)	1
1a	*Rhizopus javanicus*	46%	1% (*R*)	1
1a	*Rhizopus liquefaciens*	51%	0% (–)	n.d.
1a	*Aspergillus oryzae*	62%	12% (*R*)	1.3
1a	*Kluyveromyces marxianus*[a]	45%	2% (*S*)	1
1a	*Kluyveromyces marxianus*[b]	n.r.	0% (–)	n.d.
1a	*Bacillus coagulans*	61%	2% (*S*)	1
1b	*Rhizopus oryzae*	54%	4% (*R*)	1.1
1b	*Rhizopus javanicus*	55%	3% (*R*)	1.1
1b	*Rhizopus liquefaciens*	66%	3% (*R*)	1.1
1b	*Aspergillus oryzae*	48%	3% (*R*)	1.1
1b	*Kluyveromyces marxianus*[a]	n.r.	0% (–)	n.d.
1b	*Kluyveromyces marxianus*[b]	n.r.	0% (–)	n.d.
1b	*Bacillus coagulans*	34%	1% (*S*)	1.1
1c	*Rhizopus oryzae*	17%	3% (*R*)	1.4
1c	*Rhizopus javanicus*	25%	1% (*R*)	1.1
1c	*Rhizopus liquefaciens*	30%	2% (*R*)	1.1
1c	*Aspergillus oryzae*	34%	18% (*R*)	2.5
1c	*Kluyveromyces marxianus*[a]	n.r.	0% (–)	n.d.
1c	*Kluyveromyces marxianus*[b]	n.r.	0% (–)	n.d.
1c	*Bacillus coagulans*	86%	15% (*S*)	1.2
1d	*Rhizopus oryzae*	28%	16% (*S*)	2.8
1d	*Rhizopus javanicus*	38%	36% (*S*)	5.5
1d	*Rhizopus liquefaciens*	23%	18% (*S*)	5
1d	*Aspergillus oryzae*	56%	59% (*S*)	5
1d	*Kluyveromyces marxianus*[a]	36%	49% (*S*)	23.5
1d	*Kluyveromyces marxianus*[b]	89%	91% (*S*)	3
1d	*Bacillus coagulans*	97%	94% (*S*)	2.2
1e	*Rhizopus oryzae*	47%	2% (*R*)	1.1
1e	*Rhizopus javanicus*	48%	1% (*R*)	1
1e	*Rhizopus liquefaciens*	53%	1% (*R*)	1
1e	*Aspergillus oryzae*	63%	23% (*R*)	1.6
1e	*Kluyveromyces marxianus*[a]	n.r.	0% (–)	n.d.
1e	*Kluyveromyces marxianus*[b]	n.r.	0% (–)	n.d.
1e	*Bacillus coagulans*	n.r.	0% (–)	n.d.
1f	*Rhizopus oryzae*	n.r.	0% (–)	n.d.
1f	*Rhizopus javanicus*	72%	11% (*R*)	1.2
1f	*Rhizopus liquefaciens*	76%	1% (*R*)	1
1f	*Aspergillus oryzae*	86%	75% (*S*)	2.4
1f	*Kluyveromyces marxianus*[a]	59%	1% (*S*)	1
1f	*Kluyveromyces marxianus*[b]	82%	31% (*R*)	1.4
1f	*Bacillus coagulans*	70%	12% (*R*)	1.2
1g	*Rhizopus oryzae*	46%	57%	9
1g	*Rhizopus javanicus*	47%	78%	37
1g	*Rhizopus liquefaciens*	89%	70%	2
1g	*Aspergillus oryzae*	68%	90%	7
1g	*Kluyveromyces marxianus*[a]	51%	90%	42
1g	*Kluyveromyces marxianus*[b]	n.r.	0%	n.d.
1g	*Bacillus coagulans*	24%	6%	1.6
1h	*Rhizopus oryzae*	76%	8% (*R*)	1.1
1h	*Rhizopus javanicus*	80%	0% (–)	n.d.
1h	*Rhizopus liquefaciens*	79%	7% (*R*)	1.1
1h	*Aspergillus oryzae*	90%	12% (*R*)	1.1
1h	*Kluyveromyces marxianus*[a]	92%	28% (*R*)	1.3
1h	*Kluyveromyces marxianus*[b]	n.r.	0% (–)	n.d.
1h	*Bacillus coagulans*	88%	10% (*S*)	1.1

All enzymes tested showed no activity towards substrate **1i**.
[a] Lyophilised.
[b] Freshly prepared.

HPLC: column: Chiralpak AD-H, flow: 0.5 mL/min, UV-detection: 215 nm, solvent: *n*-heptane:2-propanol 80:20 + 0.1% TFA: 13.4 min (*R*) and 14.6 min (*S*).

1e: R^1 = Ph, R^2 = Et: see Ref. [10].

1f: R^1 = Ph, R^2 = CH=CH–Ph: ^{1}H NMR ($CDCl_3$, 300 MHz): 2.17 (s, 3H, CH_3), 6.33 (d, *J* = 15.9 Hz, 1H), 6.94 (d, *J* = 15.9 Hz, 1H), 7.29–7.61 (m, 10H, ArH); ^{13}C NMR ($CDCl_3$, 300 MHz): 21.1, 76.4, 116.4 (C≡N), 125.3, 125.6 (2C), 127.2 (2C), 128.5, 128.6, 128.8 (2C), 129.1 (2C), 129.4, 134.1, 134.6, 168.1 (C=O); elemental analysis calcd for $C_{18}H_{15}NO_2$: C, 77.9; H, 5.4; N, 5.0; found: C, 77.5; H, 5.5; N, 4.4; HRMS: *m*/*z* calcd for $C_{18}H_{15}NNaO_2$ $(M+Na)^+$ 300.0995, found 300.0985; MS: 277 (M^+), 253, 238, 235, 206, 179, 165, 140, 130, 105, 89, 77, 63, 43, 40; HPLC: column: Chiralpak AD-H, flow: 0.5 mL/min, UV-detection: 215 nm, solvent: *n*-heptane:2-propanol 80:20 + 0.1% TFA: 13.9 min (*S*) and 15.2 min (*R*).

1g: R^1, R^2 = Isatinyl: ^{1}H NMR (400 MHz, $CDCl_3$): 2.18 (s, 3H), 2.71 (br s, NH, 1H), 7.32 (dd, *J* = 7.6 and 7.6 Hz, 1H), 7.51 (dd, *J* = 8.0 and 8.0 Hz, 1H), 7.58 (d, *J* = 7.6 Hz, 1H), 8.28 (d, *J* = 8.4 Hz, 1H); ^{13}C NMR (400 MHz, $CDCl_3$): 20.2, 70.8, 112.7, 117.5 (C≡N), 120.9, 124.5, 126.5, 132.9, 140.7, 167.9 (C=O), 169.8 (C=O); elemental analysis calcd for $C_{11}H_8N_2O_3$: C, 61.1; H, 3.7; N, 12.9; found: C, 61.2; H, 3.9; N, 12.3; HRMS: *m*/*z* calcd for $C_{11}H_8N_2NaO_3$ $(M+Na)^+$ 239.0427, found 239.0428; MS: 216 (M^+), 188, 172, 157, 146, 118, 102, 90, 75, 63, 43, 40; HPLC: column: Chiralpak AD-H, flow: 0.5 mL/min, UV-detection: 215 nm, solvent: *n*-heptane:2-propanol 80:20 + 0.1% TFA: 13.5 min and 16.9 min.

1h: R^1 = 2-Thiophenyl, R^2 = Me: see Ref. [10].

1i: R^1 = 2-Furanyl, R^2 = Me: ^{1}H NMR ($CDCl_3$, 400 MHz): 2.08 (s, 3H), 2.10 (s, 3H), 6.42 (s, 1H), 6.66 (d, *J* = 3.0 Hz, 1H), 7.44 (s, 1H); ^{13}C NMR ($CDCl_3$, 400 MHz): 20.9, 24.8, 66.9, 110.4, 110.9, 116.8 (C≡N), 143.8, 148.1, 168.4 (C=O); elemental analysis calcd for $C_9H_9NO_3$: C, 60.3; H, 5.1; N, 7.8; found: C, 61.6; H, 5.3; N, 7.6; HRMS: *m*/*z* calcd for $C_9H_9NNaO_3$ $(M+Na)^+$ 202.0475, found 202.0475; MS: 179 (M^+), 164, 151, 137, 120, 110, 95, 76, 65, 43; GC: 140 °C: 1.62 min (*S*) and 1.67 min (*R*).

2.5. Synthesis of catalyst [(R, R)-salen)Ti(μ-O)]$_2$ (**4**)

The bimetallic Ti-salen complex **4** was synthesized according to literature procedures. Spectroscopic data are in agreement with those reported in literature [19,20]. $[\alpha]^{23}_D = -209$ (c 1.06, $CHCl_3$; lit [19] = −267 (c 0.0125, $CHCl_3$)).

2.6. General procedure for the synthesis of enantioenriched O-TMS-cyanohydrins (S)-3

The Ti(salen) catalyst **4** (0.5 mol%) was dissolved in dry DCM (1.5 mL) under N_2, then ketone (1 equiv.) and TMSCN (1.5 equiv.) were added. The mixture was stirred at RT for 24 h. The crude mixture was then filtered through a silica pad eluting with the solvents indicated for every product [19,21].

Synthesis of (*S*)-3b: According to the General Procedure, 182 mg (1.18 mmol) of 4-chloroacetophenone afforded 297 mg of **3b** (quantitative yield). Purification by flash chromatography was performed using heptane/EtOAc 5:1 as eluent. GC: 40 °C (20 min)–10 °C/min–250 °C (20 min): 33.42 min (major, *S*) and 33.49 min (minor, *R*); *ee* = 59%; $[\alpha]^{23}_D = -17.3$ (c 1.93, $CHCl_3$); **(*R*)-3b**: $[\alpha]^{25}_D = +29.5$ (*ee* 92%) [22]. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.21 (s, 9H), 1.85 (s, 3H), 7.39 (ABA′B′ system, *J* = 8.4 Hz, 2H), 7.49 (ABA′B′ system, *J* = 8.4 Hz, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 1.06 (3C), 33.52, 71.07, 121.25, 126.09 (2C), 128.84 (2C), 134.62, 140.73 ppm.

Synthesis of (*S*)-3c: According to the General Procedure, 240 mg (1.61 mmol) of 4-methoxyacetophenone afforded 281 mg of **3c** (70%). Purification by flash chromatography was performed using PE/EtOAc 8:2 as eluent. GC: 105 °C: 75.6 min (major, *S*) and 77.3 min (minor, *R*); *ee* = 46%; $[\alpha]^{23}_D = -9.21$ (c 1.61, $CHCl_3$); **(*R*)-3c**: $[\alpha]^{25}_D = +21.8$ (*ee* 89%) [23]. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.17 (s, 9H), 1.86 (s, 3H), 3.84 (s, 3H), 6.92 (ABA′B′ system, *J* = 8.8 Hz, 2H), 7.47 (ABA′B′ system, *J* = 8.8 Hz, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 1.09 (3C), 31.45, 55.35, 71.28, 113.87 (2C), 121.82, 126.06 (2C), 133.04, 159.77 ppm.

Synthesis of (*S*)-3d: According to the General Procedure, 365 mg (2.03 mmol) of 3,4-dimethoxyacetophenone afforded 396 mg of **3d** (70%). Purification by flash chromatography was performed

using PE/EtOAc 1:1 as eluent. GC: 40 °C (20 min)–10 °C/min–250 °C (20 min): 33.1 min (major, *S*) and 33.2 min (minor, *R*); *ee* = 19%; $[\alpha]^{23}_D = -6.13$ (c 1.26, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.18 (s, 9H), 1.87 (s, 3H), 3.91 (s, 3H), 3.92 (s, 3H), 6.87 (d, *J* = 8.4 Hz, 1H), 7.04 (s, 1H), 7.25 (d, *J* = 8.4 Hz, 1H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 1.07 (3C), 33.51, 55.93, 55.96, 71.41, 108.04, 110.81, 117.11, 121.72, 134.50, 149.02, 149.28 ppm.

Synthesis of (*S*)-3f: According to the General Procedure, 422 mg (2.03 mmol) of *trans*-chalcone afforded 584 mg of **3f** (94%). Purification by flash chromatography was performed using PE/EtOAc 1:1 as eluent. $[\alpha]^{23}_D = +5.93$ (c 1.30, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.27 (s, 9H), 6.22 (d, *J* = 15.8 Hz, 1H), 7.03 (d, *J* = 15.8 Hz, 1H), 7.62–7.23 (m, 10H) ppm. ^{13}C NMR (100 MHz): δ 1.31, 75.09, 119.70, 125.50 (2C), 127.09 (2C), 128.70 (2C), 128.75 (2C), 128.79, 128.89, 129.73, 130.93, 135.14, 140.39 ppm.

2.7. Synthesis of (S)-1b

202 mg (0.8 mmol) of **3b** were dissolved in acetonitrile (2 mL) under N_2. Acetic anhydride (150 μL, 2 equiv.) and scandium(III)triflate (4 mg, 0.011 equiv.) were added and the mixture was stirred at RT for 26 h. After removal of the solvent *in vacuo* the crude product was purified twice through flash chromatography using PE/EtOAc 8:2 as eluent to afford 109 mg of **1b** (61%). GC: 160 °C: 8.71 min (major, *S*) and 9.17 min (minor, *R*): *ee* = 58%: $[\alpha]^{23}_D = +6.7$ (c 1.49, $CHCl_3$). Analytical data as given for *rac*-**1b**.

2.8. Cleavage of the TMS-group

Synthesis of (*S*)-2c: 69 mg of **(*S*)-3c** were dissolved in 1 M HCl (3 mL) and the mixture was allowed to stir at RT for 16 h. The crude product was extracted with diethyl ether (3 × 10 mL), dried over sodium sulphate and evaporated under reduced pressure (63 mg, quantitative yield). **(*S*)-2c** was immediately acylated as described in Section 2.9.

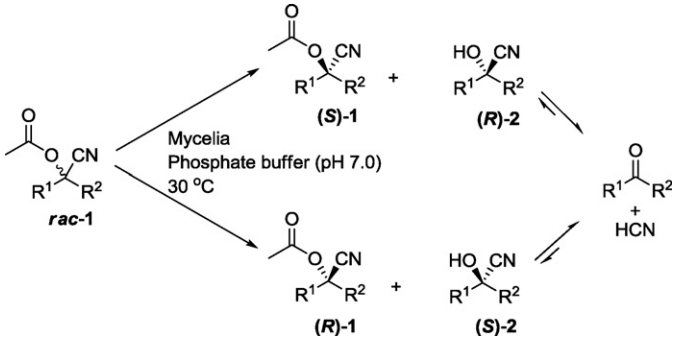
Synthesis of (*S*)-2d: 162 mg of **(*S*)-3d** were dissolved in 1 M HCl (3 mL) and the mixture allowed to stir at RT for 16 h. The crude product was extracted with diethyl ether (3 × 10 mL), dried over sodium sulphate and evaporated under reduced pressure (133 mg, quantitative yield). **(*S*)-2d** was immediately acylated as described in Section 2.9.

Synthesis of (*S*)-2f: 246 mg of **(*S*)-3f** were dissolved in MeOH (5 mL). 48% aqueous HF (67 μL, 2 equiv.) were added and the mixture was allowed to stir at RT for 16 h. A 3:1 DCM/water mixture (20 mL) was added. After stirring vigorously for 30 min, the phases were separated and the aqueous layer was extracted with DCM (3 × 15 mL). The organic layer was dried over sodium sulphate and evaporated under reduced pressure to afford **(*S*)-2f** (186 mg, 98%) as a yellow oil. The crude cyanohydrin was immediately acylated as described in Section 2.9.

2.9. General procedure for the acetylation of cyanohydrins

The crude cyanohydrin **2c**, **2d**, or **2f** (1 equiv.) was dissolved under nitrogen in dry DCM (5 mL). Pyridine (7 equiv.) and acetic anhydride (5 equiv.) were added and the mixture was stirred at RT until completion. The reaction mixture was then washed with 1 M HCl (3 × 10 mL), brine (1 × 10 mL) and then dried over sodium sulphate. Evaporation of the solvents under reduced pressure afforded the crude cyanohydrin acetate. No further purification was required.

(*S*)-1c: Following the General Procedure, **1c** (63 mg, 45% from **3c**) was obtained as a yellow oil. GC: 160 °C: 11.5 min (major, *S*) and 11.8 min (minor, *R*); *ee* = 27%; $[\alpha]^{23}_D = -1.37$ (c 1.14, $CHCl_3$). Analytical data as given for *rac*-**1c**.



Scheme 1. Hydrolytic kinetic resolution of ketone-derived cyanohydrin acetates catalysed by mycelia.

(*S*)-1d: Following the General Procedure, **1d** (125 mg, 83% from **3d**) was obtained as a yellow oil. GC: 160 °C: 19.9 min (major, *S*) and 20.2 min (minor, *R*); *ee* = 14%; $[\alpha]^{23}_D = -1.34$ (c 1.57, $CHCl_3$). Analytical data as given for *rac*-**1d**.

(*S*)-1f: Following the General Procedure, **1f** (151 mg, 75% from **3f**) was obtained as a yellow oil. HPLC: 13.8 min (major, *S*) and 15.2 min (minor, *R*); *ee* = 19%; $[\alpha]^{23}_D = +0.59$ (c 1.25, $CHCl_3$). Analytical data as given for *rac*-**1f**.

3. Results and discussion

The mycelia and other whole cells known for cell-bound carboxyl-esterase activity (the yeast *Kluyveromyces marxianus* and the bacterium *Bacillus coagulans*) were investigated as potential catalysts for the enantioselective hydrolysis of a novel class of *tert*-alcohol esters, namely α,α-disubstituted cyanohydrin acetates **1**. All of the different strains were tested either as lyophilized mycelia or freshly prepared mycelia grown on medium [12–16,18].

The hydrolytic kinetic resolutions were performed in a phosphate buffer (10 mM, pH 7.0) with a substrate concentration of 2 g/L at 30 °C (Scheme 1) analogous to our recently described screening of commercially available hydrolases [10]. Given the fact that the whole cells buffer the pH of the reaction mixture, the conversion could not be monitored by the addition of base. In addition, the products formed in the reaction, the free cyanohydrins **2a**–**i**, proved to be unstable and decomposed under the reaction conditions to their corresponding ketones, accompanied by the release of HCN (Scheme 1). Consequently, (*S*)-acetates are isolated for (*R*)-selective enzymes and *vice versa*. In order to measure the degree of conversion, *n*-dodecane and 1,3,5-triisopropylbenzene, respectively, were added as internal standards at the end of the reaction, but before the mixtures were subjected to work-up. As substrates a wide range of structurally diverse α,α-disubstituted cyanohydrin acetates **1a**–**i** were employed (Fig. 2). This allowed a full evaluation not only of the activity of the mycelia, but also of their substrate specificity.

All strains of the mycelium-bound carboxyl-esterases were found to be active towards most of the substrates, except the 2-furanyl derivative **1i**. In addition to activity, moderate to good selectivity was also observed. The *E*-values for the hydrolytic kinetic resolution were in the range 1–42 (Table 2), which is in the same range as reported earlier in the literature for *tert*-alcohols [10,11]. The highest selectivity was shown by almost all mycelia for the dimethoxy-substituted compound **1d** and for indole-2,3-dione derived cyanohydrin **1 g**. For these two substrates, all mycelia were found to be (*R*)-selective, leaving thus the (*S*)-acetate unchanged. For other substrates, a mild enantiopreference ($E < 4$) for the (*S*)-acetate was observed. The enantiopure cyanohydrin acetates were isolated by solvent extraction with low to moderate enantiomeric excesses (full details in Table 1). The differences between freshly

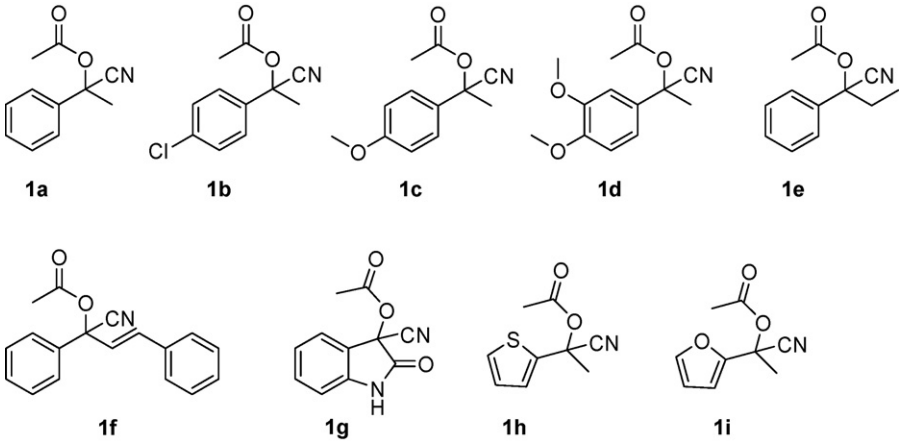


Fig. 2. Ketone-derived cyanohydrin acetates tested in the hydrolysis.

prepared and lyophilised *Kluyveromyces marxianus* as for activity and selectivity towards some substrates highlight the importance of the formulation for complex mixtures of enzymes such as whole cells. For example, the lyophilisation process may be responsible for the deactivation of some hydrolases.

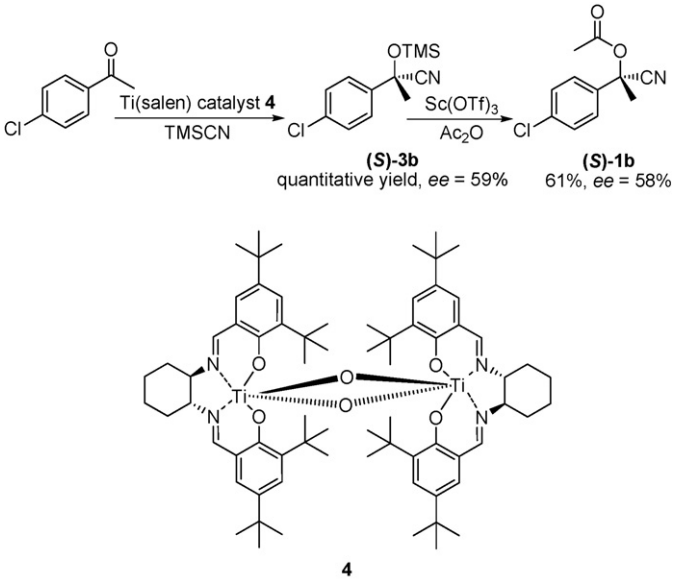
The enantioselectivity of mycelia towards compounds **1b**, **1c**, **1d**, and **1f** was determined by the chemical preparation of enantioenriched samples of these cyanohydrin acetates. In this way, the absolute configuration could be assigned to the enantiomer obtained *via* the kinetic resolution. The corresponding ketones were converted into their enantioenriched *O*-TMS-cyanohydrins (**3b**, **3c**, **3d**, and **3f**) by a Ti(salen)-complex mediated cyanosilylation [19]. Utilising the (*R*, *R*)-salen-bimetallic catalyst **4**, **(*S*)-3b** was prepared with an *ee* of 59% and an optical rotation $[\alpha]^{23}_{D}$ of −17.3. **(*R*)-3b** is known to have $[\alpha]^{25}_{D}$ = +29.5 with an *ee* of 92% [22]. Similarly, **(*S*)-3c** was obtained with an *ee* of 46% and an optical rotation $[\alpha]^{23}_{D}$ of −9.21, while **(*R*)-3c** is known to have $[\alpha]^{20}_{D}$ of +21.8 when *ee* = 89% [23]. Sc(III)triflate then catalysed the direct conversion of **(*S*)-3b** into the corresponding acetate, with retention of configuration [24] (Scheme 2).

The unknown TMS-cyanohydrins **3d** and **3f** were prepared in the same manner, assuming that the enantiopreference by the catalyst **4** towards the corresponding ketones was the same. This was furthermore confirmed upon conversion of the enantioenriched silyl derivatives **3c** and **3d** into their acetates. When analyzed by chiral GC with the same method, **1b**, **1c**, and **1d** all showed the same order of elution, the major (*S*)-enantiomer eluting first. This was also the case for **1a**, the absolute stereochemistry of which was determined prior to this study [10]. It is noteworthy that direct acetylation must be replaced by a two step procedure (cleavage of TMS, acetylation) for derivatives **3c**, **3d**, and **3f** because they all underwent Lewis acid catalysed rearrangements or deprotection

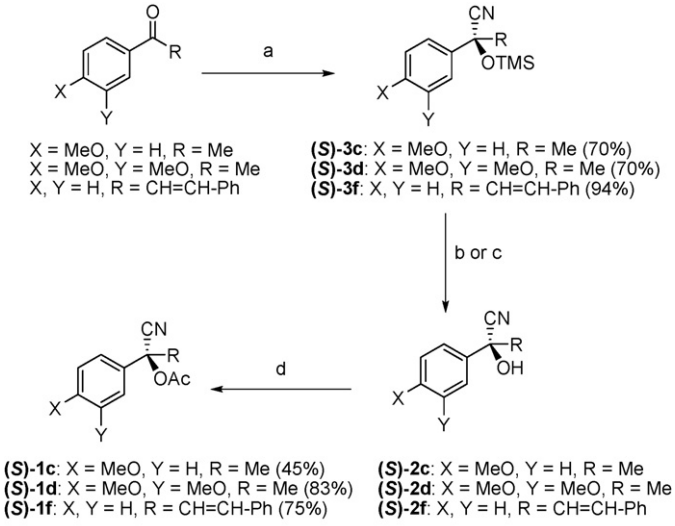
Table 2
Activity towards the different substrates, selectivity and *E*-values for hydrolytic kinetic resolutions of α,α-disubstituted cyanohydrin acetates catalysed by mycelia.

Mycelium	Activity	Selectivity, $E^{a,b}$
Rhizopus oryzae	**1a–e**, **1g–h**	**1g**, 9
Rhizopus javanicus	**1a–h**	**1d**, 5.5 (*S*), **1g**, 37
Rhizopus liquefaciens	**1a–h**	**1d**, 5 (*S*)
Aspergillus oryzae	**1a–h**	**1d**, 5 (*S*), **1g**, 7
Kluyveromyces marxianus (lyophilized)	**1a**, **1d**, **1f–h**	**1d**, **23.5** (*S*), **1g**, 42
Kluyveromyces marxianus (freshly prepared)	**1d**, **1f**	–
Bacillus coagulans	**1a–d**, **1f–h**	–

[a] Only *E*-values above 4 are reported. All details are given in Table 1.
[b] In brackets the absolute configuration of the isolated cyanohydrin acetate.



Scheme 2. Preparation of (*S*)-**1b**.



Scheme 3. Synthesis of (*S*)-**1c,d,f**.

in the presence of Sc(III)triflate (Scheme 3). No enantioselective synthesis was available for compound **1 g**.

4. Conclusion

A novel source of enzymes, carboxyl-esterases from various microbial strains, has been investigated in the hydrolytic kinetic resolution of a new type of *tert*-alcohols, α,α-disubstituted cyanohydrin acetates. All seven strains tested proved to be both active and selective towards these *tert*-alcohols, with the exception of **1i**, giving *E*-values up to 42 as the best result. Furthermore, the most enantioselective mycelia proved to be (*R*)-selective towards the tested substrates. The enzymatic toolbox to access sterically congested enantiopure cyanohydrins has thus been expanded.

Acknowledgements

JH acknowledges the National Research School Combination Catalysis (NRSC-C) for financial support. MP acknowledges the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) and IBOS (Integration of Biosynthesis and Organic Synthesis, project no. 053.63.304) for financial support. DR would like to acknowledge a TU Delft "Junior Researcher Fellowship".

References

[1] T. Purkathofer, W. Skranc, C. Schuster, H. Griengl, Appl. Microbiol. Biotechnol. 76 (2007) 309–320.
[2] J.-M. Brunel, I.P. Holmes, Angew. Chem. Int. Ed. 43 (2004) 2752–2778.
[3] J. Holt, U. Hanefeld, Curr. Org. Synth. 6 (2009) 15–37.
[4] M. North, Tetrahedron: Asymmetry 14 (2003) 147–176.
[5] D.T. Mowry, Chem. Rev. 42 (1948) 189–283.
[6] R. Kourist, P.D. de Maria, U.T. Bornscheuer, ChemBioChem 9 (2008) 491–498.
[7] K. Konigsberger, K. Prasad, O. Repic, Tetrahedron: Asymmetry 10 (1999) 679–687.
[8] H. Ohta, Y. Kimura, Y. Sugano, Tetrahedron Lett. 29 (1988) 6957–6960.
[9] H. Ohta, Y. Kimura, Y. Sugano, T. Sugai, Tetrahedron 45 (1989) 5469–5476.
[10] J. Holt, I.W.C.E. Arends, A.J. Minnard, U. Hanefeld, Adv. Synth. Catal. 349 (2007) 1341–1344.
[11] M. Wiggers, J. Holt, R. Kourist, S. Bartsch, I.W.C.E. Arends, A.J. Minnaard, U.T. Bornscheuer, U. Hanefeld, J. Mol. Catal. B: Enz. 60 (2009) 82–86.
[12] F. Molinari, K.S. Cavenago, A. Romano, D. Romano, R. Gandolfi, Tetrahedron: Asymmetry 15 (2004) 1945–1947.
[13] A. Romano, R. Gandolfi, F. Molinari, A. Converti, M. Zilli, M. Del Borghi, Enz. Microbiol. Technol. 36 (2005) 432–438.
[14] A. Romano, D. Romano, F. Molinari, R. Gandolfi, F. Costantino, Tetrahedron: Asymmetry 16 (2005) 3279–3282.
[15] D. Romano, F. Falcioni, D. Mora, F. Molinari, A. Buthe, M. Ansorge-Schumacher, Tetrahedron: Asymmetry 16 (2005) 841–845.
[16] D. Romano, V. Ferrario, F. Molinari, L. Gardossi, J.M. Sanchez-Montero, P. Torre, A. Converti, J. Mol. Catal. B: Enz. 41 (2006) 71–74.
[17] U. Hanefeld, L. Gardossi, E. Magner, Chem. Soc. Rev. 38 (2009) 453–468.
[18] A. Converti, R. Gandolfi, M. Zilli, F. Molinari, L. Binagli, P. Perego, M. Del Borghi, Appl. Microbiol. Biotechnol. 67 (2005) 637–640.
[19] Y.N. Belokon, S. Caveda-Cepas, B. Green, N.S. Ikonnikov, V.N. Krustalev, V.S. Larichev, M.A. Moscalenko, M. North, C. Orizu, V.I. Tararov, M. Tasinazzo, G.I. Timofeeva, L.V. Yashkina, J. Am. Chem. Soc. 121 (1999) 3968–3973.
[20] J.F. Larrow, E.N. Jacobsen, Y. Gao, Y. Hong, X. Nie, C.M. Zepp, J. Org. Chem. 59 (1994) 1939–1942.
[21] Y.K. Belokon, B. Green, N.S. Ikonnikov, M. North, T. Parsons, V.I. Tararov, Tetrahedron Lett. 40 (1999) 8147–8150.
[22] Y. Hamashima, M. Kanai, M. Shibasaki, J. Am. Chem. Soc. 122 (2000) 7412–7413.
[23] K. Shen, X. Liu, Q. Li, X. Feng, Tetrahedron 64 (2008) 147–153.
[24] S. Norsikian, I. Holmes, F. Lagasse, H.B. Kagan, Tetrahedron Lett. 43 (2002) 5715–5717.