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Microbial-catalysed resolution of sterically demanding cyanohydrins

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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.

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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, α,α-disubstituted cyanohydrin acetates 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.
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 (1R, 2R)-(−)-1,2-diaminocyclohexane (98%, Sigma–Aldrich) were used as received. Racemic α,α-disubstituted cyanhydrin acetates were prepared according to established procedures by cyanation, deprotection and acetylation [10].
  - The reactions were run on a scale of 2 g/L (substrate concentration).
  - Cells (400 mg) and substrate (4 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.

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).
- Kluveromyces marxianus CBS 1553 (Central Bureau voor Schimmelscultures, Utrecht, The Netherlands).

**Kluveromyces 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 rpm).

2.2. Spectroscopic data for α,α-disubstituted cyanhydrin acetates

1a: R₁ = Ph, R₂ = Me: see Ref. [10].
1b: R₁ = 4-Cl–C₆H₄, R₂ = Me: 1H NMR (CDCl₃, 300 MHz): 1.98 (s, 3H), 2.13 (s, 3H), 7.36–7.49 (m, 4H); 13C NMR (CDCl₃, 300 MHz): 20.9, 29.6, 72.7, 117.8 (C₆), 126.0 (2C), 129.2 (2C), 135.2, 136.8, 168.3 (C=O); MS: 225/223 (M⁺), 232, 210, 207, 190, 165, 138, 119, 104, 89, 77, 63, 43, 40.
1c: R₁ = 4-Me–C₆H₄, R₂ = Me: see Ref. [10].
1d: R₁ = 3,4-di-O-Me–C₆H₄, R₂ = Me: 1H NMR (400 MHz, CDCl₃): 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); 13C NMR (400 MHz, CDCl₃): 21.1, 31.0, 56.0, 56.1, 73.1, 108.1, 111.1, 117.3 (C₆), 118.3, 130.5, 149.2, 149.7, 168.4 (C=O); elemental analysis calcd for C₁₃H₁₄N₂O₄: C, 62.6; H, 6.1; N, 5.6; found: C, 62.8; H, 6.2; N, 5.5.
2.3. General procedure for the hydrolytic kinetic resolution

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, Kluveromyces marxianus.

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

1a: R₁ = Ph, R₂ = Me: see Ref. [10].
1b: R₁ = 4-Cl–C₆H₄, R₂ = Me: 1H NMR (CDCl₃, 300 MHz): 1.98 (s, 3H), 2.13 (s, 3H), 7.36–7.49 (m, 4H); 13C NMR (CDCl₃, 300 MHz): 20.9, 29.6, 72.7, 117.8 (C₆), 126.0 (2C), 129.2 (2C), 135.2, 136.8, 168.3 (C=O); MS: 225/223 (M⁺), 232, 210, 207, 190, 165, 138, 119, 104, 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: 13.4 min (R) and 14.6 min (S).

1e: R1 = Ph, R2 = Et: see Ref. [10].

1f: R1 = Ph, R2 = CH=CH=Ph: 1H NMR (CDCl3, 300 MHz): 2.17 (s, 3H, CH3), 6.33 (d, J = 15.9 Hz, 1H), 6.94 (d, J = 15.9 Hz, 1H), 7.29–7.61 (m, 10H, ArH); 13C NMR (CDCl3, 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 C18H15NO2: C, 77.9; H, 5.4; N, 5.0; found: C, 77.5; H, 5.5; N, 4.4; HRMS: m/z calcd for C9H9NNaO3 (M+Na)+ 239.0427, found 239.0428; GC: 40 ◦C/min–250 ◦C (20 min): 33.42 min (major, 1.06); 17.3 (c 1.93, CHCl3).
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%; [α]D \text{2d} ^{20} = -6.13 (c 1.26, CHCl3). \text{1H NMR} (400 MHz, CDCl3): \text{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. \text{13C NMR} (100 MHz, CDCl3): \text{δ} 1.07 (3C), 33.51, 55.93, 55.96, 71.41, 108.04, 110.81, 111.71, 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. \text{[α]}D ^{20} = +5.93 (c 1.30, CHCl3). \text{1H NMR} (400 MHz, CDCl3): \text{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. \text{13C NMR} (100 MHz): \text{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 N2. 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%; \text{[α]}D ^{20} = +6.7 (c 1.49, CHCl3). 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%; \text{[α]}D ^{20} = -1.37 (c 1.14, CHCl3). Analytical data as given for rac-1c.

(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%; \text{[α]}D ^{20} = -1.34 (c 1.57, CHCl3). 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%; \text{[α]}D ^{20} = +0.59 (c 1.25, CHCl3). 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 hydrodases [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 – disubstituted cyanohydrin acetates are isolated for (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-trisopropylbenzene, 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 1–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-estersases were found to be active towards most of the substrates, except the 2-furanyl derivative 11. 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 1g. 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 enantipure cyanohydrin acetates were isolated by solvent extraction with low to moderate enantiomeric excesses (full details in Table 1). The differences between freshly
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 hydrolys.

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 [α]_D^23 of -17.3. (R)-3b is known to have [α]_D^23 of +29.5 with an ee of 92% [22]. Similarly, (S)-3c was obtained with an ee of 46% and an optical rotation [α]_D^23 of -9.21, while (R)-3c is known to have [α]_D^23 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 enantipreference 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>
<thead>
<tr>
<th>Mycelium</th>
<th>Activity</th>
<th>Selectivity, E^a,b</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhizopus oryzae</em></td>
<td>1a–e, 1g–h</td>
<td>1g, 9</td>
</tr>
<tr>
<td><em>Rhizopus javanicus</em></td>
<td>1a–f</td>
<td>1d, 5.5 (S), 1g, 37</td>
</tr>
<tr>
<td><em>Rhizopus liquefaciens</em></td>
<td>1a–f</td>
<td>1d, 5 (S)</td>
</tr>
<tr>
<td><em>Aspergillus oryzae</em></td>
<td>1a–h</td>
<td>1d, 5 (S), 1g, 7</td>
</tr>
<tr>
<td><em>Kluyveromyces marxianus</em> (lyophilized)</td>
<td>1a, 1d, 1f–h</td>
<td>1d, 23.5 (S), 1g, 42</td>
</tr>
<tr>
<td><em>Kluyveromyces marxianus</em> (freshly prepared)</td>
<td>1d, 1f</td>
<td>–</td>
</tr>
<tr>
<td><em>Bacillus coagulans</em></td>
<td>1a–d, 1f–h</td>
<td>–</td>
</tr>
</tbody>
</table>

^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.

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### Table 2

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

<table>
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<td>–</td>
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<td><em>Bacillus coagulans</em></td>
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<td>–</td>
</tr>
</tbody>
</table>

^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.

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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 1g.

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.

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