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Improvement of a Stereoselective Biocatalytic Synthesis by Substrate and Enzyme Engineering: 2-Hydroxy-(4′-oxocyclohexyl)acetonitrile as the Model

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Abstract: Even if biocatalysis is finding increasing application, it still has to gain widespread use in synthetic chemistry. Reasons for this are limitations that enzymes have with regard to substrate range, reaction scope, and insufficient selectivity with unnatural compounds. These shortcomings can be challenged by enzyme and/or substrate engineering, which are employed to alter substrate specificity and enhance the enzyme selectivity toward unnatural substrates. Herein, these two approaches are coupled to improve the hydroxynitrile lyase catalyzed synthesis of 2-hydroxy-(4′-oxocyclohexyl)acetonitrile (**4**). The ketone functionality is masked as an enol ether, and the oxynitrilase of *Hevea brasiliensis* is engineered towards this masked substrate to give the product with a high optical purity and to drastically lower the amount of enzyme needed.

Keywords: cyanohydrins · enzyme catalysis · enzyme engineering · hydroxynitrile lyase · substrate engineering

Introduction

Numerous natural products, drugs, plant-protecting agents, and fragrances are chiral compounds that exhibit their biological activity often in only one enantiomeric form.[1] In 2000, 35 % of pharmaceutical intermediates were chiral and the number is expected to increase to 70 % by 2010.[2] Alongside chromatographic separation and chemocatalysis, biocatalysis is emerging as a key component in the toolbox of synthetic chemists.[2,3] Enzymes are attractive as asymmetric catalysts because they are typically very active and highly selective with their natural substrates. However, enzymes also have limitations, especially when performing reactions with non-natural substrates and under non-physiological conditions. Some of these shortcomings were overcome by reaction engineering, enzyme engineering, or substrate engineering.[4]

Enzyme engineering has been widely used to create improved biocatalysts. Directed evolution or rational-design approaches create new biodiversity, modifying enzyme features such as activity, stability, specificity, and even stereoselectivity.[5–8] In the case of the hydroxynitrile lyases, random mutagenesis and rational design were used to highly improve the activity and selectivity towards bulky substrates.[9–11]

In contrast, altering the substrate to increase enzyme activity and selectivity has only found application to a lesser extent. Nevertheless, different substrate engineering approaches were used successfully. Wang and Withers, for example, obtained an increased reaction rate for glycosidases by substrate-assisted catalysis.[12] Griengl and co-workers used the docking/protecting concept to direct and enhance the regio- and stereoselectivity of biohydroxylations and to broaden the substrate acceptance of the enzymes involved.[13–15] Recently, this approach was also applied to enzyme-catalyzed cyanohydrin reactions. *Hb*HNL (*Hb* = *Hevea brasiliensis*, HNL = hydroxynitrile lyase) showed a high increase in enantiopreference toward "thio-disguised" 2-butanone compared with the unmasked ketone. The enantiomeric excess of the desired product could be increased from an *ee* value of 54 % to an *ee* value of 99 %.[16]

In this study, using the enzyme-catalyzed formation of 2-hydroxy-(4′-oxocyclohexyl)acetonitrile (**4**) as a model, substrate and enzyme engineering were coupled. The results

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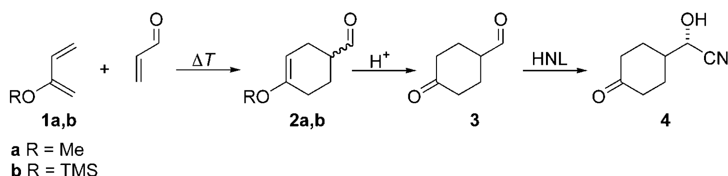
emphasize the importance of the interaction of the different methodologies available to improve biocatalytic transformations.

Results and Discussion

Starting from the 2-substituted buta-1,3-dienes **1a** and **1b**, a thermal Diels–Alder reaction was performed with acrolein as the dienophile, affording racemic **2a** and **2b**.[17,18] After treatment with aqueous HCl, 4-oxocyclohexane carbaldehyde (**3**) was obtained in moderate yields (Scheme 1).

The cyanohydrin formation catalyzed by *Hb*HNL was performed in an emulsion of 1:1 (v/v) *tert*-butyl methyl ether/aqueous buffer solution (citrate, pH 4.5) at 0°C. Only 2-hydroxy-(4′-oxocyclohexyl)-acetonitrile (**4**) was obtained, the additional carbonyl group not being converted to the corresponding cyanohydrin. The enzyme showed very low selectivity, affording product **4** with only a 10% *ee* value (Table 1, entry 1, 2*R*/2*S*:1.2). Probably, the ring oxo group, although not reacting, influences the orientation of the substrate within the active site by also interacting with the amino acids of the enzyme responsible for the fixation of the reactive aldehyde group, mainly by hydrogen bonding.



Scheme 1. Synthesis of 2-hydroxy-(4′-oxocyclohexyl)acetonitrile (**4**).

Table 1. Reaction conditions and product distribution for the conversion of **3**, **2a**, and **2b**.

Entry	Starting material	Enzyme	U mmol^{-1}	HCN equiv	(2*R*/2*S*)[a]	% Conv. to[b] 5	4
1	**3**	*Hb*HNL	2500	3.5	1/1.2	–	90
2	**2a**	blank	–	3.6	1/1	94	(36.3)[c]
3	**2a**	*Hb*HNL	4900	3.6	1/25	87	(2.0)[c]
4	**2a**	*Hb*HNL	490	3.5	1/14	84	(1.0)[c]
5	**2a**	*Pa*HNL	700	4.2	4/1	78	(n.d.)[c]
6	**2b**	blank	–	10	1/1	66	(11.8)[c]
7	**2b**	*Hb*HNL	2000	3.0	1/3	76	(7.0)[c]
8	**2b**	*Pa*HNL	850	2.0	1/1	90	(11.3)[c]

[a] Configuration at the new stereocenter. [b] Conversion calculated after acetylation. [c] Percentage of **4** present in the reaction mixture owing to spontaneous cleavage of the enol ether after the cyanohydrin reaction.

Therefore, the enzymatic cyanohydrin reaction was performed before cleaving the enol ether, masking the second carbonyl group (Scheme 2). Stability tests showed no cleavage of the acid labile enol ethers of **2a** and **b** above pH 5.0. Thus, the reactions were performed at pH values between 5.0 and 6.5, which is uncommon for enzymatic cyanohydrin reactions because the chemical background reaction is less repressed at these pH values. Nevertheless, with respect to the newly generated stereocenter for the reaction studied, *Hb*HNL showed high and moderate selectivities for substrates **2a** and **2b**, respectively (Table 1, entries 3, 4, 7) at elevated pH values. By using *Pa*HNL as a catalyst (*Pa* = *Prunus amygdalus*), as expected converse selectivity at the newly generated stereocenter was obtained, but only at a moderate level for aldehyde **2a**. With aldehyde **2b**, no particular stereopreference was displayed (Table 1, entries 5 and 8).

As shown in Figure 1 for the example of **2a** and with the *Hb*HNL enzyme, the enantiomers are converted at the same rate, indicating no chiral discrimination regarding the existing stereocenters. NMR analysis of product **5** confirmed that *Pa*HNL also makes no chiral discrimination regarding the existing center.

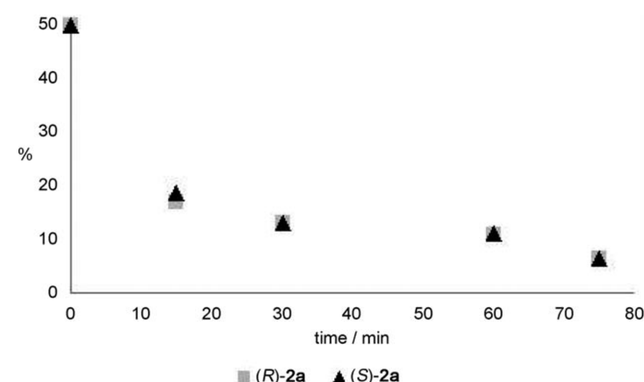
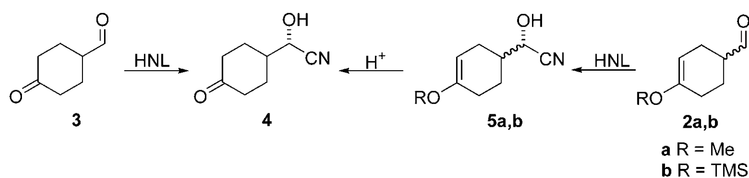


Figure 1. Consumption of (*R*)- and (*S*)-**2a** over the time period. Reaction conditions: 490 U *Hb*HNL per mmol **2a**.

Therefore, for better depiction only the combined amounts of (2*R*)- and (2*S*)-**5** are shown. In the blank reaction no stereopreference was obtained, indicating no diastereomeric induction of the already existing stereocenter (Table 1, entries 2 and 6).

The mixture of four stereoisomers of cyanohydrin **5** (Scheme 3) was converted to **4** by treatment with aqueous HCl without loss of optical purity at the cyanohydrin center, affording the desired product with 92% *ee* and 53% *ee*, starting from **2a** and **2b**, respectively (Table 1 entries 3 and



Scheme 2. Improvement of the synthesis of **4** by substrate engineering.

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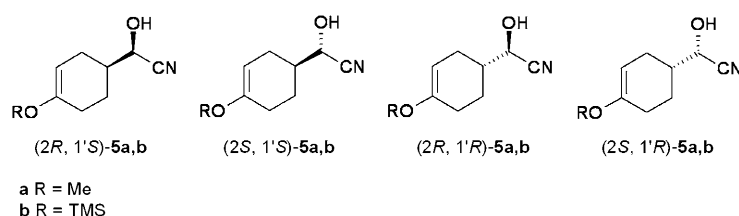

Scheme 3. Stereoisomers of the cyanohydrins **5a** and **5b**.

7). As already mentioned, the lower selectivity achieved with substrate **3** can probably be attributed to the second carbonyl functionality, which leads to differences in polarity and structure of compounds **3** and **2.**

Upon application of lower amounts of enzyme, the selectivity is affected, because the chemical background reaction is more competitive under these conditions (Table 1, entry 4). Thus, large amounts of *Hb*HNL are needed to obtain good results. To make this conversion more attractive, however, the amount of enzyme has to be lowered. *Hb*HNL muteins were chosen as a starting point. They have already been demonstrated to show good activity with bulky substrates.[9] In these enzyme variants, the tryptophan residue 128, which is positioned in the hydrophobic channel forming the narrow entrance to the enzyme's active site, is substituted to alanine and phenylalanine, respectively. Analogously, with the highly homologous HNL from *Manihot esculenta* (*Me*HNL), the substitution of corresponding tryptophan by alanine facilitates the access to the active site for bulky substrates, such as *meta*-phenoxybenzaldehyde.[10,19]

As a preliminary experiment, the performance of *Hb*HNL wild type and these muteins was examined in the cleavage reaction of **5a** and **5b**. Several single-cell generated colonies of *E. coli* TOP10F′ (pSE420HNL_WT), *E. coli* TOP10-F′(pSE420HNL_W128A), and *E. coli* TOP10-F′(pSE420HNL_W128F) were screened under different conditions (substrate solution buffer, equilibration buffer solution, time of equilibration) towards cleavage of 2-hydroxy-(4′-methoxycyclohex-3′-enyl)acetonitrile (**5a**), by using the visual colorimetric screening assay.[10] *Hb*HNL_W128A exhibited the strongest activity with this substrate, whereas *Hb*HNL_W128F showed only low activity, and HNL_WT no activity at all. Regarding the cleavage of **5b**, only *Hb*HNL_W128A showed activity. The enlarged hydrophobic tunnel to the active site of *Hb*HNL tunnel mutants may facilitate the access of these 4-subtituted cyclohex-3-ene carbaldehyde cyanohydrins to the enzyme's active center.

For comparison of the enzyme variants in the synthesis, *Hb*HNL_WT as well as the tunnel-mutants *Hb*HNL_W128A and *Hb*HNL_W128F were overexpressed in *E. coli* XL1-Blue. Formation of cyanohydrins **5a** and **b** was investigated by using the concentrated crude lysates. 4-Methoxycyclohex-3-ene carbaldehyde (**2a**) was converted in the presence of 1.8 equivalents of prussic acid and 80 U mmol^{-1} substrate. Faster conversions and higher preference for the 2*S* isomers were achieved by using *Hb*HNL_W128A as the catalyst. By contrast, *Hb*HNL_W128F showed lower conversions and moderate selectivities compared with the wild type *Hb*HNL (Figure 2a). The reaction of 4-trimethylsilyloxycyclohex-3-ene carbaldehyde (**2b**) was catalyzed with 150 U mmol^{-1} enzyme in the presence of 1.8 equivalents of HCN. Again, catalysis by *Hb*HNL_W128A displayed higher conversions and better selectivities, whereas *Hb*HNL_W128F exhibited worse performance compared with the wild type *Hb*HNL (Figure 2b).

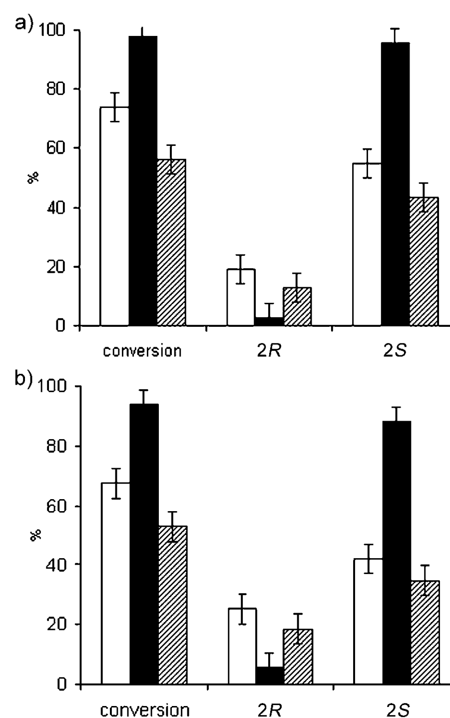

Figure 2. Conversion and product distribution of a) 4-methoxycyclohex-3-enecarbaldehyde (**2a**) with 80 U mmol^{-1} after 5 min and b) 4-trimethylsilyloxycyclohex-3-enecarbaldehyde (**2b**) with 150 U mmol^{-1} after 6 h with *Hb*HNL enzyme variants (white = wild type, black = W128A, shaded = W128F).

To further increase the activity regarding 4-methoxycyclohex-3-ene carbaldehyde (**2a**), error-prone PCR was used to introduce random mutations in the *hnl_W128 A* gene. DNA sequencing of 12 randomly picked transformants revealed that by using 0.2 mM $MnCl_2$ and 7 mM $MgCl_2$ in the PCR reaction, a mutation rate of 0.59 %, which corresponds to an average of 1–6 mutations per gene was achieved. Calculations resulted in a library size of 300 000 clones. Approximately 40 000 clones of the mutant library (MGB7) were screened for improved activity on **5a** with the colorimetric filter assay. For re-screening, 30 clones were chosen and finally 5 clones showing improved activity and containing mutations could be recovered (see Table 2). Three of these car-

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Table 2. Sequencing data from the selected clones from screening towards 4-methoxy-cyclohex-3-en carbaldehyde cyanohydrin (**5a**).

Clone	Nucleotide mutations	Amino acid mutations
mutant1	456 t→c	–
mutant 4	585 a→g, 681 t→g	–
mutant 10	–	–
mutant 13	645 a→t, 655 a→g	Glu 215→His, Ile 219→Val
mutant 21	–	–
mutant 23	58 c→t, 648 c→a	His 20→Tyr
mutant 30	91 c→t, 308 a→t, 440 a→g, 519 a→g, 560 c→t	His 31→Tyr, His 103→Leu, Lys 147→Arg, –, Pro 187→Leu

ried amino acid mutations, whereas two clones carried only silent mutations.

The starting clone HNL_W128 A and mutants 4, 13, 23, and 30 were expressed in *E. coli* XL1-Blue, and lyophilized crude cell lysates were used to study the formation of cyanohydrins **5a** and **5b**. Owing to the fast reaction observed with W128 A, only 10 U mmol^{-1} of substrate **2a** and 100 U mmol^{-1} of substrate **2b** were employed in the reactions for better comparison. High levels of conversion could be achieved with all enzyme variants. Interestingly, two of the mutants, mutant 13 and mutant 30, also showed increased selectivity towards the (2*S*)-cyanohydrin for both 4-substituted cyclohex-3-ene carbaldehydes (Table 3).

Table 3. Conversion and product distribution for enzymatic cyanohydrin formation of **2a** with 10 U mmol^{-1} after 5 min and of **2b** with 100 U mmol^{-1} after 6 h.

Enzyme	**2a** Conversion %	(*R*)/(*S*)[a]	**2b** Conversion [%]	(*R*)/(*S*)[a]
W128A	90.9	1/2.0	92.3	1/1.7
mutant 13	95.7	1/2.8	94.0	1/2.9
mutant 23	95.4	1/1.9	93.9	1/1.6
mutant 30	96.9	1/3.0	93.6	1/2.0

[a] Configuration at the new stereocenter.

To determine the effects of each of the mutations present in mutants 13 and 30 on selectivity and activity, respective mutants, each harboring only one of the mutations found in the parent clones (Gln215His, Ile219Val, His31Tyr, His103Leu, Lys147Arg, Pro187Leu) in addition to Trp128Ala, were constructed by site-directed mutagenesis. These mutants were first semiquantitatively examined in the cleavage reaction with the visible colorimetric assay. The mutant H103L showed the highest activity on substrate **5a**. Its signals arose fastest on the detection paper and, in addition, the blue spots had the strongest intensity. The parent mutants, mutant 13, mutant 30, as well as mutant Q215H showed slightly weaker activity followed by the starting clone W128A. I219V and K147R single mutants exhibited only very weak activity, whereas the P187L single mutant did not show any detectable activity in the cleavage reaction of **5a**. All "separated" mutants (Q215H, I219V, H31Y, H103L, K147R, and P187L) were expressed in *E. coli* XL1-Blue and lyophilized crude lysates were used to explore their performance in the synthesis reaction. In pre-experiments the activity of the lysates was examined towards cleavage of (*R,S*)-mandelonitrile. Only mutants H103L and Q215H showed good, and the other mutants exhibited only moderate (K147R) or very low (I219V, H31Y, P187L), activity. Nevertheless, all variants were investigated for performance in the synthesis reaction of 2-hydroxy-(4′-methoxycyclohex-3′-enyl)acetonitrile (**5a**) and 2-hydroxy-(4′-trimethylsilyloxycyclohex-3′-enyl)acetonitrile (**5b**).

The results obtained showed that the mutations Q215H and H103 L are responsible for high conversion and high selectivity of mutant 13 and mutant 30 on **2a** (Figure 3). Surprisingly, the mutation P187 L also showed high conversion and moderate selectivity, although the activity measured against the cleavage of (*R,S*)-mandelonitrile was found to be very low, and in the colorimetric filter assay no signals were obtained for the cleavage reaction of **5a**. The same mutations (Q215H and H103L) were found to be responsible for the improved performance of mutant 13 and mutant 30 in the conversion of 4-trimethylsilyloxycyclohex-3-encarbaldehyde (**2b**). Mutant Q215H showed the best results with **2b**, whereas with **2a** H103L displayed the highest *S*-isomer preference.

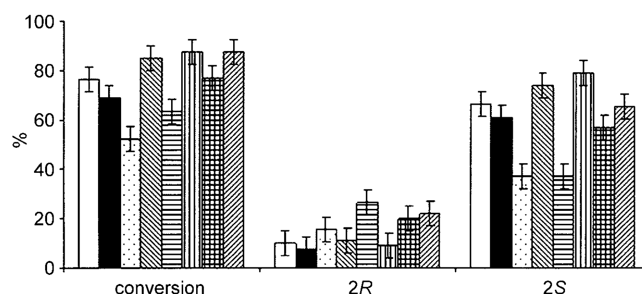

Figure 3. Comparison of conversion and selectivity for the enzymatic cyanohydrin formation of **2a** with the single mutants by using 10 U mmol^{-1} substrate. (2*R*) represents the percentage of (2*R*, 1′*RS*)-**5a** and (2*S*) the percentage of (2*S*, 1′*RS*)-**5a** present in the reaction mixture. The columns from left to right in each group of columns are: mutant B, Q215H, I219V, mutant 30, H31Y, H103L, K147R, and P187L.

In Figure 4, the location of the particular amino acid exchanges is shown with the 3D structure of *Hb*HNL. In mutant 13 (Figure 4a), both mutations (Q215H, I219V) are located on an α-helical structure. This α helix is the continuation of a loop, which bears the amino acid Asp207, which is essential for catalysis. It is assumed that the modification of this α helix by mutation at position 215 would cause a shifting of this loop region, possibly leading to a structural alteration within the active site and thereby influencing the activity of the enzyme on 4-substituted cyclohex-3-ene carbaldehydes. Three of the amino acid mutations in mutant 30 (K147R, H31Y and P187L) are located on the surface of the enzyme, whereas H103L is in the vicinity of the active site.

In all known *Hb*HNL structures, a conserved "structural" water molecule (Wat501) is found[20] that forms hydrogen

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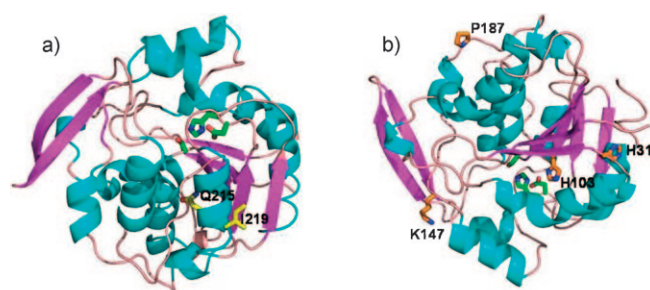

Figure 4. Ribbon representation of the 3D structure of *Hb*HNL. The catalytic triad is shown in green. Altered amino acids are shown in yellow for *mutant 13* (a) and in orange for *mutant 30* (b). The picture was prepared by using PyMol (http://www.Pymol.org/).

bonds to His103, Glu79, and the main-chain carbonyl group of His235. Furthermore, His103 interacts with the nitrogen of the main chain amino group of Asn238, which itself forms a salt bridge to Glu79. On the other hand, Glu79 creates a salt bridge to Lys236. By substitution of His103 with a nonpolar amino acid, such as leucine, these interactions are lost and this consequently leads to a change within the active site. For the P187 L mutation, which surprisingly showed good conversions in the synthesis reaction, although it was not active in the cleavage reaction, it is quite hard to predict its influence on the activity. It is placed on the surface of HNL and general structure modification may of course lead to altered activity. This example indicates that a mutation may have a different influence regarding the cyanohydrin synthesis reaction (in this case positive) and the reversed cleavage reaction (in this case negative).

To determine if the improved properties of mutants H103L and P187L are additive, the triple mutant W128A/H103L/P187L was constructed by site-directed mutagenesis. Transformants harboring the triple mutations examined for the cleavage reaction with the visual screening assay showed similar activity to mutant H103L. Investigation of lyophilized crude lysate of the triple mutant W128A/H103L/P187L by applying the same conditions as before revealed that for the transformation of **2a** a higher conversion (93 %) and *S* preference (82 %) was achieved. For the conversion of **2b**, comparable results as for the reaction catalyzed with mutant H103L were obtained. Best variants were used for reactions on a preparative scale for the conversion of the two aldehydes **2a** and **2b**. Aldehyde **2a** was converted by the triple mutant (100 $U\,mmol^{-1}$ substrate) in 1 h, yielding 95 % of the crude cyanohydrin with a (2*S*,1′*RS*)/(2*R*,1′*RS*) ratio of 95:5. On the other hand, **2b** was converted to the respective cyanohydrin with Q215H (200 $U\,mmol^{-1}$ substrate) in 2 h with 92 % yield (crude) and a ratio (2*S*,1′*RS*)/(2*R*,1′*RS*) of 93:7. Treatment of the products according to Scheme 2 affords 2-hydroxy-(4′-oxocyclohexyl)acetonitrile (**4**) in 90 % and 86 % *ee*, respectively.

Conclusion

Herein we describe an impressive example for the improvement of an important biocatalytic conversion coupling substrate and enzyme engineering. For the synthesis of 2-hydroxy-(4′-oxocyclohexyl)acetonitrile (**4**), the selectivity of the hydroxynitrile lyase from *Hevea brasiliensis* is increased by masking the carbonyl functionality as an enol ether. Furthermore, it is shown that the performance can be improved by using the already established *Hb*HNL tunnel mutant W128A. In addition, one round of directed evolution based on *Hb*HNL_W128A and subsequent screening identified two variants that show further enhanced activity and selectivity. Important mutations within these clones were identified and thus two enzyme variants were created, which allow cyanohydrins to be obtained from 4-substituted cyclohex-3-ene carbaldehydes in high yields and high selectivities with only small amounts of enzyme. The enantiomeric excess of the desired product **4** could be improved from 10 % to 90 % and 86 % starting from **2a** and **2b**, respectively, by using 10–20 times less enzyme.

Experimental Section

General: All solvents and materials not described in this section are commercially available and were appropriately purified, if necessary. *Hb*HNL and *Pa*HNL wild-type enzymes were kindly provided by DSM Fine Chemicals Austria. Reactions were monitored by TLC (Merck silica gel 60 F_{254} or aluminum oxide 60 F_{254} neutral) and the compounds were visualized by spraying with Mo reagent (10 % H_2SO_4, 10 % $(NH_4)Mo_7O_{24}\cdot 4H_2O$ and 0.8 % $Ce(SO_4)_2\cdot 4H_2O$ in water) or vanillin/H_2SO_4 solution (1 g vanillin in 1000 mL conc. H_2SO_4). Flash chromatography was performed on silica gel 60 (70–230 mesh, Merck), neutral (pH 7.0) or basic (pH 10, Brockmann activity II) aluminum oxide (Fluka). ^1H and ^{13}C NMR spectra were recorded on a VARIAN INOVA 500 MHz (^1H: 500 MHz; ^{13}C: 125 MHz) spectrometer with TMS as an internal reference. Enantiomeric purities were analyzed by chiral GC by using a Hewlett Packard 6890 instrument equipped with a FID and a Chirasil-DEX CB column (25 m × 0.32 mm, 0.25 μm film). GC–MS measurements were performed by employing a Hewlett Packard HP6890 Series-II GC system equipped with a HP 5973N mass selective detector employing methane as reactant, a HP5-MS column (30 m × 0.25 mm, 0.25 μm film) and He as carrier gas. The analytical data are described below.

Synthesis methods

2-Methoxy-1,3-butadiene (1a): As reported in the literature,[21] sodium acid sulfate (0.17 g) was added to 1,3,3-trimethoxybutane (20 mL, 123 mmol) in a three-necked round-bottom flask equipped with a thermometer, a Vigreux column with condenser, and a dropping funnel. The mixture was heated to 140–150 °C. As the reaction proceeded, the remaining 1,3,3-trimethoxybutan (60 mL, 369 mmol) was added at such a rate to keep about 20 mL of liquid in the flask. The decomposition products were collected over 5 % sodium carbonate solution. After completion, the organic phase was separated, washed with water, and dried over calcium chloride. Distillation of the crude product yielded 2-methoxy-1,3-butadiene (12.5 g, 30 %). To increase the yield, the aqueous phase was extracted with diethyl ether. The organic phase was then washed with water, dried over $CaCl_2$, and the diethyl ether was removed by distillation at atmospheric pressure. Distillation together with the pre- and post-fraction of the first distillation afforded another 4.5 g (10 %) of product. The NMR spectra showed 2,2-dimethoxybut-3-ene (see below) as an impurity in a ratio 3:1 (product/by-product), which, however, does not affect the

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Diels–Alder reaction (b.p. 73–76 °C). Spectroscopic data correspond with those reported.[22]

2,2-Dimethoxybut-3-ene: Spectroscopic data correspond to those in reference [23].

4-Methoxycyclohex-3-ene carbaldehyde (2a): As reported in the literature,[17] a mixture of 2-methoxybutadiene (10 g, 119 mmol, 1 equiv), freshly distilled acrolein (12 mL, 179 mmol, 1.5 equiv), and hydroquinone (0.5 g) in benzene (30 mL) were heated at 160 °C for 30 min in a stainless steel bomb. After cooling to room temperature, the solution was transferred and the excessive acrolein and benzene were removed by distillation at atmospheric pressure. Distillation of the crude product afforded **2a** as a colorless oil (7.5 g; 45 %; b.p. 80–85 °C (7 mbar)). ^{1}H NMR spectra correspond to those in reference [24]. ^{13}C NMR ($CDCl_3$): $\delta = 204.59$ (CHO), 155.41 (C4), 91.09 (C3), 54.24 (OCH_3), 46.30 (C1), 26.30 (C2), 23.13 (C5), 22.47 ppm (C6); MS: 141 ($[M]^+$), 125 ($[M-CH_3]$), 81 ($[M-(CHO + OCH_3)]$); Chiral GC: 120 °C, 2 min, 5 °C min^{-1}, 160 °C, 10 min; 4.67, 4.76 min, 0.45 bar H_2.

2-Trimethylsilyloxy-1,3-butadiene (1b): As reported in the literature,[25] a solution of triethylamine (55 mL, 395 mmol, 1.2 equiv) in DMF (200 mL) were heated to 70–80 °C under an argon atmosphere. Solutions of methyl vinyl ketone (30 mL, 328 mmol, 1 equiv) in DMF (25 mL) and trimethylsilyl chloride (50 mL, 394 mmol, 1.2 equiv) in DMF (25 mL) were added simultaneously over 30 min. The solution darkened from colorless to brown and a precipitate of triethylamine hydrochloride was formed. The reaction was run overnight at 80–90 °C. After cooling the reaction mixture to room temperature, it was filtered and transferred to a separating funnel containing pentane (300 mL). The solution was extracted with ice-cooled, saturated $NaHCO_3$ (1 L). The organic layer was separated and the aqueous phase was washed with pentane (2×300 mL). The combined pentane layers were extracted with water and dried over Na_2SO_4. The pentane and other volatile compounds were removed by distillation at atmospheric pressure. After distillation, **1b** was obtained as a colorless oil (20 g; 40 %; b.p. (70 mbar) 42–44 °C). The spectroscopic data are slightly different from those in the literature.[26] ^{1}H NMR ($CDCl_3$): $\delta = 6.13$ (q, $J = 10.3$, 17.1 Hz, 1H, H3), 5.40 (dd, $J = 2.0$, 17.1 Hz, 1H, H4), 5.02 (dt, $J = 2.0$, 10.7 Hz, 1H, H4′), 4.28 (s, 1H, H1), 4.29 (s, 1H, H1′), 0.18 ppm (s, 9H, $Si(CH_3)_3$); ^{13}C NMR ($CDCl_3$): $\delta = 154.89$ (C2), 134.64 (C3), 114.55 (C4), 96.47 (C1), -0.004 ppm ($Si(CH_3)_3$); MS: 171 ($[M + C_2H_5]$), 143 ($[M]^+$), 127 ($[M-CH_3]$), 73 (TMS).

4-Trimethylsilyloxycyclohex-3-ene carbaldehyde (2b): As reported in the literature,[18] freshly distilled acrolein (2.0 equiv), hydroquinone (0.02 equiv), and 2-trimethylsilyloxy-1,3-butadiene were dissolved in toluene and heated to reflux for 24 h. The solvent and unreacted starting material were removed under reduced pressure. Distillation gave **2b** as a colorless liquid (15 g; 66 %; b.p. (4 mbar) 89–92 °C). The spectroscopic data were slightly different from those reported in the literature.[18] ^{1}H NMR ($CDCl_3$): $\delta = 9.53$ (s, 1H, CHO), 4.71 (m, 1H, H3), 2.30–2.20 (m, 1H, H1), 2.12–2.08 (m, 2H, H2, H2′), 1.94–1.88 (m, 2H, H5, H5′), 1.84–1.78 (m, 1H, H6), 1.58–1.65 (m, 1H, H6′), 0.01 ppm (s, 9H, TMS); ^{13}C NMR ($CDCl_3$): $\delta = 204.07$ (CO), 150.17 (C4), 101.49 (C3), 45.34 (C1), 27.87 (C2), 23.02 (C5), 22.43 (C6); MS: 198 ($[M]^+$), 170 ($[M-CO]$), 155 ($[M + C_2H_5 - TMS]$), 127 ($[M-TMS]$), 73 ppm (TMS); chiral GC: 100 °C, 2 min, 10 °C min^{-1}, 160 °C, 10 min, 0.45 bar H_2, 7.10 min.

4-Oxocyclohexane carbaldehyde (3): A solution of 4-trimethylsilyloxycyclohex-3-ene carbaldehyde (**2b**) in aqueous HCl (2 M) was stirred for 2 h at room temperature and then extracted twice with dichloromethane. The organic layer was separated, washed with saturated $NaHCO_3$ and water, and dried over Na_2SO_4. Solvent removal under reduced pressure gave aldehyde **2c** in 55 % yield. ^{1}H NMR ($CDCl_3$): $\delta = 9.78$ (s, 1H, CHO), 2.70–2.64 (m, $J = 4.39$ Hz, 1H, H1), 2.48–2.34 (m, $J = 6.35$, 4.39 Hz, 4H, H3, H3′, H5, H5′), 2.26–2.19 (m, $J = 6.35$, 4.39 Hz, 2H, H2, H6), 2.02–1.94 ppm (m, $J = 4.88$ Hz, 2H, H2′, H6′); ^{13}C NMR ($CDCl_3$): $\delta = 209.74$ (CO), 202.42 (CHO), 47.27 (C1), 39.45 (C3, C5), 25.42 ppm (C2, C6); MS: 155 ($[M + C_2H_5]$), 127 ($[M]^+$), 109 ($[M-OH]$), 81 ($[M-(CHO+O)]$).

pH Stability of the enol ethers 2a and 2b: A buffer solution (30 mM, K_2HPO_4/citrate, pH 5.5) containing *Hb*HNL (2000 U mmol^{-1} aldehyde) was adjusted to the desired pH (4.5–7.0) and added to a solution of aldehyde in *tert*-butyl methyl ether (*t*BME). The solution was stirred at room temperature and 100 µL samples were taken at 15 and 30 min, 1, 2, 3, 4, 5, and 24 h, filtered over a Celite 545 Na_2SO_4 pad, diluted with *t*BME (1 mL), and analyzed with GC-MS: 60 °C, 2 min, 10 °C min^{-1}, 160 °C, 5 min, 1 bar He.

Synthesis and safe handling of anhydrous HCN—CAUTION: All reactions in which HCN or cyanides were involved, were performed in a well-ventilated hood. An electrochemical sensor for HCN detection was used for continuous warning. The required amount of HCN was freshly prepared by adding a saturated NaCN solution dropwise to aqueous sulfuric acid (60 %) at 80 °C. HCN was transferred in a nitrogen stream through a drying column and collected in a cooling trap at −12 °C. Waste solutions containing cyanides were treated with aqueous sodium hypochlorite (10 %). Subsequently, the pH was adjusted to 7.0 with aqueous sulphuric acid.

General procedure for the chemical synthesis of racemic cyanohydrins (blank): An aqueous buffer solution (30 mM, K_2HPO_4/citrate, 1/1 v/v) was added to a solution of aldehyde in *t*BME. The resulting mixture was stirred at 0 °C until an emulsion was formed. After the addition of freshly prepared prussic acid (3.6 equiv), the mixture was stirred at 0 °C until quantitative conversion. The emulsion was broken with Celite 545, filtered, and dried over Na_2SO_4. Evaporation of the solvent under reduced pressure yielded the crude cyanohydrins as light yellow liquids. For determination of the product distribution, a small amount was acetylated with acetic anhydride and pyridine in dichloromethane by using standard methods. The results are shown in Table 1.

2-Hydroxy-2-(4-oxocyclohexyl)acetonitrile (4): ^{1}H NMR ($CDCl_3$): $\delta =$ 4.57 (d, $J = 5.41$ Hz, 1H, H2); 2.51–1.36 ppm (m, 9H, H1′, $2 \times$H2′, $2 \times$H3′, $2 \times$H5′, $2 \times$H6′); ^{13}C NMR ($CDCl_3$): $\delta = 210.9$ (C4O), 121.1 (CN), 64.1 (C2), 40.5 (C1′), 40.2 (C3′, C5′), 27.7 ppm (C2′, C6′).

2-Hydroxy-2-(4′-methoxycyclohex-3′-enyl)acetonitrile (5a): ^{1}H NMR ($CDCl_3$): $\delta = 4.62$–4.57 (m, 1H, H3′), 4.38 (dd, $J = 10.2$, 5.9 Hz, 1H, H2), 3.51 (s, 3H, OCH_3), 2.37–1.95 (m, 6H, H1′, $2 \times$H2′, $2 \times$H5′, H6′), 1.66–1.50 ppm (m, 1H, H6″); ^{13}C NMR ($CDCl_3$): $\delta = 155.3$, 155.2 (C4′), 119.5, 119.4 (CN), 91.1, 91.0 (C3′), 65.4 (C2), 54.4, 54.3 (OCH_3), 38.9, 38.8 (C1′), 26.9, 26.8 (C6′), 25.4, 25.3 (C2′), 24.4, 24.3 ppm (C5′).

2-Hydroxy-2-(4′-trimethylsilyloxycyclohex-3′-enyl)acetonitrile (5b): Unstable.

General procedure for the enzymatic synthesis of (*S*)-cyanohydrins: An aqueous solution (1/1 v/v) that contained *Hb*HNL (ca. 250–5000 U mmol^{-1} aldehyde) in buffer solution (30 mM, K_2HPO_4/citrate, pH 5.0) was added to a solution of aldehyde in *t*BME and the resulting mixture was stirred at 0 °C until an emulsion was formed. After addition of freshly prepared prussic acid (1.8 to 3.6 equiv), the mixture was stirred at 0 °C until quantitative conversion. The emulsion was broken with Celite 545, filtered, and dried over Na_2SO_4. Evaporation of the solvent under reduced pressure yielded the crude cyanohydrin as a light yellow liquid. For the determination of the distribution of the stereoisomers, a small amount was acetylated (for results see Table 1).

General procedure for the synthesis of (*R*)-cyanohydrins: An aqueous solution (1:1 v/v) containing *Pa*HNL (ca. 600–700 U mmol^{-1} aldehyde) in a buffer solution (30 mM, K_2HPO_4/citrate, pH 5.0) was added to a solution of aldehyde in *t*BME and the resulting mixture was stirred at 0 °C until an emulsion was formed. Freshly prepared prussic acid (1.8–3.5 equiv) was added and the mixture was stirred at 0 °C until quantitative conversion occured. The emulsion was broken with Celite 545, filtered, and dried over Na_2SO_4. Solvent removal in vacuo gave the crude cyanohydrin as a light yellow liquid. For the determination of the distribution of the stereoisomers, a small amount was acetylated by using standard procedures. Results are shown in Table 1.

Treatement of 5a and 5b with HCl: A solution of **5a** or **5b** in aqueous HCl (2 M) was stirred for 4 h at room temperature and then extracted twice with dichloromethane. The organic layer was separated, washed with water, and dried over Na_2SO_4. Solvent removal under reduced pressure gave aldehyde **4** in 80–90 % yield. Spectroscopic data are given above.

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General procedure for the acetylation of cyanohydrins 4 and 5a,b: Cyanohydrins **2** were acetylated according to standard procedures with 1.5 equivalents of acetic anhydride and 1.5 equivalents of pyridine in CH_2Cl_2 overnight.

Cyano-(4′-methoxycyclohex-3′-enyl)methyl acetate: ^{1}H NMR ($CDCl_3$): δ=5.28 (dd, J=6.35, 3.42 Hz, 1H, H3′), 4.57 (m, 1H, H2), 3.51 (s, 3H, CH_3O), 2.38–1.87 (m, 6H, H1′, 2×H2′, 2×H5′, H6′), 2.17 (s, 3H, CH_3Ac), 1.66–1.51 ppm (m; 1H; H6′); ^{13}C NMR ($CDCl_3$): δ=169.5, 169.4 (CO), 155.3, 155.2 (C4′), 116.3 (CN), 90.7, 90.6 (C3′), 64.8, 64.7 (C2), 54.4, 54.3 (OMe), 36.8, 36.7 (C1′), 26.8, 26.7 (C2′), 25.4, 25.3 (C5′), 24.5, 24.3 (C6′), 20.6, 20.5 ppm (CH_3Ac); MS: 210 ([M]$^+$), 183 ([M−CN]), 168, ([M−(CN+CH_3)], 150 ([M−OAc]); chiral GC: 120°C, 2 min, 5°C min^{-1}, 160°C, 10 min, 0.45 bar H_2, 11.7 min (2*R*, 1′*RS*), 12.6 min (2*S*, 1′*RS*).

Cyano-(4′-trimethylsilyloxycyclohex-3′-enyl)methyl acetate: ^{1}H NMR ($CDCl_3$): δ=5.09 (d, J=6.34, 1H, H2), 4.63 (m, 1H, H3′), 1.97 (s, 3H, CH_3Ac), 2.12–1.57 (m, 7H, H1′, 2×H2′, 2×H5′, 2×H6′), −0.01 ppm (s, 9H, TMS); ^{13}C NMR ($CDCl_3$): δ=169.2, 169.1 (CO), 150.2, 150.1 (C4′), 116.0 (CN), 101.1, 101.0 (C3′), 64.6, 64.5 (C2), 36.2, 36.1 (C1′), 29.5 (C2′), 25.5, 25.4 (C5′), 24.5, 24.4 (C6′), 20.3 (CH_3Ac), 0.25 ppm (TMS); MS: 268 ([M]), 73 (TMS); chiral GC: 100°C, 2 min, 10°C min^{-1}, 160°C, 10 min, 0.45 bar H_2, 13.4 min (2*R*, 1′*RS*), 14.8 min (2*S*, 1′*RS*).

Cyano-(4′-oxocyclohexyl)methyl acetate: ^{1}H NMR ($CDCl_3$): δ=5.34 (d, J=5.86, 1H, H2), 2.54–2.16 (m, 8H, 2×H2′, 2×H3′, 2×H5′, 2×H6′), 2.18 (s, 3H, CH_3Ac), 1.77–1.64 ppm (dq, J=26.36, 4.89 Hz, 1H, H1′); ^{13}C NMR ($CDCl_3$): δ=209.08 (C4O), 169.23 (COAc), 115.78 (CN), 64.33 (C2), 39.85 (C3′), 39.79 (C5′), 38.60 (C1′), 27.47 (C2′), 27.62 (C6′), 20.55 ppm (CH_3Ac); MS: 196 ([M]$^+$), 169 ([M−CN]), 154 ([M−(CN+CH_3)]), 127 ([M−(CN+Ac)]; chiral GC: 100°C, 2 min, 10°C min^{-1}, 160°C, 10 min, 0.45 bar H_2, 13.8 min (2*R*), 14.9 min (2*S*).

***Hb*HNL mutein investigations with aldehyde 2a**: The lysates of the *Hb*HNL enzyme variants (10 or 80 U mmol^{-1} aldehyde) were diluted with aqueous buffer solution (30 mM, citrate, pH 5.5) to an end volume of 1.0 mL. Aldehyde **2a** (100 mg, 0.71 mmol, 1 equiv) was dissolved in *t*BME (1.0 mL), mixed with the enzyme solution in 30 mL glass vials, and stirred at 0°C for 10 min. HCN (50 µL, 1.3 mmol, 1.8 equiv) was added and the solution stirred at 0°C and 650 rpm. At the times indicated, 50 µL samples were taken and extracted with CH_2Cl_2 (300 µL). The organic layer (100 µL) was used for derivatization with acetic anhydride (10 µL) in the presence of pyridine (10 µL) and CH_2Cl_2 (500 µL) overnight at room temperature and subsequently analyzed by chiral GC.

Cyanohydrin synthesis small-scale reactions of *Hb*HNL muteins with aldehyde 2b: Preparations of the *Hb*HNL enzyme variants (80–150 U mmol^{-1} aldehyde) were diluted with aqueous buffer solution (30 mM, citrate, pH 5.5) to an end volume of 1.5 mL. Aldehyde **2b** (140 mg, 0.71 mmol, 1 equiv) was dissolved in *t*BME (1.5 mL), mixed with the enzyme solution in 30 mL glass vials, and stirred at 0°C for 10 min. HCN (50 µL, 1.3 mmol, 1.8 equiv) was added and the solution stirred at 0°C and 650 rpm. At the times indicated, 100 µL samples were taken and extracted with CH_2Cl_2 (300 µL). The organic layer (100 µL) was used for derivatization with acetic anhydride (10 µL) in the presence of pyridine (10 µL) and CH_2Cl_2 (500 µL) overnight and at room temperature and subsequently analyzed by chiral GC.

Bacterial strains and vector: *Escherichia coli* XL1-Blue (Genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′*proAB lacI^{q}ZDM15 Tn10* (Tet^r)]; Stratagene, La Jolla, CA) was used as the host for mutant libraries. Strains *E. coli* TOP 10F′ (Genotyp: F [*lacI*q Tn*10* (tetR)] *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) *ϕ*80 *lacZ* ΔM15 Δ*lacX*74 *recA*1 *deoR araD*139 Δ(*ara-leu*)7697 *galU galK rpsL* (strR) *endA*1 *nupG*) (Invitrogen, San Diego, CA) and *E. coli* XL1-Blue were used for basic genetic work. The *Hevea brasiliensis hnl*_wild-type gene as well as the *Hevea brasiliensis hnl*_tunnel mutant genes (W128A and W128F), were cloned into the plasmid pSE420 (Invitrogen, San Diego, CA) *via NcoI* and *HindIII* restriction sites. The genes are under the control of the inducible *trc* promoter (*trp-lac*), allowing strong transcription initiation for expression of the gene of interest.

Media and culture conditions: *E. coli* strains were routinely grown in Luria–Bertani (LB) media that was supplemented with ampicillin (100 mg L^{-1}) to select for the plasmids at 30°C or 37°C. LB solid media contained agar (15 g L^{-1}) and ampicillin (100 mg L^{-1}) (LB-Amp media). Alternatively, shake flask fermentations for HNL production were performed in 2*TY media (16 g L^{-1} tryptone/peptone, 10 g L^{-1} yeast extract, 5 g L^{-1} sodium chloride) supplemented with ampicillin (100 mg L^{-1}). Induction of HNL expression was induced by IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 0.1 mM in case of solid media and 0.1 mM–1 mM in case of liquid cultures.

Mutagenesis

Random mutagenesis: Random mutations of the *hnl_W128A* gene were introduced by error-prone PCR.[10,27] Plasmid pSE420 harboring the *hnl_W128A* gene served as template for amplification of the 1031 bp insert with the primer pair pSE420forward (5′-GGCACTCGACCGGAATTATC-3′) and pSE420reverse (5′-CACTTCTGAGTTCGGCATGG-3′). Increase in error rates was performed by the addition of $MnCl_2$ and increased amounts of $MgCl_2$. Reactions were performed at a volume of 50 µL containing 2 ng of template DNA (QIAprep-Mini-DNA-preparations of plasmid pSE420HNL_W128A (Quiagen)), 75 pmol of pSE420forward and 112.5 pmol of pSE420reverse primer, 20 nmol dNTPs (dNTP = deoxyribonucleotide triphosphate), 7 mM $MgCl_2$, 0.2 mM $MnCl_2$ and 2.5 U of *Taq* DNA polymerase (Fermentas) in 10x reaction buffer solution (provided with the enzyme). PCR was performed under the following conditions: 3 min at 95°C, followed by 35 cycles of incubation at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min and final incubation at 72°C for 10 min. PCR products were purified by using the QIAquick PCR Purification Kit (Qiagen) and cloned into plasmid pSE420 by using *NcoI* and *HindIII* restriction sites. Ligation-mixtures were desalted (MF-membrane filters, Millipore) prior to electro-transformation into *E. coli* XL1-Blue. An aliquot of regenerated cells was plated in different dilutions to calculate the number of independent clones within the library and the rest was plated in 200 µL portions on LB-Amp agar plates. After overnight incubation at 37°C, the cell-lawn was harvested by suspending with a Drigalski-spatula in LB liquid media supplemented with 15% glycerol, and aliquots were frozen in liquid nitrogen and stored at −70°C. The amplified mutant library was used for screening.

Site-directed mutagenesis: Separation of multiple mutations within the identified mutants (Q215H, I219V, H31Y, H103L, K147R, P187L) and creation of a triple mutant (W128A/H103L/P187L) was performed by overlap-extension PCR.[28] Six mutagenic primers containing the codons for the desired amino acids and six appropriate internal primers were designed (see Table 4). The mutagenic primers contained 8–13 nucleotides at the 5′ end complementary to the 5′ end of the corresponding internal primers. The mutagenic primers and primer pSE420reverse (5′-CACTTCTGAGTTCGGCATGG-3′) were used to generate the first PCR fragments in each case. The internal primers and the standard primer pSE420forward (5′-GGCACTCGACCGGAATTATC-3′) were used to generate the second PCR fragments. These PCR reactions were performed as follows: 10 µL 5× buffer solution (supplied with enzyme),

Table 4. Primers.

Mutagenic primers	Sequence (5′→3′)
Q215H	TACCTGAATTTCATCTCTGGC
I219V	CAACTCTGGCAAATAGAAAAC
H31Y	CTTGAGGCACTTGGCTACAAGG
H103L	CAGCTGCTGTTTTCCTCAATTC
K147R	GGAGATAACTGGATTGAGACTGG
P187L	TTAGCTAAGCGACTATTCTTC
Internal primers	
Q215H-internal	TTCAGGTAAAAATATTTCGTC
I219V-internal	GCCAGAGTTGAAATTCAGG
H31Y-internal	TGCCTCAAGGAGGGGTTTGAGC
H103L-internal	ACAGCAGCTGCAATCTTTTC
K147R-internal	TCCAGTTATCTCCTTGCCATC
P187L-internal	GTCGCTTAGCTAAAATATTTTG

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10 ng of DNA template (pSE420HNL_W128A), 20 pmol of forward and reverse primer, 20 nmol dNTPs, and 0.6 U *Phusion* DNA polymerase (Finnzymes). The reaction mixture was heated at 98 °C for 30 s, followed by 30 cycles of incubation at 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 15 s. Final incubation at 72 °C lasted for 7 min. Full length of the *hnl* gene was reconstituted by an overlap extension PCR. Therefore, the gel-purified fragments (Gel Purification Kit, Sigma) synthesized by primary PCR were mixed together and the reactions were performed as follows: 10 µL 5× buffer soltion (supplied with enzyme), 10 ng of PCR product 1, 10 ng of PCR product 2, 20 nmol dNTPs, and 0.6 U *Phusion* DNA polymerase (Finnzymes). The reaction mixtures were heated at 98 °C for 30 s, followed by 35 cycles of incubation at 98 °C for 10 s, 55 °C for 25 s and 72 °C for 55 s. Final incubation at 72 °C lasted for 7 min. After 10 cycles, the reaction was paused and 20 µL of a PCR mix containing 20 pmol of each, pSE420forward and pSE420reverse primer, were added. Thereby the entire *hnl* gene was amplified. Further components of the added PCR mix were: 5× *Phusion* buffer solution, 200 µM dNTPs and 0.6 U *Phusion* DNA polymerase. Overlap PCR products were purified over gel (Gel Purification Kit, Sigma) and cloned into pSE420. Multiple transformants were sequenced to confirm that they were harboring the right mutations.

Screening for HNL activity by a colorimetric filter assay: Screening for improved enzyme variants was performed by following the cleavage reaction of cyanohydrins **5** by using the filter assay according to Krammer et al.[10] Amplified mutant libraries were plated on LB-Amp agar plates and grown over night at 37 °C. The next day, colonies were lifted on nitrocellulose membranes (Biodyne A, 0.45 µm, Pall Corporation). For induction of HNL expression, membranes with colonies on it were transferred on LB-Amp agar plates supplemented with 100 mg L^{-1} IPTG (isopropyl-β-D-thiogalactopyranoside) and incubated for 24 h at 20 °C to avoid the formation of inclusion bodies. The membrane-blotted colonies of *E. coli* mutant libraries were equilibrated to pH 5.9 for 15 min for screening with **5a** and to pH 4.0 for 30 min for screening with **5b.** For preparation of the substrate solutions, 40 µL of **5a** was dissolved in DMF (1.5 mL) prior to the addition of 0.1 M citrate/phosphate buffer solution (3.5 mL, pH 5.3) and **5b** (20 µL) was dissolved in DMF (3 mL) prior to the addition of 0.1 M citrate/phosphate buffer solution (7 mL, pH 5.5). Instead of the permeable nylon tissue, a mosquito net was used. Alternatively, for re-screening and examination of single mutants, transformants were placed in a 96-well microtiter plates, filled with 150 µL LB-Amp media per well, with sterile toothpicks and grown for several hours or overnight at 37 °C. Mutants were stamped from the master plate directly on nitrocellulose membranes and growing, induction, and screening was performed as described above.

Enzyme preparation: *E. coli* strains were grown over night on LB-Amp agar plates and single-cell generated colonies were used to inoculate 100 mL of LB-Amp liquid media (in 300 mL baffled Erlenmeyer flasks). Pre-cultures were grown at 37 °C and 150 rpm for approximately 16 h. LB-Amp media (330 mL in 1 L baffled Erlenmeyer flasks) were inoculated with the pre-cultures to an optical density (OD_{600}) of 0.15. Further incubation was performed at 30 °C and 150 rpm. After 3–5 h at an OD_{600} of approximately 0.8, the main cultures were induced with IPTG to a final concentration of 0.1–1 mM and further incubated for 20–24 h at 20 °C and 150 rpm. The cells were harvested by centrifugation for 20 min, 5000 × g at 4 °C. The supernatant was removed and the cell pellet was dissolved in 10 mM phosphate buffer solution (15 mL; pH 6.5). Cells were disrupted by ultrasonic treatment (Branson sonifier 250) for 6 min (80 % output, 80 % duty cycle) under permanent cooling in an ice-water bath. Soluble and insoluble fractions were separated by centrifugation for 1 h at 20000 rpm and 4 °C. Soluble fractions were collected, lyophilized and redissolved in an appropriate smaller volume of deionised water to concentrate the samples.

Enzyme activity determination: HNL activity of lyophilized crude lysates was determined by spectrophotometrically following the formation of benzaldehyde as described in the literature.[10]

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