Hydroxynitrile Lyase Isozymes from *Prunus communis*: Identification, Characterization and Synthetic Applications

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Abstract: Biocatalysts originating from Badamu (*Prunus communis*) have been applied to catalyze the asymmetric synthesis of (R)-4-methylsulfanyl-mandelonitrile, a key building block of thiamphenicol and florfenicol. Here, four hydroxynitrile lyase (HNL) isozymes from Badamu were cloned and heterologously expressed in *Pichia pastoris*. The biochemical properties and catalytic performances of these isozymes were comprehensively explored to evaluate their efficiency and selectivity in asymmetric synthesis. Among them, *Pc*HNL5 was identified with outstanding activity and enantioselectivity in asymmetric hydrocyanation. Under the optimized mild biphasic reaction conditions, seventeen prochiral aromatic aldehydes were converted to valuable chiral cyanohydrins with good yields (up to 94%) and excellent optical purities (up to >99.9% ee), which provide a facile access to numerous chiral amino alcohols, hypoglycemic agents, angiotension converting enzyme (ACE) inhibitors and β-blockers. This work therefore underlines the importance of discovering the most potent biocatalyst among a group of isozymes for converting unnatural substrates into value-added products.

Keywords: biocatalysis; characterization; chiral cyanohydrins; hydrocyanation; hydroxynitrile lyase isozymes

Introduction

Hydroxynitrile lyases (HNLs) were first isolated from cyanogenic plants and are one of very few classes of biocatalysts capable of catalyzing the formation of C–C bonds selectively.[1] HNLs catalyze the cleavage of cyanohydrins to release HCN, which plays an important role in the plant defense system.[2] The reverse reaction, which is also driven by HNLs, involves the enantioselective formation of cyanohydrins from HCN and aldehydes or ketones under mild conditions. This reaction has attracted considerable interest because the hydroxy and nitrile moieties in cyanohydrins are versatile functional groups in pharmaceuticals and agrochemicals.[3]

Although HNLs were discovered more than one century ago,[4] their use was hampered by the unsatisfactory enantiomeric excess (ee) of the cyanohydrin products. This issue is mainly attributed to the occurrence of the competitive spontaneous (non-selective) addition reaction. An ideal enzymatic system for the asymmetric hydrocyanation of aldehydes and ketones should therefore consist of a potent HNL that is capable of overriding the deleterious side reaction. Several biphasic and micro-aqueous systems have consequently been designed to suppress the formation of the undesired enantiomer from the hydrocyanation reaction.[5] However, the native HNLs are expressed with low amounts in natural tissues, making it difficult to obtain sufficient enzyme for synthetic usage. Accordingly, the heterologous expression in microbial hosts is an effective alternative strategy for enzyme mass production for the chiral cyanohydrin synthesis. Successful precedent examples include (S)-selective HNLs derived from *Manihot esculenta* (*Me*HNL, cassava)[6] and *Hevea brasiliensis* (*Hb*HNL, rubber tree);[7] the (R)-selective HNLs derived from *Linum usitatissimum* (*Lu*HNL, flax)[8] and *Arabidopsis thali-
**Results and Discussion**

Although there has been a preference for acquiring complete eukaryotic HNL sequences via the construction and screening of cDNA libraries,\(^{[7a,10]}\) this type of strategy is labor intensive and time consuming. Instead, we aligned the reported gene sequences of all the HNLs isolated from *Prunus* species and designed homology primers based on several conserved regions of these genes. Genomic DNA isolated from the kernels of Badamu was used as a template to perform PCR. The amplified band was about 2300 bp and contained four independent genes. All of the *PcHNL* isoyme genes contained 3 short introns and a highly conserved signal peptide. It is noteworthy that all of these isoymes were similar to the known (R)-selective HNLs from different species of the *Prunus* family (i.e., *P. amygdalus, P. persica* and *P. mume*). The four different isoymes were therefore named after the HNLs with which they had the most in common. In this study, *P. persica* was chosen as a heterologous host for the large scale production of the HNLs. Initially, very low titers (1–2 U mL\(^{-1}\)) of recombinant HNLs were harvested by shake flask incubation. However, following redesign of the HNL genes, we observed considerable increases in the activity (four- to six-fold). Further fermentation in a 5 L bioreactor yielded 83, 190, 420 and 380 kU of *PcHNL* 1, 2, 4 and 5, respectively, per liter of yeast culture. It is noteworthy that one liter of fermentation supernatant was equal to the amount of enzyme isolated from 0.4–2 kg of defatted kernel meal or 1.5–7.5 kg of fresh kernel tissue from Badamu. Since *Pichia pastoris* only secretes very small amounts of its endogenous proteins, the use of recombinant *PcHNL* represented an efficient strategy for the rapid preparation of large amounts of these materials in high purity. The re-
combinant system also represents a low-cost alternative to long-term accumulation of native PcHNLS in the plant. This was particularly useful since the HNLs isolated from the native plant tissue showed high variability in terms of the enantiomeric purity of their cyanohydrin products.

The N-terminal His-tagged recombinant PcHNLS were concentrated by ultrafiltration and subjected to fractional precipitation with 1,2-dimethoxyethane (DME), before being purified by nickel affinity chromatography. We initially investigated the purified native PcHNLS, which consisted of a mixture of four isozymes of approximately 63 kDa in weight with different pi values (data not shown). However, all of the recombinant PcHNLS were observed as overglycosylated proteins by SDS-PAGE analysis, which was a similar result to that observed for recombinant PaHNLS (Supporting Information, Figure S2).[^21]

Micro-aqueous systems have been proven to suppress the formation of racemic addition products. However, the application of this strategy to Badamu meal afforded the desired addition product 2a in only 95% ee prior to recrystallization,[^34] which was the result of the combined performance of different isozymes present in Badamu meal. Thus we firstly measured the steady-state kinetic parameters of the isozymes towards 1a (Table 1, entries 1–4). The results revealed that the catalytic efficiency ($k_{cat}/K_m$) was in the range of 17–61 mmol s$^{-1}$ g$^{-1}$. However, when catalyzing the natural substrate mandelonitrile (2b), the catalytic efficiency of PcHNLS was 2.7–25-times more than that of 1a, as is commonly observed in dehydrocyanation. It indicates that these four isozymes exhibited lower catalytic activities towards the unnatural substrate.

The asymmetric hydrocyanation of substrate 1a was carried out in the presence of each of the four pure isozymes in a biphasic methyl tert-butyl ether (MTBE)-citrate buffer system. PcHNLS1 and PcHNLS4 both showed poor conversions (38.6% and 14.4%, respectively) and showed clear differences in the enantioselectivity (90.2% and 70.3% ee, respectively) after 12 h (Figure 1, A and C). In contrast, PcHNLS2 and PcHNLS5 performed much more effectively with respect to their conversion or enantioselectivity (Figure 1, B and D). The cleavage of racemic mandelonitrile was chosen as a model reaction for evaluating the enzymatic and physicochemical properties of the different PcHNLS. All the isozymes exhibited good thermostability, even when they were incubated at 65°C for 1 h (Supporting Information, Figure S5). Moreover, the deactivation temperature of the PcHNLS showed a positive correlation with the increased potential of the N-glycosylation motif (N-X-S/Y). PcHNLS5 showed the greatest stability of all of the isoymes tested towards acidic buffer with an optimum pH of 5.0, whereas all of the other isoymes quickly lost their activities in the acidic buffer (Supporting Information, Figure S4). The tolerance of this enzyme for MTBE was also evaluated (Supporting Information, Figure S6), and the results showed that all of the isoymes except PcHNLS5 precipitated from MTBE after they had been vigorously stirred at 30°C for 36 h. Based on the results above, PcHNLS5 was

![Table 1: Comparison of kinetic parameters of PcHNLS towards (R)-4-methylsulfonylbenzaldehyde (1a) and natural substrate mandelonitrile.](image)

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate</th>
<th>Isozyme</th>
<th>$K_m$ [mM]</th>
<th>$k_{cat}$ [s$^{-1}$]</th>
<th>$k_{cat}/K_m$ [s$^{-1}$mM$^{-1}$]</th>
<th>$V_{max}$ [μmol min$^{-1}$ mg$^{-1}$]</th>
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<tbody>
<tr>
<td>1</td>
<td>1a (R$^1$=p-MS)[^a]</td>
<td>PcHNLS1</td>
<td>2.29 ± 0.72</td>
<td>139 ± 17</td>
<td>61</td>
<td>134 ± 16</td>
</tr>
<tr>
<td>2</td>
<td>1a (R$^1$=p-MS)[^a]</td>
<td>PcHNLS2</td>
<td>1.09 ± 0.12</td>
<td>40.3 ± 1.3</td>
<td>37</td>
<td>35.0 ± 1.0</td>
</tr>
<tr>
<td>3</td>
<td>1a (R$^1$=p-MS)[^a]</td>
<td>PcHNLS4</td>
<td>1.54 ± 0.51</td>
<td>26.3 ± 2.7</td>
<td>17</td>
<td>22.0 ± 2.0</td>
</tr>
<tr>
<td>4</td>
<td>1a (R$^1$=p-MS)[^a]</td>
<td>PcHNLS5</td>
<td>2.97 ± 0.31</td>
<td>142 ± 9</td>
<td>48</td>
<td>120 ± 7</td>
</tr>
<tr>
<td>5</td>
<td>2b (R$^1$=H)[^b]</td>
<td>PcHNLS1</td>
<td>1.16 ± 0.05</td>
<td>240 ± 4</td>
<td>207</td>
<td>229 ± 3</td>
</tr>
<tr>
<td>6</td>
<td>2b (R$^1$=H)[^b]</td>
<td>PcHNLS2</td>
<td>1.47 ± 0.24</td>
<td>380 ± 31</td>
<td>258</td>
<td>327 ± 27</td>
</tr>
<tr>
<td>7</td>
<td>2b (R$^1$=H)[^b]</td>
<td>PcHNLS4</td>
<td>1.91 ± 0.19</td>
<td>818 ± 27</td>
<td>428</td>
<td>690 ± 23</td>
</tr>
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<td>8</td>
<td>2b (R$^1$=H)[^b]</td>
<td>PcHNLS5</td>
<td>6.36 ± 0.18</td>
<td>817 ± 8</td>
<td>128</td>
<td>697 ± 4</td>
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</tbody>
</table>

[^a]: Synthesis of 4-methylsulfonyl-mandelonitrile was monitored by HPLC (see the Experimental Section for details).
[^b]: Activity was determined by UV-Vis absorbance at 280 nm of benzaldehyde that is cleaved from racemic mandelonitrile.

The reaction system contained 980 μL citrate buffer (100 mM, pH 5.0), 10 μL DMSO solution of mandelonitrile with varied concentrations, and 10 μL HNL solution with an appropriate concentration to maintain the variation within 0–0.1 units.

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identified as the best isozyme in terms of its conversion and enantioselectivity, and was selected for further studies.

The main challenge in asymmetric hydrocyanation is to control the non-selective spontaneous reaction, which will deteriorate the optical purity of the product. One strategy for avoiding this possibility is to complete the reaction as soon as possible prior to the accumulation of the undesired enantiomer. With this in mind, we investigated the synthesis of (R)-2a using different amounts of lyophilized crude recombinant PcHNL5, which was prepared in a biphasic reaction system (Figure 2). For the initial attempt, a 2–10 g L⁻¹ of HNL loading resulted in a reasonable conversion (94.7–97.3%) of 1a and with 98.6–99.6% ee of 2a after 12 h. However, reducing the catalyst loading to 1 g L⁻¹ led to a decrease in substrate conversion as well as ee deterioration of 2a (95.5% ee) due to the accumulation of the racemic product. Based on a compromise between reactive performance, cost, and enantioselectivity, we selected an HNL loading of 5 g L⁻¹ for further optimization.
The pharmaceutical industry not only requires optically pure chemicals to simplify the synthesis of biologically active compounds, but also to decrease the amount of energy consumed during the synthesis of these compounds. Biphasic asymmetric hydrocyanation reactions are generally conducted at low temperatures (4–10°C) to suppress the formation of racemic addition products. However, the use of low temperatures is often accompanied by a considerable decrease in enzymatic activity. As shown in Table 2, the performance of PcHNL5 was encouraging when the reaction temperature was elevated from 15 to 25°C (entries 1–3). These conditions gave good conversions (94–96%) and the desired product 2a was also achieved with excellent optical purity (>99% ee) over a much shorter time period (3.5 h versus 7 h).

Lowering the pH of the aqueous phase is also an alternative strategy for suppressing the occurrence of non-selective addition reactions. However, a lower pH did not show a significant improvement in terms of isolated yield and enantioselectivity (Table 2, entry 4). In contrast, setting the pH of the aqueous phase to 6 resulted in a lower optical purity of the cyanohydrin product. To increase the atom economy of the target reaction whilst also reducing the cost of the toxic cyanide reagent, the loading concentration of aldehyde at the same equivalent of the cyanide source was investigated as well. When the loading of 1a was increased from 0.2 to 4 M with an HNL loading of 5 g per mol of 1a, the aldehyde could almost completely converted within 4 h (Table 2, entries 6, 7, and 8). However, when the molar ratio of HCN to the aldehyde was less than 2, the conversion was stalled at around 80% and both the isolated yield and optical purity of the product declined even when the reaction time was prolonged to 24 h (entries 8 and 9). Compared with the micro-aqueous hydrocyanation catalyzed by defatted kernel meal from *P. communis*, the optimal reaction conditions directly (Table 2, entry 7) afforded 2a with an STY of up to 343.4 g L⁻¹ d⁻¹ in much higher optical purity without recrystallization.

This newly developed asymmetric hydrocyanation was further applied to a wide range of aldehydes at room temperature (Table 3). The majority of the cyanohydrins are intermediates for the preparation of bioactive drugs such as semi-synthetic cephalosporins (antibacterial agents), clopidogrel (anticoagulant), cycloprothrin (insecticide), hypoglycemic agents, ACE inhibitors, and β-blockers. Most of the substituted benzaldehydes evaluated in the current study were readily converted to the corresponding cyanohydrins with good yields (90–95%) and excellent optical purity (95% to >99.9% ee). However, ortho-chlorobenzaldehyde (1c) gave a much lower optical purity (83.5% ee) than the corresponding meta- and para-substituted counterparts under the current optimal conditions (entry 2). In contrast to the engineered PaHNL5 variants or AtHNL, *PcHNL5* exhibited a lower selectivity towards benzaldehydes bearing an electron-withdrawing group at the ortho-position of their benzene ring, whereas substrates bearing an ortho-substituted electron-donating group (1d) gave 95% ee (entry 3). Furthermore, longer reaction times were needed for the complete conversion of substrates bearing bulkier substituents (entries 6, 15–17). Notably, *PcHNL5* showed a much higher substrate preference to 2-naphthaldehyde (1q) than 1-naphthaldehyde (1p) (entries 16 and 17), while *PmHNL* which also tolerated a wide range of aldehydes showed little difference in catalyzing these two bulky aldehydes. However, it showed much lower enantioselectivity (entries 12–14) for substrates bearing a heterocyclic substituent, compared to the results when using defatted bitter almond meal (mixture of

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**Table 2. Optimizing the reaction parameters of *PcHNL5*-catalyzed asymmetric hydrocyanation of 1a.**

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<td>80</td>
<td>95</td>
<td>73</td>
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</table>

[a] The reaction mixture, containing 10–100 mg lyophilized crude *PcHNL5* preparation (12.6 U/mg), 1–5 mmol 1a, 2.5 mL 2M HCN dissolved in MTBE and citrate buffer (100 mM, pH 5.0) up to 5 mL, was agitated at 15–25°C.
[b] Concentrations were calculated by the total volume.
[c] Determined by chiral HPLC.
[d] Determined by chiral HPLC after acetylation of the resultant cyanohydrins.
[e] Isolated yield after purification with silica gel column chromatography.
isozymes of *Prunus amygdalus* in a micro-aqueous system. To summarize, this *Pc*HNL5-catalyzed system could broadly effect the asymmetric hydrocyanation of aromatic aldehydes for the synthesis of chiral cyanohydrins.

Conclusions

Several recombinant isozymes from *Prunus communis* were prepared and evaluated in terms of their catalytic properties and synthetic performances for the preparation of (R)-4-methylsulfanylmandelonitrile. *Pc*HNL5 was identified as the best isozyme because of its high tolerance towards MTBE, as well as its outstanding catalytic efficiency. This isozyme could therefore be used to catalyze a wide range of existing asymmetric hydrocyanation reactions. The optimization of the reaction parameters for this isozyme resulted in the development of a room-temperature, single isozyme-catalyzed hydrocyanation system for the enantioselective synthesis (up to 99.9% ee) of valuable (R)-cyanohydrins in good yields (up to 95%). Notably, this work demonstrates that the evaluation of biocatalytic properties in combination with synthetic performance can yield insights on understanding of the proficiency of a biocatalyst system. This report therefore represents an effective process for identifying unknown biocatalysts for the efficient conversion of unnatural substrates into structurally diverse products.

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**Table 3. Substrate scope of the asymmetric hydrocyanation by *Pc*HNL5 at room temperature.**

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate 1 [R&lt;sup&gt;1&lt;/sup&gt;=]</th>
<th>Time [h]</th>
<th>Conv. [%]&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ee [%]&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Yield [%]&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Entry</th>
<th>Substrate 1 [R&lt;sup&gt;1&lt;/sup&gt;=]</th>
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<th>Conv. [%]&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ee [%]&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Yield [%]&lt;sup&gt;c&lt;/sup&gt;</th>
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[a] The reaction system containing 50 mg lyophilized crude *Pc*HNL5 preparation (12.6 U/mg), 2 mmol aldehyde, 2.5 mL 2M HCN dissolved in MTBE and citrate buffer (100 mM, pH 5.0) up to 5 mL, was agitated at 25°C.

[b] Determined by chiral HPLC.

[c] Isolation yield after silica gel column chromatography.

[d] The product was racemic.
Experimental Section

General

Badamu seeds were purchased from Kashgar, Xinjiang Province, China. E. coli Top10 and Pichia pastoris X33 were chosen as cloning and heterologous expression hosts, respectively. Plasmids pMD-19T and pPICZαA were used for cloning and expression, respectively. Benzaldehyde and NaCN were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 4-Methyliobiobenzaldehyde (97%), 2-chlorobenzaldehyde (99%) and 4-methoxybenzaldehyde (98%) were purchased from Tokyo Chemical Industry (Shanghai, China). 2-Methoxybenzaldehyde (98%) and furfural (99.5%) were purchased from Aladdin (Shanghai, China). 3-Pyridinecarboxyaldehyde (97%), 1-naphthaldehyde (97%), 1,4-benzodioxan-6-carboxaldehyde (98%) and mandelonitrile (98%) were purchased from J&K Chemicals (Beijing, China). 3-Phenoxybenzaldehyde (98%), 2-thienaldehyde (97%) and phenylpropyl aldehyde (95%) were purchased from Energy Chemical (Shanghai, China). 3-Chlorobenzaldehyde (98%), 2-naphthaldehyde (98%), 4-methylbenzaldehyde (97%), phenylacetalddehyde (95%) and trimethylstil cyanide (96%) were purchased from Macklin (Shanghai, China). Unless specified otherwise, all the other chemical reagents were also obtained commercially, with the highest purity available, and used without further purification. All the aldehydes were freshly washed with saturated KHC03 solution, dried, and stored under argon. 1H NMR and 13C NMR spectra were measured on a Bruker Avance spectrometer (400 MHz) using tetramethylsilane (TMS) as an internal standard.

Cloning and Heterologous Expression of PcHNLs

Genomic DNA of Prunus communis was isolated from kernels by phenol-chloroform extraction. Primers were designed according to known conserved coded sequences of all Rosaceae HNLs to get full length gene sequence. For more details see the Supporting Information. Each isozyme gene was ligated into pPICZαA by restrict enzymatic digestion (EcoRI and Not I) with N-terminal 6xhis-tag and transferred into Pichia pastoris X33. Competent cells, transformation and multiple integrant transformants were prepared according to the Pichia Expression Kit (Invitrogen, USA). The positive Mut+ transformant with highest HNL activity was selected for further 5-L fermentation.

Purification of PcHNLs

The HNL preparations were produced in a 5-L bioreactor, concentrated and chromatographically purified. The protein concentration was determined according to the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific™, USA). The calculation of the molarity of PcHNL was based on FAD spectral scan, more details are shown in the Supporting Information, and the protein purity was examined by 10% SDS-PAGE. All of the subsequent steps were performed at 4°C.

Fermentation broth was centrifuged at 6,000 x g for 20 min to remove the biomass. The supernatant was concentrated by ultrafiltration until the concentration of proteins was higher than 2 mg/mL if it is necessary. Prior to the further purification procedure, the concentrated HNL preparations were diluted and concentrated repeated with citrate buffer (20 mM, pH 5.5) to remove pigment and ions in the culture. Such procedures were carried out by a Labscale™ Tangential Flow Filtration System (Merck Millipore, German) equipped with a 30 kDa cut off module (Pellicon® XL, 50 cm², Millipore, Germany).

After 30–60% fractional precipitation with DME, the yellow precipitant (HNL) was dissolved in iced binding buffer A (20 mM sodium phosphate, 500 mM NaCl, 5 mM 2-mercaptoethanol, pH 7.4) and loaded onto a Ni-NTA column. Most of the unbound proteins were removed by washing the column with 10 column volumes of binding buffer A. Then HNL was eluted with 5–100% elution buffer B (20 mM sodium phosphate, 500 mM imidazole, 500 mM NaCl, 5 mM 2-mercaptoethanol, pH 8.0). It is easy to observe the yellow color of PcHNLs due to the cofactor FAD in the desired elution fragments. The fragments containing highly pure PcHNLs were collected, concentrated and desalted with buffer C (20 mM sodium phosphate, 20% glycerol, v/v, pH 7.4).

Lyophilized HNL Preparation

To prepare lyophilized HNL for asymmetric hydrocyanation, all steps were the same as the purification procedure except for 30–60% fractional precipitation with DME. Desalted HNL preparations were placed at –80°C overnight, lyophilized under vacuum at –60°C, collected and stored at 4°C until use.

Assay of Enzymatic Kinetics

The synthesis of 2a from 1a was monitored by HPLC in a 0.5-mL scale reaction which employed 1M citrate buffer (pH 4.2) to suppress the spontaneous hydrocyanation. HNL samples were diluted in the above buffer, containing 0.2–30 mM 4-methylsulfanylbenzaldehyde, 1% DMS0 (v/v) and 100 mM NaCN, and incubated at 25°C for 1 min. The reactant was extracted with one reaction volume of MTBE, then analyzed by HPLC using a Shimadzu LC-2010A liquid chromatograph equipped with an OD-H column (250 mm x 4.6 mm, 5 μm particle size, Daicel) and a UV detector. For each data point, the rate of non-enzymatic reaction as the control was also measured and subtracted from the total value.

HCN-MTBE Formation

Caution: Because hydrogen cyanide and sodium cyanide are extremely toxic, all experiments related to cyanide should be performed in a well-ventilated hood! All cyanide waste should be decontaminated by saturated FeCl3/FeSO4. It is suggested that all the apparatus should be soaked in 10% hydrogen peroxide for 12 h before use.

HCN-MTBE should be used as freshly prepared. Based on Pohl’s method, 0.98 g sodium cyanide was mixed homogeneously with 2 mL water and 5 mL MTBE at 0°C, then 2.5 mL 4M sulfuric acid was added slowly dropwise. The mixture was kept stirring for about 15 min. After separation, 5 mL MTBE were added to extract HCN from the residual aqueous phase. The combined organic layers were transferred into a small dark bottle and stored at 0°C.
General Procedure for Enzymatic Reactions

The activity of lyophilized HNL powders was determined according to Hanefeld’s work. HNL was firstly diluted in 100 mM citrate with an appropriate pH value. Aldehyde (the total volume of aldehyde and aqueous phase makes up half of the system) was added as follows, then the reaction was started with the addition of 2 M HCN-MTBE. The reaction was traced by thin-layer chromatography (TLC) until no more product was formed. Then the reaction was forced to terminate by ethyl acetate (EA) extraction, the combined organic phase was washed by aqueous saturated FeCl$_3$ until the color of FeCl$_3$ remained unchanged, dried over Na$_2$SO$_4$, and evaporated under reduced pressure, and purified by fast chromatography on silica gel.

Racemic Cyanohydrins Standard and Acetylated Derivative for HPLC Analysis

Cyanohydrin synthesis: based on the work of Ohkuma et al. 5 mmol of aldehyde were added in a 25-mL glass vessel and mixed with 1 equiv. trimethylsilyl cyanide on an ice bath. The reaction was started by adding one drop of 100 mM LiCl dissolved in tetrahydrofuran (THF), warmed spontaneously to room temperature and further stirred for 1 h. Then 5 mL EA were added, and the TMS group was removed by mixing vigorously with 1 N HCl at room temperature. The reaction mixture was extracted by EA, washed in sequence with saturated KHCO$_3$ and NaCl solutions, dried over anhydrous Na$_2$SO$_4$, evaporated, and purified by column chromatography on silica. All of the racemic cyanohydrins obtained by the above method were fully characterized by $^1$H NMR spectroscopy and used as HPLC standards.

Acetylation of products: 1 mmol of cyanohydrin was diluted with 2 mL dichloromethane, 400 µL acetic anhydride, 5 mg 4-dimethylaminopyridine (DMAP), and 200 µL pyridine were added in that order and stirred. Then after being diluted with 10 mL EA, aqueous saturated CuSO$_4$ solution and water were used to remove pyridine and redundant acetic anhydride, respectively. The organic phase was dried over anhydrous Na$_2$SO$_4$, evaporated, and purified by silica column chromatography. All of acetylated racemic cyanohydrins obtained were fully characterized by $^1$H NMR spectroscopy and used as HPLC standards.

Determination of the Biotransformation Conversion and Enantiometric Excess

At the scheduled time, 50 µL samples from the reaction mixture were taken and extracted by MTBE (450 µL), the organic layers were mixed with 80 µL acetic anhydride, 50 µL pyridine, 1 mg DMAP, agitated for 2 h, then washed with aqueous saturated CuSO$_4$ solution and water, dried over anhydrous Na$_2$SO$_4$. Then all the MTBE was evaporated and exchanged with n-hexane/2-propanol (see ratio in the Supporting Information, Table S1), and analyzed by HPLC.

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References


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