Babeş-Bolyai University
Faculty of Chemistry and Chemical Engineering
Doctoral School of Chemistry
Enzymology and Applied Biocatalysis Research Center

Doctoral thesis

PROTEIN ENGINEERING OF PHENYLALANINE AMMONIA-LYASES FOR THE SYNTHESIS OF UNNATURAL L-PHENYLALANINE ANALOGUES

PhD candidate: Raluca Bianca TOMOIAGĂ
Scientific supervisor: Prof. Habil Dr. Monica-Ioana TOȘA

Cluj-Napoca
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Prof. Habil Dr. Monica Ioana TOȘA – Babeș Bolyai University, Cluj-Napoca, Romania

President:

Prof. Habil Dr. Csaba PAIZS – Babeș Bolyai University, Cluj-Napoca, Romania

Reviewers:

Prof. Em. Dr. Florin-Dan IRIMIE – Babeș-Bolyai University, Cluj-Napoca, Romania

Prof. Dr. László POPPE – Budapest University of Technology and Economics, Hungary

Assoc. Prof. Dr. Lígia O. MARTINS – NOVA University, Lisbon, Portugal

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2024

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Key words: biocatalysis, phenylalanine ammonia-lyase, phenylalanine, unnatural amino acids, protein engineering, rational design, saturation mutagenesis, high-throughput assay, L-amino acid oxidase

OBJECTIVES

The main objective of the thesis was to ***develop biocatalysts for the synthesis of high-value di-substituted phenylalanine derivatives, focusing on the enhancement of the catalytic potential of phenylalanine ammonia-lyases (PALs)*** by the application of various protein engineering techniques, including rational design and semi-rational saturation mutagenesis, with further insights gained from data mining approaches. The thesis outlines a set of objectives categorized into three distinct chapters, each fulfilling a different aspect of the overall goal:

OBJECTIVE 1: High-throughput assays development for screening saturation mutagenesis-derived phenylalanine ammonia-lyase libraries

The development of a high-throughput solid-phase assay designed for screening protein-engineered libraries of phenylalanine ammonia-lyases (PALs)in the ammonia addition route and a liquid-phase assay targeting the ammonia elimination reaction were envisaged. The solid-phase assay involved coupling the PAL library with the ancestral L-amino acid oxidase (AncLAAO-N1), leveraging the production of hydrogen peroxide during the regeneration of the FAD cofactor essential for the LAAO-catalyzed reaction. A peroxidase was also incorporated into the system, which reacted with the generated H_2O_2 to oxidize an organic substrate, producing a colour change that marks the colony containing the active PAL variant. For implementation, initial assessments of the compatibility between the substrate scope of PAL and the novel ancestral L-amino acid oxidase AncLAAO-N1 were also targeted. For the liquid-phase assay, crude cellular extracts were used as the enzyme source, making it essential to optimize various lysis and reaction conditions before validation.

OBJECTIVE 2: Protein engineering of phenylalanine ammonia-lyases for enhanced activity on challenging substrates

We targeted the identification of phenylalanine ammonia-lyase variants active towards 3,4-dimethoxy *trans*-cinnamic acid and 3-bromo 4-methoxy *trans*-cinnamic acid to produce the synthetically valuable L-amino acids 3,4-dimethoxy L-phenylalanine and 3-bromo 4-methoxy L-phenylalanine. These PAL variants were developed through a rational design approach and derived from saturation mutagenesis libraries, conducting multiple rounds of iterative saturation

mutagenesis at key residues within the catalytic site of phenylalanine ammonia-lyase from *Petroselinum crispum*.

OBJECTIVE 3: Unveiling the biocatalytic potential of aromatic ammonia-lyase from *Loktanella atrilutea*

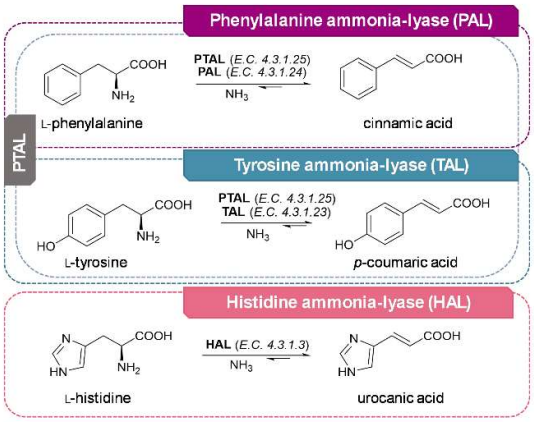
The characterization of the substrate scope and the biocatalytic potential of the aromatic ammonia-lyase from *Loktanella atrilutea*, which possesses unusual activity towards 3,4-dimethoxy *trans*-cinnamic acid is targeted. This research aimed to characterize this distinct subclass of aromatic ammonia-lyases by homologue mining, investigating their evolutionary relationships and substrate profile comparison with known phenylalanine ammonia-lyases, tyrosine ammonia-lyases, and histidine ammonia-lyases.

CHAPTER 1

1. INTRODUCTION

Biocatalysis stands out as one of the leading methodologies that adhere to the Principles of Green Chemistry[1], using enzymes as catalysts to create environmentally friendly and sustainable chemical processes, especially in the food and pharmaceutical industries. Advances in enzyme discovery and engineering have expanded the use of biocatalysis in synthesizing complex molecules, including active pharmaceutical ingredients (APIs).[2] Ammonia-lyases, particularly phenylalanine ammonia-lyases, are highlighted for their versatility in producing enantiopure natural and unnatural amino acids, playing a crucial role in developing novel synthetic routes for pharmaceutical compounds.

Aromatic amino acid ammonia-lyases (AALs) use an unusual highly electrophilic heterocycle to convert their substrates, represented by the post-translationally formed 3,5-dihydro-5-methylidene-4H-imidazol-4-one (MIO) prosthetic group[3]. From this category, ***histidine ammonia-lyase***[4] (EC 4.3.1.3, HAL) ***tyrosine ammonia-lyase***[5] (EC 4.3.1.23, TAL), ***phenylalanine ammonia-lyases***[6] (EC 4.3.1.24, PAL) and ***phenylalanine***



Scheme 1 Reversible non-oxidative elimination of ammonia catalyzed by phenylalanine ammonia-lyases (purple), tyrosine ammonia-lyases (dark turquoise), histidine ammonia-lyases (pink) and dual phenylalanine/tyrosine ammonia-lyase (gray).

ammonia-lyases with dual PAL/TAL activity[7] (EC 4.3.1.25, PTAL) catalyze the reversible elimination of ammonia from the corresponding aromatic L-amino acid yielding urocanic acid, *p*-coumaric acid and cinnamic acid, respectively (**Scheme 1**).In recent years, aromatic ammonia-lyases, particularly PALs, have gained popularity as versatile catalysts for the synthesis of substituted amino acids or as biotherapeutic enzymes. A plethora of applications[8–15] have been described involving these enzymes, both independently and within the framework of chemo-enzymatic cascade catalysis, their biocatalytic potential being directed towards the synthesis of unnatural phenylalanine derivatives.

1.1. Unnatural aromatic ammino acids

Unnatural amino acids serve as crucial building blocks, providing conformational constraints, molecular scaffolds, and pharmacologically active compounds.[16,17] Unnatural amino acids are structurally similar to the proteinogenic ones; however, their distinct chemical and biological characteristics resulting from both their unusual side groups and stereochemistry, make them highly attractive as biologically active molecules.[18,19]

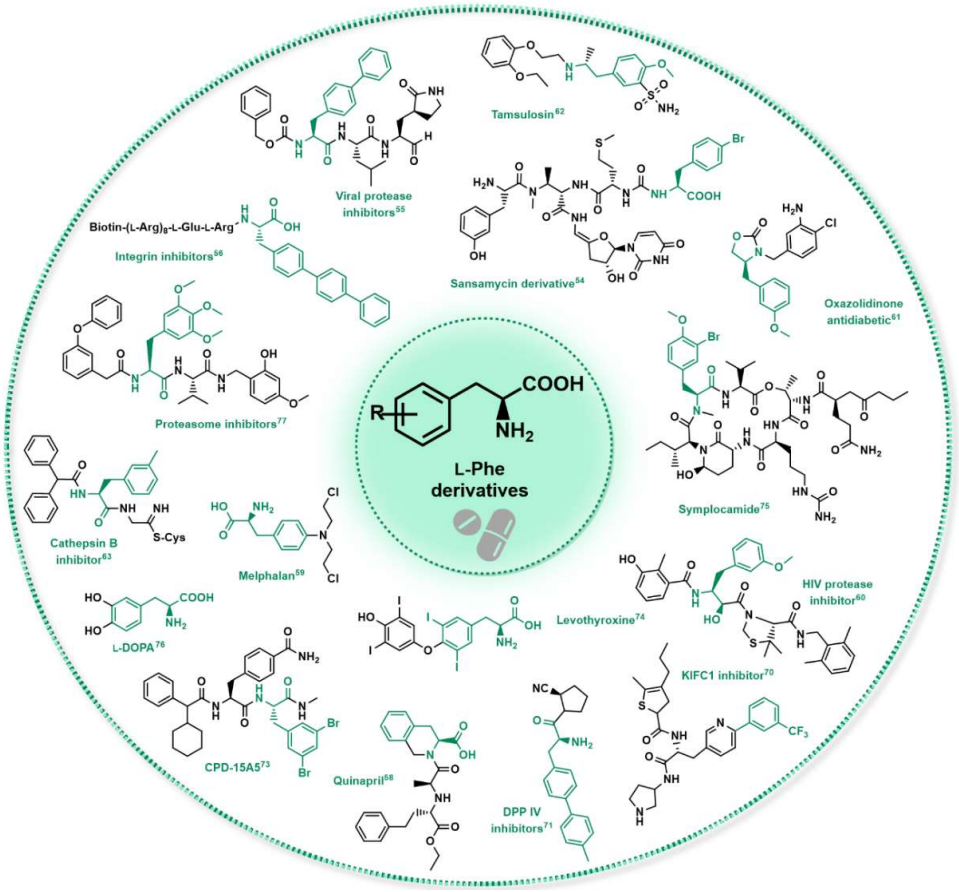


Figure 1. Representative examples of APIs, drug and drug candidates containing L-Phenylalanine derivatives (the phenylalanine moiety is highlighted in green).

The utilization and production of optically pure enantiomers of both D- and L-phenylalanine, as well as their analogues, has become of significant interest. This is particularly due to their essential role in synthesizing peptidomimetics[13,20,21], peptides, and inhibitors targeting various biomolecular interactions[22,23], with representative APIs using L-phenylalanine derivatives being presented in **Figure 1**. For the synthesis of optically pure L-phenylalanines, the asymmetric hydroamination of *trans*-cinnamic acids, catalyzed by phenylalanine ammonia-lyases, also the focus of the current thesis, is one of the most attractive biocatalytic routes. This method offers 100% atom economy, an improved E-factor, enhanced space-time yield, and the elimination of cofactor regeneration systems.[24–26]

1.2. Phenylalanine ammonia-lyases

Phenylalanine ammonia-lyases (PALs, EC 4.3.1.24) catalyze the conversion of L-phenylalanine (L-Phe) to *trans*-cinnamic acid (*t*-CA) by a non-oxidative deamination. The reversible reaction, the ammonia addition, is also possible, using excess ammonia, at an elevated pH, thus, shifting the equilibrium towards the stereoselective formation of L-Phe.

PALs together with HALs, TALs and PTALs (**Scheme 1**) are wildly distributed across diverse origins. Phenylalanine ammonia-lyases are present both in eukaryotes and prokaryotes. While they are mainly distributed in higher plants[27–29], they can also be found in some fungi[30], yeasts[31,32], a few bacteria[33–35], certain algae[36] or amoeba[37]. The most studied enzymes of this family are PAL from *Petroselinum crispum* (*Pc*PAL)[38–40], PAL from *Arabidopsis thaliana (At*PAL)[41,42], PAL from *Rhodotorula glutinis (Rg*PAL)[43], PAL from *Rhodosporidium toruloides (Rt*PAL)[44] and PAL from *Anabaena variabilis (Av*PAL)[33,37,45].

1.2.1. Molecular architecture and functional properties of PALs

PALs typically exist as homotetramers, with around 52% of residues consisting of α-helixes and only 5% of β-strands.[28,33,46] However, some reports also indicate the presence of heterotetrameric forms of PAL.[30,47] The homotetramer formation is primarily driven by the interaction among large hydrophobic surfaces present on the subunits.[48] Each tetramer consists of four identical subunits with the active site of each subunit being

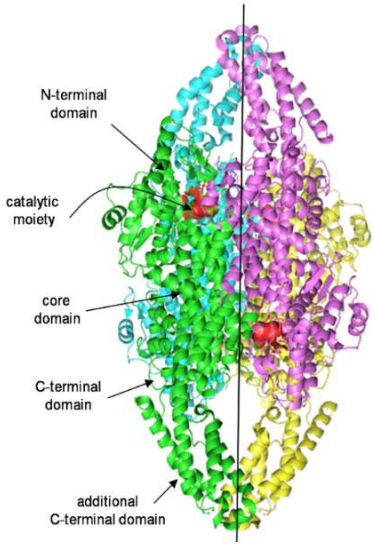


Figure 2. Homotetramer of *Pc*PAL with monomeric chains individually coloured and representing all domains and the catalytic moiety (red surface).

surrounded by three other PAL monomers.[4] Each of the four PAL monomers consists of a rigid central core domain, a globular N-terminal domain, and an elongated C-terminal domain. Certain plant and fungal PALs have been proven to possess an additional C-terminal multi-helical domain, in contrast to their bacterial homologues (**Figure 2**). This domain may contribute to the precise regulation of enzyme activity and its correlation with the phenylpropanoid biosynthesis in these organisms, through mechanisms such as substrate channelling or overall destabilization of the protein structure.[28,49]

1.2.1.1. Catalytic site of PALs

The catalytic site of PAL enzymes consists of two main regions: a hydrophilic region and a hydrophobic region. The hydrophilic region includes residues like Arg354 that form hydrogen bonds with the substrate's carboxylic group, stabilizing it in the catalytic center. The hydrophobic region accommodates the substrate's aromatic ring, primarily through hydrophobic interactions, which influence substrate specificity (**Figure 3A**).A key feature of this site is the MIO (3,5-dihydro-5-methylidene-4H-imidazol-4-one) group, essential for enzyme function and post-translationally formed the the three amino acid side chains Ala-Ser-Gly (ASG): Ala202-Gly204 in PAL from *Petroselinum crispum*.[50] (**Figure 3B**). Specific residues near the aromatic ring, such as Phe/Leu in *Pc*PAL, determine enzyme activity, with mutations like His88 to Phe in *Rs*TAL and Phe144 to His in *At*PAL1 switching enzyme specificity from TAL to PAL or vice versa.

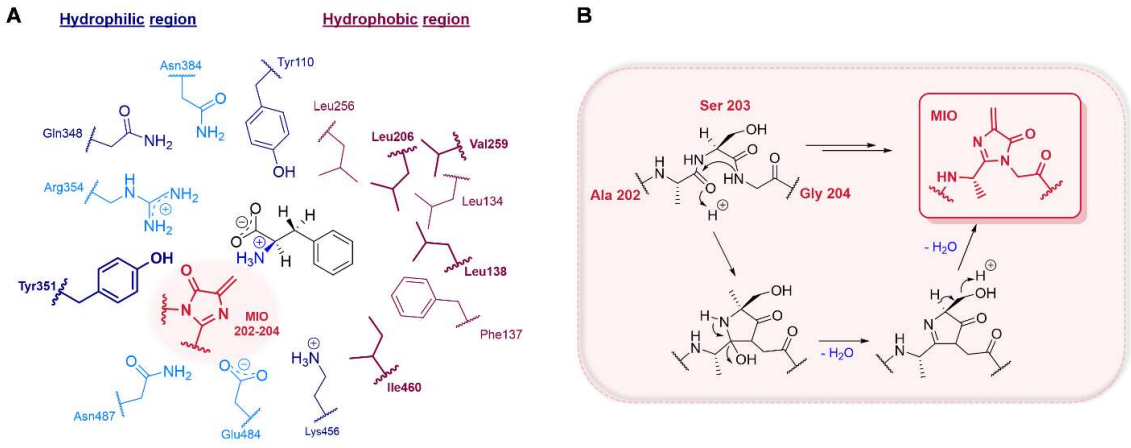


Figure 3. Catalytic site of PAL enzymes: illustration featuring PAL from *Petroselinum crispum*. (**A**) Catalytic site of *Pc*PAL, highlighting the hydrophilic (blue) and the hydrophobic (purple) substrate binding regions, as well as the MIO electrophilic prosthetic group (red). Residues marked in bold are positioned below the plane of the substrate, non-bold residues are situated above the substrate plane, while light blue residues are depicted within the same plane as the substrate. (**B**) Schematic depiction of the post-translational formation of the MIO moiety, involving cyclization and double dehydration of Ala202-Ser203-Gly204 sequence of the representative *Pc*PAL.

1.2.2. Substrates scope and protein engineering of PALs for producing unnatural phenylalanine derivatives

Wild-type PALs from different organisms exhibit a wide substrate scope thus, providing synthetic accessibility of various unnatural L-phenylalanine derivatives, which are difficult to emulate by the chemical synthesis. Among the characterised PALs, PAL from the bacterium *Anabaena variabilis*[51] and the plant *Lactuca sativa*[52]or *Petroselinum crispum*[38,40,53] are the most promiscuous. Additionally, extensive efforts have been undertaken to enhance PALs applicability by expanding their substrate scope through protein engineering,[38–40,45,54–56] aiming for their industrial scale usage (**Figure 4**).

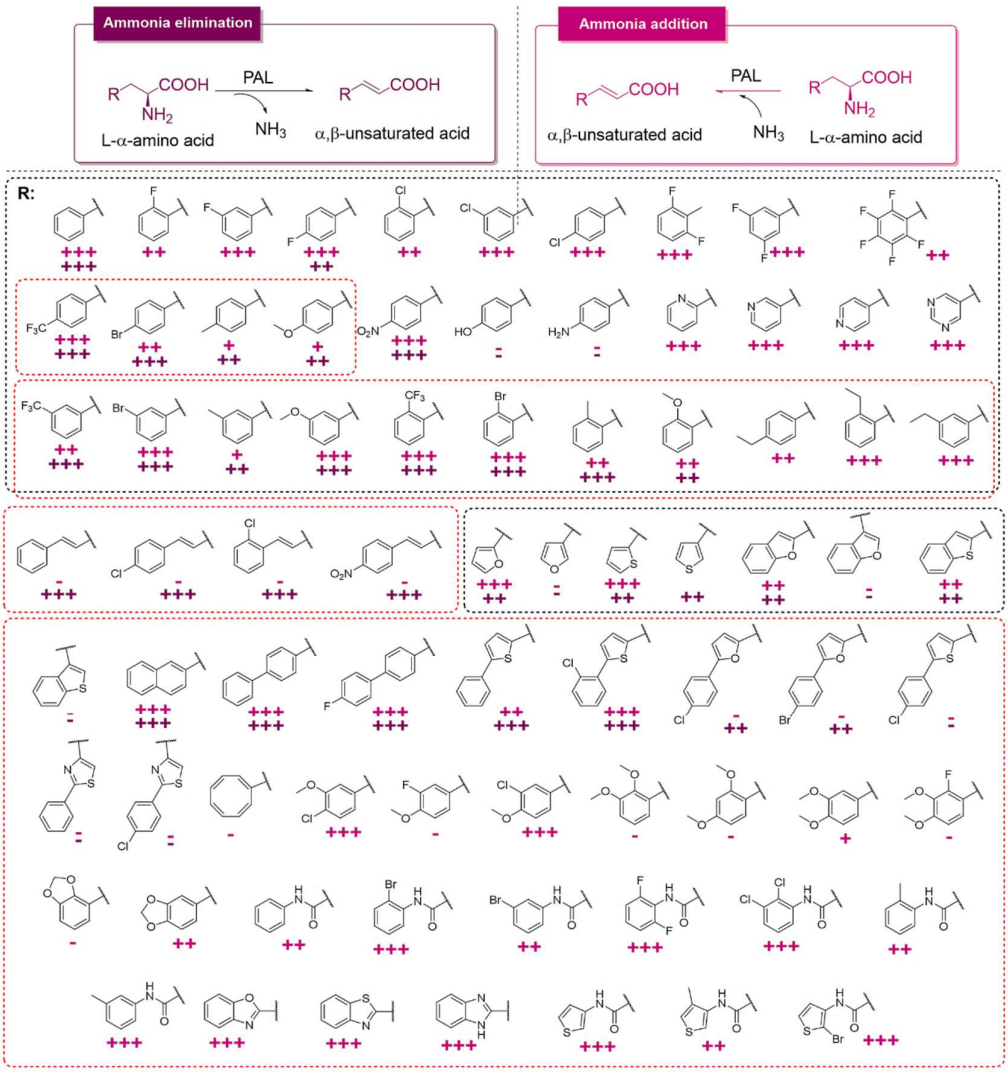
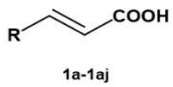


Figure 4. Substrate panel tested for wild-type and engineered PALs in ammonia elimination (purple) and ammonia addition (pink) reactions. Substrates transformed by wild-type PALs are framed with black dotted lines and those transformed by engineered PALs are highlighted in red dotted lines, while those double framed are transformed by wild-type PAL, with their activity being increased by protein engineering. The conversion levels for each reaction are categorized as follows: "–" (0%), "+" (1-30%), "++" (31-70%), and "+++" (>70%).

SUBSTRATE INDEX FOR SUBSTRATES USED ACROSS CHAPTERS

Substrate for ammonia addition



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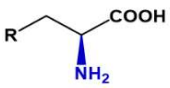
Substrate for ammonia elimination



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Natural substrates of PAL/ TAL/ HAL

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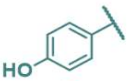

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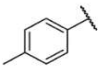
para-substituted substrates



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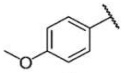

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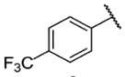

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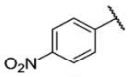

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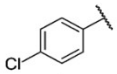

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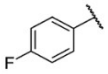

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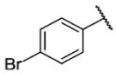

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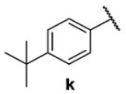

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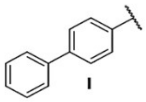

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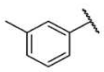
meta-substituted substrates



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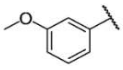

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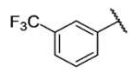

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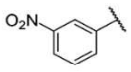

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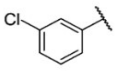

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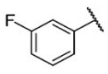

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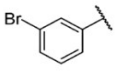

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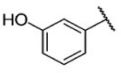

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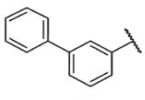

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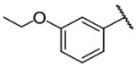

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***ortho*-substituted substrates**

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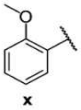

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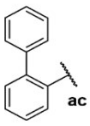

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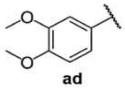
multi-substituted substrates



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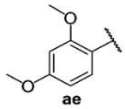

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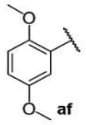

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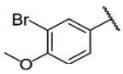

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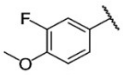

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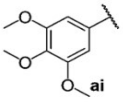

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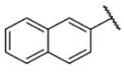


aj

<u>Guide to substrates across chapters</u>

Substrates used in Chapter 2:
a,d,f,i,j,l,m,n,o,r,s,u,v,w,y,ad,ae,ag,ah,ai

Substrates used in Chapter 3:
e,n,s,ad,ag,ah

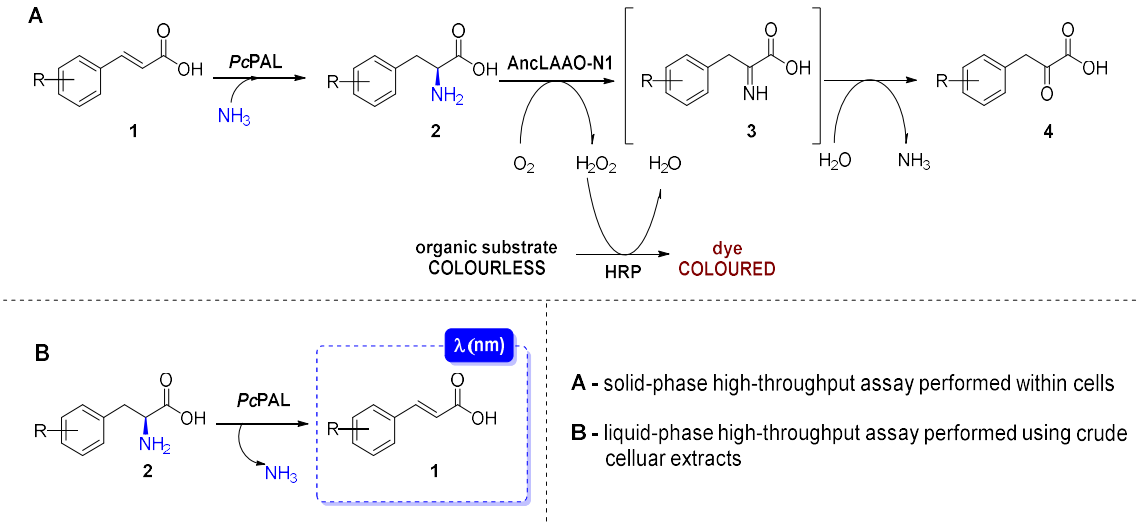
Substrates used in Chapter 4:
a,b,c,d,e,f,g,h,k,l,m,n,o,p,q,t,u,w,x,y,z,aa, ab,ac,ad,ae,af,ag,ah,ai,aj

CHAPTER 2

2. HIGH-THROUGHPUT ASSAY FOR SCREENING SATURATION MUTAGENESIS DERIVED PHENYLALANINE AMMONIA-LYASE LIBRARIES

2.1. Results and Discussion

Two high-throughput assays were developed to efficiently screen extensive directed evolution-derived PAL libraries using recombinant expression in *E. coli*. In the solid-phase assay, PAL libraries are co-expressed with the ancestral L-amino acid oxidase (AncLAAO-N1)[57], where LAAO oxidizes the L-phenylalanine derivative obtained from the ammonia addition to the cinnamic acid, producing hydrogen peroxide. This is detected through a horseradish peroxidase (HRP)-mediated colorimetric reaction, highlighting positive PAL variants (**Scheme 2A**). In the liquid-phase assay, cells are lysed, and the crude extract is used to monitor the ammonia elimination reaction from the phenylalanine derivative, with cinnamate production tracked spectrophotometrically (**Scheme 2B**).



Scheme 2. General reactions set-up for the solid-phase high-throughput PAL-LAAO-HRP-based assay (**A**) and the liquid-phase high-throughput assay using crude PAL cellular extracts (**B**).

In our aim to test the two high-throughput assays for screening PAL libraries, we selected as model substrates those having electron-donating substituents grafted on the aromatic ring of phenylalanine, since they are known to be poorly transformed by PAL enzymes.[38,55] Thus, using *p*-methoxy-cinnamic acid (*p*-MeO-*t*-CA) and *p*-methoxy-L-phenylalanine (*p*-MeO-L-Phe) as substrates, the rationally engineered I460V-*Pc*PAL variant with reported activity towards these substrates, could be used as positive control within the library screens.[38]

2.1.1. Liquid-phase UV- based phenylalanine ammonia-lyase-activity assay

2.1.1.1. Evaluating and optimizing cell lysis conditions for efficient PAL-activity screening

To induce cell lysis, a range of chemical agents, including Tween-20, Triton X-100, lysozyme, polymyxin B sulfate, glucose, and glycerol solutions were employed. The effectiveness of each lysis solution was evaluated by measuring the activity of the crude extract within the ammonia elimination reaction of *p*-methoxy-L-phenylalanine, catalyzed by I460V-*Pc*PAL (**Figure 4A**).

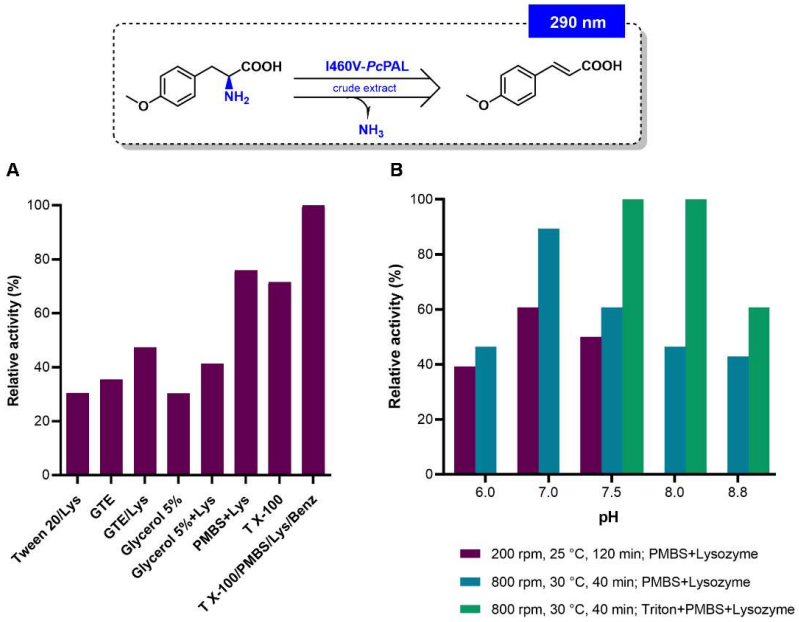


Figure 4. Relative activity of I460V-*Pc*PAL towards *p*-MeO-Phe after lysis (**A**) *lane 1* – Tween-20/Lys (1% Tween-20, 10 mM imidazole, 0.5 mg/mL lysozyme, 400 U/mL DNAse I, 50 mM $NaH_2PO_4{\cdot}2H_2O$ pH 8.0, 300 mM NaCl); *lane 2* – GTE buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris.HCl pH 8); *lane 3* – GTE / Lys lysis buffer (50 mM glucose, 10 mM EDTA, 0.5 mg/mL lysozyme, 25 mM Tris.HCl pH 8.0);) *lane 4* – 5% glycerol lysis buffer (5 % glycerol, 100 mM NaCl, 1 mM DTT, 50 mM Tris pH 7.5; *lane 5* – 5% glycerol / Lys lysis buffer (5 % glycerol, 0.5 mg.mL lysozyme, 1 mM DTT, 50 mM Tris.HCl pH 7,5, 100 mM NaCl); *lane 6* – PMBS / Lys buffer (1 mg/mL PMBS, 0.5 mg/mL lyzozyme, 20 mM Tris pH 8.8, 50 mM NaCl); *lane 7* – Triton X-100 buffer (2% Triton X-100, 20 mM Tris pH 8.8, 50 mM NaCl); *lane 8* – T X-100 / PMBS / Lys buffer (2% Triton X-100, 1 mg/mL PMBS, 0.5 mg/mL lyzozyme, 25 U/mL Benzonase, 20 mM Tris pH 8.8, 50 mM NaCl). (**B**) Optimization of the lysis buffer using two sets of lysis agents 1 mg/mL PMBS, 0.5 mg/mL lysozyme and 0.36 mM PMBS, 0.07 mM lysozyme, 2% Triton X-100, 25 U/mL Benzonase, respectively, under different pHs and lysis conditions. Abbreviations: Lys., PMBS, T X-100 and Benz. denote for lysozyme, polymyxin B sulphate, Triton X-100 and Benzonase.

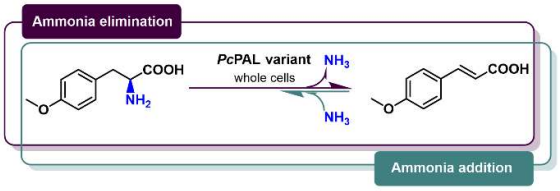
A two-component PMBS-lysozyme lysis buffer was identified as optimal from the initial screening, and its efficiency was further improved by optimizing the pH, incubation temperature and stirring speed (**Figure 4B**). The optimal lysis conditions were shown when a Tris.HCl solution with pH 7.0 containing 0.5 mg/mL lysozyme, 1 mg/mL PMBS, performed for 40 min at 800 rpm and

30 °C. This provided a homogenous and clear cell lysate with low UV-background which later proved to be applicable for the high-throughput PAL-activity screens (**Figure 4B**, *lane 4*).

2.1.1.2. Validation of the UV-assay for the high-throughput screen of PAL-activity

After determining the optimal lysis conditions, the saturation mutagenesis library containing randomized residue 460 of *Pc*PAL using NNK degeneracy (generated as described in *Chapter 3, Section 3.2.2.1.*) was screened in a high-throughput manner to assess its ammonia elimination activity towards *p*-MeO-Phe.

Within the library screening experiments the inactive wild-type *Pc*PAL was included as a negative reference and the I460V-*Pc*PAL active variant as positive control. The results obtained from the liquid phase assay and validated by HPLC reaction monitoring, revealed ten hits, with the rationally designed I460V mutation found in four selected colonies, while five novel beneficial mutations, I460C, I460L, I460W, I460T, I460S, were also revealed (**Figure 5**).



*Pc*PAL variant	c_E (%)	c_A (%)
I460V	82	37
I460C	4	n.a.
I460W	4	n.a.
I460L	8	5
I460S	100	37
I460T	100	40

Figure 5. Ammonia elimination from *p*-methoxy-L-phenylalanine and ammonia addition to *p*-methoxy-*trans*-cinnamic acid using the induced whole-cell biocatalysts of the corresponding *Pc*PAL variants showing increased activity within the liquid-phase high-throughput activity screens.

The results indicated limitations of the liquid-phase assay, as variants like I460C, I460W, and I460L, which show improved activity in the ammonia elimination reaction, do not necessarily perform well in the hydroamination reaction. This underscores the need for assays specifically targeting the ammonia addition reaction. However, the improved variants from the I460NNK-*Pc*PAL library offer a well-characterized control library for validating the solid-phase assay.

2.1.2. Solid-phase LAAO-based phenylalanine ammonia-lyase activity assay

Once the liquid-phase UV-assay was in place, the feasibility of the solid-phase assay also needed to be evaluated. In this case, initial steps were imperative. Firstly, the expression and purification of the ancestral L-amino acid oxidase (AncLAAO-N1) was optimized, followed by the assessment of the compatibility of its substrate scope with the substrate domain of PALs. Subsequently, the determination of the catalytic constants of AncLAAO-N1 was also essential to ensure that the bottleneck within the system remains the ammonia addition catalyzed by PAL,

rather than the oxidation of the produced L-amino acid. Further, the assay set-up was optimized, while the validation of the assay was performed screening the same I460NNK-*Pc*PAL library for activity towards the model substrate, *p*-MeO-cinnamic acid, in the ammonia addition reaction.

2.1.2.1. Expression/purification of AncLAAO-N1

For the production of AncLAAO-N1 two plasmid constructs were employed: one construct in pET-28a(+) enabled the fusion of the enzyme with a C-terminal His_6-tag, while the other, in pET-15b, facilitated the expression of AncLAAO-N1 fused with an N-terminal His_6-tag. After several rounds of purification of both the C-terminally His-tagged and N-terminally His-tagged AncLAAO-N1, a homogeneous N-terminal His_6-tagged AncLAAO-N1 with minimal impurities was obtained using Ni-NTA affinity chromatography.

The size-exclusion chromatography (SEC) of AncLAAO-N1 revealed the presence of two oligomerization states (**Figure 6A**) associated to the dimeric and octameric forms, with the octamer displaying a 6.3-fold higher specific activity measured within the oxidation of L-Phe (**Figure 6B**) and an increase in the T_m with approximately 8 °C (T_m = 56 °C), compared to the dimeric form (T_m = 48 °C) (**Figure 6C**).

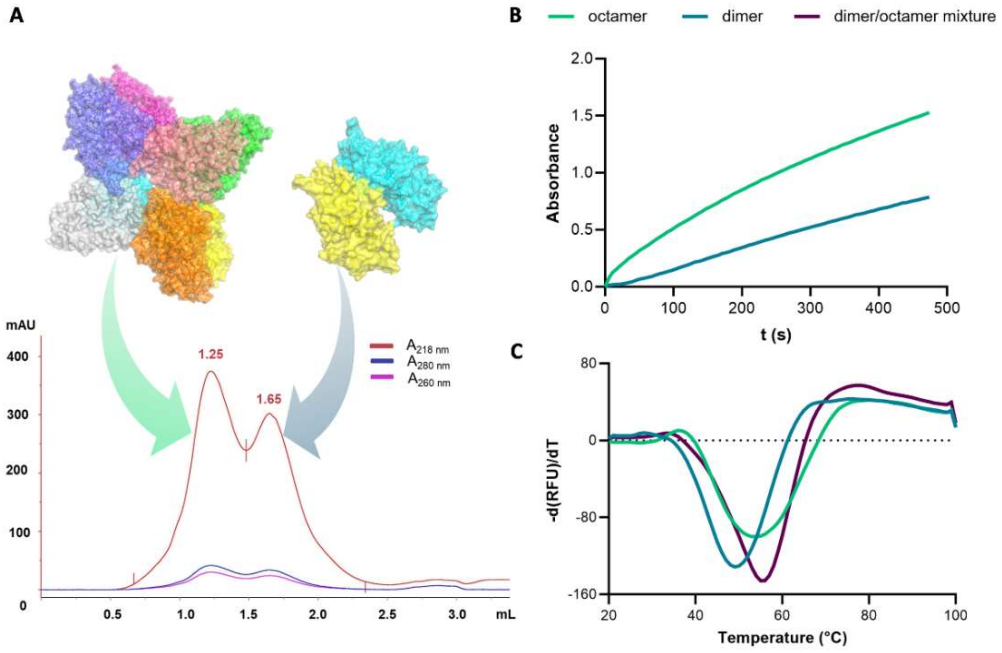
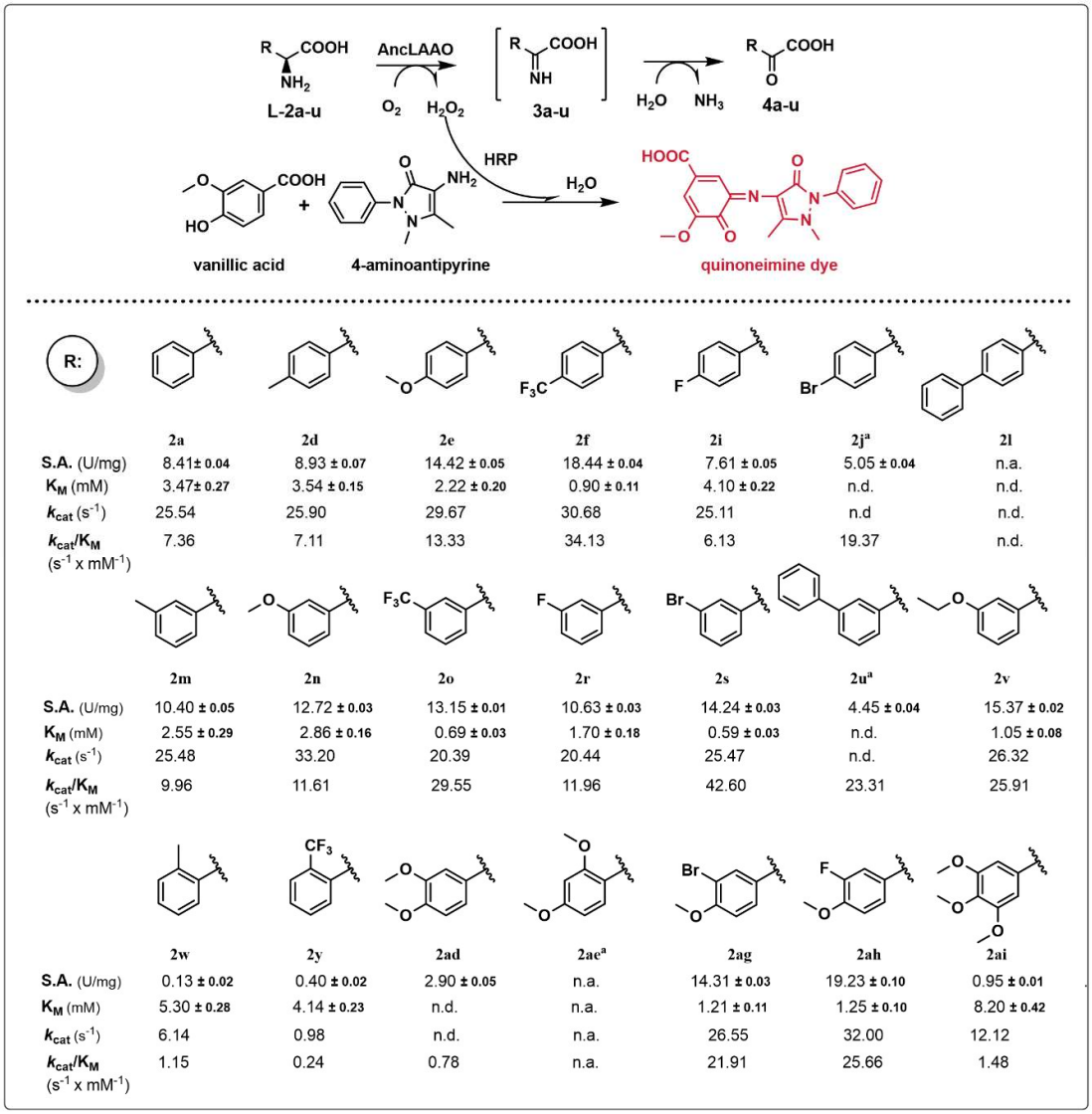


Figure 10. (**A**) Size exclusion chromatography of AncLAAO-N1 showing the presence of an octameric (1.23 mL) and a dimeric (1.65 mL) form; (**B**) Activity measurement for L-Phe using the dimeric and octameric fractions; (**C**) Thermal unfolding profiles of the different oligomeric forms of AncLAAO-N1.

2.1.2.2. Substrate scope of AncLAAO_N1

To assess the compatibility of AncLAAO-N1's substrate scope with that of both wild-type and engineered phenylalanine ammonia-lyases, required for its potential application within the PAL-activity screening, an extensive substrate profiling of AncLAAO-N1 was undertaken. Thus, a variety of *ortho*-, *meta*-, and *para*- mono-substituted, as well as di-, tri-substituted phenylalanines or their biarylic analogues were tested within the LAAO mediated oxidation (**Figure 7**).



	2a	2d	2e	2f	2i	2j[a]	2l
S.A. (U/mg)	8.41± 0.04	8.93 ± 0.07	14.42 ± 0.05	18.44 ± 0.04	7.61 ± 0.05	5.05 ± 0.04	n.a.
K_M (mM)	3.47± 0.27	3.54 ± 0.15	2.22 ± 0.20	0.90 ± 0.11	4.10 ± 0.22	n.d.	n.d.
k_{cat} (s^{-1})	25.54	25.90	29.67	30.68	25.11	n.d	n.d.
k_{cat}/K_M (s^{-1} x mM^{-1})	7.36	7.11	13.33	34.13	6.13	19.37	n.d.

	2m	2n	2o	2r	2s	2u[a]	2v
S.A. (U/mg)	10.40 ± 0.05	12.72 ± 0.03	13.15 ± 0.01	10.63 ± 0.03	14.24 ± 0.03	4.45 ± 0.04	15.37 ± 0.02
K_M (mM)	2.55 ± 0.29	2.86 ± 0.16	0.69 ± 0.03	1.70 ± 0.18	0.59 ± 0.03	n.d.	1.05 ± 0.08
k_{cat} (s^{-1})	25.48	33.20	20.39	20.44	25.47	n.d.	26.32
k_{cat}/K_M (s^{-1} x mM^{-1})	9.96	11.61	29.55	11.96	42.60	23.31	25.91

	2w	2y	2ad	2ae[a]	2ag	2ah	2ai
S.A. (U/mg)	0.13 ± 0.02	0.40 ± 0.02	2.90 ± 0.05	n.a.	14.31 ± 0.03	19.23 ± 0.10	0.95 ± 0.01
K_M (mM)	5.30 ± 0.28	4.14 ± 0.23	n.d.	n.a.	1.21 ± 0.11	1.25 ± 0.10	8.20 ± 0.42
k_{cat} (s^{-1})	6.14	0.98	n.d.	n.a.	26.55	32.00	12.12
k_{cat}/K_M (s^{-1} x mM^{-1})	1.15	0.24	0.78	n.a.	21.91	25.66	1.48

Figure 7. The substrate library tested for the AncLAAO-N1 catalyzed oxidative deamination and the corresponding kinetic parameters determined by the HRP-coupled spectrophotometric assay. (a – Complete Michaelis-Menten curves could not be determined due to low solubility of the substrate, instead k_{cat}/K_M constants were determined at substrate concentrations at least 10-fold lower than the estimated K_M, n.a. – no activity detected, n.d. – could not be determined).

The kinetic parameters of AncLAAO-N1 in the oxidative deamination of ring-substituted phenylalanines revealed high catalytic efficiency towards substrates mono-, di-, or multiple-substituted in the *meta*- or *para*-positions, while *ortho*-substituted substrates were poorly or not transformed.

2.1.2.3. Assay set-up and validation

The assay set-up was initiated by coupling the AncLAAO-N1 catalyzed oxidation with the PAL-mediated ammonia addition reaction, using the horseradish peroxidase (HRP) detection system (**Figure 8**). During the assay initiation the active PAL variants identified previously from the *Pc*PAL I460NNK saturation mutagenesis library were used as positive controls, representing active PAL variants for the ammonia addition of the model substrate, *p*-methoxy-cinnamic acid.

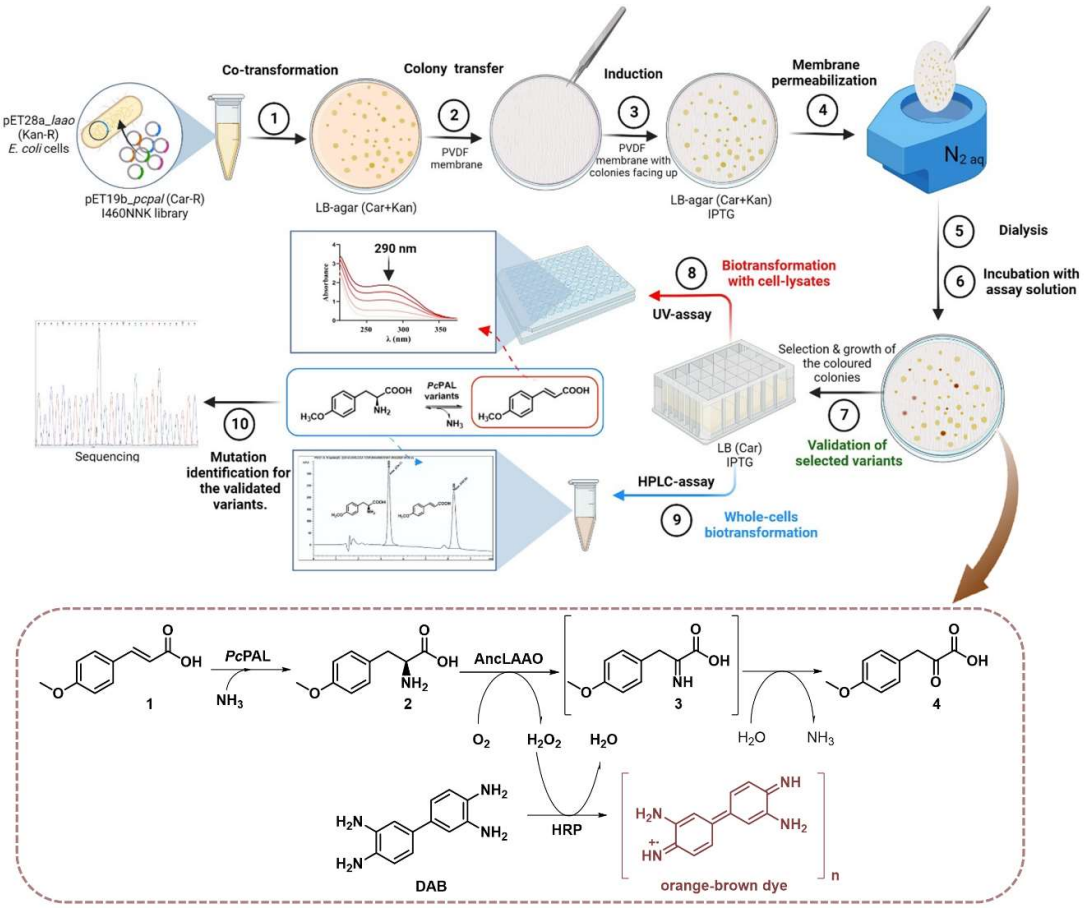
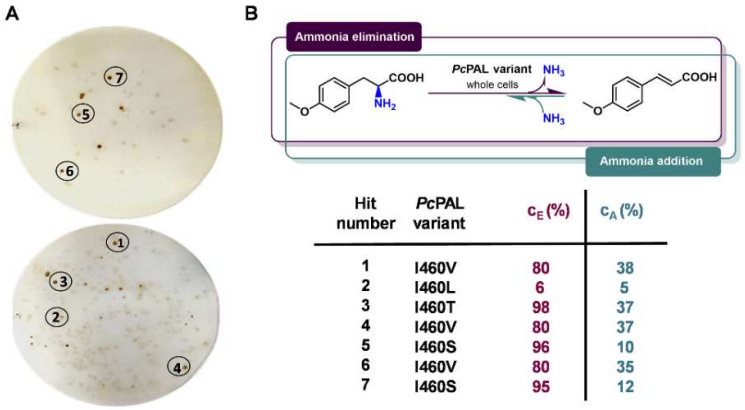


Figure 8. General procedure for the solid-phase, LAAO-based PAL-activity assay

Performing the solid-phase assay on the obtained library, the qualitative assessment of PAL activity in each colony could be easily carried out by visual inspection, allowing the identification and selection of the best performing variants based on their orange-brown colour intensities (**Figure 9A**). Strikingly, the best performing variants, I460V, I460T, I460S and I460L

(**Figure 9B**), all corresponded to the variants previously identified by the UV-assay from the *Pc*PAL I460NNK-library.

These results demonstrate that the solid-phase assay effectively highlights the potential of AncLAAO-N1 and aids in identifying superior PAL variants. Moreover, while most of the currently used PAL-assays rely on the ammonia elimination reaction, the developed solid-phase procedure allows the direct identification of improved variants within the synthetically valuable ammonia addition reaction.



Hit number	*Pc*PAL variant	c_E (%)	c_A (%)
1	I460V	80	38
2	I460L	6	5
3	I460T	98	37
4	I460V	80	37
5	I460S	96	10
6	I460V	80	35
7	I460S	95	12

Figure 9. Results from the screening of the *Pc*PAL-I460NNK library. (**A**) Assay membranes obtained from screening the I460NNK-library with the AncLAAO-PAL coupled assay; (**B**) Conversion values of the sequenced hits within the PAL-mediated ammonia elimination and ammonia addition reaction routes.

2.2. Conclusions

Two high-throughput PAL activity assays were developed and compared using the saturation mutagenesis-derived I460NNK-*Pc*PAL library and *p*-methoxy L-phenylalanine and *p*-methoxy *trans*-cinnamic acid as model substrates. The liquid-phase UV-assay, targeted the ammonia elimination reaction catalyzed by *Pc*PAL, and the solid-phase assay, which coupled PAL with the ancestral L-amino acid oxidase (AncLAAO-N1), focused on the ammonia addition.

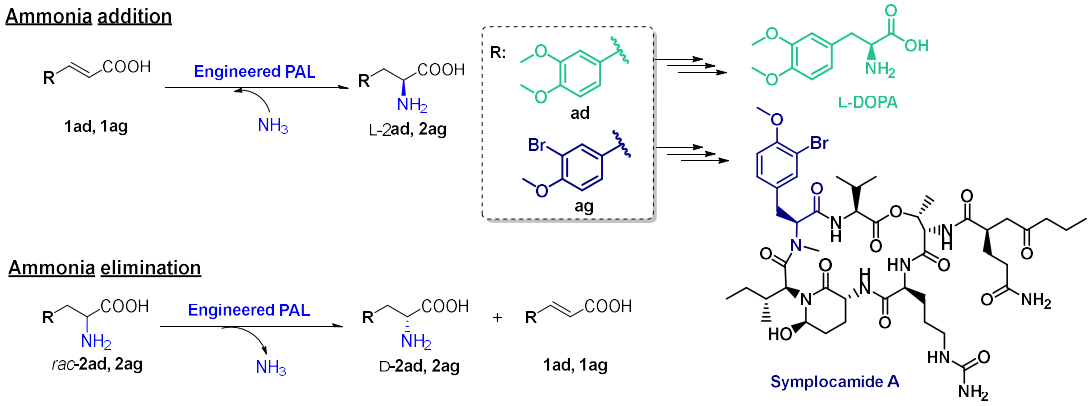
The liquid-phase assay identified six variants with increased activity in ammonia elimination, I460V, I460T, I460S, I460L, I460W, I460C, with I460W and I460C showing no activity in ammonia addition, highlighting one limitation of the liquid-phase assay. Conversely, the solid-phase assay, leveraging AncLAAO-N1, proved effective for high-throughput screening of ammonia addition. Improved PAL variants were successfully identified and validated, matching those found in the liquid-phase assay, but with the added benefit of directly targeting the valuable ammonia addition reaction. This approach underscores AncLAAO-N1's utility in identifying high-efficiency PAL variants and enhances screening capabilities for reactions with synthetic importance.

CHAPTER 3

3. PROTEIN ENGINEERING OF PHENYLALANINE AMMONIA-LYASES FOR ENHANCED ACTIVITY ON CHALLENGING SUBSTRATES

3.1. Results and discussion

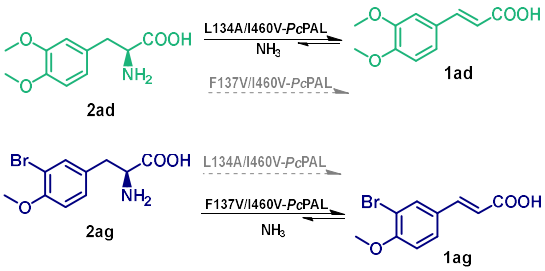
Aiming to engineer phenylalanine ammonia-lyases (PALs) for challenging substrates, such as 3,4-dimethoxy-L-phenylalanine and 3-bromo-4-methoxy-L-phenylalanine, essential for the synthesis of L-DOPA[58] and Symplocamide A[59] (**Scheme 3**), initial strategies focused on mono-substituted versions of these substrates. These preliminary results guided the identification of PAL variants active towards the targeted di-substituted substrates. Both rational design and saturation mutagenesis approaches were used and compared for their effectiveness.



Scheme 3. Targeted PAL activity towards 3,4-dimethoxy- (**1ad**, **2ad**) and 3-bromo 4-methoxy- (**1ag**, **2ag**)-substituted substrate analogues, yielding high value products, with **2ad** and **2ag** serving as key synthons for API synthesis.

3.1.1. Rational design for challenging substrates

Previous research expanded *Pc*PAL's substrate scope to include bulkier phenylalanine derivatives with mono-substituents, achieving moderate conversions with specific residue substitutions, including L134A, I460V and F137V.[38–40] Building on these results, residues L134, F137, and I460 were selected for mutagenesis to improve the activity towards the two di-substituted substrates.



Scheme 4. Ammonia elimination and ammonia addition reaction of **1ad**,**2ad** and **1ag**,**2ag** using the rationally designed double variants L134A/I460V-*Pc*PAL and F137V/I460V-*Pc*PAL.

Double mutants L134A/I460V and F137V/I460V showed enhanced activity within both the ammonia elimination and addition reactions of towards 3,4-dimethoxy-(**1ad**, **2ad**) and 3-bromo

4-methoxy-(**1ag**, **2ag**) substrates (**Scheme 4**), with L134A/I460V achieving up to 17% conversion and F137V/I460V reaching 15% conversion in the ammonia addition reaction of 3,4-dimethoxy *trans*-cinnamic acid and 3-bromo 4-methoxy *trans*-cinnamic acid.Despite these improvements, the ammonia addition reaction remained low. Thus, a second approach, involving the saturation mutagenesis at these rationally selected residues, was implemented.

3.1.2. Saturation mutagenesis–based approach

Given that the rational design approach was previously validated across PALs of different origins,[38,42] establishing a robust methodology for the saturation mutagenesis approach was necessary. Consequently, the optimization of the saturation mutagenesis approach was initially conducted using the I460NNK-*Pc*PAL library, followed by the implementation of Combinatorial Active-site Saturation Test (CAST-ing)[60] and iterative saturation mutagenesis (ISM)[61,62] to tailor PAL variants for the two challenging substrates.

3.1.2.1. Site-saturation mutagenesis methodology

For effective mutagenesis, one based on the partially overlapping primers[63] (**Figure 10A**) and the other on the Megaprimer method[64] (**Figure 10B**). The partially overlapping primer approach showed improved efficiency over the traditional QuickChange method, however, the Megaprimer method, especially with small megaprimers, resulted in a library with better base distribution and less amino acid bias of the NNK degeneracy. Consequently, the small-megaprimer approach was chosen for its superior quality and consistency in generating diverse protein libraries.

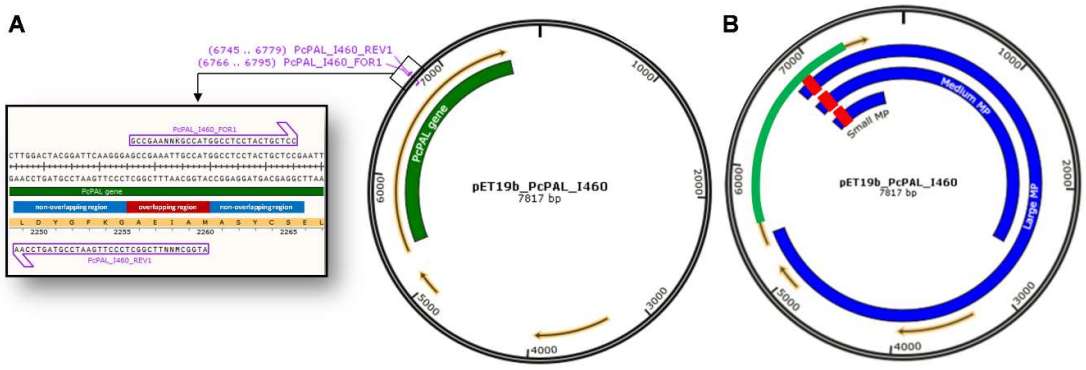


Figure 10. Saturation mutagenesis using the (**A**) Partially overlapping procedure (**B**) Megaprimers procedure, representing the gene encoding *Pc*PAL (green) the pET19b-vector backbone (black), and the megaprimers of different, small, medium, large lengths (blue). In the first PCR stage the forward mutagenic primer (red) and the reverse, non-mutagenic, primer anneal to the template resulting the amplified sequences of the corresponding, large, medium and small-sized megaprimers. In the second PCR stage through annealing the two single stranded DNA of the megaprimers the mutated plasmid products are amplified.

3.1.2.2. *Saturation mutagenesis for challenging substrates*

The Combinatorial Active-site Saturation Test (CAST-ing) combined with iterative saturation mutagenesis (ISM) strategy, aimed to address the limitations of the previous rational design, by targeting the previously three key catalytic residues from *Pc*PAL: L134, F137 and I460, together with L138. Despite extensive mutagenesis and screening (**Figure 11**), combining the four catalytic residues in iterative rounds, the rational design approach consistently yielded the most effective variants. For the two di-substituted substrates, the rationally designed variants L134A/I460V and F137V/I460V proved to be the most active, with saturation mutagenesis not surpassing the performance of these rationally engineered variants. These results underscore that while saturation mutagenesis can generate diverse variant libraries, it reveals a restricted mutational diversity for the substrate specificity-modulator residues L134, F137, and I460 in *Pc*PAL.

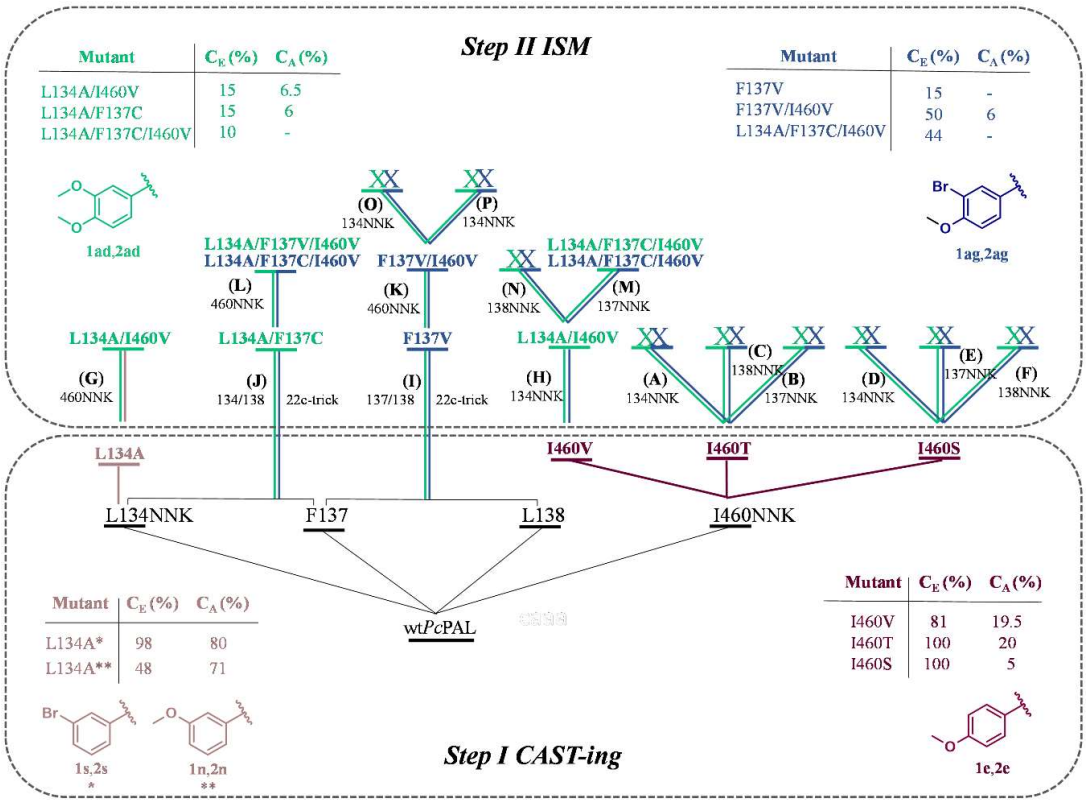


Figure 11. Saturation mutagenesis strategy employed for PAL-activity generations towards 3,4-dimethoxy L-phenylalanine (**2ad**) and 3-methoxy-4-bromo L-phenylalanine (**2ag**). Image was adapted from Tomoiagă *et.al.* 2023[65]

3.1.3. Combining protein engineering with natural diversity

In previous studies, aimed to extend the rational mutational strategy for enhanced PAL-activity towards mono-substituted substrates in PALs of different origin, it was observed that the

degree of the catalytic efficiency towards a particular substrate varies even among PALs having identical catalytic site.[42] This variation illustrates that PAL-activity is influenced by factors beyond catalytic site differences and highlighted the importance of conducting activity screens with PALs from various origins within the development of efficient PAL-biocatalysts. Accordingly, in our next attempt to generate active PALs for the targeted challenging substrates, we tested the effect of applying the identified beneficial mutations on various PALs of different origins.

While the best-performing *Pc*PAL variants, L134A/I460V and F137V/I460V, demonstrated enhanced activity however, their catalytic efficiency was still limited. Therefore, these beneficial mutations were imprinted to PALs of different origins to investigate potential additional improvements on PAL-activity towards 3,4-dimethoxy-(**1ad**, **2ad**) and 3-bromo 4-methoxy-(**1ag**, **2ag**) substrates. Variants of PALs from plant *Arabidopsis thaliana* (*At*PAL) and bacterial origin *Anabaena variabilis* (*Av*PAL) demonstrated higher catalytic efficiency than their homologous *Pc*PAL variants (**Figure 12**). Additionally, the engineered PALs were compared with a novel aromatic ammonia-lyase from *Loktanella atrilutea* (*La*AAL), a close relative of the metagenome-derived aromatic ammonia-lyase AL-11[66], which has been recently reported to possess atypically high activity towards substrates with electron-donor aromatic substituents. *La*AAL indeed outperformed the engineered *Pc*PAL, *At*PAL and *Av*PAL variants in the production of 3,4-dimethoxy-L-phenylalanine, while showing no activity towards 3-bromo-4-methoxy derivatives.

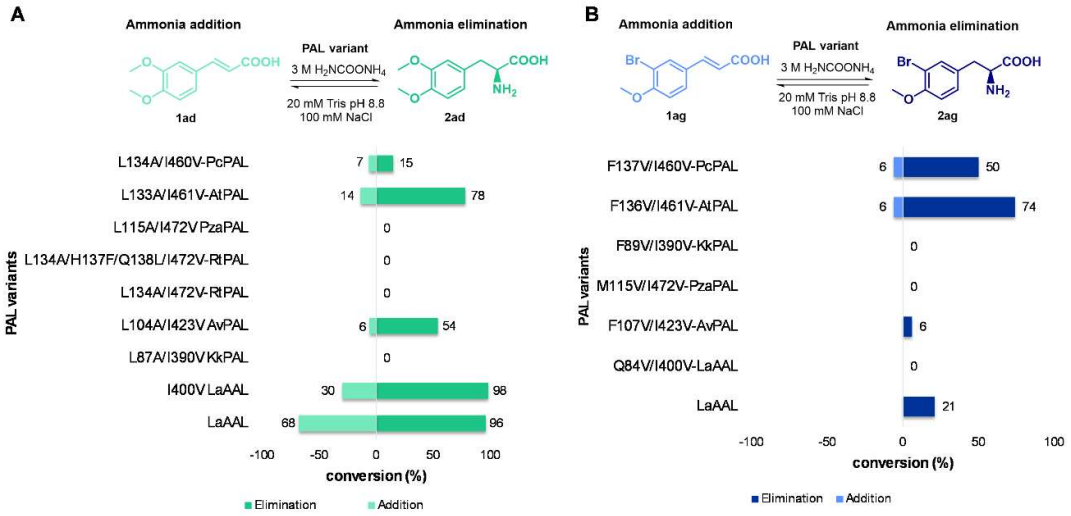


Figure 12. Conversions achieved using engineered PALs from different origins as whole-cell biocatalysts in ammonia addition and ammonia elimination reactions of (**A**) **1ad**, **2ad** and (**B**) **1ag**, **2ag**.

While the engineered variants of *Pc*PAL, *At*PAL, and *Av*PAL demonstrate comparable high conversions for ammonia elimination of **2ad**, *La*AAL exhibits superior catalytic performance in several key areas. Specifically, *La*AAL achieves a significantly higher conversion for ammonia

addition to 3,4-dimethoxy *trans*-cinnamic acid **1ad** compared to the best-performing engineered variant L133A/I461V-*At*PAL, despite having a lower substrate affinity as indicated by its higher K_M values. *La*AAL's higher turnover numbers, suggest that substrates accommodate in a manner that enhances catalytic turnover, rivalling the natural catalytic efficiencies observed in wild-type PALs, highlighting *La*AAL's potential as a highly efficient biocatalyst for 3,4-dimethoxy substrates. The attempts to transfer the unique array of selectivity modulator residues from *La*AAL to the well-characterized PALs failed to enhance their activity towards the targeted substrates, while *La*AAL's activity was reduced when applying the rational design approach successful for the well-characterized PALs. These results indicate that other tailoring strategies are necessary for *La*AAL-like aromatic ammonia-lyases, which may represent a different PAL subclass with naturally evolved modifications to the reaction and substrate scope.

3.2. Conclusions

Engineering of *Pc*PAL using saturation mutagenesis and rational design yielded variants with improved activity towards challenging substrates, such as 3-bromo-4-methoxy-L-phenylalanine and 3,4-dimethoxy-L-phenylalanine. The best-performing variants, L134A/I460V and F137V/I460V still showed limited catalytic efficiency. Transferring these mutations to PALs of different origins, the corresponding PAL variants from *Arabidopsis thaliana* (*At*PAL) and *Anabaena variabilis* (*Av*PAL) exhibited higher efficiency compared to those from *Pc*PAL. However, the aromatic ammonia-lyase from *Loktanella atrilutea* (*La*AAL), a close relative of AL-11, demonstrated superior performance with 3,4-dimethoxy-L-phenylalanine but none with 3-bromo-4-methoxy derivatives. Attempts to imprint *La*AAL's catalytic site to other PALs or to apply the rational strategy of the known PALs to *La*AAL did not provide any variants with enhanced activity towards the two di-substituted substrates.

CHAPTER 4

4. UNVEILING THE BIOCATALYTIC POTENTIAL OF AROMATIC AMMONIA-LYASE FROM *LOKTANELLA ATRILUTEA*: HOMOLOGUE MINING AND ANCESTRAL RELATIONSHIP ANALYSIS

4.1. Results and discussion

The characterization the substrate scope and the biocatalytic potential of the aromatic ammonia-lyase from *Loktanella atrilutea* was considered, focusing on its utility in the synthetically valuable ammonia addition reaction pathway, using as model substrate 3,4-dimethoxy *trans*-cinnamic acid. Seeking to expand the knowledge of this potential distinct subclass of aromatic ammonia–lyases and enhance their applicability in the production of

valuable chemical compounds, we explored its substrates scope and investigated its evolutionary relationships within the MIO-enzyme family.

4.1.1. Initial ammonia-lyase activity screens

The screening of *La*AAL activity involved measuring the initial velocities of the purified enzyme towards the natural substrates of aromatic ammonia-lyases, L-Phe, L-Tyr and L-His and also for the highly valuable 3,4-dimethoxy-L-phenylalanine (**2ad**), selected as model substrate and comparing these activities with those of known PALs (**Figure 13**). As observed before, in *Chapter 3*, *La*AAL showed unusually high activity for 3,4-dimethoxy-L-phenylalanine, compared to the aromatic ammonia-lyases natural substrates, with much lower activity for L-Phe, L-Tyr and no activity for L-His.

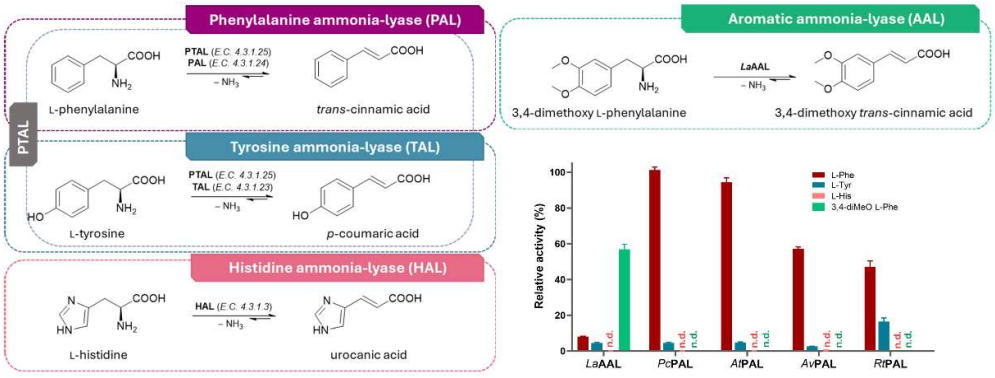


Figure 13. Initial tests for assessing the natural reaction of *La*AAL towards the natural reactions of PALs, TALs, HALs and synthetically valuable model reaction used to determine the optimal conditions for the *La*AAL-catalyzed reactions; Relative activities of the *La*AAL and other well characterized PALs towards the natural substrates L-Phe, L-Tyr, L-His and model substrate 3,4-dimethoxy L-Phe.

4.1.2. The effect of pH on the *La*AAL-catalyzed ammonia elimination

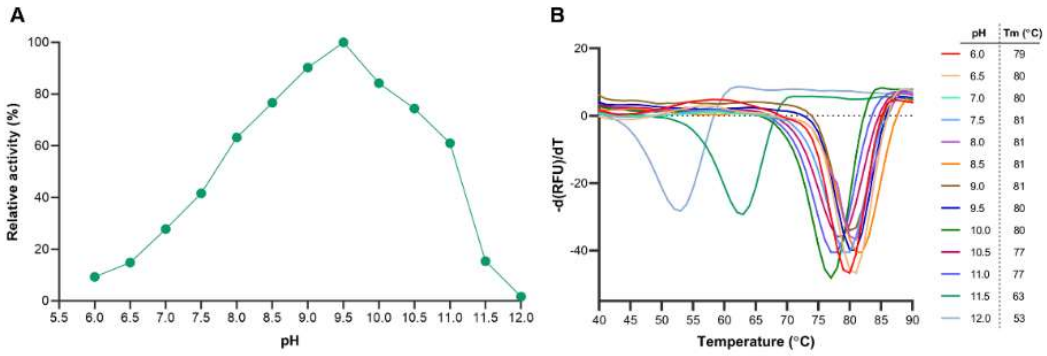


Figure 14. The pH optimum of the *La*AAL-mediated ammonia elimination reaction from **2ad**: (**A**) Relative initial velocities of the *La*AAL-catalyzed ammonia elimination reaction in buffers of different pH; (**B**) Melting curves and melting temperatures (T_M) of *La*AAL in the tested buffers of different pH (in all cases standard deviations of duplicate measurements were < 0.4 °C)

The recombinantly expressed and purified *La*AAL exhibited optimal activity for the ammonia elimination reaction from 3,4-dimethoxy-L-phenylalanine (**2ad**) at pH 9.5 (**Figure 14A**). This optimal pH is comparable to that of some recently identified TALs.[67,68] is higher than the pH optima for most plant[48,52,69]-derived and bacterial[70] PALs, but lower than the extreme pH optimum of thermophilic *Rx*PAL[35]. Additionally, *La*AAL maintains its protein fold and thermal stability across a broad pH range (6.5 to 11.0), with significant denaturation and reduced activity occurring only at pH values above 11.5 (**Figure 14B**).

4.1.3. The effect of ammonia source

*La*AAL exhibits optimal activity for ammonia addition to 3,4-dimethoxy *trans*-cinnamic acid (**1ad**) at pH 9.0 in 4 M NH_4OH, with significant activity decrease at higher pH levels, particularly above pH 10.0 (**Figure 15A**). In contrast, *La*AAL demonstrates superior stability and higher activity in 4 M ammonium carbamate at pH 9.5 compared to 4 M NH_4OH.

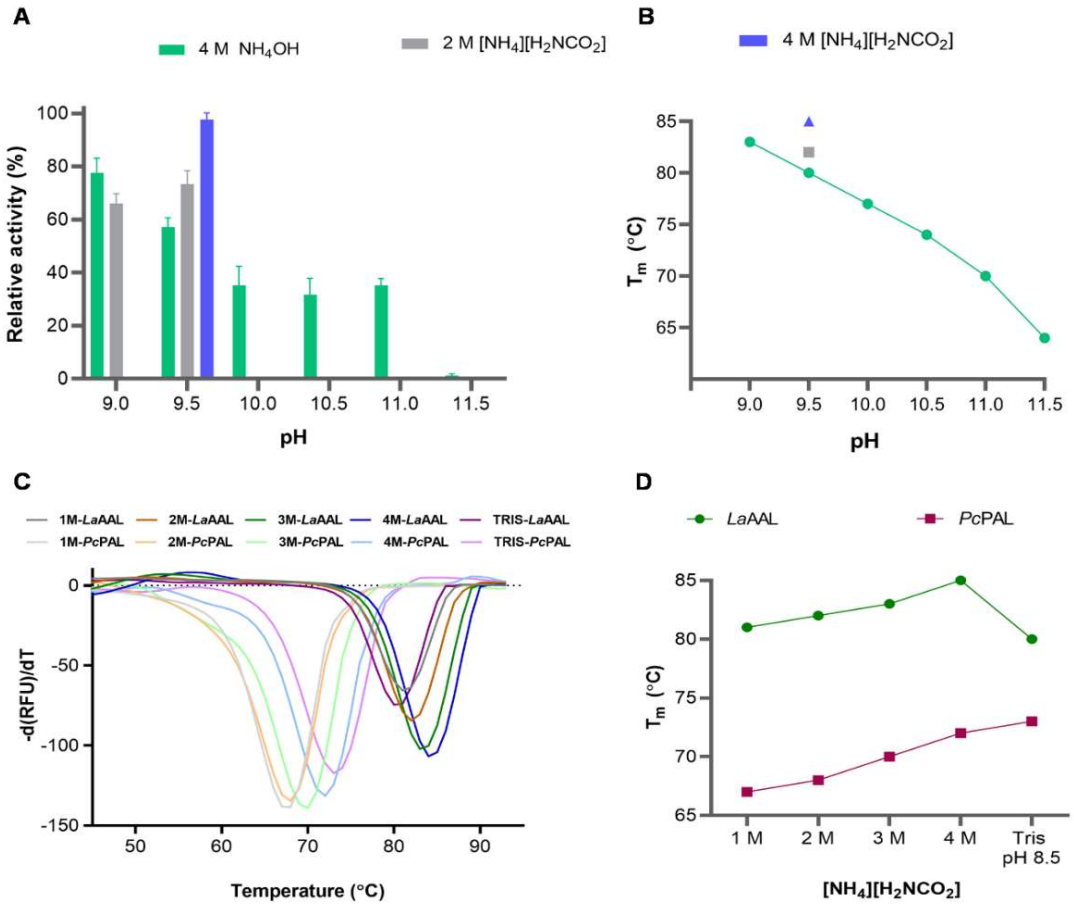


Figure 15. Determination of the pH optimum of the *La*AAL-mediated ammonia addition reaction of **2d**: (**A**) Relative initial activities for the ammonia addition reactions performed in 4 M ammonium hydroxide of different pH and ammonium carbamate of unadjusted pH (~9.5) and of pH 9.0. (**B**) The melting temperature (T_m) of *La*AAL in the ammonia-buffers of different pH. (**C**) Thermal unfolding profiles of *La*AAL and *Pc*PAL in ammonium carbamate 1 M – 4 M of unadjusted pH

of ~9.5 in comparison with the one registered in 20 mM Tris.HCl buffer of pH 8.5. (**D**) Variation of melting temperature (T_m) of *La*AAL and *Pc*PAL in ammonium-carbamate buffers (1 M–4 M) and Tris-buffer, pH 8.5. All standard deviations for the T_m values determined in duplicate were < 0.3 °C.

4.1.4. The effect of temperature on the activity of *La*AAL

The evaluation of *La*AAL's thermostability and activity revealed that while the enzyme's activity increases with temperature up to 65°C in the ammonia elimination reaction (**Figure 16A**), its operational stability is crucial for sustained synthetic applications, especially when targeting the reverse ammonia addition reaction. *La*AAL maintains around 80% of its initial activity after 4 hours of incubation in 4 M ammonium carbamate, at temperatures below 45°C, with a $T_{50}^{240\ min}$ of 55.6°C (**Figure 16B**). However, higher temperatures lead to rapid activity loss, making 30°C the optimal temperature for achieving high conversion rates in ammonia addition reactions (**Figure 16C**). This suggests that although *La*AAL is more active at slightly higher temperatures, 30°C provides the best balance between enzyme stability and efficiency for synthetic applications.

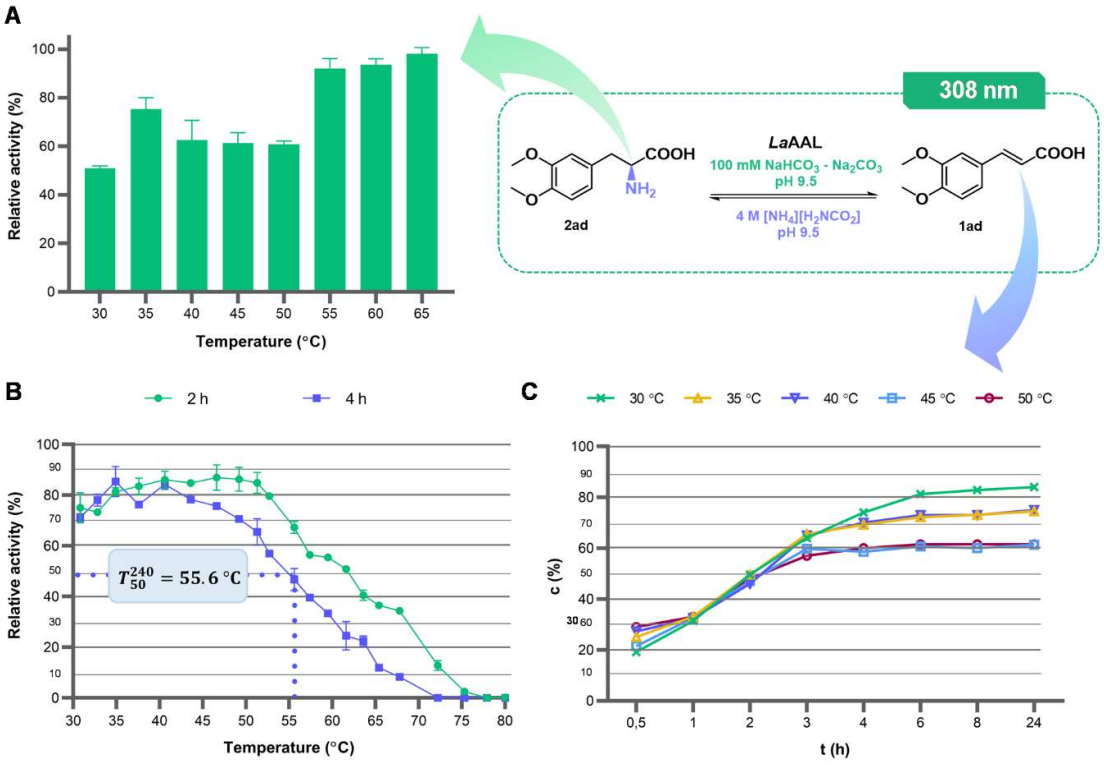


Figure 16. The effect of temperature on the activity and operational stability of LaAAL: (**A**) relative activities for the ammonia elimination of 3,4-dimethoxy-L-Phe **2ad** performed at various temperatures with no prior incubation period; (**B**) residual activity of LaAAL after 2 h– and 4 h–incubation in 4 M ammonium-carbamate at different temperatures ranging from 30-80 °C; (**C**) the time conversion-profiles of the *La*AAL-mediated ammonia addition reactions of **1ad**, performed at temperatures of 30, 35, 40, 45, 50 °C in 4 M ammonium-carbamate.

4.1.5. Substrate scope of *La*AAL in the ammonia addition reaction

The catalytic versatility of *La*AAL in the ammonia addition reaction shows significant variability based on the nature and position of substituents on cinnamic acid substrates. Generally, substrates with electron-donating groups, particularly in the *para*-position, exhibit lower conversion rates, indicating that such substituents negatively impact the enzyme's catalytic efficiency. Conversely, substrates with electron-withdrawing groups tend to show higher conversions, especially when multiple substitutions are present, although no clear correlation between the electronic effects of substituents and catalytic efficiency was observed (**Figure 17**).

The steric effects of substituents were rationalized for some substrates, with certain *ortho*- and *meta*-substituted cinnamic acids showing favourable interactions within the active site, leading to higher conversions. However, the influence of electronic properties remains less understood, highlighting the need for advanced modelling approaches to fully elucidate the underlying mechanisms.

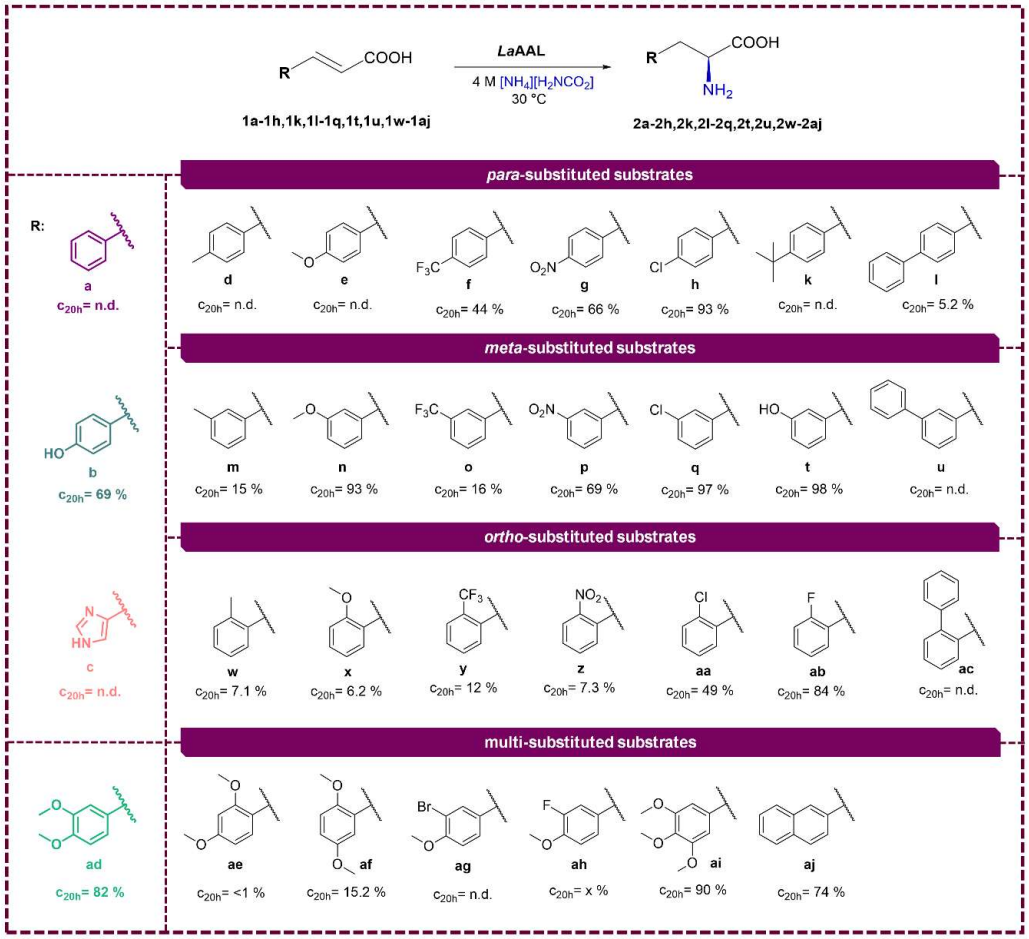


Figure 17. The substrate library evaluated within the *La*AAL-catalyzed ammonia addition.

Moreover, the kinetic data suggest that the ammonia elimination route is the most favoured reaction route by *La*AAL, exhibiting higher turnover rates and specificity constants compared to the ammonia addition reaction, as observed for 3,4-dimethoxy cinnamic acid (**1ad**) (**Table 1**). This preference aligns with observations in other aromatic ammonia-lyases (AALs), further complicating efforts to define the natural substrate of *La*AAL. Among the tested substrates, *La*AAL demonstrated the highest specificity for 3-methoxy-L-phenylalanine (**2n**) and L-2-naphthylalanine (**2aj**), with specificity constants (k_{cat}/K_M) much higher than for substrates like L-phenylalanine. Specifically, *La*AAL shows higher specificity for L-Tyr compared to L-Phe and demonstrates significant activity in ammonia addition reactions with *p*-coumaric acid while being inactive towards *trans*-cinnamic acid and L-His.

Table 1. Enzyme kinetic parameters obtained in the ammonia elimination reaction catalyzed by *La*AAL

Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1} \times mM^{-1}$)
L-Phe **2a**	5.88 ± 0.55	0.51 ± 0.01	0.09
L-*m*-Tyr **2t**	0.29 ± 0.03	0.63 ± 0.01	2.14
3-MeO-L-Phe **2n**	0.27 ± 0.03	1.72 ± 0.04	6.42
3,4-MeO-*t*-CA **1ad**	4.75 ± 0.80	0.81 ± 0.09	0.17
3,4-MeO-L-Phe **2ad**	1.11 ± 0.08	1.14 ± 0.03	1.02
3,4,5-MeO-L-Phe **2ai**	2.59 ± 0.22	0.77 ± 0.01	0.30
3-F-,4-MeO-L-Phe **2ah**	6.26 ± 0.78	0.14 ± 0.02	0.02
L-2-naphtylalanine **2aj**	–	–	6.43
L-Tyr **2b**	–	–	3.62

4.1.6. Classification of *La*AAL

The characterization of the substrate specificities of *La*AAL together with the kinetic data suggest that *La*AAL is likely to belong to the TAL (E.C. 4.3.1.23) subclass of aromatic ammonia-lyases. However, *La*AAL's unique substrate scope and catalytic fingerprint, especially its high specificity for *meta*-substituted substrates, indicate that it might have evolved to accommodate substrates beyond those typically catalyzed by known TALs.

The distinct catalytic site residues and the broader substrate versatility suggest that *La*AAL, along with newly AALs, identified through homologue mining and sharing diverse catalytic fingerprints (**Table 2**), may represent a distinct evolutionary branch of ammonia-lyases with potential novel functionalities. These characteristics, along with the distribution of AALs in bacteria from marine and symbiotic environments hints at a specialized role for *La*AAL and related enzymes, potentially contributing to unique metabolic pathways and offering new insights into biocatalysis and enzyme evolution. Notably, the bacterial-derived AALs cluster within the same clade as the bacterial-origin PALs. This clustering indicates a closer evolutionary relationship to the cyanobacterial PALs, specifically *Av*PAL and *Np*PAL, which suggests that these AALs are more likely to have evolved from bacterial PALs rather than from bacterial-origin TALs, such as *Rc*TAL, of which clade is distal (**Figure 18**).

Table 1. Multiple sequence alignment of the catalytic site residues of *La*AAL with homologous AALs. Conserved residues are shown in bold, while distinct catalytic fingerprints are highlighted in various colours. MIO-forming residues are highlighted with pink background, and residues distinct in the hydrophilic region are highlighted in grey. Microorganisms from the Proteobacteria phylum are highlighted in green, and those from the Actinobacteria phylum are highlighted in yellow.

NCBI acces ID	Origin	Identity (%)								
*La*AAL			55	81 85	144 148	196 199	288 291 294	324	396 400	425 429
WP_178352722	*Loktanella atrilutea*	100	.Y.	.ALWQH.	.ASGDL.	.LALN.	.QDRYSLR.	.N.	.KSLHI.	.EMYNQ.
QOV09174.1	uncultured bacterium	97.45	.Y.	.ALWQH.	.ASGDL.	.LALN.	.QDRYGIR.	.N.	.KSLHI.	.EMYNQ.
WP_212536965	*Thetidibacter halocola*	85.40	.Y.	.ALWQH.	.ASGDL.	.LALN.	.QDRYGIR.	.N.	.KSLHI.	.EMYNQ.
WP_163479703	*Geodermatophilus sabuli*	60.59	.Y.	.ALWQH.	.ASGDL.	.LALN.	.QDRYSLR.	.N.	.KSLQI.	.EMYNQ.
MBL0935101	*Rhizobiaceae*	67.61	.Y.	.AVWQH.	.ASGDL.	.LALN.	.QDRYSLR.	.N.	.KSLQI.	.EQFNQ.
WP_051375809	*Aliihoeflea sp. 2WW*	67.65	.Y.	.AVWQH.	.ASGDL.	.LALN.	.QDRYSLR.	.N.	.KSLQI.	.EQFNQ.
WP_311507619	*Novosphingobium sp.MMS21-SN21R*	65.25	.Y.	.ALWQH.	.ASGDL.	.LALN.	.QDRYSLR.	.N.	.KSLQI.	.EQFNQ.
HXR88911	*Steroidobacteraceae*	64.27	.Y.	.ALWQH.	.ASGDL.	.LALN.	.QDRYSLR.	.N.	.KSLQL.	.EQFNQ.
MCI5076028	*Oncola*	73.08	.Y.	.ALWQH.	.ASGDL.	.LALN.	.QDRYGLR.	.N.	.KSLHV.	.EMYNQ.
MCG8858434	*Salaquimonas*	70.44	.Y.	.ALWQH.	.ASGDL.	.LALN.	.QDRYGLR.	.N.	.KSLHV.	.EMYNQ.
WP_185801870	*Parasphingopyxis sp. GrpM-11*	60.07	.Y.	.ALWQH.	.ASGDL.	.LALN.	.QDRYSLR.	.N.	.KSLQV.	.EQFNQ.
PZN81218	*Pseudomonadota*	63.12	.Y.	.AVWQH.	.ASGDL.	.LALN.	.QDRYSLR.	.N.	.KSLQV.	.EQFNQ.
MBO6716438	*Rhizobiaceae*	64.58	.Y.	.AMWHH.	.ASGDL.	.LALN.	.QDRYSLR.	.N.	.KSLQI.	.EQFNQ.
MCG8115566	*Mesorhizobium*	65.95	.Y.	.AMWHH.	.ASGDL.	.LALN.	.QDRYSLR.	.N.	.KSLQI.	.DQFNQ.
WP_169285622	Chelativorans sp. ZYF759	65.75	.Y.	.AMWHH.	.ASGDL.	.LALN.	.QDRYSLR.	.N.	.KSLQI.	.DQFNQ.
MCB1344270	*Paracoccaceae*	61.02	.Y.	.ALWHH.	.ASGDL.	.LALN.	.QDRYSLR.	.N.	.KSLQL.	.EQFNQ.
WP_068297293	*Pararhodobacter*	56.41	.Y.	.ALWHH.	.ASGDL.	.LALN.	.QDRYSLR.	.N.	.KSLQL.	.EQFNQ.
WP_225294365	*Cereibacter sphaeroides*	56.04	.Y.	.ALWHH.	.ASGDL.	.LALN.	.QDRYSLR.	.N.	.KSLQL.	.EQFNQ.
PVE48351	*Pararhodobacter aggregans*	56.00	.Y.	.ALWHH.	.ASGDL.	.LALN.	.QDRYSLR.	.N.	.KSLQL.	.EQFNQ.
MBV9883721	*Sphingomonadaceae*	66.30	.Y.	.ALWHH.	.ASGDL.	.LALN.	.QDRYSLR.	.N.	.KSLQL.	.EQFNQ.
WP_104045095	Arthrobacter sp. ZGTC41	61.70	.Y.	.ALWTH.	.ASGDL.	.LALN.	.QDRYGIR.	.N.	.KSLGV.	.EQYNQ.
MBV8104808	*Hyphomicrobiales*	64.29	.Y.	.SLWHH.	.ASGDL.	.LALN.	.QDRYSLR.	.N.	.KSLQI.	.EQFNQ.
KAB2913557	*Hyphomicrobiales*	62.73	.Y.	.SLWHH.	.ASGDL.	.LALN.	.QDRYSLR.	.N.	.KSLQL.	.EQFNQ.
MBZ0140866	*Pseudorhodoplanes*	62.55	.Y.	.SLWHH.	.ASGDL.	.LALN.	.QDRYSLR.	.N.	.KSLQL.	.EQFNQ.

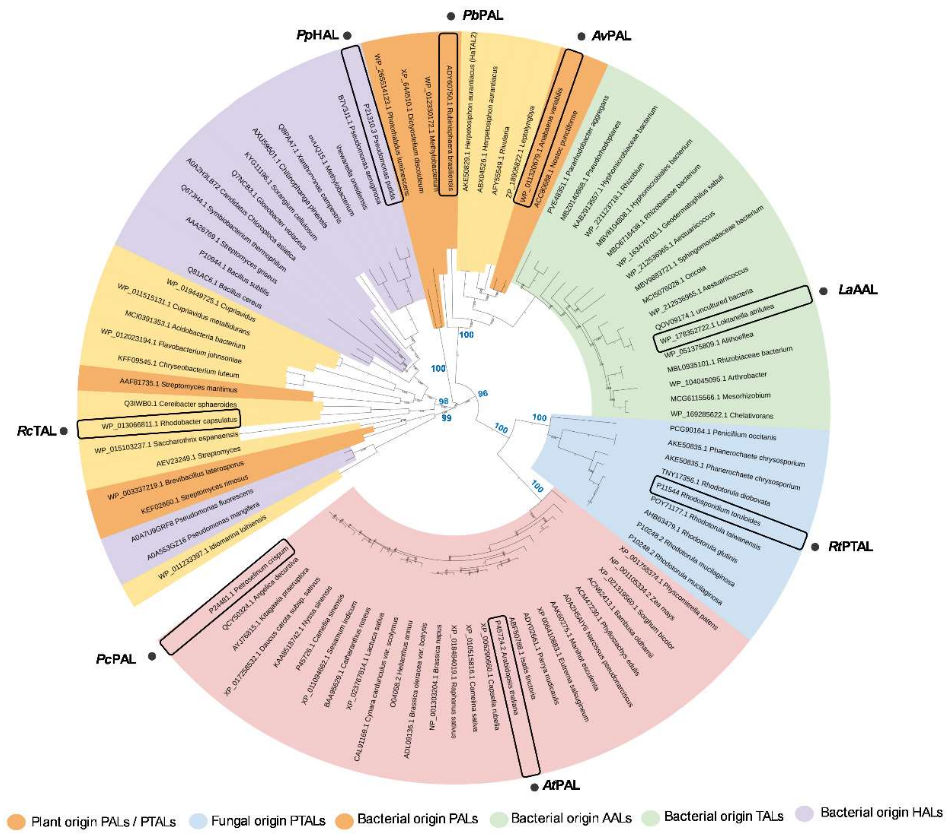


Figure 18. Dendrogram for the phylogenetic analysis of different representatives of PALs, PTALs, TALs, HALs and the putative AALs. Bootstrap values for the main branches are coloured in blue and were zoomed out for clarity.

Nevertheless, further studies, including gene knockout and metabolomic profiling, are necessary to fully understand *La*AAL's native substrate and biological role.

4.2. Conclusions

In this study, we characterized the substrate scope and biocatalytic potential of the aromatic ammonia-lyase from *Loktanella atrilutea* (*La*AAL). The enzyme demonstrated reduced activity towards canonical AAL substrates such as L-Phe, L-Tyr, and L-His, while showing significant efficacy with 3,4-dimethoxy-L-phenylalanine. Using 3,4-dimethoxy *trans*-cinnamic acid as model substrate *La*AAL exhibited optimal activity at a higher pH of 9.5 in 4 M ammonium carbamate. Based on the conversion values and the operational stability of *La*AAL during the several hours reaction time, 30 °C was found as the optimal reaction temperature. The substrate scope analysis highlighted the catalytic adaptability of *La*AAL in the hydroamination of diverse cinnamic acids, especially for *meta*-substituted and/or di-/multi-substituted analogues, with structural modelling exposing steric clashes between the substrates *ortho*-substituted and catalytic site residues. The kinetic data revealed a predilection of *La*AAL for the ammonia elimination, while supporting its classification as a tyrosine ammonia-lyase (TAL, E.C. 4.3.1.23), among the currently existing aromatic ammonia-lyase family. However, phylogenetic analysis suggests *La*AAL evolved from bacterial PALs, and its distinct substrate specificity and genomic context point to a novel functional role, potentially expanding the known diversity of AALs.

5. GENERAL CONSLUSIONS

This thesis makes significant contributions to the field of phenylalanine ammonia-lyases (PALs) by developing a comprehensive protein engineering toolkit, which includes both rational design and saturation mutagenesis techniques, along with high-throughput assays for screening enzyme libraries. Key outcomes include the identification of novel PAL homologues with enhanced activity against challenging substrates, highlighting the potential for further biocatalytic applications.

One of the major achievements was the development of high-throughput screening methods, including a liquid-phase UV assay for ammonia elimination and a solid-phase PAL-AncLAAO-coupled assay targeting the ammonia addition reaction, which proved effective in identifying PAL variants with improved activity. The existence of a high-throughput assays was critical for exploring and optimizing the activity of PALs against challenging di-substituted substrates, such as 3-bromo-4-methoxy-L-phenylalanine and 3,4-dimethoxy-L-phenylalanine.

Engineering efforts using both saturation mutagenesis and rational design revealed specific mutations in PALs from *Petroselinum crispum* (*Pc*PAL), *Arabidopsis thaliana* (*At*PAL), and *Anabaena variabilis* (*Av*PAL), which enhanced their activity for the two di-substituted substrates, though with some limitations. The engineered PALs showed improved, but still limited, catalytic efficiency, suggesting the substitution pattern of key active site residues in PALs is strongly constrained.

The identification and characterization of the aromatic ammonia-lyase from *Loktanella atrilutea* (*La*AAL), revealed its unique biocatalytic potential, especially for substrates with electron-donating groups, such as 3,4-dimethoxy-L-phenylalanine and 3,4-dimethoxy *trans*-cinnamic acid, *La*AAL outperforming engineered PALs in the ammonia addition to the synthetically valuable 3,4-dimethoxy *trans*-cinnamic acid,

Despite it functional characteristics suggest *La*AAL might belong to the subclass of TALs, phylogenetic analysis positioned *La*AAL and its homologues closer to bacterial PALs, suggesting an evolutionary link to cyanobacterial PALs rather than TALs. The distinct attributes of *La*AAL, including its broad substrate scope, unique genomic clustering and diverse homologues, indicate a potential for discovering novel AALs with functions beyond current classifications.

In conclusion, this research advances the understanding of PALs and related AALs, providing new tools for protein engineering and paving the way for the discovery of novel biocatalysts with enhanced capabilities. The work highlights both the possibilities and limitations of current engineering approaches, setting the stage for future innovations in the field.

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