BABEŞ-BOLYAI UNIVERSITY

CLUJ-NAPOCA

Faculty of Chemistry and Chemical Engineering

Biotransformations of primary amines and amino acids

– PhD Thesis Abstract –

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General introduction

Over the last few years biocatalysis registered a major development, being turned to get a wide range of compounds with important practical applications in medicine, pharmaceutical, chemical, food and agrochemical industry. The identification and the study of these compounds structure required the development and the use of new modern and effective methods of synthesis. The major improvement of enzymatic procedures consists in achieving highly enantioselective biotransformation. Furthermore, the biocatalysts are easily handled and in most of the cases do not imply special working conditions, the reactions go off with increased speed and efficiency, and, extremely important, they are environmentally friendly since the use of hazardous substances is avoided.[1]

The results of the scientific research activity presented in this PhD thesis and directed to stereoselective biotransformation of chiral compounds with amino functional group are structured in two main directions.

The first part introduces the chemoenzymatic methods for stereoselective synthesis of new phenylthiazole-based ethanamines and amides, variously substituted, preparing four pairs of enantiomers of high yields and enantiomeric excesses.

In the second part it was elaborated an efficient protocol to express and purify a prokaryotic and two eukaryotic ammonia-lyases and a prokaryotic aminomutase.

The resulted enzymes were characterized in terms of stability at different pH values and temperature, by monitoring the influence of metal ions and polar organic solvents on ammonia-lyase activity from *Petroselinum crispum*. Subsequently, the affinity of the substrate and the rate of ammonia-lyase reaction were determined for both L-phenylalanine and the newly synthesized substrates. (S)-β-phenylalanine was obtained starting from (S)-α-phenylalanine through *Pa*PAM and *Pc*PAL enzymes usage. Afterwards, we got at preparative scale both (D, L) enantiomers of phenylalanine substituted in the *ortho*-, *meta*- and *para*- positions with $–NO_2$.

Part I. *Candida antarctica* lipases acting as versatile catalysts for the synthesis of enantiopure (*R*)- and (*S*)- phenylthiazole-based ethanamines and amides

Keywords: enzymatic kinetic resolution, biotransformation, lipases, phenylthiazole amines, amide hydrolysis

Chapter I.1 Introduction

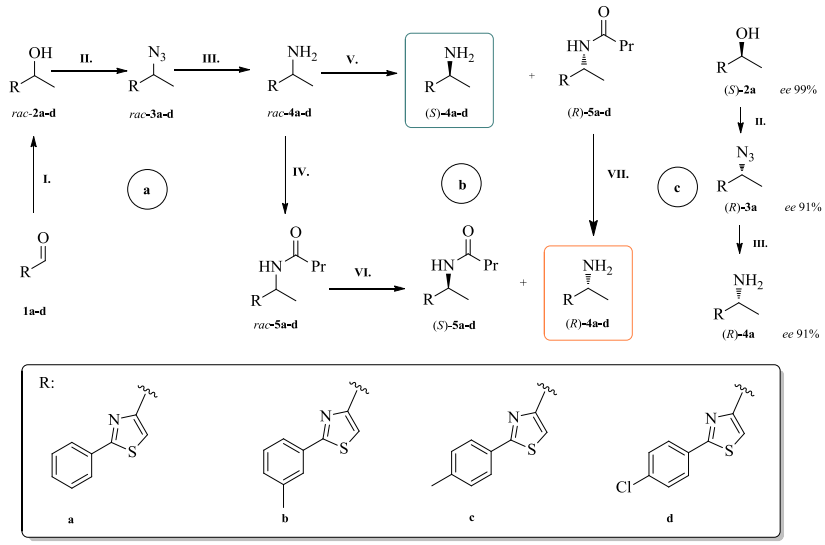
– literature data –

Chapter I.2 Aims of the study

In the first part of the study, the main objective consisted in developing a new original and efficient method necessary to obtain valuably intermediates used in organic synthesis.

In order to gain both enantiomers of phenylthiazole-based ethanamines and the corresponding amides, as well as their substituents in *meta*- and *para*- position, under a well-established screening procedure, the optimum reaction conditions were determined. It required the selection of a proper solvent and a nucleophilic agent that increase the catalytic activity and the selectivity of the lipases used in the enantiomeric kinetic resolution of racemic mixture.

Scheme I.2.1 depicts the steps performed in order to obtain the desired enantiomeric forms.



Scheme I.2.1 Synthesis and biotransformations of the studied 1-(2-phenylthiazol-4-yl)ethanamines and ethanacetamides.

Reagents and conditions:

I. CH_3MgI, diethyl ether
II. $(PhO)_2PON_3$/toluene
III. Zn/NH_4Cl, H_2O/ethanol
IV. $CH_3(CH_2)_2COCl$/DMAP/pyridine/DCM
V. CaL-B/ethyl *n*-butirate/ACN, 23 ^oC
VI. CaL-B/H_2O, 45 ^oC
VII. CaL-A/H_2O, 45 ^oC

Chapter I.3 MATERIALS AND METHODS

Chapter I.4 RESULTS

I.4.1 Chemical synthesis

According to the methods described in literature, the preparation of the *rac*-**4a–d** amines involved in the biotransformation starts from the corresponding aldehydes. The **1a-d** aldehydes were used as raw material for the synthesis of racemic *rac*-**2a-d** alcohols *via* a Grignard reaction.[2] Further, the alcohols obtained in the previous stage were transformed into racemic amines *rac*-**4a–d** through azide derivatives *rac*-**3a–d**.[3]

The synthesis of the racemic amides *rac*-**5a–d** was performed by chemical acylation of *rac*-**4a–d** with butyryl chloride in dichloromethane, in presence of pyridine and a catalytic amount of DMAP.

I.4.2 Enzymatic kinetic resolution

I.4.2.1 Analytical scale enzymatic *N*-acylation of *rac*-4a–d

In order to find the optimal reaction conditions for the *N*-acylation reaction a series of free or immobilized commercially available lipases were tested. The tested solvents were selected as they are frequently used in organic synthesis and industry and are known for their compatibility with this class of enzymes.

Following the enzymatic screening and the analysis of chromatograms carried out on HPLC, the CRL, PS and AK lipases proved to be catalytically inactive even after 28 h, while CaL-A showed either no activity or poor selectivity in MTBE with isopropyl *n*-butyrate and ethyl *n*-butyrate.

The highest enantioselectivity ($E \gg 200$) and reactivity (c = 50%) were registered after 16 hours in the *N*-acylation reaction of *rac*-**4a** with ethyl *n*-butyrate as acyl donor in dry ACN as solvent in presence of CaL-B.

I.4.2.2 Preparative scale enzymatic *N*-acylation of *rac*-4a-d

The preparative *N*-acylation of *rac*-**4a-d** was performed in the presence of the most efficient enzyme, Novozyme 435, with ethyl *n*-butyrate in dry ACN. The highly enantioselective *N*-acylations of the reactive (*R*)-**4a–c** ($E \gg 200$) were all completed ($c \sim 50\%$) in 16 hours. Lower selectivity and activity of the enzyme was observed when the chlorinated *rac*-**4d** was subjected to the same biotransformation ($E = 125$, $c \sim 46\%$ after 16 hours).

The unreacted (*S*)-**4a–d** and the obtained (*R*)-**5a–d** enantiomers were isolated at close to 50% theoretical yields (93–97% from the theoretical amounts at 50% conversion) in highly enantiopure forms (*ee* 82–99%) (**Table I.4.2.2.1**).

Table I.4.2.2.1 Preparative scale Novozyme 435 mediated *N*-acylation of *rac*-**4a-d** (t = 16 hours)

Compound	η [a] (%)	***ee*** **(%)**	$[\alpha]_D^{25}$ [b]	**mp** (°C)	***E***
(*S*)-**4a**	97	> 99	-6.2	–	» 200
(*R*)-**5a**	97	> 99	+175.9	110±1	
(*S*)-**4b**	95	> 99	-8.4	–	» 200
(*R*)-**5b**	95	> 99	+164.2	113±1	
(*S*)-**4c**	93	> 99	-18.3	–	» 200
(*R*)-**5c**	96	> 99	+148.8	135±1	
(*S*)-**4d**	93	82	-11.0	–	125
(*R*)-**5d**	93	96	+151.2	130±1	

[a] 50% of the racemates taken as 100% theoretical yields
[b] 10^{-1}deg×cm^2 ×g^{-1}; c = 1.0 mg/mL; $CHCl_3$ for (*R*)-**5a-d** amides, CH_3OH for (*S*)-**4a-d** amines

I.4.2.3 Kinetic resolution of *rac*-5a-d and deprotection of (*R*)-5a-d by lipase catalyzed hydrolysis

Nowadays, amide hydrolysis by serine proteases became a subject of study for researchers. They attributed to the hydrogen bond formed between the substrate's amide nitrogen and the enzyme or the substrate itself, thus facilitating the nitrogen inversion during the catalytic process, the hydrolysis reaction.[4] In what concerns lipases, some of them lack this particular hydrogen bond and, subsequently, the ability to efficiently cleave amides. However, previous

results showing that lipases CaL-A[5, 6] and CaL-B[7] can hydrolyze amides in water, motivate us to study the hydrolysis of the arylethanamides using lipases as biocatalysts.

In order to obtain the opposite enantiomeric forms of the enantiomerically enriched 1-(2-phenylthiazol-4-yl)ethanamines, the lipase-mediated kinetic resolution of *rac*-**5a–d** was investigated, due to the fact that in general lipases keep their enantiopreference in the hydrolysis reactions.

To this extent, the analytical scale hydrolytic reactions were performed in water at room temperature (23 °C), and at 45 °C using four enzymes for the selective hydrolysis of the model compound *rac*-**5a**. It is worth mentioning that the addition of any co-solvent was not necessary.

The results obtained after 30 hours showed that the hydrolytic reactions mediated by CaL-B were highly selective, the best activity being obtained for Novozyme 435 at 45°C (**Table I.4.2.3.1**, entry 1). CaL-A on Celite proved to be highly active, giving the highest reactivity at 45 °C, but non-stereoselective catalyst for these transformations (**Table I.4.2.3.1**, entry 4).

Table I.4.2.3.1 CaL-A and CaL-B catalyzed analytical scale hydrolysis of *rac*-**5a** in water (t = 30 hours)

Entry	**Enzyme**	**Temperature** (ºC)	***c*** (%)	ee_s (%)	ee_p (%)	***E***
1	CaL-B (Novozyme 435)	23	28	37	> 99	> 200
		45	**45**	**80**	**> 99**	**> 200**
2	CaL-B (Chiral Vision)	23	39	64	> 99	> 200
		45	44	78	> 99	> 200
3	CaL-A free	23	14	3	18	1
		45	31	9	20	2
4	CaL-A on Celită	23	55	5	4	1
		45	**75**	**18**	**6**	**1**

Following the results obtained in the analytical scale, the preparative hydrolysis reactions of *rac*-**5a-d** were performed at 45 °C using Novozyme 435 as biocatalyst. The values obtained from experimental data indicates that the reactions were enantioselective and ensure the acquiring of (*R*)-amine and (*S*)-amide with very good enantiomeric excesses and yields close to 50%. The enantioselective hydrolytic reactions were completed in 80 hours for *rac*-**5a**,**b** and 90 hours for *rac*-**5c**,**d** (**Table I.4.2.3.2**).

Another way to provide amines (*R*)-**4a-d,** implies deprotection of amides (*R*)-**5a-d** by CaL-A on Celite. This time also, the reactions were performed in water at 45 °C. After 60 hours they were considered finished, yielding quantitatively the corresponding enantiomerically enriched (*R*)-**4a–d** amines.

Table I.4.2.3.2 Preparative scale Novozyme 435 mediated hydrolysis of *rac*-**5a-d** (t = 30 hours)

Compound	η[a] (%)	***ee*** (%)	$[\alpha]_D^{25}$ [b]	***E***
(*R*)-**4a**	96	> 99	+6.4	» 200
(*S*)-**5a**	96	> 99	-176.4	
(*R*)-**4b**	93	> 99	+8.5	» 200
(*S*)-**5b**	93	98	-164.8	
(*R*)-**4c**	94	> 99	+18.8	» 200
(*S*)-**5c**	96	96	-147.4	
(*R*)-**4d**	91	98	+10.8	> 200
(*S*)-**5d**	95	94	-149.7	

[a] 50% of the racemates taken as 100% theoretical yields
[b] 10^{-1}deg×cm^2 ×g^{-1}; c = 1.0; CH_3OH for amines, $CHCl_3$ for amides

I.4.3 Determination of the absolute configuration

The absolute configuration of the new synthetized products was determined by comparing the rotation direction of plane polarized light with the values submitted in the literature. Also the (*R*)-enantiopreference of CaL-B shown here proved to be in accordance with the enantiopreference observed before for the enzyme in *N*-acylations of arylethanamines.[8,9]

Experimentally, the synthesis of *rac*-**2a** through the method described above, using CaL-B and vinyl acetate, led to (*S*)-**2a**.[10] The subsequent transformations of (*S*)-**2a** to (*R*)-**4a** did not significantly affect the enantiopurity of the involved compounds.

Chapter I.5 Conclusions

In this study a new efficient and ecological procedure for the synthesis of enantiomerically enriched (*R*)- and (*S*)-1-(2-phenylthiazol-4-yl)ethanamines was developed, except for the chlorinated compound.

We found that the lipase B from *Candida Antarctica* (Novozyme 435) is the proper catalyst for both enantioselective *N*-acylation and hydrolysis.

The deprotection of (*R*)-**5a–d** to the corresponding (*R*)-**4a–d** was achieved faster using CaL-A immobilized on Celite, without affecting the optical purity.

As summary of this part, eight new compounds with good yields, subsequently characterized from the structure point of view, were obtained and analyzed.

Part II Expression and purification of three phenylalanine ammonia lyase and one aminomutase involved in the biotransformation of L-phenylalanine and their unnatural analogues

Keywords: phenylalanine ammonia-lyase, phenylalanine aminomutase, protein expression and purification, stability, biocatalysis

In the last few years, the researchers focused on studying phenylalanine ammonia-lyase enzyme (PAL). It is considered one of the most important enzymes involved in the metabolism of plants, where it redirects the flow of carbon atoms resulted from the protein synthesis in order to get phenolic compounds. Furthermore, PAL has the capability to connect primary metabolism - the shikimate pathway - to secondary metabolism – the phenylpropanoid pathway.[11]

The ability of PAL to catalyze the conversion of L-Phe into non-toxic compounds, in the absence of additional cofactors, encouraged the scientists to pay attention to this enzyme due to its therapeutic potential in the treatment of phenylketonuria.[12, 13] It can also assist the synthesis of antifungal and antimicrobial agents, and the biosynthesis of antibiotics and some anticancer drugs.[14,15]

Therefore, an efficient isolation and purification of the concerned enzyme allow us to realize a complete study that contains information about the protein conformation, the substrate specificity, and also about its interaction with other substrates.

Chapter II.1 INTRODUCTION

– literature data –

Chapter II.2 Aims of the study

The research activity was focused on five directions:

1. the development of an efficient protocol in order to realize the expression and purification of three phenylalanine ammonia-lyase, two of eukaryotic origin (isolated from *Petroselinum crispum*–***Pc*PAL** and *Rhodosporidium toruloides*–***Rt*PAL**) and one of prokaryotic origin (*Anabaena variabilis*–***Av*PAL**), and also one prokaryotic aminomutase (*Pantoea agglomerans*–***Pa*PAM**)
2. the enzyme characterization from the stability point of view at different pH values and temperatures, as well as the study of various additives influence on lyase activity of *Pc*PAL.
3. the determination of the substrate affinity regard to the three ammonia-lyase for both L-Phe as well for the new synthetized substrates.
4. the preparation of (*S*)-β-phenylalanine in presence of *Pa*PAM and *Pc*PAL enzymes.
5. the study of phenylalanine interaction, substituted in *ortho*-, *meta*- and *para*- position with –NO_2, with two new phenylalanine ammonia-lyases: *wt-Pc*PAL and MIO_less *Pc*PAL, followed by the formation at preparative scale of both (D-, L-) amino acids.

Chapter II.3 MATERIALS AND METHODS

Chapter II.4 RESULTS

II.4.2 The introduction of plasmids into Rosetta(DE3)pLysS competent cells

The *E. coli* Rosetta(DE3)pLysS host strain was successfully transformed with the genes of interest. After the insemination on LB agar plates which contain the appropriate antibiotic, the *Pc*PAL, *Av*PAL, *Rt*PAL and *Pa*PAM microorganisms grew up under individual "S" colonies. Subsequently, they have been used for the expression of these proteins.

II.4.3 Expression and purification of proteins

Following the expression step, in which the protein expression was induced by addition of 0.1 mM IPTG at an OD_{600} = 0.7-0.8 at a low temperature, the purification of the four proteins trough Ni-NTA affinity chromatography succeed.

In what concerns the protocol used for the protein purification, the first step involved the cell membrane destabilization and subsequent purification of the enzyme labeled with 10XHis by affinity chromatography. This method is intensively used due to its efficiency and involves a relatively small number of steps. Eventually, all the samples collected during the purification process were analyzed by SDS-PAGE electrophoresis (**Figure II.4.3.1**).

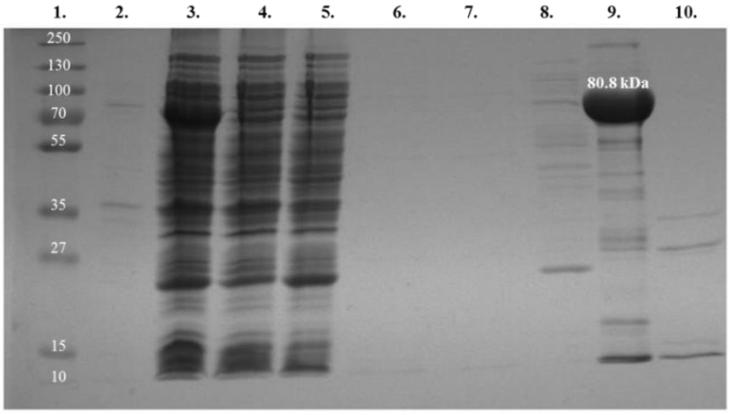


Figure II.4.3.1 SDS-PAGE gel performed in order to analyze the *Pc*PAL protein purification steps.

1- molecular weight size marker: PageRuler Plus Prestained Protein Ladder from Thermo Scientific,
2- the fraction containing the cell debris, **3**- bacterial lysate, **4**- flow through,
5- fraction LS, **6**- fraction HS, **7**- fraction LS, **8**- fraction 0.02 M IM,
9- fraction 0.50 M IM containing the purified protein, **10**- fraction 1.00 M IM.

The number 9 fraction (**Figure II.4.3.1**) denotes a 90-95% purity of *Pc*PAL protein; the percentage value was visually assessed.

The other three proteins, *Rt*PAL, *Av*PAL and *Pa*PAM were purified following the same protocol.

II.4.4 Determination of optimal pH

Since the enzyme activity is closely related to the environment pH, the next step consisted in the determination of the optimal pH for the phenylalanine ammonia-lyase enzymes.

Subsequent to the spectrophotometric measurements, the maximum of lyase activity for *Pc*PAL was reached around 8.8 (**Figure II.4.4.1.A.**). The other two enzymes, *Rt*PAL and respectively *Av*PAL, reach the optimum pH at 8.3 for the fungal enzyme (**Figure II.4.4.1.B.**) and respectively at 8.0 for the cyanobacterial enzyme (**Figure II.4.4.1.C.**).

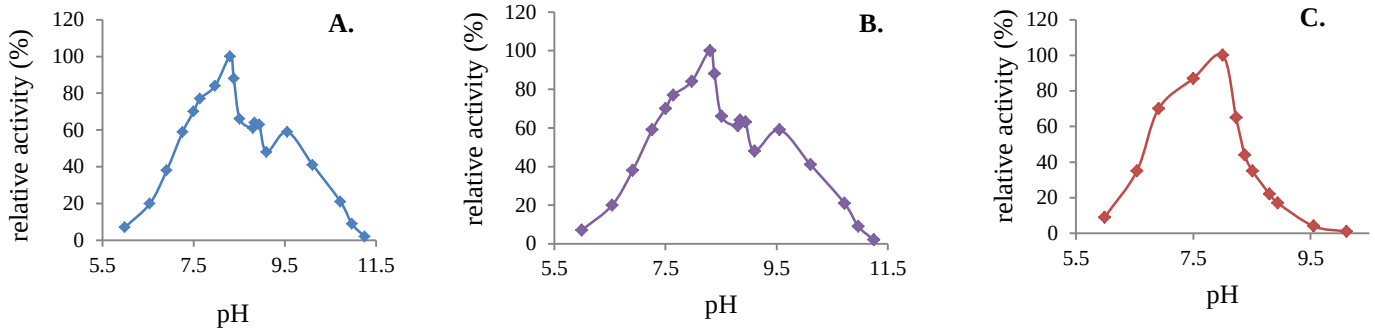


Figure II.4.4.1 The effect of pH on liase activite isolated from

A. *Petroselinum crispum*, **B.** *Rhodosporidium toruloides*, **C.** *Anabaena variabilis*

II.4.5 Determination of thermal stability

The thermal stability of the enzymes originary from *P. crispum*, *R. toruloides*, *A. variabilis* and *P. agglomerans* was determined using the Thermofluor assay.

The measurements were carried out both in the presence and in the absence of the ligand, in this case, the cinnamic acid. **Table II.4.5.1** includes the values obtained for the thermal stability of the four proteins. The melting temperature was determined by reading the inflection point value from the melting curve obtained from the experimental values (**Figure II.4.5.1**).

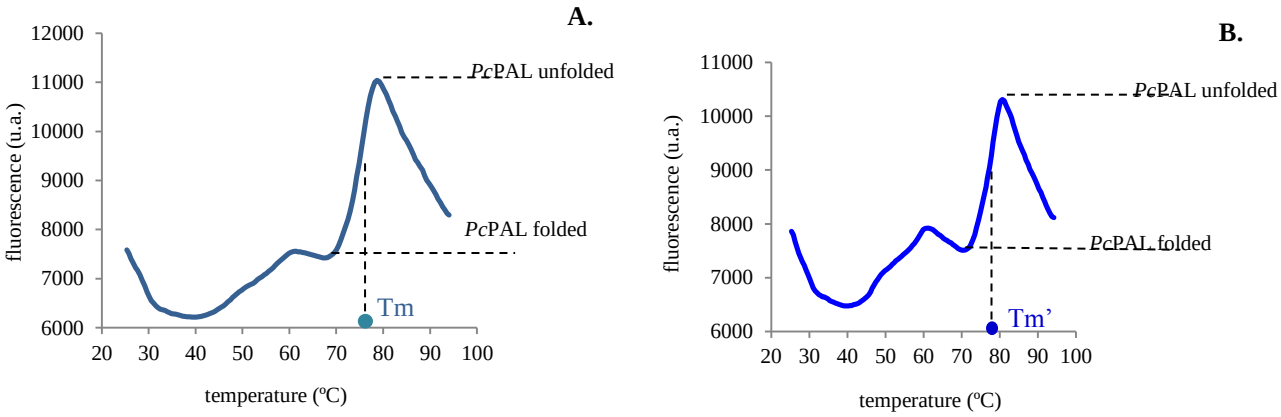


Figure II.4.5.1 Stability curves obtained for *Pc*PAL

A. in absence of cinnamic acid and **B.** în presence of cinnamic acid

Table II.4.5.1 Determination of thermal stability

Potein	**T** (ºC)	**T'** (ºC)
*Pc*PAL	76.0	78.0
*Rt*PAL	73.5	74.0
*Av*PAL	45.0	45.2
*Pa*PAM	72.0	73.0

T (ºC) – temperature values for samples without cinnamic acid

T' (ºC) – temperature values for samples containing cinnamic values

The T_m value for the prokaryotic enzyme *Av*PAL is about 31 ºC lower compared to the melting temperature of the eukaryotic enzyme *Pc*PAL and 27ºC lower than the *Rt*PAL T_m. These values indicate that *Av*PAL enzyme is less stabile compared to the other three enzymes, fact that is contrary to the assumption that the C-terminal extension domain serve to destabilize the eukaryotic enzymes.[16]

II.4.6 The influence of temperature on *Pc*PAL activity having L-phenylalanine as substrate

Table II.4.6.1 Variation of K_M and v_{max} depending on temperature

T (ºC)	$\mathbf{K_M}$ (µM)	$\mathbf{v_{max}}*10^{-2}$ (µM/s)	$\mathbf{v_{max}}/\ \mathbf{v_{max\ L\text{-}Phe}}$ (–)
30	49.5	6.83	1.00
45	119.0	6.83	1.00
50	179.0	9.11	1.33
55	193.0	9.11	1.33
60	243.0	13.7	2.00

From the experiments results we conclude that, once the temperature increases, the enzyme affinity for the substrate decreases, while the reaction rate rises.

II.4.7 The influence of several additives on *Pc*PAL activity

II.4.7.1 The effect of metal ions on *Pc*PAL activity

The investigations performed in the presence of various metal ions revealed the inhibitory effect of Zn^{2+} and Cu^{2+} ions, which at 2.0 mM concentration led to enzyme activity decrease by 50%. Contrary, other metal ions like Mn^{2+} and Co^{2+} proves to have a positive effect, enhancing the lyase activity at different concentrations (**Table II.4.7.1.1**)

Table II.4.7.1.1 *Pc*PAL residual activity determination in presence of metal ions

Metallic ion	**Residual activity** (%) (10 mM L-Phe)		
	0.5 mM	**1.0 mM**	**2.0 mM**
Li^+	92	92	91
Na^+	114	101	100
K^+	110	108	106
Mg^{2+}	73	70	75
Ba^{2+}	83	83	80
Mn^{2+}	**160**	119	119
Co^{2+}	90	109	**151**
Cu^{2+}	91	72	44
Zn^{2+}	88	78	48

II.4.7.2 The effect of organic solvents on *Pc*PAL activity

After the incubation of *Pc*PAL in 0.1 M Tris for 5 min at 30 ºC the reaction was initiated by the addition of the substrate and subsequently of the substrate together with polar organic solvents. The acrylate formation was spectrophotometrically monitored at 290 nm for 5 min. **Table II.4.7.2.1** contains the results of the residual activity. The ammonia-lyase activity was enhanced by approximately 27% in the presence of DMSO and respectively 25% in the presence

of MeOH. On the other hand, a high percentage of ACN and 1-PrOH led to the enzyme activity decrease at half.

Table II.4.7.2.1 *Pc*PAL residual activity determination in presence of organic solvents

Organic solvent	**Residual activity** (%) (10 mM L-Phe)		
	1%	**5%**	**10%**
1-PrOH	109	96	65
2-PrOH	110	109	99
MeOH	125	124	115
EtOH	113	110	114
ACN	99	78	56
DMSO	127	126	123

II.4.7.3 The simultaneous use of several additives on *Pc*PAL activity

The following studies were focused to determine the kinetic parameters K_M and v_{max} in the reactions whereby MeOH was combined with DMSO, EtOH with DMSO, and also, in the reaction involving Mn^{2+} with MeOH and DMSO.

Table II.4.7.3.1 Determination of kinetic parameters in the reaction catalyzed by *Pc*PAL having L-Phe as substrate

	$\mathbf{K_M}$ (μM)	$\mathbf{v_{max}}*10^{-1}$ (μM/s)	$\mathbf{v_{max}}/\mathbf{K_M}*10^{-4}$ (s)
Control	385	1.00	2.60
DMSO	350	1.00	2.86
EtOH	368	1.00	2.72
EtOH:DMSO	276	1.11	4.00
MeOH	362	1.11	3.07
MeOH:DMSO	132	1.00	7.58
MeOH:DMSO:Mn	201	1.11	5.52

Tabel II.4.7.3.2 Determination of kinetic parameters in the reaction catalyzed by *Pc*PAL having *rac*-2-amino-3-(tiophen-2-yl)propanoic acid as substrate

	K_M (μM)	$v_{max}*10^{-1}$ (μM/s)	v_{max}/K_M*10^{-4} (s^{-1})	Conversion after 6 hours
Control	935	0.33	0.35	43
DMSO	983	0.33	0.34	42
EtOH	5110	1.00	0.20	43
EtOH:DMSO	299	1.67	5.60	44
MeOH	1230	0.33	0.27	43
MeOH:DMSO	176	1.43	8.13	45
MeOH:DMSO:Mn	220	1.43	6.50	41

In both cases, the experimental results indicate that the enzyme originary from parsley shows higher affinity for the combination of MeOH with DMSO, followed by MeOH:DMSO and Mn^{2+}. Good results were also obtained for the association of EtOH with DMSO.

II.4.8 The effect of 6a-g and 7a-b on lyase activity of the three enzymes

The lyase activity of the three enzymes (*Pc*PAL, *Av*PAL, *Rt*PAL) was tested in presence of various substrates, different from L-Phe. Thus, benzofuran, phenylthiophenes and its derivatives dissolved in 0.1 M Tris-HCl at the optimum pH were added to the enzyme which was previously incubated for 5 min at 30 ºC. Product formation was monitored at wavelengths of acrylates were the corresponding amino acids does not show absorption. **Tables II.4.8.2-4** summarizes the kinetic parameters, K_M and v_{max}, and the ratio between the relative maximum speed and the speed obtained for the unsubstituted derivatives.

Table II.4.8.2 Kinetic data of *Pc*PAL in presence of *rac*-**6a-g**

Entry	Substrate	K_M (μM)	$v_{max*}10^{-3}$ (μM/s)	v_{max}/v_{maxBF} (–)
1	**6a**_amino acid	248	16.10	1.000
2	**6b**_amino acid	8000	29.00	0.181
3	**6c**_amino acid	1770	0.30	0.018
4	**6d**_amino acid	133	0.15	0.009
5	**6e**_amino acid	882	2.92	0.182
6	**6f**_amino acid	903	0.17	0.011
7	**6g**_amino acid	3240	5.19	0.323

The values obtained for the *rac*-**6a-g**, indicates that the highest affinity was achieved for 5-methyl-benzofuran-alanine (*rac*-**6d**), even higher than in the case of unsubstituted substrate. The high value of K_M indicates the appearance of a steric hindrance of these compounds during their docking to the catalytic site of the enzyme, the affinity depending on both the substituents nature and their electronic effect.

Concerning the v_{max}, 5-methyl-benzofuran was slowly transformed, even if it shows high affinity for the substrate, while 5-nitro and 7-ethyl-benzofuran present a high value of the reaction speed.

According to the results included in **Table II.4.8.3** and **Table II.4.8.4** it can be observed that PAL's from *Rhodosporidium toruloides* and *Anabaena variabilis* accept a small number of heteroarilalanine compared to *Pc*PAL.

Table II.4.8.3 Kinetic data of *Rt*PAL in presence of *rac*-**6a-b** and *rac*-**6e**

Entry	Substrate	K_M (μM)	$v_{max}*10^{-3}$ (μM/s)	v_{max}/v_{maxBF} (-)
1	**6a**_amino acid	2670	8.0	1.000
2	**6b**_amino acid	22200	0.8	0.100
3	**6e**_amino acid	123	0.9	0.111

Table II.4.8.4 Kinetic data of *Av*PAL in presence of rac-**6a** and *rac*-**6e**

Nr.	Substrate	K_M (μM)	$v_{max*}10^{-3}$ (μM/s)	v_{max}/v_{maxBF} (-)
1	**6a**_amino acid	101	7.6	1.000
2	**6e**_amino acid	389	3.0	3.947

Next, it was investigated the effect of the temperature upon the interaction of phenyl-tiophen-2-yl-alanines, *rac*-**7a** și *rac*-**7b**, with PAL from parsley. The results show a significant increase in the affinity of the enzyme along with temperature rises (**Table II.4.8.5**).

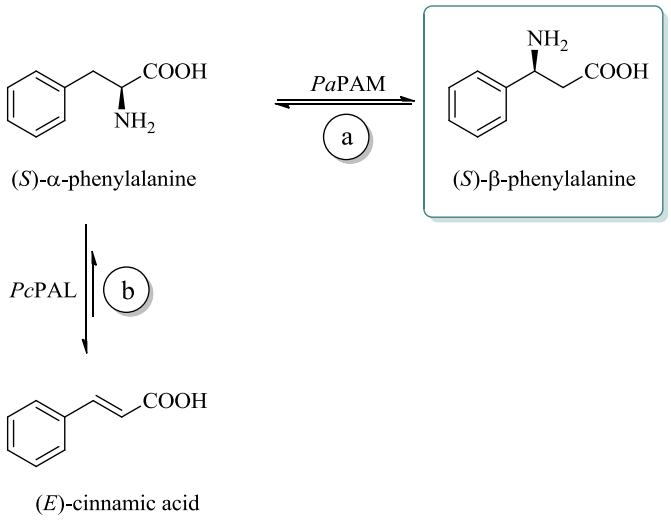
Tabel II.4.8.5 Kinetic data for the interaction of *rac*-**7a-b** with *Pc*PAL

Substrate 7a_amino acid				**Substrate 7b_**amino acid			
T (ºC)	**K_M** (μM)	**v_{max}*10^{-3}** (μM/s)	**v_{max}/v_{maxFT}** (-)	**T** (ºC)	**K_M** (μM)	**v_{max}*10^{-3}** (μM/s)	**v_{max}/v_{maxFT}** (-)
30	97	3.3	1.00	30	181	100.0	1.00
40	36	3.3	1.00	40	150	140.0	1.40
45	221	10.0	3.03	45	170	200.0	2.00
50	423	10.0	3.03	50	195	160.0	1.60
55	86	16.0	4.85	55	134	250.0	2.50
60	61	50.0	15.15	60	97	330.0	3.30

II.4.9 Preparation of (*S*)-β-phenylalanine

The (*S*)- enantiomer of β-phenylalanine was obtained by joining two steps (**Scheme II.4.9.1**). In the first stage, *Pa*PAM catalyzes the conversion of (S)-α-phenylalanine to (S)-β-phenylalanine. After 29 hours the reaction reached a 50% conversion, in accordance with the equilibrium concentration.

In the second part, (S)-α-phenylalanine was completely converted to cinnamic acid by addition of *Pc*PAL enzyme, resulting (*S*)-β-phenylalanine.



Scheme II.4.9.1 Preparation of (*S*)-β-phenylalanine using *Pa*PAM and *Pc*PAL

The High Performance Liquid Chromatography (HPLC) conduced with Agilent 1200 by using a Chiralpak ZWIX(+) column was used in order to monitorize the reactions.

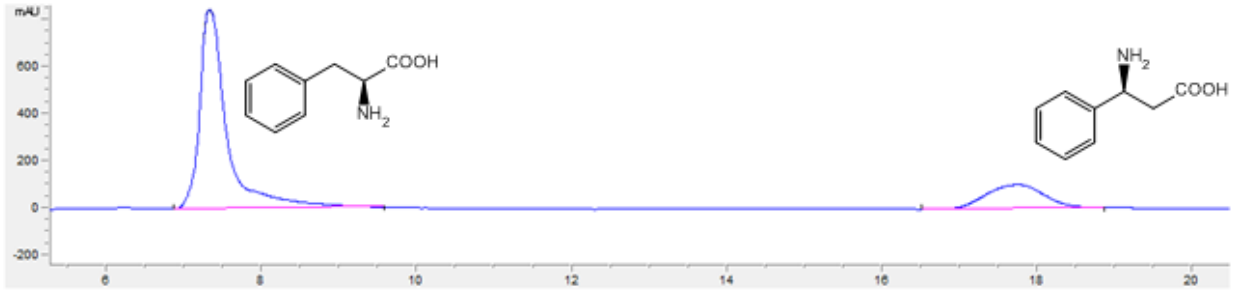


Figure II.4.9.1 The HPLC chromatograms obtained after 2 hours, for the enzymatic isomerization of (*S*)-β-phenylalanine in presence of *Pc*PAM, c = 22%

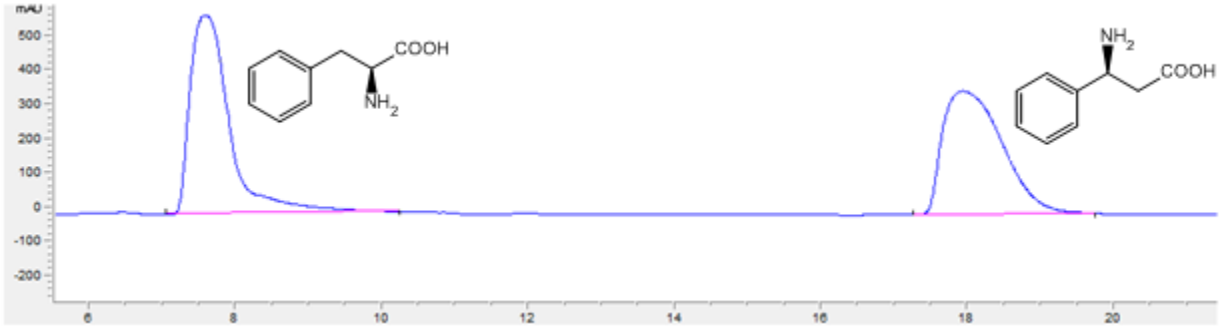


Figure II.4.9.2 The HPLC chromatograms obtained after 29 hours, for the enzymatic isomerization of (*S*)-β-phenylalanine in presence of *Pc*PAM, c = 50%

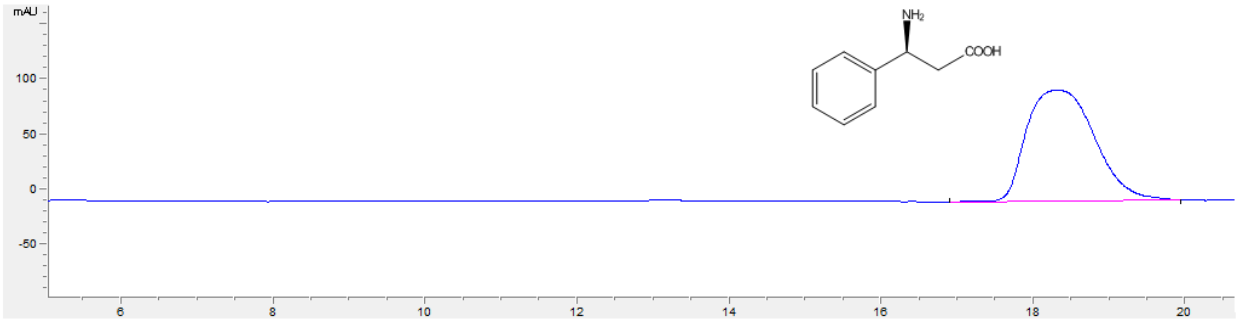


Figure II.4.9.3 The HPLC chromatograms obtained after 24 hours, for the ammonia elimination reaction from (*S*)- α-phenylalanine in presence *Pc*PAL, c = 100%

II.4.10 The interaction of *wt-Pc*PAL and MIO_less *Pc*PAL with *rac*-8a-c

After determining the effect of *rac*-**6a-g** and *rac*-**7a-b** on the purified ammonia-lyase, the next step consisted on studying the interaction of phenylalanine substituted in position 2, 3 and 4 with $–NO_2$ group with two new phenylalanine ammonia-lyases: *wt-Pc*PAL and MIO_less *Pc*PAL. The two enzymes are a gift from Prof. Janos Rétey, Karlsruhe University, Germany. Thereafter, we obtained at preparative scale both enantiomers of the unnatural amino acids *rac*-**8a-c** (**Scheme II.4.10.1**).

In order to determine the kinetic parameters, the measurements were based on spectrophotometrically determination of the formed acrylates at wavelengths, where the corresponding amino acids do not show absorption. The enzymatic assays were carried out at 30 °C in 0.1 M Tris-HCl (pH 8.8) by varying substrate concentration. **Table II.4.10.1** contains the K_M values and the maximal velocity values (v_{max}) acquired for *rac*-**8a-c,** relative to that of L-phenylalanine (v_{maxPhe})

Table II.4.10.1 The influence of *rac*-**8a-c** on the activity of *wt-Pc*PAL and MIO less_ *Pc*PAL

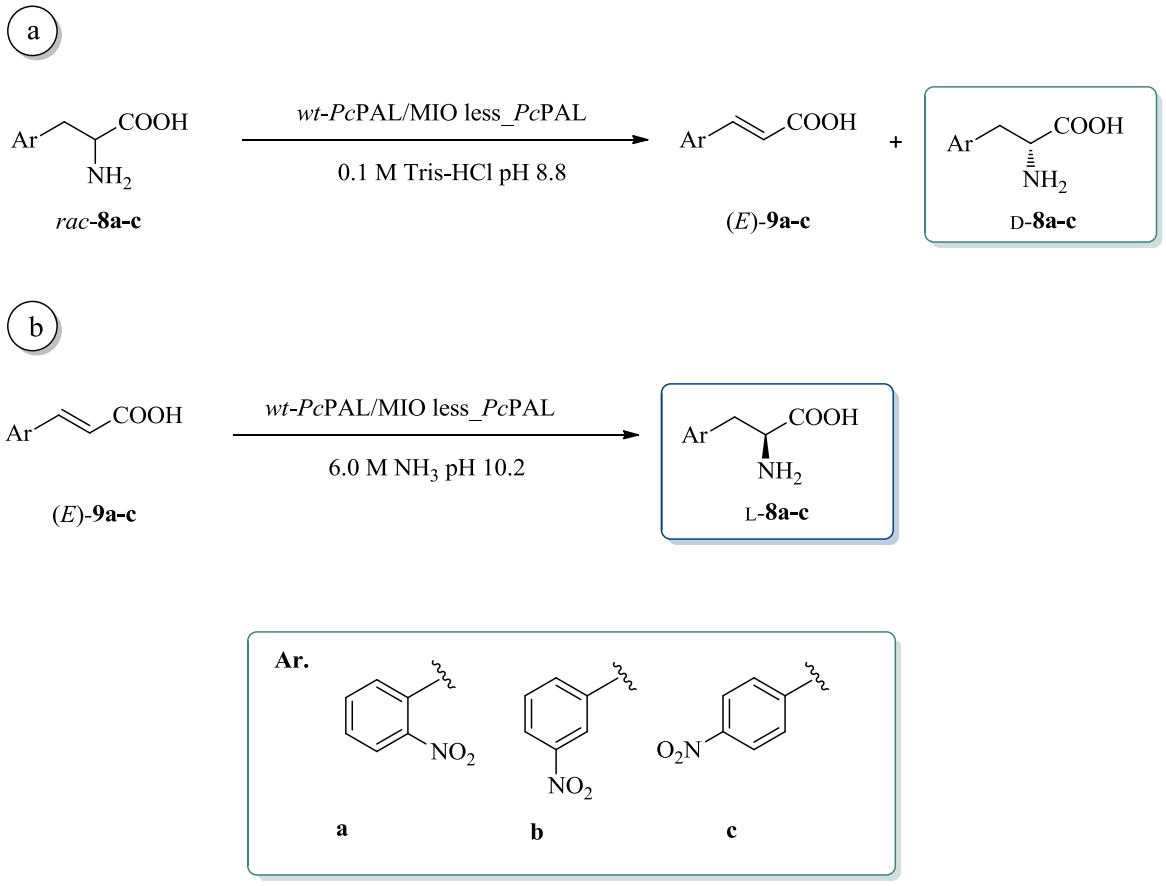
Kinetic constants for *Pc*PAL				**The relative velocities**
Substrate	**K_M** (μM)	**v_{max}/v_{maxPhe}** (–)	**λ** (nm)	**$v_{max\ PcPAL}/v_{max\ MIO\ less\_PcPAL}$** (–)
L-Phe	33	1.00	290	413
rac-**8a**	268	0.57	243	244
rac-**8b**	65	0.21	260	52
rac-**8c**	296	0.86	340	411

The results indicate that for *rac*-**8a** and *rac*-**8c** the transformations take place faster compared to nitrophenilalanine substituted in *meta*- position. Instead, the affinity of the enzyme for the above mentioned substrates is approximatively four times lower reported to *rac*-**8b**.

In order to investigate the interaction of MIO less_*Pc*PAL with the three nitrophenylalanines and with L-Phe, similar experiments were performed. Due to its small activity (about 400 times lower for the natural substrate compared to *wt-Pc*PAL), it was possible

to determine only the relative maximal velocities (**Table II.4.10.1**). For reliable results it was necessary to use a 10 times higher concentration of MIO less_*Pc*PAL than those of *wt-Pc*PAL.

This study reveals that both mentioned enzymes successfully mediate the nitrophenylalanine deamination.



Scheme II.4.10.1 Biotransformation mediated *wt-Pc*PAL and MIO less_*Pc*PAL in order to obtain (D-, L-)enantiomers of **8a-c**

Based on the results obtained by the kinetic measurements, the synthesis of D-**8a-d** at preparative scale was performed by incubating at 30 ºC the corresponding racemates (0.5 mM) dissolved in 0.1 M Tris-HCl pH 8.8 with different amounts of *wt-Pc*PAL. The progress of the reaction was monitored by HPLC using a Chirobiotic TAG column (**Table II.4.10.2**). The complete transformation of L-amino acids, followed by pH adjustment to 1.5, and the

inactivation and removal of the enzyme from the reaction mixture by filtration, led to the isolation and purification of D-amino acids through cation exchange column.

The synthesis of L-amino acids is based on the stereoconstructive reversible reaction that involves the ammonia addition on achiral unsaturated precursors. In order to obtain the desired enantiomer, *wt-Pc*PAL was added in the reaction mixture which also contains 0.5 mM (*E*)-**8a-c** dissolved in 6 M ammonia solution (pH 10.2). This time also, the reaction occurred under inert atmosphere at 30°C. HPLC was used to monitorize the reaction (**Table II.4.10.2**). The isolation and purification of L-**8a-c** enantiomers were performed under the same conditions previously mentioned.

Table II.4.10.2 Preparative scale synthesis of D- and L- nitrophenylalanines in presence of *wt-Pc*PAL

Substrate	**Product** [a]	η[b] (%)	***wt-Pc*PAL**[c] (UI)	**Time** (days)	$[\alpha]_D^{25}$ [d]
(*E*)-**9a**	L-**8a**	79	2	3	-11.5
(*E*)-**9b**	L-**8b**	65	5	3	-13.4
(*E*)-**9c**	L-**8c**	88	1	3	-7.8
rac-**8a**	D-**8a**	45	3	3	+11.4
rac-**8b**	D-**8b**	45	7	5	+13.3
rac-**8c**	D-**8c**	46	3	2	+7.7

[a] *ee* > 98% in all cases

[b] yields for the isolated products

[c] 1 UI/mg *wt-Pc*PAL

[d] in H_2O, at 20 °C

Similar experiments involving the biotransformation of *rac*-**8a-d** and (*E*)-**8a-c** at preparative scale were also conducted for MIO less_*Pc*PAL. The yields obtained in the ammonia elimination reaction, catalyzed by the mutant ammonia lyase, were 20-30% lower even after 7 days, while the ammonia elimination from *rac*-**8a-c** was not possible.

Chapter II.5 Conclusions

The second part of this work materialized through attaining with a high grade of purity the ammonia-lyases from *Petroselinum crispum*, *Rhodosporidium toruloides*, *Anabaena variabilis* and the aminomutase from *Pantoea agglomerans*.

In what concerns the stability based on the pH of the environment, we found that the optimum pH for ammonia-lyases is situated in the basic domain. Thermofluor measurements indicate that, among the four studied enzymes, the ammonia lyase from *Anabaena variabilis* has the lowest value of Tm, 45 ºC, while the others present good stability around 72-76 ºC.

The results obtained for the reaction that involves metal ions on the activity of ammonia-lyase from *Petroselinum crispum* revealed that Mn^{2+} and Co^{2+} enhanced the enzyme activity up to 60% and 50%, while Zn^{2+} and Cu^{2+} ions decreased the enzyme activity. All the other cations: Li +, Na +, K +, Mg 2+, Ba2 + did not significantly affect the enzyme activity or they showed a moderate inhibitor.

Among the tested organic solvents, 1% MeOH and 1% DMSO enhance the *Pc*PAL activity. On the other hand, a high percentage of ACN and 1-PrOH decreases the enzyme activity at half. Based on the earlier results, the determined K_M and v_{max} kinetic parameters show high affinity and reactivity for the combination of MeOH with DMSO. Good results were also obtained for the association of MeOH:DMSO and Mn^{2+}.

The subsequent studies were supposed to determine the substrate affinity and the reaction rate of the three ammonia-lyases (*Pc*PAL, *Av*PAL, *Rt*PAL) for L-Phe and for the unnatural analogues: benzofuran and phenylthiophenes. The results confirm that the enzymes can successfully be used as biocatalyst.

The (*S*)- enantiomer of β-phenylalanine was obtained in the presence of *Pa*PAM and *Pc*PAL starting from (S)-α-phenylalanine.

In the end, the results attained in the experiments performed in the presence of *wt-Pc*PAL and MIO less_ *Pc*PAL consolidate the assumption that the architecture of the catalytic site of PAL is responsible for the stereoselectivity of the enzymatic reactions, while the prosthetic MIO is responsible to activate the substrate without its direct involvement in the ammonia addition/elimination process.

LIST OF PUBLICATIONS

I. Scientific publication

1. M.I. Toșa, J. Brem, A. Mantu, F.D. Irimie, C. Paizs. J. Rétey, *ChemCatChem* **2012**, *5*, 779–783.
2. A. Radu, M.E. Moisă, M.I. Toșa, N.D. Dima, V. Zaharia, F.D. Irimie, *J. Mol. Catal. B: Enzymatic* **2014**, *107*, 114–119.

II. Conference publications:

1. M.E. Moisă, M.A. Naghi, A. Mantu, F.D. Irimie, *CaL-B mediated synthesis of optically pure (R)- and (S)-ethyl 3-hydroxy-3-(2-aryl-thiazol-4-yl)propanoates*, 9th International Conference "Students for Students" 10-13 May, 2012, Cluj-Napoca, Romania.

2. A. Mantu, A. Varga, B. Nagy, M.I. Toșa, F.D. Irimie, C. Paizs, *Expression and Purification of Various Types of PAL and PAM*, 13th Symposium and Summer School on Bioanalysis, 27 June-7 July, 2013, Debrecen, Hungary.

3. M.E. Moisă, A. Radu, F.D. Irimie, *Lipases as versatile catalysts for the enzymatic kinetic resolution of 1-heteroaryl-ethanamines*, 11th International Conference "Students for Students" 9-13 April, 2014, Cluj-Napoca, Romania.

4. A. Radu, D. Weiser, M.I. Toșa[1], F.D. Irimie, L. Poppe, C. Paizs, *The Influence of Several Additives on the Phenylalanine Ammonia Lyase Activity*, International Conference "Biotransformations for Pharmaceutical and Cosmetic Industry" 23-24 October, 2014, Warsaw, Polonia.

5. S.D. Tork, A. Radu, C. Paizs, *Medium engineering for enhanced biocatalytic power of various PAL enzymes*, 12th International Conference "Students for Students" 23-26 May, 2015, Cluj-Napoca, Romania.

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