BABEȘ-BOLYAI UNIVERSITY

CLUJ-NAPOCA

Faculty of Chemistry and Chemical Engineering

Doctoral School of Chemistry

Stereoselective Additions/Eliminations and Biodiesel Synthesis by Single Walled Nanotubes Immobilized Enzymes

PhD Thesis Abstract

PhD candidate: Judith-Hajnal VÁRI (married BARTHA-VÁRI)

Scientific advisor: Prof. Dr. Eng. Florin Dan IRIMIE

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Jury:

President: Prof. Cristian SILVESTRU - Babeş-Bolyai University, Cluj-Napoca

Scientific advisor: Prof. Florin Dan IRIMIE - Babeş-Bolyai University, Cluj-Napoca

Reviewers: Prof. Francisc PÉTER - Politehnica University, Timişoara

Assoc. Prof. Niculina HĂDADE - Babeş-Bolyai University, Cluj-Napoca

Prof. Valentin ZAHARIA - Iuliu Haţieganu University of Medicine and Pharmacy, Cluj-Napoca

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Keywords: functionalized single walled carbon nanotubes, immobilization, biotransformations, biodiesel synthesis

Introduction

1.1 Enzyme immobilization

Enzymes are used as biocatalyst on a large scale on many areas, such as the industry of fine chemicals, pharmaceuticals,[1] food and beverage, cosmetics and for the production of biofuel.[2] They are biocompatible and can be obtained from renewable sources, the enzymatic processes being environmental friendly and cost effective.[3] However enzymes are unstable and their retain and reuse is difficult, which can hamper their application in industrial processes.[4] The immobilization of enzymes can overcome these drawbacks resulting biocatalysts with reusability and long-term stability.[5] Besides the improved stability and reusability of the immobilized enzyme, immobilization may also enable favorable changes in temperature or pH optimum, co-immobilization with other enzymes, and can also reduce the risk of contamination.[4] Moreover, by different immobilization techniques the enzymes enantioselectivity may also be improved.[6] However, additionally to these advantages immobilization can cause mass transfer limitations, may reduce the enzymes activity, may cause small distortion in the enzymes structure, altering the final properties of the enzyme, and can also produce increase of the costs.[7] The main strategies of enzyme immobilization can be summarized into three categories: binding to a support (carrier), crosslinking and encapsulation.

The immobilization method is unique for each enzyme, and should be chosen by the best compromise between the advantages and disadvantages mentioned above.

When performing an immobilization the properties of the **carrier material** should also be taken into account, since their characteristics have a strong influence on the immobilized enzymes performance.[8]

1.2 Carbon nanotubes

Carbon nanotubes are formed from carbon atoms which are structured in layers of graphene rolled into the shape of a cylinder.[9]

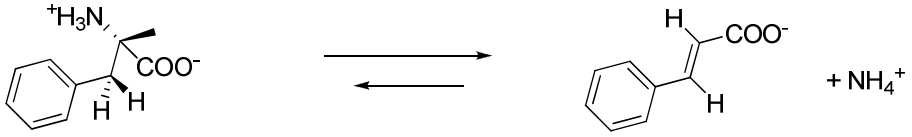
Nanotubes can be categorized in two groups: single-walled and multi-walled carbon nanotubes (SwCNTs and MwCNTs). MwCNTs are composed of concentrically displayed SwCNTs.[10]

The use of carbon nanotubes ranges from large scale structures in automobiles to nanometer scale electronics.[11] Carbon nanotubes are used primarily in composites[12] but also in tensile strength fibers and fire resistant materials.[13] Due to their unique structural, mechanical,

electrical, electrochemical properties there are also gaining interest in biological applications[14] used in interaction mainly with proteins aiming to develop efficient biosensors. Carbon nanotubes are widely used for the immobilization of biomacromolecules, exploiting their electrical, mechanical, thermal properties and general biocompatibility.[15]

1.3 Phenylalanine ammonia lyase (PAL)

Ammonia-lyases are acting on C-N bonds to catalyze the formation of α, β-unsaturated bonds by elimination of ammonia from their substrates.[16] Phenylalanine ammonia-lyases (PAL EC 4.3.1.24, EC 4.3.1.25) are homotetrameric enzymes performing the non-oxidative deamination of L-phenylalanine into (*E*)-cinnamic acid[17] (**Scheme 1**), the precursor of various phenylpropanoids, such as lignins, flavonoids and coumarins,[18] but at high pH and ammonia concentration also the reverse reaction is possible, yielding L-phenylalanine.[19]



Scheme 1 Non-oxidative deamination of phenylalanine

1.4 Candida Antractica Lipase B (CaL-B)

The yeast *Candida antarctica* has been originally isolated in Antarctica. It produces two lipases, CaL-A and CaL-B.[20] These lipases had been purified and characterized.[21] The three dimensional structure and the amino acid sequence of CaL-B has been resolved by Uppenberg et al., revealing that it is composed of 317 amino acids, folding similarly to other hydrolases.[22]

The pH optimum of CaL-B is 7, but the enzyme is stable in the 3.5- 9.5 pH range. Its denaturation temperature is approximately 50-60 ^{o}C.[23]

The active site of CaL-B contains the Ser105-His224-Asp187 catalytic triad, which is common to all serin-hydrolases,[24] an oxyanion hole (which stabilizes the transition state) and a stereospecificity pocket.[25] The oxyanion hole represents a spatial arrangement of 3 hydrogen-bond donors, 2 from the backbone amides of Gln106 and Thr40 and one from the side chain of Thr40. The stereospecificity pocket, gives CaL-B high substrate selectivity towards secondary alcohols.[24]

1.5 Amano Lipase from Pseudomonas fluorescens (L-AK)

L-AK has been characterized by the Amano researchers. It has a molecular weight of 33 kDA. The pH optimum of the enzyme is 8 while the optimum temperature is 60°C. The solution of L-AK is stable in a wide pH range (4 - 10).[26] It was shown that used in the alcoholysis of triglycerides it has a high tolerance toward ethanol and methanol.[27,28]

2 Results and discussion

2.1 Immobilization of phenylalanine ammonia-lyase from *Petrolselinum crispum* (*Pc*PAL) on functionalized single-walled carbon nanotubes for stereoselective biotransformations in batch and in continuous-flow modes

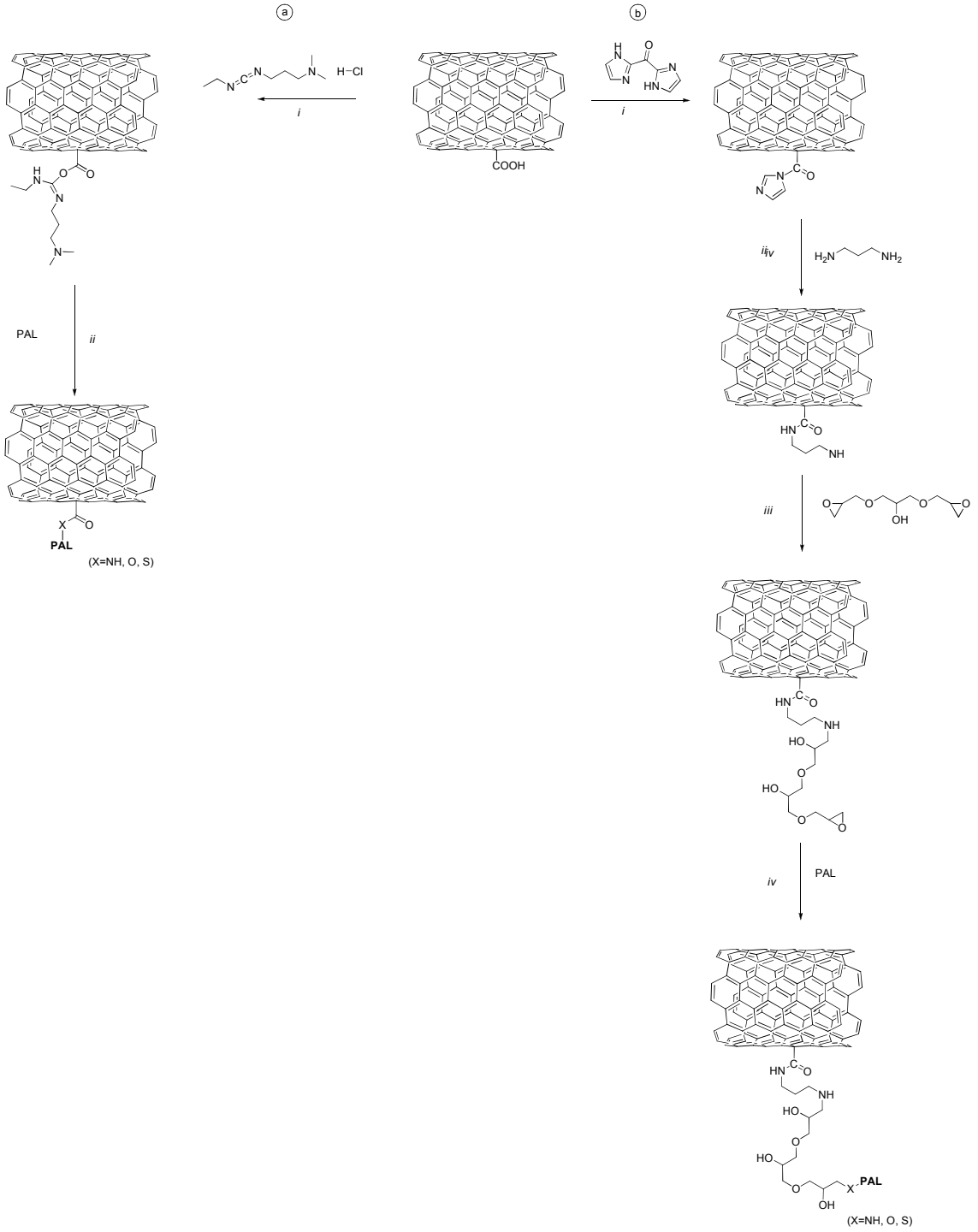
2.1.1 Aims of the study

The aim of the study represents the covalent immobilization of *Pc*PAL on functionalized SwCNTs ($SwCNT_{COOH}$ and $SwCNT_{NH2}$) and testing the activity and reusability of the resulted immobilized enzyme preparations in reactions carried out in batch and also in continuous-flow modes.

2.1.2 Covalent immobilization of *Pc*PAL on carboxylated single-walled carbon nanotubes

The covalent immobilization of *Pc*PAL on commercially available carboxylated SwCNTs was achieved by two different approaches. In the first immobilization procedure 1-ethyl-3-(3-dimethylaminopropyl)carbo-diimide hydrochloride, EDAC.HCl, was used to activate the carboxylic groups from the surface of functionalized nanotube (**Scheme 2a**). Although this immobilization method provided high immobilization yields, 89.5% of the enzyme bound to support, and the biocatalyst obtained by this method ($SwCNT_{COOH}$-PAL^{I}) was stable in the ammonia elimination reaction from **1,** it proved to be not stable enough under much harsher conditions of the ammonia addition reaction to **2** performed in a 6 M NH_3 solution **(Table 1)**. The reason of this behavior could be explained assuming that the immobilization procedure was only partially covalent, immobilization by adsorbtion also occurred.

The next goal was to create covalently immobilized SwCNT-PALs with higher stability. For this purpose glycerol diglycidyl ether (GDE), was used to attach *Pc*PAL onto the surface of 1,3-diaminopropane-functionalized $SwCNT_{COOH}$ (**Error! Reference source not found.**). The obtained product ($SwCNT_{COOH}$-PAL^{II}) was characterized by reproducibility and high immobilization yield, (95.5% of the enzyme bound to $SwCNT_{COOH,}$ activity yield 61±1.3%, determined by L-Phe at 30 °C, for 10 minutes in Tris buffer, at pH = 8.8).

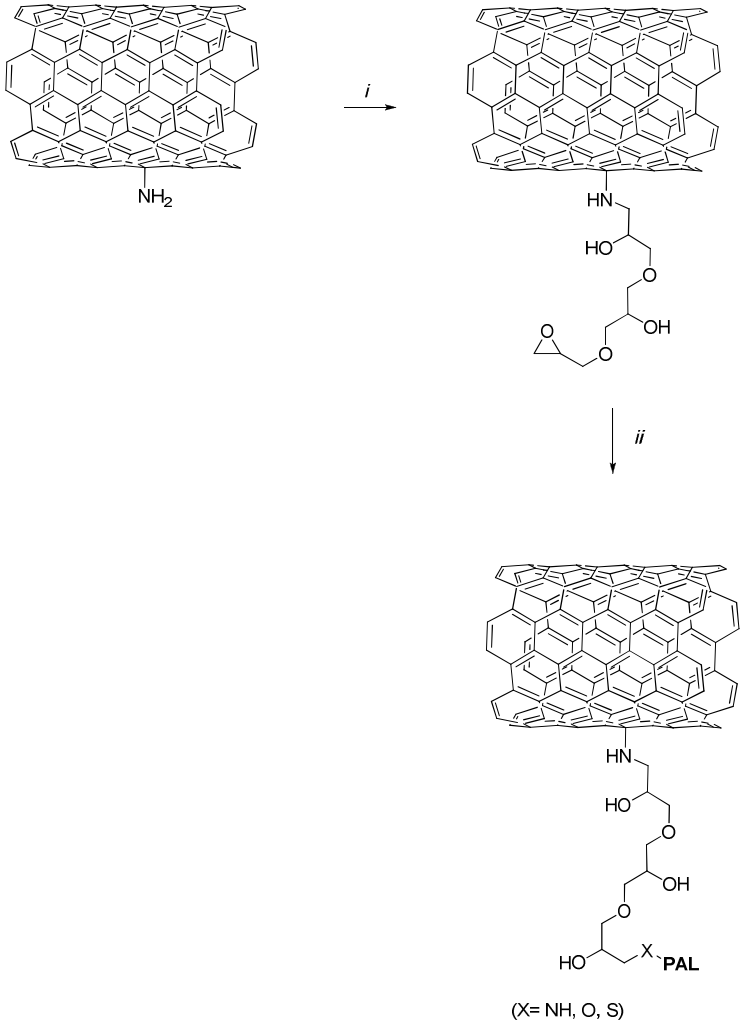


Scheme 2 Immobilization of *Pc*PAL on $SwCNT_{COOH}$ a) by EDAC activation: *i*) EDAC.HCl in Tris buffer (0.1M, pH 8.8); *ii*) *Pc*PAL in Tris buffer (0.1M, pH 8.8), b) via GDE bazed linker: *i*) CDI in CH_2Cl_2; *ii*) $H_2N(CH_2)_3NH_2$ in water *iii*) glycerol diglycidyl ether in CH_2Cl_2; *iv*) PAL in Tris buffer (0.1M, pH 8.8)

2.1.3 Covalent immobilization of PAL on $SwCNT_{NH2}$

$SwCNT_{NH2}$ was obtained by the amination of the commercially available single-walled carbon nanotubes with urea in a microwave assisted reactor.

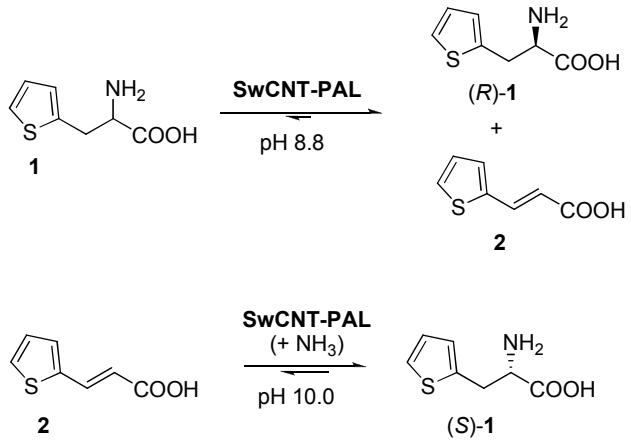
For the covalent immobilization of *Pc*PAL on functionalized $SwCNTN_{H2}$ a similar approach was applied like in case of the $SwCNT_{COOH}$. Thus, glycerol diglycidyl ether (GDE) was used as linker to attach the enzyme onto the surface of $SwCNT_{NH2}$ (**Scheme 3**). Reproducible and high immobilization yield (97 % of the *Pc*PAL bound to $SwCNT_{NH2}$) characterized the product ($SwCNT_{NH2}$-PAL).



Scheme 3 Immobilization of PAL on $SwCNT_{NH2}$ *i*) CDI in CH_2Cl_2; *ii*) PAL in Tris buffer (0.1 M Tris buffer, pH 8.0)

2.1.4 Biocatalytic behavior of SwCNT-PALs in stereoselective biotransformations of 2-amino-3-(thiophen-2-yl) propanoic and 3-(thiophen-2-yl)acrylic acid in batch mode

*Pc*PAL presents selectivity in the ammonia elimination reaction from 2-amino-3-(thiophen-2-yl) propanoic acid **1** and in the ammonia addition reaction to 3-(thiophen-2-yl)acrylic acid **2.**[29] The preservation of the enantiopreference offers the possibility of obtaining both enantiomers. The SwCNT-PALs immobilized onto the carboxy- and amino functionalized $SwCNT_{COOH}$ and $SwCNT_{NH2}$ surface were tested as biocatalysts in the ammonia elimination from **1** and ammonia addition to **2** (**Scheme 4**) in batch mode, at room temperature.



Scheme 4 SwCNT-PALs in the ammonia elimination reactions from **1** and ammonia addition reactions to **2**.

$SwCNT_{COOH}$-PALs and $SwCNT_{NH2}$-PALs showed high conversions in the kinetic resolution of racemic **1** close to the theoretically possible 50% conversion. Reusability experiments showed no significant loss of the immobilized enzymes initial activity (**Table 1**). Under the harsher conditions for ammonia addition reaction to **2** (6 M ammonia, pH 10), the immobilized enzyme preparations were less durable (**Table 1**). Among the two forms of the carboxy nanotube supported enzymes, $SwCNT_{COOH}$-PAL^{II} enabled the highest number of recycling, retaining more than 85% of its initial activity after 4 reuses (**Table 1** and **Figure 1**). The enzyme preparation obtained by immobilization of *Pc*PAL on amino functionalized CNTs showed even higher activity at the addition reaction to **2 (Table 1)**, but it was much less stable in the recycling experiments.

Table 1 Conversion of the reactions catalyzed by the two forms of *Pc*PAL immobilized on $SwCNT_{COOH}$ and *Pc*PAL immobilized on $SwCNT_{NH2}$ (25 °C, 17 h)

Run	Elimination from **1** (pH 8.8, Tris-buffer) Conversion (%)			Addition to **2** (4.5 mM in 6 M NH_3, pH 10.0) Conversion (%)		
	$SwCNT_{COOH}$-PAL^{I}	$SwCNT_{COOH}$-PAL^{II}	$SwCNT_{NH2}$-PAL	$SwCNT_{COOH}$-PAL^{I}	$SwCNT_{COOH}$-PAL^{II}	$SwCNT_{NH2}$-PAL
1	48.4	49.2	49.6	36.9	36.7	64.1
2	49.2	48.5	48.5	24.2	37.2	41.3
3	49.0	48.8	48.8	3.8	36.2	45.1
4	49.3	47.4	47.4		34.2	16.4
5	45.6	46.3	46.3		17.5	6.4
6	43.7	42.4	42.4		5.7	
7	42.5	42.3	42.3		2.2	

These conditions of ammonia addition reactions to **2** (6 M ammonia, pH 10) proved to be too harsh also for the most durable $SwCNT_{COOH}$-PAL^{II} thus the ammonia concentration was decreased to 3 M and 2 M. At the lower ammonia concentrations $SwCNT_{COOH}$-PAL^{II} and $SwCNT_{NH2}$-PAL exhibited significantly higher stability. In a 2 M ammonia solution more than 80% of the initial enzyme activity was retained even after 12 reuses in case of the $SwCNT_{COOH}$-PAL^{II} and more than 82% of the initial activity in case of $SwCNT_{NH2}$-PAL after 12 cycles (**Figure 1** and **Figure 2**).

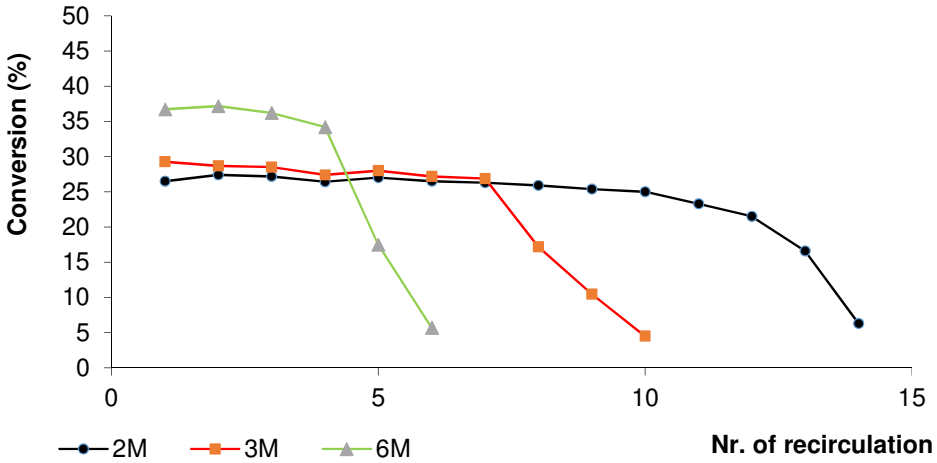


Figure 1 Reusability of SwCNT$_{COOH}$-PAL^{II} in batch mode for the ammonia addition reaction to 3-(thiophen-2-yl)acrylic acid **2** (4.5 mM) at different ammonia concentrations (2 M, 3 M and 6 M; 25 °C, 17 h, pH 10.0).

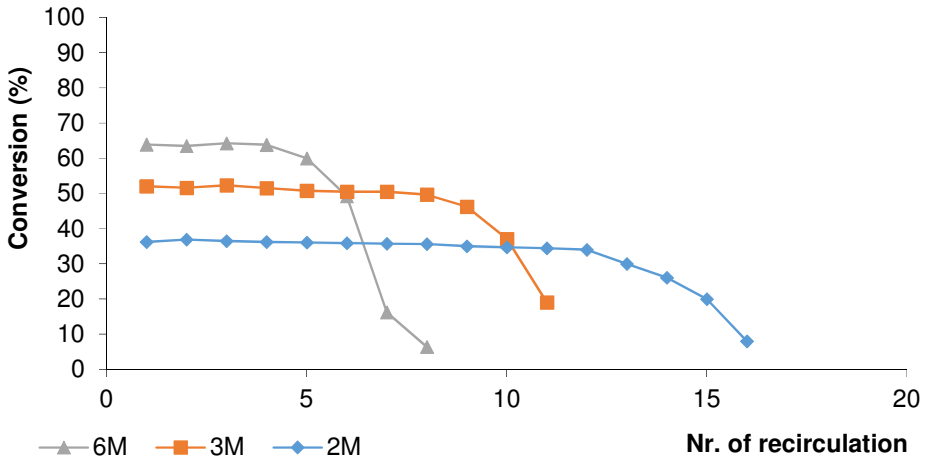


Figure 2 Reusability of SwCNT$_{NH2}$-PAL in batch mode for the ammonia addition reaction to 3-(thiophen-2-yl)acrylic acid **2** at different ammonia concentrations (2 M, 3 M and 6 M; 25 °C, 17 h, pH 10.0).

In order to increase the durability of the immobilized enzyme in the ammonia addition to **2**, when the enzyme's apparent deactivation was observed, the biocatalyst obtained through immobilization on *Pc*PAL on SwCNT$_{NH2}$ (SwCNT$_{NH2}$-PAL) was washed and kept in phosphate buffer (pH=6) for several hours, at room temperature, shaking at 1250 rpm. After this washing procedure, the enzyme regained its initial activity in the ammonia addition reaction for several cycles (**Figure 3**). When the apparent inactivation occurred again, the washing procedure was repeated.

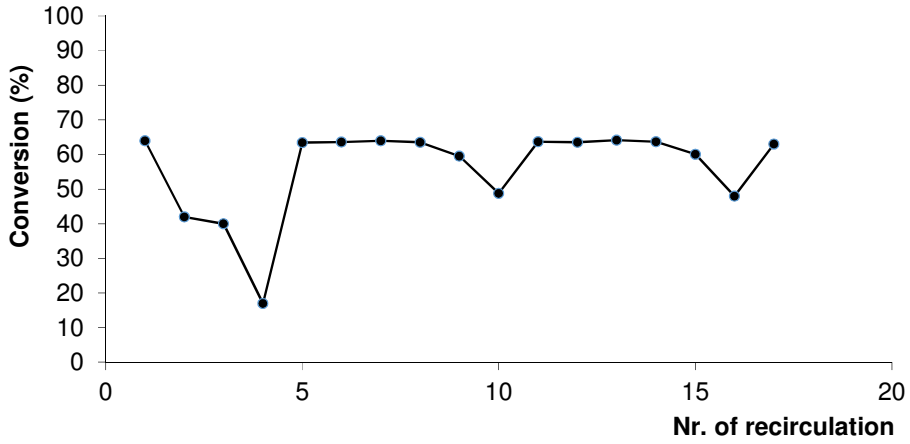


Figure 3 Ammonia addition reaction to **2**; washing the enzyme with phosphate buffer (pH 6) when the apparent inactivation is observed

Next, when performing the ammonia addition reaction mediated by SwCNT$_{NH2}$-PAL (6 M ammonia solution, pH=10), after each reuse, the enzyme was washed with phosphate buffer (pH=6), and kept in this solution for several hours at room temperature, shaking at 1250 rpm. In these conditions the enzyme preserved more than 90% of its initial activity even after 25 cycles (**Figure 4**).

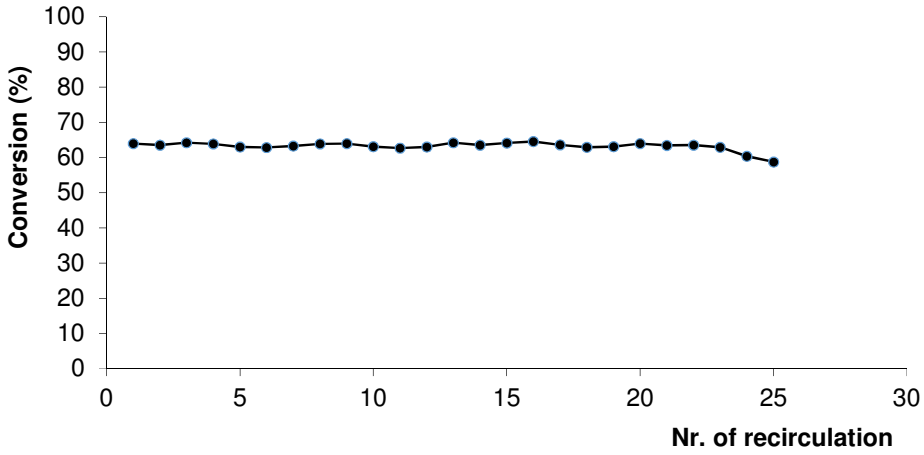


Figure 4 Ammonia addition reaction to **2**; washing the enzyme with phosphate buffer (pH 6) after each reuse

2.1.5 Ammonia addition to 3-(thiophen-2-yl)acrylic acid 2 in continuous-flow reactor with backpressure

Since the initial studies of the ammonia addition reaction to 3-(thiophen-2-yl)acrylic acid **2** in a continuous-flow packed bed microreactor indicated an apparent inactivation of the enzyme, which can be attributed to the bubble formation, in further studies a 15 bar backpressure was used. Under these conditions apparent inactivation of the immobilized *Pc*PAL was not observed. Continuous-flow ammonia addition reaction to **2** (2 M ammonia solution) with backpressure was studied in a SynBioCart system filled with the immobilized enzyme. The bioreactor was placed in the column thermostat of a HPLC system with full control of temperature and pressure (**Figure 5**).

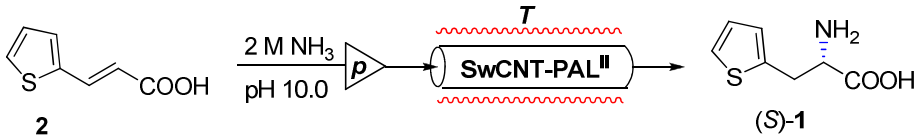


Figure 5 Ammonia addition to 3-(thiophen-2-yl)acrylic acid **2** catalyzed by SwCNT$_{COOH}$-PAL^{II} in a continuous-flow packed-bed microreactor (flow 0.1 mL/min)

The first goal was to compare the specific reaction rate of ammonia addition reaction to **2** with the most durable $SwCNT_{COOH}$-PAL^{II} biocatalyst at 30 °C in batch and also in continuous-flow mode.

The specific reaction rate (r_{flow}) in a continuous-flow reactor is a measure of the biocatalyst's productivity. r_{flow} can be obtained from the product concentration ([P], μmol mL^{-1}), the flow rate (f, mL min^{-1}) and the mass of the biocatalyst (m_e, g) {r_{flow}= ([P] × f) / m_e}.[30]

A stirred batch reaction can also be described by the specific reaction rate (r_{batch}). This value can be calculated from the amount of the product (n_p, μmol), the reaction time (t, min), and the mass of the biocatalyst (m_e, g) {r_{batch}= n_p / (t × m_e)}.[30]

The batch and the continuous-flow mode reactions in 2 M ammonia (set to pH 10, at 30 °C) were compared at 58% conversion (which is far enough from the > 90% equilibrium conversion of the ammonia addition to **2**[31] when the ammonia concentration is above 1 M). The specific reaction rate with $SwCNT_{COOH}$-PAL^{II} in continuous-flow system (r_{flow}= 2.39 μmol min^{-1} g^{-1}) was significantly higher than that in the batch experiment (r_{batch}= 1.34 μmol min^{-1} g^{-1}). By taking the known enzyme content of $SwCNT_{COOH}$-PAL^{II} into account, the productivity of phenylalanine ammonia lyase (in 2 M ammonia, pH 10.0, 58% conversion, 30°C,) was 27.5 μmol min^{-1} g^{-1} in continuous-flow mode and 15.4 μmol min^{-1} g^{-1} in the batch mode. Due to the beneficial mass transfer situation, the productivity of PAL in $SwCNT_{COOH}$-PAL^{II} in the continuous-flow system was higher than the specific activity of the native phenylalanin ammonia lyase (25.8 μmol min^{-1} g^{-1}) using the same substrate (**2**) in batch mode with the same conditions.

The influence of the temperature on the ammonia addition reaction to **2** was also investigated in the 30 - 80 °C temperature range (**Figure 6**).

The conversions of the ammonia addition to **2** in the continuous-flow microreactor remained constant after 72 hours even at temperatures up to 60 °C. These results are indicating an improved durability of the immobilized enzyme (**Figure 6**).

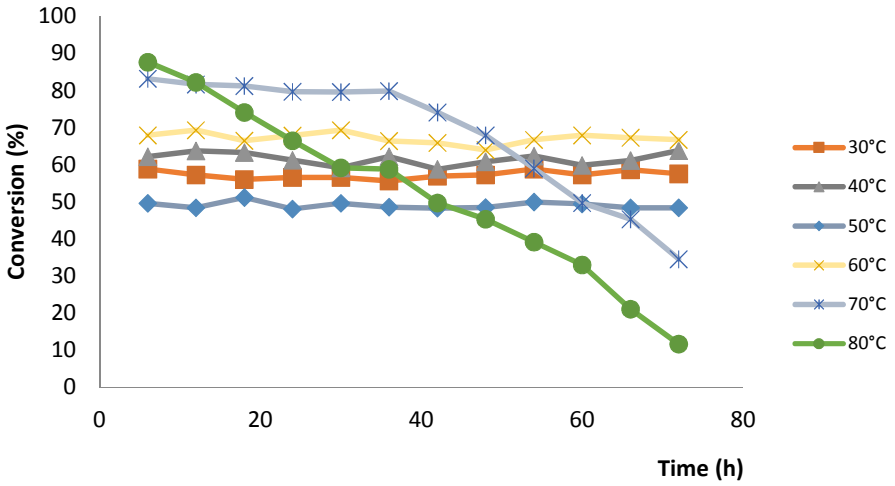


Figure 6. The effect of the temperature between 30 – 80 ° on the long term stability in ammonia addition to 3-(thiophen-2-yl)acrylic acid **2** mediated by $SwCNT_{COOH}$-PAL^{II} in a continuous-flow microreactor, under 15 bar backpressure.

2.1.6 Investigation of the ammonia addition reaction to 3-(thiophen-2-yl)acrylic acid *2* catalyzed by $SwCNT_{NH2}$-PAL in a continuous-flow packed-bed microreactor

To avoid the unwanted bubble formation, the ammonia addition reaction to **2** in continuous flow-mode mediated by $SwCNT_{NH2}$-PAL was also conducted under a 15 bar backpressure, and no apparent inactivation of the enzyme was observed for future experiments.

For the ammonia addition to **2** mediated by $SwCNT_{NH2}$-PAL, the same system was used as in case of $SwCNT_{COOH}$-PAL^{II} (SynBioCart). The bioreactor was placed in the column thermostat of a HPLC system with full control of temperature and pressure (**Figure 7**).

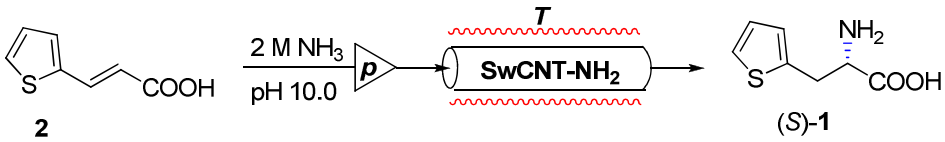


Figure 7 Ammonia addition to 3-(thiophen-2-yl)acrylic acid **2** (4.5 mM) catalyzed by $SwCNT_{NH2}$-PAL in a continuous-flow packed-bed microreactor (flow rate: 0.1 mL min^{-1}, column: 30 mm × 3 mm ID;).

As in case of the $SwCNT_{COOH}$-PAL^{II} catalyzed reaction, the first goal was the comparison between the specific reaction rate of ammonia addition reaction to **2** at 30°C in batch and in continuous-flow mode.

The specific reaction rate using $SwCNT_{NH2}$-PAL for the continuous-flow reactor (r_{flow}= 3.2 µmol min^{-1} g^{-1}) was also significantly higher than that in case the batch reaction (r_{batch}= 1.6 µmol min^{-1} g^{-1}), exceeding also the values obtained in the case of $SwCNT_{COOH}$-PAL^{II}. Next, the

influence of the temperature on the ammonia addition reaction to **2** was studied in the temperature range of 30-80 °C (**Figure 8**).

In the $SwCNT_{NH2}$-PAL catalyzed ammonia addition to **2** the biocatalysts behavior was similar with the $SwCNT_{COOH}$-PAL^{II}s behavior, the conversions remained constant after 72 h of operation at temperatures up to 60°C. The conversion rates reached higher values with the $SwCNT_{NH2}$ biocatalyst. (**Figure 8**).

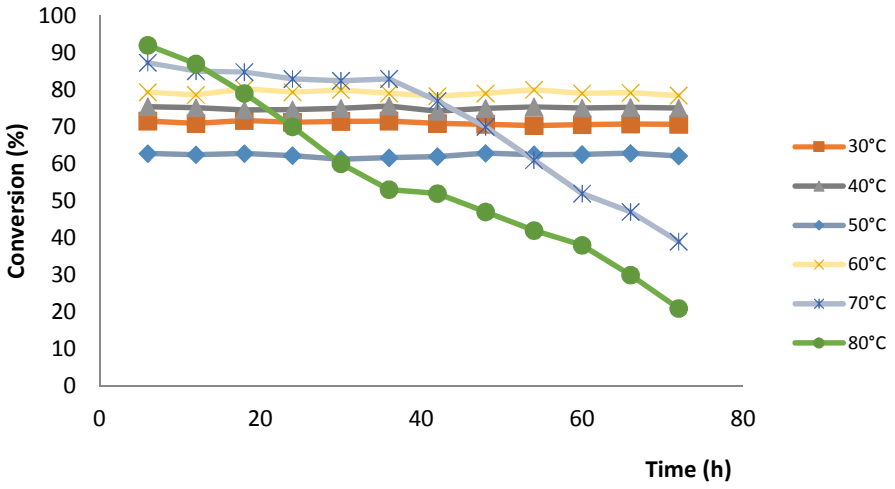


Figure 8. Temperature effect between 30 – 80 °C on the long term stability in ammonia addition to 3-(thiophen-2-yl)acrylic acid **2** catalyzed by $SwCNT_{NH2}$-PAL in a continuous-flow microreactor, under 15 bar backpressure.

2.1.7 Conclusions

This study demonstrates that carboxy-functionalized and amino-functionalized single-walled carbon nanotubes ($SwCNT_{COOH}$ and $SwCNTN_{H2}$) can be used efficiently as nanostructured supports for covalent immobilization of phenylalanine ammonia-lyase from *Petroselinum crispum.* In case of the carboxy functionalized single walled carbon nanotubes the most durable enzyme preparations were obtained with glycerol diglycidyl ether (GDE) as a linker molecule for enzyme immobilization. In case of the amino functionalized nanotube the immobilization of *Pc*PAL was also obtained using GDE. Both of the biocatalyst ($SwCNT_{COOH}$-PAL^{II} and $SwCNT_{NH2}$-PAL) prepared in this way proved to be efficient in preparing (*R*)-2-amino-3-(thiophen-2-yl)propanoic acid (*R*)-**1** by kinetic resolution of racemic **1** and its enantiomer (*S*)-**1** by the enantiotop selective ammonia addition onto (*E*)-3-(thiophen-2-yl)acrylic acid **2**. Recycling studies demonstrated that $SwCNT_{COOH}$-PAL^{II} can preserve more than 80% of its original activity even after 7 cycles in ammonia elimination reaction from **1** and 12 cycles in ammonia addition reaction to **2** in 2 M NH_3, while $SwCNT_{NH2}$-PAL proved to be an even more stable biocatalyst, preserving more than 82% of its initial activity after 12 cycles in the ammonia

addition reaction whit 2 M NH_3. Moreover, when washing and keeping the $SwCNT_{NH2}$-PAL in a 6 M buffer for several hours before recycling it, the enzyme retained more than 90% of its initial activity even after 25 cycles in ammonia addition to **2** (in 2 M NH_3). Studies using a $SwCNT_{COOH}$-PAL^{II} and $SwCNT_{NH2}$-PAL-filled packed-bed continuous-flow microreactor in the 30-80 °C temperature range revealed that unaltered enzyme activity for ammonia addition reaction to **2** can be maintained for at least 72 h, up to 60 °C temperature in 2 M ammonia solution in a packed-bed continuous-flow microreactor in case of both enzyme preparations.

2.2 Functionalized nanotubes supported lipases for biodiesel synthesis

2.2.1 Nanobioconjugates of functionalized single-walled carbon nanotubes and *Candida antarctica* lipase B in biodiesel production

2.2.1.1 Aims of the study

The aim of this study was the immobilization of lipase B from *Candida antarctica* (CaL-B) on functionalized single walled carbon nanotubes, and the use of the immobilized preparations for the production of biodiesel from sunflower oil.

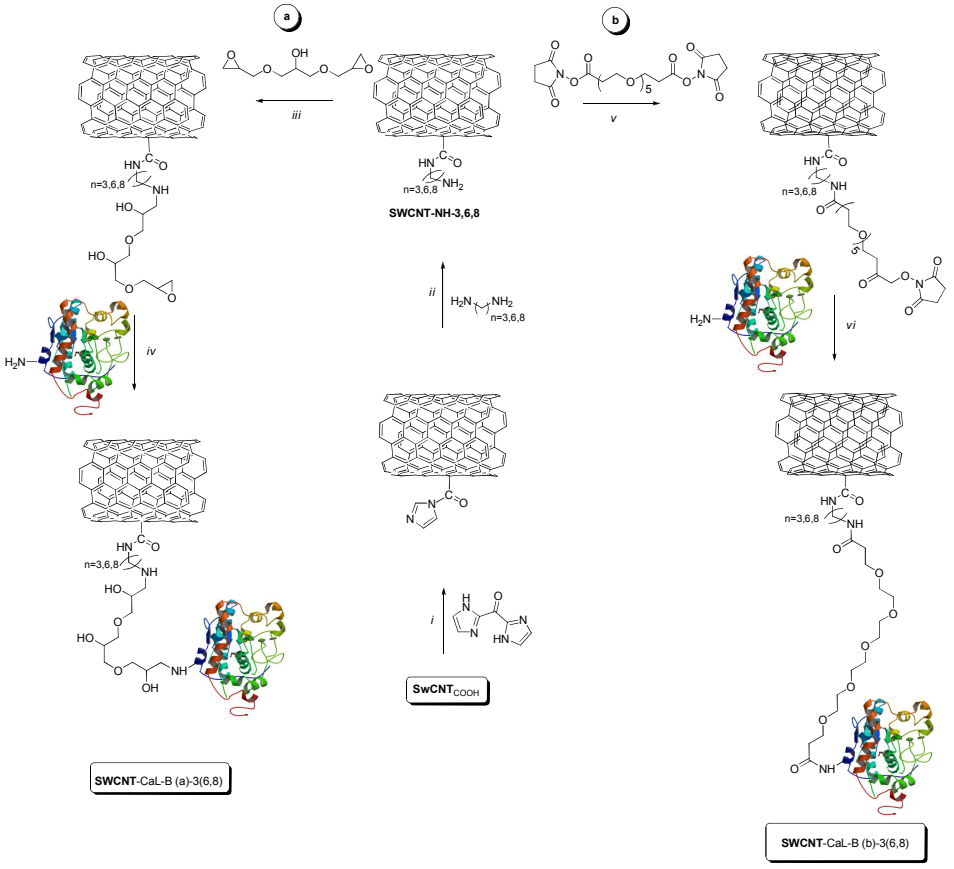
2.2.1.2 Covalent immobilization of CaL-B on single walled carbon nanotubes

The commercially available $SwCNT_{COOH}$ was activated with carbonyldiimidazol (CDI), followed by a coupling with alkyldiamines with different lengths (1,3-diaminopropane, 1,6-diaminohexane and 1,8 diamonooctane), yielding the amino functionalized single walled carbon nanotubes with different lengths (SwCNT-NH-3, SwCNT-NH-6, SwCNT-NH-8). Next, two different types of crosslinkers were used (glycerol diglycidyl ether, GDE and PEGylated bis (sulfosuccinimidyl)suberate, $BS(PEG)_5$ to activate the SwCNT-NHs in order to create the linkage of the amino groups from the enzyme surface to the support. The enzyme immobilization was carried out in the presence and in the absence of Tween 80, a non-ionic surfactant (**Scheme 5**).

In all the immobilization protocols reproducible and high immobilization yields were obtained (> 99% of the enzyme bound to $SwCNT_{COOH}$). The enzyme loading was of 0.33 mg protein per mg of $SwCNT_{COOH}$.

2.2.1.3 Transesterification of sunflower oil with SwCNT-CaL-B

To test the SwCNT-CaL-B immobilized enzyme preparations the transesterification reaction of sunflower oil was performed. With the model enzyme preparate, SwCNT-CaL-B(a)-6, obtained through immobilization with 1,6-hexanediamine and glycerol diglycidyl ether as crosslinker in presence of the non-ionic surfactant the optimal reaction conditions were determined.



Scheme 5 Immobilization of CaL-B on SWCNT$_{COOH}$. a) immobilization route with GDE: i) CDI in CH_2Cl_2; ii) $H_2N(CH_2)_3NH_2$/$H_2N(CH_2)_6NH_2$/$H_2N(CH_2)_8NH_2$ in water; iii) glycerol diglycidyl ether in CH_2Cl_2; iv) CaL-B in PBS buffer (20 mM Na_2HPO_4, 150 mM NaCl, pH 7); b) Immobilization via BS(PEG)$_5$: i) CDI in CH_2Cl_2; ii) $H_2N(CH_2)_3NH_2$/$H_2N(CH_2)_6NH_2$/$H_2N(CH_2)_8NH_2$ in water; v) BS(PEG)$_5$ in DMSO; vi) CaL-B in PBS buffer (20 mM Na_2HPO_4, 150 mM NaCl, pH 8).

2.2.1.4 The effect of organic solvents upon the ethanolysis reaction

The transesterification reactions were performed in several organic solvents, and the results indicated that acetonitrile is the best solvent for the biodiesel synthesis mediated by the model biocatalyst, SwCNT-CaL-B(a)-6. In this case after 4 hour reaction time the conversion reached 63% (**Table 2**).

Table 2 The effect of the nature of the solvent on the ethanolysis of sunflower oil mediated by SwCNT-CaL-B(a)-6 after 4 hour reaction time.

Entry	Solvent	Conversion (%)
1	EtOH	20.7
2	ACN	63.5
3	*t*-BuOH	30.3
4	DIPE	12.1
5	*t*-BME	9.51
6	1,4-Dioxane	<5
7	Heptane	<5
8	Hexane	<5
9	Octane	<5
10	Isooctane	<5
11	CH_2Cl_2	<5
12	CCl_4	<5
13	DMF	<5
14	THF	<5
15	Acetone	<5
16	EtOAc	<5

2.2.1.5 The effect of water content on biodiesel production

The SwCNT-CaL-B(a)-6 mediated transesterification reactions of sunflower oil were carried out by adding different amount of water (0-5 vol. %,) to the reaction mixtuer. Reactions carried out with low amount of water (0.1- 0.5 vol. %) presented higher conversion values, while by increasing the water content, the enzyme activity decreased significantly (**Table 3**).

Table 3 The effect of water content on the biodiesel production (after 3 h reaction time)

Entry	Water content (vol. %)	Conversion (%)
1	0	56.9
2	0.1	59.2
3	0.5	58.5
4	1	49.5
5	1.5	46.7
6	2	38.5
7	3	34.6
8	4	42.3
9	5	34.9

2.2.1.6 The effect of substrate-solvent ratio on the ethanolysis of sunflower oil

The substrate concentration has also a great influence upon the enzymes activity. Determining the optimal substrate content in the reaction mixture is important regarding the cost-efficacy of the process. By varying the sunflower oil content in the ethanolysis reactions catalyzed by SwCNT-CaL-B(a)-6, the highest conversion was obtained at a substrate-solvent ratio of 1:5 (w/v) (**Table 4**).

Table 4 The effect of substrate concentration upon the biodiesel production (after 3 h reaction time)

Entry	Substrate- ACN *ratio* (w/v)	Conversion (%)
1	1:1	6.9
2	1:2	17.5
3	1:5	58.9
4	1:10	51.5
5	1:20	52.2
6	1:25	52.1
7	1:50	37.2

2.2.1.7 The effect of ethanol concentration on biodiesel production

The amount of nucleophile could also significantly influences the reaction rate of the biocatalytic transesterification processes.[32] The initially fixed, 1:5 oil:ethanol molar ratio was varied, and the results showed that by lowering the ethanol content the conversion value grows, while at higher ethanol content the enzyme activity was significantly lowered. The highest conversion value was obtained using the minimal stoichiometric oil-etanol ratio (1:3) (**Table 5**).

Table 5 The effect of oil:ethanol molar ratio on the ethanolysis process (after 3 h reaction time)

Entry	Oil-ethanol molar *ratio*	Conversion (%)
1	1:1	46.7
2	1:3	59.7
3	1:5	58.3
4	1:10	38.7
5	1:20	42.2
6	1:50	20.7

2.2.1.8 The effect of crosslinker type and length and surfactant presence on the transesterification reaction

The catalytic activity of the variously immobilized CaL-B preparates was tested using the optimized conditions determined previously. The immobilized preparations without the use of

Tween-80 (**Table 6**, entry 2,7) showed lower enzyme activity than those immobilized in presence of the surfactant (**Table 6**, Entry 1,6). The shorter (1,3-diaminopropane) linker provided superior enzyme activities while the use of longer linkers displayed a negative effect upon enzyme efficacy (**Table 6**, entry 1, 5).

The immobilization procedure with the shorter diglycidyl ether crosslinker (**Table 6**, entries 1-4) provided better results in all cases the, than those with the longer ($BS(PEG)_5$) crosslinker (**Table 6**, entries 5-11).

Table 6 The effect of immobilization type on biodiesel production after 4 h reaction time (*immobilization without Tween-80)

Entry	Immobilized enzyme	Linker	Conversion (%)
1	SwCNT-CaL-B(a)-3	1,3-diaminopropane	79.9
2	SwCNT-CaL-B(a)-3*	1,3-diaminopropane	30.3
3	SwCNT-CaL-B(a)-6	1,6-diaminohexane	63.2
4	SwCNT-CaL-B(a)-8	1,8-diaminooctane	20.4
5	SwCNT- CaL-B(b)-3-1	1,3-diaminopropane	37.2
6	SwCNT-CaL-B(b)-3-2	1,3-diaminopropane	50.8
7	SwCNT-CaL-B(b)-3-2*	1,3-diaminopropane	20.2
8	SwCNT- CaL-B(b)-6-1	1,6-diaminophexane	30.1
9	SwCNT-CaL-B(b)-6-2	1,6-diaminohexane	39.7
10	SwCNT- CaL-B(b)-8-1	1,8-diaminooctane	21.1
11	SwCNT- CaL-B(b)-8-2	1,8-diaminooctane	27.6

2.2.1.9 The effect of temperature

Upon investigation of the temperature effect of the ethanolysis reaction catalyzed by SwCNT-CaL-B(a)-3, the optimal temperature was found to be 35^0C (**Figure 9**).

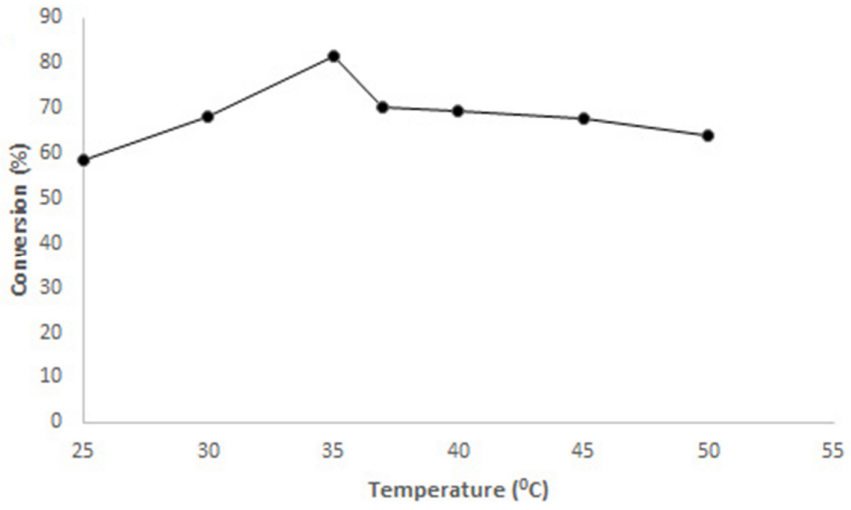


Figure 9 . The temperature effect on the enzymatic transesterification reaction after 3 hours reaction time

2.2.1.10 Reusability of the biocatalyst

The reusability of the lipases is a very important aspect, since their high-cost. Since the covalently immobilized enzyme cannot be washed from the nanosupport, it is suitable for long term usage. The reusability of SwCNT-CaL-B(a)-3 in a repeated batch procedure was tested using the optimal conditions.

10 repeated batches were performed, while the biocatalysts retained more than 90% of its initial activity (**Figure 10**).

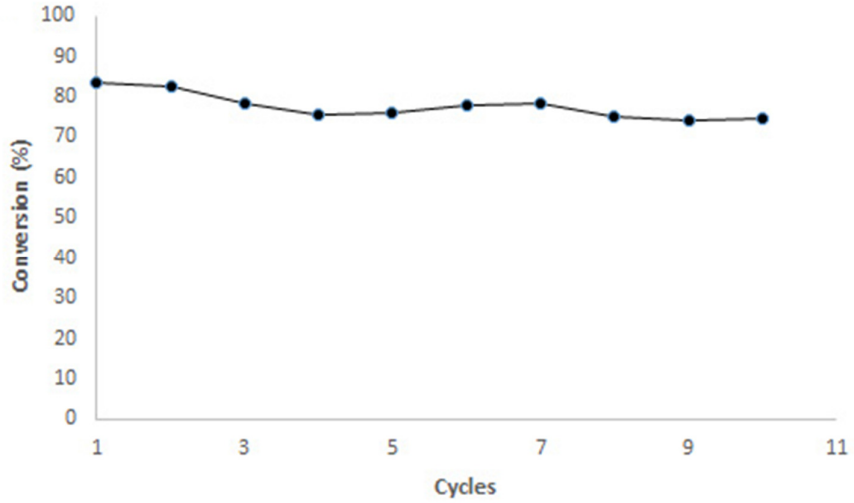


Figure 10 The reusability study of the $SwCNT_{COOH}$-CaL-B(3)-a using 4 hours batch runs.

2.2.1.11 Time-profile of the transfesterification reaction

The time course profile of the reaction shows that the reaction proceeds fast in the first four hours, (reaching a conversion of 83.4%), after that the conversion increasement slows down (reaching 93.1% after 16 hours) (**Figure 11**).

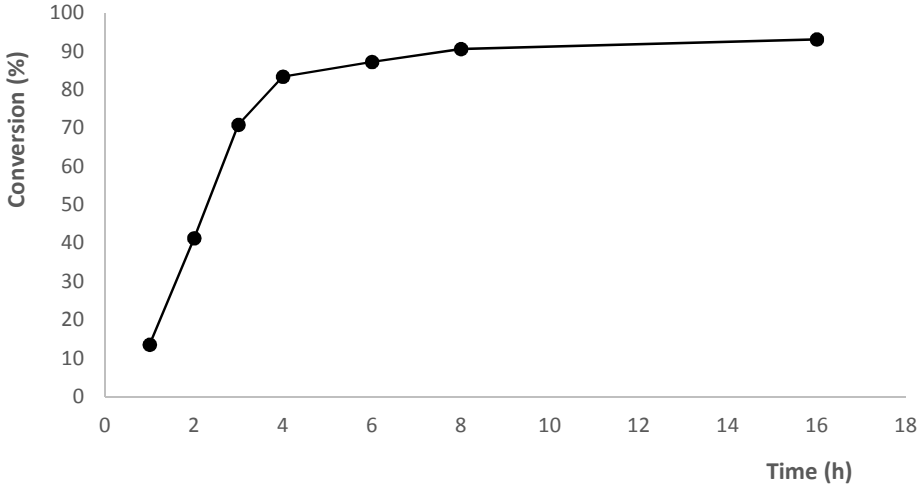


Figure 11 The time course profile of the sunflower oil transesterification reaction using the optimal reaction conditions

2.2.1.12 Conclusions

The covalent immobilization of lipase B from *Candida antarctica* on carboxy-functionalized single-walled carbon nanotubes ($SwCNT_{COOH}$) provided a useful biocatalyst for biodiesel production. The linker length had a strong influence on the enzyme activity, more active immobilized preparates were obtained using shorter linkers. SwCNT-CaL-B(a)-3, the most active enzyme preparat obtained by the immobilization of CaL-B with glycerol diglycidyl ether on propane-1,3-diamine functionalized $SwCNT_{COOH}$ provided high conversion for the ethanolysis of sunflower oil. Recycling studies demonstrated that SwCNT-CaL-B(a)-3 has high operational stability (preserving more than 90% of its original activity even after 10 reaction cycles).

2.2.2 Nanobioconjugates of Amano lipase from *Pseudomonas fluorescens (L-AK)* and functionalized single-walled carbon nanotubes in biodiesel production

2.2.2.1 Aims of the study

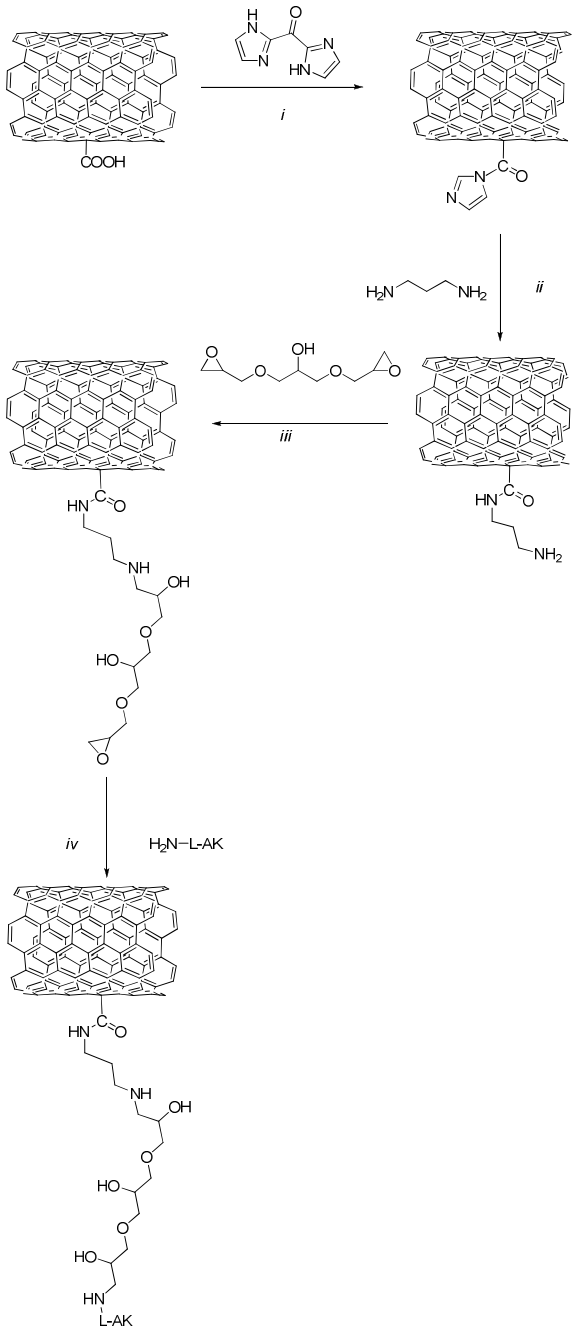
This study aims the covalent immobilization of Amano lipase from *Pseudomonas fluorescens* (L-AK) on carboxylated single-walled carbon nanotubes ($SwCNT_{COOH}$) for the preparation of biocatalysts with improved properties in continuous-flow applications.

2.2.2.2 Covalent immobilization of L-AK on $SwCNT_{COOH}$

The immobilization of L-AK was achieved using the optimum conditions from the immobilization of CaL-B.

The commercially available activated $SwCNT_{COOH}$ with carbonyldiimidazol were coupled with 1,3-propilenediamine yielding the amino-functionalized $SwCNT_{COOH}$. Using glycerol diglycidyl ether (GDE) as crosslinker amine-functionalized supports were activated for the linkage of the amine groups from the enzyme surface to the activated support. The L-AK immobilization was performed in the presence of the non-ionic surfactant, Tween-80 (**Scheme 6**). Furthermore, the enzyme loading was tested with different support-enzyme ratios.

In all cases reproducible and high immobilization yields (> 99% of the L-AK bound to $SwCNT_{COOH}$) characterized the product.



Scheme 6 Immobilization of L-AK on $SwCNT_{COOH}$. with GDE: *i*) CDI in CH_2Cl_2; *ii*) $H_2N(CH_2)_3NH_2$ in water; *iii*) glycerol diglycidyl ether in CH_2Cl_2; *iv*) L-AK in PBS buffer (20 mM Na_2HPO_4, 150 mM NaCl, pH 7)

2.2.2.3 Transesterification of sunflower oil with SwCNT-L-AK

Next, the activity of SwCNT-L-AK immobilized enzyme preparations was also tested in the ethanolysis of sunflower oil. The optimal reaction conditions were determined performing

several analytical scale screening, using as model enzyme preparate the SwCNT-L-AK obtained with an enzyme loading of 0.33mg enzyme/mg enzyme preparation.

2.2.2.4 The effect of organic solvents upon biodiesel production

Performing the transesterification reactions in various solvents, it was found that isooctane is the best solvent since the conversion reached high values (87 %) after 4 h (**Table 7**)

Table 7 The effect of the nature of the solvent on the ethanolysis of sunflower oil mediated by SwCNT-L-AK after 4 hours reaction time.

Entry	Solvent	Conversion (%)
1	ACN	53.74909
2	CH_2Cl_2	2.829617
3	EtOH	86.78548
4	Hexane	67.80793
5	Izooctane	87.74641
6	t-Bme	31.71333
7	t-BuOH	42.17548

2.2.2.5 The effect of water content on biodiesel production

Performing the SwCNT-L-AK catalyzed ethanolysis of sunflower oil, by varying the water content between 0-5 vol.%, as in case of the CaL-B, the results found showed that the highest enzyme activity is achieved with no water content. (**Table 8**).

Table 8 The effect of water content on the biodiesel production (after 4 h reaction time)

Entry	Water content (vol. %)	Conversion (%)
1	0	84.0525
2	0.1	82.98121
3	0.5	63.50525
4	1	54.4212
5	1.5	34.95314
6	2	16.09766
7	3	48.85216
8	4	73.0505
9	5	49.17327

2.2.2.6 The effect of the enzyme loading on the transesterification reactions

Performing the reactions with enzyme preparates with different loading of enzyme it was shown that the enzyme preparate with the 1:2 support- enzyme ratio had the highest activity (**Table 9**).

Table 9 The effect of the enzyme loading on the activity of the immobilized biocatalyst (after 3 h reaction time)

Entry	Support-Enzyme ratio (w/w)	Conversion (%)
1	2:1	53.57741
2	1:1	72.6089
3	1:2	85.57113
4	1:5	67.52561

2.2.2.7 The effect of ethanol concentration on biodiesel production

Further, by varying the initially fixed oil:ethanol molar *ratio* (1:5), it was found that the 1:5 molar ratio provides the higher conversion rats (**Table 10**).

Table 10 The effect of oil:ethanol molar ratio on the ethanolysis process (after 3 h reaction time)

Entry	Oil-ethanol molar *ratio*	Conversion (%)
1	1:1	65.2
2	1:3	88.8
3	1:5	91.5
4	1:10	89.4
5	1:20	90.4
6	1:50	84.4

2.2.2.8 Production of biodiesel in a continuous-flow reactor

The continuous-flow biodiesel production was performed in SynBioCart columns, filled with $SwCNT_{COOH}$-L-AK, attached to the pump module of an Agilent LC 1100 HPLC. The column was placed vertically, to prevent the formed glycerol byproduct to attach to the immobilized enzyme, thus disabling the substrate to reach the biocatalyst.

First, the flow rate was set to 0.35 mL/min, a value where the initial conversion of the trasesterification reaction was approximately 70%. For investigation of the long term stability of the immobilized enzyme upon the ethanolysis of the sunflower oil, samples were withdrawn after different time intervals and analyzed. The results show continuous decrease of the enzymes initial activity in the first 60 hours, after which the biocatalysts activity remains constant for at least 40 hours (**Figure 12**).

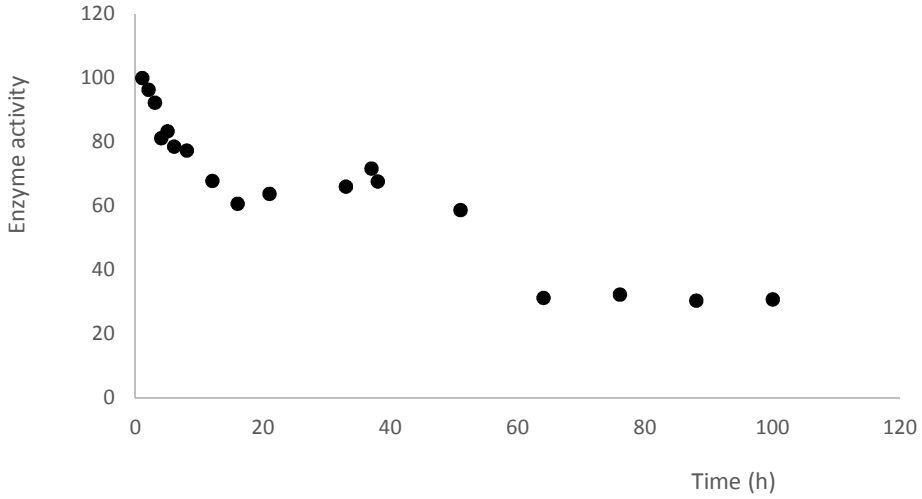


Figure 12 Time dependent behaviour of the $SwCNT_{COOH}$-L-AK in the continuous-flow biodiesel syntheis

2.2.2.9 Conclusions

The covalent immobilization of Amano lipase from *Pseudomonas fluorescens* on carboxy-functionalized single-walled carbon nanotubes proved to be a useful biocatalyst for biodiesel production. After several optimization rounds (including the solvent screening, the water content screening, the ethanol concentration and the enzyme loading effect), the conversion reached 90% using isooctane as solvent. Moreover, the obtained enzyme preparation has potential application in continuous-flow biodiesel synthesis.

3 General conclusions

The present thesis describes the usefulness of functionalized single walled carbon nanotubes (SwCNT) as support material for the covalent immobilization of phenylalanin ammonia lyase from *Petroselinum crispum* (*Pc*PAL), lipase B from *Candida antarctica* (CaL-B) and Amano lipase from *Pseudomonas Fluorescens* (L-AK).

The covalent immobilization of *Pc*PAL on carboxy- and amino functionalized single walled carbon nanotubes ($SwCNT_{COOH}$ and $SWCNT_{NH2}$) demonstrates for the first time the usefulness of the obtained biocatalyst ($SwCNT_{COOH}$-PAL^{II} and $SwCNT_{NH2}$-PAL) for stereoselective biotransformations both in batch mode and in continuous-flow reactors. The durability and temperature dependent properties and of SwCNT-PALs were also characterized.

Through the covalent immobilization of two lipases, lipase B from *Candida antarctica* (CaL-B) and Amano lipase from *Pseudomonas fluorescens* (L-AK) on carboxylated single-walled carbon nanotubes stable and highly efficient biocatalyst were obtained with application in biodiesel production.

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5 List of publications

1. Bartha-Vári, J.H., Toşa, M.I., Irimie, F.D., Weiser, D., Boros, Z., Vértessy, B.G., Paizs, C., Poppe, L. Immobilization of phenylalanine ammonia-lyase on single-walled carbon nanotubes for stereoselective biotransformation in batch and continuous-flow modes. *ChemCatChem,* **2015**, 7, 1122-1128, I.F.= 4.556

2. Bencze, L.C., Bartha-Vári, J.H., Katona, G., Toşa, M.I., Paizs, C., Irimie, F.D. Nanobioconjugates of Candida antarctica lipase B and single-walled carbon nanotubes in biodiesel production. *Bioresource Technol*, **2016**, 200, 853–860, I.F.=4.494

Conference presentations

1. Bartha-Vári, J.H., Bencze, L.C., Toşa, M.I., Paizs, C., Irimie, F.D., Functionalized Nanotubes Supported Lipases for Biodiesel Synthesis, *Young Researchers' International Conference on Chemistry and Chemical Engineering,* 12nd-14th May, **2016**, Cluj-Napoca, Romania – oral presentation
2. Bartha-Vári, J.H., Nagy, E.Z., Gal, C.A., Bencze, L.C., Toşa, M.I., Irimie, F.D., Abaházi, E., Poppe, L., Paizs, C., CaL-B Immobilized on Single Walled Carbon Nanotubes as Efficient Biocatalyst for the Kinetic Resolution of 1-(Hetero)aryl –Ethanols, COST Action CM1303 "*SysBiocat" Training School,* 27th- April-1st May, 2016, Certosa di Pontignano, Italy - poster
3. Vári, J.H., Varga, A., Poppe, L., Irimie, F.D., Paizs, C., Covalent Immobilization of Phenylalanine Ammonia Lyase on Functionalized Single Walled Carbon Nanotubes, *COST Action CM1303 "SysBiocat" Training School,* 28th May-1st June, **2014**, Certosa di Pontignano, Italy - poster
4. Vári, J.H., Varga, A., Poppe, L., Irimie, F.D., Paizs, C., Covalent immobilization of Phenylalanine Ammonia Lyase on Functionalized Single-Walled Carbon Nanotubes, *COST Action CM1303 "SysBiocat‖ Kick-off Workshop,* 10th-14th April, **2014**, Madrid, Spain – oral presentation
5. Vári, J.H., Poppe, L., Irimie, F.D., Paizs, C., Fenil-alanin ammónia-liáz (PAL) és Candida Antarctica lipase B (CaL-B) kovalens immobilizálása funkcionalizált egyfalú szén nanocsövön (SWNT), *19th International Conference on Chemistry,* 21st – 24th November, 2013, Baia-Mare, Romania - oral presentation

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