A Comprehensive Overview of Novel Hydroxynitrile Lyases

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Abstract

Hydroxynitrile lyases (HNLs) are a heterogeneous family of enzymes that are of particular interest because of their structurally unique categories, a wide range of immobilisation techniques and procedures, and a wide range of sources with varying degrees of enantiopurity and enantioselectivity. Cupin, which contains a new type of HNL from bacteria, the lipocalin superfamily, which has HNLs from millipedes, and the + barrel fold superfamily, which contains HNL from a fern, have all been discovered in recent decades. Their biochemistry has been deciphered, and engineering efforts have been made to boost their productivity, purity, and activity. These remarkable enzymes opened up a new vista in the field of industrial catalysts since they are actively used in the synthesis of crucially important agrochemicals, medicines, physiologically active substances, and chemo-enzymatic follow-up procedures. This review focuses on recent advances, evolutionary history, and recombinant engineering of HNL from the previous decade.

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Abstract

Hydroxynitrile lyases (HNLs) are a heterogeneous family of enzymes that are of particular interest because of their structurally unique categories, a wide range of immobilisation techniques and procedures, and a wide range of sources with varying degrees of enantiopurity and enantioselectivity. Cupin, which contains a new type of HNL from bacteria, the lipocalin superfamily, which has HNLs from millipedes, and the + barrel fold superfamily, which contains HNL from a fern, have all been discovered in recent decades. Their biochemistry has been deciphered, and engineering efforts have been made to boost their productivity, purity, and activity. These remarkable enzymes opened up a new vista in the field of industrial catalysts since they are actively used in the synthesis of crucially important agrochemicals, medicines, physiologically active substances, and chemo-enzymatic follow-up procedures. This review focuses on recent advances, evolutionary history, and recombinant engineering of HNL from the previous decade.

Keywords: Hydroxynitrile lyases, Cyanohydrins, Cupin superfamily, Lipocalin superfamily, Mandelonitrile.

Introduction

Hydroxynitrile lyases catalyse the conversion of cyanohydrins to hydrogen cyanide, aldehydes, or ketones. (Kassim & Rumbold, 2014; Dadashipour & Asano, 2011; Sharma *et al.*, 2005). Plants, arthropods, and bacteria all have HNLs (Hussain *et al.*, 2012; Wiedner *et al.*, 2014; Dadashipour *et al.*, 2015). As a universal defense system against pathogens and herbivores, HNLs are part in the cyanogenic pathway. Cyanogenic glycosides such as amygdalin and prunasin are found in plants and are stored in various tissues, where they are released as hydrogen cyanide when the tissue is damaged (Dadashipour *et al.*, 2011). Similarly, HNL releases mandelonitrile as an arthropod defence molecule (Dadashipour *et al.*, 2015). The function of HNLs in bacteria is still unknown. They can synthesize -cyanohydrins and execute the reverse condensation reaction, which results in C-C bonds being formed. Cyanohydrins are the building blocks for a wide variety of reactions as well as compounds used in the pharmaceutical, agrochemical, and cosmetic industries (Gruber-Khadjawi *et al.*, 2012; Lanfranchi *et al.*, 2013; Purkarthofer *et al.*, 2007; Winkler *et al.*, 2012). New enzymes with outstanding features are constantly sought after because (R)- and (S)-selective HNLs are used in biocatalysis reactions. Despite the fact that cyanogenic defence mechanisms are used by over 3,000 plant species and that cyanogenic activity varies by family, only a few amino acid sequences have been documented to have proven HNLs activity and are found in common gene and protein sequences (Hickel, 1997; Hernández *et al.*, 2004; Asano *et al.*, 2005; Kassim *et al.*, 2014). Understanding the amino acid sequence of the enzyme, on the other hand, is critical for recombinant expression and its engineering. Current HNLs have drawbacks such as low stability (Okrob, 2012), low activity (Hajnal *et al.*, 2013), limited flexibility in expression host selection (Weis. *et al.*, 2004), and low expression level (Dadashipour *et al.*, 2015), all of which have an impact on large-scale applications.

Novel sources of Hydroxynitrile lyases

Despite the fact that several HNLs have been discovered and employed as biocatalysts in industry, scientists have continued to explore for new enzymes in nature (Fig 1). Low-throughput, GC, or HPLC tests made previous screening of these unique enzymes difficult. For direct profiling of HNL activities in plants, Zheng *et al.* (2021) devised a chromogenic-based high-throughput screening (HTS) technique.

In the same investigation, HNL was isolated from an invading millipede, *Chamberlinius hualienensis*, a cyanide-emitting arthropod. ChuaHNL was pH and temperature stable, had broad substrate specificity for aromatic carbonyl aldehydes, and could be used to make enantioselective mandelonitrile. In the production of (R)-mandelonitrile, the enzyme had five times the functional activity of industrial PaHNL. Physicochemical property analysis and logical protein engineering have been used to build industrial biotechnology in a sustainable manner for decades (Purkarthofer *et al.*, 2007). Millipede HNL was used as a template for isolating successful and promising enzymes, as well as tailoring enzymes through rational protein engineering, both of which are critical for the commercial synthesis of fine compounds and pharmaceuticals (Dadashipour *et al.*, 2015). In *Parafontaria laminata* (PlamHNL) millipedes, another R- specific HNL was found and recombinantly expressed in *Pichia pastoris* and *Escherichia coli* (Nuyt *et al.*, 2020). From the seeds of *Eriobotrya japonica* (loquat), a novel R-selective FAD containing hydroxynitrile lyase was discovered and isolated. The enzyme had a FAD prosthetic group and was exceptionally pH and temperature stable. The enzyme could degrade both aromatic and aliphatic aldehydes, and it favoured smaller substrates than larger ones (Ueatrongchit *et al.* 2008). PmHNL, a FAD hydroxynitrile lyase from *Prunus mume* seed, was isolated, purified, and recombinantly produced in *Pichia pastoris*, and showed activity over a broad temperature and pH range (Fukuta *et al.* 2011).

Pratish *et al.* (2011) discovered heteromeric R-specific HNLs in *Abrus precatorius* (rosary pea) seeds. Hajnal *et al.* (2013) identified and successfully expressed a manganese-dependent R- selective hydroxynitrile lyase from the bacterium *Granulicella tundricola* (GtHNL) in *E. coli*. In the crystallographic analysis of the HNL crystal, a cupin fold was identified. A conserved barrel domain is found in the cupin protein superfamily (Lyskowski *et al.* 2012). Enzymes and non-enzymatic storage proteins are both members of the superfamily. GtHNL exhibited neither sequence nor structural relationship to any other HNL and lacked HNL-specific motifs; hence cupins were the first of a new class of HNLs. Through database mining, another highly efficient novel cupin R- selective HNL, *Acidobacterium capsulatum* (AcHNL), was found. Due to their excellent

yield on recombinant production in *Escherichia coli* and increased activity, enantioselectivity, stability, and substrate specificity, these novel cupin HNLs have emerged as a powerful catalyst (Wiedner *et al.* 2014).

From the young leaves of *Nandina domestica* Thunb., two distinct R selective HNLs were isolated. The enzymes were different since there was no FAD or carbohydrate. Purified HNLs had molecular weights of 24.9 kDa and 28.0 kDa, respectively, and were designated as NdHNL-S and NdHNL-L. NdHNL-L demonstrated higher functional activity for mandelonitrile production than other plant HNLs, and its amino acid sequence was unique. At pH 4.0 and temperatures below 55°C, the enzyme catalysed the synthesis of enantioselective (R)-cyanohydrins and showed great activity and stability (Isobe *et al.* 2018). Although a few new sources of hydroxynitrile lyases have recently been described, HNL is a non-homologous isofunctional enzyme, making identifying new HNL a difficult endeavour (Fig 2).

Evolutionary facet of hydroxynitrile lyases

Nakano and Asano (2015) designed the INTMSAlign tool to investigate protein engineering and evolution. They utilised the algorithm to make functional proteins, and three S-selective hydroxynitrile lyase (S-HNL)-active protein sequences were generated. According to sequencing and biochemical characterization of the generated S-HNLs, neutral mutations increase during the evolution of S-HNLs from a low activity form to a high-activity native form. Overall, the findings showed that software may be used to forecast protein evolution, notably across families like S-HNLs and esterases, in addition to constructing entire sequences.

Because catalytic promiscuity is a valuable but undesired enzyme characteristic, it's difficult to identify reactive promiscuous enzymes in environment. Several primordial enzymes are thought to have been indiscriminate in action because they were at evolutionary branch points of new enzymes. Devamani *et al.* (2016) put this theory to the test by rebuilding ancestral enzymes at four points when HNLs diverged from esterases. Both enzymes have the identical α/β -hydrolase-fold, but the reaction forms and mechanisms are different. The ancient enzymes showed more catalytic promiscuity than their successors when tested with esterase and lyase substrates. They discovered that ancient esterases are far more inclined than modern esterases to catalyse a lyase reaction, and ancestral HNLs are more inclined than modern HNLs to catalyse ester hydrolysis. The HNL1 progenitor enzyme was notably promiscuous during the transition from esterase to hydroxynitrile lyases, catalysing both hydrolysis and lyase reactions with a wide range of substrates (Devamani *et al.* 2016).

In some aspects, the modern rubber tree HNL (HbHNL) differs from HNL1. Catalytic promiscuity has been linked to a diverse active site conformation, a rich biochemical system in the active site interior, and similar transition states. Ester hydrolysis and cyanohydrin cleavage have slightly different transition states and processes. The active site validation alterations can be seen in the x-ray crystallographic structure, such as the substrate binding site residues being more flexible. During ester hydrolysis, serine in the catalytic site acts as a nucleophile, whereas during cyanohydrin cleavage, it behaves as a base. HNL1's ancestral hydroxynitrile lyase has a three-fold higher catalytic promiscuity than modern HbHNL, which is most likely attributable to HNL1's bigger substrate binding site.

The larger binding site allows the flexible substrate p-nitrophenyl acetate (pNPAC), and both of them include aryl groups, however the active catalytic disposition of these groups differs. If the catalytic site is separated into an alcohol binding domain and an aryl binding domain, the aryl group is located in the alcohol binding domain by pNPAC's productive alignment, while the aryl group is located in the aryl binding domain by mandelonitrile's productive alignment. Because a separate substrate orientation is required, a broad active site that can handle both orientations is required, resulting in enhanced catalytic promiscuity. In contrast to HbHNL, the x-ray structure of HNL1 shows a large active site; this difference explains for HNL1's three-time higher ester hydrolysis propensity (Jones *et al.*, 2020). Higher catalytic promiscuity was also linked to a bigger active site in the alkaline phosphatase family (Barrozo *et al.*, 2015).

The enhanced thermostability of revived ancestral proteins favours the reconstruction of stabilising substitutions (Trudeau *et al.*, 2016; Wheeler *et al.*, 2016; Williams *et al.*, 2006). Because stabilising residues are more likely to be kept in subsequent generations, identifying these residues can be used to precisely determine the ancestral residue. Unstable residues, on the other hand, are more prone to loss in descen-

dants and hence disregarded when determining ancestral series. As proteins diverge, destabilising residues are altered, resulting in stabilizing residues to counter the impact of substitutions that improve additional properties (Tokuriki & Tawfik, 2009). As a result of this propensity, any unstable residues discovered in ancestral proteins are unlikely to be conserved in modern proteins, and hence cannot be accurately predicted in a reconstructed antecedent. As a result, ancestral proteins like HNL1 might be rebuilt, and they could be more stable than their real predecessor (Jones *et al.* . 2020).

The structural and functional properties of HNLs are distinct. The catalytic mechanisms of the HbHNL and AtHNL, which have opposite enantioselectivity against mandelonitrile, have been determined to be unique. To determine out how this change happened, they built ancestor-like synthetic active-sites in each subdomain. HbHNL kept (S)-selectivity, whereas AtHNL' with identical active site residues retained (R)-selectivity. (A unique mechanism is suggested by (S)-selective composite active-site mutants lacking Lys236 and Thr11, which are required for the conventional (S)-HNL pathway. This new mechanism, which isn't found in modern enzymes, was discovered by simulation. As a result, the last common ancestor of both HbHNL and AtHNL is likely to have used an unknown mechanism rather than the AtHNL-like mechanism. With the same catalytic residues, several pathways are possible, and residues present on the exterior of the active site have a significant impact on mechanism and enantioselectivity (Jones *et al.* . 2017).

Hydroxynitrile lyases are non-homologous isofunctional enzymes that have evolved through convergent evolution (Omelchenko *et al.* . 2010). A unique HNL enzyme was isolated from the fern *Davallia tyermannii* , and crystallographic analyses indicated that it has a Beta v1-like protein structure, a novel catalytic core, and HNL activity. To find expressed genes, researchers employed normalised transcriptomes from *D. tyermanii* leaves and croziers. Proteins from active tissues were separated using anion exchange chromatography, followed by a native PAGE coupled to HCN detection assay. After isolating the enzymatically active fractions, the protein was digested with trypsin and analysed using LC-MS. The nucleotide transcriptome database of *D. tyermannii* was compared to peptide data. In *E. coli* , the sequences were recombinantly expressed and HNL activity was evaluated; isotig02643 had the best chance of being an HNL-active protein. The translated nucleotide transcriptome database revealed three additional remarkably similar sequences to the reported HNL; these gene sequences were amplified from genomic DNA by PCR and their nucleotides validated. After *E.coli* recombinant synthesis, the HNL activity assay revealed activity of all three isoenzymes, and four proteins were encoded. The uniqueness of DtHNL was investigated using BLAST. The derived DtHNL is distinct from other HNL groups, as evidenced by its low identity and sequence coverage (Lanfranchi *et al.* , 2017).

Padhi et al. (2010) used only two amino acid modifications to turn a mutant variant of a plant esterase, SABP2, into a hydroxynitrile lyase. The capacity of SABP2-G12T-M239K to catalyse ester hydrolysis was lost, while it gained the ability to catalyse cyanide release from mandelonitrile. This modification also accelerated the reversible reaction, resulting in the formation of mandelonitrile with low enantioselectivity. Mandelonitrile lysis has a specificity constant 13,000 times higher than the unaided reaction and is only 1300-fold lesser efficient than HbHNL.

HNLs recombinant expression and protein engineering

HNLs have been shown to be quite common in nature, and a variety of microorganisms have been identified, the majority of which have been extensively purified and characterised so far. These enzymes have distinct conformations, molecular weights, pH optimum conditions, and kinetic properties (Table 1). Saturation mutagenesis and random mutagenesis were employed by Asano and Kawahara (2011) to construct S-selective HNL in *Baliospermum montanum* . Two created BmHNL double mutants, H103C and N156G, showed improved enantioselectivity and specificity for (S)-mandelonitrile production. Nuyt *et al.* . (2017) evaluated the influence of glycosylation on the hydroxynitrile lyase of *Passiflora edulis* (PeHNL). The gene was recombinantly expressed in *Pichia pastoris* and *E. coli* . In a biphasic system, wild type HNL and recombinant expressed HNL from *Pichia pastoris* outperformed HNL from *E. coli* and the deglycosylated N105Q mutant of *Pichia pastoris* expressed HNL in terms of pH, thermal stability, and solvent tolerance. The results demonstrated that the stability profile of HNL may be enhanced by employing a better enzyme

expression system and N-glycosylation (Nuykert *et al.* 2017a).

According to an X-ray crystallographic investigation of *Prunus communis* HNL (PcHNL), the side chain of Leucine at 331 position can be fine-tuned to vary the substrate flexibility of large and rigid benzo-ketal aldehyde. This allowed Zheng *et al.* (2020) to structure-guided redesign HNL by creating an L331A variant that was used in the chemo-enzymatic synthesis of (R)-salmeterol (adrenoreceptor agonist) and obtained >99% ee optically pure drug form with 54% yield. Nuykert *et al.* (2020) showed that *Parafontaria laminata* (PlamHNL) millipede HNLs had wider substrate selectivity than other millipede HNLs and may be utilised to make 2-chloromandelonitrile, a critical step in the antithrombotic medication clopidogrel. According to the X-ray crystal structure of PlamHNL, despite having comparable catalytic residues, the binding cavity produced by PlamHNL and *Chamberlinius hualienensis* is distinct. The PlamHNL-N85Y mutant had 98.2 percent ee purity of 2-chloromandelonitrile and a 91 percent conversion rate, compared to 90 percent ee purity and 76 percent conversion rate for the wild type enzyme (Nuykert *et al.* 2020).

The HNL coding gene of *P. edulis f. flavicarpa* (PeHNL-Ny) was successfully cloned and expressed to yield recombinant PeHNL-Ny (Asano *et al.* 2005). For KCN and benzaldehyde, the presence of glycan on PeHNL improves catalytic performance without reducing substrate affinity (Nuykert *et al.* 2017). Non-glycosylated PeHNL appears to be less stable than glycosylated PeHNL, according to evidence. The structure of PeHNL, as well as a glycosylation site on the protein, improves stability by stabilising the prominent elastic state at the carboxy-terminal. When a 15-amino-acid-residue C-terminal truncated form of PeHNL was made and expressed in *E. coli*, it produced an enzyme that was not glycosylated. When non-glycosylated and C-terminally truncated mutant PeHNL107 is expressed in *P. pastoris*, it enhances stability and reusability to the same extent as glycosylated native PeHNL (Nuykert *et al.* 2017b).

The ChuaHNL of cyanogenic millipedes exhibited maximum activity for (R)-mandelonitrile synthesis, as well as strong enantioselectivity and stability. HNLs have been found and characterised in eleven cyanogenic millipedes from the Xystodesmidae and Paradoxosomatidae families. HNLs are found in all cyanogenic millipedes and according to sequencing research, they may have evolved from a single common ancestor gene. HNL from a different millipede *Parafontaria tonominea* when expressed in *E. coli* Shuffle T7, showed strong specific activity for the stability and synthesis of (R)-mandelonitrile throughout a wide temperature and pH range. The presence of disulfide bond gives millipede HNLs their stability. Due to unavailability of the millipedes genome sequences, the ChuaHNL did not exhibit relation to any protein present in the database (Dadashipour *et al.* 2015). Yamaguchi *et al.* (2018) isolated an HNL gene from one of these cyanogenic millipedes and sequenced complementary DNA. They designed degenerate primers for amplification of cDNAs of HNL. Five out of eleven HNL cDNA displayed expression in *E. coli*, and these transgenic cells encoding HNL were employed as enzymes for (R)-mandelonitrile production (Yamaguchi *et al.* 2017).

Zhai *et al.* 2019 investigated the impact of glycosylation on the synthesis of Histidine tagged ChuaHNL in the *P. pastoris*. The codon sequence of the ChuaHNL gene was examined, and it was discovered that the ChuaHNL gene's codons have a high codon bias for *P. pastoris*, which could be a major bottleneck in the efficient synthesis of His-ChuaHNL in recombinant expression system. As a result, the ChuaHNL gene's codons were altered to *P. pastoris*-favorable codons. (Sinclair & Choy, 2002). Hundred codons from ChuaHNL were replaced in this codon optimization, accounting for 61.7 % of all residues. The codon optimization His-Syn-ChuaHNL strain produced 86 times more than His-Ori-ChuaHNL. These findings suggested that the codon's translation competence is a crucial component in increasing ChuaHNL production in the heterologous host *P. pastoris* (Zhai *et al.* 2019).

Reaction mechanism of HNLs

HNLs catalyse a range of reactions that convert chiral cyanohydrins to hydrocyanic acid, aldehyde, or ketone (Sharma *et al.* 2005). The reaction mechanism and catalytic residues participating of these R-selective HNLs active sites is shown in Fig 3. Zhu *et al.* (2015) studied the reaction mechanism of AtHNL using a quantum mechanics/MM approach and proposed two types of response mechanisms. According to the calculations, the cyanohydrin hydroxyl group proton transfer take place first, subsequently the C1-C2 bond

breaks and rotation of the cyanide ion takes place, and finally, cyanide ion captures a proton from His236 via Ser81. Because the free energy barriers for breaking C1-C2 bonds and protonation of cyanide ions are similar (12.0 kcal/mol), they both contribute to the rate-limiting step. Path B, with a maximal free energy barrier of ~13kcal, deprotonates the cyanohydrin hydroxyl group while simultaneously cleaving the C1-C2 bond. Both paths have similar free energy barriers, therefore either one can be chosen. The reaction's general acid/base is reflected in the catalytic triad formed by His236, Ser81, and Asp208, and the generated cyanide ion is stabilised by Ser81's hydroxyl group and the amine-groups of Ala13 and Phe82 (Zhu *et al.* . 2015).

Motojima *et al.* . (2018) proposed a response mechanism for the lowest HNL in *Passiflora edulis* (PeHNL). PeHNL is a member of the dimeric $\alpha\beta$ barrel superfamily, which is distinguished by two alpha dimers sandwiching a central β barrel. The $-\text{OH}$ group of (R)-mandelonitrile forms H bonds with His8 and Tyr30 at the binding site, whereas the nitrile group is orientated to the carboxyl group of Glu54 in the docked structure of PeHNL with (R)-mandelonitrile. The catalytic dyad produced by His8-Asn101 is needed for the enzymatic process, according to mutational studies. The hydrogen bond between His-N1 and Asn101-O1 is shorter in the PeHNL-(R)-mandelonitrile complex, increasing the basicity of His8 and allowing it to accept a proton from the OH group of (R)-mandelonitrile. When the cyanide generated by the nitrile group accepts one proton from the protonated His8, HCN is formed. As a result, the His8 in PeHNL's active site behaves as both a general acid and a basic in the process (Motojima *et al.* . 2018).

Motojima *et al.* . (2021) reported a structural analysis of natural ChuaHNL as well as a response mechanism. They looked at the enzyme in the absence of a ligand (1.6), as well as with acetate (1.5), cyanide ions (2.1), thiocyanate (1.55), and iodoacetate inhibitors (1.55). ChuaHNL appears to be a member of the lipocalin superfamily based on its crystal structure. In the docked model of ChuaHNL with its substrate (R)-Mandelonitrile, the hydroxyl group makes hydrogen bonds with Arg38 and Lys117, while the nitrile group creates hydrogen bonds with Arg38 and Tyr103. Lys117 behaves as a base to take a proton from the $-\text{OH}$ group of cyanohydrins, while Arg38 serves as an acid to give a proton to the CN^- ion during the bond fission reaction, according to mutational studies. During the cyanohydrin production process, the opposite will happen (Motojima *et al.* . 2021).

Zhao *et al.* . (2014) deduced the reaction mechanism of *Hevea brasiliensis* HNLs (HbHNL) using *ab initio* quantum mechanics and molecular dynamics simulations. Ser80, His235, and Asp207 are the catalytic triads of HbHNL's active site, which is buried inside. The substrate can reach the active location through a small route. Catalytic reactions have been discovered to occur in two steps. In the first stage, proton abstraction (double proton transfer) and carbon-carbon bond splitting occurs in quick, coordinated reactions. The second stage involves the production of HCN, which is a rate-determining process. The protonation state of Lys236 has a minimal impact on enzyme function, but it has a significant impact on the catalytic process as a whole. Trp 128 residues act as a gate that adjusts continuously during substrate delivery and HCN release. The Trp128 indole ring acts as a "door" that can be open or closed. As a result of all of this, Helix D's conformation shifts from a α -helix to a loop shape. The enzyme releases energy when it binds to a substrate, and product release takes only a little amount of energy (4.0 kcal mol⁻¹), making the reaction efficient overall (Zhao *et al.* . 2014).

Synthesis of cyanohydrins

A combination of cassava plant (*Manihot esculenta*) (S)-specific HNL, nitrile hydratases, and nitrilases was utilised to efficiently produce chiral 2-hydroxycarboxamides and 2-hydroxycarboxylic acids from non-chiral aldehydes/ketones and cyanide (van Rantwijk & Stolz, 2015). By combining a (R)-specific HNL with nitrilase or nitrile hydratase enzymes, an isofunctional system for generating (R)-2-hydroxycarboxamides and (R)-2-hydroxycarboxylic acids was created (Muller *et al.* ., 2021). Recombinant *E. coli* clones were used to study the synthesis of (R)-mandelic acid and (R)-mandelic acid amide, and strains were generated that expressed a (R)-specific hydroxynitrile lyase from *Arabidopsis thaliana* as well as the arylacetone nitrilase from *Pseudomonas fluorescens*. Recombinant strains expressing an acid-tolerant HNL variant and an amide-forming nitrilase variant were produced. In mildly acidic pH values, whole cell catalysts expressing both the (R)-specific HNL and the wild-type nitrilase converted benzaldehyde and cyanide preferentially to (R)-mandelic acid with ee-

values greater than 90%. The acid and amide producing catalysts transformed chlorinated benzaldehydes to chlorinated mandelic acid/chlorine mandelic acid amides when coupled with cyanide (Muller *et al.* , 2021).

PlamHNL from *Parafontaria laminata* millipedes was cloned and expressed as a host from cDNA. There are 8 Cys residues found in millipede HNLs that generate functional disulfide bonds and are required for HNL folding, activity, and stability (Yamaguchi *et al.* , 2018). PlamHNL was produced in *E. coli* and the stereoselectivity for (R)-2-chloromandelonitrile production was enhanced after bacteria were subjected to a computational-directed evolution strategy. Because the enzyme was stable and the gene allowed for continued rational evolution in *E. coli* , PlamHNL can be employed to produce enantiomerically pure cyanohydrins using structural analyses (Nuyler *et al.* , 2020)

Future perspective

Only a few decades ago, genes for (S) and (R)-HNLs were discovered and expressed, and new enzymes are discovered all the time. Several international patent applications typically include manufacturing, improved variants, novel enzymes from various sources (plants, microorganisms, arthropods, etc.), and applications of both (S)- and (R)-HNLs for numerous chemical synthesis. Several enzymes, such as LuHNL, novel and adaptable bacterial HNLs, and synthetic esterases with HNL activity, have become fundamental research targets in addition to patent-protected major enzyme sources. Since then, HNL catalysis has opened up a plethora of new opportunities for a wide range of industrial applications.

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Table 1. The biochemical and kinetic properties of novel hydroxynitrile lyase sources

Source	Enantioselectivity	Techniques	Subunit composition	Molecular weight	Optimum Temperature and pH	Kinetic parameter	Specific activity	Purification Fold	Reference
<i>Abrus precatorius</i> (rosary pea) Seeds	R-selective	Ion exchange chromatography Gel filtration chromatography	Heteromer	Native molecular mass 205 kDa Subunits: 42.0 kDa and 36.5 kDa	30°C with 0.1 M sodium citrate buffer (pH 5).	$K_m = 13\text{mM}$ $V_{max} = 625\text{U/mg}$	577 Umg^{-1}	9 fold	Pral et al.
<i>Acidobacterium capsulatum</i> (expressed in <i>E. coli</i>)	R-selective	Anion exchange chromatography Size exclusion chromatography	Homotetramer	14.25 kDa	pH 5.5, 25°C	NA	0.69 Umg^{-1}	2% yield	Wang et al.
<i>Baliospermum mon-tanum</i> Leaves	S-selective	Ammonium sulfate fractionation column chromatographies	Homodimer	60-65 kDa non-FAD enzyme (gel filtration) 29.5 kDa	pH 5, 20 °C	$k_{cat} = 98/\text{s}$	178 Umg^{-1}		Datta et al.

Source	Enantioselectivity	Techniques	Subunit composition	Molecular weight	Optimum Temperature and pH	Kinetic parameter	Specific activity	Purification Fold	Reference
<i>Chamberlinius hualienensis</i>	R-selective	Ammonium sulfate precipitation Ion exchange chromatography Hydrophobic interaction chromatography Gel filtration	Homodimer	47.3 kDa Gel filtration, 25kDa SDS/PAGE	pH 4.9 , 35 °C	V _{max} =406 micro-mol/min/mg K _{cat} =142/s K _m =8.7mM K _{cat} /K _m =16.4/mM/s	7420 U _{mg} ⁻¹	1230 fold, 3.35% Yield	Das et al.
<i>Eriobotrya japonica</i> (Loquat) Seeds	R selective	Ammonium sulfate fractionation Ion exchange DEAE-Toyopearl chromatography Concanavalin A Sepharose 4B chromatography	Monomer	Molecular weight-72 kDa (gel filtration) and 62.3 kDa (SDS-gel electrophoresis)	40 °C pH 5.5	K _m =161 μM K _{cat} /K _m =348 /s/mM	31.5 U _{mg} ⁻¹	49-fold purification, 36% yield	Ueda et al.
<i>Granulicella thridicola</i> (expressed in <i>E. coli</i>)	R-selective	Anion exchange chromatography Size-exclusion chromatography	Two homotetramer	~15kDa	pH 4.5, 5°C	K _m =10.3 ± 2.1mM k _{cat} =0.03 s ⁻¹ V _{max} 0.12 +- 0.009 U*mg ⁻¹	NA	NA	Haralal

Source	Enantioselectivity	Techniques	Subunit composition	Molecular weight	Optimum Temperature and pH	Kinetic parameter	Specific activity	Purification Fold	Reference
<i>Nandina Nandina domestica</i> Thunb (leaves)	R-selective	Ammonium sulfate fractionation Anion exchange chromatography	Homodimer	46–47 kDa Native 24.9 kDa -NdHNL-S; 28.0 kDa NdHNL-L in SDS	pH-4.0, 15°C	Km =12.5 mM Vmax =1230 $\mu\text{mol min}^{-1}\text{mg}^{-1}$	990 U mg^{-1}	10.5 fold, 11% yield	Isco et al
<i>Passiflora edulis</i>	R-selective		Monomer	15kDa SDS-PAGE and 18kDa by gel filtration	pH 4, 10°C		136 U mg^{-1}		Ue et 2019
<i>Phlebodium aureum</i> (Fern)	R-selective	Anion-Exchange Chromatography, Chromatofocusing, Gel-Filtration Chromatography, Anion-Exchange Chromatography, Cation Exchange Chromatography,	homomultimer	20kDa	Ph-6.5, 35-50 °C	Km 0.83 mM, Vmax 60.1 $\mu\text{mol ml}^{-1}\text{min}^{-1}$	19,000 U mg^{-1}	1,600 fold, 21% yield	Wa et 1997

Source	Enantioselectivity	Techniques	Subunit composition	Molecular weight	Optimum Temperature and pH	Kinetic parameter	Specific activity	Purification Fold	Reference
<i>Prunus amygdalus turcomanica</i> seeds	R-selective	Ammonium sulfate fractionation Hydrophobic interaction chromatography, Gel filtration chromatography	homotetramer	25 kDa	pH- 6.0, 25 °C	K _m 0.38 mM, V _{max} 197.0 U mg ⁻¹	40 U mg ⁻¹	14 Fold, 19% yield	Alal.
<i>Prunus armeniaca</i>	R-selective	Ammonium sulfate fractionation, Gel permeation Chromatography, Ion-Exchange Chromatography	heteromultimer	40 , 37 kD, Native 360 kDa	pH 4.75, 25 °C	K _m of 3.76 mM, V _{max} 188.4 μmol mg ⁻¹ min ⁻¹	141 units mg ⁻¹	8.1 fold, 18.2 % yield	As an and Bh (2010)
<i>Prunus mume</i> (Japanese apricot) Seeds	R-selective	Ammonium sulfate fractionation and chromatographic steps	Monomer	Molecular weight- 58kDa	Broad Temperature and pH range		220 U mg ⁻¹		Fu et al. 2010

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