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The combi-CLEA approach: enzymatic cascade synthesis of enantiomerically pure (*S*)-mandelic acid

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ABSTRACT

Enantiomerically pure (*S*)-mandelic acid was synthesised from benzaldehyde by sequential hydrocyanation and hydrolysis in a bienzymatic cascade at starting concentrations up to 0.25 M. A cross-linked enzyme aggregate (CLEA) composed of the (*S*)-selective oxynitrilase from *Manihot esculenta* and the non-selective nitrilase from *Pseudomonas fluorescens* EBC 191 was employed as the biocatalyst. The nitrilase produces approx. equal amounts of (*S*)-mandelic acid and (*S*)-mandelic amide from (*S*)-mandelonitrile under standard conditions, but we surprisingly found that high (up to 0.5 M) concentrations of HCN induced a marked drift towards amide production. By including the amidase from *Rhodococcus erythopolis* in the CLEA we obtained (*S*)-mandelic acid as the sole product in 90% yield and >99% enantiomeric purity.

1. Introduction

Cascade conversion—combined (catalytic) procedures without recovery of intermediate products—is considered an important future methodology for reducing the environmental footprint of organic synthesis.[1] Industrial chemoenzymatic synthesis of enantiomerically pure 2-hydroxycarboxylic acids[2] (see Fig. 1) presents an interesting example within this context. Route A involves enzymatic synthesis of the enantiomerically pure (*R*)- or (*S*)-cyanohydrin, employing the appropriate hydroxynitrile lyase (HnL, oxynitrilase, E.C. 4.1.2.10), followed by hydrolysis with strong acid.[3] Alternatively (route B), a dynamic kinetic resolution of the chemically synthesised cyanohydrin in the presence of an enantioselective nitrilase (NLase, E.C. 3.5.5.1) can be employed.

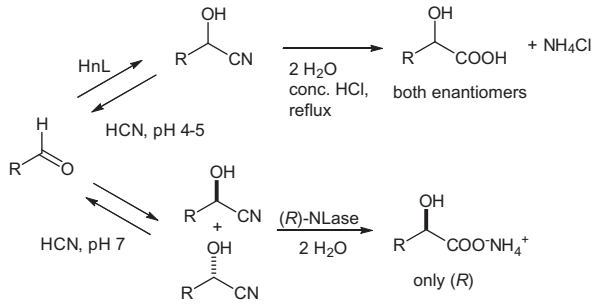

Figure 1. Synthetic routes to enantiomerically pure 2-hydroxyacids, via hydroxynitrile lyase (HnL, oxynitrilase) catalysed enantioselective hydrocyanation (route A) or, alternatively, dynamic kinetic resolution mediated by an (*R*)-specific nitrilase (NLase, route B).

Both routes suffer from limitations and disadvantages. In view of the sustainability issues it is worth noting that route A generates copious quantities of salt. It is not compatible, moreover, with sensitive functional groups, due to the harsh hydrolysis conditions. Route B, which is quite efficient and is industrially employed in the multiton-scale industrial synthesis of (*R*)-mandelic acid,[4] is restricted to (*R*)-2-hydroxyacids since, so far, no NLase with preference for (*S*)-cyanohydrins has been identified.[5]

Combining the enzymatic steps—hydrocyanation and nitrile hydrolysis—into a bienzymatic cascade is particularly advantageous in the synthesis of (*S*)-2-hydroxyacids and would avoid the weaknesses of route A—salt production and incompatibility with hydrolysable groups. As an additional bonus, the cascade would lessen (but not entirely obviate) concerns with regard to racemisation of the cyanohydrin and would pull an incomplete hydrocyanation equilibrium towards complete conversion.

An efficient cascade requires any potential incompatibilities with regard to pH and reaction medium to be resolved; as will become clear, such issues are particularly relevant here. HnL mediated hydrocyanation, on the one hand, is preferably carried out at pH <5 to suppress the competing spontaneous hydrocyanation.[6,7] It is common practice, moreover, to conduct enzymatic hydrocyanation

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in biphasic aqueous-organic[6] or micro-aqueous medium[8] to reduce the background reaction even further. Most NLases, on the other hand, have an optimum pH of 7–9 and do not tolerate organic solvents well.[9]

These incompatibilities can be solved by immobilising a suitable HnL and NLase as cross-linked enzyme aggregates (CLEAs),[10] preferably in a combi-CLEA, as we have demonstrated in our proof-of-principle paper.[11] Alternatively, a whole-cell biocatalyst expressing both enzymes may be employed.[12] High conversions and near-quantitative enantioselectivities were demonstrated with the combi-CLEA but from the synthetic viewpoint the reactant concentrations were rather low. Increasing these to >0.1 M would be desirable but may not be trivial, since NLase inhibition by aldehydes and HCN has been reported.[12b,13] A further shortcoming of the cascade procedure, as originally published, is the formation of amide as a major side product,[11,14] which detracts from the green as well as the practical value of the procedure.

In the present Letter we will report the application of the combi-CLEA approach to the cascade synthesis of (*S*)-mandelic acid at synthetically relevant concentrations and address the formation of amide side product as well.

2. Results and discussion

Our research was focused on the cascade synthesis of (*S*)-mandelic acid, as it is a more challenging target than the (*R*)-enantiomer, which is already produced in an efficient chemoenzymatic procedure.[4] The HnL from *Manihot esculenta* (MeHnl) was the obvious choice as (*S*)-selective hydrocyanation biocatalyst, on account of its operational stability[8c] and easy availability. A survey of readily available NLases revealed that only the NLase from *Pseudomonas fluorescens* EBC191[15] (PfNLase) converted (*R*)- and (*S*)-mandelonitrile at comparable rates[16,17] whereas other NLases were (*R*)-selective to a varying extent.[5,17] PfNLase was also exceptional in maintaining activity down to pH 5.5[16,17] and is obviously the enzyme of choice for our purpose. Since proper immobilisation is a crucial success factor, we set out to investigate the immobilisation of MeHnL and PfNLase in a combi-CLEA.

2.1. Preparing a combi-CLEA

A cross-linked enzyme aggregate of MeHnL and PfNLase was prepared using the protocol developed for the NLase,[10c] which involves precipitation with 1,2-dimethoxyethane and cross-linking with dextran polyaldehyde, followed by reduction with sodium borohydride. The relative amounts of MeHnL and PfNLase in the CLEA were based on experiments with separate biocatalysts.[11] The activity recoveries of the enzymes were measured separately (see Table 2). We used synthetic rather than—more convenient—photometric assays because the outcome of these may be too low, due to diffusion limitation in the CLEA at photometric reactant concentrations.[8c] 57% Of the HnL starting activity was recovered in the combi-CLEA, using a hydrocyanation assay of *trans*-cinnamic aldehyde.[8c,18] This outcome is much lower than the 93% recovery that we achieved using an optimised CLEA protocol,[8c] but here our objective was to maintain the activity of the more sensitive NLase.[10c] Indeed, 90% of the NLase starting activity was recovered in the combi-CLEA, which is important to avoid accumulation of cyanohydrin in the reaction mixture.

2.2. Cascade synthesis of (*S*)-mandelic acid

Preliminary experiments have been performed with 10 mM benzaldehyde (**1**, see Fig. 2) starting concentration and a fourfold excess of HCN[11] to reduce enzymatic dehydrocyanation to a minimum.[8c] These reactions were performed at pH 5.5 as a compromise between maintaining the enantiomeric purity of the HCN adduct (**2**) and NLase activity.[11,16] (*S*)-Mandelic acid ((*S*)-**3**) was produced with high enantiomeric purity (cf. Table 1). Significant amounts of (*S*)-mandelic amide ((*S*)-**4**) were also formed and we have previously shown that amide formation from (*S*)-**2** is a major side-reaction in the presence of PfNLase, whereas it is barely significant with (*R*)-**2**.[14] This amide side-product formation, which severely detracts from the green as well as the practical value of the procedure, will be addressed below.

Table 1
Cascade synthesis of (*S*)-mandelic acid[a]

1 (mM)	HCN (mM)	Time (h)	**2** (mM)	**3** (mM)	ee (%)	**4** (mM)
10	50	1.6	<0.2	6.0	99	4.0
25	125	1.6	<1	14	99	10
42	210	1.6	<1	23	96	18
83	415	1.6	8	36	96	40
250	750	2.0	<1	111	96	155

[a] Reaction conditions: combi-CLEA prepared from MeHnL (14.1 mg) and semi-purified PfNLase (4.8 mg) in 20 mM citrate buffer pH 5.5 (2 mL); **1** and HCN as appropriate in diisopropyl ether (4.7 mL) at 25 °C. Product concentrations are from HPLC analysis.

The operational stability of the combi-CLEA was assessed in a wash-and-reuse cycle. The recovered enzymatic activities in the CLEA (see Fig. 3) were calculated from the reaction progress. An activity loss was observed after the fourth cycle, which is tentatively ascribed to mechanical losses as the biocatalyst particles became difficult to spin down at this point.

Fast and nearly complete conversion of **1** and **2** was still observed when the starting concentration of **1** was increased to the synthetically more relevant value of 0.25 M and the enantiomeric purity of the products was ⩾96% (see Table 1). As before, the reactions were performed in a biphasic aqueous-organic medium to suppress the uncatalysed hydrocyanation while preventing precipitation of the products.

It became apparent from the results in Table 1 that the yield of (*S*)-**4** increased at higher starting concentrations, from 40% at 10 mM **1** to 58% at 0.25 M, which came to us as a surprise but was also observed with a whole cell biocatalyst.[12b] One could surmise, by analogy with the pH effects on the hydrolysis of phenylacetonitrile,[14] that the high final concentration of **3** induces an upward pH shift.[19] If this were the case, the enantiomeric purity of the products would be expected to suffer, as it is highly sensitive to small pH changes.[11] Since this was not observed, it would rather

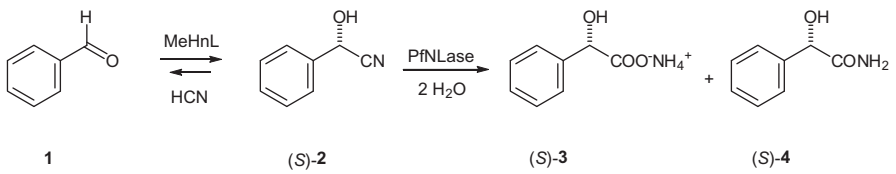

Figure 2. Bienzymatic procedure for the synthesis of (*S*)-2-mandelic acid (**3**), using the (*S*)-specific MeHnL and the non-specific PfNLase in tandem.

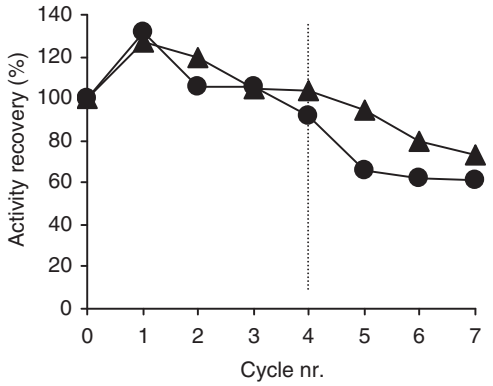

Figure 3. Activity recovery of MeHnL (▲) and PfNLase (●) in the combi-CLEA upon recycling and reuse. The CLEA particles resisted spinning down at Cycle nr. >4.

seem that an inhibitory effect of the reactants is involved (see later).

2.3. Effects of high reactant concentrations on PfNLase

The shifting acid/amide ratio mentioned above prompted us to investigate the possible inhibition or deactivation of PfNLase by high reactant concentrations in some detail. It is worth noting here that inhibition by aldehydes and HCN of PfNLase[12b] as well as the NLase from *Alcaligenes faecalis*[13] has been reported.

Exposing the combi-CLEA to 10 mM **1** for 1 h resulted in a slight (10%) loss of NLase activity, increasing to 20% activity loss at 250–500 mM **1**, as measured in the hydrolysis of phenylacetonitrile (data not shown). Hence, it would seem that **1** has little intrinsic effect on PfNLase if protected in a combi-CLEA.

HCN presented a more complicated picture as a low (25 mM) concentration had little effect, whereas 125 mM HCN after 1 h of preincubation caused 45% activity loss in the NLase (see Fig. 4a). At 450 mM HCN deactivation was nearly complete after 1 h of preincubation and could not be recovered by washing with aqueous buffer. When the residual activity was measured over time, it became clear that free PfNLase was deactivated faster than the combi-CLEA (Fig. 4b).

The effects of HCN on the activity and selectivity of PfNLase were investigated in some more detail in the hydrolysis of (*S*)-**2**. Reactions were performed in the presence of a PfNLase CLEA with 0–400 mM added HCN, using the same biphasic system that was employed in the cascade synthesis. An effect on the reaction rate became apparent (Fig. 5a), but complete deactivation was not observed, even after >2 h exposure to 400 mM HCN. It would seem, hence, that (*S*)-**2** partially protects the enzyme against HCN-induced deactivation.

We also observed (Fig. 5b) that amide production became more prominent at increasing HCN concentrations, in agreement with our results presented in Table 1 and Ref. 12b. Hence, we confidently conclude that the selectivity drift in the cascade synthesis is due to interference of HCN with the NLase. Whether the phenomenon is general with NLases and a wider range of nitrile substrates will be the subject of future research.

2.4. In situ amide hydrolysis

The trend, noted above, towards increased amide production at synthetically relevant concentrations prompted us to design a solution. A fairly obvious approach is to conduct the reactions in the presence of an amidase and convert (*S*)-**4** into the desired product (*S*)-**3**. Preliminary tests showed that racemic **4** was completely hydrolysed in the presence of penicillin acylase as well as the amidase from *Rhodococcus erythopolis* MP50[20,21] (RheAMase). Both enzymes, when added to the cascade reaction mixture, accordingly mediated the in situ hydrolysis of all **4** into **3**, demonstrating their compatibility with the reaction conditions. The lack of enantioselectivity, implicated by the test results, is an important aspect when considering the possible application of the cascade method to (*R*)- as well as (*S*)-hydroxy acids.[17] A similar reasoning prompted us to focus the further development on RheAMase on account of its relaxed substrate specificity,[20] in contrast to penicillin acylase, which is restricted to phenylacetic acid derivatives.

2.5. Optimising precipitation and cross-linking

Precipitation of RheAMase with a range of common agents,[8c] followed by redissolution in aqueous buffer, revealed that the activity recovery was acceptable only with acetonitrile, 1,2-dimethoxyethane, *tert*-butyl alcohol and saturated aqueous ammonium sulfate. Subsequently, the newly formed aggregates were incubated in the precipitant for up to 20 h and sampled at appropriate intervals. With ammonium sulfate the activity recovery upon redissolution of the samples was a constant 45% of the starting activity, whereas in the other media a nearly complete loss of activity after 2 h of exposure was observed. Hence, ammonium sulfate was the precipitant of choice for the preparation of the amidase CLEA.

These results made it necessary to adapt the procedure for preparing a CLEA of MeHnL and PfNLase. An incubation/redissolution test as described above showed excellent activity retention of MeHnL, whereas aggregates of PfNLase rapidly lost activity upon >2 h incubation in saturated ammonium sulfate. These data,

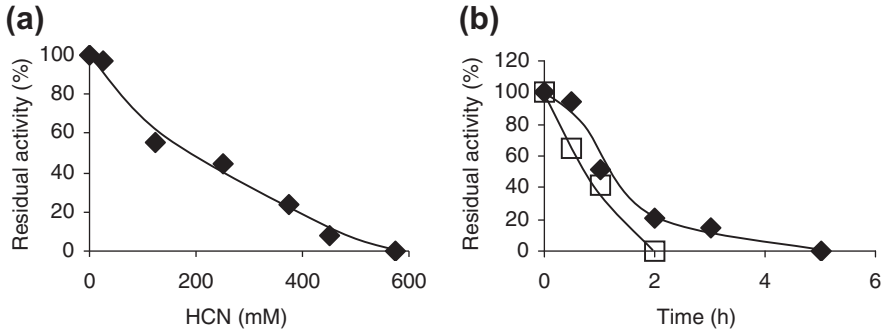

Figure 4. Residual activity of PfNLase upon exposure to HCN in the hydrolysis of phenylacetonitrile; (a) CLEA, 1 h incubation; (b) effects of 125 mM HCN versus time on the residual activity; CLEA (♦), free enzyme (□).

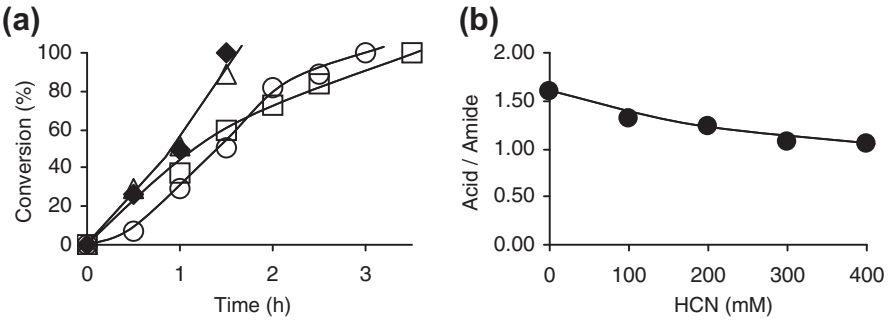

Figure 5. Effects of HCN on the hydrolysis of 10 mM (*S*)-2 in the presence of PfNLase CLEA in 70:30 DIPE-buffer pH 5.5 at rt; (a) time-course, HCN 0 mM (♦), 200 mM (△), 300 mM (○), 400 mM (□); (b) acid/amide ratio versus HCN concentration.

although somewhat disappointing, served as a basis for the cross-linking experiments.

Single enzymes (MeHnL, PfNLases and RheAMase) were aggregated with saturated ammonium sulfate solution and subsequently cross-linked with dextran polyaldehyde solution at various concentrations for 2–23 h. Schiff's base bonds in the cross-links were made permanent by reduction with cyanoboro hydride, which is required by the NLase.[10c] We found that cross-linking times should be limited to 2 h, since the activity recovery (see Fig. 6a–c) of all three enzymes suffered when it was prolonged. MeHnL and PfNLase showed little tendency to leach, as shown by the minute activity recovery from the supernatants. RheAMase, in contrast, required a dextran polyaldehyde concentration of 3 mg mL^{-1} to suppress leaching (see Fig. 6d). At this concentration the recovery of NLase and amidase activity (>70%) was good while the activity recovery of MeHnL (40%) was acceptable.

2.6. A triple CLEA

Rational optimisation of the protocol for a triple CLEA of MeHnL, PfNLase and RheAMase requires enzyme-specific non-spectrophotometric assay techniques. More specifically, the HCN addition product resulting from the HnL assay should not be consumed by the NLase, and the NLase assay substrate should not be prone to dehydrocyanation; neither should it produce an amide.

The MeHnL in the triple CLEA was assayed in a synthetic hydrocyanation of *trans*-cinnamic aldehyde, as before.[18] Phenylacetonitrile was adopted as the NLase assay substrate since it is not sensitive to dehydrocyanation, neither is amide produced in the presence of PfNLase. The AMase was assayed in the hydrolysis of **4**. A triple CLEA was prepared by successively precipitating MeHnL, PfNLase and RheAMase in saturated ammonium sulfate, followed by cross-linking with 2.5 mg mL^{-1} of dextran polyaldehyde and subsequent cyanoborohydride reduction. The activity recovery was 45% (MeHnL), 58% (PfNLase) and 57% (RheAMase).

Our common practice, up to here, was to store the CLEAs in the reaction buffer (20 mM citrate buffer pH 5.5) ready for immediate use. Monitoring the activity of the enzymes in the CLEA revealed this practice to be far from optimal, although acceptable for storage over a few days (see Fig. 7a). MeHnL in the CLEA rapidly lost 35% of the starting activity but the deactivation of PfNLase upon storage at pH 5.5 for >10 d was particularly serious. We fortunately found that the precipitation medium, saturated ammonium sulfate, is also the optimum storage medium in which the activity retention

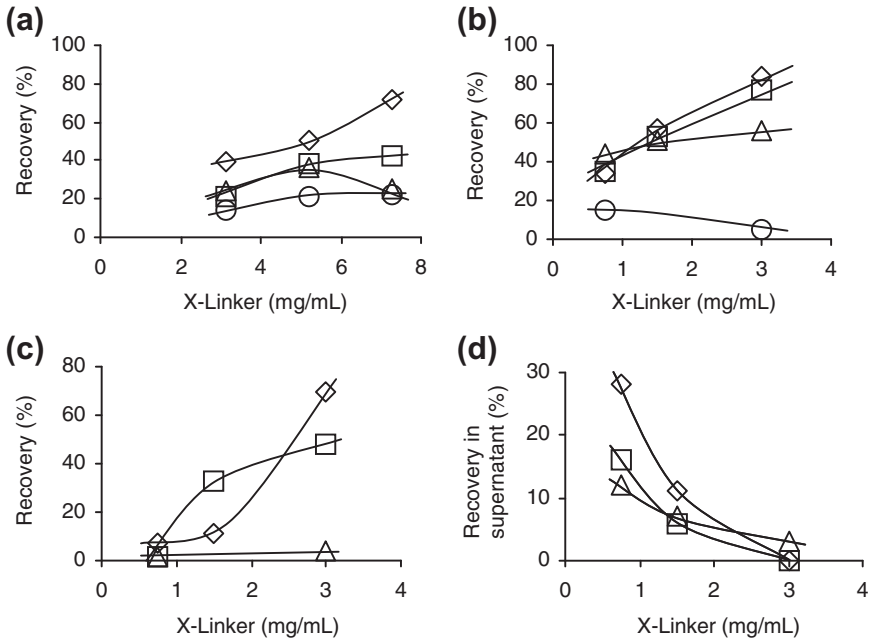

Figure 6. Effects of the dextran polyaldehyde concentration and cross-linking time on the activity recovery in the CLEAs; (a) MeHnL, (b) PfNLase, (c) RheAMase, (d) leaching of RheAMase activity from the CLEA. Cross-linking time: 2 h (◇), 4 h (□), 6 h (△), 23 h (○).

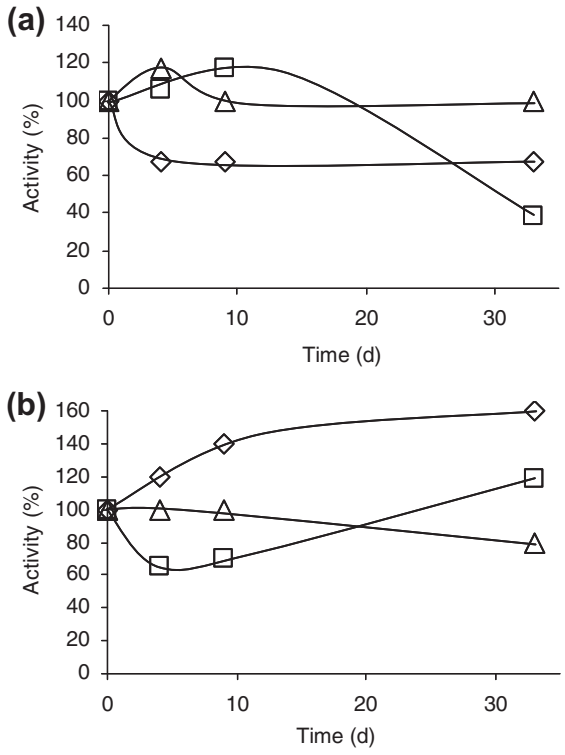

Figure 7. Storage stability of MeHnL (◇), PfNLase (□), RheAMase (△) in the triple-CLEA at 0 °C; (a) citrate buffer pH 5.5; (b) saturated $(NH_4)_2SO_4$.

is satisfactory over one month (Fig. 7b). Remarkably, CLEA-stabilised PfNLase was stable in saturated ammonium sulfate, whereas non-crosslinked aggregates rapidly lost activity (see above).

Hydrocyanation of **1** in the presence of the triple CLEA resulted in a near-quantitative conversion into (*S*)-mandelic acid. Some transient accumulation of **4** was observed (see Fig. 8 for comparison with the two-enzyme-CLEA), which required a somewhat prolonged reaction time but >99% enantiomeric purity of the product was maintained nevertheless.

3. Conclusion

Enantiomerically pure (*S*)-mandelic acid was produced from benzaldehyde at 0.25 M starting concentration and HCN, using a bienzymatic cascade employing a combi-CLEA of *Manihot esculenta* hydroxynitrile lyase and the nitrilase from *Pseudomonas fluorescens* EBC191. The conversion was fast and complete but the production of large amounts of (*S*)-mandelic amide is a major drawback. This latter problem was convincingly solved by including an amidase in the CLEA, which proved that employing a triple CLEA in synthetic biocatalysis is a viable option. We surprisingly found that the selectivity of the nitrilase drifts towards amide at high HCN concentrations.

4. Experimental section

4.1. Materials and methods

Semi-purified (*S*)-hydroxynitrile lyase from *M. esculenta* (MeHnL, E.C. 4.1.2.10, protein content 88 mg mL^{-1}) was obtained from Jülich Fine Chemicals (Jülich, Germany), which has terminated delivery. Soluble MeHnL is available from Sigma-Aldrich and ASA Spezialenzyme GmbH (URL: http://www.asa-enzyme.de). MeHnL CLEA was received from CLEA Technologies B.V. as a gift. Nitrilase from *P. fluorescens* EBC191 (PfNLase, E.C. 3.5.5.1), heterologously expressed in *Escherichia coli* JM109, was obtained as described.[16] The cell-free extract contained 34 mg mL^{-1} of protein. Amidase from *Rhodococcus erthropolis* MP50 (E.C. 3.5.1.4) was obtained from heterologous expression in *E. coli* as described.[21]

Phenylacetonitrile and phenylacetic acid were obtained from Merck-Schuchardt, (±)-mandelic amide 97% from Alfa Aesar; (*R,S*)-mandelic acid and *trans*-cinnamic aldehyde +99% were from Acros Organics; benzoic acid, 1,2-dimethoxybenzene +99% and di-isopropylether (DIPE) were purchased from Fluka; these compounds were used as received.

Dextran polyaldehyde solution (3.2%, 2.5 mg mL^{-1}) was obtained by oxidation of Dextran 100–200 kDa (Serva Feinbiochemica) as described.[10c]

A stock solution of HCN in DIPE was prepared as previously described.[8c] WARNING: sodium cyanide and HCN are highly poisonous. They should be handled in a fume cupboard with a good draught. It is strongly advised to keep an HCN alarm switched on.

(*S*)-Mandelonitrile ((*S*)-**2**): *Me*HNL CLEA (CLEA Technologies B.V., 100 mg) and 20 mM citrate buffer pH 5.5 (1 mL) were added to DIPE (100 mL) in a round-bottomed flask. The mixture was vigorously stirred at 0 °C until a fine suspension was obtained. HCN (80 mL, 1.98 M solution in DIPE) was added, followed by a solution of benzaldehyde (10 g, 0.09 mol) in DIPE (100 mL). The mixture was stirred at room temperature.

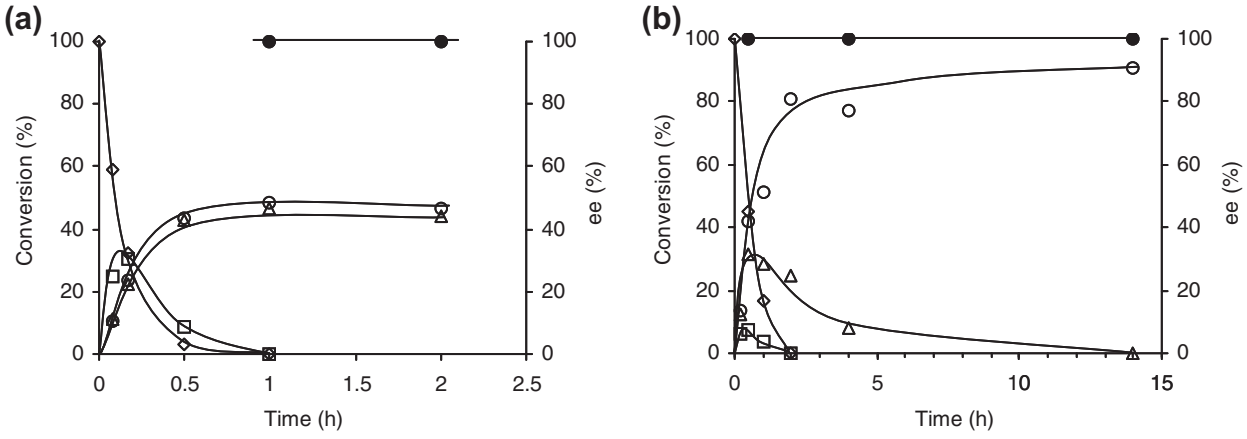

Figure 8. (a) Bienzymatic synthesis of (*S*)-mandelic acid from benzaldehyde (10 mM) and HCN (50 mM) in the presence of a MeHnL and PfNLase combi-CLEA biocatalyst; (b) with a triple CLEA of MeHnL, PfNLase and RheAMase. Reaction in 90:10 DIPE-buffer pH 5.5 at rt. Conversion: benzaldehyde (**1** , ◇), (*S*)-mandelonitrile (**2** , □), (*S*)-mandelic acid (**3**, ○), (*S*)-mandelic amide (**4** , △); ee: **3** (●).

The progress of the reaction and the ee of the product were measured by HPLC using a Waters 510 pump and a Waters 468 variable wavelength detector at 215 nm, equipped with a Daicel 5 μ 4.6 × 250 mm Chiralcel OB-H column, eluent heptane-isopropyl alcohol (95:5, v/v) with TFA (1%, v/v) at 1 mL/min at 40 °C. Retention times: benzaldehyde 7.32 min, (*R*)-(**2**) 15.72 min, (*S*)-**2** 16.77 min. Complete conversion was obtained after 8 h. The reaction mixture was filtered to remove the biocatalyst, dried over Na_2SO_4 and concentrated in vacuo at 50 °C until the last traces of HCN had been removed. (*S*)-**2** (11.81 g, 94%, 98.0% ee) was obtained as a colourless oil.

2-Hydroxy-4-phenyl-*trans*-3-butenenitrile (modified procedure[22]): to freshly distilled *trans*-cinnamaldehyde (0.02 mol) and 0.1 M phosphate buffer pH 7.5 in a magnetically stirred reactor a solution of HCN in DIPE (300 mL, 2 M) was added dropwise. After standing overnight equilibrium had been reached (>90% conversion); the cyanohydrin was extracted into CH_2Cl_2–hexane (50:50) and dried over $MgSO_4$. The solvent was evaporated in vacuo until crystallisation occurred spontaneously. The crystals were collected and dried. The structure was confirmed by ^{1}H NMR.

4.2. Analysis

4.2.1. Compound characterisation

Intermediates and products were characterised by comparison with an authentic sample. Full spectral data are available in the literature: mandelonitrile (**2**),[23] mandelic acid (**3**) ^{1}H and ^{13}C NMR,[24] MS,[25] mandelic amide (**4**),[26] 2-hydroxy-4-phenyl-*trans*-3-butenenitrile.[27]

4.2.2. HPLC (general procedures)

The progress of all of the reactions was monitored by HPLC, using either a Waters 590 pump and a Waters 486 Tunable Absorbance Detector at 215 nm or a Waters Alliance 2695 Separation Module and a Waters 2487 Dual Wavelength Absorbance Detector at 215 nm. The reaction products were identified by comparison with an authentic sample.

Procedure A: 4.6 × 50 mm Merck Chromolith™SpeedROD RP-18e column, eluent acetonitrile–H_2O (5:95, v/v) with heptafluorobutyric acid (0.03%, v/v) and ammonium formate buffer pH 2.3 (30 mM) at 1 mL min^{-1}.

Procedure B: 4.6 × 50 mm Merck Chromolith™SpeedROD RP-18e column, eluent acetonitrile–H_2O (10:90, v/v) with trifluoroacetic acid (0.1%, v/v) at 1 mL min^{-1}.

The enantiomeric purity of mandelonitrile (**2**) and its hydrolysis products was measured by HPLC using a Waters 515 pump and a Waters 486 Tunable Absorbance Detector at 215 nm and a 4.6 × 250 mm Chiralpak AD-H column; eluent hexane–isopropyl alcohol (80:20, v/v) with TFA (0.1%, v/v) at 0.6 mL min^{-1}.

4.2.3. Protein contents

The total protein concentration of cell-free extracts was assayed according to the standard Bradford procedure.[28] The samples were measured on a Shimadzu UV-240IPC spectrophotometer.

4.2.4. Activity assays (general procedures)

MeHnL: To a 1.5 mL Eppendorf tube containing 20 mM citrate buffer pH 5.5 (0.5 mL), enzyme solution (0.3 mg), DIPE (0.4 mL), 1,2-dimethoxybenzene (internal standard, IS) and HCN (100 mM) were added. Finally, *trans*-cinnamic aldehyde (10 mM) was added. The reaction was shaken (QInstruments ThermoTWISTER) at 25 °C while kept closed to prevent the escape of HCN. Samples were taken from each phase and analysed by HPLC (procedure B). One unit (U) of MeHnL will produce 1 μmol of 2-hydroxy-4-phenyl-*trans*-3-butenenitrile per min.

PfNLase: To a 1.5 mL Eppendorf tube containing 20 mM citrate buffer pH 5.5 (0.98 mL), NLase solution (10 μL, 100 × prediluted; 0.003 mg protein), 1,2-dimethoxybenzene (IS) and 2-phenylacetonitrile (10 mM) were added. Samples were taken and analysed by HPLC (procedure B). One unit (U) of PfNLase will hydrolyse 1 μmol of 2-phenylacetonitrile per min.

Amidase: To a 1.5 ml Eppendorf tube containing 20 mM citrate buffer (0.98 mL), amidase solution (0.195 mg protein) and 1,2-dimethoxybenzene (IS) was added (*R,S*)-mandelic amide ((*R,S*)-**4**, 10 mM). Samples were taken and analysed by HPLC (procedure B).

4.3. Combi-CLEA preparation and activity assay

4.3.1. Combi-CLEA preparation and assay

MeHnL (8.8 mg) and semi-purified PfNLase (3 mg) were added to a mixture of dextran polyaldehyde solution (0.5 mL), 0.5 M phosphate buffer pH 7.5 (0.25 mL) and 1,2-dimethoxyethane (1 mL, aggregation agent). The resulting mixture was stirred overnight at 4 °C and centrifuged. The pellet was resuspended in 0.1 M sodium bicarbonate solution (40 mL) containing sodium borohydride (1 g L^{-1}) to reduce Schiff's bases and stirred for 45 min at 40 °C. The CLEA was washed three times with Milli-Q water (6 mL), resuspended in 20 mM citrate buffer pH 5.5 and stored at 4 °C.

The kinetic assays were carried as described above with regard to substrates and reaction conditions. The results are compiled in Table 2.

Table 2
The activity recovered in the combi-CLEA

	Reaction rate (μmol min^{-1} (mg protein)$^{-1}$)	
	Free enzyme	Combi-CLEA
MeHnL	0.41	0.23
PfNLase	13.5	12.2

4.4. Cascade synthesis of (*S*)-mandelic acid ((*S*)-2) in the presence of the combi-CLEA

4.4.1. Combi-CLEA preparation

MeHnL (70.4 mg) and semi-purified PfNLase (24 mg) were added to a mixture of dextran polyaldehyde solution (4 mL), 0.5 M phosphate buffer pH 7.5 (2 mL) and 1,2-dimethoxyethane (8 mL, precipitant). The resulting mixture was stirred overnight at 4 °C and centrifuged. The pellet was resuspended in 0.1 M sodium bicarbonate solution (40 mL) containing sodium borohydride (40 mg) to reduce Schiff's bases and stirred for 45 min at 40 °C. The CLEA was washed three times with Milli-Q water (40 mL), resuspended in 20 mM citrate buffer pH 5.5 (5 mL) and stored at 4 °C.

4.4.2. Cascade reaction

The reactions were carried out in a 10 mL thermostatted glass reactor equipped with a magnetic stirrer at 25 °C. Freshly prepared combi-CLEA in 20 mM citrate buffer pH 5.5 (1.0 mL) was diluted with the same buffer (1 mL). Then, appropriate amounts of DIPE and benzoic acid (IS) were added. Benzaldehyde (**1**) was added to a concentration of 10, 25, 42, 83 or 250 mM, and stock solution of HCN to a concentration of 50, 125, 210, 415 or 750 mM; concentrations of **1** and HCN are with respect to the total volume. The total reaction volumes were 6 mL and consisted of 70% organic phase. Samples were withdrawn periodically to monitor the progress of the reactions. For quantitative measurements 20 μL of the lower (aqueous) phase and 100 μL of organic phase were

mixed with acidified mobile phase. DIPE was evaporated under vacuum, and the residue was analysed by reverse-phase HPLC as described above. The samples for enantiomeric purity measurements were extracted from the aqueous phase into ethyl acetate and then analysed as described above.

4.5. Operational stability of the combi-CLEA: catalyst recycling

The reactions were carried out in 1.5 mL Eppendorf tubes on a QInstruments ThermoTWISTER comfort shaker at 25 °C. To 20 mM citrate buffer pH 5.5 (450 μL), combi-CLEA suspension was added (50 μL, 0.24 U MeHnL and 10.1 U PfNLase). DIPE (465 μL), veratrole (IS), HCN (50 mM) and **1** (10 mM) were added. The total reaction volume was 1 mL and consisted of 50% organic phase. After 30 min, the reaction was spun down at 10×10^3 min^{-1} for 3 min; the pellet was washed twice with citrate buffer and used in the next reaction cycle.

In each reaction run a sample was withdrawn after 5 min to determine the enzyme activities; a sample was taken after 30 min to check the progress of the reaction. For each sample 10 μL aliquots of each phase were mixed with acidified mobile phase (980 μL) and analysed by reverse-phase HPLC as described above, except that ACN–H_2O: (10:90, v/v) with TFA (0.1%, v/v) was used as the mobile phase.

4.6. Effects of reagents at high concentrations

4.6.1. Inhibition experiments

Benzaldehyde: The reactions were carried out in 1.5 mL Eppendorf tubes on a QInstruments ThermoTWISTER comfort shaker at 25 °C. To 20 mM citrate buffer pH 5.5 (280 μL), combi-CLEA suspension (20 μL, 2.8 U PfNLase) was added. Subsequently, DIPE and **1** (10–500 mM) were added; the organic phase was 70% of the total volume. After 1 h, veratrole (IS) and 2-phenylacetonitrile were added to start the NLase activity assay. Samples were withdrawn periodically from both phases and analysed on reverse-phase HPLC as described above.

Effects of HCN concentration: The reactions were carried out in 1.5 mL Eppendorf tubes on a QInstruments ThermoTWISTER comfort shaker at 25 °C. To 20 mM citrate buffer pH 5.5 (250 μL), combi-CLEA suspension (50 μL, 7.0 U NLase) was added. Subsequently DIPE and HCN (25–575 mM) were added. The organic phase was 70% of the total volume. After 1 h veratrole (IS) and 2-phenylacetonitrile were added to start the NLase activity assay. Samples were withdrawn periodically from both phases and analysed on reverse-phase HPLC as described above.

Effects of 125 mM HCN over time: Combi-CLEA was suspended in 20 mM citrate buffer pH 5.5, HCN (125 mM) and DIPE (70% v/v) were added; the organic phase was 70% (v/v) of the total volume. Samples from the aqueous phase (containing the CLEA) were withdrawn at appropriate intervals. These were diluted with 20 mM citrate buffer pH 5.5 (920 μL), veratrole (IS) and 2-phenylacetonitrile (10 mM) were added and the reaction was monitored over time, using reversed-phase HPLC as described above.

4.6.2. Effects of HCN on PfNLase selectivity

PfNLase CLEA: A solution of semi-purified PfNLase (15 μL, 0.63 mg protein) was added to a cold, saturated ammonium sulfate solution (135 μL, pH 8.7). The aggregates were cross-linked for 2 h with dextran polyaldehyde solution (3.2%, 15 μL) and subsequently reduced with $NaCNBH_3$ (1.5 mg, dissolved in a minimum volume of water) for 1 h.

The CLEA was isolated by centrifugation at 13×10^3 min^{-1} (74 mg), washed three times with cold 10 mM phosphate buffer pH 8.7 and stored as a suspension in 1 ml of saturated ammonium sulfate solution at 0 °C. The activity recovery (see procedure below) was 35.8%.

Activity assay: to 0.5 M phosphate buffer pH 8 at 25 °C (1 mL) were added 2-phenylacetonitrile (10 μmol), veratrole (IS, 10 μmol) and 20 μL of the 100× diluted CLEA suspension. The reaction was monitored by reversed phase HPLC as described above.

Hydrolysis of (S)-mandelonitrile: a suspension of PfNLase CLEA (74 mg in 1 mL saturated ammonium sulfate solution) was centrifuged and the solid was resuspended in 20 mM citrate buffer pH 5.5 (0.5 mL).

(*S*)-**2** (6 μL, 0,05 mmol) and benzoic acid (IS, 5 mg, 0.043 mmol) were dissolved in 3.5 mL of DIPE, then 1.3 mL of citrate buffer was added. 70 μL of organic phase and 30 μL of aqueous phase were withdrawn with a syringe to determine the initial conditions. The reaction was initiated by adding 200 μl of the CLEA suspension and the mixture then stirred at 25 °C. Samples were withdrawn periodically to check the progress of the reaction: 30 μL of the lower (aqueous) phase and 70 μL of organic phase were mixed with acidified mobile phase. DIPE was evaporated under vacuum, the sample spun down, and analysed by reversed phase HPLC as described above.

The hydrolysis of (*S*)-**2** in the presence of HCN was carried out in the same way, except that an appropriate amount of a stock solution of HCN in DIPE (1.98 M) was added, while reducing the volume of additional DIPE to maintain a constant 30:70 aqueous–organic ratio.

4.7. Triple CLEA preparation and activity assay

4.7.1. Aggregation study

Samples of MeHNL (0.88 mg protein, 20 μL), PfNLase (0.23 mg protein, 7.5 μL and MP50 amidase (0.44 mg protein, 7.5 μL) were separately aggregated in 90% (v/v) aqueous aggregation reagent in 0.5 mL Eppendorf tubes. After an appropriate interval the aggregate was spun down (11×10^3 min^{-1}), the pellet was redissolved in 20 mM citrate buffer pH 5.5 or 0.1 M phosphate buffer pH 7 as appropriate and assayed for activity as described above. The effects of the aggregation reagent on the activity recovery with RheAMase are shown in Figure 9.

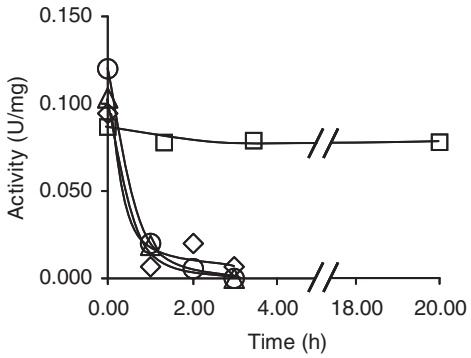

Figure 9. Activity recovery of RheAMase upon precipitation, incubation of the aggregates in the precipitant and redissolution in buffer. Ammonium sulfate (□), acetonitrile (○), 1,2-dimethoxyethane (△), *tert*-butyl alcohol (◇).

4.7.2. Single enzyme CLEAs

MeHnL: Enzyme samples (1.34 mg protein, 15 μL) were aggregated in cold, saturated ammonium sulfate solution (pH 8.7, 135 μL) in 1.5 mL Eppendorf tubes. The aggregates were cross-linked by the addition of appropriate amounts of dextran polyaldehyde solution and shaken (QInstruments ThermoTWISTER). After the appropriate interval sodium cyanoborohydride (5.2 mg dissolved in a minimum amount of water) was added and shaking

was continued for 1 h. The CLEA was spun down and three times resuspended and centrifuged. The CLEA as well as the cross-linking supernatant were assayed for activity as described above, except that the assay substrate was benzaldehyde.

PfNLase: CLEAs were prepared from 0.45 mg (15 μL) enzyme samples as described above (1.5 mg sodium cyanoborohydride). The activity assay was as described above for soluble PfNLase.

MP50 amidase: CLEAs were prepared from 0.88 mg (15 μL) enzyme samples as described above (1.5 mg sodium cyanoborohydride). The activity assay was carried out as described for the soluble amidase.

4.7.3. Semi-optimised triple CLEA

MeHnL (22 mg protein, 0.25 mL), PfNLase (17 mg protein, 0.5 mL) and MP50 amidase (49 mg protein, 2.5 mL) were aggregated in cold, saturated ammonium sulfate solution pH 8.7 (24.5 mL). The aggregate was cross-linked by the addition of 3.2% dextran polyaldehyde solution (2.2 mL) followed by magnetic stirring for 2 h at rt. Sodium cyanoborohydride (412 mg dissolved in a minimum amount of water) was added; the suspension was shaken for another 60 min and centrifuged at 4×10^3 min^{-1}. The CLEA was washed in three resuspension–centrifugation cycles, using in 25 mL 20 mM citrate buffer pH 5.5 (25 mL) at 4 °C. The triple-CLEA was stored on ice as a suspension in citrate buffer.

Storage stability: Samples of the triple CLEA as prepared above were washed three times with 20 mM citrate buffer pH 5.5 or saturated ammonium sulfate solution (pH 8.7) and suspended in these media at 0 °C. The residual enzyme activities were measured as described above.

4.7.4. Cascade synthesis of (*S*)-mandelic acid ((*S*)-2) in the presence of the triple CLEA

Triple CLEA as prepared above (30 μL) was added to 20 mM citrate buffer pH 5.5 (0.47 mL). To the CLEA suspension were added successively DIPE (0.39 mL), HCN (0.2 M), veratrole (10 mM) and **1** (10 mM); concentrations are with respect to the total volume.

Samples (10 μL) were withdrawn periodically and mixed with acidified mobile phase. DIPE was evaporated under vacuum and the residue was analysed by reverse-phase HPLC as described above. The samples for enantiomeric purity measurements were extracted from the aqueous phase into ethyl acetate and then analysed as described above.

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