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CONTINOUS FLOW ENZYMATIC DYNAMIC KINETIC RESOLUTIONS FOR STEREOSELECTIVE BIOORGANIC PROCESSES

OPTIMIZATION OF THE DINAMIC KINETIC RESOLUTION (DKR) PROCESS IN CONTINUOUS MODES

Since immobilized biocatalysts can be reused and kept a long time and they can be easily manipulate, immobilization represents an important domain of bioengineering. The most relevant parameters determining the efficacy of the biocatalysts are: selectivity, specificity, catalytic activity and stability[1]. Immobilization generally improves all these properties and enables the retention and reuse of the enzyme[2]. All the immobilization methods present advantages and disadvantages; choosing the immobilization method requires a rigorous analyses based on the structure of the transformed substrate and the reaction conditions.

Recent studies conducted with commercial lyophilized or immobilized biocatalysts[1] have shown that due to the large variety of substrates, enzymes and conditions there isn't an ideal method for enzyme immobilization.

The retain and reuse of the enzymes are often complicated in the conventional processes, the application of enzymes being hampered by the lack of operational stability and the difficulty in the work-up processes.

Encapsulation of enzymes in matrices is one of the most general techniques of immobilization. This method does not cause chemical or structural modifications of the enzymes. Moreover, the thermal stability and solvent compatibility generally increases, but may appear *diffusional limitations.*

Binding the biocatalyst to a supportis the mostfrequently used method in case of the mesoporous support materials; the adsorbtion and the covalent binding are also being studied. The properties of immobilized biocatalyst depend on the nature of the functional group and the linker's length (in case of the covalent binding). The porous support has the advantage of an easy manipulation and retain and a relatively high surface, the presence of the pores increases the *diffusional limitations*.

By immobilization on nanoparticles (NP) the diffusional barriers are being minimized and the proteins are available on a large surface. The suspension of the NP doesn't settle, it behaves similar to a homogeneous liquid, thus the separation of particles in order to reuse is being difficult.

Nanotubes, especially carbon nanotubes (CNTs) with nano-sized diameter and micron-sized length offers a support with large available surface, low diffusion limitation and easy recovery. Thus, CNTs are widely used for the immobilization of biomacromolecules, exploiting their mechanical, thermal, electrical properties and general biocompatibility. Biologic applications of enzymes often contain as key steps the immobilization of enzymes on CNTs by covalent or noncovalent bonds1. Glycerol diglycidyl ether (GDE) can be used not only as a crosslinking agent to obtain cross-linked enzyme aggregates (CLEAs), but also as a linker in order to attach the enzymes on the surface of the CNTs functionalized with diamines. Thus the enzyme preparations with micron sized particles can be used as biocatalysts in fixed- bed reactors.

The important characteristics of lipases regarding their industrial applications (ecologic technologies, a small number of operation phases, superior operational stability, increased production capacity and the possibility of retain and reuse) are generally superior after immobilization.

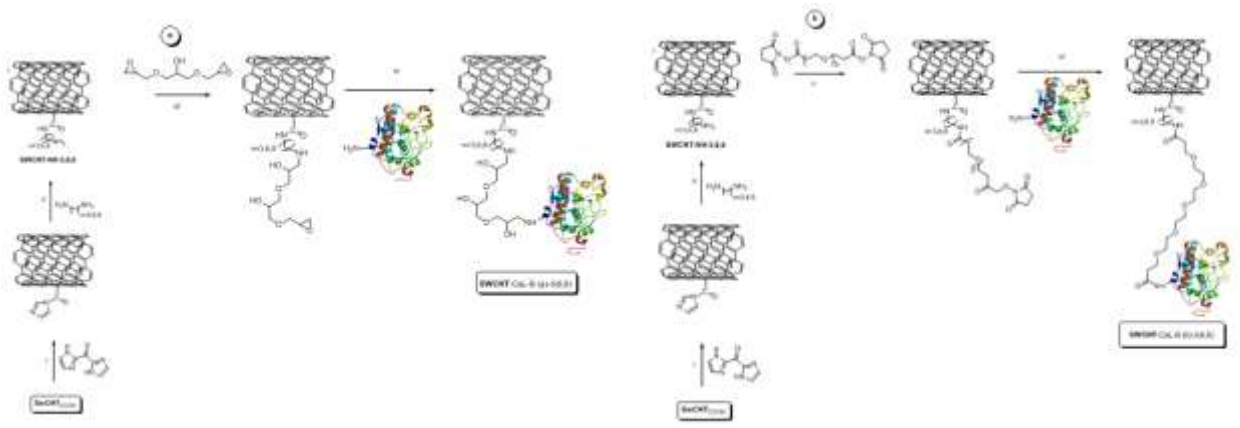
A: Covalent immobilization of lipase B from *Candida antarctica* (CaL-B) on single walled carbon nanotubes functionalized with carboxy groups ($SWCNT_{COOH}$)

Two methods have been applied for the immobilization of CaL-B on $\mathbf{SWCNT_{COOH}}$ (Scheme 1):

1. Immobilization via glycerol diglycidyl ether (GDE), route a
2. Immobilization via bis-N-succinimidyl-(pentaethylene glycol) ester (BS(PEG)5), route b

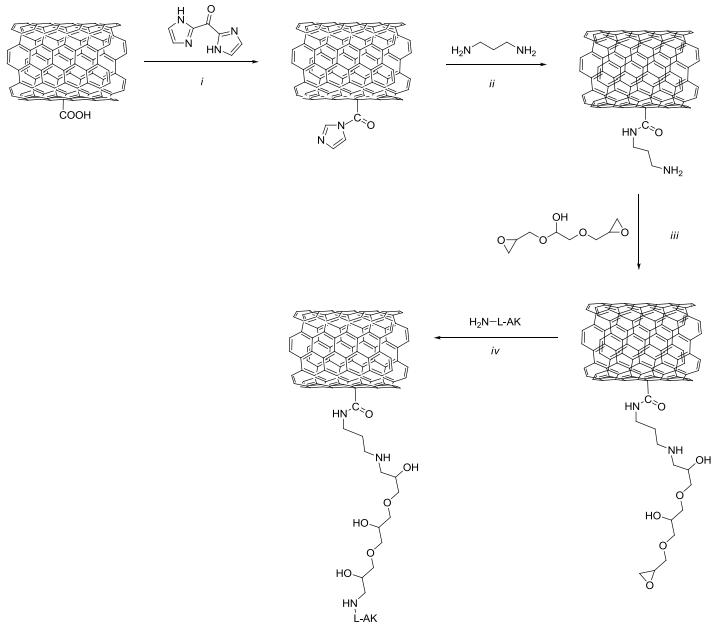
a)*i*) CDI/CH_2Cl_2; *ii*) $H_2N(CH_2)_nNH_2$/water, n=3,6,8; *iii*) glycerol diglycidyl ether /CH_2Cl_2; *iv*) CaL-B/ PBS buffer (20 mM Na_2HPO_4, 150 mM NaCl, pH 7);

b) *i*) CDI/ CH_2Cl_2;*ii*) $H_2N(CH_2)_nNH_2$/water, n=3,6,8; *v*) $BS(PEG)_5$/DMSO ; *vi*) CaL-B/ PBS buffer (20 mM Na_2HPO_4, 150 mM NaCl, pH 8);



Scheme 1. Immobilization strategies of Cal-B on $SWCNT_{COOH}$

B. Covalent immobilization of Amano lipase from *Pseudomonas fluorescens* (L-AK) on single walled carbon nanotubes functionalized with carboxy groups ($SWCNT_{COOH}$)



i) CDI/ CH_2Cl_2

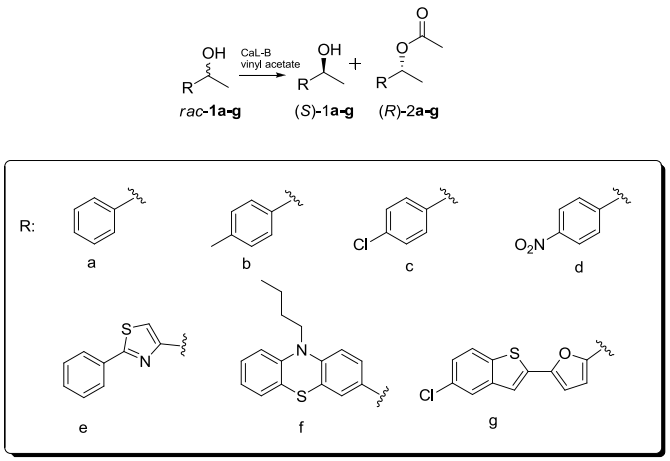
ii) $H_2N(CH_2)_3NH_2$/water;

iii) glycerol diglycidyl ether/CH_2Cl_2;

iv) L-AK in PBS buffer (20 mM Na_2HPO_4, 150 mM NaCl, pH 7).

Scheme 2. Immobilization of L-AK on $SWCNT_{COOH}$

The immobilized enzyme preparations were tested in the kinetic resolution of various chiral secondary alcohols (Scheme 3), in a continuous flow reactor, using phenylethanol as model substrate (Figure 1).



Scheme 3. EKR of various secondary alcohols mediated by lipase immobilized on $SWCNT_{COOH}$

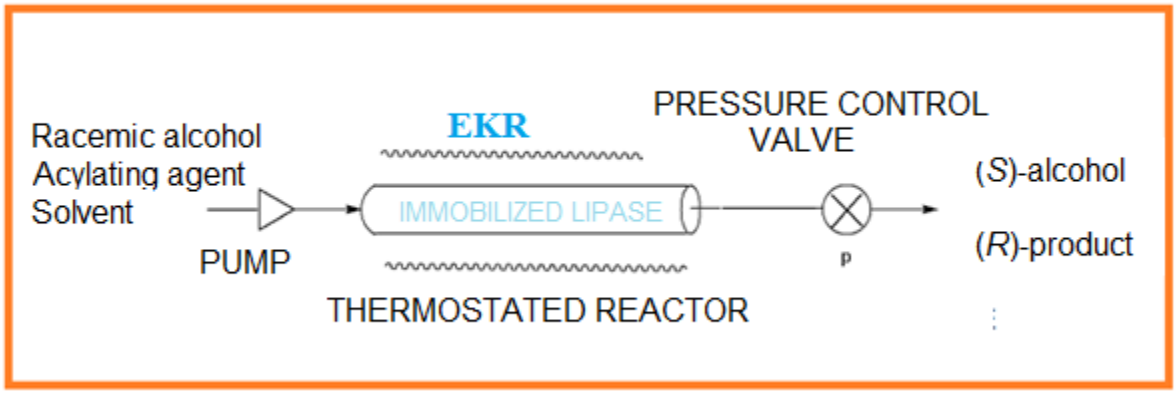


Figure 1. Testing the enzymatic processes in continuous flow reactors

Enzymatic acylation in batch reactions

1 mg of racemic alcohol was dissolved in 500 µL of organic solvent. In the mixture 10 µL vinyl acetate and 1 mg enzyme was added. The reaction mixture was shaken (1350 rpm) at room temperature. The reactions were monitored by gas chromatographic separations using specific conditions for each substrate or on a Chiralpak IB HPLC column (Table 1).

Determining the optimal immobilization procedure

The immobilized enzymes activity on the two types of nanosupport was determined by theenzymatic acylation of the modelsubstrate, *rac*-**1a.** The enzyme immobilizedon carbon

nanotubesby the second approach proved to be the most efficient catalyst in the acylation reaction of the model substrate. The reaction was performed on two other substrates, *rac*-**2d,g,** and the results were in accordance with those observed in case of the model substrate, thus further experiments were performed using this enzyme preparation.

Table 1. Chromatographic separation conditions on chiral columns

Alcohol	**Separation method**	**Temperature (°C) or eluent (hexan:IPA, v/v)**	**Retention time (min)**
rac-**2a**(*R*/*S*)	GC	120	5.5/5.9
rac-**1a**(*S*/*R*)	GC	120	7.4/7.6
rac-**2b**(*R*/*S*)	GC	130	6.1/6.4
rac-**1b**(*R*/*S*)	GC	130	6.5/6.8
rac-**2c** (*S*/*R*)	GC	120-160 (2.6°/min)	9.4/9.8
rac-**1c** (*R*/*S*)	GC	120-160 (2.6°/min)	11.3/11.6
rac-**2d**(*S*/*R*)	HPLC-IB	87:13	8.8/12.4
rac-**1d**(*S*/*R*)	HPLC-IB	87:13	15.4/19.8
rac-**2e**(*R*/*S*)	HPLC-IB	95:5	6.2/6.7
rac-**1e**(*S*/*R*)	HPLC-IB	95:5	11.9/13.8
rac-**2f**(*R*/*S*)	HPLC-IB	90:10	5/5.9
rac-**1f**(*R*/*S*)	HPLC-IB	90:10	9.5/12.9
rac-**2g**(*S*/*R*)	HPLC-IB	95:5 (for 8 min), 90:10 (20 min)	6.2/6.7
rac-**1g**(*S*/*R*)	HPLC-IB	95:5 (for 8 min), 90:10 (20 min)	11.9/13.8

The effect of the enzyme loading

Performing the acylation reaction with enzyme preparations bearing different loading of enzyme it was shown that the enzyme preparation with the 1:2 support- enzyme ratio had the highest activity.

Table 2. The effect of the enzyme loading on the activity of the immobilized biocatalyst (after 30 h reaction time)

Support material	**Support-enzyme *ratio* (w/w)**	**ee_s(%)**	**c (%)**
$SWCNT_{COOH}$	2 : 1	72	42
	1 : 1	69	41
	1 : 2	71	42
	1 : 5	32	24

The effect of the linker length upon the reaction

The shorter (1,3-diaminopropane) linker provided superior enzyme activities while the use of longer linkers displayed a negative effect upon enzyme efficacy.

Table 3. The effect of the linker length upon the reaction

Linker	**Conversion (%)**
1,3-diaminopropane	45
1,6-diaminohexane	31
1,8-diaminooctane	41

Effect of the temperature

Upon investigation of the temperature effect on the acylation reaction of the modelssubstrate catalyzed by CaL-B immobilized on carbon nanotubes, the optimal temperature was found to be 60^0C .

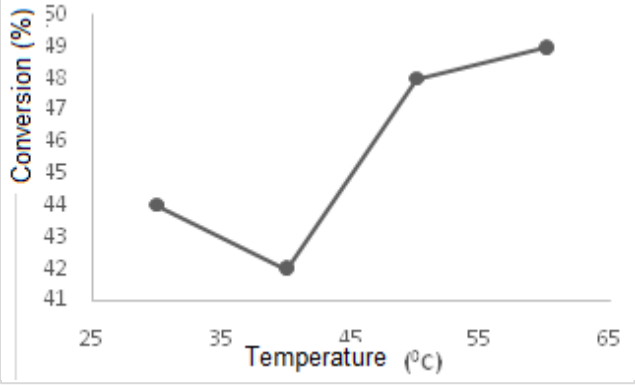


Figure 2. Temperature effect upon the acylation reaction of the model substrate after 1 h reaction time

THE OPTIMUM CONDITIONS FOR DKR PROCESS IN CONTINUOUS FLOW. COMPARATIVE STUDY OF THE CONTINUOUS OPTIONS

In order to optimize the DKR process in continuous flow using the preliminary data obtained for the process conducted in batch mode, different configurations were tested, where the enzymatic resolution unit and the racemization unit, both containing the immobilized catalyst (the enzyme and the racemization agent) were connected according to Scheme 4-6. Both units were thermostated, in order to obtain reproducible results and to perform a study of this parameter upon the DKR process.

As reactors SynBioCart (30 mm×3 mm ID PTFE) colums were used, which were filled with the immobilized catalyst (120 mg CaLB-SWCN$_{COOH}$ or 110 mg of immobilized racemization agent) in fixed bed, using as sealing system teflon and metallic sieves seals which permit the uniform distribution of the flow.

Feeding the reactor with a constant flow (0.3 mL/min) was achieved using a quaternary HPLC pump (the pump module of an Agilent LC 1150 system) and the backpressurewas controlled by a regulation valve.

In order to elaborate the optimum conditions for stereoselective synthesis in dynamic kinetic resolution (DKR) of the chiral compounds (cyanohydrins, amino acids and heterocyclic amines) in

systems operating with tubular reactors in continuous mode, three distinct configurations were used. In this way, in the cascade reactor system of enzymatic kinetic resolution (Figure 4), using the immobilized enzyme preparations, with the reacemization units containing immobilized chemical agents the optimum immobilized enzyme and immobilized racemization agent ratio could be determined. First, the operation flow was established for a known substrate concentration in order to achieve a total enentiomeric resolution (50 % conversion, the complete transformation of one enantiomer of the substrate).

Further the racemization process of the remaining substrate was tested. Using the previously fixed flow in the resolution reactor, the required quantity of immobilization agent could be determined for the complete racemization, thus after the second unit, the composition of the reaction mixture in enantiopure product: racemic substrate was 1:1, M/M.

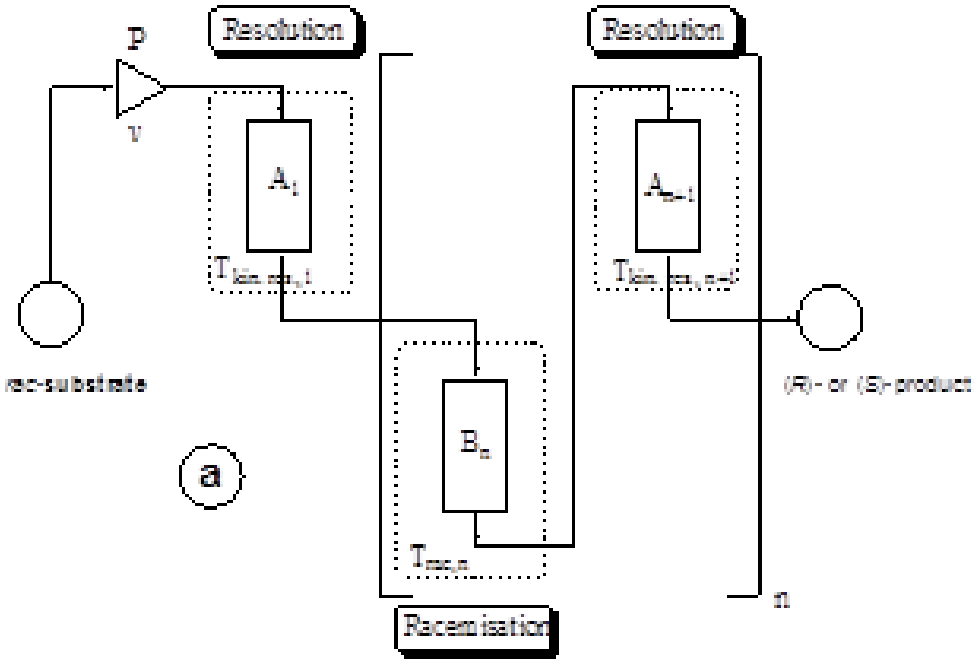


Figure 4. Optimization of the DKR parameters in continuous mode

Folowing the same principle, at the same flow rate, the enzyme and the racemization agent loading of the next resolution-racemization unit was determined, in order to achieve the composition of the reaction mixture of enantiopure product:racemic substrate of 3:1, M/M.

The maximum number of the tested cascadeunit consisted of 3 resolution reactors and 3 racemization reactors.

The results of the experiments conducted for the 3 classes of compounds enabled the determination of the ratio of the biocatalyst and the racemization agent.

Table 4. The DKR parameters in continuous mode for the 3 classes of compounds

Process	**Biocatalyst/ racemization agents on support ratio (m/m)**
DKR cyanohydrins	CaL-A on celite/ diethyl aminoethanol covalently immobilized on carbon nanotubes 1:3, m/m
DKR amine	Novozym 435/ Pd on aluminium oxide 1:1, m/m
DKR oxazolone (for amino acids)	Novozym 435/ diethyl aminoethanol covalently immobilized on carbon nanotubes 5:1, m/m

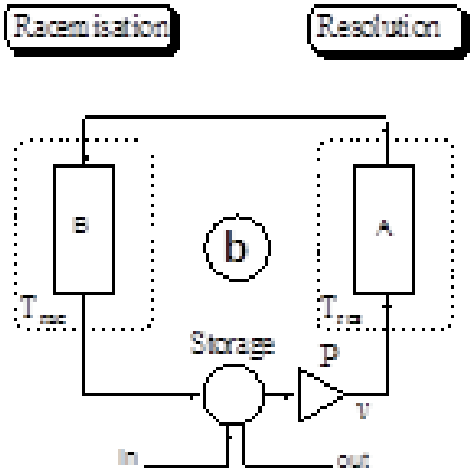


Figure 5. Continuous DKR in cascade reactors

Further the testing of the DKR reactions in a continuous system with recycle followed, using two reactors (a reactor for enzymatic kinetic reaction and one reactor for racemization) (Figure 5).

Next the efficiency of the obtained parameters were monitored in order to achieve total conversion of the substrates in one enantiomer of the product such that during the process the substrate to remain racemic.

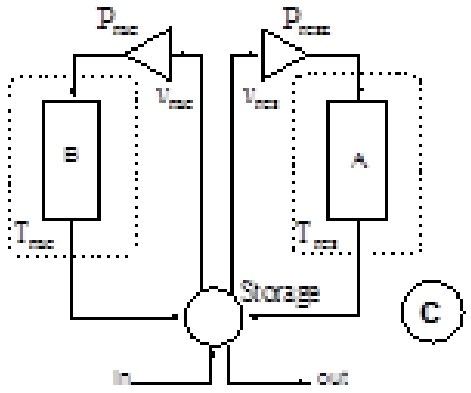
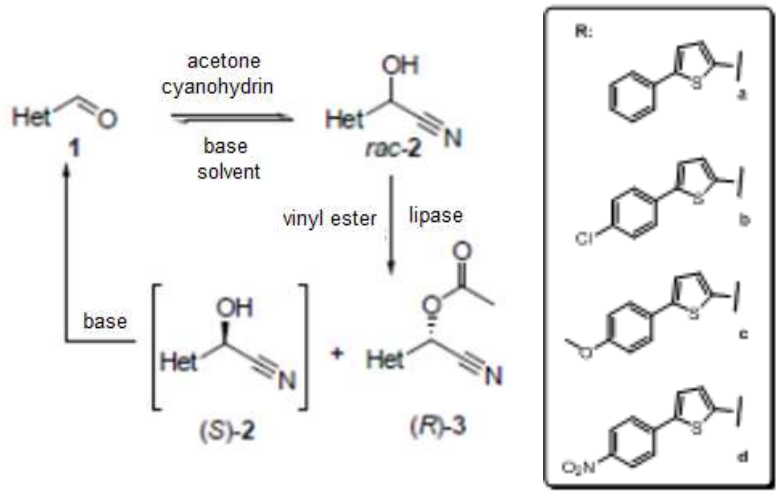


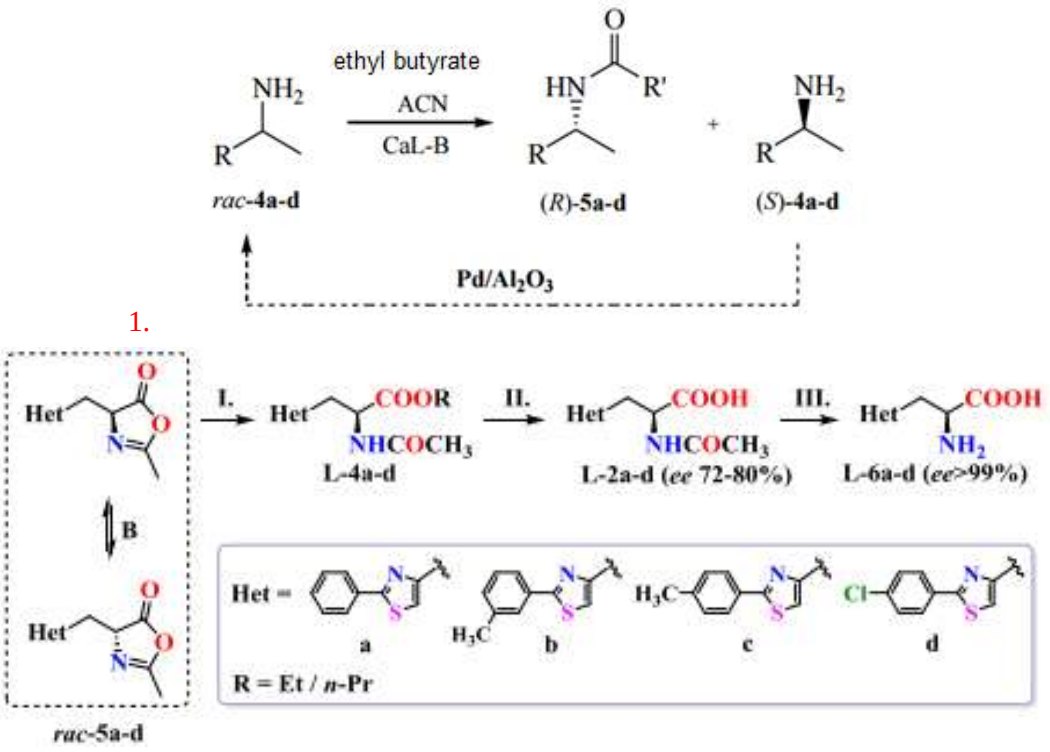
Figure 6.Continuous DKR using reactors in parallel arrangement

With the obtained data the DKR was tested for all 3 cases using reactors in aparallel arrangement (Figure 6) in continuous mode with recirculation. In this case both reactors (the resolution and the racemization reactor) had the same catalyst loading (enzyme and racemization agent). By adjusting the flow rate on the two branches of the system the efficient operation of the system was achieved, the velocity of the enzymatic kinetic resolution increased, maintaining the racemic character of the substrates during the process.

The studied enzimatic kinetic resolution processes are presented in Scheme 4-4.



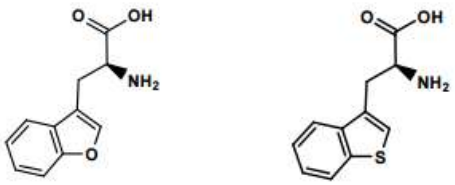
Scheme 4.Enzymatic kinetic resolution of the cyanohydrins



Scheme 5. Dynamic enzymatic kinetic resolution of amines and amino acids

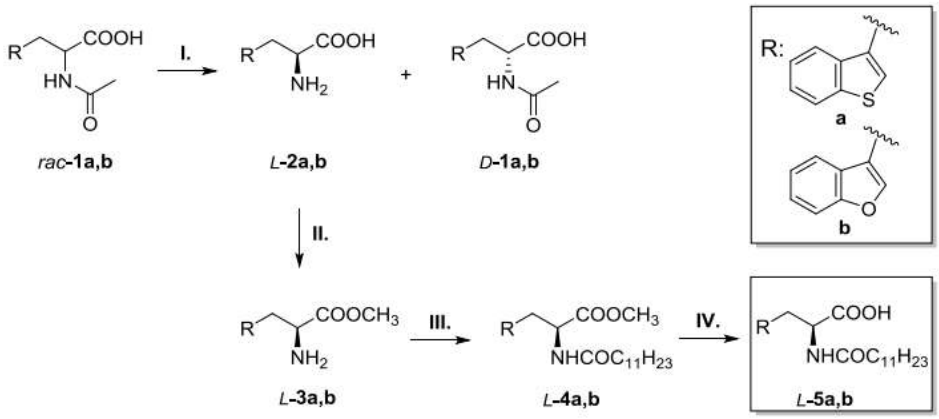
BIOCATALYSIS AS A TOOL OF FUNDAMENTAL RESEARCH

To explain the selection process using the proteinogen amino acids (present in the methabolic reserveoir and probiotics) genetic code, two enantiopure L-Tryptophane analogs (benzofuranyl and benzothiophenyl derivatives) as free amino acids and their *N*-acylated derivatives were synthetized.[i]



Anayzing the differences beetween HOMO – LUMO orbitals and their reactivity, the importance of this correlations and the selection process was demonstrated.

Enantiopure N-dodecanoyl-*L*-(benzofuran-3-yl)alanine (NDo-BFA, *L*-**5b** in Scheme 1) and N-dodecanoyl-*L*-(benzo[*b*]thiophen-3-yl)alanine (NDo-BTA, *L*-**5a**) were synthesized from the non-natural amino acids *L*-(benzofuran-3-yl)alanine (BFA, *L*-**2b**) and *L*-(benzo[*b*]thiophen-3-yl)alanine (BTA, *L*-**2a**) as starting material. The enantiopure amino acids had been obtained from racemic N-acetyl amino acids (*rac*-**1a,b**) by enantiospecific Acylase I mediated hydrolysis, followed by preparative ion-exchange column chromatography (*31*). The synthetic strategy is summarized below (Scheme 6).



Schema 6. Synthesis of enantiopure nonnatural L-Trp amino acids analogs through EKR mediated by Acylase 1.

Synthesis of *L*-3a,b (*L*-methyl 2-amino-3-(heteroaryl)propanoates). Freshly distilled chlorotrimethylsilane (2 equivalents) was added dropwise to the solution of amino acid *L*-**2a,b** (~100 mg) in dry methanol (15 mL) under magnetic stirring at room temperature. After completion of the reaction (as monitored by TLC, ~24 h), the reaction mixture was concentrated in vacuum to give the product *L*-amino acid methyl ester *L*-**3a,b** as white solid. Yield: 87%, 93%.

Synthesis of *L*-4a,b (*L*-methyl 3-(heteroaryl)-2-dodecanamidopropanoates). 4-*N*,*N*-Dimethylaminopyridine in pyridine (DMAP, 1%, 2 equivalents) and dodecanoyl chloride (2

equivalents) were added to a solution of the *L*-amino acid methyl ester *L*-**3a,b** in dry dichloromethane (15 mL). The reaction mixture was refluxed until completion of the reaction (as monitored by TLC, ~3 h). After cooling down to room temperature, the organic phase was extracted with 10% HCl, then with 2 M Na2CO3 and finally with water. The isolated organic layer was dried over anhydrous Na2SO4, the solvent was evaporated in vacuum, and the crude product was purified by column chromatography on silica gel with dichloromethane:methanol (95:5, v/v) as eluent. *L*-**4a,b** was obtained as yellow semi-solid. Yield: 76%, 79%.

Synthesis of *L*-5a,b (*L*-3-(heteroaryl)-2-dodecanamidopropanoic acids). A 2 M Na2CO3 solution was added to a solution of *L*-**4a,b** in acetonitrile and refluxed for 2 h. After cooling down to room temperature, concentrated HCl was added dropwise on ice until the pH reached 1.5, when the product started to precipitate. The suspension was kept in the refrigerator for 2 h, after which the precipitated solid was filtered off and dried by lyophilization to obtain the product *L*-**5a,b**. Yield: 88%, 91%.

Analytical procedures. The identity and purity of *L*-**5a,b** were determined by 1H/13C-NMR spectroscopy and atmospheric pressure chemical ionization (APCI) mass spectrometry. The enantiopurity of *L*-**2a,b** and *L*-**4a,b** (in lieu of *L*-**5a,b**) were verified by chiral high-performance liquid chromatography (HPLC) (Agilent 1200, Santa Clara, CA, USA) on Chiralpak columns (Daicel Corporation, Osaka, Japan) using *rac*-**2a,b** and *rac*-**4a,b** as standards. To obtain these racemic standards, 100 mg of *rac*-**1a,b** was suspended in 10% HCl and refluxed for 5 h. After evaporation in vacuum, and the residual solid was washed with diethyl ether and dried, affording *rac*-**2a,b** as white solid. Racemic *rac*-**2a,b** was further subjected to the same transformations as the enantiopure *L*-**2a,b** to obtain *rac*-**4a,b**. The enantiomeric separation of *rac*-**2a,b** was performed on a Chiralpak ZWIX(+) column (250 × 4.0 mm) using acetonitrile:methanol[50 mM formic acid, 25 mM diethylamine]:water 49:49:2 (v/v/v) as mobile phase for *rac*-**2a**, and acetonitrile:methanol[50 mM formic acid, 25 mM DEA]:water 70:28:2 (v/v/v) as mobile phase for *rac*-**2b** (flow in both cases: 1 mL/min). The separation of *rac*-**4a,b** was accomplished on Chiralpak IA and IB columns (250 × 4.6 mm). As mobile phase, hexane:2-propanol 80:20 (v/v) was used for *rac*-**4a** (flow: 1 mL/min, Chiralpak IA column), and hexane:2-propanol 95:5 (v/v) was used for *rac*-**4b** (flow: 1 mL/min, Chiralpak IB column). The analysis revealed that the chemical transformations to which *L*-**2a,b** was subjected to yield *L*-**4a,b** did not affect the enantiopurity of the involved compounds (*ee* of *L*-**4a,b**: >99%, >99%).

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