BABEŞ-BOLYAI UNIVERSITY
CLUJ-NAPOCA

Faculty of Chemistry and Chemical Engineering
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Phenylalanine ammonia-lyases and 2,3-aminomutases in biocatalytic stereoselective reactions

PhD Thesis Abstract

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List of Contents

Chapter I. General Introduction

The use of enzymes as biocatalysts for the preparation of novel compounds has received steadily increasing attention over the past few years and found increasing application in many areas, especially in the synthesis of pharmaceutical and fine chemical targets.[1]

The major advantage of using enzymes (isolated enzyme or whole-cell) in biocatalytic transformations are their chemo-, regio- and stereospecificity, as well as the mild reaction conditions that can be used.[2]

Enzymes are compatible with each other; several biocatalytic reactions can be carried out in a reaction cascade in a single flask. Thus, in order to simplify reaction processes, sequential reactions are feasible by using multienzyme systems.[3] Like all catalysts, enzymes work by lowering the activation energy of a reaction, thus dramatically increasing the rate of the reaction. The enzymatic reaction's rates are millions of times faster than comparable non-enzymatic reactions rates.[4]

Enzymes, being promiscuous with respect to the substrates accepted or to the catalyzed reaction type, are very interesting biocatalysts due to their capability to convert a wide range of substrates.[5]

Biocatalysis is becoming more and more the method of choice for the preparation of some chiral molecules in the chemical and pharmaceutical industry. Enantiomerically pure amino acids, amino alcohols, amines and alcohols are particularly important classes of compounds for the synthesis of many active pharmaceutical and agrochemical products.
Moreover, optically active α-amino and β-amino acids are fundamental building blocks for the synthesis of a wide range of bioactive natural products, including drugs. A very attractive enzymatic route~~s~~ using ammonia-lyases (AL) and 2,3-aminomutases (AM) has been explored for the synthesis of enantiomerically pure, non-natural α- and β-amino acids.

This thesis describes the isolation, characterization and application of phenylalanine ammonia-lyases (PALs) and phenylalanine 2,3-aminomutases (PAMs) as biocatalysts in stereoselective reactions.

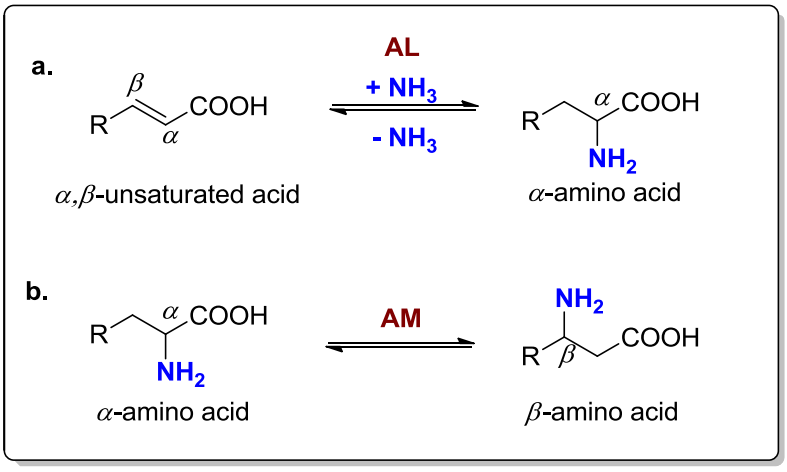
Literature Review

I.1. Ammonia-lyases

Lyases (EC 4) are defined as enzymes that catalyze the breaking of various chemical bonds (C–C, C–O, C–N) by other means than hydrolysis and oxidation, usually forming a double bond or adding groups to double bonds. Lyases utilize a range of prosthetic groups which include coenzyme B-12, dipyrromethane cofactor, pyridoxal-phosphate or the 3,5-dihydro-5-methylidene-4*H*-imidazol-4-one (MIO) prosthetic groups.[6]

Aromatic amino acid ammonia-lyases catalyze the reversible non-oxidative deamination of *α*-amino acids, yielding *trans*-*α*-, *β*-unsaturated acids and ammonia (**Scheme 1a**).[7]

In some cases elimination is followed immediately by addition and hence the overall of the reaction is apparently a substitution. If there is evidence of an unsaturated intermediate, the enzyme is nevertheless classified as a lyase. The intramolecular lyases are a small group of enzymes, classified as isomerases (EC 5), including aminomutases.[8] Aminomutases catalyze the isomerisation of *α*-amino acids to *β*-amino acids (**Scheme 1.b**).



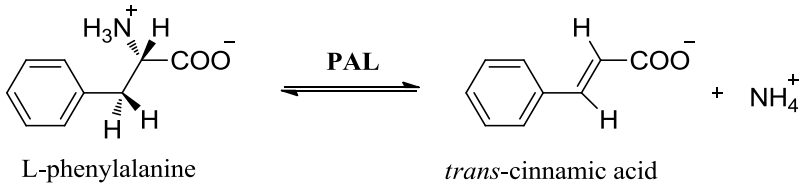
Scheme 1. a. Ammonia-lyase catalyzed reaction. **b.** 2,3-aminomutase catalyzed reaction.

AMs and ALs are members of the class I lyase-like family, that includes tyrosine ammonia-lyases (TAL)[9], phenylalanine ammonia-lyase (PAL), histidine ammonia-lyases (HAL)[10], tyrosine 2,3-aminomutases (TAM)[11], phenylalanine and 2,3-aminonutase (PAM) (**Scheme 2.**). All these enzymes rely on a protein-derived cofactor: the 3,5-dihydro-5-

methylidene-4*H*-imidazol-4-one (MIO), generated autocatalytically from three active site residues: Ala/Thr-Ser-Gly, forming a MIO signature motif.[12]

I. 1.1. Phenylalanine ammonia-lyase (PAL)

Phenylalanine ammonia-lyase catalyze the non-oxidative elimination of ammonia from L-phenylalanine (**Scheme 3.**) to yield *trans*-cinnamic acid.



Scheme 3. PAL catalyzed deamination of L-phenylalanine

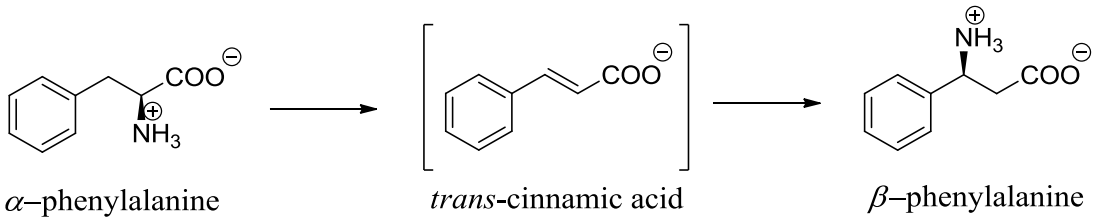
PALs are ubiquitous in plants and represent the first enzyme in the phenylpropanoid biosynthetic pathway. *Trans*-cinnamic acid is an important intermediate in the biosynthesis of various phenylpropanoids such as: lignin, flavonoids and coumarins.[13]

PALs are also commonly found in fungi, but only a few examples of prokaryotic PALs are known. The rarity of PAL in bacteria can be explained by the fact that phenylpropanoids rarely occur in these organisms.

I. 1.2. Phenylalanine 2,3-aminomutase (PAM)

The 2,3-aminomutases differ from the ammonia-lyases: they shuffle the α-amino group to the β position. As they are closely related in structure, active site and sequence, it is likely that the same mechanism guides the reactions catalyzed by this enzymes class.[14]

Phenylalanine 2,3-aminomutases (PAMs) remove the amino group from naturally occurring α-phenylalanine to produce β-phenylalanine via *trans*-cinnanic intermediary (**Scheme 5.**).[15]



Scheme 5. PAM catalyzed isomerization reaction of α-phenylalanie to β-phenylalanine

This transformation has important industrial applications, in the production of chiral phenylalanine derivatives,[16] as well as in the production of pharmaceutical product such as the anticancer drug Taxol[17] and the antibiotic Andrimid[18], which is produced by the pathogenic Gram-negative bacteria *Pantoea agglomerans*.[19]

I. 2. Structure of phenylalanine ammonia-lyase and phenylalanine 2,3-aminomutase

PALs are homotetrameric proteins consisting of four identical monomer subunits.[20] The four subunits are tightly interconnected and organized around an up-down bundle.[21] The homotetramer contains four active sites, with three distinct monomers participating in formation of each catalytic site.[20]

Ammonia-lyases are predominantly α-helical proteins; each monomer should be subdivided into four domains. The first one is the catalytic MIO domain, containing the prosthetic MIO group. The amino acids sequence which codifies this prosthetic group is strictly conserved. The second domain is the core domain and the third one is known as the "shielding-domain". These domains are connected with mobile loops. Each MIO-cofactor is anchored to each monomer by non-covalent bonding to three central α-helices, which are coiled together and form a rigid central core and a funnel into the active site.20

Structural studies on PALs showed a characteristic difference between prokaryotic[22] and eukaryotic PAL20 (and also in PAM) enzymes. In plants and also in yeasts PALs is an approximately 120-residue long *C*-terminal multi-helix domain (shielding domain), which is absent in bacterial PALs.

PAMs have similar construction to the phenylalanine ammonia-lyases, each subunit contains an active site. At the end of this bundle is located the active site, which resides at the interfaces between three of the monomers in the tetramer, and includes residues from all three monomers.

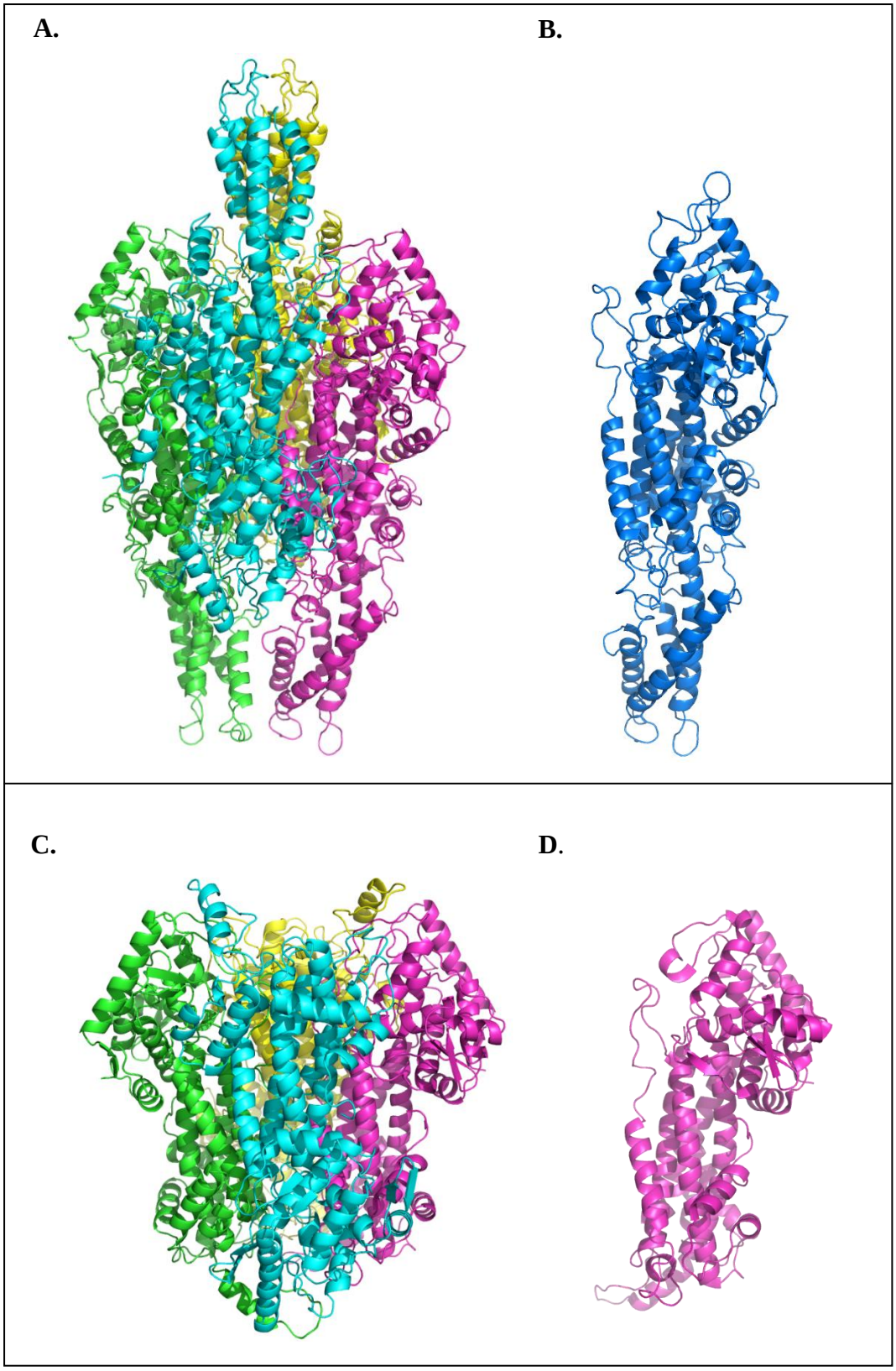


Figure 1. Structure of phenylalanine ammonia-lyase from *Petroselinum crispum* (*Pc*PAL) and phenylalanine 2,3-aminomutase from *Pantoea agglomerans* (*Pa*PAM). **A**. tetramer structure of *Pc*PAL, **B.** monomer structure of *Pc*PAL, **C**. tetramer structure of *Pa*PAM, **D.** monomer structure of *Pa*PAM

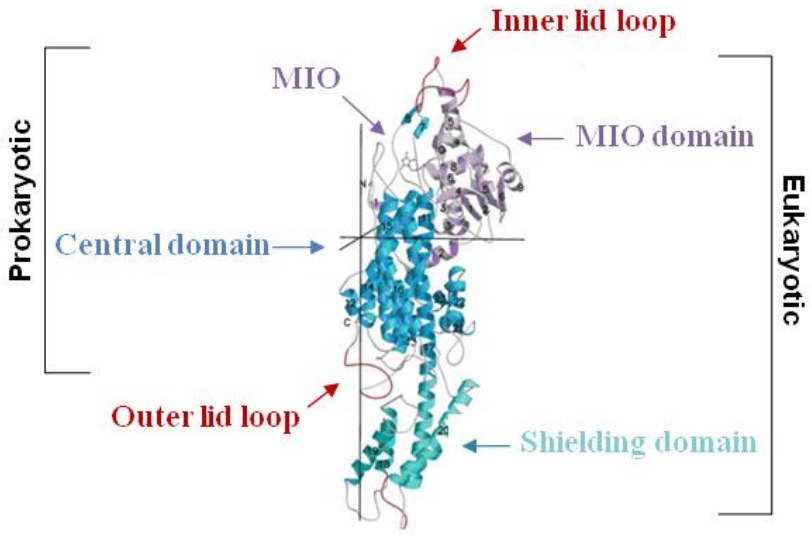
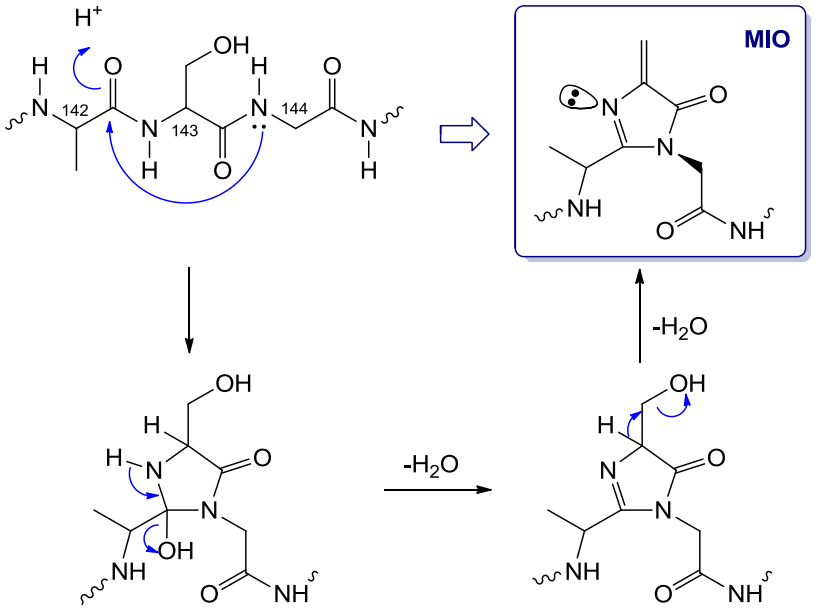


Figure 2. PAL monomer structure. Eukaryotic vs. prokaryotic structure with the three domain: **dark blue** - central domain, **light blue** – Shielding domain, **purple**- MIO domain and **red** - mobile loops (Inner lid loop and Outer lid loop)

I. 2.1. MIO electrophilic group

The availability of the first crystal structure of histidine ammonia-lyase[14] from *Pseudomonas putida* established that ammonia-lyases and 2,3-aminomutases possess an unusual modified active site cofactor named 3,5-dihydro-5-methylidene-4*H*-imidazol-4-one (MIO), an electrophilic group.[14]

Firstly, it was suggested by Hanson and Havir (1970), that the electrophile is produced by a posttranslational dehydration of a Ser, forming a dehydroalanine.[23] One of the most extensively characterized ammonia-lyases is from the bacteria *Pseudomonas putida* (*Pp*HAL); the MIO-cofactor was first identified from the X-ray crystal structure of this enzyme, solved in 1999 by Schwede *et al.*, revealing that not dehydroalanine, but a prosthetic 3,5-dihydro-5-methylidene-4*H*-imidazol-4-one (MIO) is the catalytically essential electrophilic group.[14] MIO is formed by autocatalytic cyclization and dehydration of an internal Ala-Ser-Gly tripeptid, present within the active site of the enzyme[24] (**Scheme 6**), a strictly conserved motif, acting as electrophile in the reaction mechanism, found in all ammonia-lyases and 2,3-aminomutases identified to date.[25] In case of PAM from *Pantoea agglomerans* as well as from *Streptomyces maritimus* MIO is formed by Thr-Ser-Gly, Ala being replaced by Thr.[26]

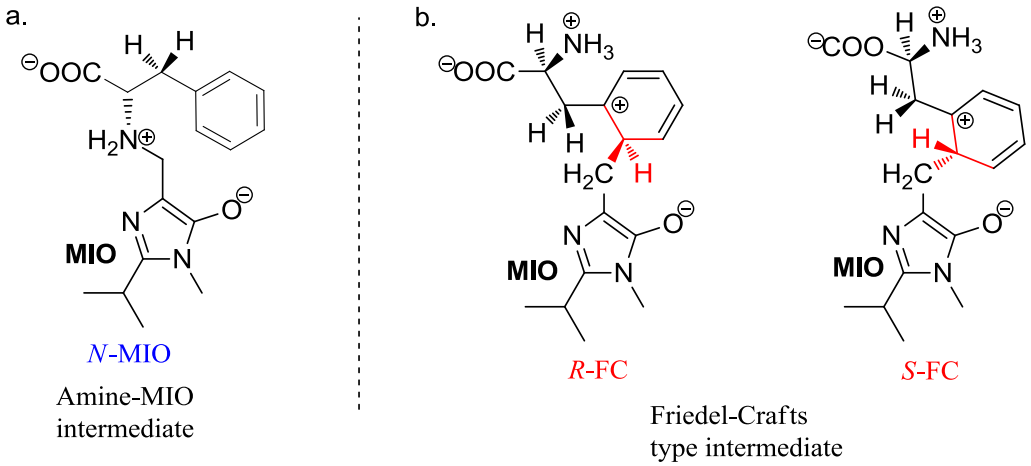


Scheme 6. Formation of MIO electrophilic group in *Pp*HAL

I. 3. Reaction mechanism

The reaction catalyzed by phenylalanine ammonia-lyases and 2,3-aminomutases represents a mechanistic challenge, regarding how ammonia is eliminated from the aromatic amino acids. It is still not clearly understood the enzymatic reaction mechanism**.**

Two debated mechanisms have been proposed for the MIO-catalyzed elimination of ammonia observed in the ammonia-lyase family.[27]



Scheme 7. Two proposed intermediates in the PAL catalyzed L-phenylalanine deamination reaction. **a.** *N*-MIO intermediate (Hanson – Havir),[23] **b.** Friedel-Crafts type intermediate (Rétey)[28]

The first one is based on an *N*-MIO cofactor intermediate in the elimination pathway and the second on a Friedel-Crafts type reaction. In both proposed mechanisms, MIO forms a covalent intermediate with the phenylalanine substrate, which is subsequently deprotonated at the β-position.

I. 4. Application of PAL and PAM

The product of the PAL reaction is *trans*-cinnamate, the key precursor of the phenylpropanoids such as lignin, flavonoids, and coumarins. Thus PAL lies at the branch point of primary and secondary metabolism of plants and its importance makes it a *target for herbicides.*

Phenylalanine ammonia-lyase has also potential *therapeutic application* as PAL can convert L-phenylalanine to *trans*-cinnamic acid, which is a harmless metabolite and it does not require a cofactor; it is therefore considered as a candidate for phenylketonuria treatment.[29]

In comparison with HAL, PAL tolerates a diverse selection of substrates. Therefore PALs represent potentially attractive *biocatalysts* for the synthesis of enantiomerically pure non-natural L-amino acids from substituted cinnamic acid analogues and ammonia. PALs have the advantage to use readily available prochiral cinnamic acid derivatives as starting materials, and they do not rely on any external cofactors or recycling systems, making them particularly suitable as industrial biocatalysts.

The aims of this study

The aims of this study are as follows:

1. Expression and characterization of the highly alkalophilic phenylalanine ammonia-lyase from *Rubrobacter xylanophilus* (*Rx*PAL)

In this study, the identification of a novel PAL from thermotolerant bacterium *Rubrobacter xylanophilus* (*Rx*PAL), cloning the synthetic gene and characterization of the novel enzyme are described.

2. Development of biocatalytic synthesis mediated by phenylalanine ammonia-lyase and 2,3-aminomutase

- **Investigation of the stereoselectivity of *Pc*PAL under unconventional conditions** - Several factors that can influence the enantioselective synthesis of *(ortho-, meta-, para-*)-nitro-phenylalanine mediated by whole cells as well by purified MIO-containing and inactive MIO-containing *Pc*PALs were analyzed. First, the behavior of the enzymes on the ammonia concentration was investigated. The influence of the pH on the *Pc*PAL catalyzed biotransformations was also inspected.

- **A novel method for the immobilization of *Pc*PAL – Bis-epoxide Cross-Linked Enzyme Aggregates (CLEA)** - The usefulness of glycerol diglycidyl ether (GDE) as a convenient bis-epoxy-type cross-linker in the preparation of CLEAs and co-CLEAs of phenylalanine ammonia-lyase from *Petroselinum crispum*, which improves the characteristics of the biocatalysts was investigated

- ***Pa*PAM catalyzed biotransformation** – The catalytic activity of phenylalanine 2,3-aminomutase from *Pantoea agglomerans* (*Pa*PAM) towards various racemic α- and β-arylalanines were examined. Effects of the nature and the position of aromatic substituents on the reaction equilibrium were also discussed.

Chapter II. Expression and characterization of phenylalanine ammonia-lyase from thermophilic *Rubrobacter xylanophilus* (*Rx*PAL)

II. 1. Introduction

As described in the literature review, even if PALs are frequently found in plants and in fungi, where they have an essential role in forming phenylpropanoids, the occurrence of the bacterial PAL is very rare. The rarity of PAL in bacteria may be explained by the fact that phenylpropanoids are not essential for bacteria.

This chapter describes the identification and characterization of a novel PAL from thermotolerant bacterium *Rubrobacter xylanophilus* (*Rx*PAL). Therefore, the synthetic gene was cloned and the encoded *Rx*PAL enzyme was characterized at different pH values; a point mutation was also constructed within the characteristic ASG tripeptide involved in the formation of the MIO moiety.

II. 2. Results and discussion

II. 2.1. Identification, expression and purification of *Rx*PAL

To the best of our knowledge, PAL enzyme has not been yet characterized from the Gram-positive, thermophilic and radiotolerant *Rubrobacter xylanophilus* strain. After identifying the supposed PAL-coding gene in *Rubrobacter xylanophilus* by screening the genomes of bacteria for members of the ammonia-lyase family online using the BLAST and Clustal W programs, this gene was synthesized for *Escherichia coli* host strains with an optimized codon usage.

The recombinant *Rx*PAL was overexpressed as an *N*-terminal His_6-tagged protein in a *E. coli* TOP 10 strain.

The expression and the purification of *Rx*PAL from *Rubrobacter xylanophilus* was successfully performed; the obtained purified enzyme showed a high degree of electrophoretic purity as shown in SDS-PAGE.

II. 2.2. The pH optimum of the *Rx*PAL

The enzymatic activity of *Rx*PAL was spectrophotometrically monitored, at 290 nm by detecting the formation of the *trans*-cinnamic acid product, starting from its natural substrate, L-phenylalanine. First, the optimal pH of the enzyme was identified. The measurements were performed in different buffer solutions, in the pH range of 6.5-12, maintaining the ionic strength and the temperature constant.

The rate of the *Rx*PAL catalyzed reaction slowly increased up to pH 8.5. Between 8.5 and 11.5 enzyme activity rapidly increased, reaching a maximum at pH 11.5 (**Figure 3**); after 11.5 the activity abruptly dropped, probably due to protein denaturation.

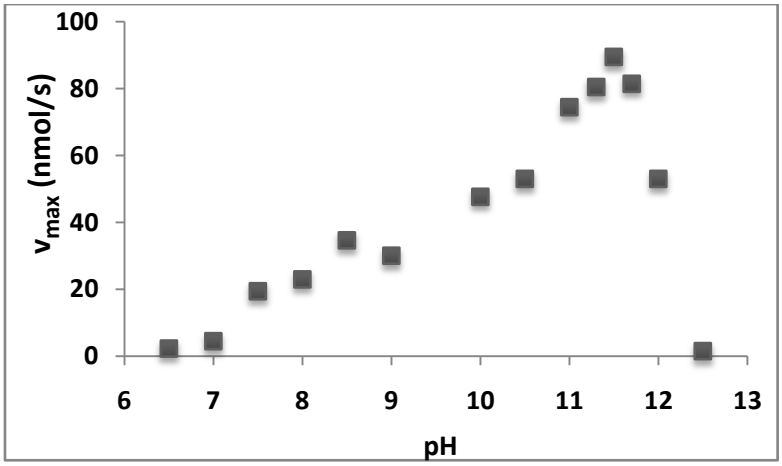


Figure 3. pH profile of the *Rx*PAL. Data indicate a local pH optimum around pH 8.5, and a global optimum at pH 11.5

II. 2.3. The thermal stability of the enzyme

The thermal stability of the *Rx*PAL was determined by thermofluorometric measurements at a concentration of 0.5 mg/mL enzyme. First, in order to determine the optimal pH regarding the melting temperature (Tm), measurements were performed in buffer solutions 100 mM, in the pH range 6-12. From the pH profile (**Figure 6**), it can be observed that the maximum melting temperature is reached in the 8-8.5 pH range and is approximately 64°C.

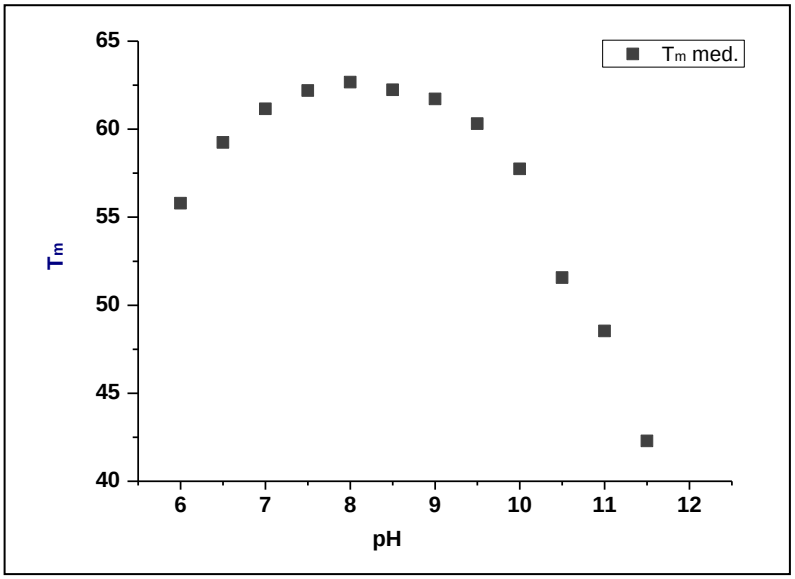


Figure 4. Melting temperature profile of *Rx*PAL in different pH conditions

II. 2.4. Circular dichroism measurement to estimate the secondary structure of *Rx*PAL

In order to investigate the proportions of different secondary structure elements in *Rx*PAL, far UV CD spectroscopy was applied. The far UV CD spectra (**Figure 7**) clearly indicated that *Rx*PAL is associated with high content of α-helical secondary structures, as the spectra show the corresponding characteristic double maxima at 208 and 222 nm.

The far UV CD spectra measured at different pH values retained the characteristics of the double maxima at 208 and 222 nm wavelength values. Hence, based on the far UV CD spectra, we conclude that the overall secondary structure of *Rx*PAL is well preserved up to pH 11.0.

Near UV CD measurements (**Figure 8**) were also carried out as the fine spectral details in this wavelength range are diagnostic for conformational changes. **Figure 8** shows that there are two major peaks in the near UV CD spectrum of *Rx*PAL, at 289 and 298 nm, characteristic for tryptophan and tyrosine residues, respectively.

Both of these major peaks are well observable in the protein spectrum up to pH 11.0, although at this pH, the relative heights of the two peaks are somewhat altered: the peak at the higher wavelength, putatively associated with tryptophan residues, is smaller and also presents a slight red-shift.

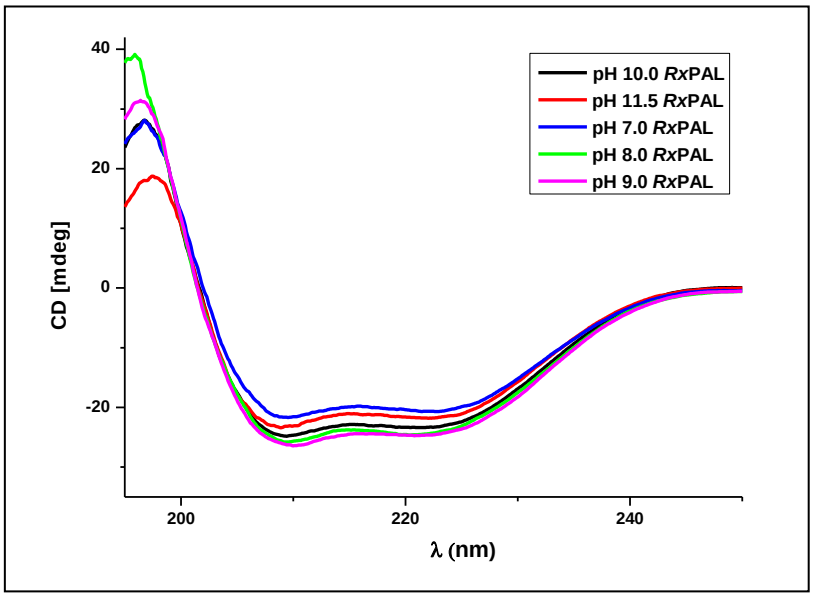


Figure 7. Circular dichroism spectra of *Rx*PAL recorded in the far UV (195–250 nm)

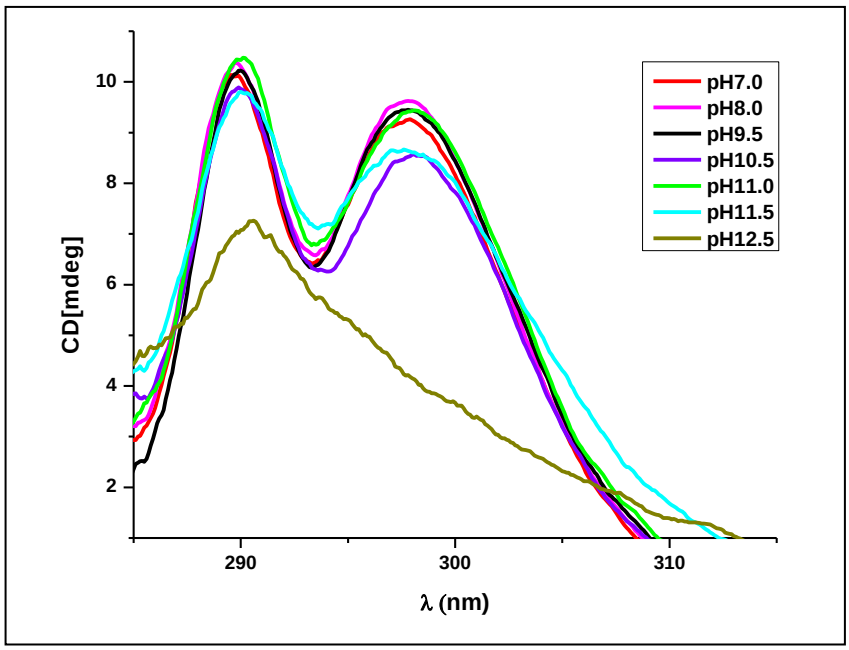


Figure 8. Circular dichroism spectra of *Rx*PAL recorded in the near UV (285–310 nm)

The spectral characteristics of tryptophan residues are especially sensitive to changes of the protein microenvironment. These spectra provided additional convincing information on the integrity of the tertiary structure between pH 6.5–11.0, confirming the stability of the enzyme at highly alkaline pH (**Figure 8**), and also showed that at pH 11.0 some slight conformational changes may already be initiated. At pH 12.5, CD spectra (**Figure 8**)

indicated the partial denaturation of the protein in agreement with the results of activity measurements.

II. 2.5. Kinetic measurements

Kinetic constants for *Rx*PAL catalyzed the deamination of the natural substrate (L-phenylalanine) to *trans*-cinnamic acid were measured spectrophotometrically in triplicate.

Table 2. Kinetic parameters for purified *Rx*PAL enzyme

K_M(mM)	v_{max}(mMs^{-1})	k_{cat}(s^{-1})	k_{cat} / K_M ($mM^{-1}s^{-1}$)
0.37	$1.67*10^{-4}$	0.083	0.22

II. 2.6. Monitoring the presence of the catalytically essential MIO group in the structure of *Rx*PAL

The catalytically essential MIO moiety in PAL enzymes was shown to generate a characteristic peak in the near-UV domain.[30] The amino acid sequence of *Rx*PAL also contains the ^{152}ASG^{154} sequence motif that was shown to be involved in the formation of the MIO group in ammonia-lyases. In order to investigate the presence and function of this motif in *Rx*PAL, we designed the S153A mutant, disrupting thereby the MIO group formation. The S153A *Rx*PAL mutant showed practically a total loss of enzymatic activity in deamination of L-Phe. UV spectra of the wild-type *Rx*PAL and of its S153A mutant were investigated lacking the MIO group, in order to prove their presence and to estimate the amount of this group in this highly alkalophilic enzyme (**Figure 9**). Results clearly showed that the wild type *Rx*PAL possessed the MIO-characteristic UV absorbance peak, being absent in the spectrum of the S153A mutant. The presence of the catalytically essential MIO in *Rx*PAL was determined by UV difference spectra of the active enzyme and the S153A mutant at several pH values, between 6.5–11.0. This fact provided a direct spectroscopic evidence for the formation of the MIO group with involvement of the ^{152}ASG^{154} sequence segment in *Rx*PAL.

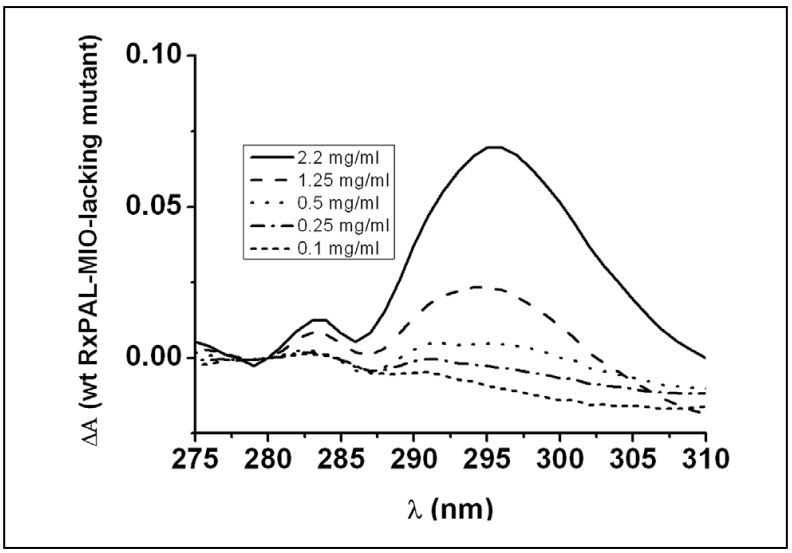


Figure 9. Mutation of wild type *Rx*PAL at the putative MIO site erases the MIO-specific spectroscopic signal. Difference spectra for wild type and S153A mutant *Rx*PALs were recorded at different protein concentrations.

II. 3. Conclusion

Bioinformatics' tools proved to be useful for the identification of novel PALs from thermotolerant bacteria, as we demonstrated by the recognition, expression and characterization of a novel PAL of the true thermophile *Rubrobacter xylanophilus* (*Rx*PAL).

The recombinant *Rx*PAL protein was successfully purified by Ni-NTA affinity chromatography. The activity assay of *Rx*PAL with L-phenylalanine at various pH values exhibited a local maximum at pH 8.5 and a global maximum at pH 11.5. The novel PAL enzyme also presents a good thermal stability. Circular dichroism (CD) studies showed that *Rx*PAL is associated with an extensive α-helical character (far UV CD) and has two distinctive near-UV CD peaks. These structural characteristics were well preserved up to pH 11.0. Due to these properties, *Rx*PAL has the potential to be exploited as biocatalyst in stereoselective synthetic biotransformation under extreme conditions or as therapeutic enzyme in the treatment of phenylketonuria or leukemia.

Chapter III. Biocatalytic synthesis mediated by *Pc*PAL and *Pa*PAM

III. 1. Investigation of the stereoselectivity of *Pc*PAL under unconventional conditions

III. 1.1. Introduction

As previously mentioned, phenylalanine ammonia-lyase from parsley (*Pc*PAL) belongs to the class of enzymes containing 4-methylideneimidazole-5-one (MIO) as a prosthetic group, generated post-translationally from three active site residues, Ala-Ser-Gly and it is responsible for the conversion of L-phenylalanine into *trans*-cinnamic acid.

Amination reactions were typically performed in 5-6 M NH_4OH, pH 10.0 and deamination reactions in 100 mM Tris buffer, pH 8-9, at 30 °C. In earlier publications PALs mediated synthesis of non-natural amino acids have been reported to proceed with >98 % enantioselectivity.[31,32]

Lovelock *et al*. examined the activity and enantioselectivity of *Anabaena variabilis* PAL (*Av*PAL), a bacterial PAL, towards a broad range of non-natural substrates and compared this activity and selectivity with the eukaryotic *Rg*PAL and *Pc*PAL in 5 M NH_4OH, pH 9.5.[33] They observed that the *enantiomeric excesses* of the substrates bearing electron-withdrawing substituents decrease in time for all three PALs, suggesting that they are able to catalyze the formation of the D-enantiomers of electron-deficient structures.[33]

Herein, we examined the variation of the ammonia concentration on the enantioselectivity of *Pc*PAL in the synthesis of *ortho-*, *meta-*, *para*-nitro-phenylalanine, mediated by whole cells as well by purified wild type *Pc*PAL enzyme. Furthermore, the effect of pH on the reaction and also the inactive MIO-containing *Pc*PAL enzyme mediated reactions were examined.

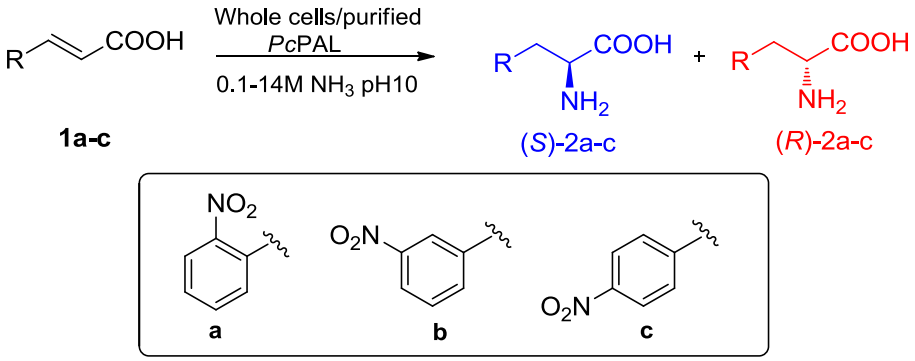
III. 1.2. Results and discussion

The conversion of *ortho-*, *meta-*, *para*-nitro-cinnamic acids into their corresponding amino acids was carried out using *E. coli* BL21(DE3)pLysS whole cells expressing *Pc*PAL enzyme and also purified MIO-containing and the inactive ($NaBH_4$ reduced) MIO-containing *Pc*PAL.

Several factors that seem to have notable influence on the enantioselectivity of the nitro-phenylalanine synthesis mediated by whole cells as well as by purified wild type-*Pc*PAL and inactive MIO-containing *Pc*PAL, were analyzed, including the effects of the ammonia concentration (0.1-14 M) on the reaction rate for amination and the pH effect on the deamination reactions.

III. 1.2.1. The effect of ammonia concentration on the amination reaction catalyzed by whole cells and purified *Pc*PAL

First, the behavior of the whole cells and purified *Pc*PAL catalyzed reactions depending on the ammonia concentration was investigated (**Scheme 10**). The reactions were monitored by chiral HPLC.



Scheme 10. *Pc*PAL mediated amination of *trans ortho-*, *meta-*, *para*-nitro-cinnamate undergoes with poor stereoselectivity

We also observed that the synthesis of *ortho-*, *meta-*, *para*-nitro-phenylalanine underwent with low stereoselectivity, suggesting that *Pc*PAL is also able to catalyze the formation of the D-enantiomer besides the L-enantiomer, as described by Lovelock *et al.* Surprisingly, the enantiomeric excess (*ee*) of the amino acid products **2a-c** decreased significantly in the presence of low concentration of ammonia (0.1-5 M) with both whole cells and purified *Pc*PAL biocatalysts. Over 5 M NH_3 concentrations the *ee* value is near the maximum and above 8 M NH_3 it is considered invariable. (*R*)-amino acid formation is prominent especially for *ortho* and *para* nitro-substituents respectively. At 0.5 and 1 M ammonia concentrations, the (*R*)-enantiomer occurred predominantly at early stages of the reactions. The *ee* value of the formed *ortho*-nitro-phenylalanine is higher when purified *Pc*PAL was used as catalyst, as compared with the whole cells *Pc*PAL. Contrary, when *para*-nitro-phenylalanive was synthesized the whole cell catalyst proved to be the most selective

one. For both type of catalyst (whole cell and single enzyme) the ammonia addition showed highest enantioselectivities when *meta*-nitro-cinnamate was used as substrate.

Interestingly, in the case of *para* substituted derivative, the purified enzyme transformation has lower selectivity as compared with whole cells catalyzed reaction.

The enantiomeric excess is presented in **Table 3** and **Table 4** respectively. (The obtained HPLC chromatograms for the *ortho*-nitro-derivative are presented in **ANNEXES, Figure A5**)

Tabel 3. The dependence of enantioselectivity with ammonia concentration for whole cell catalyzed biotransformations, after 3 h.

NH_3(M)	(*S*)-2a	(*S*)-2b	(*S*)-2c
pH 10	ee_S (%)	ee_S (%)	ee_S (%)
0.1	**-30.7**	42.2	**-23.2**
0.5	**-18.3**	68.8	**-7.7**
1	9.1	74.4	19.0
2	46.3	82.2	47.4
3	63.3	80.1	57.9
4	64.6	80.9	58.0
5	65.3	81.3	69.7
6	82.2	82.5	70.5
8	84.3	84.9	72.7
14	88.7	85.6	73.9

Table 4. The dependence of enantioselectivity with ammonia concentration for purified *Pc*PAL mediated biotransformations after 3 h

NH_3 (M)	(*S*)-2a	(*S*)-2b	(*S*)-2c
pH10	ee_S (%)	ee_S (%)	ee_S (%)
0.1	**-2.6**	40.9	**-38.9**
0.5	13.9	49.8	**-24.6**
1	27.9	64.4	**-8.2**
2	54.9	70.2	6.9
3	56.7	70.8	35.3
4	58.1	76.6	34.7
5	69.6	76.7	37.7
6	73.9	80.7	44.2
8	79.5	80.6	51.7
14	85.0	84.7	54.7

III. 1.2.3. The influence of the pH on the amination reaction

The influence of the pH on the *Pc*PAL catalyzed biotransformation was also inspected, when purified enzyme was used in buffered 1 M NH_3 in the pH range 7.0-10 (**Table 5**). By decreasing the pH, the enantioselectivity of the ammonia addition increased, but the reactions proceeded slower. At pH 7.0 the enzyme did not presents any activity even after 12 hours. A slight activity of *Pc*PAL at pH 7.5 was detected when the *ortho* substituted derivative was used as subtrate.

When the same enzyme was incubated with *meta* and *para* nitro cinnamates no activity was detected for pH < 8.5. When *ortho*-nitro-phenylalanine formation was investigated the enzyme catalyzed reaction showed maximum selectivity at pH 8, while for *meta*- and *para*-nitro-phenylalanine the same optimum was found at pH 8.5. For all cases, further increase of pH caused the continuous decrease of stereoselectivity.

Table 5. The influence of pH upon stereoselectivity for purified *Pc*PAL mediated ammonia adition to **1a-c**, after 12 h

(1M NH_3)	**(*S*)-2a**	**(*S*)-2b**	**(*S*)-2c**
pH	*ee*$_S$(%)	*ee*$_S$(%)	*ee*$_S$(%)
7.5	>95	-	-
8	89.7	-	-
8.5	74.3	87	73.95
9	40.6	84	50.8
9.5	29.7	53.2	26
10	24.6	46.2	-1.7

III. 1.2.4. MIO-containing and reduced MIO-containing *Pc*PALs

Further on, we investigated the necessity of presence of the super electrophilic MIO group in the catalytic center when PAL catalyzed the ammonia addition onto nitro cinnamates. It is also questionable if MIO could influence the stereoselectivity of the reaction. The inactivation of the MIO-group was performed reducing it with $NaBH_4$. The resulted reduced MIO-containing enzyme had complete loss of activity towards the amination of cinnamic acid and deamination of L-phenylalanine, but retained low activity level towards the nitro-phenylalanines.

Monitoring the enantiopurity of the formed *para*-nitro-phenylalanine using *wt*- and reduced MIO-containing PALs (**Table 6**), it was found that in the presence of the reduced

enzyme higher enantioselectivities of the product were detected compared with those formed with *wt*-PAL, than the MIO-containing enzyme.

Table 6. Enantioselectivities for the amination of *para*-nitro-cinnamate catalyzed by wt- and inactive MIO-containing *Pc*PALs, after 12 h

NH_3 (M) pH 10	**wt- *Pc*PAL ee_S (%)**	**$MIO_{inactive}$ *Pc*PAL ee_S (%)**
	12h	12h
1	-9.64	73.51
3	61.03	80.92
8	67.51	83.80

III. 1.3. Conclusion

Based on our results, we concluded that the reaction conditions can have a significant effect on the *ee* of the obtained amino acid products, suggesting that *Pc*PAL is able to catalyze the formation of both L- and D-enantiomers of electron-deficient structures.

The ammonia concentration and the pH, play a remarkable role in the enzyme enantioselectivity as much as the presence of MIO prosthetic group.

III. 2. Novel methods for *Pc*PAL immobilization: Bisepoxide Cross-Linked Enzyme Aggregates

III. 2.1. Introduction

Immobilized enzymes possess many benefits when compared to their soluble counterparts. Selectivity, specificity, catalytic activity, and enzyme stability are key factors that affect the efficiency of biocatalysts.[2,34,35] Various immobilization techniques may improve enzyme properties in several ways, such as by increasing rigidity of the enzyme, by preventing subunit dissociation through multipoint, multisubunit immobilization, and/or by generating a favorable environment for the enzyme.[36]

Cross-linked enzyme aggregates (CLEAs) were developed as simple and robust immobilization tools. They are generally prepared through precipitation with a suitable solvent in the absence of any carrier and then cross-linking of the aggregates formed by a multifunctional cross-linker such as glutaraldehyde (GA). In addition to the factors investigated most frequently in CLEA optimization studies (choice of solvent, amount of GA, and addition of surfactants and other chemicals), other aldehyde cross-linkers or co-immobilization of the active enzyme with an inert protein such as BSA were used.[37]

Poly(glycidyl ethers), such as glycerol diglycidyl ether (GDE) and poly(ethylene glycol) diglycidyl ether, are widely used as additives for cross-linking polymers bearing amine, hydroxy, or carboxy groups. Bis-epoxides, such as GDE, are useful cross-linkers in forming CLEAs owing to their ability to form stable bonds under mild conditions not only with the amine groups of lysine but also with the sulfur- and oxygen containing residues of cysteine, tyrosine, aspartate, or glutamate. Furthermore, GDE is an inexpensive, partially water-soluble bisepoxy compound and it is less toxic than GA.

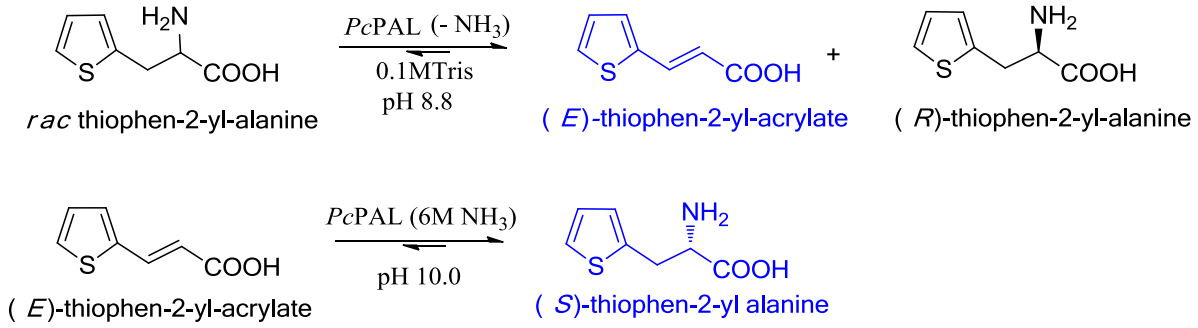
In this study the usefulness of GDE as a convenient bisepoxy-type cross-linker for the preparation of CLEAs and co-CLEAs of phenylalanine ammonia-lyase from *Petroselinum crispum* was investigated, for improving the characteristics of the biocatalysts.

III. 2.2. Results and discussion

III. 2.2.1. Stereoselective biotransformations with *Pc*PAL CLEAs

PAL-CLEAs and PAL–BSA co-CLEAs were prepared from purified recombinant PAL (from *Petroselinum crispum*) and tested in ammonia elimination and addition reactions, respectively. It was demonstrated earlier that the reaction of *rac*-thiophen-2-yl-alanine with

nonimmobilized purified *Pc*PAL is faster than the elimination from the natural substrate L-phenylalanine.[38] Therefore, the behavior of PAL–BSA co-CLEAs from *Petroselinum crispum*, with either GA or GDE as cross-linkers, were investigated further in the elimination reaction and also ammonia additions, using *rac*-thiophen-2-yl-alanine and thiophen-2-yl-acrylate as substrates (**Scheme 11**).



Scheme 11. Biotransformation of *rac*-thiophen-2-yl-alanine and (*E*)-thiophen-2-yl-acrylate with *Pc*PAL biocatalysts

Although both GA- and GDE–PAL CLEAs were active as biocatalysts in the elimination reactions from *rac*-thiophen-2-yl alanine, none of them were active in the ammonia addition to the thiophen-2-yl acrylate. In contrast, the GA- and GDE–PAL–BSA co-CLEAs were active both in the ammonia elimination from *rac*-thiophen-2-yl alanine and in the ammonia addition to thiophen-2-yl acrylate (**Figure 11.B**).

Recycling studies indicated remarkable stability differences between GA- and GDE–PAL–BSA co-CLEAs (**Figure 11**). Although the GDE–PAL–BSA co-CLEAs retained significantly their initial activities after the third reaction cycle even in the alkaline medium used for ammonia addition to thiophen-2-yl acrylate (6M ammonia solution, pH 10.0; **Figure 11. B**), the GA–PAL–BSA co-CLEAs were found to be completely inactivated after the first cycle.

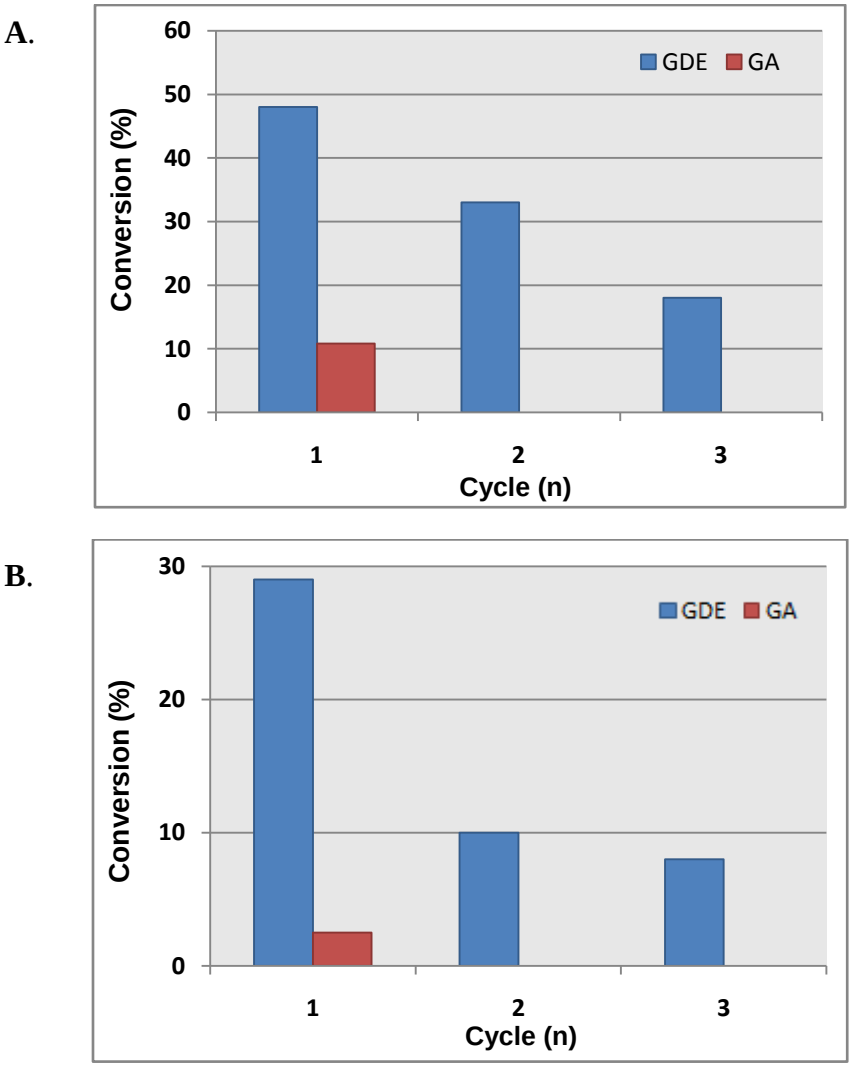


Figure 11. Recycling of PAL–BSA co-CLEAs with GDE and GA in **A.** ammonia elimination from *rac*-thiophen-2-yl-alanine and **B.** ammonia addition to thiophen-2-yl-acylate.

III. 2.3. Conclusion

Glycerol diglycidyl ether (GDE) was used as a new and efficient bisepoxide-type cross-linker for the cross-linked enzyme aggregates (CLEAs) of phenylalanine ammonia-lyases from *Petroselinum crispum*.

It can be concluded that GDE cross-linking increases not only the mechanical stability of the CLEAs formed but also the operational stability compared with those of the GA CLEAs.

The GDE CLEAs were efficient and robust biocatalysts surpassing the performance of the corresponding glutaraldehyde CLEAs. The use of GDE and similar bisepoxide cross-linkers widen the scope of the cross-linking technology for enzyme immobilization.

III. 3. Phenylalanine 2,3-aminomutase from *Pantoea agglomerans* (*Pa*PAM) catalyzed biotransformation

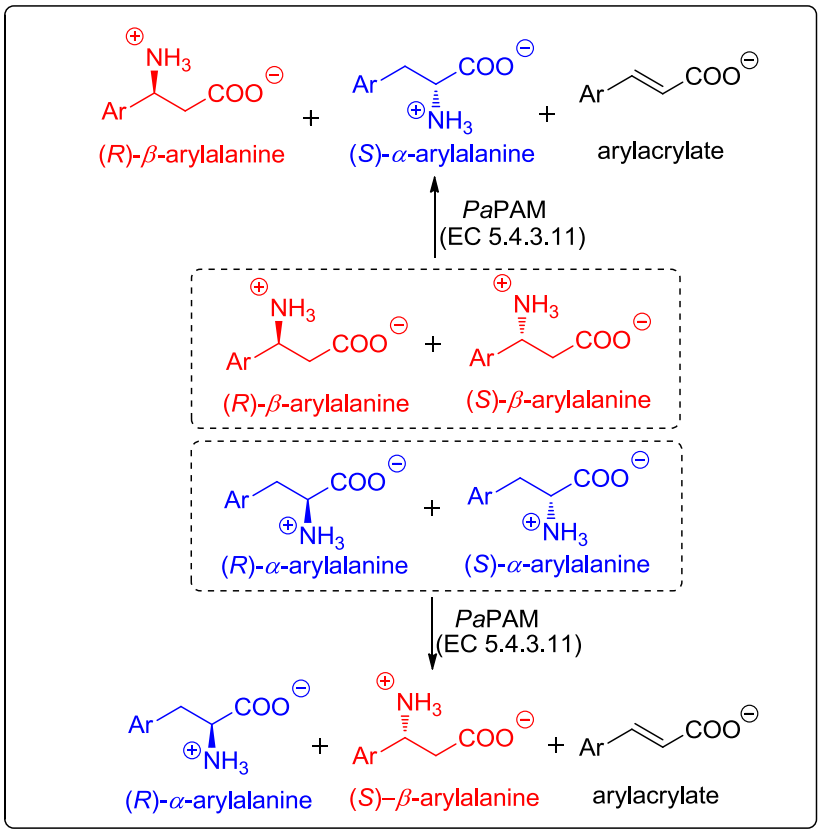
Nowadays, there is an increased demand for the optically pure *β*-amino acids due to their importance in pharmaceutical industry and peptide synthesis.[39,40] A considerable number of catalytic asymmetric methods have been investigated for the synthesis of optically pure *β*-amino acids.

An attractive approach for the synthesis of enantiomerically pure, non-natural *β*-amino acids involves the use of phenylalanine 2,3-aminomutase (PAM). There are two kinds of PAM according to their stereochemical preference, the (*R*)-*β*-phenylalanine forming PAM (EC 5.4.3.10) of plant origin and the (*S*)-*β*-phenylalanine forming PAM (EC 5.4.3.11) of bacterial origin.

The broad range of aromatic and heteroaromatic amino acids tolerated as substrates of these enzymes was also exploited for the preparation of a wide range of non-natural aryl and heteroaryl *α*- and *β*-amino acids.[25,41,] Phenylalanine 2,3-aminomutase from *Taxus canadensis* was used for the partial biotransformation of (*S*)-*α*-phenylalanine and its derivatives into their (*R*)-*β*-isomer counterparts[42] and also to catalyze the asymmetric amination reaction of (*E*)-cinnamic acid producing a mixture of optically pure (*S*)-*α*- and (*R*)-*β*-phenylalanine.[15] Wanninayake et al. have explored some non-natural amino acids which served as suitable amino group donor substrates. The amino group of these donor substrates was transferred intermolecularly to another aryl-acrylate skeleton by *Tc*PAM to form *α*- and *β*–arylalanine mixtures. More recently, Weise et al. investigated the EncP from *Streptomyces maritimus* in the animation reaction of several aryl-substituted *trans*-cinnamic analogues. It was found that EncP converts a range of arylacrylates to a mixture of (*S*)-*α*- and (*S*)-*β*-arylalanines. Regioselectivity of the enzyme proved to be significantly affected by the ring substituents by varying with their electronic effects, nature and position, and it was improved by protein engineering.

Substrate specificity of phenylalanine 2,3-aminomutase from *Pantoea agglomerans* (*Pa*PAM) was tested for a wide range of (*S*)-arylalanines.[43] It was shown, that the catalytic efficiency of *Pa*PAM and the formation of aryl-acrylate byproducts are significantly influenced by the electronic effects of the aromatic rings' substituents. It was observed that the *meta*-substituted (*S*)-*α*-phenylalanines were faster transformed than the corresponding *ortho*- or *para*-substituted isomers. Recently it was shown that recombinant whole cell *E. coli*

expressing *Pa*PAM could also produce enantiopure (*S*)-*α*-arylalanines from (*S*)-*β*-arylalanines.[44]



Scheme 12. Literature overview of PAMs-catalyzed reactions: **A.** Ammonia addition catalyzed by *Tc*PAM or EncP (*Sm*PAM); **B.** Transformation of (*S*)-*α*-arylalanines by *Tc*PAM and *Pa*PAM; **C.** Synthesis plan proposed by us starting from racemic mixtures

In this study, we determined the optimal conditions for high level enzyme expression in *E. coli* and we also investigated the catalytic activity of *Pa*PAM starting from both *α*- and *β*-arylalanines. Several racemic *α*- and *β*-(hetero)arylalanines were tested, reaction equilibrium and substrate-products distributions were determined by ^{1}H and ^{19}F NMR spectroscopy and the enantiomeric purities were determined by chiral HPLC methods.

III. 3.2. Results and discussions

III. 3.2.1. Optimization of *Pa*PAM expression

First, it was investigated the effect of different experimental conditions on the expression of *Pa*PAM in order to obtain high purity and yields for the enzyme which can be used as biocatalyst for the preparation of enantiomerically pure (*S*)-*β*- and (*S*)-*α*-amino acids.

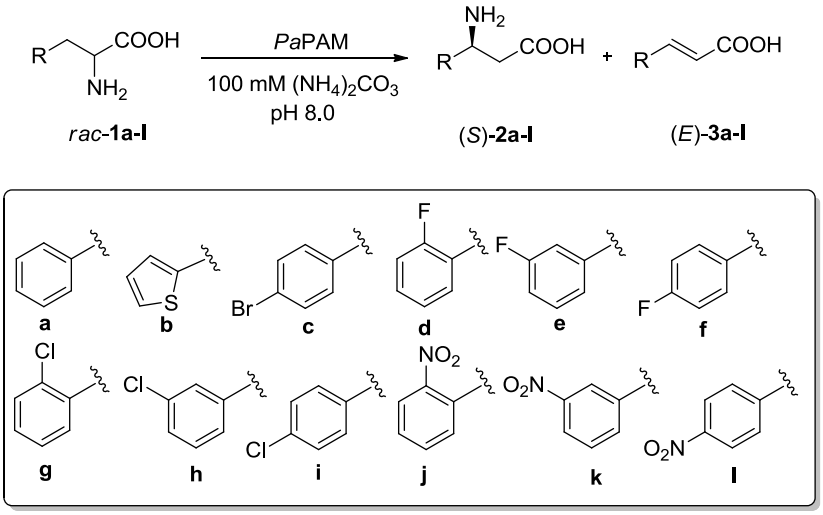
The results showed that induction with 0.1 M IPTG was sufficient to induce the expression of *Pa*PAM, which is 10 times less than normally used IPTG concentration. Moreover, 15 hour post-induction incubation at 25°C was found to be optimal for producing a higher level of *Pa*PAM. Reducing the imidazole concentration to 350 mM and adding protease inhibitor cocktail improved the stability of the yielding enzyme. Furthermore, electrophoretically pure recombinant *Pa*PAM enzyme preparation was obtained using Ni affinity chromatography.

III. 3.2.2. Transformation of racemic *α*- and *rac-β* arylalanines with *Pa*PAM

III. 3.2.2.1. Transformation of racemic *α*-arylalanines with *Pa*PAM

The catalytic efficiency of phenylalanine 2,3-aminomutase from *Pantoea agglomerans* for several racemic *α*-arylalanines (*rac*-**1a-l**) was investigated. According to the previous results, *Pa*PAM catalyzed apparently no transformation of the *ortho*-substituted (*S*)-*α*-phenylalanines.[43] Our study starting from racemic *α*-arylalanines *rac*-**1a-l** (**Scheme 13**) confirmed the validity of this observation also for the racemic mixtures (**Table 7**). *α*-(*ortho*-chlorophenyl)- (*rac*-**1g**) and *α*-(*ortho*-nitrophenyl)alanines (*rac*-**1j**) did not result in any detectable product in their reactions with *Pa*PAM. In the case of *α*-(*ortho*-fluorophenyl)alanine (*rac*-**1d**) bearing the smallest halogen atom fluorine as substituent, however, moderate mutase-activity was observed resulting in the formation of enantiopure (*S*)-*β*-(*ortho*-fluorophenyl)alanine [(*S*)-**2d**].

Conversion of the *meta*-substituted-*α*-phenylalanines (*rac*-**1e,k**) with *Pa*PAM within 20 h was moderately faster than that of the *ortho*-substituted substrate (*rac*-**1d**), but still slower than with racemic *α*-phenylalanine (*rac*-**1a**).



Scheme 13. *Pa*PAM-catalyzed transformation of *rac*-*α*-arylalanines

Comparison of the conversions of the *para*-substituted-*α*-phenylalanines (*rac*-**1c,f,i,l**) with *Pa*PAM for 20 h indicated that from *para*-fluoro- (*rac*-**1f**) and *para*-bromo-*α*-phenylalanine (*rac*-**1c**) about 1.2 times higher conversions were reached than from the *para*-chloro- (*rac*-**1i**) or *para*-nitro-*α*-phenylalanine (*rac*-**1l**). Reactions of *α*-arylalanine bearing thiophen-2-yl group as a small heteroaromatic moiety (*rac*-**1b**) with *Pa*PAM were also investigated. It was found that the conversion of racemic *α*-(thiophen-2-yl)alanine (*rac*-**1b**) was similar to racemic *α*-phenylalanine (*rac*-**1a**).

Table 7. Composition of the reaction mixtures obtained from *rac*-**1a-l** *α*-arylalanines with *Pa*PAM (after 20 h, determined by NMR, the ^{1}H NMR spectra are shown in **ANNEXES**, **Figure A11**)

Entry	Substrate	x_1	$x_{(S)\text{-}2}$[a]	x_3
1	*rac*-**1a**	0.66	0.28	0.06
2	*rac*-**1b**	0.68	0.17	0.15
3	*rac*-**1c**	0.79	0.21	0
4	*rac*-**1d**	0.8	0.07	0.13
5	*rac*-**1e**	0.77	0.19	0.04
6	*rac*-**1f**	0.77	0.20	0.03
7	*rac*-**1g**	1	0	0
8	*rac*-**1h**	0.84	0.13	0.03
9	*rac*-**1i**	0.90	0.10	0
10	*rac*-**1j**	1	0	0
11	*rac*-**1k**	0.74	0.26	0
12	*rac*-**1l**	0.89	0.06	0.05

[a] *ee* >98%;

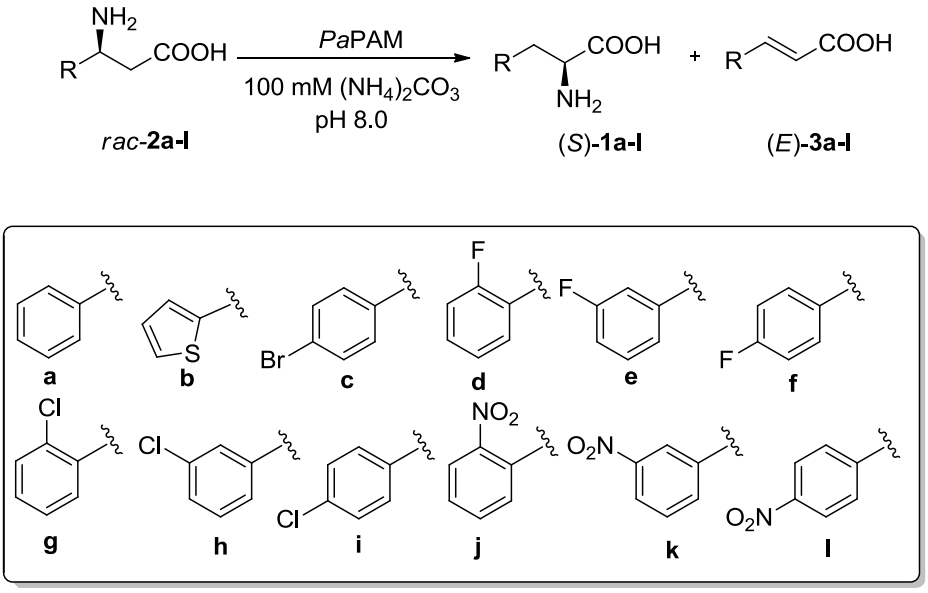
Previously, based on the study of initial reaction rates of the *α*- or *β*-phenylalanines, negligible ammonia-lyase activity was predicted for *Pa*PAM at low temperatures (under 30 °C). In our study, however, formation of substantial amount of (*E*)-arylacrylates was observed in many cases (**3a,b,d,e,f,h,l** in **Table 7**). The lyase-activity of *Pa*PAM was observed in nearly all of the other reactions investigated, except with *para*-bromo- (*rac*-**1c**), *para*-chloro (*rac*-**1i**) and *meta*-nitro-*α*-phenylalanine (*rac*-**1k**) as substrates. Relatively high amounts of arylacrylates were formed in the reactions of racemic *α*-phenylalanine (*rac*-**1a**), *α*-(thiophen-2-yl)alanine (*rac*-**1b**) and *ortho*-fluoro-*α*-phenylalanine (*rac*-**1d**) bearing the smallest aromatic moieties.

These results with the racemic *α*-arylalanines (*rac*-**1a-l**) indicated that the mutase- and/or lyase-activity of *Pa*PAM were considerably affected by the nature of the aromatic moiety of the substrates. Importantly, the high enantiomeric excess of the products [>98% *ee* for (*S*)-**2a-f,h,i,k,l** by HPLC] indicated the high stereoselectivity of the *Pa*PAM-catalyzed isomerization in the *α*- to *β*- direction. This high stereoselectivity of the *Pa*PAM-catalyzed isomerization of the (*S*)-*α*- and (*S*)-*β*-arylalanines was a major benefit compared to the not fully stereoselective *Sm*PAM-catalyzed isomerizations often resulting in formation of non-enantiopure products.

III. 3.2.2.2. Transformation of racemic *β*-arylalanines with *Pa*PAM

In order to test the synthetic availability of the opposite enantiomeric products, our study was extended to the reactions from racemic *β*-arylalanines *rac*-**2a-l** (**Scheme 14** and **Table 8**). In contrast to the *ortho*-substituted *α*-phenylalanines bearing large substituents (*rac*-**1g,j**) which were apparently not accepted as substrates by *Pa*PAM, all the racemic *ortho*-substituted *β*-phenylalanines in this study (*rac*-**1d,g,j**) were transformed smoothly. On the other hand, *Pa*PAM was apparently inactive towards *meta*- and *para*-substituted *β*-phenylalanines bearing large electron withdrawing substituents (*rac*-**2h,i,k,l**). The conversions of racemic *β*-(thiophen-2-yl)alanine (*rac*-**2b**) and *meta*-fluoro-*β*-phenylalanine (*rac*-**2e**) with *Pa*PAM were higher than that of racemic *β*-phenylalanine (*rac*-**2a**). The racemic *para*-fluoro-*β*-phenylalanine (*rac*-**2f**) was converted similarly as racemic *β*-phenylalanine (*rac*-**2b**), while *para*-bromo-*β*-phenylalanine (*rac*-**2c**) was transformed to the *α*-isomer (*S*)-**1c** and a small amount of *para*-bromocinnamate (**3c**) at significantly lower conversion as *rac*-**2b**. In all cases, except from the racemic 2-nitro-*β*-phenylalanine (*rac*-**2j**), the reactions catalyzed by *Pa*PAM proceeded with formation of a small amount of arylacrylate byproduct (**3a-g**) besides the enantiopure (*ee* >98%) (*S*)-*α*-isomer [(*S*)-**3a-g**]. The transformation from the

racemic 2-nitro-*β*-phenylalanine (*rac*-**2j**) was exceptional not only because the lack of the byproduct (**3j**) but also due to the lack of stereospecificity during the isomerization resulting in non-enantiopure product ($ee_{(S)\text{-}\mathbf{1j}}$= 92%).



Scheme 14. Transformation of *β*-arylalanines catalyzed by *Pa*PAM

Table 8. Composition of the reaction mixtures obtained from *rac*-**2a-l** *β*-arylalanines with *Pa*PAM (after 20 h, determined by NMR, the ^{1}H NMR spectra are shown in **ANNEXES**, **Figure A12**)

Entry	Substrate	x_2	$x_{(S)\text{-}1}$[a]	x_3
1	*rac*-**2a**	0.71	0.28	0.01
2	*rac*-**2b**	0.69	0.27	0.04
3	*rac*-**2c**	0.91	0.08	0.01
4	*rac*-**2d**	0.61	0.37	0.02
5	*rac*-**2e**	0.68	0.25	0.07
6	*rac*-**2f**	0.76	0.23	0.01
7	*rac*-**2g**	0.54	0.40	0.06
8	*rac*-**2h**	1	0	0
9	*rac*-**2i**	1	0	0
10	*rac*-**2j**	0.87	0.13[b]	[0]
11	*rac*-**2k**	1	0	0
12	*rac*-**2l**	1	0	0

[a] *ee* >98% when not stated otherwise, [b]*ee* = 92% [d] not observed

III. 3.2.2.3. Effects of the pH and ammonia concentration on the *Pa*PAM-catalyzed isomerization of racemic *α*- and *β*-(thiophen-2-yl)alanine

Because the conversions from racemic *α*- and *β*-(thiophen-2-yl)alanine (*rac*-**1b** and *rac*-**2b**) with *Pa*PAM were similar as from the natural substrates *α*- and *β*-phenylalanine (*rac*-**1a** and *rac*-**2a**) but the degree of the (*E*)-3-(thiophen-2-yl)acrylate by-product (**3b**) formation was higher than that of (*E*)-cinnamate (**3a**) (**Table 7** and **Table 8**), these two substrates were investigated further by modifying the pH or ammonia concentration in the biotransformations. Altering the pH of the buffer solution in the range of 7–9 did not result in significant differences in conversions (data not shown), unlike changing the ammonia concentration in the range of 50–1000 mM which influenced the reaction compositions (**Table 9**).

Analysis of the *Pa*PAM-catalyzed reactions from racemic *α*-(thiophen-2-yl)alanine (*rac*-**1b**) at various $(NH_4)_2CO_3$ concentrations at 20 h showed, that the increasing $(NH_4)_2CO_3$ concentration above 100 mM resulted in decreasing conversion to (*S*)-*β*-(thiophen-2-yl)alanine (*S*)-**2b** and elimination product **3b**. At 1 M $(NH_4)_2CO_3$ concentration both (*S*)-*β*-(thiophen-2-yl)alanine (*S*)-**2b** and arylacrylate (**3b**) production decreased to 12 %.

Interestingly, when biotransformations were started from the racemic *β*-(thiophen-2-yl)alanine (*rac*-**2b**) the best conversion to (*S*)-*α*-(thiophen-2-yl)alanine (*S*)-**1b** (27 %) was achieved at 100 mM buffer concentration. At higher $(NH_4)_2CO_3$ concentrations decreased conversions to (*S*)-*α*-(thiophen-2-yl)alanine (*S*)-**1b** and elimination product **3b** were observed.

Table 9. Composition of the reaction mixtures obtained from *rac* *α*- or *β*-(thiophen-2-yl)alanine (*rac*-**1b** or *rac*-**2b**) with *Pa*PAM at various ammonium carbonate concentrations (after 20 h, determined by NMR)

Entry	Substrate	$c_{(NH_4)_2CO_3}$ (mM)	x_{1b}	x_{2b}	x_{3b}
1	*rac*-**1b**	50	0.68	0.17	0.15
2	*rac*-**2b**	50	0.19	0.76	0.05
3	*rac*-**1b**	100	0.68	0.17	0.15
4	*rac*-**2b**	100	0.27	0.69	0.04
5	*rac*-**1b**	200	0.72	0.16	0.12
6	*rac*-**2b**	200	0.23	0.75	0.02
7	*rac*-**1b**	1000	0.76	0.12	0.12
8	*rac*-**2b**	1000	0.13	0.84	0.03

The $(NH_4)_2CO_3$ concentration-dependency study of the *Pa*PAM-catalyzed reactions from racemic *α*- and *β*-(thiophen-2-yl)alanine (*rac*-**1b** and *rac*-**1b**, respectively) indicated that the elevated ammonia concentrations had negative impact on the rate of isomerization as well as on the equilibrium of ammonia elimination as side reaction from both reaction directions.

III. 3.3. Conclusion

According to our results, it can be concluded that the selectivity and activity of *Pa*PAM towards *α*- and *β*-arylalanines is mainly influenced by the position of the substituents on the phenyl ring. The enzyme catalyzed the synthesis of the corresponding (*S*)-*β*-enantiomer from *para*- and *meta*-substituted *α*-phenylalanines substantially better than that from o*rtho*-substrates, whereas in the case of *rac-β*-counterparts it was fully inactive for the *meta*- and *para*-substituted derivatives, but the *ortho-β*-analogues were transformed with good conversions.

Chapter IV. General Conclusion

The study presented in this thesis describes the isolation, characterization and application of phenylalanine ammonia-lyases and phenylalanine 2,3-aminomutases as biocatalysts in stereoselective reactions.

A novel PAL from thermotolerant bacteria *Rubrobacter xylanophilus* was identified, successfully cloned, expressed and characterized. The pH dependence of the enzymatic activity was assessed and two optimal were found, at pH 8.5 and 11.2, respectively. Based on structural characterization with far-UV CD, it was shown that the enzyme is structurally well preserved up to pH 11.5. Circular dichroism (CD) studies showed that *Rx*PAL is associated with an extensive α-helical character (far UV CD) and two distinctive peaks (near-UV CD).

It was investigated the most used and characterized phenylalanine ammonia-lyase, *Pc*PAL from *Petroselium crispum* in unconventional conditions, using as substrate the *ortho*-, *meta*-, *para*-nitro-phenylalanine. It was observed that the reaction conditions have a significant effect on the *ee* of the obtained amino acid; we demonstrated that *Pc*PAL is able to catalyze the formation of both L- and D-enantiomers of electron-deficient structures. Modification of the ammonia concentration and the value of pH play a remarkable role in the enzyme enantioselectivity, as much as the presence of MIO prosthetic group.

A new cross-linked enzyme aggregates (CLEAs) method was developed, using GDE as a convenient bisepoxy-type cross-linker GDE for the preparation of CLEAs. We compared the traditionally used GD-PAL-CLEA with the GDE-PAL-CLEA. The simple GA- and GDE-PAL-CLEA presented no activity in the addition reactions, in contrast the GA- and GDE–PAL–BSA co-CLEAs were active both in the ammonia elimination of rac-thiophen-2-yl alanine and in the ammonia addition to thiophen-2-yl acrylate. The co-immobilization of the *Pc*PAL enzyme with BSA proved to be beneficial, as demonstrated by the obtained results.

Recycling studies indicated that the GDE–PAL–BSA co-CLEAs retained a significant portion of their initial activities after the third reaction cycle even in the alkaline medium used for ammonia addition to thiophen-2-yl acrylate however the GA–PAL–BSA co-CLEAs were found to be completely inactivated after the first cycle.

The selectivity and activity of *Pa*PAM towards α- and β-arylalanines is mainly influenced by the position of the substituents on the phenyl ring. The enzyme catalyzed the

synthesis of the corresponding (*S*)-*β*-enantiomer from *para*- and meta-substituted α-phenylalanines substantially, better than that from *ortho*-substrates, whereas in the case of *rac*-*β*-counterparts it was fully inactive for the *meta*- and *para*-positions, but the *ortho*-*β*-analogues were transformed with good conversions.

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List of Publications

Articles

1. A. Varga, G. Bánóczi, B. Nagy, L. C. Bencze, Á. Gellért, F. D. Irimie, J. Rétey, L. Poppe, C. Paizs. Influence of the aromatic moiety in *α*- and *β*-arylalanines on their biotransformations with phenylalanine 2,3-aminomutase from *Pantoea agglomerans, Amino acids,* **2016.** – submitted

2. A. Varga, A. Filip, L. C. Bencze, P. Sátorhelyi, E. Bell, B. G. Vértessy, L. Poppe, C. Paizs. Expression and purification of recombinant phenylalanine 2,3-aminomutase from *Pantoea agglomerans, Studia Universitas Babeș-Bolyai, Chemia,* **2016**,- accepted

3. D. Weiser, A. Varga, K. Kovács, F. Nagy, A. Szilágyi, B. G. Vértessy, C. Paizs, L. Poppe, Bisepoxide cross-linked enzyme aggregates - New immobilized biocatalysts for selective biotransformations, *ChemCatChem*, **2014**, *6*, 1463-1469.

4. K. Kovács, G. Bánóczi, A. Varga, I. Szabó, A. Holczinger, G. Hornyánszky, I. Zagyva, C. Paizs, B. G. Vértessy, L. Poppe. Expression and Properties of the Highly Alkalophilic Phenylalanine Ammonia-Lyase of Thermophilic *Rubrobacter xylanophilus, PLoS ONE,* **2014**, *9*:e85943

Posters:

1. A. Varga, B. Nagy, M. Miklós, F. D. Irimie, L. Poppe, C. Paizs, *29th Annual Symposium of The Protein Society*, 22th-26th July, **2015**, Barcelona, Spain.
2. A. Varga, B. Nagy, F. D. Irimie, C. Paizs, *14th Symposium and Summer School on Bioanalysis*, 28th June - 6th July, **2014**, Bratislava-Smolenice, Slovakia.
3. A. Varga, B. Nagy, C. Paizs, L. Poppe, *COST Action CM1303 "SysBiocat" Training School*, 28th May-1st June, **2014**, Certosa di Pontignano, Italy. – oral presentation.
4. A. Varga, B. Nagy, C. Paizs, L. Poppe, *COST Action CM1303 "SysBiocat" Kick-off Workshop,* 10th-14th April, **2014**, Madrid, Spain
5. A. Varga, A. Radu, E. Kókai, G. B. Vértessy, L.Poppe, C. Paizs, *19th International Conference on Chemistry*, 21th - 24th November, **2013**, Baia-Mare, Romania.- oral presentation
6. A. Varga, E. Kókai, B. G. Vértessy, L. Poppe, C. Paizs, *Budapest Biostruct Course on Basics in Protein Crystallization and Crystallography,* 30th August - 3th September, **2013**, Budapest, Hungary.-oral presentation
7. A. Mantu, A. Varga, B. Nagy, M. I. Toșa, F. D. Irimie, C. Paizs, *13th Symposium and Summer School on Bioanalysis*, 27th June – 7th July, **2013**, Debrecen, Hungary.

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