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# Asymmetric Retro-Henry Reaction Catalyzed by Hydroxynitrile Lyase from *Hevea brasiliensis*

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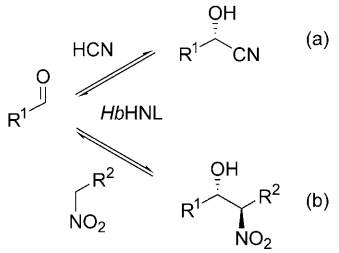
Hydroxynitrile lyase from *Hevea brasiliensis* (*Hb*HNL) is a promiscuous biocatalyst that, besides the native cyanohydrin reaction, also catalyzes the asymmetric Henry reaction yielding (*S*)-β-nitroalcohols with high enantiomeric excess. Since the Henry reaction is reversible, the enzyme can be also utilized for the production (*R*)-enantiomers by means of resolution of racemic β-nitroalcohols. Herein the biocatalytic retro-Henry reaction is studied using the cleavage of 2-nitro-1-phenylethanol as a model system. The main problem that prevents high levels of conversion or high *ee* values during the cleavage of the β-nitroalcohol is the formation of benzaldehyde, which is known to be a strong enzyme inhibitor. The product inhibition is overcome by performing the biocatalytic retro-Henry reaction in the presence of HCN, which reacts in situ with benzaldehyde and converts it to the less-inhibitive mandelonitrile. By using such a reaction cascade, it was possible to conduct the resolution practically to completion (95% *ee*, 49% conversion). Furthermore, the catalyst productivity achieved during the resolution was ten times higher than that in the *Hb*HNL-catalyzed synthesis of (*S*)-2-nitro-1-phenylethanol by condensation of benzaldehyde and nitromethane.

## Introduction

Hydroxynitrile lyase from *Hevea brasiliensis* (*Hb*HNL, EC 4.1.2.39) belongs to the industrially important group of enzymes catalyzing asymmetric C–C bond forming reactions. The high practical value of this biocatalyst is due to its exceptional stereoselectivity and wide substrate range in the *Hb*HNL-catalyzed condensation between aldehydes and HCN (Scheme 1 a).[1] The products of this biotransformation are enantiopure cyanohydrins, which are attractive as chiral precursors for various pharmaceuticals and agrochemicals.[2] Recently Purkarthofer et al.[3] discovered that *Hb*HNL exhibits so-called catalytic promiscuity;[4] besides the cyanohydrin reaction, it also catalyzes the related nitroaldol (Henry) reaction, in which aldehydes are condensed with nitroalkanes instead of hydrocyanic acid (Scheme 1 b). Although the specific activity of *Hb*HNL in this promiscuous reaction is very low (ca. 0.02 U mg$^{-1}$), the enzyme's stereoselectivity (up to 99% *ee*) is as high as in the cyanohydrin reaction.[5] The optically active β-nitroalcohols obtained as products of this biotransformation, similarly to cyanohydrins, can be utilized as synthetic chiral building blocks.[6] Moreover, these substances are more stable and less toxic than the respective cyanohydrins. Unfortunately, only the (*S*)-enantiomers can be produced in this manner, while, to date, *Hb*HNL is the only enzyme known to catalyze the asymmetric Henry reaction. However, the reversibility of the nitroaldol condensation[6b,7] enables in principle the application of *Hb*HNL also for the production of complementary (*R*)-enantiomers by means of kinetic resolution of racemic β-nitroalcohols, which are easily accessible by the base-promoted unselective Henry reaction. Such a racemic resolution might make the enzyme enantioflexible (suitable for production of either (*S*)- or (*R*)-enantiomers on demand) and could be a valuable extension of *Hb*HNL's synthetic utility. The aim of the current study was to evaluate the scope and limitations of the biocatalytic retro-Henry reaction in view of its application for the racemic resolution of β-nitroalcohols.




**Scheme 1.** Catalytic promiscuity of *Hb*HNL: the enzyme catalyzes asymmetric (a) cyanohydrin and (b) Henry reactions.

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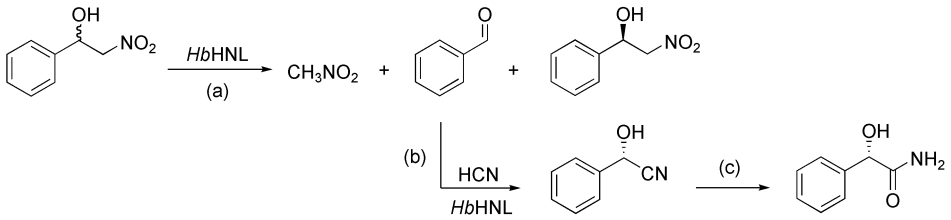
## Results and Discussion

### Resolution of 2-nitro-1-phenylethanol in a two-phase system

Racemic 2-nitro-1-phenylethanol (NPE) was selected as a model substrate in this study (Scheme 2). The preparative-scale resolution of (*rac*)-NPE was performed in a biphasic aqueous–organic system with methyl *tert*-butyl ether as an organic solvent, as in the original procedure used for the synthesis of (*S*)-NPE.[5] As expected, *Hb*HNL did catalyze the cleavage of (*S*)-NPE while the (*R*)-isomer remained in solution (Figure 1). The resolution was, however, not efficient. After 24 h, the *ee* value of the remaining NPE reached only 16%. Furthermore, denaturation of the protein was also detected. The reason for such a low conversion of (*S*)-NPE might be the benzaldehyde formed during the process. This substance is a strong competitive inhibitor for *Hb*HNL[8] in the cyanohydrin reaction and, moreover, has a negative impact on enzyme stability; the half-life of *Hb*HNL in 20 mM citrate–phosphate buffer (pH 5.0) in the presence of 10 mM of benzaldehyde was around 50 min compared to 450 min in the absence of benzaldehyde.[9]

Nevertheless, the enantiopurity of the remaining NPE could be increased by adding fresh *Hb*HNL. For this purpose, the organic phase, containing enantioenriched NPE, was separated at the end from the aqueous solution of the exhausted enzyme, washed with aqueous $NaHSO_3$ to remove the formed benzaldehyde, and then used for the consecutive batch by mixing with fresh *Hb*HNL solution. After the second batch, the *ee* value of the remaining NPE had reached 33%, and after the third, 57%. In consequence, by continuing this procedure the enantiopurity of NPE may be further increased, albeit alongside concurrent yield decline and enzyme consumption.




**Scheme 2.** a) Resolution of NPE catalyzed by *Hb*HNL; b) overcoming product inhibition by converting benzaldehyde to mandelonitrile; c) biocatalytic hydratation of mandelonitrile to mandelic acid amide might further increase the process performance.

### Resolution of NPE in a one-phase system

In order to inspect the biocatalytic retro-Henry reaction closer under minimized deactivation of *Hb*HNL, the resolution of NPE was performed in homogeneous aqueous solution at pH 6.0 and small initial substrate concentration. The reaction progress curves obtained at four different enzyme loadings (Figure 2) were fitted to the following kinetic model, which assumes that benzaldehyde is a competitive inhibitor for *Hb*HNL:

$$\begin{aligned}
\frac{d[BA]}{dt} &= v + k_b[NPE] \\
\frac{d[NPE]}{dt} &= -\frac{d[BA]}{dt} \\
\frac{d[(S)-NPE]}{dt} &= -v - k_b[(S)-NPE] \\
v &= \frac{V_{max}[HbHNL][(S)-NPE]}{K_{M,(S)-NPE}\left(1+\frac{[BA]}{K_{I,BA}}\right)+[(S)-NPE]}
\end{aligned} \quad (1)$$

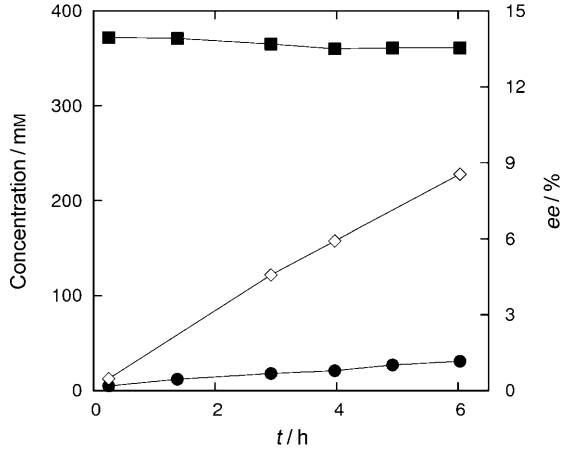



**Figure 1.** Time course of the resolution of NPE in 1:2 v/v mixture of pH 6.0 phosphate buffer and methyl *tert*-butyl ether at an enzyme/substrate ratio of 88 mg/mmol, room temperature. *BA* benzaldehyde. ■ NPE; ● BA; ◇ *ee*.

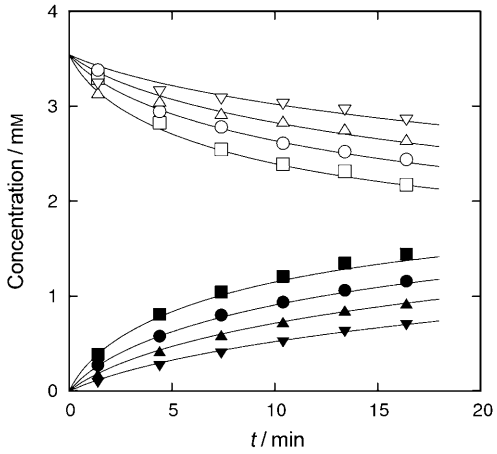



**Figure 2.** Progress curve analysis of concentration profile of NPE (open symbols) and benzaldehyde (closed symbols) during the cleavage of 3.5 mM NPE in 50 mM pH 6.0 phosphate buffer at 25 °C. Concentrations of *Hb*HNL were 0.5 (▲), 1.0 (▼), 2.1 (●) and 4.2 mg mL$^{-1}$ (■). Solid lines correspond to numerically simulated reaction progress.

where [BA], [NPE] and [(*S*)-NPE] are molar concentrations (mM) of benzaldehyde, (*rac*)- and (*S*)-NPE, respectively; [*Hb*HNL] is the concentration of enzyme(mg mL$^{-1}$); $v$ is the rate of enzyme catalyzed reaction, mM min$^{-1}$; $K_{i,BA}$ is the inhibition constant for benzaldehyde, mM; $V_{max}$ is the maximum reaction rate for the enzyme (U mg$^{-1}$); $K_{M,(S)-NPE}$ is the Michaelis constant for (*S*)-NPE (mM); $k_b$ is the rate constant of the spontaneous retro-Henry reaction (min$^{-1}$), which takes place in the absence of *Hb*HNL. The chosen model also implies that (*R*)-NPE and nitromethane have negligible inhibition effects on *Hb*HNL under the applied conditions. The rate constant $k_b = 5.3 \times 10^{-4}$ min$^{-1}$ was evaluated in a separate experiment performed in the absence of *Hb*HNL and was treated as invariant during the fitting procedure.

The estimated inhibition constant for benzaldehyde, $K_i =$ 0.37 mM, was of the same order of magnitude as that determined by Bauer et al. ($K_i = 0.8$ mM[8]), confirming that this substance is also a strong inhibitor in the retro-Henry reaction. Furthermore, the determined Michaelis constant for (*S*)-NPE, $K_{M,(S)-NPE} = 2.6$ mM, was close to that for (*S*)-mandelonitrile in the cyanohydrin reaction ($K_M = 1.55$ mM[8]), indicating that the β-nitroalcohol binds as well to *Hb*HNL as the native cyanohydrins. The calculated maximal reaction rate $V_{max}$ was found to be dependent on *Hb*HNL loading (Figure 3); at higher concentrations, the enzyme was less active. This phenomenon could be explained by either the presence of any tightly bound inhibitor in the enzyme stock solution[10] or by aggregation of *Hb*HNL molecules to homotrimers or homotetramers,[11] which could have lower turnover numbers. Remarkably, under the applied conditions the estimated enzyme's maximal reaction rate for the cleavage of NPE was in the range 0.16–0.27 U mg$^{-1}$, that is, one order of magnitude higher than for NPE synthesis (0.02 U mg$^{-1}$, estimated from the data of Gruber-Khadjawi et al.[5]).

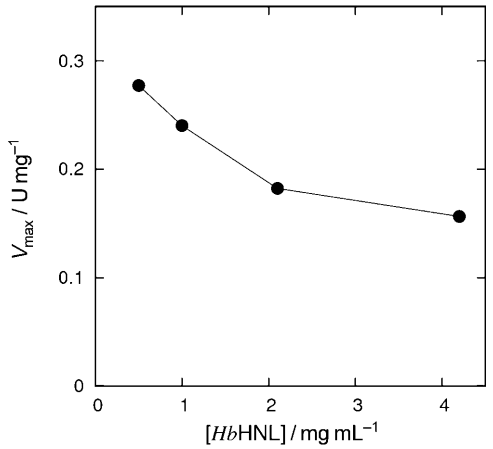



**Figure 3.** Dependence of estimated maximal reaction rate $V_{max}$ on *Hb*HNL concentration.

### Resolution of NPE at different pH

As mentioned above, during biocatalytic resolution of NPE the spontaneous retro-Henry reaction, which always runs in the background of the enzymatic transformation, should be taken into account. Since this side reaction is unselective, that is, both enantiomers of NPE are cleaved at the same rate, it has a negative influence on the resolution by decreasing the yield of the remaining enantiomer of β-nitroalcohol. A similar problem occurs in the *Hb*HNL-catalyzed cyanohydrin reaction and it is overcome by performing the process at pH 5, where the spontaneous reaction is practically suppressed[12] and the enzyme remains sufficiently stable.[9] To find the optimal pH value for the biocatalytic retro-Henry reaction, the cleavage of NPE was conducted in the pH range 4.2–7.5 in the presence of *Hb*HNL and also without the biocatalyst. The measured initial rates indicated that at pH < 6 the spontaneous reaction was practically suppressed, whereas at pH > 6.5 it was significantly enhanced (Figure 4). The rate of enzymatic reaction, which was estimated as a difference between the rates of overall and background reactions, revealed nearly linear pH dependence in the studied pH range. Therefore, pH 6 seems to be a good compromise for the resolution. Interestingly, by extrapolation, the maximal activity of *Hb*HNL in NPE cleavage should be at pH ≥ 7.5, in contrast to the biocatalytic cleavage of cyanohydrins where the enzyme activity as a function of pH is described by a bell-shape curve with maximum at around pH 5.[12]

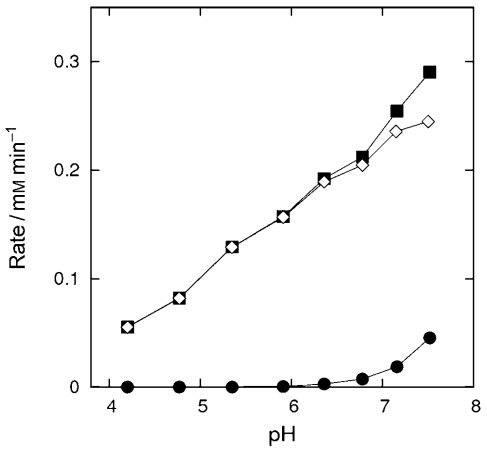



**Figure 4.** Influence of pH on reaction rates of biocatalytic (◇) and spontaneous (●) cleavage of NPE (5.3 mM) in 50 mM citrate–phosphate buffer at 25 °C; [*Hb*HNL] = 0.8 mg mL$^{-1}$; ■ = overall reaction rate.

### Overcoming product inhibition

To assess the strength of the product inhibition effect one may use the Lee–Whitesides criterion χ, defined as a ratio between the inhibition constant $K_i$ for the product and the Michaelis constant $K_M$ for the respective substrate.[13] If χ > 1, then the reaction would proceed efficiently until a high degrees of conversion was attained; otherwise the product inhibition would

drastically decrease the catalyst productivity.[14] The inhibition effect expressed as a relative reaction rate $v/v_0$, where $v_0$ is the reaction rate at the beginning of the reaction, can be evaluated as a function of $\chi$ and conversion $X$ according to Equation (2):

$$\frac{v}{v_0} = \frac{1 - X}{1 - X(1 - \chi^{-1})} \quad (2)$$

which is derived from the assumption that the enzyme exhibits zero-order kinetics in the whole substrate concentration range.

For benzaldehyde $\chi = K_{i,BA}/K_{M,(S)-NPE} = 0.37/2.6 = 0.14 < 1$. That is, when during the biocatalytic cleavage of (*S*)-NPE 20% conversion is achieved, the reaction rate would already drop to 40% of the initial value (Figure 5). Hence, to achieve a better

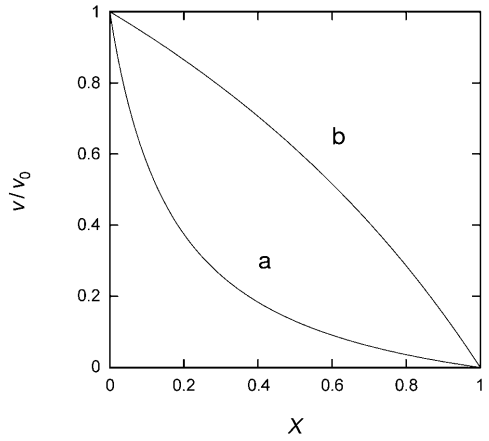



**Figure 5.** Relative reaction rate $v/v_0$ as a function of conversion $X$ during the biocatalytic cleavage of (*S*)-NPE. The lines were simulated by Equation (2) assuming product inhibition by (a) benzaldehyde, $\chi = 0.14$) or (b) (*S*)-mandelonitrile ($\chi = 1.6$[8]).

catalytic performance in the *Hb*HNL-catalyzed racemic resolution of NPE, one product inhibition by benzaldehyde must be overcome. A feasible way to do this is to design a reaction cascade in which the inhibitor formed as a product in the first step is converted to another substance in the second step.[15] To implement this approach herein, the biocatalytic retro-Henry reaction was coupled with the cyanohydrin reaction (Scheme 2b).

This choice was of a special interest, because *Hb*HNL can catalyze both steps of the reaction cascade. When NPE is cleaved in the presence of HCN, benzaldehyde could be constantly removed from the reaction mixture by its conversion into (*S*)-mandelonitrile, which is a much weaker inhibitor ($K_i$ = 4.2 mM,[8] $\chi = 1.6$). Whereas the activity of the enzyme in the cyanohydrin reaction is significantly higher than in the retro-Henry reaction, it was expected that the concentration of benzaldehyde during the whole process would be almost zero. Indeed, the addition of HCN did accelerate the cleavage of NPE catalyzed by *Hb*HNL (Figure 6). The threefold excess of HCN in respect to NPE was enough to keep benzaldehyde

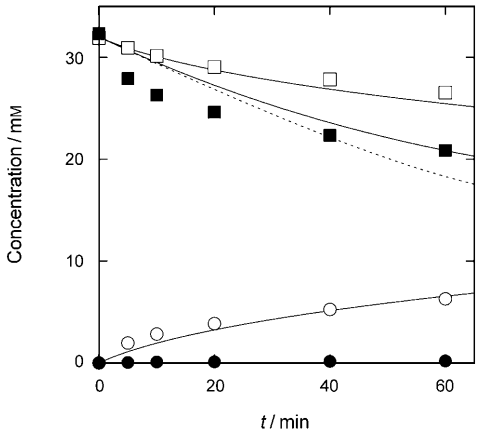



**Figure 6.** Concentration profile for NPE (■) and benzaldehyde (●) during racemic resolution of NPE by *Hb*HNL (0.9 mg mL$^{-1}$) in the presence of 0.1 M HCN in 50 mM pH 6.0 phosphate buffer at room temperature. Open symbols correspond to control experiment performed in the absence of HCN. Solid lines correspond to numerical simulations of reaction progress. Dashed line corresponds to hypothetical reaction progress without product inhibition.

concentration less than 0.2 mM, where its inhibition effect was negligible.

The plot of the enantiomeric excess of the remaining substrate as a function of its conversion (Figure 7) almost coincided with the theoretical curve constructed for the enantiomeric

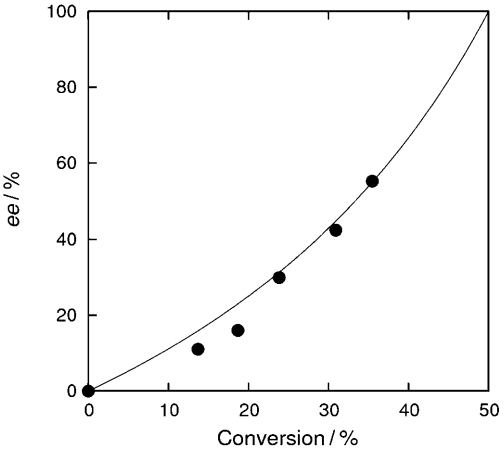



**Figure 7.** Enantiomeric excess of remaining (*R*)-NPE as a function of conversion with respect to the racemic substrate. Solid line corresponds to the enantiomeric ratio $E = \infty$.

ratio $E = \infty$,[16] that is, for the case when exclusively the (*S*)-enantiomer is cleaved. It implies that also in the presence of hydrocyanic acid the spontaneous retro-Henry reaction remained practically suppressed at pH 6.

The reaction progresses were also numerically simulated using the aforementioned kinetic model [Equation (1)]. During the simulation of the racemic resolution in the presence of HCN, it was assumed that the concentration of benzaldehyde remained zero over the whole reaction period, and that the enzyme was inhibited only by the forming (*S*)-mandelonitrile,

which acts as a competitive inhibitor. Additionally, a progress curve was simulated for the hypothetical racemic resolution in which no product inhibition takes place ($K_i = \infty$; Figure 6, dashed line). The simulated and experimental progress curves were in a good agreement. For the reaction cascade, the deviation between the predicted and the measured concentrations of NPE, that is especially visible for the first half of the process, actually did not exceed 10%. The simulated progress curve for the hypothetical racemic resolution without product inhibition (Figure 6, dashed line) reveals that, in the designed reaction cascade, the inhibition of *Hb*HNL by mandelonitrile is still significant and thus needs to be targeted during the optimization to achieve better process performance.

To investigate the scope of the proposed approach, the racemic resolution of NPE (70 mM) was conducted at various concentrations of HCN and *Hb*HNL (Table 1). In the tested concentration range, hydrocyanic acid, in contrast to *Hb*HNL loading, did not have any significant influence on the conversion and the *ee* of NPE remaining in the reaction mixture after one hour. This result suggests that HCN does not inhibit the enzyme in the retro-Henry reaction. Remarkably, at an enzyme/substrate ratio equal to 100 mg mmol$^{-1}$ and 1 M HCN (Entry 5, Table 1) the resolution was practically complete after 2 h (95% *ee*, 49% conversion), which corresponds to a catalyst productivity (defined here as kilograms of product produced per hour per kilogram of catalyst) of $Q = 0.4$ kg kg$^{-1}$ h$^{-1}$. For comparison, for the synthesis of NPE using the original procedure of Purkarthofer et al.,[3] $Q = 0.04$ kg kg$^{-1}$ h$^{-1}$. Such an increase in catalyst productivity results from the kinetic preference of *Hb*HNL for the retro-Henry reaction that is indicated by the significantly higher $V_{max}$ value for the enzyme in the cleavage of NPE in comparison to the synthesis.

**Table 1.** Resolution of NPE at various concentrations of *Hb*HNL and HCN.

| Entry | [HCN] [M][a] | [*Hb*HNL] [mg mL$^{-1}$] | *ee* [%][b] | Conversion [%][c] |
|---|---|---|---|---|
| 1 | 0.28 | 1.5 | 39 | 28 |
| 2 | 0.55 | 1.5 | 42 | 30 |
| 3 | 1.1 | 1.5 | 40 | 29 |
| 4 | 1.1 | 3.6 | 64 | 39 |
| 5 | 1.1 | 7.3 | 85(95)[d] | 46(49)[d] |

[a] Reaction conditions: [NPE] = 70 mM, 50 mM pH 6.0 phosphate buffer, room temperature, $t = 1$ h; [b] determined by chiral HPLC; [c] with respect to racemic NPE; estimated from *ee* value assuming that concentration of (*R*)-NPE remained constant; [d] values in parentheses refer to conversion after 2 h.

However, the removal of benzaldehyde in situ is accompanied by the problem of separation of mandelonitrile and (*R*)-NPE at the end of the process. These substances have similar $R_f$ values on silica, thus rendering conventional column chromatography inapplicable. In this case, high vacuum distillation can be a useful alternative, since the boiling points of the two products are quite different (100–110 °C for NPE and 40–50 °C for mandelonitrile at 5 Pa). Another possibility to separate both products is selective hydrogenation of the mixture over Pd/C in ethanol at room temperature and atmospheric pressure. In this case, NPE would be reduced without racemization to the respective aminoalcohol,[3] which, after acidification, could be easily separated from the remaining mandelonitrile in the form of a salt (the reduction of mandelonitrile over Pd/C proceeds only in the presence of a strong acid).[17]

To increase the catalyst productivity *Q* further and to facilitate the separation of the remaining (*R*)-NPE from the coproduct, mandelonitrile may be removed in situ in another consecutive biotransformation by converting it to a substance that does not inhibit *Hb*HNL and can be easily separated from the remaining (*R*)-NPE. The biocatalytic hydratation of mandelonitrile to mandelic acid amide catalyzed by a nitrile hydratase[18] could be the reaction of choice (Scheme 2c), since, according to our preliminary experiments, this amide does not inhibit the enzyme (data not shown) and may be separated from NPE by conventional column chromatography.

## Conclusions

The biocatalytic retro-Henry reaction extends the synthetic utility of *Hb*HNL also in the production of (*R*)-β-nitroalcohols through enzymatic resolution of their racemic mixtures. In contrast to the *Hb*HNL-catalyzed direct synthesis of (*S*)-enantiomers, the resolution process may be limited by product inhibition and enzyme denaturation caused by aldehydes formed during the cleavage, as was shown for the resolution of racemic NPE. It prohibits the achievement of high levels of conversion and consequently *ee* values in a single batch.

Nevertheless, this problem can be partially overcome by performing racemic resolution in the presence of HCN, which reacts with aldehydes to yield less-inhibitive cyanohydrins. By using this approach, it was possible to conduct the racemic resolution of NPE in a single batch to high conversion (up to 95% *ee* of the remaining (*R*)-enantiomer). Furthermore, thanks to the significantly higher $V_{max}$ value of *Hb*HNL in the retro-Henry reaction than in the Henry reaction, the catalyst productivity achieved during the resolution of (*rac*)-NPE was ten times higher than that of the *Hb*HNL-catalyzed synthesis of (*S*)-NPE. The application of the proposed reaction cascade to the preparative racemic resolution of NPE was, however, limited by the problem of separation of the formed mandelonitrile from the remaining chiral nitroalcohol. Although this drawback remains to be satisfactorily resolved, it is believed that it might be overcome by expanding the cascade with the biocatalytic hydratation of the nitrile to the corresponding amide.

## Experimental Section

### Enzyme and chemicals

The enzyme *Hb*HNL (99% purity, 73 mg mL$^{-1}$, activity in mandelonitrile cleavage = 4500 U mL$^{-1}$, stabilized by 30 ppm sodium azide) was kindly provided by Prof. Griengl (Research Center Applied Biocatalysis, Graz, Austria). The chemicals used in this work were commercially available, with the exception of (*rac*)-2-nitro-1-phenylethanol and HCN. Benzaldehyde was distilled in vacuo prior to usage.

NPE was synthesized according to the following procedure; benzaldehyde (1.1 g, 10 mmol), nitromethane (6.1 g, 100 mmol) and triethylamine (1.0 g, 10 mmol) were mixed and incubated overnight at 4 °C. The solvent was then removed under reduced pressure (2 kPa, 40 °C) and the yellow residue was purified by column chromatography on silica gel using cyclohexane/ethyl acetate mixture (16:1 v/v) as an eluent. After solvent evaporation, NPE was isolated as a colorless oil (1.2 g, 7.3 mmol, 73% yield). $^1$H NMR (500 MHz, $CDCl_3$): $\delta = 2.86$ (br, 1H, OH), 4.49 (dd, $^2J = 13.2$ Hz, $^3J = 2.9$ Hz 1H, C*H*$HNO_2$), 4.58 (dd, $^2J = 13.2$ Hz, $^3J = 9.8$ Hz, 1H, CH*H*$NO_2$), 5.43 (dd, $^3J = 9.8$ Hz, $^3J = 2.9$ Hz, 1H, C*H*OH), 7.33–7.39 ppm (m, 5H); $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta = 71.2$, 81.4, 126.2, 129.2, 129.3, 138.3 ppm.

Hydrocyanic acid was prepared by reaction of NaCN with $H_2SO_4$ (70%) in a well-ventilated hood and used within hours.

### Resolution of NPE in two-phase system

A sealed 10 mL glass vial was loaded with a solution in *tert*-butyl methyl ether (4 mL) containing NPE (0.27 g, 1.6 mmol) and *Hb*HNL solution in 50 mM pH 6.0 phosphate buffer (2 mL, 70 mg mL$^{-1}$). The reaction mixture was stirred with a Teflon-coated magnetic stirrer at 700 rpm at room temperature. To monitor the reaction progress, the stirring was stopped at different time points and the mixture was left still for several minutes to let the phases separate. Aliquots (50 μL) were withdrawn from the organic layer, diluted with methanol (950 μL) and analyzed by HPLC. After 24 h the reaction was stopped. To remove benzaldehyde formed during NPE cleavage, the separated organic layer was washed twice with 5 mL of $NaHSO_3$ solution (10%) and twice with 50 mM pH 6.0 phosphate buffer. The organic layer was then used for a consecutive batch with fresh *Hb*HNL solution (2 mL, 70 mg mL$^{-1}$). In this manner, two consecutive batches were carried out at the same reaction conditions as for the first batch, except that reaction times were increased to 48 h.

## Resolution of NPE in one-phase system

The reactions were performed in sealed 10 mL glass vials in 50 mM pH 6.0 phosphate buffer at 25 °C. Citrate-phosphate buffer (50 mM) was used for the study of NPE cleavage at varied pH. The experiments involving HCN were conducted in a well-ventilated hood at room temperature. To follow the reaction kinetics, the aliquots (50 μL) were withdrawn from the reaction mixtures at different time points, quenched in 2 M HCl (950 μL) and centrifuged at 6000 rpm for 20 min to remove the precipitated protein; the supernatants were analyzed by HPLC.

### HPLC analysis

The concentrations of NPE and benzaldehyde were determined by using a KNAUER chromatograph equipped with a UV detector set to 225 nm, and MERCK RP-8 column (5 $\mu$m, 0.4 cm ø × 12.5 cm) eluted at 30 °C with 67 mM $KH_2PO_4$ buffer/methanol mixture (65:35 v/v) at 2 mL min$^{-1}$. Retention times: NPE 2.3 min; benzaldehyde 3.7 min. Under the applied HPLC conditions, the concentration of mandelonitrile could not be monitored, because its peak overlapped with that of NPE. However, it did not significantly perturb NPE quantification since the optical absorbance of mandelonitrile at 225 nm was negligible compared to that of the β-nitroalcohol.

The enantiomeric excess of NPE was determined by HPLC analysis on Chiralcel OD-RH column (0.46 cm ø × 15 cm) eluted at 5 °C with water/methanol mixture (35:65 v/v) at 0.2 mL min$^{-1}$. Retention times: (*S*)-NPE 35 min; (*R*)-NPE 38 min.

### Progress curve analysis

Fitting of experimentally observed concentration profiles to the kinetic model was implemented in Matlab r2007a (Mathworks) utilizing *lsqnonlin* and *ode45* routines.

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**Keywords:** biocatalysis · catalytic promiscuity · Henry reaction · lyases · racemic resolution

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