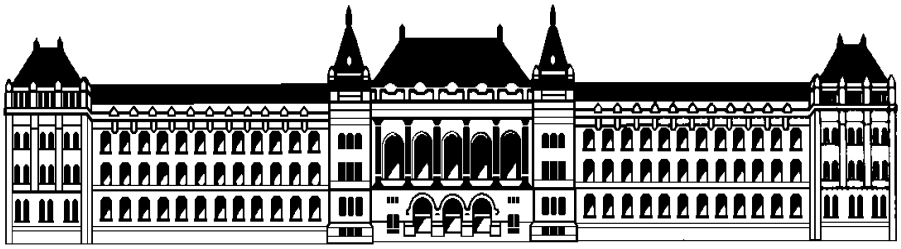

BUDAPEST UNIVERSITY OF TECHNOLOGY AND ECONOMICS
FACULTY OF CHEMICAL TECHNOLOGY AND BIOTECHNOLOGY
GEORGE A. OLAH DOCTORAL SCHOOL

DEVELOPMENT OF BIOCATALYSTS FOR PRODUCTION OF ENANTIOPURE COMPOUNDS

PHD THESISBOOK

AUTHOR: PÁL CSUKA

SUPERVISOR: DR. LÁSZLÓ POPPE

DEPARTMENT OF ORGANIC CHEMISTRY AND TECHNOLOGY

2021

1. Introduction

The topic of my dissertation belongs to the biotechnology and its diverse parts. Biotechnology have been developing together with human and technological area of our life. The biotechnological sector influences highly the national economy through the important sectors of food industry, agricultural industry, pharmaceutical industry, energy industry, chemical industry and environmental protection. The traditional biotechnology based on the fermentation technologies. It deals with the production of organisms and create natural products. The modern aspect of the biotechnology begins from the gene technology. The opportunity of change and influence genes and take in another organism creates the chance to develope highly in the biotechnology. This novelty opened the opportunity for research fields which uses recombinant proteins. This dynamic development created novel individual scientific areas such as: enzymology, immunology, molecular diagnostics or bioinformatics. Figure 1. shows the main parts of biotechnology.

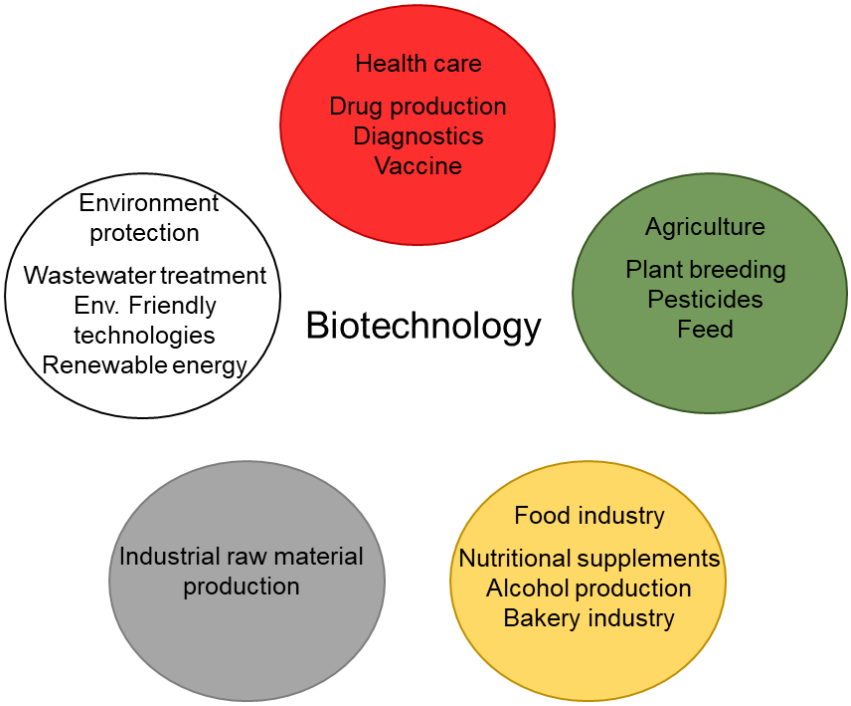

Figure 1. Sections of biotechnology

My research topic focuses on different types of biocatalysts which are usable in diverse technologies and create products in highly enantiopure form. We investigated a lipase enzyme for kinetic resolution of amines to create amides in high enantiopurity, which are raw material for the creation of potential tyrosine kinase inhibitors. Furthermore we investigated whole-cell yeasts which are well usable for stereoselective ketone reduction using its oxidoreductase enzymes to create (*S*)-alcohols in high enantiopurity. We investigated six yeast strains with three different testsubstrate in orthogonal designed experiments to determine the optimal reaction conditions. After the optimization we characterized the yeast strains by activity in wide pH range, by reaction temperature and by the (*S*)-alcohol production in different substrate concentrations. We also identified novel MIO-enzymes (aromatic ammonia-lyases and 2,3-

aminomutases) and after the searching with homology in databases we predicted their natural substrates. After the production we want to characterize the enzymes and we investigated their utilization. Our goal was to use this enzymes in kinetic resolutions and stereoselective ammonia addditions to create amino acid derivatives in high enantiopurity.

2. Background

2.1. Kinetic resolution with lipase B from *Candida antarctica*

The lipase B from *Candida antarctica* (*Ca*LB) is an industrially relevant biocatalyst for hydrolysis, esterification and acylation reactions. The *Ca*LB enzyme are used in immobilized form.[1] The biocatalyst utilization are realizable in organic solvents for kinetic resolution of amines and alcohols. The acylation reactions were investigated with aromatic and alphalitic amines and alcohols also: 2-butylamine, 1-phenylethylamine, 2-phenyl-1-propylamine, indole, butan-2-ol and with several acylating agents: ethyl acetate, isopropyl acetate, ethyl methoxiacetate, isopropyl butanoate, or butyl acetate.[2,3] The kinetic resolution of racemic 1-phenylaethylamine is resolved with ethyl methoxiacetate acylating agent by the patent of BASF. There is the possibility to separate the produced (*R*)-amide and the non responsive (*S*)-amine by boiling point. The products are in high enantiopurity.[4]

2.2. Stereoselective synthesis of alcohols with yeasts with ketoreductase activity

The ketoreductases belong to the oxidoreductase enzyme family. They catalyze the reduction of ketones into secondary alcohols or the oxidation of these alcohols into ketones. The NAD(P)H cofactors are essential for the catalyzation, which provides the hydrogen transportation in the reactions. In case of the carbon chains are different by the ketone group there is the possibility to produce two different enantiomer by the reduction. The preferable enantiomer production or consumption depends on the enzyme. This preference depends on the enzyme structure and the binding of the cofactor and the substrate. The ketoreductases are widespread enzymes in nature and they are responsible for many metabolite production in different methabolic pathways. Therefor one organism contains lots of ketoreductases and their properties may be different. The most conspicuous properties are the substrate acceptance. [5,6] The utilization of whole-cell yeast strains are effective biocatalyst because their production by fermentation is a simple technology. The major part of the yeasts are non-dangerous, if the safety protocols and regulations are complied. The whole-cell yeast have the possibility to

[1] G. Hellner, Z. Boros, A. Tomin, L. Poppe, *Adv. Synth. Catal.* **2011**, *353*, 2481–2491.
[2] Z. Boros, P. Falus, M. Márkus, D. Weiser, M. Oláh, G. Hornyánszky, J. Nagy, L. Poppe, *J. Mol. Catal. B Enzym.* **2013**, *85–86*, 119–125.
[3] M. Oláh, S. Suba, Z. Boros, P. Kovács, M. Gosselin, C. Gaudreault, G. Hornyánszky, *Period. Polytech. Chem. Eng.* **2018**, *62*, 519–532.
[4] A. Schmid, J. S. Doderick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholt, *Nature* **2001**, *409*, 258–268.
[5] G. W. Huisman, J. Liang, A. Krebber, *Curr. Opin. Chem. Biol.* **2010**, *14*, 122–129
[6] S. F. Haq, A. P. Shanbhag, S. Karthikeyan, I. Hassan, *Microb. Cell Fact.* **2018**, 1–14.

realize the cofactor regeneration in the cell parallel with the bioreduction. The yeast strains are widespread biocatalyst by different reaction types, such as: ketoreduction, C=C reduction, hydrolytic reaction and C-C covalent bond formation.[7,8]

2.3. Identification and utilization of MIO-enzymes

The MIO-enzymes are aromatic ammonia-lyases and 2,3-aminomutases which have a posttranslational forming catalytic group (3,5-dihydro-5-methylidene-4*H*-imidazol-4-one; MIO group). The enzymes catalyze the reversible non-oxidative ammonia elimination and *α,β*-isomerization. This catalytic moiety are required for all MIO-enzyme. It is proved with single point mutations of the essential presence of the MIO forming ASG or TSG amino acid triad. The mutations causes the total loss of thecatalytic activity.[9,10]

To the MIO-enzymes belong three main ammonia-lyase group, but there are also overlappings. The main enzyme groups are the phenylalanine ammonia-lyases (PAL), tyrosine ammonia-lyases (TAL) and histidine ammonia-lyases, but there already have been described several the phenylalanine/tyrosine ammonia-lyases. The 2,3-aminomutases: phenylalanine 2,3-aminomutase (PAM) and tyrosine 2,3-aminomutase (TAM) catalyze the *α,β*-isomerization in enantioselective form. Figure 2 describe the grouping of the MIO-enzyme family.

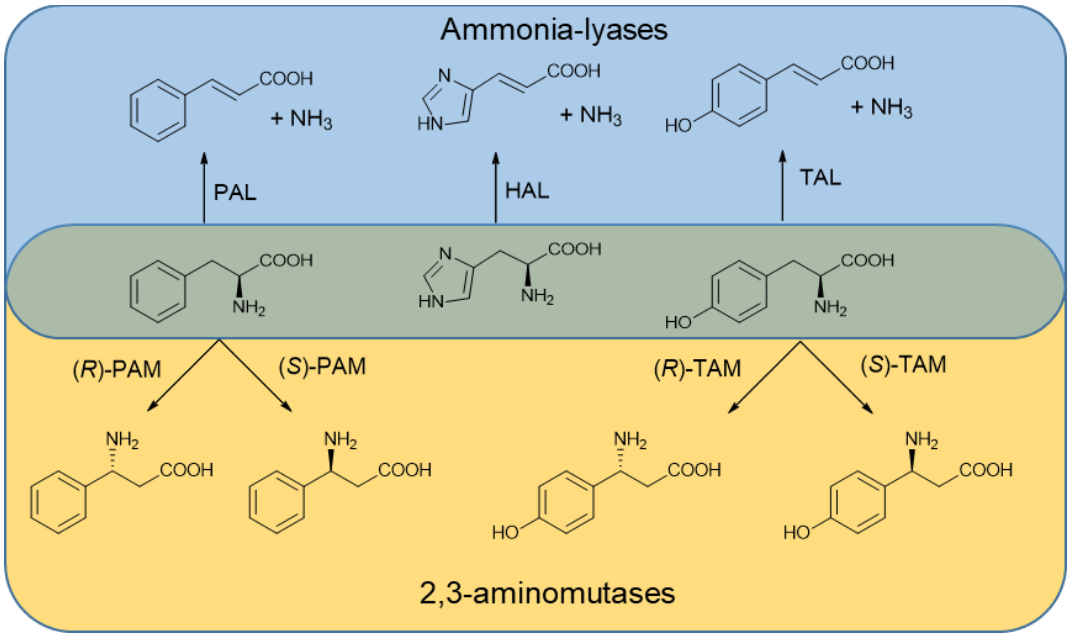

Figure 2. Classification of MIO-enzymes based on the catalyzed reaction

[7] D. Kuhn, L. M. Blank, A. Schmid, B. Bühler, *Eng. Life Sci.* **2010**, *10*, 384–397.
[8] B. Pscheidt, A. Glieder, *Microb. Cell Fact.* **2008**, *7*, 1–36.
[9] L. Poppe, *Curr. Opin. Chem. Biol.* **2001**, *5*, 512–524.
[10] F. Parmeggiani, N. J. Weise, S. T. Ahmed, N. J. Turner, *Chem. Rev.* **2018**, *118*, 73–118.

3. Experimental section

3.1. Kinetic resolution of *rac*-1-phenylethylamine

Racemic 1-phenylethylamine *rac*-**1** (50 mg, 0.41 mmol), *Ca*LB N435 (100 mg) and alkyl 2-cyanoacetate (ethyl 2-cyanoacetate **2a**, 23 mg, 0.5 equiv.; or isopropyl 2-cyanoacetate **2b,** 26 mg, 0.5 equiv.) were added to a mixture of toluene-THF 1:1 (2 mL) and the resulting mixture was shaken (400 rpm) at room temperature for 4 h (sampling at 1, 2, 3 and 4 h was performed). At the end of the reaction, the biocatalyst was filtrated off and the filtrate was evaporated in vacuum. The residue was dissolved in 5% HCl (7.5 mL) and the solution extracted with dichloromethane (3×12 mL). The organic phase was dried and evaporated to give pure (*R*)-**3**. To the aqueous phase was added 40% NH_3 solution (5 mL) and it was extracted with dichloromethane (3×12 mL). The organic phase was dried and evaporated to give unreacted (*S*)-1-phenylethanamine (*S*)-**1**.

To a mixture of toluene-THF 1:1 (2 mL) were added racemic 1-phenylethylamine *rac*-**1** (50 mg, 0.41 mmol), *Ca*LB N435 (100 mg) and ethyl 2-cyanoacetate **2a** (Method A: 23 mg, 0.5 equiv.; Method B: 46 mg, 1 equiv.; Method C: 23 mg, 0.5 equiv., further 11.5 mg, 0.25 equiv. after 2 h and further 11.5 mg, 0.25 equiv. after 4 h) and the resulting mixture was shaken (400rpm) at room temperature for 24 h (sampling at 2, 4, 6 and 24 h was performed). The reaction mixture were processed as described before.

3.2. Optimization of ketoreduction with design of experiments (DOE)

Optimization of the bioreduction conditions for the selected model ketones **6a-c** were planned with the DOE module of Statistica software. The following factors were investigated: lyophilized biocatalyst amount, co-substrate volume, buffer strength, temperature, and pH. The DOEs were designed and evaluated with Statistica 13 software for every strain–substrate combinations. The results were assessed with the Effect Estimates and Fitted Surface visualization. The first run of the optimization was designed in a $2^{(5-2)}$ design with foldover and triplicates in the center points. The curvatures and the significances of effects and interactions were evaluated. The investigated parameters have been considered to have no significant effect on the conversion or enantiomer excess if the p-values were above 0.05. Then, a second run of optimization with DOE was designed with 2^2 or 2^3 full factorial model with triplicates in the center points. The values of the significant reaction parameter were adjusted based on the results of the first optimization round. If the curvature check showed significant result ($p<0.05$) the DOE was completed to a DOE of 3^2. In the quadratic models both the linear and the quadratic effects were evaluated.

Reactions with the strains WY1, WY4 and WY12 were done in 5 mL volume, reactions with the strains WY2, WY7 and WY11 were performed in 1 mL volume. Reactions were carried out at 6 mg mL^{-1} substrate concentration in sodium phosphate buffer with lyophilized yeast cells as biocatalysts in the presence of 2-propanol as cosubstrate. The reaction mixtures were shaken at 300 rpm for 4 h except for the productivity profiling where the samples were taken after 15 min.

3.3. Identification and utilization of MIO-enzymes

3.3.1. Identification and production of MIO-enzymes

The potential gene of MIO-enzymes were identified in the NCBI database with blastp searches with specified MIO sequence fragments. The UniProt Align tool was used for sequence analysis and comparison to other aromatic ammonia-lyase and 2,3-aminomutase sequences [*Ustilago maydis* PAL (*Um*PAL, Q96V77), *Rubrobacter xylanophilus* PAL (*Rx*PAL, Q1AV79), *Anabaena variabilis* PAL (*Av*PAL, Q3M5Z3), *Petroselinum crispum* PAL (*Pc*PAL, P24481), *Aradopsis thaliana* PAL (*At*PAL, P35510), *Sorghum bicolor* PAL (*Sb*PAL, C5XXT9), *Rhodobacter sphaeroides* TAL *(Rs*TAL, Q3IWB0), *Rhodosporidium toruloides* TAL (*Rt*TAL, P11544), *Pseudomonas fluorescens* XAL (*Pf*XAL, K0W9Y7), *Pseudomonas fluorescens* HAL (*Pf*HAL, K0WDT6), *Pseudomonas putida* HAL (*Pp*HAL, P21310), *Pseudomonas fluorescens* PAM (*Pf*PAM, K0WLD2), *Pantenoa agglomerans* PAM (*Pa*PAM, Q84FL5), *Taxus canadensis* PAM (*Tc*PAM, Q6GZ04), *Chondromyces crocatus* TAM (*Cc*TAM, Q0VZ68) and *Streptomyces globisporus* TAM (*Sg*TAM, Q8GMG0)].

The recombinant protein production was performed with *E. coli* Rosetta (DE3)pLysS cells which contains the plasmid of the protein genes in pET-19b expression vector. The proteins carrying *N*-terminal His_{10}-tag. For the expression step, a colony of the transformed plasmid was grown overnight at 37 °C in 50 mL of Luria-Bertani (LB) medium containing carbenicillin (50 μg mL^{-1}) and chloramphenicol (30 μg mL^{-1}). A volume of 0.5 L LB medium was inoculated with 1 V/V% of the overnight culture and grown until the optical density at 600 nm (OD_{600}) reached 0.6 - 0.7 at 37 °C. 0.1 mM IPTG was added to the cells to induce protein production. In the expression phase by *Pf*HAL, *Pf*XAL and *Pf*PAM the temperature was reduced to 25 °C for 16 h and by *Pza*PAL the temperature was reduced to 28 °C for 6 h and the cultures were shaken at 180 rpm.

3.3.2. Utilization of MIO-enzymes in bioconversations

Biotransformations with *Pf*PAM (50 μg mL^{-1}) contained the substrate 2 mM of L-phenylalanine (*S*)-**8a** , 4 mM of *rac*-*α*-phenylalanine *rac*-**8a**, 4 mM of *rac*-*β*-phenylalanine *rac*-**9a** and 2 mM of cinnamic acid **10a** in the following buffers: 100 mM Tris, pH= 8.5 for the aminomutase reactions; and 4 M ammonium carbamate pH=9.0 for the ammonia addition reactions. The reactions were performed at 30°C and the samples were taken after 1; 3; 5; 18 and 24 h.

The ammonia elimination reaction mixtures containing 5 mM of the racemic phenylalanines *rac*-**8a-s** in 100 mM TRIS buffer pH= 8.5 and purified *Pza*PAL or *Pc*PAL (50 μg mL^{-1}, either) in 1 mL reaction volume were incubated at 30 °C. Samples were taken after 17 and 168 h. The reaction mixtures containing substituted cinnamic acids (**10a-s**, 5 mM) in 3 M ammonium carbamate pH= 9.1 supplemented with *Pza*PAL (50 μg mL^{-1}) or in 6 M ammonium solution pH= 10 supplemented with *Pc*PAL (50 μg mL^{-1}) were incubated at 30 °C. Samples were taken after 17 and 168 h.

4. Results

4.1. Kinetic resolution of *rac*-1-phenylethylamine for potentital tyrosine kinase inhibitors

We produced the racemic and enantiopure forms of the intermedier amide of potential tyrosine kinase inhibitors in chemoenzymatic system with chemical acylation and kinetic resolution with *Ca*LB. The chemoenzymatic system represents on Figure 3.

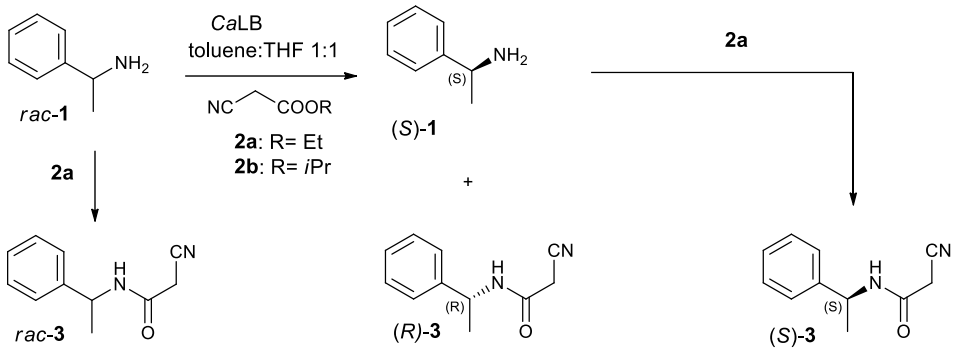

Figure 3. Production of racemic and enantiopure amides from *rac*-1-phenylethylamine in chemoenzymatic route

We investigated for the effective kinetic resolution with various modes of acylating agent addition. The increased amount of acylating agent was helpful for higher conversion but decreased the enantiopurity with the increasing reaction rate of chemical acylation. The increased volume of the acylating agent increased the enantiopurity of the remaining (*S*)-1 amine because it causes the change of equilibrium and increase the reaction rate. We compared three method of the kinetic resolution. The "A" method: we used 0.5 eq. acylating agent, by the "B" method: we used 1 eq. acylating agent and by "C" method: we applied a dosage mode (0.5 eq. + 0.25 and 0.25 eq. in 2 and 4 h time addition). Samples were take after 2; 4; 6 and 24 h, the Table 1. represents the results of the kinetic resolution.

Acylating agent addition	Reation time [h]	Conversion [%]	$ee_{(R)\text{-}3}$ [%]	$ee_{(S)\text{-}1}$ [%]	U_{spec} [μmol min^{-1} g^{-1}]
"A" method	2	26	99.9	35.9	9.10
	4	**29**	**99.9**	**40.4**	**4.96**
	6	30	99.9	41.8	3.38
	24	32	99.9	46.4	0.91
"B" method	2	44	99.9	78.8	15.18
	4	48	99.3	90.1	8.19
	6	49	99.0	94.8	5.61
	24	50	98.2	98.5	1.44
"C" method	2	27	99.9	37.5	9.40
	4	41	99.5	69.9	7.10
	6	47	99.1	89.1	5.44
	24	**50**	**98.9**	**99.3**	**1.44**

Table 1. Addition methods by kinetic resolution of rac-1-phenylethylamine; A method: 0.5 eq. acylating agent; B method: 1 eq. acylating agent; C method: addition method [0.5 eq. +0.25 eq. (2 h) +0.25 eq. 4 h)]; bold: optimal method

The results showed the best choice for the production of (*R*)-**3** amide is the "A" method with 4 h reaction time. We achieved 29% conversion rate and 99.9% enantiomeric excess. The specific activity of the biocatalyst was better than the increased reaction time. The prolonged reaction time do not increase the conversion as much. For the production of (*S*)-**1** amine the "C" method for 24 h reaction time is the good acylating agent addition mode. The starting amount of 0.5 eq. acylating agent do not accompanied the chemical acylation of (*S*)-**1** amine. The increased amount of the acylating agent consume almost the (*R*)-**1** amine. The remained amine have 41% conversion rate with 99.3% enantiomeric excess. We produced the (*S*)-**3** amide from the kinetic resolution remained amine with chemical acylation in high enantiopurity.

4.2. Stereoselective ketoreduction with whole-cell yeasts

We investigated the yeast strains with three different substrate: phenylacetone **6a**, acetophenone **6b** and 2-heptanone **6c** (Figure 4). The optimization of the ketoreductions were planned with $2^{(5-2)}$ designed experience (DOE) with foldover experiments and three times repeated centerpoint experiments. In the first optimization cycle we gathered the information if the substrate is not accepted from the yeast strain and the production of the alcohol is not possible. We investigated this five factor in the designed experience: amount of biocatalyst, amount of 2-propanol cosubstrate, buffer strength, reaction temperature, buffer pH.

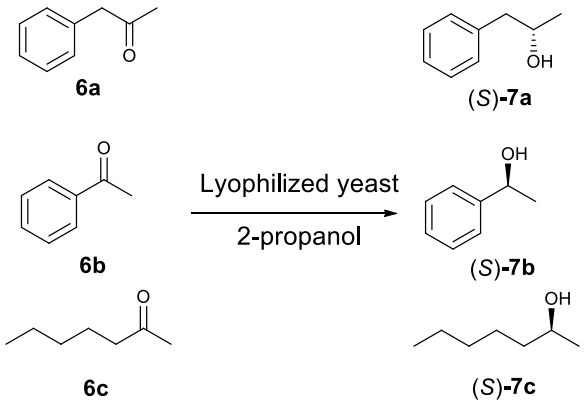

Figure 4. Ketone reductions with lyophilized yeasts whole-cell biocatalysts

The results showed from the first DOE the acetophenon **6b** is not a natural substrate for *Candia norvegica* (WY4). *Candida guillermondi* (WY7), they only accepted the alphalitic 2-heptanone **6c** as substrate. The other yeast strains (*Pichia carsoni* (WY1), *Lodderomyces elongisporus* (WY2), *Debaromyces fabryi* (WY11) and *Candida parapsilosis* (WY12)) catalyzed the stereoselective reduction of all the three investigated ketone. We produced the (*S*)-alcohol in high enantiopurity, if the conversion achieved the 10% conversion rate the enantiomeric excess were above 99.5% ($ee_{(S)\text{-}\mathbf{7a\text{-}c}}$.> 99.5 %,). We evaluated the effect of the factors by the first cycle of DOE and we generated and modified the second cycle of DOE just with the significant factors for the higher conversion rate. The optimized reaction settings includes in the Table 2.

Yeast strain	Substrate	Reaction parameter				Conversion [%]
		Lyophilized yeast [mg mL^{-1}]	Cosubstrate [V/V%]	Temperature [°C]	pH [-]	
WY1	**6a**	40	3	35	7	97
	6b	40	4	30	7	83
	6c	40	4	30	7	84
WY2	**6a**	30	4	30	8	74
	6b	30	2	30	7	38
	6c	30	4	30	8	75
WY4	**6a**	40	8	30	7	81
	6c	40	8	30	7	83
WY7	**6c**	30	4	30	8	26
WY11	**6a**	30	4	27	7	68
	6b	30	2	27	7	35
	6c	30	8	30	7	84
WY12	**6a**	20	3	35	7	97
	6b	20	4	30	7	82
	6c	20	4	35	7	83

Table 2. Optimal reaction parameters in the reaction optimum by the stereoselective ketoreductions with whole-cell; * buffer strength 64 mM

4.3. Identification of MIO-enzymes

We scanned and identified MIO-enzymes in sequence databases with local alignment searches. The results gave the opportunity to find genes and amino acid sequences of potential novel aromatic amino acid ammonia-lyases and 2,3-aminomutases. The highest similarities and identities were found in the sequence part of the MIO catalytic group forming amino acids, which also allowed us to identify the amino acid sequences as a MIO-enzymes. The MIO group is autocatalytically forming from the ASG amino acid triad. About this ten amino acid long sequence have 80-90% identity by the MIO-enzymes, and found in all MIO-enzyme (Figure 5. orange). The TSG amino acid triad in the MIO forming sequence part predicts a bacterial 2,3-aminomutase enzyme. By the sequence number 137-138 are the amino acids which are important for the substrate selectivity, it decleares the natural amino acid acceptance by ammonia-lyase activity. The PALs have FL motif (Figure 5. light blue), the TALs have HL or HQL motif (Figure 5. dark green), and SH motif is characteristic to HALs (Figure 5. turquoise). Therefore we predicted that we found one HAL enzyme with the SH motif, and we found 84% sequence identity with the HAL from *Pseudomonas putida*, it is a strong confirm to our prediction. We found by the *Pf*XAL in the postion 137-138 FH, which amino acids appear in all ammonia-lyases (HAL, TAL and PAL) from the MIO-enzyme family. We predicted that this enzyme accepted and catalyze the ammonia elimination from the L-phenylalanine, L-tyrosine and also the L-histidine. To our best knowledge this is the first organism that conatins three other type of MIO-enzymes (HAL, PAM and XAL).

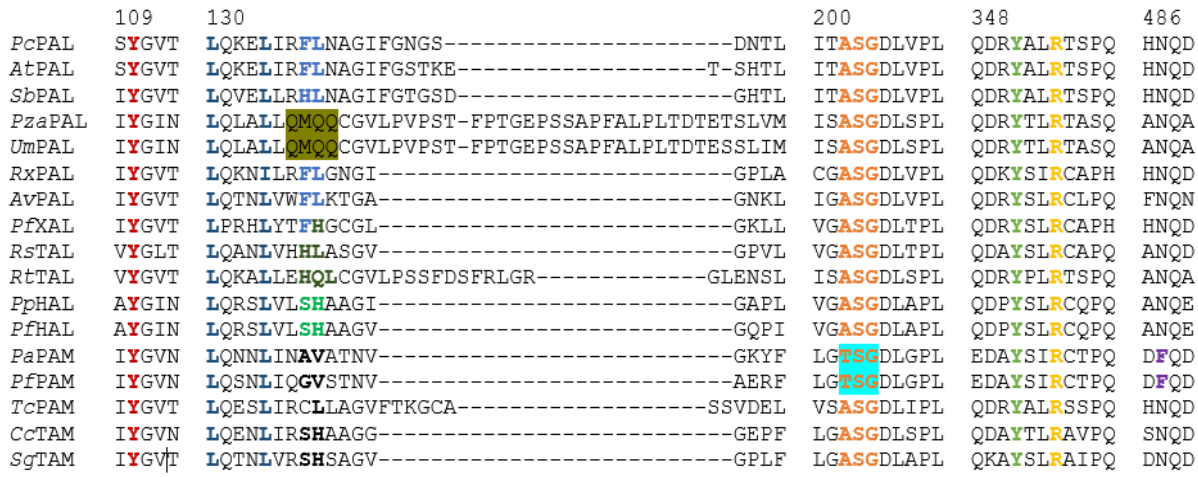

Figure 5. Sequence alignment of the highly conserved catalytic domains and the aromatic binding pocket region of the MIO-enzymes; red: catalytical tyrosine; dark blue: conserved part of the aromatic binding pocket; light blue: PAL characteristic aromatic binding region; dark green: TAL characteristic aromatic binding region; turquize: HAL characteristic aromatic binding region; orange: MIO catalytic group ; orange with turquize: bacterial PAM MIO catalytic group; light green: MIO stabilizing amino acid; yellow: arginine responsible for binding the substrate by carboxylic group; purple: responsible for (*S*)-PAM

We investigated the natural substrate acceptance of the novel ammonia-lyases in ammonia elimination reactions. The test were performed in accordance with our predictions for the relation between the substrate acceptance and the amino acid motifs in the substrate binding site. We investigated the substrate acceptance with the following L-amino acids: L-phenylalanine, L-tyrosine, L-histidine, L-tryptophan, L-asparagine, L-aspartic acid, L-glutamine. The enzymes accepted only the aromatic amino acids excluding the L-tryptophan, whose heteroaromatic ring does not fit in the binding pocket. The *Pf*HAL catalyze selectively the ammonia elimination from the L-histidine. The *Pf*XAL catalyze the ammonia elimination in accordance to our prediction from the L-phenylalanine, L-tyrosine, L-histidine, in order the activity decreasing. The *Pza*PAL accepted just the L-phenylalanine as natural substrate. Our predictions were correct for the relation between the sequence motif in the aromatic binding site and the natural substrate acceptance.

4.4. Utilization of MIO-enzymes in bioconversions

We investigated the isomerization reaction of *α*- és *β*-phenylalalnine by *Pf*PAM. We specified the *Pf*PAM as a full selective (*S*)-2,3-aminomutase. The enzyme catalyzed the production of (*S*)-*β*-phenylalanine (*S*)-**9a** in 100% enantiomeric excess from the (*S*)-*α*-phenylalanine (*S*)-**8a**. The enzyme also have some ammonia-lyase activity which is concluded from the indication of cinnamic acid **10a** in the reaction mixture. The rate of (*S*)-**9a** in the equilibrium are between 52.5-55% (Table 3. line 1.). The (*S*)-**9a** produced selectively in the kinetic resolution of *rac*-**8a**. The ammonia-lyase activity is reduced by the inhibition of the (*R*)-**8a** in the equilibrium the rate of **10a** was just 4%. The (*R*)-**8a** is an inhibitor (it can bind in the active site, but have no effect) by the ammonia-lyase activity and by the 2,3-aminomutase activity (Table 3. line 2.). The kinetic resolution of *rac*-**9a** have slower reaction rate, the product

formation rate is lower and the cinnamic acid **10a** formation also decreased. The reaction mixture in the equilibrium contained only 1% cinnamic acid (Table 3. line 3.). We investigated the *Pf*PAM in ammonia addition reactions also, started from cinnamic acid **10a** in ammonia rich conditions. First formed (*S*)-**8a** product will formed into (*S*)-**9a** final product (Table 3. line 4). We can declare the *Pf*PAM have high enantioselective (S)-2,3aminomutase activity and also some ammonia-lyase activity. The enzyme was usable in addition reactions as well, and was able to form enantiopure *α*- or *β*-phenylalanine (*S*)-**8a** or (*S*)-**9a**.

Entry	Substrate	Reaction time [h]	**9a** [%]	**8a** [%]	**10a** [%]
1	(*S*)-**8a**	1	52.5	36.9	10.6
		3	53.7	31.9	14.4
		5	54.8	30.5	14.7
		18	56.9	24.1	19.0
		24	55.0	25.0	20.0
2	*rac*-**8a**	1	19.0	80.1	0.9
		3	24.1	74.3	1.6
		5	26.2	71.3	2.3
		18	28.0	68.0	4.0
		24	27.8	68.2	4.0
3	*rac*-**9a**	1	91.6	7.9	0.5
		3	89.7	9.5	0.8
		5	87.9	11.3	0.8
		18	87.4	11.7	0.9
		24	87.2	11.8	1.0
4	**10a**	1	4.7	3.1	92.2
		3	8.1	5.7	86.2
		5	13.6	9.2	77.2
		18	31.0	23.3	45.7
		24	33.1	24.8	42.1

*Table 3. Pf*PAM catalyzed bioconversions: isomerization of (*S*)-phenylalanine, kinetic resolution of *rac*-*α*-phenylalanine, kinetic resolution of *rac*-*β*-phenylalanine and stereoselective ammonia addition onto cinnamic acid

The biocatalytic properties of *Pza*PAL were investigated by kinetic resolution of *rac*-**8a-s** using the ammonia elimination reactions. The biocatalytic performance of *Pza*PAL was compared to the already well-characterized *Pc*PAL (Table 4. A). The experiments included several L-phenylalanine derivatives, substituted on the aromatic ring in three positions (ortho, meta and para) with fluoro- **8b-d**, chloro- **8e-g**, nitro- **8h-j**, bromo- **8k-m**, methyl- **8n-p** and methoxygroup **8q-s**. High enantiomer selectivity was observed in most of the *Pza*PAL catalyzed kinetic resolutions except for the ammonia elimination from the racemic ortho- and para-nitro derivatives *rac*-**8h,j** and para-bromo derivative *rac*-**1m**. In the case of attempted kinetic resolutions with *Pza*PAL using phenylalanines substituted with electron-donating

groups methyl *rac*-**8n-p** and methoxy *rac*-**8q-s** reactions proceeded with moderate, low or even negligible conversion, but with perfect enantiomer selectivity for *rac*-**8n**. The different aminoacids (asparagine and methionine with QMQQ motif) in the aromatic binding pocket can causes the higher entatiomselectivity by the *Pza*PAL.

Most of the ortho substituted cinnamic acid derivatives **10b,e,h,k,n** were proper substrates in the *Pza*PAL catalyzed selective ammonia addition, with the exception of ortho-methoxy derivative **10q** which was not transformed at all. The *Pza*PAL catalyzed ammonia addition onto substituted cinnamic acid derivatives **10a-e,g,k,l,n** yielded the corresponding enantiopure L-phenylalanine derivatives (*S*)-**8a-e,g,k,l,n** with variable conversions. Excellent conversions (>90%) were achieved with the ortho-fluoro, metha-fluoro and orto-chloro substituted cinnamates **10b,c,e** within 17 h, and with the ortho-bromo substituted cinnamate after 168 h. The absent or negligible conversion (< 5% within 168 h) observed with substituted cinnamic acids **10i,m,o–s**.

A

Substrate *rac*-**8a-s**	17 h reaction time *Pza*PAL Conversion ($ee_{(R)\text{-}8a\text{-}s}$) [%]	17 h reaction time *Pc*PAL Conversion ($ee_{(R)\text{-}8a\text{-}s}$) [%]	168 h reaction time *Pza*PAL Conversion ($ee_{(R)\text{-}8a\text{-}s}$) [%]	168 h reaction time *Pc*PAL Conversion ($ee_{(R)\text{-}8a\text{-}s}$) [%]
rac-**8a**	49 (>99)	49 (>99)	49 (>99)	**54** (>99)
rac-**8b**	49 (>99)	52 (>99)	53 (>99)	**69** (>99)
rac-**8c**	49 (>99)	52 (>99)	52 (>99)	**63** (>99)
rac-**8d**	51 (>99)	**54** (>99)	51 (>99)	**59** (>99)
rac-**8e**	48 (98)	49 (>99)	50 (>99)	**64** (>99)
rac-**8f**	47 (88)	51 (>99)	50 (>99)	**61** (>99)
rac-**8g**	44 (82)	**53** (>99)	50 (>99)	**64** (>99)
rac-**8h**	**35** (43)	**67** (>99)	**62** (>99)	**82** (>99)
rac-**8i**	21 (26)	**63** (>99)	40 (64)	**91** (>99)
rac-**8j**	**56** (88)	**96** (>99)	**78** (94)	**99** ([a])
rac-**8k**	43 (79)	49 (>99)	49 (99)	**58** (>99)
rac-**8l**	29 (39)	50 (>99)	47 (87)	**60** (>99)
rac-**8m**	**18** (17)	50 (>99)	38 (58)	**53** (>99)
rac-**8n**	50 (>99)	49 (>99)	50 (>99)	**52** (>99)
rac-**8o**	10 (12)	29 (45)	18 (23)	48 (>99)
rac-**8p**	1 ([a])	47 (94)	6 (7)	48 (>99)
rac-**8q**	0 ([a])	**7** (6)	0 ([a])	**25** (30)
rac-**8r**	20 (25)	48 (>99)	35 (53)	51 (>99)
rac-**8s**	0 ([a])	**22** (25)	0 ([a])	**37** (57)

B

Substrate **10a-s**	17 h reaction time *Pza*PAL Conversion ($ee_{(S)\text{-}8a\text{-}s}$) [%]	17 h reaction time *Pc*PAL Conversion ($ee_{(S)\text{-}8a\text{-}s}$) [%]	168 h reaction time *Pza*PAL Conversion ($ee_{(S)\text{-}8a\text{-}s}$) [%]	168 h reaction time *Pc*PAL Conversion ($ee_{(S)\text{-}8a\text{-}s}$) [%]
10a	41 (>99)	81 (>99)	80 (>99)	81 (98)
10b	94 (>99)	96 (97)	96 (>99)	95 (92)
10c	93 (>99)	89 (97)	93 (>99)	89 (97)
10d	11 (>99)	78 (>99)	51 (>99)	78 (98)
10e	98 (>99)	97 (94)	98 (>99)	98 (70)
10f	5 (91)	93 (97)	20 (96)	93 (93)
10g	3 (>99)	92 (>99)	9 (>99)	91 (96)
10h	32 (92)	98 (89)	45 (92)	98 (82)
10i	2 (78)	80 (89)	4 (89)	89 (78)
10j	78 (92)	97 (75)	91 (92)	97 (62)
10k	41 (>99)	97 (>99)	95 (>99)	97 (95)
10l	1 ([a])	92 (>99)	5 (>99)	93 (95)
10m	0 ([a])	88 (>99)	1 ([a])	90 (>99)
10n	27 (>99)	84 (>99)	74 (>99)	84 (99)
10o	0 ([a])	44 (>99)	0 ([a])	74 (97)
10p	0 ([a])	34 (>99)	0 ([a])	55 (>99)
10q	0 ([a])	21 (>99)	0 ([a])	49 (>99)
10r	0 ([a])	58 (>99)	0 ([a])	71 (>99)
10s	0 ([a])	0 ([a])	0 ([a])	0 ([a])

Table 4. Kinetic resolution (A) and stereoselective ammonia addition (B) with *Pza*PAL and *Pc*PAL enzymes for production substituted amino acid derivatives in high enantio pure form

The *Pza*PAL and *Pc*PAL catalyzed the effective ammonia elimination and addition reactions of various substituted amin acid derivatives. This two enzyme together gives a kit which give the opportunity to produce (*R*)- or (*S*)-amino acid derivative in high enantiopurity.

5. Thesis points

1. I realized the kinetic resolution of racemic 1-phenylethylamine with lipase B from *Candida antarctica* and ethyl 2-cyanoacetate acylating agent. I produced the (*R*)-2-cyano-*N*-1-phenylethylacetamide via kinetic resolution, and (*S*)-2-cyano-*N*-1-phenylethylacetamide with the acylation of the remaining (*S*)-1-phenylaethylamine in high enantiopurity, which are chiral base materials for further synthesis. (I.)

2. I planned orthogonal design of experiments for the optimization of stereoselective synthesis for (*S*)-alcohols with lyophilized whole-cell yeast strains (*Pichia carsonii*, *Lodderomyces elongisporus*, *Candida norvegica*, *Candida guillermondi*, *Debarommyces fabryi* és *Candida parapsilosis*) with the consumption of 2-propanol as cosubstrate and from the starting material as aromatic and alphalytic ketones. I produced the (*S*)-alcohols with the lyophilized whole-cell biocatalysts in high conversion and with excellent enantiopurity. (IV.) I performed the production of (*S*)-1-phenylpropane-2-ol and (*S*)-heptane-2-ol in preparative scale with *Debarommyces fabryi* yeast strain. (IV.)

3. I identified three novel MIO-enzyme from the *Pseudomonas fluorescens* R124 bacterial strain with similarity sequence alignments. To our best knowledge this is the first organism that contains three other type of MIO-enzymes. (II.) I proposed to the enzyme function and substrate acceptance based on the amino acid motifs and I proposed the enzyme classification also for the novel enzymes. I proved the natural substrate acceptance of the produced enzymes and I identified a histidine ammonia-lyase (*Pf*HAL), a phenylalanine/tyrosine/histidine ammonia-lyase (*Pf*XAL) and a phenylalanine 2,3-aminomutase (*Pf*PAM).(II)

4. I discovered the first MIO-enzyme (*Pf*XAL) which catalyzes the ammonia elimination from three natural aromatic amino acid (L-phenylalanine, L-tyrosine and L-histidine). I proposed a new enzyme class in the ammonia-lyase family: phenylalanine/tyrosine/histidine ammonia-lyase. (II.)

5. I proved the (*S*)-selectivity of *Pf*PAM. The production of enantiopure (*S*)-*β*-phenylalanine is realizable from (*S*)-*α*-phenylalanine in isomerization reaction of *Pf*PAM. I created (*R*)-*β*-phenylalanine in high enantiomeric excess via the kinetic resolution of racemic *β*-phenylalanine with *Pf*PAM. (II.)

6. I identified a novel phenylalanine ammonia-lyase (*Pza*PAL) from the psychrophilic *Pseudozyma (Candida) antarctica* strain with similarity sequence alignments. (III) We demonstrated that *Pza*PAL completed with PAL from *Petroselinum crispum* (*Pc*PAL) are usable for the production of D-amino acid derivatives (substituted on the aromatic ring in ortho, metha and para position with fluoro-, chloro-, bromo-, methoxi-, and methylgroup), as the remaining product from the racemic amino acid derivatives. We also demonstrated that *Pza*PAL and *Pc*PAL catalyze the stereoselective ammonia addition onto ortho, metha and para fluoro-, chloro-, bromo-, methoxy- and methyl-substituted phenylacrilates in ammonia rich condition to produce L-phenylalanine derivatives in high enantiomeric excess. (III)

6. Potential application

The whole-cell yeast strains were useful biocatalysts for the production of enantiopure alcohols from prochiral ketones. The enantiopure alcohols are valuable building blocks of various pharmaceuticals or chemical base materials. The use of *Pichia carsonii, Lodderomyces elongisporus, Candida norvegica, Candida guillermondi, Debaromyces fabyi* and *Candida parapsilosis* as biocatalysts widens the usage of the potential biocatalysts.
The MIO-enzymes are well-usable biocatalysts for the production of amino acids and their derivatives in enantiopure form with kinetic resolution or stereoselective ammonia addition reactions. The enantiopure amino acid derivatives are valuable base materials for chemical industry as building blocks of pharmaceuticals.

7. Publications

Scientific achivements of the thesis are based on the following articles:

I. P. Csuka, Z. Boros, L. Örfi, J. Dobos, L. Poppe, G. Hornyánszky: Chemoenzymatic route to Tyrphostins involving lipase-catalyzed kinetic resolution of 1-phenylethanamine with alkyl cyanoacetates as novel acylating agents *Tetrahedron: Asymmetry,* **2015**, *26(12-13),* 644-649. DOI: 10.1016/j.tetasy.2015.04.013 (95%, IF: 2.1, idéző: 7(4/3))

II. P. Csuka, V. Juhász, S. Kohári, A. Filip, A. Varga, P. Sátorhelyi, L.C. Bencze, H. Barton, C. Paizs, L. Poppe: *Pseudomonas fluorescens* Strain R124 Encodes Three Different MIO Enzymes *ChemBioChem*, **2018**, *19(4),* 141-418. DOI: 10.1002/cbic.201700530 (75%, IF: 2.6, idéző: 5(4/1))

III. A. Varga, P. Csuka, O. Sonesouphap, G. Bánóczi, M.I. Tosa, G. Katona, Z. Molnár, L.C. Bencze, L. Poppe, C. Paizs: A novel phenylalanine ammonia-lyase from *Pseudozyma antarctica* for stereoselective biotransformations of unnatural amino acids *Catal Today*, **2021**, 366, 185-194. DOI: 10.1016/j.cattod.2020.04.002 (80%, IF: 5.8, idéző: 2 (1/1))

IV. P. Csuka, L. Nagy-Győr, Z. Molnár, C. Paizs, V. Bódai, L. Poppe: Characterization of yeast strains with ketoreductase activity for bioreduction of ketones *Period. Polytech. Chem. Eng.* **2021**, DOI: 10.3311/PPch.17429 Accepted (80%; IF: 1.6)

Articeles not included in the thesis:

V. Varga, Z. Bata, P. Csuka, D.M. Bordea, B.G. Vértessy, A. Marcovici, D.F. Irimie, L. Poppe, L.C. Bencze: A Novel Phenylalanine Ammonia-Lyase from *Kangiella Koreensis Studia UBB Chemia*, **2017**, *3,* 293-308. DOI: 10.24193/subbchem.2017.3.25 (10%, IF: 0.3)

VI. L. Nagy-Győr, M. Lacatus, D. Balogh-Weiser, P. Csuka, V. Bódai, B. Erdélyi, Z. Molnár, G. Hornyánszky, C. Paizs, L. Poppe: How to Turn Yeast Cells into a Sustainable and Switchable Biocatalyst? On-Demand Catalysis of Ketone Bioreduction or Acyloin Condensation *ACS Sustainable Chem Eng,* **2019**, *7,* 19375-19383. DOI: 10.1021/acssuschemeng.9b03367 (20%, IF:7.6)

Lectures:

P. Csuka, V. Juhász, A. Filip, A. Varga, S. Kohári, P. Sátorhelyi, L.C. Bencze, C. Paizs, L. Poppe: *Pseudomonas fluorescens* encodes three MIO-enzyme *Fiatal Biotechnológusok Országos Konferenciája (FIBOK)* **2018. március 28-29**. Budapest, Magyarország

Posters:

P. Csuka, L. Nagy-Győr, Z. Boros, C. Paizs, B. Erdélyi, V. Bódai, L. Poppe: Whole-cell ketoreductases for stereoselective bioreduction of ketones *Training School SysBiocat,* **2016. április 27-30.** Certosa di Pontignano, Siena, Olaszország

P. Csuka, V. Juhász, A. Filip, A. Varga, S. Kohári, P. Sátorhelyi, L.C. Bencze, C. Paizs, L. Poppe: *Pseudomonas fluorescens* encodes three MIO-enzyme *BioTrans, 13th International Symposium on Biocatalysis and Biotransformation* **2017. július 9-13**. Budapest, Magyarország

P. Csuka, K. Gyulai, E. Sánta-Bell, D. Balogh-Weiser, L. Poppe: Immobilization studies on aromatic ammonia-lyase from *Pseudomonas fluorescens, BioTrans, 14th International Symposium on Biocatalysis and Biotransformation* **2019. július 7-11**. Gröningen, Hollandia