A high-throughput screening assay for hydroxynitrile lyase activity

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A high-throughput screening assay for hydroxynitrile lyase activity accepting a wide range of HNL-substrates is presented, which is useful either for enzyme fingerprinting or screening of huge variant libraries generated in metagenome or directed evolution approaches.

Hydroxynitrile lyases (HNLs) naturally occur in plants integrated in microbial and herbivore defense mechanisms by HCN-release due to cyanohydrin cleavage. Some HNLs are biochemically characterized in detail concerning substrate specificity and enantioselectivity. The HNLs from *Prunus* species, like *P. amygdalus* (bitter almond)[1a] and *P. mume* (Japanese apricot)[1b] as well as the HNL from *Linum usitatissimum* (flax)[1c] show (*R*)-selectivity, whereas the other HNLs from *Sorghum bicolor* (millet),[1d] *Hevea brasiliensis* (para rubber tree)[1e] and *Manihot esculenta* (cassava)[1f] show (*S*)-selectivity towards different cyanohydrins. Since the reverse reaction is also catalyzed by HNLs, these enzymes are valuable catalysts for the synthesis of cyanohydrins, which are versatile chiral building blocks in the pharmaceutical and agrochemical industries.[1] Apart from their availability, the application of HNLs is often restricted by their substrate range and low stability under technical conditions.

Novel or improved HNLs can be found by screening plants for appropriate enzymes,[2] by directed evolution, rational design, or by metagenomic approaches.[3] However, one major necessity for all these strategies is a simple and powerful high-throughput screening (HTS) assay to identify potential novel or better performing HNLs. So far HNL-activity has usually been determined by GC- or HPLC-analysis,[2a] which is not practical in high-throughput. In addition, a spectrophotometric assay based on HCN-detection using the well-known König reaction[4,5] has been described. However, this assay was applied in a total reaction volume of 10 mL which is not suitable for high-throughput screening approaches. Furthermore, only acetone cyanohydrin has been used so far as a substrate.[6] Another spectrophotometric assay for activity towards benzaldehyde cyanohydrin is available, detecting the increase in absorption of the released benzaldehyde at 280 nm wavelength.[7] Although this assay is applicable for high-throughput, it is restricted to aromatic substrates only and requires microtiter plates (MTPs) which are transparent in the UV. Furthermore, recently a colony assay, based on NADH-fluorescence has been developed to detect HNL-activity towards benzaldehyde cyanohydrin.[8]

Here, we describe an HNL assay in MTP format (200 μL), which allows screening in high-throughput using automated pipetting workstations. The broad applicability of the assay is demonstrated using different aliphatic and aromatic cyanohydrins (Fig. 1); two of them were achiral, four of them were used as a racemic mixture and furthermore benzaldehyde cyanohydrin was applied as (*R*)- and (*S*)-enantiomers to detect the enantioselectivity of recombinant HNL from *Manihot esculenta* (MeHNL) which was used as a model.

The assay consists of two parts, first the biotransformation step yielding HCN by cyanohydrin cleavage; subsequently HCN is detected spectrophotometrically at 600 nm wavelength in MTPs (Fig. 1). The crucial parameter in the cyanohydrin cleavage reaction is the pH, because many cyanohydrins decompose at pH > 6.0, whereas on the other hand the enzyme activity and stability

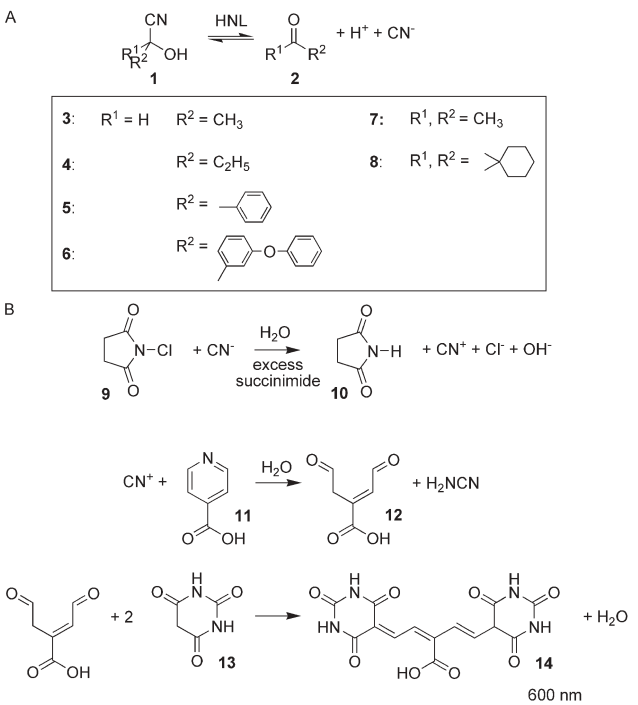

Fig. 1 Schematic overview of the assay system. A: Biotransformation step. The cyanohydrin **1** is enzymatically converted to a carbonyl compound **2** and HCN. Six different cyanohydrins (**3**: acetaldehyde cyanohydrin, **4**: propionaldehyde cyanohydrin, **5**: benzaldehyde cyanohydrin, **6**: 3-phenoxybenzaldehyde cyanohydrin, **7**: acetone cyanohydrin, **8**: cyclohexanone cyanohydrin) were tested with MeHNL. B: Cyanide-determination step (modified according to Markley *et al.*[12]): cyanide anions are oxidized by *N*-chlorosuccinimide **9** (stabilized with succinimide **10**) to cyanide cations, which react with isonicotinic acid **11** forming a dialdehyde **12**, which is coupled to two molecules of barbituric acid **13** to form the dye **14** which is measured spectrophotometrically at 600 nm.

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are significantly impaired at pH < 5.0. Therefore, assaying cyanohydrin cleavage at pH 5.0 to 5.5 is a good compromise.[6,7]

The assay is sufficiently sensitive to screen HNL-libraries using crude cell extracts. For the purpose of library screening, first the enzymatic reaction using *E. coli* crude cell extracts containing over-expressed MeHNL is performed, thereby a certain amount of cyanide is liberated from the cyanohydrin substrate. Therefore, 140 μL citrate–phosphate buffer pH 5.0, 10 μL of HNL containing crude cell extracts and 10 μL cyanohydrin solution (final concentration 15 mM) are mixed and incubated at room temperature for 5 min. By addition of 10 μL of mix I (*N*-chlorosuccinimide **9**/succinimide **10**) the biotransformation step is stopped,[9] thereby oxidizing the liberated CN^- to CN^+. After 2 min the colorimetric detection step is started by adding 30 μL of mix II (isonicotinic acid **11**/barbituric acid **13**).[10] Subsequently, the rate of color formation is measured spectrophotometrically over 20 min at 600 nm using a microtiter plate reader. The dye **14** is stable for at least 2 hours. Barbituric acid **13** is applied instead of the alternatively used dye compound 3-methyl-1-phenyl-5-pyrazolone in HCN-detection,[11,12] because the latter is unsuitable at pH-values below 7.[13] Isonicotinic acid is a well suited alternative to the widely used pyridine.[6]

Instead of measuring the spectrophotometric properties of the resulting aldehyde or ketone, the major advantage of this assay is the possibility of analyzing HNL-activity towards virtually any cyanohydrin by detecting the liberated HCN. This makes the assay suitable for a vast substrate screening as well as for detection of new or improved activities in enzyme libraries obtained by rational design or directed evolution. We have used our HTS-assay to determine MeHNL-activity towards six different aromatic and aliphatic cyanohydrins. All substrates were converted by MeHNL with **5** and **7** being the best substrates. The high selectivity of MeHNL towards the (*S*)-enantiomer of benzaldehyde cyanohydrin **5** is obvious when (*R*)-**5** and (*S*)-**5** are used separately in the assay (Fig. 2).

Furthermore, the assay allows calculation of specific enzymatic activity, since color development in the HCN-detection step is proportional to the amount of cyanide in the solution. Time dependent cyanohydrin conversion was calculated based on a

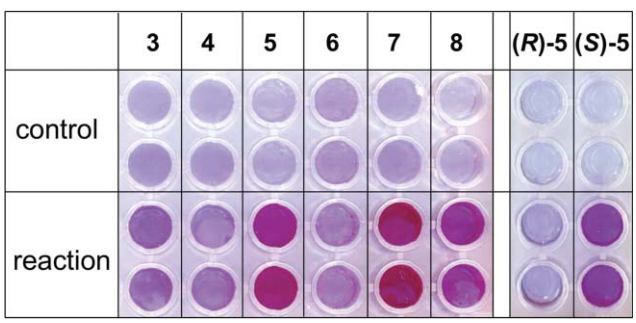

Fig. 2 Spectrophotometric detection of hydroxynitrile lyase activity. Microtiter plate with different substrates **3–8** (see Fig. 1). Control: autolysis of the respective cyanohydrin (without enzyme). For reactions 10 μL of *E. coli* crude cell extracts containing over-expressed MeHNL were used. Faint blue to purple color represents an increasing amount of cyanide. The application of enantiomerically pure substrates can be used to estimate the enantioselectivity of the biocatalyst as demonstrated in the case of enantiomerically pure (*R*)- and (*S*)-benzaldehyde cyanohydrin **5**.

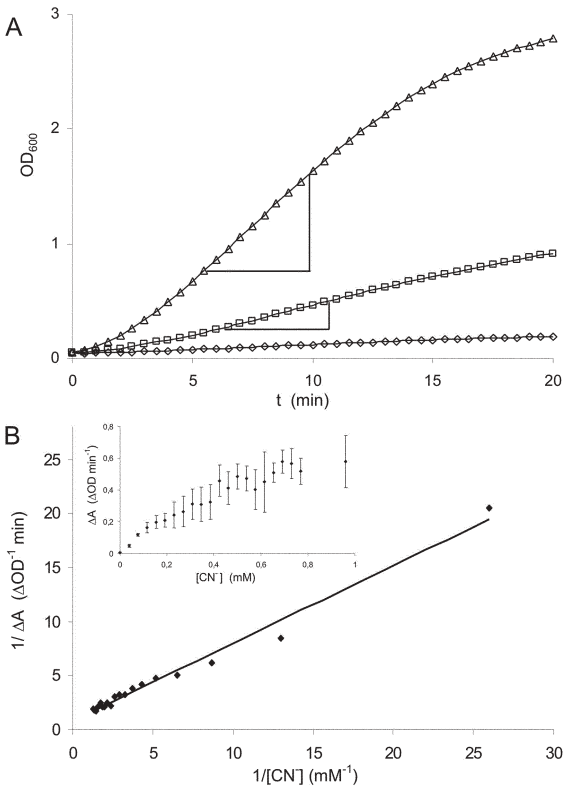

Fig. 3 Spectrophotometric detection of acetone cyanohydrin **7** cleavage using different amounts of purified MeHNL (–□– 50 ng, –△– 250 ng). A: The increase in absorbance at 600 nm over 20 min is shown. The amount of liberated cyanide is calculated from the linear part of the curve. Autolysis of the respective cyanohydrin is detected in a control without enzyme (–◇–) and subtracted from the slope values of the samples. B: Hyperbolic cyanide calibration curve and linearization by double reciprocal presentation.

cyanide standard curve ($K_2[Zn(CN)_4]$) (Fig. 3A) by correlating the rate of color formation at 600 nm with the cyanide concentration. For this purpose the hyperbolic standard curve was linearized in a double reciprocal diagram (Fig. 3B). For purified MeHNL[14] the calculated specific activity for acetone cyanohydrin **7** was 130 ± 30 U/mg, which is consistent with data from the literature, giving values between 92 U/mg and 260 U/mg[15] depending on the assay conditions. Comparison of the specific activities towards different substrates in Table 1 clearly demonstrates the highest catalytic activity of MeHNL towards the natural substrate acetone cyanohydrin. However, it must be taken into account that substrates **3–6** were applied as racemic mixtures containing 50% of the non-favored enantiomer, whereas substrates **7** and **8** are achiral.

Table 1 Results of cyanohydrin cleavage catalyzed by MeHNL. Substrates **3–8** (15 mM, see Fig. 1) were incubated with purified MeHNL[14]

Substrate	Specific activity [U/mg]
3	1.3 (±0.4)
4	0.4 (±0.2)
5	19.1 (±4.9)
6	0.1 (±0.04)
7	130.0 (±30.0)
8	1.0 (±0.4)

In summary, a novel HCN-based high-throughput screening assay for HNL activity was developed. The assay is useful to detect activity and enantioselectivity of HNLs theoretically towards any cyanohydrin substrate. Limitations might occur in the case of hydrophobic substrates due to poor water solubility. This problem can be overcome by the use of emulsifying agents like gum arabic. As tested, the increased turbidity has no influence on the formation and spectrophotometric detection of the dye (data not shown). Therefore, the assay is useful for both preparing enzyme fingerprints and screening large variant libraries generated in metagenome or directed evolution approaches. The assay is highly sensitive; at least 5 ng of purified MeHNL representing 1 mU of enzyme activity was reliably detectable in the assay. Furthermore, the assay is robust and easy to handle without the necessity of expensive equipment; however, it is possible to automate the test by using pipetting robots in order to increase the sample throughput.

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