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REVIEW

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Hydroxynitrile Lyases: Insights into Biochemistry, Discovery, and Engineering

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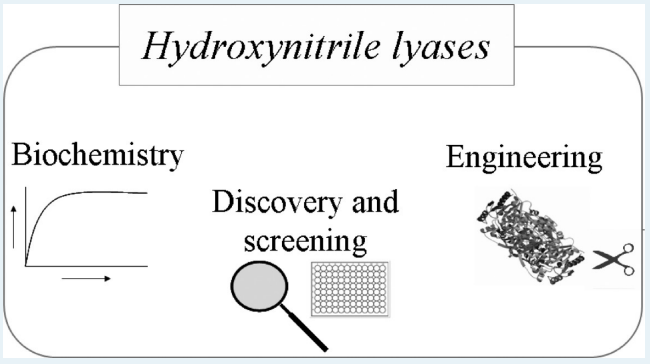
ABSTRACT: Hydroxynitrile lyases are valuable enzymes for asymmetric synthesis of cyanohydrins. These hydroxyl and nitrile−containing compounds are being used in production of very useful pharmaceuticals, agrochemicals, and other biologically active compounds using chemical or chemoenzymatic follow-up reactions in industry. Although a huge amount of information exists on the reaction parameters of these enzymes, including stability to pH and organic solvents, yield, reaction time, and valuable data on the enantiopurity of their products, cyanohydrins, there is a lack of update on the biochemistry, discovery, and engineering of the HNLs. Therefore, in the Introduction, we will have a look into these enzymes, cyanohydrins, and aldoxime-nitrile pathways. A brief view of functional groups and several examples of cyanohydrin-based chemicals and pharmaceuticals will also be described. Then we will present characteristics of many *S*- and *R*-selective HNLs with comparative tables for several enzymatic properties under biochemistry section. The methods of screening and discovery of these enzymes both from nature and a library of mutants will be described as well as their potential in the synthesis of chemicals. Cloning and expression of new HNLs will also be described under the discovery section. A pool of successful applications of protein engineering methods and the subsequent improvement in the properties of mutant HNLs will be reviewed in detail afterward.



KEYWORDS: cyanohydrins, directed evolution, hydroxynitrile lyase screening, protein/enzyme engineering, HNL screening/discovery

1. INTRODUCTION

1.1. Synthesis of Cyanohydrins by Biocatalysis. Enzymes catalyze the synthesis of chiral intermediates with high chemo-, regio-, and enantioselectivity that are in high demand for bulk preparations of pharmaceuticals and fine chemicals using environmentally friendly methods. Carrying out the enzymatic reactions at room temperature and under atmospheric pressure makes it possible to avoid undesired problems originating from extreme conditions. Immobilization and reusability of cells and enzymes are big advantages. In addition, overexpression and alteration of properties is possible for enzymes by protein engineering techniques.[1,2] In many cases, a combination of chemical synthesis and biocatalysis can be an efficient strategy to produce fine chemicals.[3] Hydroxynitrile lyases (HNLs) play an important role in the synthesis of chiral cyanohydrins. There is free room for chemical synthesis of sterically demanding compounds, although aldehydes and some ketones have been synthesized by biocatalytic processes.[4]

Considering the literature on HNLs reveals much data from different aspects of these enzymes. Even though current information on the chemical reactions is dominant, there are remarkable data on the biochemistry and other features of these valuable biocatalysts. Previous major reviews have summarized the potential of methods and reactions for synthesis of the cyanohydrins, which are valuable intermediates in follow-up synthesis of pharmaceuticals, agrochemicals, and other chemicals.[5−11] It seems there are some missing areas of HNLs, including reviews on updated biochemistry, discovery, and engineering features. Although there are robust HNLs at the present time, there is still a demand for improved reaction conditions, new chemicals, and new reactions that might be catalyzed by these enzymes. Newly discovered HNLs also could be a basis for engineering and achieving better biocatalysts for the above-mentioned purposes. Therefore, we will have a quick view into the chemistry of cyanohydrins in this article, although the main focus will be on the biochemistry, discovery, and engineering of hydroxynitrile lyases from both groups (*R*- and *S*-selective HNLs) (Figure 1).

2.2. General Information on Hydroxynitrile Lyases. Lyases (EC 4) are categorized into (1) carbon−carbon, (2) carbon−oxygen, (3) carbon−nitrogen, (4) carbon−sulfur, (5) carbon−halide, (6) phosphorus−oxygen, and other lyases. Hydroxynitrile lyases (HNLs) belong to the aldehyde lyases, which are categorized under C−C lyases. HNLs (EC 4.1.2.*X*, *X* = 10, 11,

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46, 47; Table 1) are of special interest in biocatalysis because they are being used mainly for production of pure enantiomers of cyanohydrins (α-hydroxynitriles), which are key intermediate molecules in the production of ranges of chemicals, including α-hydroxy acids, primary and secondary β-hydroxy amines, aziridines, α-hydroxy aldehydes or ketones, α-hydroxy esters, α- and β-amino alcohols, 3-ethanolamines, α-aminonitriles, α-azidonitriles, etc.

Early research on oxynitrilase was carried out by the German chemists Justus von Liebig and Friedrich Wöhler in 1837, who named an extract of bitter almonds as "emulsin", which could cause release of HCN from the cyanogenic glycoside amygdalin. Emulsin contained an oxynitrilase (HNL) and a β-glucosidase.[12] The field grew during more than one century, and despite high synthetic potential of chiral cyanohydrins in synthesis of pharmaceuticals, agrochemicals and biologically active compounds, intensive research on this field is relatively young.

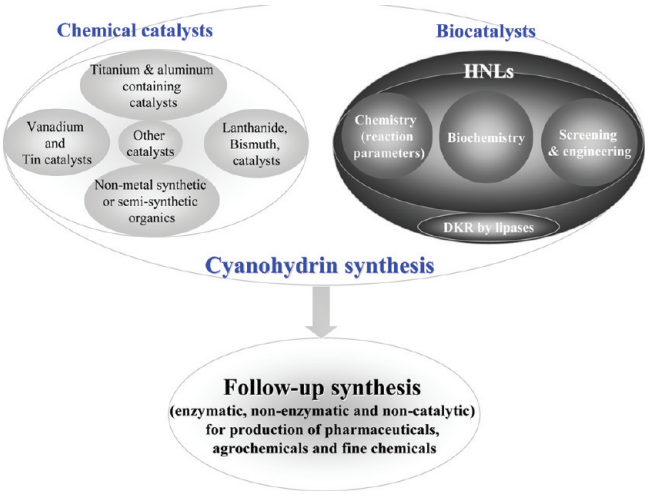


Figure 1. Synthesis of cyanohydrin by chemocatalysis and biocatalysis. This review will give a pool of synthesized chemicals in the follow-up reactions and mainly will discuss the biochemistry, discovery, and engineering of these versatile enzymes (DKR: dynamic kinetic resolution).

There were three EC numbers for HNLs: EC 4.1.2.10, 11, and 37 until EC 4.1.2.39 was created in 1999, although it was deleted and its content transferred to EC 4.2.1.37 in 2007. In an updated classification by IUBMB in 2011, the current EC numbers of HNL have been defined as EC 4.1.2.10, EC 4.1.2.11, EC 4.1.2.46, and EC 4.1.2.47. Further reclassification of these enzymes is expected, especially following the discovery of new HNLs.

2.3. Aldoxime-Nitrile Pathway, Cyanogenesis, and Hydroxynitrile Lyases. The microbial aldoxime-nitrile pathway, the involved enzymes, and the main chemical compounds have recently been reviewed in detail,[15] and a comparison of this pathway with that of plants was presented in 2009.[16]

The aldoxime-nitrile pathway is different between plants and microorganisms in terms of both chemical molecules and involved enzymes. In microorganisms, the main enzymes include aldoxime dehydratase, nitrile hydratase, amidase, and nitrilase, which convert the aldoxime, step by step, to carboxylic acid (Scheme 1a).

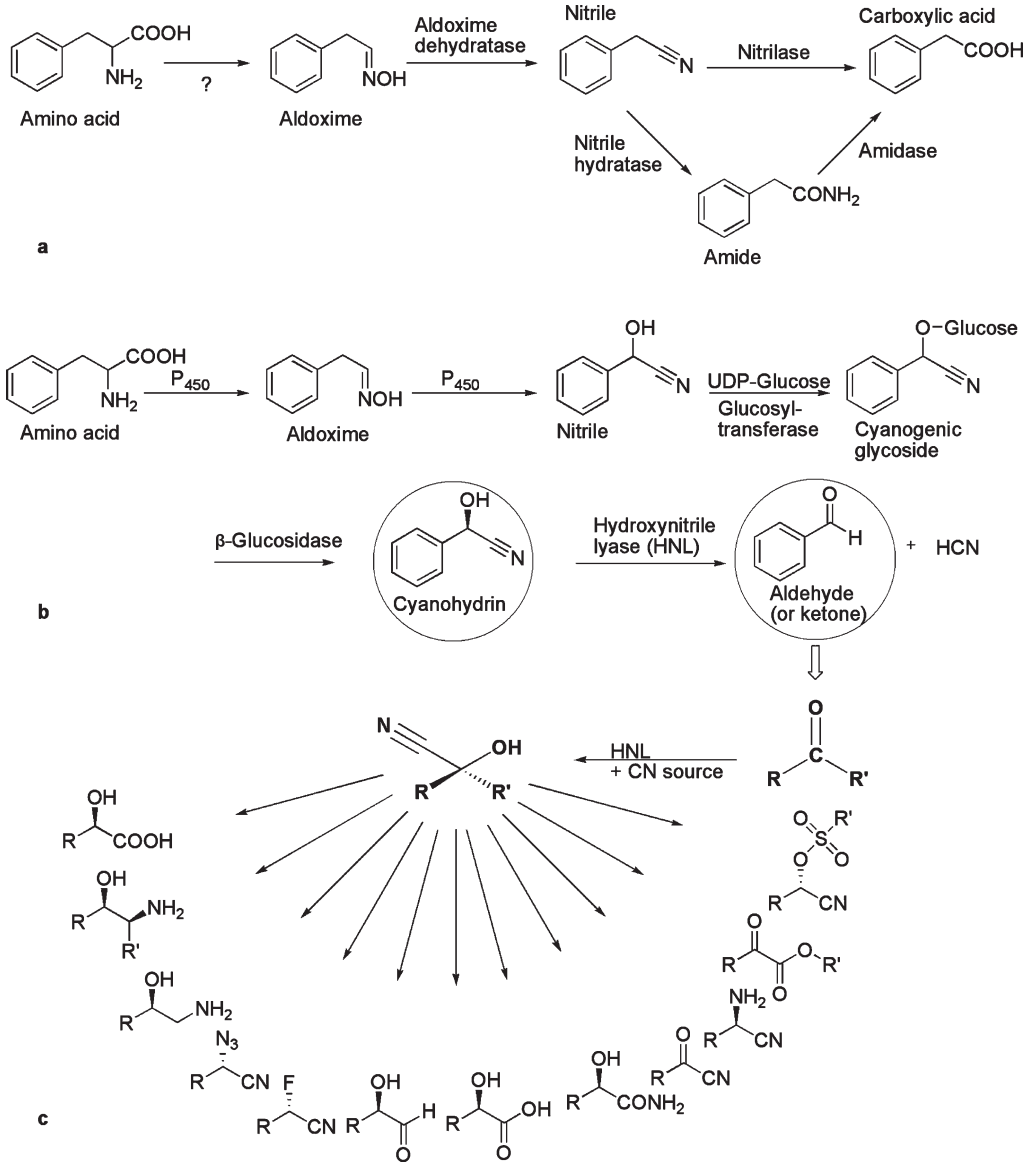
In plants, usually there are enzymes with monooxygenase activity, such as P450 (CYPs), that catalyze the earlier steps in the pathway, starting usually from the biogenetic precursors hydrophobic aliphatic amino acids (such as L-tyrosine, L-phenylalanine, L-valine, L-isoleucine, L-leucine) producing cyanogenic glycosides in the cells: first P450 produces an aldoxime compound by N-hydroxylation of amino group of the starting amino acid, followed by decarboxylation and dehydration.[17] Another P450 catalyzes the synthesis of an α-hydroxynitrile through nitrile formation by dehydration and hydroxylation of α-carbon as shown by Møller et al during their in vitro reconstruction of the biosynthetic pathway of cyanogenic glycoside dhurrin in *Sorghum bicolor* L. Moench.[18] Glycosylation of the α-hydroxynitrile is catalyzed by a UDP−glucose glucosyltransferase, resulting in the cyanogenic glycoside, which is the cyanohydrin substrate of hydroxynitrile lyase in the last step, where it is cleaved to the corresponding aldehyde or ketone and hydrogen cyanide (Scheme 1b). HCN is used as a defense system and also as a nitrogen source.[19] There is also chemical decomposition of the

Table 1. EC number[a], Names and Reactions of the HNLs

EC	systematic name	accepted name	reaction	other names
4.1.2.10	(*R*)-mandelonitrile benzaldehyde-lyase (cyanide-forming)	(*R*) -mandelonitrile lyase	(*R*)-mandelotiitrile = cyanide + benzaldehyde	(*R*)-hydroxynitrile lyase, D-oxynitrilase, (*R*)-oxynitrilase, *Pa*HNL, *At*HNL, *Pe*HNL, *Fa*HNL, etc.
4.1.2.11	(*S*)-4-hydroxymandelonitrile 4- hydroxybenzaldehyde lyase (cyanide- forming)	hydroxy mandelonitrile lyase	(*S*)-4-hydroxymandelonitrile = cyanide + 4-hydroxybenzaldehyde	hydroxynitrile lyase, oxynitrilase, *Sorghum* hydroxynitrile lyase, etc.
4.1.2.46[b]	(2*R*)-2-hydroxy-2-methylbutanenitrile butan-2-one lyase (cyanide-forming)	aliphatic (*R*)-hydroxynitrile lyase	(2*R*)-2-hydroxy-2-methylbutan enitrile = cyanide + butan-2-one	(*R*)-HNL, *Lu*HNL, (*R*)-oxynitrilase, etc.
4.1.2. 47[c]	(*S*)-cyanohydrin lyase (cyanide-forming)	(*S*)-hy droxynitrile lyase	(1) an aliphatic (*S*)-hydroxynitrile = cyanide + an aliphatic aldehyde or ketone (2) an aromatic (*S*)-hydroxynitrile = cyanide + an aromatic aldehyde	(*S*)-oxynitrilase, α-hydroxynitrile lyase, hydroxynitrile lyase, acetone-cyanohydrin lyase, *Hb*HNL, *Me*HNL, etc.

[a] According to the latest update from IUBMB in 2011.[13] BRENDA Enzyme Database has also suggested some new temporary categorizations for hydroxynitrile lyases, including former EC 4.1.2.10, 11, 37 and temporary categories of EC 4.1.2. B4, B5 and B6.[14] [b] Similar to former EC 4.1.2.37. [c] Similar to former EC 4.1.2.39.

Scheme 1. Aldoxime-Nitrile Pathways in (a) Microorganisms and (b) Plants and (c) Potential Application of Cyanohydrins As Key Intermediates in Industry[a]



[a] HNLs condense the aldehydes or ketones with a cyanide source and synthesize α-hydroxynitriles, which are versatile chiral intermediates for ranges of valuable chemicals and pharmaceuticals.

cyanohydrins in various cyanogenic plant species, that is, cyanogenesis happens in many plants without involvement of hydroxynitrile lyases and only by slow chemical reaction.

Although cyanohydrins or α-hydroxynitriles are synthesized in plants through cleavage reaction of cyanogenic glycosides using β-glucosidases, they are synthesized using a reversible reaction of hydroxynitrile lyases in industry. These compounds have attracted much attention as precursors in follow-up reactions (Scheme 1c). In Table 3, there are examples of the chemical and pharmaceutical products that have been synthesized from cyanohydrins and show the importance of these key intermediates, as well.

The number of cyanogenic plants in the literature varies from >2650[20,21] to >3000 species in recent reports,[22] although the number of the hydroxynitrile lyases are quite limited, as illustrated in Figure 2. Cyanogenesis is not restricted to plant species. For example, it has been reported in several bacteria (e.g., *Chromobacter violaceum*, *Psudomonas* sp.), cyanobacteria (e.g., *Anacyctis nidulans*), microalgae (e.g., *Chlorella* sp.), fungi (e.g., *Fusarium* sp.), etc.[23−25]

The plant pathogenic bacterium *Xylella fastidiosa*, found to have the only microbial gene encoding HNL so far, was described in 2009 following the genome mining for these enzymes. Characteristics of this recombinant enzyme have been included in the comparative Table 4 of *R*-selective HNLs in this review.[26]

There are almost 100 cyanogenic glycosides and related nitrile glycosides that have been studied in detail, categorized properly and reported by Lechtenberg and Nahrstedt in 1999. Prunasin, amygdalin, dhurrin, and linamarin are among the most famous cyanogenic glycosides in higher plants.[20] We will compare the details of the properties of the HNLs in the Biochemistry section of this review.

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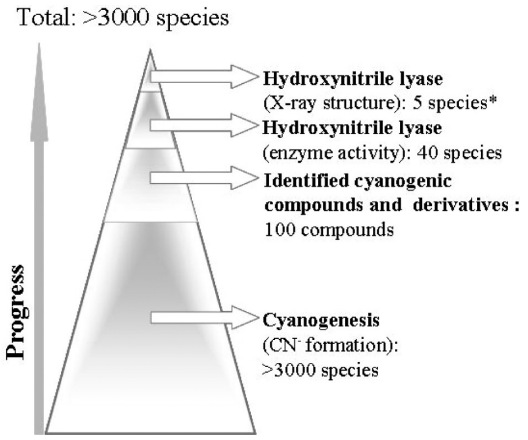


Figure 2. Comparison of a number of cyanogenic plants, known cyanogenic compounds, hydroxynitrile lyases and crystallized enzymes from both *R*- and *S*-selective hydroxynitrile lyases (also see Tables 4 and 5). *See Table 2.

Table 2. Crystal Structures Deposited in Protein Data Bank for These Five FAD-Dependent and Independent HNLs

Hydroxynitrile lyase	Organism	Structure	Protein Data Bank ID
*S-Me*HNL	*Manihot esculenta*		1DWO, 1DWP, 1DWQ, 1E89,1E8D, 1EB8, 1EB9
*S-Hb*HNL	*Hevea brasiliensis*		1QJ4, 1SC9,1SCI, 1SCK, 1SCQ, 1YAS, 1YB6, 1YB7, 2G4L, 2YAS, 3C6X, 3C6Y, 3C6Z, 3C7O, 3YAS, 4YAS, 5YAS, 6YAS, 7YAS
*S-Sb*HNL	*Sorghum bicolor*		1GXS
*R-Pa*HNL	*Prunus dulcis* (*P. amygdalus*)		1JU2, 3GDN, 3GDP
*R-At*HNL	*Arabidopsis thaliana*		3DQZ

Crystal structures of five HNLs, including three *S*-selective enzymes *Me*HNL, *Hb*HNL, and *Sb*HNL and two *R*-selective enzymes, *Pa*HNL and *At*HNL, have been solved and deposited in the Protein Data Bank until mid-2011 (Table 2).[27,28]

2. CHEMISTRY OF CYANOHYDRINS

The main aspects of these enzymes have been highlighted by researchers in the terms of chemical reactions, including improving reaction conditions, yield, and ee percent, as well as catalyzing new reactions and introducing new substrates with potential applications in industrial processes. Cyanohydrins are being synthesized by *R*- and *S*-selective HNLs and, in some cases, by lipases in dynamic kinetics resolutions. Chemical or metal catalysts have priority in synthesis of chiral cyanohydrins when the starting carbonyl compounds are not (or are poorly) accepted by the enzymes as substrates, especially in the case of sterically demanding aldehydes and ketones (Figure 1). The reactivity of the carbonyl group in ketones is affected by higher steric hindrance and lower electrophilicity.[10]

2.1. Functional Groups of Cyanohydrins. The functional groups of cyanohydrins provide a wide variety of functionalized

Figure 3. General structure of a cyanohydrin molecule.

units for further transformations in follow-up chemical or chemo-enzymatic reactions for synthesis of valuable intermediates or pharmaceuticals and agrochemicals (Figure 3).

2.1. A. Nitrile Group. This group in cyanohydrins can undergo hydrolysis, solvolysis, and reduction; therefore, ranges of products are synthesized by this property, a few of which are listed in Table 3.

2.1. B. Hydroxyl or Alcohol Group. To suppress or avoid instability, degradation and racemization, O-protection can be used for the synthesized cyanohydrins. The hydroxyl group also can be used for inversion of configuration.

2.1. C. Reactions at the carbon center. Inversion of the carbon center also provides a range of opportunitites for follow-up reactions for the cyanohydrins.

2.2. Examples of Chemicals Synthesized from Cyanohydrins in Follow-up Reactions. As we have mentioned earlier in this article, there is much information regarding the methods of cyanohydrin synthesis by both chemocatalytic and biocatalytic reactions. Therefore, to avoid repetition, we present various examples of cyanohydrin-containing chemicals, their cyanohydrin precursors, and applications in Table 3. Using low pH values or organic solvents can increase the enantiomeric excess in the aqueous media.[29] Immobilization of these enzymes on solid supports, such as cellulose, was introduced in 1966, when (*R*)-mandelonitrile and some other cyanohydrins were continuously synthesized by native *Pa*HNL from HCN and very pure benzaldehyde in a methanol−water mixture.[30] A combination of HNL immobilization with the various organic solvents and biphasic systems of buffer-organic solvents resulted in high enantioselectivity and shorter reaction times in industry.[4]

Another new option is the application of ionic liquids, which has successfully been used for (*R*)-*Pa*HNL- and (*S*)-*Hb*HNL catalyzed reactions, introduced by Griengl and co-workers in 2004.[31] It is also necessary to state the potential and application of CLEAs (cross-linked enzyme aggregates), which display an enhanced stability to organic solvents and can be easily reused.[32] These four HNLs are being used in preparative scales: (*R*)-*Pa*HNL (from almonds), (*S*)-*Sb*HNL (from *Sorghum*), (*S*)-*Me*HNL (Cassava) and (*S*)-*Hb*HNL (rubber tree). HNL from *Prunus dulcis* (*Prunus amygdalus* or almonds) is a very robust enzyme in both natural and recombinant forms (isoform 5 in *Pichia pastoris*). The enzyme accepts a wide range of carbonyl compounds as substrate, including various aromatic and aliphatic aldehydes and ketones and is being used in the industrial processes of cyanohydrin synthesis. In addition, (*R*)-*Lu*HNL is available from commercial suppliers; its plant source is flax (*Linum usitatissimum*).

Among (*S*)-HNLs, the enzyme from *Sorghum* accepts only aromatic and heteroaromatic substrates and not prepared in recombinant form, whereas *Me*HNL and *Hb*HNL have a similar broad substrate specificity. We have very recently described a novel (*S*)-*Bm*HNL, which has a different substrate specificity in comparison with these two industrial enzymes in aqueous phase,

Table 3. Examples of Pharmaceuticals, Agrochemicals, and Biologically Active Compounds Starting from Enzymatically Synthesized Cyanohydrins[a]

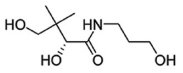
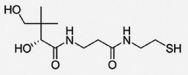
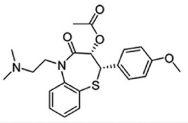
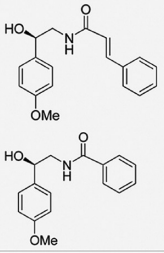
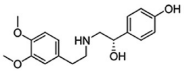
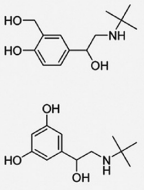
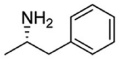
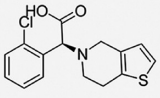
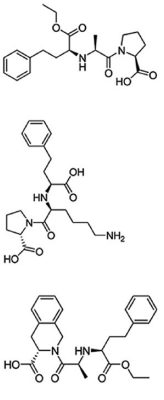
Entry	Chemical		Precursor cyanohydrin	Property or application [b]	Reference
	Name	Structure			
1	(*R*)-panthenol		(*R*)-pantolactone	A bactriocide (F)	34
2	(*R*)-pantetheine		(*R*)-pantolactone	Growth factor (*Lactobacillus bulgaricus* Factor or LBF) (F)	34
3	diltiazem		*Threo*-3-Aryl-2,3-dihydroxypropanoic acids derived from mandelonitrile	Cardiac drug (calcium channel blocker) (F)	35
4	(-)-aegeline (up) (-)-tembamide (down)		(*R*)-hydroxy-2-(4-methoxyphenyl) acetonitrile or *para*-methoxy or *para*-allyloxybenzaldehyde	Natural hydroxyl amides having insecticidal and adrenaline-like activity (aegeline shows hypoglycemic activity) (F)	36-37
5	(-)-denopamine		*para*-methoxy or *para*-allyloxybenzaldehyde	Cardiac drug, a β-receptor agonist for treatment of congestive heart failure (F)	37
6	Adrenalin and ephedrine-like compounds [(-)-salbutamol (up), (-)-terbutaline (down), Albuterol]		*para*-methoxy or *para*-allyloxybenzaldehyde;2-furaldehyde cyanohydrin, or TMS-ether of mandelonitrile	β-adrenoceptor agonists for treatment of asthma (a bronchodilator) (F) Biologically active 1,2-amino alcohols (F)	4, 37-39
7	*S*-amphetamines		2-amino-1-aryl alcohols	Psychoactive, Ecstasy, medically important drugs (F)	40
8	Clopidogrel (Plavix)		(*R*)-2-chlorobenzaldehyde cyanohydrin	Inhibiting blood clots (anti-platelet) (F)	41
9	ACE inhibitors [prils e.g., enalapril (up), lisinopril (middle), benzapril, cilazapril, quinapril (down)]		(*R*)-3-phenylpropionaldehyde cyanohydrin (*R*)-2-hydroxy-4-phenylbutyronitrile	Inhibition of angiotensin-converting enzymes (F)	42

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Table 3. Continued

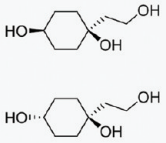
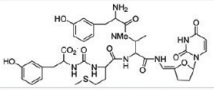
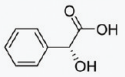
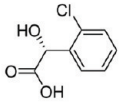
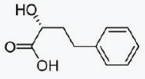
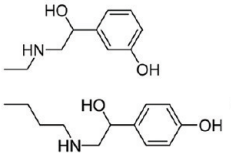
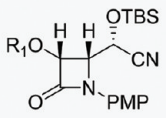
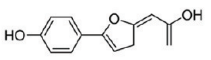
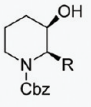
Entry	Chemical		Precursor cyanohydrin	Property or application [b]	Reference
	Name	Structure			
10	Rengyol (up) and isorengyol (down)		4-hydroxycyclohexanones	Anti-inflammatory and antibacterial effects (F)	43
11	Mureidomycin A		deoxysugars containing (*S*)-butenal cyanohydrins	Inhibitor of bacterial peptidoglycan synthesis (F)	44
12	*R*-mandelic acid		*Racemic* mandelonitrile	Antibacterial agent such as semi-synthetic cephalosporins and optical resolving agent (F)	45
13	(*R*)-2-chloromandelic acid		(*R*)-2-chloromandelonitrile	Chiral building block of an antithrombotic agent (F)	46-47
14	(*R*)-2-hydroxy-4-phenylbutyric acid		(*R*)-2-hydroxy-4-phenylbutyronitrile	Key intermediate for the synthesis of ACE-inhibitors (F)	46
15	Pharmaceuticals such as Etilefrine (up), bamethan (down) and denopamine		Chiral amino alcohols	β-blockers (F)	48
16	Polyhydroxylated piperidines (azasugars or iminosugars)		β-lactam cyanohydrin hybrides	Inhibition of glycosidases and glycoprotein-processing enzymes, carbohydrate-mediated diseases and viral infections; Sialidase inhibitors (F)	49
17	Stemofurans e.g., 2-(4-hydroxyphenyl)-6-hydroxybenzofuran		2-hydroxybenzaldehyde cyanohydrin	Cosmetics and pharmaceutical formulations, e.g., in a shampoo (F)	50
18	2,3-substituted piperidine (Nonpeptidic tachykinin receptor antagonists and cardiovascular agents)		(*R*)-(+)-5-bromo-2-hydroxypentanitrile	Physiologically active compounds (F)	3

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Table 3. Continued

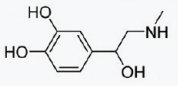
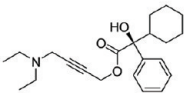
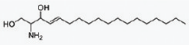
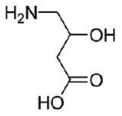
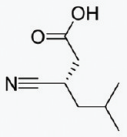
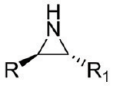
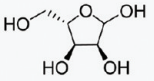
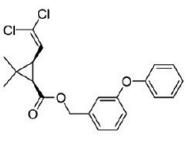
Entry	Chemical		Precursor cyanohydrin	Property or application [b]	Reference
	Name	Structure			
19	Antibiotics, antihypertensives, antitumors, fungisides, insecticides	Various structures	Chiral heterocycles aminofuranones and hydroxyfuranones	Pharmaceutical and agrochemical activity (F)	51
20	2-amino-1-phenyleth-anols (e.g., adrenaline)		2-amino-, 2-halo-, 2-azido-1-phenylethanols, 2-hydroxycarboxylic acids	Important adrenergic drugs (F)	52
21	(*S*)-Oxybutynin		1-cycloalkyl-1-hydroxy-1-ph-enyl cyanohydrin	Antimuscarinic agent (F)	53
22	Sphingosines		2-octenal cyanohydrin	Unsaturated amino alcohol present in phospholipids of nervous tissue and cell membranes (F)	54
23	GABOB		Pentafluorobenzaldehyde cyanohydrin	A derivative of the neurotransmitter GABA (F)	55
24	(3*S*)-3-cyano-5-meth-yl hexanoic acid		*racemic* isobutylsuccinonitrile	Key chiral intermediate for the preparation of Pregabalin, a GABA analog for epilepsy, neuropathic pain, fibromyalgia, etc (F)	56
25	2,3-disubstututed *trans*-aziridines		Cyanohydrins of benzaldehyde, 4-bromobenzaldehyde, other commercially available aldehydes	Naturally occurring mitosanes (e.g., mitomycins A, B, C; porfiromycin and mitiromycin) contain an aziridine ring essential for antitumor activity. They also show antibiotic activity (F)	57
26	Pentoses (from L-sugars)		Cyanohydrins of 4-*O*-protected 4-hydroxybut-2-enals	L-sugars are building blocks of nucleoside analogues having potential antiviral and antitumor activity (F)	58
27	Pyrethroids (e.g., Fenvalerate Aα, cypermethrin, deltamethrin, permethrin, the figure)		(*S*)-phenoxybenzaldehyde cyanohydrins, as alcoholic moiety	Important synthetic insecticides (I)	59-60

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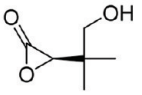
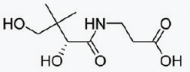
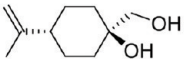
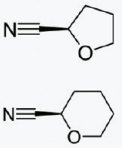
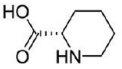
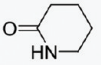
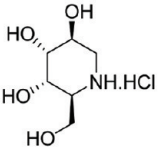
Table 3. Continued

Entry	Chemical		Precursor cyanohydrin	Property or application [b]	Reference
	Name	Structure			
28	13-(*S*)-HODE [(*S*)-coriolic acid][c]		(*E*)-2-octenal cyanohydrin	Anti-rice blast activity (inhibits spore germination of the blast fungus) (A)	61
29	(*R*)-pantolactone		Hydroxy pivalaldehyde (*R*)-cyanohydrin	Chiral building block and precursors for pantolactone derivatives (B)	62
30	(*R*)-pantothenic acid (Vitamin B5)		(*R*)-pantolactone	Part of coenzyme A (B)	34
31	Monoterpens (e.g., *cis*-*p*-menth-8-ene-1, 7-diol)		4-isopropylcyclohexanone cyanohydrin	Biologically active compounds (B)	63
32	(*R*)-2-cyanotetrahydr-ofuran; (*R*)-2-cyanotetrahydr-opyran		ω-bromocyanohydrins	Common structural components of biologically active materials (B)	3
33	Piperidine ring forms (e.g., optically active coniine and (*S*)-pipecolic acid)		ω-bromocyanohydrin	Biologically active materials (B)	3
34	Piperidones		(*S*)-Methyl 4-cyano-4-(methoxymethoxy) butanoate	Building blocks for biologically active compounds (B)	64
35	L-altro-1-Deoxynojirimy-cin, D-allo-1-Deoxynojirimy-cin, D-galacto-1-Deoxynojiri-mycin		*O*-TBDPS-protected 2-hydroxypent-3-enenitrile	Iminosugars, potentially applicable to synthesis of inhibitors of enzymes involved in metabolism of glucosylceramide (B)	65

[a] Only chemicals for which their cyanohydrin precursors are synthesized by the enzymatic procedures have been included in this table. [b] A, agrochemical; B, biologically active compound; I, insecticide; F, pharmaceutical. [c] 13-(*S*)-HODE: (*S*)-13-hydroxyoctadeca-(9*Z*,11*E*)-dienoic acid.

although it shows a high level of amino acid identity with them. There were 11 new substrates for this (*S*)-HNL in the report.[33]

3. BIOCHEMISTRY OF THE HNLS

Hydroxynitrile lyases originated from various ancestors among the protein classes. They have different primary structures, and all catalyze the same reaction; therefore, they have been considered as an example of the convergent evolution.[27,66]

Amino acid sequence alignment of the known HNLs classifies them into four superfamilies:

(i) Esterase−lipase superfamily including HNLs from *Manihot esculenta* ((*S*)-*Me*HNL), *Hevea brasiliensis*

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Table 4. Detailed Characteristics of *R*-Selective Hydroxynitrile Lyases

property	*R*-selective HNL[a]										
	*Pa*HNL	*Ps*HNL	*Pm*HNL	*Pl*HNL	*Ej*HNL	*At*HNL	*Fa*HNL	*Lu*HNL	*Pe*HNL	*Xf*HNL	*Pla*HNL
plant source, tissue and family	*P. dulcis* (*P. amygdalus*), seeds, *Rosaceae*	*P. serotina*, seeds, *Rosaceae*	*P. mume*, seeds, *Rosaceae*	*P. lyonii*, seeds, *Rosaceae*	*E. japonica*, seeds, *Rosaceae*	*A. thaliana*, ?, *Bracicaceae*	*Phlebodium aureum*, leaves, *Polypodiaceae*	*L. usitatissimum* flax, seedlings. *Linaceae*	*Passiflora edulis*, leaves and rinds, *Passifloraceae*	*X. fastidiosa* (plant-pathogenic bacterium)	*Prunus laurocerasus*, seeds, *Rosaceae*
EC number (4.1.2.-)	10	10	10	10	10	10	10	46	?	10?	10
isoenzymes	5	5	at least 3	1	1	1	at least 3 (A, B, C)	1?	1?	probably only 1	3
protein length (no. of aas)	563	559−576	576	?	552	258	?	422	?	251	?
molecular mass (kDa by gel filtration)	72 ± 2	57−59 (and 70 in hypocotyl and epicotyl)	57 (natural isoform 2), 62 and 97 (after and before deglycosylation	50	72	50	168 ± 30 (to 40) for A and C	80−82, 87	18	61.9	60
enzyme structure: M_r of subunit (number)	61 000 (monomer)	63 000 (monomer)	58 100 (monomer)	59 000	62 300 (monorner) (60 100 predicted)	29 000 (dimer)	20 000 (homo-multimer)	2 × 42 000 (rec. 43 000 and 45 780)	15 000	2 × 30 400	?
glycosylation	yes	yes	yes	yes	yes	no	no	no	yes	?	yes
cofactor	FAD	FAD	FAD	FAD	FAD	no	no	Zn^{2+}	no	no	FAD
crystallization	yes	no	yes	no	no	yes	no	?	no	no	no
active site residues	Cys328, Lys361, Tyr457, His459, Ser496, His497	?	such as *Pa*HNL?	?	?	Ser, Asp, His	?	?	?	Ser, His, Asp?	?
natural substrate/ substrate specificity	(*R*)-mandelonitrile/wide range of carrbonyl compounds, including aromatics and aliphatic aldehydes and ketones	(*R*)-mandelonitrile/ ?	(*R*)-mandelonitrile/ aromatic and aliphatic aldehydes and ketones	(*R*)-mandelonitrile/ ?	? /ahphatic and aromatic aldehydes	(*R*)-mandelonitrile?/ aromatic and aliphatic aldehydes and ketones	(*R*)-mandelonitrile/ aromatic and heterocyclic carbonyls, but poor acceptance of aliphatic carbonyls	acetone and 2-butanone cyanohydrins/ only aliphatic aldehydes and kelones	(*R*)-mandelonitrile/ aromatic and aliphatic (e.g., benzaldehyde, 2-pentanone, ect.)	mandelonitrile used for assay	(*R*)-mandelonitrile/ ?
inhibitors	$AgNO_3$ iodoacetamide, diethyl dicarbonate, PMSF, diisopropy1 fluorophosphate, methy-*p*-(3-aminipropoxy)-benzoate	benzoic acid, *p*-hydroxybenzyl alcohol, benzyl-alcohol (isoforms 4, 5)	iodoacetate, 2-iodoacetamide, Ag^+, Hg^{2+}, PMSF, PCMB, diethyl pyrocarbonate	$AgNO_3$, $HgCl_2$, iodoacetamide, benzoic acid	$CuSO_4$, $HgCl_2$, $AgNO_3$, 2ME, PMSF, iodoacetate	?	diethyl pyrocarbonate	*o*-phenanthroline, DEP	?	?	?
molecular cloning and expression in	*P. pastoris*	none (all amino acid sequences are deduced)	*P. pastoris*[b]	none	predicted, UniProt	*E. coli*	none	*E. coli*, *P. pastoris*	none	*E. coli*	none
pH (range and optimum)	3−11, 5−6	?/6−7 (isoforms 4, 5)	4−7, 4.5	4−7, 5.5	4−6, 5.5	4.75−6.5, 5.75−6.5	3.5−7.5, 6.2	3−6, 5.5	3−7, 4	?, 7 (assay)	?, 5.5−6.0

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Table 4. Continued

property		*R*-selective HNL[a]										
		*Pa*HNL	*Ps*HNL	*Pm*HNL	*Pl*HNL	*Ej*HNL	*At*HNL	*Fa*HNL	*Lu*HNL	*Pe*HNL	*Xf*HNL	*Pla*HNL
temperature (range and optimum, °C)		?, 25 (assay)	?/23.3 (assay condition)	0−65, 25−35	?	15−60, 40	5−50, 35	?, 35−40	?, 25 (assay)	5−50, 10	?, 25 (assay)	?
homologies/ protein family		GMC oxidoreductase superfamily	GMC oxido-reductase superfamily	GMC oxido-reductase superfamily	?	GMC oxidoreductase superfamily	esterase−lipase superfamily	?	MDR superfamily	N-terminus is different from all know HNLs[c]	estrase−lipase super family	?
selected K_m value and substrates (mM)		0.29 and 0.59 (mandelonitrile), 0.15 (benzaldehyde)	0.17 (mandelo-nitrile)	5.3 (bezalde-hyde)	0.093 (mandelo-nitrite)	0.161 (mandelo-nitrile)	1.4 and 6.0 (mandelo-nitrile and benzaldehyde	0.83 (mandelo-nitrile)	2.5 and 1.9 (natural and recombinant for acetone cyanohydrin), 1.25 (2-butanone cyanohydrin)	?	?	?
specific activity (μmol min^{-1} mg^{-1}) (C, cleavag reaction; S, synthesis - reaction)		272(*p*-hydroxy-mandelonitrile), 64 and 71.5 (mandelonitrile) (C)	24 580−34 130 (mandelonitrile for isoforms 1−5) (C)	220 and 129 (natural and recombinant isoform 1 for benzaldehyde) (S)	112 × 10^{-3} (mandelo-nitrile) (C)	46.5 (mandelo-nitrile) (C)	80 and 15.3 (mandelonitrile and benzaldehyde) (C and S)	19 000 (mandelo-nitrile) (C)	34.1 and 53 (natural) and 40 and 76 (recombinant) for acetone cyanohydrin (C)	136 (mandelo-nitrile) (C)	43 (mandelo-nitrile) (C)	?
stability	general	?	4 °C for 11 days: 12% loss; lyophilization + lactose increased stability up to a few months	4 °C for 6 months, only 10% loss of activity	4 °C: 6 months no loss of activity	4 °C: one month no loss of activity	very low activity at pH < 5, Sorbitol and saccharose stabilize the enzyme	?	highly stable at 4 °C for 45 days, no loss of activity; highly stable at 6−11 °C during storage	stable at 4 °C, stable in organic solvents (MTBE, DBE, HEX, DIPE)	?	?
	pH	3−11 (60−100% original activity)	?	3−9	?	3−9	unstable under pH 5, linear inactivation during 5 h in pH 5.4	?	half-life times, h (1 h, pH 4, stability increase for Eupergit-immobilized)	?	4.9−7.8	?
	temp (°C)	>95% at 56 °C, >80% at 65 °C (1 h), fast inactivation during 30 min at 75 °C	?	0−60	?	0−70	half life times, h (>96 for 0−10 °C, 80 for 20, 33 for 30, and <10 for higher)	?	?	?	?	?
references		69,108,114,115,122,129	116−118	113[d]	137	124,125	132, 149	122	29, 66, 119, 121	126[d]	26	7

[a] Other plant (*R*)-HNLs: *P. domestica*, *P. spinosa*, *P. avium*, *P. persica* (these enzymes were reported by Becker and Pfeil in 1966 and it was found that these enzymes from different members of *Prunaceae* exhibited identical immunological behavior, molecular weight, specific activity and absorption spectra, while showed differences in the electrophoretic behavior probably because of differences in the protein sequences);[30] *P. armeniaca*, *Ch. japonica*, *M. communis*, *Cydomia oblonga* (Quince seeds) (these enzymes were reported by Gerstner and Pfeil in1972 and their enzymological properties were precisely studied. For example, they exhibited a molecular mass of 58, 75, 75, and 82 kDa, respectively. They reported some of already discovered hydroxynitrile lyases in this study, too. It is interesting the authors reported 2 isoenzymes for *M. communis* and *Ch. japonica*; 3 isoenzymes for HNLs from *P. amygdalus*, *P. persica* (60 kDa), and *P. spinosa* (58 kDa); 4 isoenzymes for *P. armeniaca* and *P. avium* (58 kDa), and finally 6 isoenzymes for *P. domestica* with a molecular mass of 58 kDa);[138] *P. capuli* and *M. americana*;[139] *P. serotina* var. *capuli*, *C. melo* (melon seeds), *P. sapota*;[140] *S. aucuparia* and *Ch. sinensis*;[123] *V. sativa* L., common vetch;[141] *P. armeniaca*, Shakarpara cultivar (apricot);[142] *P. pseudoarmeniaca* [143] [b] Isoforms 3 and 4 were cloned and sequenced but could be expressed neither in *E. coli* nor in *P. pastoris*. [c] The first 20 amino acid sequence of N-terminus of this small enzyme exhibited no similarity to those of other known HNLs until now. Instead, it showed a 55% amino acid identity to a hypothetical protein from *Plasmodium falciparum* 3D7 (accession number AE014830 by NCBI Blast and FASTA databases). [d] Unpublished work from the Asano group. A new classification of the IUBMB categorized it under EC 4.2.1.10, although the N-terminal shows a new sequence and might be among a new category of these enzymes, which could be clarified by cloning and sequencing of the corresponding gene. Abbreviations for the inhibitors: PCMB, *p*-chloromercuribenzoate; DEP, diisopropyl fluorophosphate; 2ME, 2-mercapthoethanol; PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropyl fluorophosphate.

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Table 5. Characteristics of *S*-Selective Hydroxynitrile Lyases

property		*S*-selective HNL[a]				
		*Sb*HNL[b]	*Xa*XNL	*Hb*HNL	*Me*HNL	*Bm*HNL
plant source, tissure and family		*S. bicolor* L. (epicotyl, leaf, mesophyl shoot, seedlings, *Poaceae*)	*X. americana* L. (leaf, *Olacaceae*)	*H. brasiliensis* (tropical rubber tree, leaf, *Euphrobiaceae*)	*M. esculenta* Crantz (Cassava, leaf, petiole, root, stem, *Euphrobiaceae*)	*B. montanum* (Willd.)Muell. Arg. (leaf, *Euphrobiaceae*)
EC number (4.1.2.)		11	11	47	47	47
isoenzymes		3	3 or more	1	3 or more	probably 2
	protein length (no. of aas)	366, 510	?	257	258	263
	mol mass (kDa by gel filtration)	95−105,108 ± 3, 180	38 (lyase2, ≥90% activity), 108 (lyase1, ≤10% activity)	100−105	50, 92−124	60−65
enzyme structure	M_r of subunit (no.)	heterotetramer (α2, β2) (30−33 and 22−25)	36.5 (lyase2), 4 × 29 (lyase1)	2 or 4 × 30	2 (or 4) × 28−30	2 × 29.5
	glycosylation	yes	yes (only lyase2)	no	no	no
	cofactor	no	no	no	no	no
	crystallization	yes	no	yes	yes	in process
	active site residues	Ser, His, Asp	?	Ser, His, Asp	Ser, His, Asp	Ser, His, Asp
natural substrate/ substrate specificity		*p*-hydroxymandelonitrile (from Dhurrin)/ only aromatic aldehydes	(*S*)-mandelonitrile and 4-hydroxy-mandelonitrile	acetone cyanohydrin/ aliphatic and aromatic aldehydes and ketones	acetone cyanohydrin/ aliphatic and aromatic aldehydes and ketones	? /aromatic and aliphatic aldehydes and ketones
inhibitors		$CuSO_4$, $Fe(SO_4)_2$, $HgCl_2$, benzoic acid	$AgNO_3$, GSH, PCMS	DFP, DEP, acetone, diethyl dicarbonate, diisopropyl fluorophosphate, hexafluoroacetone, mandelonitrile, rhodanide, etc.	DFP, PMSF, acetone, benzaldhyde, PMSF, propanol, propionaldehyde, phenol, etc.	$AgNO_3$, PMSF, PCMB, AS_2O_3, acetone, acetone cyanohydrin, phenol, rhodanid etc.
molecular cloning and expression in		*E. coli* (inactive)	none	*E. coli*, *S. cerevisiae*, *P. pastoris*	*E. coli*, *P. pastoris*, *S. cerevisiae*	*E. coli*
pH (range and optimum)		3.5−6.5, 5−6	?, 5.5	?, 5.0−5.7	4.5−6.5, 5−5.5	4−6.5, 5
temperature (range and optimum, °C)		0−35, 35	?, ?	?, 25	0−50 and 25−70, 40	0−60, 20
homologies/protein family		peptidase S10 superfamily, serine carboxypeptidase	?	esterase−lipase superfamily	esterase−lipase superfamily	esterase−lipase superfamily

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Table 5. Continued

property		*S*-selective HNL[a]				
		*Sb*HNL[b]	*Xa*XNL	*Hb*HNL	*Me*HNL	*Bm*HNL
selected K_m value and substrates (mM)		0.55 and 0.7 (4-hydroxymandelonitrile), 0.79 (D,L-hydroxymandelonitrile)	0.28 (lyase 2) and 11 (lyase 1) for racemic mandelonitrile)	0.8 (2-methyl-2-hydroxybutyronitrile), 0.7 (acetone cyanohydrin)	0.625 (4-hydroxymandelonitrile, mutant W128A), 0.93 (CN), 4(acetone cyanohydrin), 1.4 (mandelonitrile, wild-type enzyme)	7.5 (benzaldehyde), 12.5 (4-biphenyl carboxaldehyde), 15.7 (piperonal), 0.87 (mandelonitrile)
specific activity (μmol min^{-1} mg^{-1}) (C, cleavage reaction; S: synthesis reaction)		188 and 227 (4-hydroxymandelonitrle) (C)	453 (*rac*-mandelonitrile) (C)	86.9 (acetone cyanohydrin) (C)	36.1, 47.5, 91.6 (acetone cyanohydrin) (C)	227 (piperonal) (S), 52 (benzaldehyde) (S), 49.3 (mandelonitrile) (C)
stability	general	−15 °C, 3 months, no loss of activity	stable at 4 °C and RT for 1 month	stable at 4 °C for a few months	up to 1 year at 4 °C without loss of activity	precipitaion begins after 1 month of incubation on ice
	pH	2−11 (40−100%), 5−6.5	?	5−10, very stable above pH 6.0	3−11	2.4−8
	temperature	>95% at 50 °C, >65% at 65 °C, >40% at 70 °C (all for 1 h)	?	30 °C a few hours; half-life at 40 °C and 100 mM buffer up to 60 h; inactivation at 70 °C	45−70 (mutant G113S, loses activity up to 80% at 70 °C)	0−65
references		69,72,128−130,144	136	133,134,145,146	22,68,73,147	33,123

[a] Other plant (*S*)-HNLs: *S*-selective oxynitrilases reported by Rosenthaler (1913) as enzymes in leaves of *Taraktogenos blumei* and *Achillea millefolium*;[148] *Sambacus nigra*;[119] *A. cherimolia*, and *A. squamosa*.[140] [b] *Sb*HNL, synonym of *Sv*HNL (*S. vulgare*). Abbreviations for the inhibitors: PCMB, *p*-chloromercuribenzoate; DEP, diisopropyl fluorophosphate; 2ME, 2-mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropyl fluorophosphate.

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((*S*)-*Hb*HNL), *Baliospermum montanum* ((*S*)-*Bm*HNL), and *Arabidopsis thaliana* ((*R*)-*At*HNL);

(ii) Peptitades−S10 superfamily: serine carboxypeptidase, including HNL from *S. bicolor* ((*S*)-*Sb*HNL)

(iii) Medium-chain reductase/dehydrogenase (MDR) superfamily, including HNL from *L. usitatissimum* ((*R*)-*Lu*HNL)

(iv) Glucose−methanol−choline (GMC) oxidoreductases N and C superfamily including HNLs from *P. dulcis* or *P. amygdalus* (*R*-*Pa*HNL), *Prunus serotina* (*R*-*Ps*HNL), and *Prunus mume* (*R*-*Pm*HNL) (Tables 4 and 5).[67]

FAD-dependent HNLs (iv), being mainly glycosylated monomeric enzymes possessing (*R*)-mandelonitrile as in vivo substrate, are of similar molecular weight and serologically related.[68] Two main domains have been found in the structure of *Pa*HNL (almond HNL): FAD-binding and substrate-binding domains. FAD-dependent proteins usually have a signal peptide in their primary structures. First, it was thought that FAD was involved in catalysis of *R*-*Pa*HNL, but the idea was refuted by investigations of Conn and co-workers.[69] It is generally accepted that FAD has an important role in stability[70] because the enzyme shows HNL activity only in the presence of this cofactor and glycosylation maintains its functional structure.[72]

Non-FAD HNLs form a rather heterogeneous group of enzymes differing in size, subunit composition, and the substrate specificity.[68] Three classes of these proteins were introduced above (i−iii). The reaction mechanism for these enzymes has been better understood than FAD-dependent HNLs, especially by extensive investigations of (*S*)-*Hb*HNL and (*S*)-*Me*HNL, including X-ray crystallography and mutagenesis. The common active triad Ser, His, and Asp (nucleophile−histidine−acid) together with the side chain of a conserved Thr residue have been considered to play important roles in the process of catalysis (Scheme 2A, B).[73−75]

Hereafter, we have some highlights on HNLs, and finally, two precise Tables 4 and 5 containing gathered information on 5 *S*- and 11 *R*-selective HNLs. The remaining cases have been listed under each corresponding table with some brief descriptions.

3.1. (*R*)-Hydroxynitrile Lyases ((*R*)-HNLs). *(R)-PaHNL.* The first description of HNL from almond (*P. dulcis* or *P. amygdalus*) in asymmetric synthesis was reported by L. Rosenthaler in 1908.[76] "Emulsin" was studied for its enzymatic content,[77,78] cleavage of amygdalin,[79−82] cyanohydrin synthesis,[77,83] and cleavage.[84] Hydroxynitrile lyase was described as an ideal catalyst, and emulsin was found to affect the reaction velocity. HNL promoted cyanohydrin synthesis and cleavage equally.[85] There is a gap until the 1960s, when researchers purified the (*R*)-HNLs from the *Prunaceae* plant family and used the purified enzymes to synthesize aliphatic, aromatic, and heterocyclic aldehyde cyanohydrins. Ultimately, the cyanohydrin products were converted to D-α-hydroxy acids by acid saponification.[30] It became possible to synthesize the (*R*)-mandelonitrile with a yield of 95% and optical purity of 94% using (*R*)-*Pa*HNL combined with cellulose-based ion exchangers.[86−88]

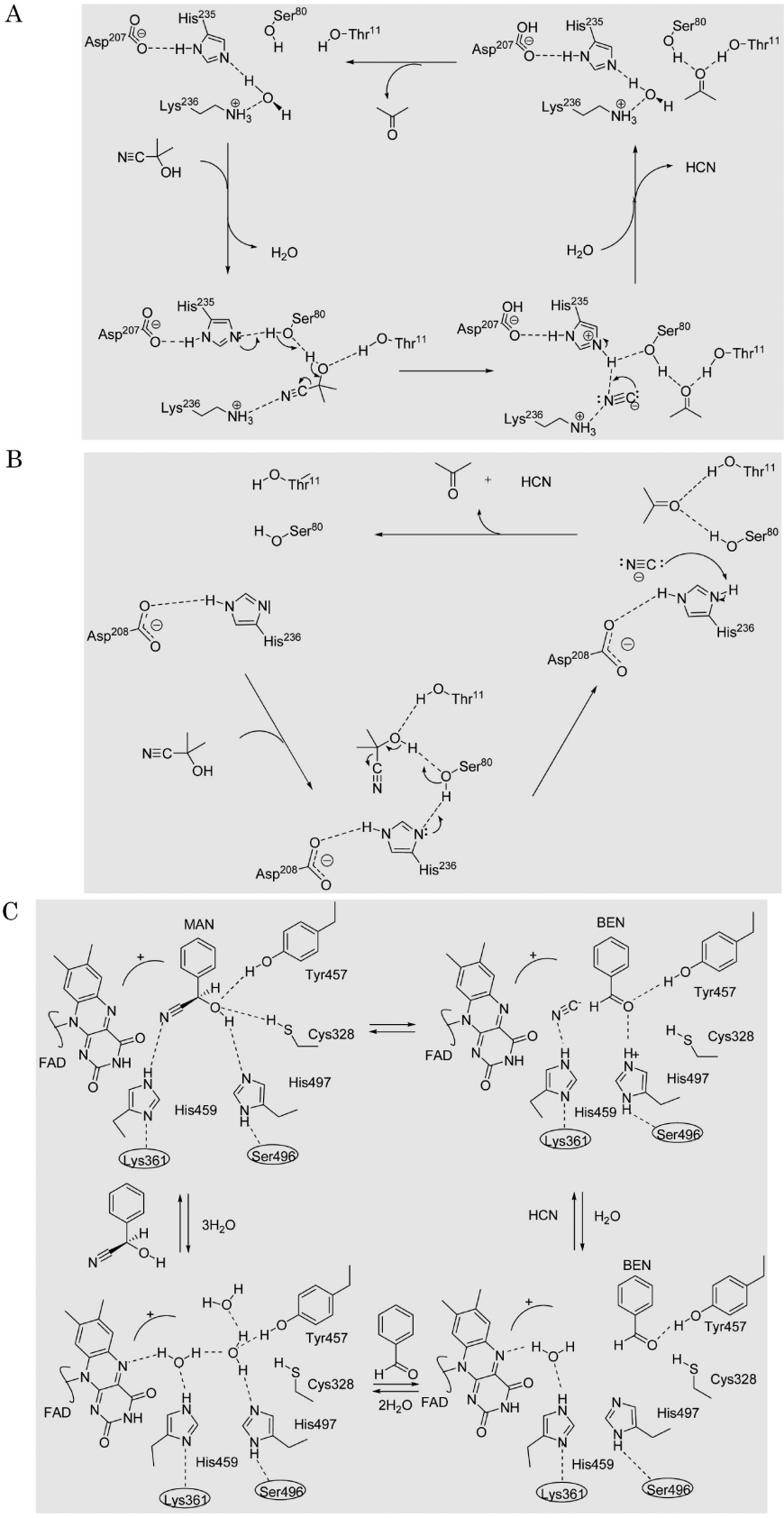
Although many research groups have studied the occurrence and structures of cyanogenic glycosides in plants,[89−101] hydroxynitrile lyases and their mechanism of action for years,[102−108] there was another gap from a biocatalysis point of view until, after many years, Effenberger and co-workers employed this enzyme for synthesis of highly pure enantiomeric cyanohydrins in 1987. Using this enzyme, they suppressed the chemical reaction in addition of HCN to aldehydes. The enantiomeric excess up to 99 and 86% were achieved in ethyl acetate and water/ethanol, respectively.[109] It took more than 150 years, after the description of emulsin in 1837, to have the first information from the crystal structure of mandelonitrile lyase from almonds. Three isoenzymes were crystallized, and isoform III was obtained in a resolution of 2.6 Å.[110] To have new insights into the mechanism of reactions catalyzed by this robust *R*-selective enzyme in industry, continuous attempts have been made until now, although it seems that more time is needed to clarify the remaining unsolved part of the reaction mechanism.[27,70,111,112]

In one of the latest proposed mechanisms, researchers investigated the reversible cleavage of the mandelonitrile, according to the structural, mutational, and available biochemical evidence. The reaction proceeds via general acid/base catalysis through residue His497 (Scheme 2C).[27] The cyanide is stabilized by the overall positive electrostatic potential in the active site region: His497 is proposed to act as a general base that abstracts the proton from the hydroxyl group of mandelonitrile, which, on the basis of the model built, is within hydrogen-bonding distance to His497, Cys328, and Tyr457. The interaction network around the hydroxyl group suggested that His497−Nε2 is unprotonated, since both Cys328 and Tyr457 act as hydrogen bond donors to the hydroxyl group of mandelonitrile. Covalent modification of cysteine328, which previously was thought to be involved in the process, destroyed catalytic activity because the residue is very close to the substrate binding site. The second active site histidine, His459, is probably responsible for protonation of the cleaved cyanide ion. Because the hydroxyl group of mandelonitrile is located far from the N5 atom of flavin, it is suggested not to be directly involved in the reaction mechanism. These researchers concluded that FAD is an evolutionary remainder originating from an oxidoreductase precursor that is necessary for the structural integrity of FAD-HNLs, such as *Pa*HNL. The positive potential at the active site facilitates cyanide ion formation, which is mainly provided by Arg194, Arg300, Lys361, and Arg182.[27] Figure 4 illustrates a sequence alignment of HNLs from *Prunus* species, signal peptides, and the residues involved in the catalysis.

Various methods were employed to purify the (*R*)-*Pa*HNL from its natural source, almond, including regular purification methods,[69] although it was also purified by a polyethylene glycol−citrate aqueous two-phase system continued by ultrafiltration and anion-exchange chromatography on a Q-Sepharose column with a yield of 57%.[114] An inhibitor of the enzyme, methyl *p*-(3-aminopropoxy) benzoate, was synthesized and covalently attached onto Sepharose as a solid matrix, and then the enzyme was purified on the basis of this affinity in a simple step with high yield.[115] The characteristics of the purified *Pa*HNLs have been included in Table 4. The enzyme is available commercially in both natural and recombinant forms from companies such as AppliChem and Jülich Fine Chemicals (Codexis).

(R)-PsHNL, (R)-LuHNL, and (R)-FaHNL. *Ps*HNL was found to have five isoenzymes (1−5) in black cherry (*Prunus serotina*) and high amino acid sequence identical to the *Pa*HNL, the almond enzyme. These five isoenzymes were isolated by concanavalin A-Sepharose 4B chromatography and chromatofocusing. Kinetics and molecular properties of two predominant forms, 4 and 5, were studied in 1986; however, there were no significant differences between the K_m, optimum pH, stability, and inhibitors for these two studied enzymes.[116] These enzymes were studied in a biochemical and molecular biology level in the black

Scheme 2. (A−C). Proposed Mechanisms of Catalysis for (*S*)-HNLs (A: H*b*HNL; B: *Me*HNL) and *R*-HNL (C: P*a*HNL)[a]



[a] MAN, mandelonitrile; BEN, benzaldehyde.[27,75]

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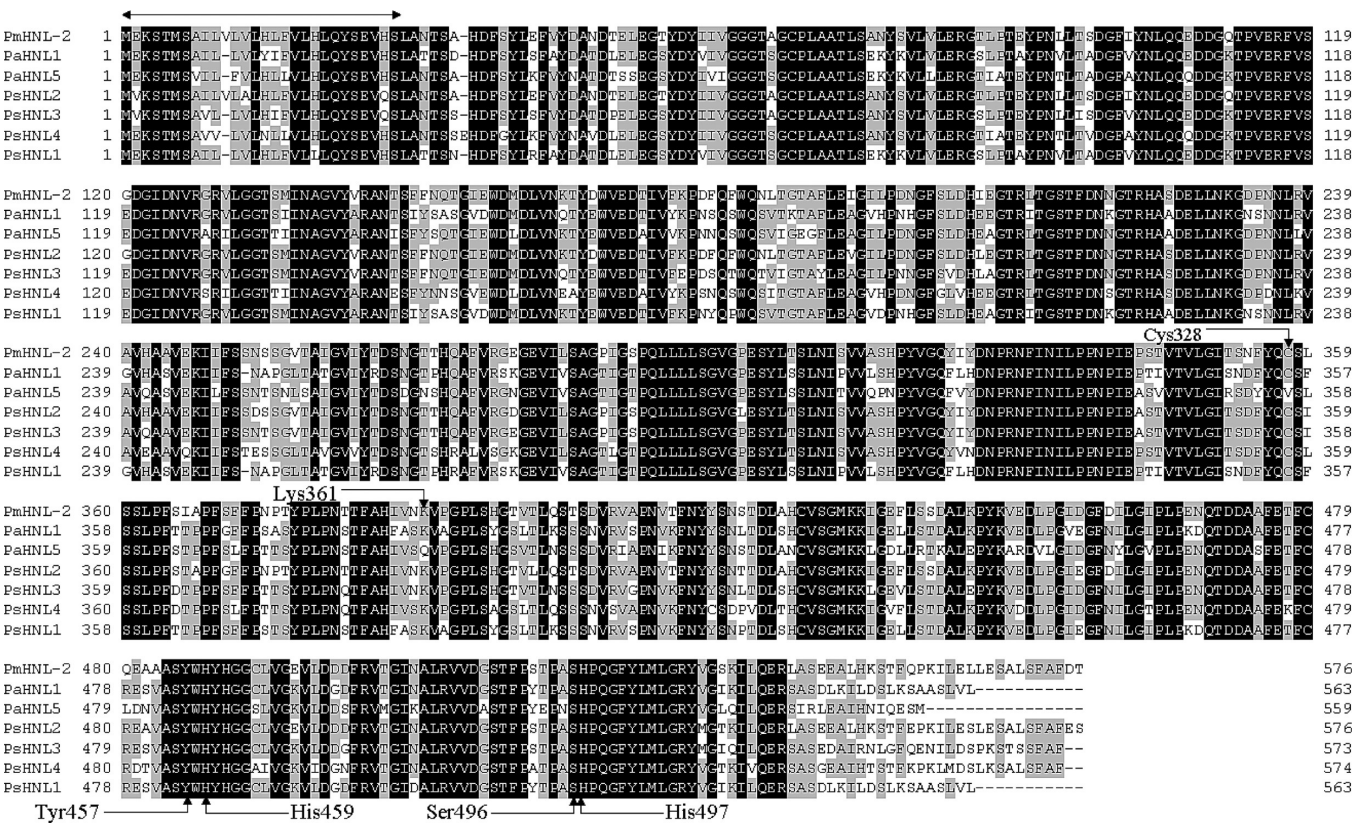

Figure 4. Sequence alignment of major (*R*)-HNLs from genus *Prunus* (*Pm*HNL, HNL from *P. mume*; *Pa*HNL, HNL from *P. amygdalus*; *Ps*HNL, HNL from *P. serotina*). The residues involved in the reaction catalyzed by *Pa*HNL have been shown in the figure. Residues 1−27 are the signal sequence of these proteins (↔).[27,113]

cherry in 1997−1999. It was found that all five isoenzymes showed very similar genomic DNA structure, containing FAD-binding site, N-glycosylation sites, N-terminal signal sequence (first 27 amino acids) (Figure 4), and three short conserved introns. The authors concluded that these five genes derived from a common ancestral gene and are members of a gene family.[117,118]

(R)-LuHNL. This acetone cyanohydrin lyase was first purified from young seedlings of flax (*L. usitatissimum*) by Conn et al., in 1988. They used regular purification methods to isolate the enzyme up to 136-fold with a recovery yields of 21%. The enzyme was characterized and found to be a non-FAD-dependent HNL. It showed no activity toward mandelonitrile and 4-hydroxymandelonitrile and was inhibited by the high concentrations of acetone cyanohydrin, its natural substrate. In another study, the enzyme *Lu*HNL was purified with an improved yield (up to 60%) and specific activity toward acetone cyanohydrin using ion exchange, hydrophobic interaction, and gel permeation chromatographies. The enzyme acted on various aliphatic aldehydes and ketones, but no aromatic carbonyl substrates.[29] The first gene cloning and sequencing of the enzyme was reported in 1997. The kinetic parameters of the histidine-tagged recombinant enzyme and the natural one were evaluated to be similar. The authors also presented a scheme suggesting the convergent evolution for the various HNLs, based on the sequence comparison of the known enzymes.[66] Low expression of the enzyme in *E. coli* was the main barrier to further characterization and mutational analyses; therefore, the gene for this Zn^{2+}-containing HNL was cloned as a myc-His-tagged *Lu*HNL-cDNA and expressed in the methylotrophic yeast *P. pastoris* (myc probably was used for persistent expression of the *hnl* gene and also for the detection of the expressed enzyme by specific anti-*Lu*HNL antibodies). The recombinant enzyme showed kinetic parameters similar to the natural *Lu*HNL and was found to have 2−4 mol of zinc ion in 1 mol of the enzyme. The enzyme was also used for synthesis of 16 (*R*)-cyanohydrins from carbonyls and HCN with a low to excellent yield and ee percent.[120] In another attempt to solve the low recombinant protein expression problem of *Lu*HNL in *E. coli*, cDNA cloning and expression of a 45.8 kDa *Lu*HNL was reported as an N-terminal His-tagged fusion protein in a thioreductase-deficient strain of *E. coli* at 28 °C in 1998. The specific activity of the one-step purified enzyme was improved to 75.7 U/mg toward acetone cyanohydrin as substrate. In this work, the structurally and catalytically important residues for both substrate and coenzyme binding domains were identified as well as the ligands of important Zn^{2+}.[121] (*R*)-*Lu*HNL is available from Jülich (Codexis).

(R)-FaHNL. The enzyme was purified and characterized from the fern *Phlebodium aureum* by Wajant et al. in 1995. The enzyme was purified 1600-fold with a specific activity of 19 000 U/mg toward (*R*)-mandelonitrile as a substrate and was studied for the kinetic parameters. The enzyme was used for synthesis of a few (*R*)-cyanohydrins, in which it was interestingly found that it catalyzed addition of cyanide to thiophen-2-aldehyde in excellent yield and ee percent with immobilized enzyme under a biphasic system superior to (*R*)-HNL from almond.[122]

(R)-PmHNL, (R)-EjHNL, and (R)-PeHNL. These enzymes are of new sources for (*R*)-HNLs which were discovered and reported

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by Asano and co-workers in 2005.[123] (*R*)-HNL from *P. mume* (*Pm*HNL) is FAD-dependent, inhibited by sulfhydryl reagents, exhibits high sequence homology with *Ps*HNL and *Pa*HNL, and shows substrate specificity similar to *Pa*HNL. *Pm*HNL was purified to homogeneity from the plant source. The natural enzyme was used to synthesize ranges of cyanohydrins. The enzyme was expressed in *P. pastoris* as a secretory protein.[113] (*R*)-HNLs from *Eriobotrya japonica* (*Ej*HNL) and *Passiflora edulis* or passion fruit (*Pe*HNL) were also purified, characterized and used properly in the synthesis of ranges of (*R*)-cyanohydrins in biphasic systems.[124−126] Earlier than our report on *Ej*HNL, synthesis of some (*R*)-cyanohydrins was reported by (*R*)-oxynitrilase from *Eriobotrya* L. in a microaqueous reaction system (Table 4).[127] There will be more information on these newly discovered enzymes under the screening section of this review. Comparative information on 11 (*R*)-HNLs have been included in the Table 4, as well.

3.2. (*S*)-Hydroxynitrile Lyases. *(S)-SbHNL.* HNL from *S. bicolor* was first described in 1961 during a study of biosynthesis of dhurrin in *Sarghum vulgare* (var. Honey Drip). The presence of a cyanohydrin catalyzing enzyme was observed, and the enzyme was purified 175-fold and partially characterized. The authors also introduced a spectrometric assay method that could monitor decomposition of the *p*-hydroxymandelonitrile.[128] The enzyme was purified and characterized by other groups in 1992[129] and 1993.[130] Finally, it was cloned and sequenced by Wajant and co-workers, although the gene was expressed as an inactive form in *E. coli*, probably because of lack of post-translational processing.[131] Currently, (*S*)-*Sb*HNL is available from Sigma.

(S)-MeHNL. HNL from *M. esculenta* was first purified from acetone powder of young leaves of cassava to 277-fold in 1994; the enzyme was characterized and its cDNA was sequenced for the first time among the (*S*)-HNLs. It was reported that the enzyme has a molecular mass of 92 000 kDa and, it was suggested, a homotrimeric structure containing S−S bond. The enzyme had three isoforms, based on the isoelectric focusing, but they were neither glycosylated nor FAD-dependent proteins. The protein showed a sigmoidal curve in a low concentration of substrate acetone cyanohydrins, 0−30 mM in the total range of 0−300 mM.[22] A typical procedure was used for purification of this HNL from dehydrated leaves of *M. esculenta* up to 241-fold. Analysis of the purified fractions under reducing and nonreducing SDS−PAGE showed a single band of ∼30 kDa, a proof of homogeneity of the *Me*HNL, and the molecular mass of the native enzyme was calculated to be 124 kDa by gel filtration using Superdex 200, suggesting a homotetrameric structure. There was no serological relationship between this acetone cyanohydrin lyase and those of known HNLs until that time.[68] In a recent work, fast equilibration of dimers and tetramers were suggested for the structure of (*S*)-*Me*HNL in solution. On the basis of the dimensions calculated for the enzyme *Me*HNL (99.6 Å × 85.4 Å × 49.2 Å), it has also been suggested that the enzyme resembles a platelet more than a globular shape in solution, and this would be the reason for the migration pattern in the size exclusion chromatography.[132] (*S*)-*Me*HNL can be purchased from evocatal and Jülich (Codexis).

(S)-HbHNL. HNL from *H. brasiliensis* was partially purified in 1989, during the studies on significance of hydroxynitrile lyase in rapid cyanogenesis. *Hb*HNL increased the rate of HCN liberation up to 20-fold, indicating the importance of the enzyme in fast release of HCN and efficient cyanogenesis.[133] *Hb*HNL was then purified from frozen leaves of the plant *H. brasiliensis* to 106-fold in 1996. The enzyme showed only one band on the reducing (DTT plus SDS) and nonreducing (only SDS) SDS−PAGE, suggesting a lack of intermolecular disulfide bonds, and exhibited a molecular mass of 100−105 kDa in gel filtration. A dimer or tetramer structure was proposed for the enzyme, and kinetic parameters were calculated on the basis of a typical Michaelis−Menten substrate saturation curve for acetone cyanohydrin up to 300 mM. Later on, a dimeric structure was revealed for the enzyme in a phosphate buffer pH 6.5 using small-angle X-ray scattering (SAXS) based on the data collected from both concentrated (46 mg/mL) and 5- and 10-fold diluted samples.[134] The enzyme was cloned and functionally expressed in *E. coli* and *Saccharomyces cerevisiae*. The active site residues also were determined by site-directed mutagenesis.[135] The enzyme (*S*)-*Hb*HNL is commercially available from evocatal and Jülich (Codexis).

S-XaHNL. Lyases 1 and 2 were isoenzymes of the (*S*)-HNL from *Ximenia americana*, which had been purified and characterized in 1989 (Table 5).[136] We will discuss successful research of the newly discovered *S*-selective enzyme (*Bm*HNL) in the screening section.[33]

4. SCREENING FOR DISCOVERY OF NEW AND POTENT HNLS

A successful screening should focus on what is new in the screening: substrate, gene, protein, property, screening source, method, etc.[16] One of the most important items in the screening for each enzyme, including the HNL, is finding out a new source possessing new catalytic or biochemical properties. Therefore, it does not matter which method we use to achieve this goal. In the case of HNLs, it is obvious that exploring for the cyanide production, for instance, by plants might not be a perfect choice, since there are more than 3000 cyanogenic plant species, whereas the number of reported HNLs (characterized and non-characterized) is about 40 enzymes (less than 2% of cyanogenic organisms including plants, insects, and microbial species). Therefore, as shown in Figure 2, there are many cyanogenic plants that have no HNL activity, although noncyanogenic plants, such as model organism *A. thaliana*, have been found to have an *R*-selective HNL.[149] The majority of cyanogenic plants use slow chemical decomposition of cyanohydrins in vivo without interference of the HNLs.[150]

In addition, more efficient spectrometric, GC, or HPLC assay methods have been developed for enzyme assay in recent decades. In recent years, two main methods have been used for screening of these enzymes from nature, including the "real enzymatic activity" seeking and genome database information. Although until now, the majority of the discovered HNL enzymes have been found using the activity detection-based method, the quite young genome-mining method is expected to be more important for this purpose in the future. Indeed, there is a desire for finding new sources of HNLs to expand the current substrate range and for possibly catalyzing new reactions by these enzymes. In addition, each of the already known or new HNLs could be suitable for protein engineering and creation of new or tailor-made enzymes.

4.1. Activity Detection-Based Screening. The traditional experimentally based method relies on screening of the mainly cyanogenic plants (as well as other organisms) and detects the "real HNL activity". Almost all of the HNLs have been discovered from early times until now on the basis of various traditional

methods based on spectrometers, HPLC, GC, and other instruments.

Rosenthaler studied the distribution of emulsin-like enzymes among preparations of a great variety of plants to investigate whether they decompose the amygdalin to release HCN and yield optically active nitriles. There were also two new sources of the S-HNLs: *Taraktogenos blumei* (leaves) and *Achillea millefolium* (flowers). A noticeable number of plants found as new sources of the HNLs in later studies were listed in the study of Rosenthaler almost one century ago.[148]

Pfeil et al. (1966 and 1972) introduced several new sources of the flavoenzyme hydroxynitrile lyases from plants and studied their biochemical and immunological properties in detail and in comparison with the HNL from almond. The enzymes were isolated from the seeds of 10 plants belong to the *Rosaceae* family, including genus *Prunus* and the flowers of *P. spinosa*. The authors used both polarimetric and spectrometric methods to assay the hydroxynitrile activity. In the polarimetric method, the degree of rotated light was used for the detection of reaction product, (*R*)-mandelonitrile. The reaction was performed in a 25 mL flask, including 1 mL of 10 mM highly distilled benzaldehyde as the bottom layer, 1 mL of hydroxynitrile lyase solution (1 mg/mL), and HCN dissolved in ethanol. The mixture was shaken until it was clear, and the reaction was started in the ethanol/acetate buffer at 20 °C.

In the photometric method, a decrease in the intensity of crotonaldehyde was measured in a synthetic reaction of cyanohydrin from HCN and aldehyde at 304 nm. All of these enzymes were catalyzed for both the synthesis and decomposition of (*R*)-mandelonitrile.[30,138] The discovered and studied HNLs are listed in Tables 4 and 5. In another attempt in 1997, plant extracts from various tissues were found to have HNL activity only in decomposition of the mandelonitrile. The assay method was photometric monitoring of benzaldehyde produced during the cleavage of mandelonitrile.[151]

Hereafter, some new screening experiments will be reviewed, and the properties or reactions catalyzed by the discovered enzymes will also be highlighted. Hernandez and co-workers screened for HNLs among edible vegetables and fruits in 2004.[140] Their traditional method for the discovery of new enzymes was a screening among the plant species based on these criteria: plants having well-known cyanogenic glycosides, taxonomically related to other known HNLs, and readily available. The seeds and leaves of the selected plants were obtained from commercially available fresh fruits and garden trees, then the materials were blended with acetone three times to remove water, greasy materials, and pigments. Diisopropyl ether-extracted KCN was added to the air-dried materials containing the enzyme preparation in citrate buffer and benzaldehyde as substrate. The resulting mixture was stirred for 24 h at room temperature, dried over Na_2SO_4, and filtrated.

Enantiomeric excess and conversion percentage of the evaporated filtrate were determined by HPLC and ^{1}H NMR, respectively. The leaves and seeds of already known sources of HNL such as *P. amygdalus* (almond) and *P. serotina* var. *capuli* (capulin) were used as positive controls. The authors reported a few new sources for HNLs, including *Annona cherimolia* (cherimoya) and *Annona squamosa* (sugar-apple) for *S*-selective enzymes and *Cucumis melo* (melon, a noncyanogenic plant) and *Poiteria sapota* (mamey) as sources for *R*-selective HNLs. It is noteworthy that *P. serotina* var. *capuli* and *M. americana* were identified as sources of (*R*)-oxynitrilase in a similar manner described above in 1998.[139]

A massive screening was carried out by Asano and co-workers among 163 species from 74 cyanogenic and noncyanogenic plant families in 2005. The authors prepared a homogenate of leaves or seeds of each plant and established a simple HPLC-based HNL activity measurement. Following these experiments, several new HNL sources were found, including a few *R*- and only one *S*-selective enzyme (~4%). Bodies of plants, including leaves, roots, shoots, spikes, and rhizomes, were mainly collected from the Botanic Gardens of Toyama, Toyama City, Japan (Figure 5, sections 4.1.1 and 2). Plant seeds were cracked to release the soft kernels, and the kernels were crushed by a homogenizer at 4 °C in 10 mM potassium phosphate buffer (KPB). The suspension was filtered through cheesecloth and was then centrifuged to precipitate the debris, which resulted in a crude enzyme preparation. The crude enzyme was concentrated by ammonium sulfate fractionation and used for HNL assay by HPLC. Alternatively, other plant materials mentioned earlier were placed in a few layers of cheesecloth, frozen by soaking in liquid nitrogen, and cracked to release smaller pieces of these materials. The latter was converted into finer pieces or powder via grinding by mortar and pestle on ice (Figure 5, section 4.1.3). The ground materials were suspended in KPB and stirred overnight at 4 °C. The crude enzyme was prepared after centrifugation and concentration of the homogenate by filtration. The homogenates of 156 out of 163 plant species showed no activity in a cyanohydrin synthesis assay using benzaldehyde as substrate (Figure 5, section 4.1.5). Six new sources of HNLs were identified, including leaves and seeds of *Passiflora edulis* and leaves of *E. japonica*, *P. mume*, *Chaenomeles sinensis*, and *Sorbus aucuparia* for (*R*)-HNL and *B. montanum* as the only *S*-selective HNL source in this study. Specific activity of the enzyme in the homogenates varied between 0.63 U/mg for the *B. montanum* and 16.3 U/mg for *E. japonica*, which are lower than that of kernels of almond (*P. dulcis*, 73.5 U/mg) under the same conditions. The HPLC method established in this study sensitively determines activity, *S* or *R* configuration, and enantiomeric excess, which is not possible to perform by spectrometric methods.[123]

The Asano group has continued to study the discovered HNLs in the above-mentioned massive screening, and we will have some highlights on the newly found enzymes and their properties with emphasis on the "synthesis" of cyanohydrins in these studies. As shown in Figure 5, after reconfirmation of the HNL activity, the next step could be purification of the enzyme (section 4.1.6). An ammonium sulfate fractionation and various column chromatographies are regular purification steps of the enzyme and have been used for four newly discovered (*R*)-*Pm*HNL, (*R*)-*Ej*HNL, (*R*)-*Pe*HNL, and (*S*)-*Bm*HNL. In the case of the enzymes with glycosylation, application of the columns such as concanavalin A is recommended. (*R*)-*Pm*HNL and (*S*)-*Bm*HNL were purified to homogeneity from the corresponding plant; their N-terminal amino acid sequences were determined (Figure 5, section 4.1.8); cDNA and genomic DNA cloning was carried out (Figure 5, section 4.1.7, 9−10); and finally, their genes were heterologously expressed in *P. pastoris* and *E. coli*, respectively.[33,113] Two others, *Ej*HNL and *Pe*HNL, as well as two former enzymes were purified to homogeneity and characterized in detail.[124−126] The main features of these purified and characterized enzymes from the natural sources or in the recombinant forms have been included in Tables 4 and 5.

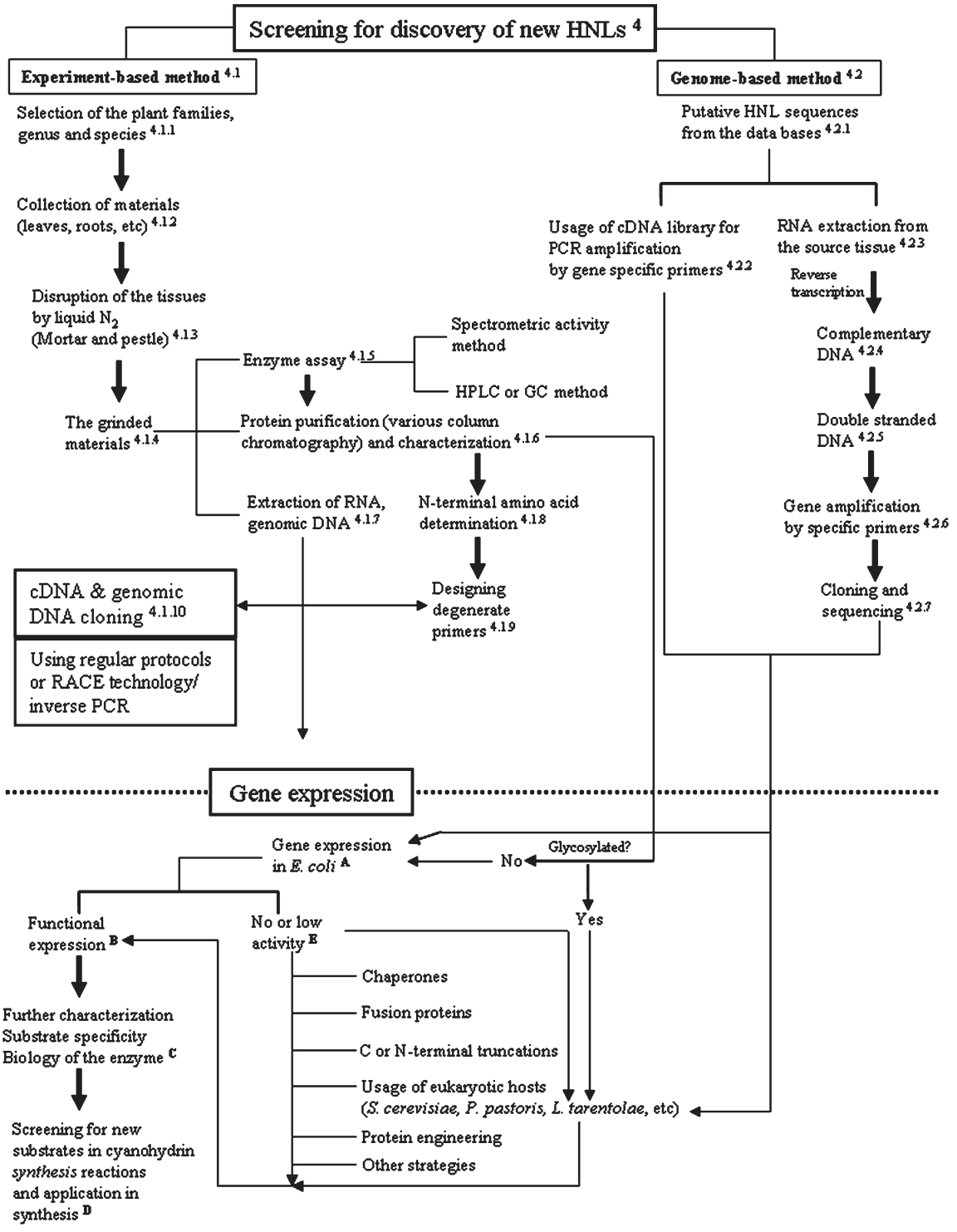

Figure 5. HNLs: from screening and discovery (upper part) to functional expression and characterization (lower part). The numbers and letters correspond to the information given in the text.

*R-Pm*HNL exhibited very broad substrate specificity: a screening was carried out among mono-, di-, and poly substituted benzaldehydes; heteroaromatic and polycyclic aldehydes; aliphatic aldehydes; and methylketones. Overall, more than 100 carbonyl compounds were used as potential substrates in the synthesis of *R*-selective cyanohydrins by the new enzyme. The enzyme synthesized (*R*)-cyanohydrins from all categories mentioned earlier with excellent enantioselectivity.

Preparative scales of cyanohydrin synthesis for selected aromatic aldehydes (>20 substrates) and aliphatic aldehydes and methyl ketones (>10 substrates) were also carried out by (*R*)-*Pm*HNL under a biphasic system (citrate buffer and an organic solvent, such as DIPE, TBME, or DBE). These synthesis reactions resulted in an ee up to 99% and a yield of 96% for the former group and an ee up to 96% with a yield up to 72% for the latter group.[150] Since aliphatic aldehydes were shown to be accepted as

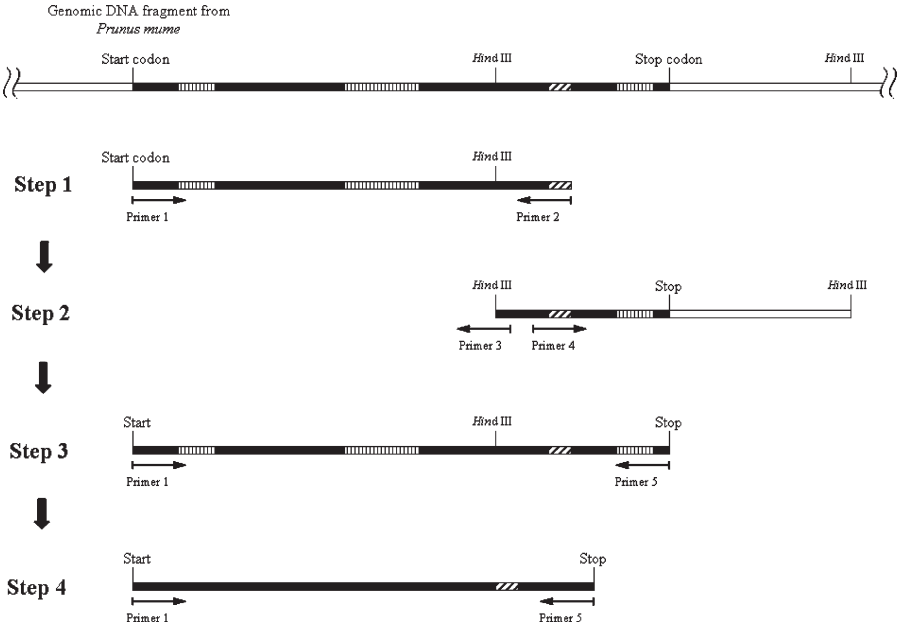

Figure 6. Cloning strategy for genomic DNA and cDNA encoding *Pm*HNL2. Black area: gene encoding *Pm*HNL2. Striped area: smino acid residues (494YWYHGG498, sequence information from *Pa*HNL1), including FAD-binding and putative active site residues. Hatched line box: introns. Two *Hind*III sites were found in the flanking region of the gene. Step 1: amplification of part of the initial sequence of the gene. Step 2: amplification in part of the terminal sequence of the gene using inverse PCR. Step 3: amplification of full-length genomic DNA encoding the enzyme. Step 4: amplification of full-length cDNA encoding the enzyme.[113]

excellent substrates compared with the aliphatic methyl ketones, a larger number of these carbonyl compounds having different structural features were screened as potential substrates. The enzyme (*R*)-*Pm*HNL has a striking similarity to the (*R*)-*Pa*HNL in substrate specificity (chemical yield and enantioselectivity). In addition, there are some unique new substrates that can be exclusively accepted by the (*R*)-*Pm*HNL.[152] Despite this high cyanohydrin synthetic potential of *Pm*HNL, there was a problem of active protein production in *E. coli* (Figure 5, sections A and E), which was not unexpected because the native enzyme is glycosylated, but *E. coli* cannot provide this post-translational service to the expressed proteins. However, finally, the problem was solved by functional secretory expression of the *Pm*HNL in the methylotrophic yeast *P. pastoris*. Here, we represent a simplified scheme for the cloning strategy for genomic DNA and cDNA encoding *Pm*HNL2 in Figure 6.[13]

The second example of these enzymes comprises purification from the plant, cloning, and expression in *E. coli* of HNL from the plant *B. montanum*. A screening has also been performed among 100 carbonyl compounds as potential substrates for this enzyme. Chemical parameters and kinetics of the cyanohydrin synthesis for 20 selected substrates were studied for this only *S*-selective enzyme discovered following the massive screening. The enzyme was primarily purified from *B. montanum* with regular column chromatographies, although it was isolated from a native-PAGE in the last step. As an (*S*)-HNL, its activity was very low in the homogenate and, therefore, harder to purify than the (*R*)-HNLs.

There was a problem of expression of the *Bm*HNL in *E. coli*, although it was not glycosylated and exhibited high identity to (*S*)-*Hb*HNL and, especially, (*S*)-*Me*HNL. These latter two could already be expressed in *E. coli*, even though their expression level was not desirable (Figure 5, sections A and E). The problem of "no gene expression" in *E. coli* was solved for the enzyme with a screening among a few expression vectors, and it was found that the gene could be expressed as a fusion protein using both pColdI and pRSET-B vectors. From the strategies mentioned for solving the problem of the low activity, C-terminal truncation was found to be effective in enhancing the productivity of the (*S*)-*Bm*HNL in culture medium (Figure 5, section E).

The recombinant enzyme was then purified to homogeneity and used for a detailed characterization. Substrate specificity of the enzyme is quite different from two other (*S*)-HNLs (*Me*HNL and *Hb*HNL), although there is a high sequence homology between them. On the basis of a model built, there are differences among residues around the active site area and also the substrate channel, which is thought to be the probable reason for a new profile for substrates of *Bm*HNL. Piperonal was identified as the best substrate for synthesis of cyanohydrins in aqueous systems. The enzyme and its C-terminally truncated forms exhibited a molecular mass of 60−66 kDa, indicating dimeric structures. The ee percent and yield of cyanohydrin synthesis by the enzyme are expected to be improved using biphasic systems.[33]

Availability of large enough amounts of these enzymes is a prerequirement for designing and carrying out experiments to find new substrates and also catalyzing new reactions, which has not been reported before. Therefore, as Figure 5 represents (section B), functional expression of the enzyme is a key step in the biocatalysis and synthesis of HNLs. In the case of some HNL enzymes, the preference would be expression in hosts other than *E. coli*, such as *S. cerevisiae* or *P. pastoris*, which have already been used for industrially used enzymes, such as *Pa*HNL; *Hb*HNL; *Me*HNL; and recently, for *Pm*HNL (section E). Briefly, flexibility in usage of various methods to access large amounts of these enzymes is a critical point. One of these tools is protein engineering, which we will be discussing regarding its applications in the next sections.

In addition to the above-mentioned examples of screening for the new HNLs, a new (*R*)-HNL from common vetch (*Vicia*

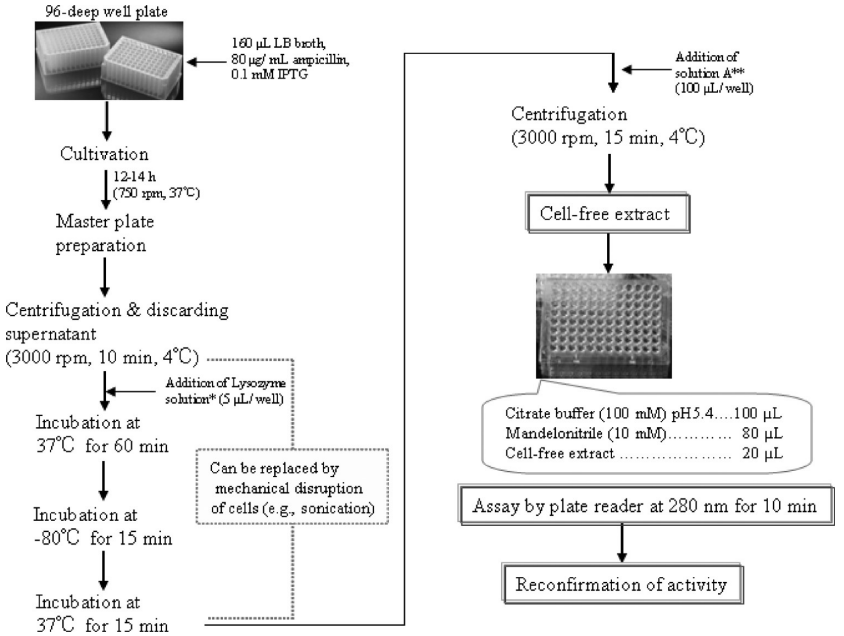

Figure 7. The high throughput screening method used for the generation of highly in vivo soluble mutants of *Me*HNL in *E. coli*[158,159] (*) Lysozyme solution contained 10 mM EDTA and 10 mg/mL lysozyme in 100 mM potassium phosphate buffer (pH 7.0). (**) Hypotonic solution consisted of 10 mM potassium phosphate buffer (pH 7.0) and 5 mM $MgCl_2$. Three cycles of −80 and 37 °C were used for preparation of cell-free extracts.

sativa L.) was introduced in 2006. The pulverized meal of the plant containing the enzyme was used for synthesis of several cyanohydrins with good to excellent yield and ee percent, although the discovery method was not described.[141] In another attempt, Nanda et al. used almost the same method as Asano group to search among the *Rosacea* family in a northern territory of India in 2009 and found a few (*R*)-HNL sources, including the new source *P. armeniaca* Shakarpara cultivar (apricot). They used a biphasic reaction mixture containing the new enzyme to catalyze synthesis of δ,ε-unsaturated cyanohydrins with very good to excellent yield and ee percent.[153] Another new source of the (*R*)-HNL was introduced from *Prunus pseudoarmeniaca*. The acetone-extracted powder of the homogenate of kernels was dried at room temperature and dissolved in phosphate buffer, the suspension was centrifuged, and the crude enzyme preparation was fractionated by ammonium sulfate and immobilized on the Eupergit C and C250 L. The free and immobilized enzyme was used to study the effect of pH and temperature on cleavage of mandelonitrile to benzaldehyde using a spectrometric method. The immobilized enzyme showed a higher K_m value than other known HNLs and could be reused 25 times with little loss of activity.[143]

4.2. Genome-based Screening. In comparison with the traditional method of screening for new HNLs, which relies on detection of real HNL activity, the genome-based strategy is easier and more straightforward. Although the chance of picking a sequence having HNL activity still is low, it is expected to increase in coming years, as we expect for other enzymes. We will have a look into two successful cases of genome mining for discovery of the new HNLs.

(*R*)-Hydroxynitrile lyase from noncyanogenic model plant *A. thaliana* is the first successful example of genome-based search for the HNL activity. During studies of structure−function relationships of α/β-hydrolases, several putative (*S*)-HNLs similar to *Hb*HNL and *Me*HNL were cloned and expressed in *E. coli*, and only one of them (AAN13041) showed activity toward mandelonitrile and some other cyanohydrins. This enzyme was named as the first (*R*)-HNL with an α/β-hydrolase fold. The subsequent study revealed that the enzyme has broad substrate specificity in synthesis of cyanohydrins, including various aromatic and aliphatic aldehydes and ketones.[149]

In the case of this enzyme, it is quite easier to have the cDNA, because the cDNA library of the *A. thaliana* is already commercialized and available (Invitrogen, Carlsbad, CA; Figure 5, section 4.2.2). This cDNA library has been used to clone and express the enzyme and subsequently in synthesis of (*R*)-β-nitro alcohols by the (*R*)-*At*HNL in a potassium phosphate and *n*-butyl acetate biphasic system as the first (*R*)-HNL-catalyzed Henry reaction. An enantiomeric excess up to 95% was achieved in this study. *At*HNL showed an optimum for the reaction at pH 7.0; however, it was quite unstable at pH values lower than 5.0.[154] This is an example of catalysis of an unusual reaction by the HNLs, that is, enzyme promiscuity, as (*S*)-*Hb*HNL does in the synthesis of the *S* enantiomers of these valuable chemicals[155] and also a retro-Henry reaction, as reported by the same group.[156] The crystal structure of the *At*HNL has been solved (PDB 3DQZ), and the enzyme has already been made available commercially from suppliers such as Sigma-Aldrich and evocatal GmbH.

As Figure 5 shows (section 4.2.2), there is no need for primary purification of the enzyme from plant (or other sources), N-terminal amino acid determination, and cDNA cloning steps. Then the gene can be expressed in a host, based on the existing information. If it is thought to be a glycosylated protein, eukaryotic hosts are better choices than *E. coli*. On the other hand, in some cases, the purified and characterized natural enzyme is in hand, but there are difficulties in preparation of cDNA and even genomic DNA for cloning purposes. Therefore, the putative gene sequence will be very useful for cloning and expressing the enzyme in a suitable host. In another scenario, the sequence of the gene is available (Figure 5, sections 4.2.3−7), but

cDNA should be prepared from the genomic DNA. This is slightly more complicated than the former situation but still much easier than the regular cDNA cloning. The gene then can be sequenced and amplified by the specific primers and subsequently be cloned and expressed in a suitable host.

The second example of the discovered HNL by genome-mining is also an *R*-selective enzyme, which was also the first bacterial hydroxynitrile lyase reported thus far. *X. fastidiosa* is a Gram-negative bacterium pathogenic to many economically important plants and is transferred by various vector insects. A conserved hypothetical protein sequence that could be a putative α-hydroxynitrile lyase enzyme was found. The bacterial genome contains no introns; therefore, cloning of the gene is possible using the gene-specific primers, that is, with skipping the cDNA cloning (it was done like Figure 5, section 4.2.2). The gene SCJ21.16 (XFa0032) was expressed in *E. coli* BL21 (DE3), and the (*R*)-*Xf*HNL was purified using a single nickel−Sepharose step and used for detailed characterization. Its main studied properties are included in Table 5.[26] There are also examples of very highly soluble expression of the putative HNL genes from *Oryza sativa* in *E. coli*, which show no HNL activity, either in synthesis or in decomposition of several cyanohydrins.[157]

4.3. High Throughput Screening of Libraries of Mutant HNLs. Following generation of a library, there are thousands of mutants that cannot be tested in a cyanohydrins synthesis or decomposition reaction by spectrometry, HPLC, or GC; therefore, a method of screening is necessary.

(I) The method illustrated in Figure 7 was used for high-throughput screening of highly in vivo soluble mutants of *Me*HNL in *E. coli* by Asano and co-workers. The clones were cultivated in Luria−Bertani broth, and 96-well plates were incubated at 37 °C for overnight. Cell disruption was performed by a combination of lysozyme and freeze-thaw cycles (replaceable by mechanical cell disruption). Next, centrifugation provided a cell-free extract ready to assay in the presence of citrate buffer containing racemic mandelonitrile. The cleavage reaction of the mandelonitrile was employed as the screening test to detect produced benzaldehyde, although the more sensitive HPLC detection was used for the positive clones in comparison with the wild-type enzyme:[158] the regular test tube level cultivation and subsequent activity measurement was done for the preselected mutants. If a high activity was reconfirmed, the protein expression profiles were monitored in soluble and insoluble fractions by SDS-polyacrylamide gel electrophoresis. Thousands of colonies can be screened with this method in a relatively short time. To prevent damage caused by produced benzaldehyde to the plate reader, the plates can be covered with a layer of transparent plastic.[158,159] Despite the method can be used only for aromatic substrates, it did work very well for finding more active and soluble mutants in this study.

(II) However, another sensitive high throughput screening method was introduced in 2006 enable to detection of HNL activity, accepting both aromatic and aliphatic substrates. In the first step, the cyanohydrin was cleaved by the cell-free extract of *E. coli* containing HNL in pH 5−5.5, yielding HCN: A mixture of citrate−phosphate buffer, cell-free extract, and cyanohydrin was incubated for 5 min at room temperature, and the reaction was stopped by a mixture of *N*-chlorosuccinimide + succinimide, which oxidizes released CN^- to CN^+. Addition of a mixture of isonicotinic acid + barbituric acid allows monitoring the color formation for 20 min at 600 nm. The method uses the basics of a previously introduced protocol for cleavage of acetone cyanohydrin[160] by replacing isonicotinic acid with pyridine and performing the reaction in a 96-well plate, which enables it to be applied for screening purposes.[161]

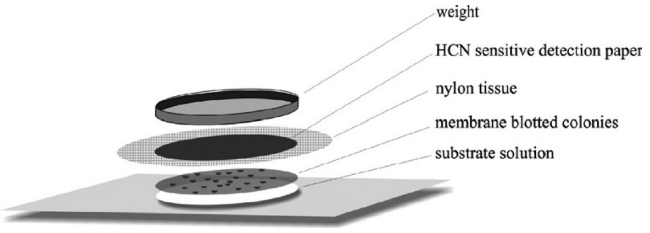

Figure 8. The sandwich test used to detect HNL activity at the colony level.[162]

(III) A sandwich test was introduced as a detection method of gaseous HCN liberated by colonies of *E. coli* expressing HNL in 2007, where racemic mandelonitrile and (*S*)-3-phenoxybenzaldehyde cyanohydrin were used as substrates. After copying the colonies that appeared following the mutagenesis, the membrane-blotted colonies of the mutants were equilibrated on a filter-paper sheet already incubated in 50 mM citrate−phosphate buffer, pH 6.5. As Figure 8 shows, membrane-blotted colonies were incubated upside down on the bottom of the sandwich with the substrate solution, allowing the colonies to be in direct contact with the substrate. A permeable nylon tissue separated it from the upper layer, which was a HCN-sensitive detection paper. A glass disk was used to keep the detection paper in uniform proximity to the blotted colonies on the membrane. The detection paper was already soaked in a mixture of copper-(II) ethyl acetate and 4,4′-methylenebis. Following the HCN's reaching the detection paper, the blue color will appear which reflects HNL activity.[162]

(IV) To screen the colonies of *Pa*HNL5 synthesizing the (*R*)-pantolactone, a library of saturation mutagenesis on several points of the gene was generated in plasmid pGAPZA. For each mutation point, more than 200 transformants of *P. pastoris* were tested for improved conversion toward hydroxypivalaldehyde at pH 2.4 by a colorimetric method. After 5 days of cultivation, the resulting grown cells were separated by centrifugation, and the supernatant was used for the assay: The supernatant was mixed with citrate−phosphate buffer, pH 2.4; substrate solution; and NaCN to start the reaction. The plate was covered by a seal of aluminum foil to prevent HCN evaporation. Magnetic stirring was used for agitation of the mixture for 1 h under a well ventilated hood, then the reaction was stopped by addition of H_2SO_4. The reaction mixture was diluted and subsequently used to screen the conversion by fluorometric analysis using 4-hydrazino-7-nitrobenzofurazane (NBDH) to detect the remaining aldehyde. The increasing amount of fluorescent hydrazone was monitored.[163]

5. ENGINEERING OF HYDROXYNITRILE LYASES

Since there are many studies on the potential active site residues and mechanism of catalysis of these enzymes, such

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mutagenesis cases will be excluded from this article, mainly Thr11, Glu79, Ser80, Cys81, Asp207/208, and His235/236 for (*S*)-HNLs and mainly His459 and His497 for (*R*)-HNLs. Therefore, mutations used for improving the properties of these enzymes will be discussed hereafter.

5.1. Engineering of *R*-Selective HNLs. pH instability of the first recombinant (*R*)-*Pa*HNL-5 was overcome using a secretory signal of *S. cerevisiae*, a mutation of Leu1Gln, and expression in methylotrophic yeast *P. pastoris* (Table 6, entry 1). The enzyme exhibited a higher productivity up to 4.5-fold. *Pa*HNL5-Leu1Gln (with and without the additional mutation Ala111Gly) showed much higher half-life values than the deglycosylated *Pa*HNL and the enzyme prepared from almond seeds. Overglycosylation of the enzyme is not the only parameter offering the improved property, since there was only a slight decrease in pH stability after treatment with endopeptidase H at 37 °C. Docking simulation of (*R*)-2-chloromandelonitrile in the active site of *Pa*HNL-5 identified the residues near the chloro substituents of this substrate with unfavorable steric hindrance, especially Ala111 (Table 6, entry 2). Activity of mutant *Pa*HNL-Ala111Gly was enhanced strongly in the synthesis of 2-chloromandelonitrile by this method along with production of a large amount of the stable recombinant enzyme in the yeast *P. pastoris*. These studies resulted in the first multiton scale production of cyanohydrins by HNLs.[164]

Both chemocatalytic and biocatalytic production methods of cyanohydrin precursors of prils had disadvantages; especially, the maximum enantioselevtivity reached by the *Pa*HNL was about 90%. To produce this precursor of ACE inhibitors (prils, see Table 3) from the 3-phenyl propionaldehyde by the *Pa*HNL, Glieder et al. (Table 6, entry 3) generated 12 mutants based on the structure of the enzyme and engineering of the active site area. The authors modeled the complexes of both *S* and *R* enantiomers of the substrate with the *Pa*HNL5. The interaction of the enzyme showed differences with the alkyl group of the substrate, which was oriented to Ala111 and Val360 in the *S* and *R* enantiomers of the substrate 3-phenyl propionaldehyde. The substrate-biding site of the enzyme was redesigned by exchanging the residues of various sizes while retaining hydrophobicity.

Val360 plays an important role such that exchanging with Ile enables the mutant enzyme to catalyze the reaction with an improved enantiomeric excess and conversion up to 98% and accelerate the transformation up to 6-fold with a low ratio of enzyme/substrate in a water-based system. Smaller amounts (10- to 30-fold) of the mutant Val360Ile were needed for this reaction. In addition, a very small amount of the Val360Ile was enough to catalyze the cyanation reaction of the recalcitrant substrate *trans*-cinnamaldehyde to its corresponding (*R*)-cyanohydrin with an ee of 98% in only 3 h.[165] Despite the improved catalytic property of the double mutant *Pa*HNL-Leu1Gln, Ala111Gly, the amount of secretory protein in the medium was decreased. Therefore, the reasons for a lower expression of the enzyme following the mutations were investigated, in which the LC−MS/MS analysis of the deglycosylated and tryptically digested *Pa*HNL5 showed a modified residue near the Ala111Gly, that is, Asn110Asp. This deamination is a result of post-translational modification (PTM), apparently triggered by the Ala111Gly. It was suggested that this PTM resulted in a delayed processing and secretion of the mutant protein into the culture medium. In addition, the result of this research reconfirmed the important and unique role of Ala111Gly for enhanced turnover rates of the substrate 2-chlorobenzaldehyde. This is an example indicating that unexpected modifications can happen to the recombinant proteins and may affect the quality of the final product.[166]

(*R*)-Pantolactone was efficiently synthesized by semirational redesign of acid-stable *Pa*HNL5, in which the conversion of sterically hindered aliphatic aldehydes was targeted. All hydrophobic residues of the substrate-binding pocket were selected as candidates for site-saturation mutagenesis (Table 6, entry 4), including Phe72, Val113, Val317, Val329, Leu331, Leu343, and Val360. An overlap extension PCR was used for generation of a saturation library of the *Pa*HNL5 gene in plasmid pGAPZA transformed in *P. pastoris*. More than 200 transformants were tested for improved conversion toward hydroxypivalaldehyde for each mutation in 96-well format at pH 2.4 by a colorimetric method. The primary obtained mutants were more precisely screened by a GC method for the enhanced enantiomeric excess, which ultimately resulted in the mutant Val317Ala. This mutant exhibited significant improvement in enantioselectivity and retained the low-pH stability, although the new mutation could cause negative effects on the synthesis of the formerly introduced bulky substrate, (*R*)-2-chlorobenzaldehyde. The conversion rate of the mutant enzyme *Pa*HNL-Val317Ala rose >2.5-fold, and there also was a 4-fold increase in the enantiomeric excess toward hydroxypivalaldehyde to 87% in 2.5 h. Increasing the reaction time to 20.5 h increased the conversion rate 87%, but there was a slight decrease in the enantiopurity for this mutant of *Pa*HNL5 with this longer reaction time. Reduction of hydrophobic interactions with the wrong enantiomer was thought to be the reason for the enhancement of the stereoselectivity. A preparative scale for this reaction was performed with the mutant Val317Ala in a 100 mL reactor at 4 °C to get 88% yield and 96% ee.[62]

A directed evolution approach was employed to enhance the *Pa*HNL5 gene expressed in *P. pastoris* because the enzyme was prepared in large amounts in this host rather than *E. coli*. First, an error-prone PCR was performed on the already engineered *Pa*HNL5/Leu1Gln/Ala111Gly gene under elevated $MgCl_2$ concentration. Then an overlap extension PCR-based strategy was used to construct a glyceraldehyde 3-phosphate dehydrogenase (GAP) promoter-driven expression cassette containing the gene. The host was directly transformed with this linear cassette, resulting in 2000−6000 transformants/μg of DNA, which subsequently were tested in 96-well plates for cleavage of the racemic mandelonitrile monitored photometrically at 280 nm. The second small library of mutants resulted in 500 transformants that were subsequently were screened. Finally, the better mutants were compared for enantioselective synthesis of 2-chloromandelonitrile. Six mutants based on the *Pa*HNL5-Leu1Gln, Ala111Gly enhanced the ee from 94% to 99%, and the conversion rate was improved from 70% to 95% at 10 °C over a 2 h reaction. Triple mutant Leu1Gln, Ile108Met, Ala111Gly provided a doubled reaction time. The above-mentioned mutant plus Asn3Ile and two silent mutations exhibited a conversion of 94% in only 2 h. Turnover frequency (TOF) was improved for the selected mutants of the new mutations in comparison of the already engineered *Pa*HNL5-Ala111Gly for different sterically hindered monosubstituted benzaldehydes. The best mutant (Leu1Gln, Asn3Ile, Ile108Met, Ala111Gly, Pro85Pro, and Ser432Ser) could synthesize (*R*)-2-chloromandelonitrile in a recovery yield and ee of almost 93 and 99%, respectively. The reaction was performed under a water-based biphasic system in 4 h reaction time (Table 6, entries 5−7).[167]

5.2. Engineering of *S*-Selected HNLs. (*S*)-*Me*HNL was subjected to the mutagenesis experiments based on the rational

Table 6. Brief Description of the Mutants or Engineered HNLs That Exhibited Improved Characteristics

entry	mutant (s)	enzyme	property and engineering method	reference(s)
1	Leu1Gln	(*R*)-*Pa*HNL5	enhanced productivity in *P. pastoris*	164
2	Leu1Gln, Ala111Gly, (Asn110Asp)	(*R*)-*Pa*HNL5	enhanced low-pH stability in synthesis of (*R*)-2-chloromandelonitrile (rational design)	164
3	Val360Ile	(*R*)-*Pa*HNL5	enhanced enantioselectivity in synthesis of cyanohydrin precursor of ACE inhibitors (rational design)	165
4	Val317Ala	(*R*)-*Pa*HNL5	A 2.5-fold elevation in conversion and 4-fold in ee percent in synthesis of hydroxypivalaldehyde cyanohydrin (semirational design)	163
5	Leu1Gln, Ala111Gly, Asn3Ile	(*R*)-*Pa*HNL5	15% increase in specific activity (directed evolution)	167
6	Leu1Gln, Ala111Gly, Pro85Pro, Ser432Ser	(*R*)-*Pa*HNL5	30% increase in specific activity by these two silent mutations (directed evolution)	167
7	triple mutant Leu1Gln, Ile108Met, Ala111Gly or another mutant Leu1Gln, Asn3Ile, Ile108Met, Ala111Gly, Pro85Pro, Ser432Ser	(*R*)-*Pa*HNL5-Leu1Gln, Ala111Gly	improved ee, yield, and turnover frequency (k_{cat}) for synthesis of (*R*)-2-chloromandelonirtile for the already engineered enzyme (directed evolution)	167
8	Trp128Tyr, Trp128Leu, Trp128Ala, Trp128Cys	(*S*)-*Me*HNL	The same or decreased specific activity to natural substrate, but dramatic increase toward the unnatural substrate 4-hydroxy-mandelonitrile + enhanced ee and especially the conversion rate for the sterically demanding aromatic substrates, such as 3-phenoxy-benzaldehyde and unsaturated cinnamaldehyde and 2-hexenal (in addition to Trp128Cys); Trp128Ala increased ee (90 → 97%) and yield (67 → 82%) for 3-phenolpropanol in DIPE; Trp128Leu showed a dramatic increase in ee percent and yield toward two ethyl ketones (rational design)	168, 169
9	Gly113Ser	(*S*)-*Me*HNL	2−3 fold increased productivity in *E. coli*; increased specific activity; increased pH (slight) and temperature stability, probably because of higher β-sheet content following this single substitution (structure-based mutagenesis)	170
10	Trp128 mutants	(*S*)-*Me*HNL	The small, substituted residues such as Ala could invert the stereo-selectivity of the enzyme so that they could synthesize the *R* enantiomers from the racemic mixture substrates, such as 2-phenylpropionaldehyde and 2-phenylbutylaldehyde	171
11	Trp128Ala	(*S*)-*Me*HNL	the mutant catalyzed the synthesis of 3-phenoxybenzaldehyde cyanohydrin at an unusually high pH value in a two-phase system faster than the wild type and with a smaller amount of enzyme	172
12	Lys176Pro, Lys199Pro, Lys224Pro	(*S*)-*Me*HNL	increased in vivo solubility of the enzyme in *E. coli*, increased specific activity in the cell-free extract (higher productivity), minor secondary structural changes, and kinetic parameters in purified form (structure-guided mutagenesis)	158, 159
13	His103Leu, His103Val, His103Ile, His103Met, His103Cys	(*S*)-*Me*HNL	increased in vivo solubility of the enzyme in *E. coli*, increased specific activity in cell-free extract (higher productivity), minor secondary structural changes, and kinetic parameters in purified form (directed evolution)	158, 159
14	Trp128Ala + His103Leu, Gln215His	(*S*)-*Hb*HNL	high conversion and selectivity toward 4-substituted cyclohex-3-ene carbaldehydes using small amounts of the enzyme in comparison with *Hb*HNL-Trp128Ala (rational design and directed evolution)	176
15	Gly12Thr, Met239Lys	SABP2 (salisylic acid binding protein 2 from *N. tabacum*)	changing two critical amino acids generated HNL activity, despite no common mechanistic steps between esterases and hydroxynitrile lyases (rational design)	177

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design by Effenberger et al., in 2002. They generated a single mutant Trp128Ala and studied its crystal structures in the presence and absence of the substrates. According to the crystal structure of the wild-type *Me*HNL, substrates should pass a narrow entrance channel to access the deeply buried active site of the enzyme (Ser80, Asp207, and His236) (Table 6, entry 8). Precise study revealed that the mutation did not change the overall structure of the active-site tunnel or those of catalytic residues; however, it changed a narrow and blocked substrate channel to a wide-open cleft. The resulting structural modification caused higher accessibility of the active site to the bulkier substrates. The wall of this channel contains 11 hydrophobic amino acids.[168]

Indeed, the channel was capped by a Trp128 residue and was shown to have an important role as a substrate determinant. Primarily, the Trp128 was replaced with four other amino acids with diminished steric demand, including Ala, Cys, Leu, and Tyr. These mutations resulted in mainly the same or decreased specific activity toward the natural substrate of the enzyme, that is, acetone cyanohydrin, while showing highly enhanced specific activity in decomposition of the unnatural substrate 4-hydroxymandelonitrile, 85-, 180-, 445-, and 935-fold increases for Trp128Tyr, Trp128Leu, Trp128Ala, and Trp128Cys, respectively.

However, the main purpose of the study was to investigate the role of this residue on acceptance of the bulky substrates, such as a valuable precursor of pyrethroids, that is, 3-phenoxybenzaldehyde cyanohydrin (see Table 3). All of the mutants showed excellent enantiomeric excess to the nine substituted benzaldehydes (aromatics), like the wild type did, although there were higher conversion rates than the wild-type *Me*HNL. Especially, the sterically demanding substrates 4-methylbenzaldehyde and 3-phenoxybenzaldehyde cyanohydrins were synthesized with excellent ee percent and very high yield (up to 97−98%) by the mutants adsorbed onto nitrocellulose in diisopropyl ether (DIPE), exhibiting a priority compared with the wild-type enzyme.

In contrast, the substrate specificity was not dependent on the bulkiness of the channel mutants for aliphatic saturated aldehydes. All mutants, with the exception of Trp128Cys, exhibited slight enhancement of the already excellent ee percent for the unsaturated cinnamaldehyde and a higher conversion rate than the wild-type *Me*HNL. The same pattern was observed for another unsaturated substrate, 2-hexenal, although the enhancement of the conversion rates were even higher than the above-mentioned substrates (4-methylbenzaldehyde and 3-phenoxybenzaldehyde) for these mutants. Mutant Trp128Ala could increase the ee (90 to 97%) and yield (67 to 82%) for the 3-phenylpropanol in the DIPE. Trp128Leu exhibited a dramatic increase in the ee and conversion rates for two ethyl ketones in this study, but all mutants showed the same rates for all other tested methyl or ethyl ketones. There was no significant difference between the results of synthesis of 3-phenoxybenzaldehyde cyanohydrin by the wild-type *Me*HNL and mutant Trp128Ala in citrate buffer and water/MTBE two-phase system.[169]

Gly113 is located on the cap domain of *Me*HNL and is completely exposed to the molecular surface of the enzyme (PDB 1DWP). A site-directed mutagenesis was performed, and mutant Gly113Ser was generated, which exhibited 2−3-fold higher productivity than the wild-type enzyme expressed in *E. coli* (Table 6, entry 9). Significant differences occurred in the content of the secondary structure of the enzyme following the mutation, as proved by circular dichroism spectroscopy. This single mutation resulted in a complete conformational change, which increased its rigidity, ultimately to an improved functionality and higher specific activity compared with the wild-type enzyme. Characterization of the mutant enzymes revealed that they are almost the same for several parameters, such as purification steps, oligomeric state based on the gel filtration, and typical Michaelis−Menten kinetic. Temperature stability of the mutant *Me*HNL-Gly113Ser was increased, which led the authors to correlate it with the higher content of the β-sheets following this single substitution. The mutant also exhibited a slightly higher stability to low pH values, which makes the enzyme more favorable for the synthesis of cyanohydrins under these conditions.[170]

Effenberger and co-workers investigated the change of stereoselectivity of HCN addition to α-and β-substituted aldehydes containing the stereocenters by the Trp128 mutants of *Me*HNL. The Trp128 mutants adsorbed onto nitrocellulose with decreasing sizes of amino acids wild type > Trp128Tyr > Trp128Leu > Trp128Cys > Trp128Ala were used to synthesize cyanohydrins (Table 6, entry 10). The wild type exhibited high *S* selectivity toward racemic 2-phenylpropionaldehyde, but the stereoselectivity changed dramatically with the size of the substituted residues. *Me*HNL and *Me*HNL-Trp128Tyr showed *S* selectivity in synthesis of the racemic 2-phenylbutyraldehyde cyanohydrin, although stereoselectivity changed for smaller substituted amino acids. *R* selectivity dominates by reactions of Trp128Ala and Trp128Val. The authors successfully rationalized the inversion of the *S* stereoselectivity of the wild-type *Me*HNL to *R* selectivity in the *Me*HNL-Trp128Ala using crystal structure data and molecular modeling.[171] Therefore, as we have seen, not only regular properties of the HNLs can be improved but also the selectivity of the enzymes might be changed using the engineering techniques and subsequent screening among the generated mutants. Another interesting lesson from this research is a multipurpose screening among the library of generated mutants, as Effenberger and co-workers have done on the Trp128 substitutes so far. Medium to high level of amino acid identity and structural homology among the HNLs from each of four categories mentioned at the beginning of this article gives a high chance to reproduce and subsequently to enhance the improvements of others.

High temperature and pH values support the occurrence of a nonenzymatic reaction and consequently result in the formation of racemic cyanohydrins. Therefore, to suppress these undesired nonenzymatic reactions, the above-mentioned parameters should be reduced, but enzymes exhibit a decreased activity following the reduction of temperature and pH values, and it is necessary to use large amounts of these biocatalysts for the reactions, which in turn will cause higher costs. A method of synthesis of cyanohydrins was developed for *Me*HNL and *Me*HNL-Trp128Ala under unusually high pH values for 3-phenoxybenzaldehdye as the substrate (Table 6, entry 11).[172] The method is mainly applicable for the substrates that exhibit a slow nonenzymatic reaction. The authors correctly pointed out that there are enzyme assays based on cleavage of the cyanohydrins, whereas the resulting data are being used for discussion of the synthesis reaction. They emphasized the "synthesis" reaction of cyanohydrins monitored by a spectrometric assay method. The optimum pH value was found to be 8.0 for the synthesis of 3-phenoxybenzaldehyde cyanohydrin using this method with a two-phase system of diisopropyl ether (DIPE) buffer. Both

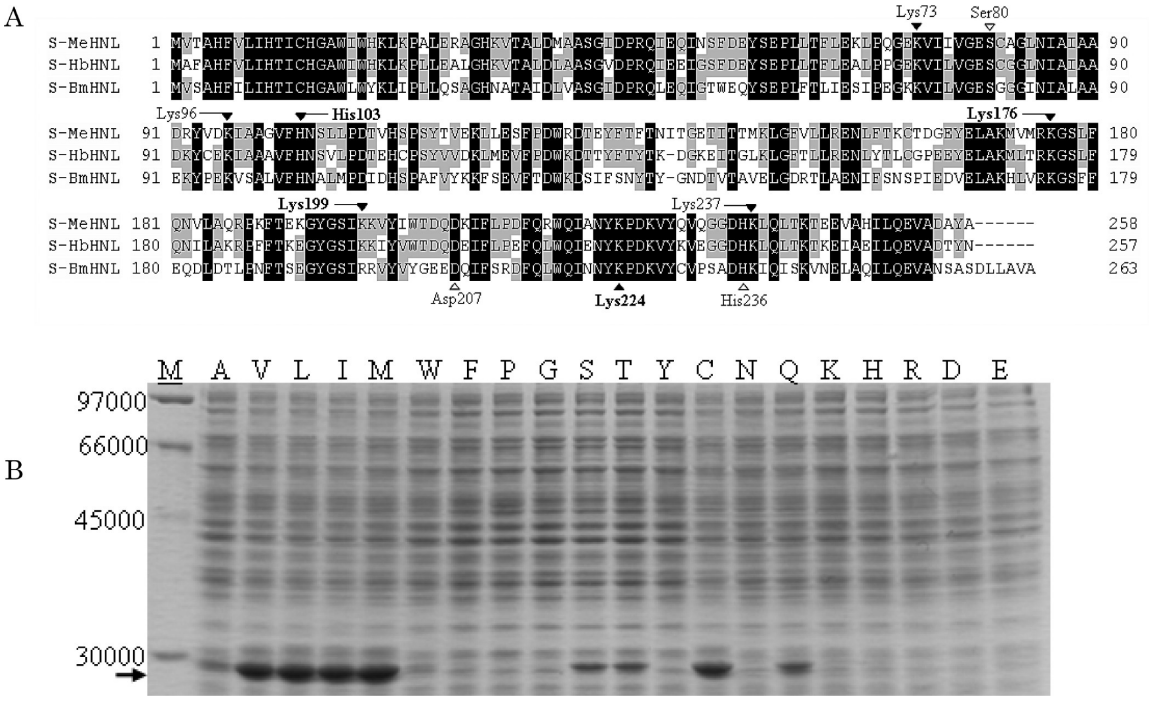

Figure 9. (A) Sequence alignment between three (*S*)-HNLs. The active site residues, five surface Lys residues, and the deeply buried residue His103 have been shown in this figure. (B) Soluble fractions of the *Me*HNL-His103 mutants for 20 L-amino acids. The location of the expressed enzyme is shown with the arrow. The amount of soluble expression of the mutants was highly enhanced in comparison with the wild-type *Me*HNL (His103). His103Gln, Thr, Ser, and Ala also showed improved in vivo solubility following the substitution in this deeply buried residue.[158,159]

wild-type and mutant Trp128Ala reached a 97% conversion rate and ~85% ee in 24 h. The mutant *Me*HNL-Trp128Ala catalyzes the reaction significantly faster (6 h) with a smaller amount of the enzyme needed, compared with the wild type.[172]

Asano and co-workers have introduced interesting results on the in vivo solubility of the overexpressed *Me*HNL in *E. coli* by structure-guided mutagenesis (Table 6, entry 12) and directed evolution (Table 6, entry 13) in 2005.[158] There are 15 out of 20 Lys residues conserved between the (*S*)-*Hb*HNL and (*S*)-*Me*HNL (Figure 9A). These residues are thought to have a role in the structure−function of the enzyme. On the other hand, there might be a relationship between the number of the Lys residues and the aldehyde resistance as the substrate of the enzyme.[173,174] First, five (i) conserved, (ii) solvent-exposed Lys residues (iii) located on the coils close to β-sheets (73, 96, 176, 199, and 224) were selected, and subsequent site-saturation mutagenesis resulted in a higher solubility for the Pro-substituted mutants at positions 176, 199, and 224 of the enzyme in this versatile host. After a screening step (section 4.3.I and Figure 5), more soluble mutants were selected on the basis of an activity assay by HPLC and subsequent SDS−polyacrylamide gel electrophoresis of the soluble and insoluble fractions. It was more interesting when the mutations were combined to obtain higher levels of solubility and activity. The double mutants (i) Lys176Pro, Lys199Pro; (ii) Lys176Pro, Lys224Pro; and (iii) Lys199Pro, Lys224Pro exhibited higher specific activity than the soluble single mutants in cell-free extract, and ultimately, triple mutant (iv) Lys176Pro, Lys199Pro, Lys224Pro showed the highest level of activity, improved up to an 8-fold increase. The expression profile in soluble and insoluble fractions were completely reversed in the mutant, which has been mentioned as a rare phenomenon among the proteins overexpressed in *E. coli* at 37 °C so far. On the basis of these promising results, the authors used a directed evolution strategy by random mutagenesis of the *hnl* gene and screened the following library of mutants. Ultimately, the mutant His103Leu was found with a very high activity and in vivo solubility at 37 °C. Subsequent site-saturation mutagenesis on the deeply buried His103 residue, which was located near the active site triad (Ser80, Asp207, His235), resulted in other highly soluble mutants, mainly substituted for hydrophobic amino acids with aliphatic side chains, such as Leu, Val, Ile, and Cys. These mutants showed an enhanced specific activity up to more than 18-fold in the cell-free extract, in comparison with the wild-type enzyme in the "synthesis" of cyanohydrin mandelonitrile (Figure 9B).[158,159]

The authors compared the specific activity of the all Lys-Pro and 20 His103 mutants of both the plant codon and *E. coli*-optimized codon, in which almost the same patterns were observed for the counterpart mutants. Overall, improvement in the specific activity level of the plant codon was higher than that of the *E. coli*-optimized codon.[159] Catalytic efficiency of the mutants was found to be 0.8−1.6-fold toward benzaldehyde (in synthesis) and 0.9−1.9-fold for the mandelonitrile (in decomposition) for the selected purified enzymes, including His103Met, His103Leu, His103Tyr, Lys176Pro, and triple mutant Lys176Pro, Lys199Pro, Lys224Pro as well as the wild-type enzyme. The K_m value was almost half that of the wild type for the highly soluble His103 Leu and the inactive mutant His103Tyr, and the parameter was not affected so much for other tested mutants toward benzaldehyde.

Decreased values of K_m were observed for the mutants His103Met and His103Leu. It is noteworthy that none of the mutants showed an increase in specific activity in their purified form toward benzaldehyde, although a slight increase was

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observed in the specific activity of the triple Lys-Pro mutant toward the mandelonitrile. Slight secondary structural changes were observed for the mutants, based on the CD and FT-IR data.

Fluorescence studies showed a slight increase for the triple Lys-Pro mutant without a red shift, but there were a higher intensity and red shift for the three tested His103Tyr, His103Leu, and His103Met, indicating some intramolecular rearrangements, leading to tertiary structural changes. The mutants showed some level of differences in their temperature and pH stability in comparison with the wild-type enzyme, (*S*)-*Me*HNL. The authors suggested that the enhanced activity and solubility are the results of mutations that led to fewer inclusion bodies being formed, that is, increased solubility with larger numbers of active *Me*HNL molecules produced.

The newly synthesized enzymes overcame intrinsic and extrinsic factors preventing correct folding following both Lys to Pro and His103 mutations. This higher activity results in the higher specific activity in the cell-free extract.[159] This is an example how mutations can positively interfere with the process of in vivo protein folding and generate very highly soluble enzymes from an originally low functionally expressed (eukaryotic) protein in *E. coli*. In addition, the wild-type and mutant His103Leu were comparatively expressed in prokaryotic and eukaryotic expression systems in which they exhibited two distinct expression profiles. In prokaryotic hosts, including *E. coli* and an *E. coli*-based cell-free translation system, the wild-type and highly soluble mutant showed different levels of expression and HNL activity level, but both of these genes were expressed in almost the same level in eukaryotic systems, such as *P. pastoris*, *Leishmania tarentolae*, and wheat germ cell-free translation systems.[175]

*Hb*HNL-Trp128Ala exhibited priority for synthesis of 2*S* isomers of 4-methoxycyclohex-3-ene carbaldehyde cyanohydrin and 4-trimethylsilyloxycyclohex-3-ene cyanohydrin in comparison with the wild type and Trp128Phe. Griengl et al. attempted to increase the activity of the enzyme toward the 4-methoxycyclohex-3-ene carbaldehyde employing an error-prone PCR of *hnl*-Trp128Ala (Table 6, entry 14). Five out of 30 preselected colonies exhibited improved activity, and 2 of them contained silent mutations. Two mutants showed higher conversion and selectivity containing multimutation sites. In an interesting coincidence with research of the Asano group (mentioned above), the authors found His103Leu (and Gln215His) as responsible residues for high conversion and selectivity of the corresponding mutants toward the target substrate. Mutants generated with these selected mutations could synthesize cyanohydrins from 4-substituted cyclohex-3-ene carbaldehydes in high yield and high selectivity with small amounts of the enzyme *Hb*HNL in comparison with the wild-type enzyme.[176]

As an example of active site engineering, Kazlauskas and coworkers[177] switched an esterase to a hydroxynitrile lyase (Table 6, entry 15). Despite major mechanistic changes between the esterases and hydroxynitrile lyases (of EC 4.1.2.11 and 4.1.2.46), their active triad shares serine, aspartic acid, and histidine and an oxyanion hole, on the basis of the crystal structures. Because the residues have different roles in these two groups of enzymes, esterases catalyze the hydrolysis reaction, but hydroxynitrile lyases do C−C bond formation. It seems that the amino acid sequences of the structurally related *Hb*HNL, *Me*HNL, SABP2 (salisylic acid binding protein 2 from *N. tabacum*), and PFE (Aryl esterase from *P. fluorescens*) showed more changes than the structures during the evolution. A structure-based alignment of amino acids revealed only 19 conserved residues, including the catalytic triad, but it was expectable that these two esterases do not have the catalytically important Thr and Lys residues of HNLs.

Modeling studies and calculations of interaction energies using substrate mandelonitrile suggested a higher possibility for HNL activity by double or triple mutations of the SABP2 rather than the PFE. The authors also calculated the free energy of unfolding of various mutants using the FoldX force field, which showed lower stability and, consequently, lower correct folding for the mutants with a higher number of mutation points. The mutants of SABP2 and PFE exhibited decreased esterase activity (to 0−6.5% of the original activity). The mutant SABP2-Gly12Thr, Met239Lys showed the HNL activity of 20 mU/mg and a ratio of 22 for HNL/esterase activity. As wild-type *Me*HNL showed about 2300 mU/mg activity and a ratio of 7500, therefore, the engineered enzyme showed only 340-fold less activity in the cleavage reaction of mandelonitrile as the substrate. The authors proved that it is possible to create HNL activity from the other members of the superfamily, and this is remarkable from a scientific point of view, especially considering the role of computer modeling and rational design used in this work as gateways for future studies. It was demonstrated that only two substitutions can dramatically change the reaction mechanism of the enzymes. The authors found their results in accordance with gene duplication as a mechanism of evolution, since a few point mutations can enable a dramatically different reaction mechanism.[177] Table 6 represents examples of the engineered HNLs described in this review.

6. CONCLUSING REMARKS

What we want to emphasize is highlighting the importance of screening and discovery of the valuable enzymes hydroxynitrile lyases in Nature's tool box and transfer them to the chemist's tool box! Then the engineering methods can be employed to modify these new as well as already existing biocatalysts to access more potent and "better" enzymes for various synthetic purposes. These attempts may target many substrates and catalyze the new reactions or even a single valuable reaction because despite industrial applications for a very limited number of the HNLs, there are still necessities to improve the enzymes and to optimize their properties to the specific demands in industry. The methods of screening can be improved both for discovery of the new HNLs and creation of better properties for the currently in use biocatalysts by protein engineering strategies. All attempts should be set up to establish less expensive and more efficient processes with high selectivity and recovery, and ultimately methods that are safe to the planet.

In this review, we gave many examples of chemicals derived from cyanohydrins, which have important applications as pharmaceuticals, agrochemicals, and biologically active materials. Then the biology and biochemistry of these enzymes were reviewed, and the *R*- and *S*-selective HNLs were compared in detail through two precise Tables 4 and 5. The screening of the enzyme covered traditional activity detection-based; genome-based; and finally, high-throughput screening for protein engineering purposes. We have also described some information derived from the newly discovered HNLs in this article. The last part contains examples of protein engineering on both (*R*)- and (*S*)-HNLs accompanying an easy-to-use table summarizing the main data in each case.

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