

Aminated Single-walled Carbon Nanotubes as Carrier for Covalent Immobilization of Phenylalanine Ammonia-lyase

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RESEARCH ARTICLE

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Abstract

A new and efficient immobilized form of phenylalanine ammonia-lyase (PAL) was obtained by covalent linkage onto amino functionalized single-walled carbon nanotubes (SwCNT_{NH₂}) as carrier. The catalytic properties of the resulted nanostructured biocatalyst (SwCNT_{NH₂}-PAL) were tested in the kinetic resolution of racemic 2-amino-3-(thiophen-2-yl)propanoic acid **1** by ammonia elimination and in the enantioselective addition of ammonia onto (*E*)-3-(thiophen-2-yl)acrylic acid **2**. SwCNT_{NH₂}-PAL was a durable biocatalyst in batch mode for ammonia elimination from **1** (>85% of original activity after 7 cycles) and in ammonia addition to **2** (>70% of original activity after 3 cycles in 6 M NH₃, pH 10.0). The ammonia addition onto **2** was also studied in a continuous-flow microreactor packed with SwCNT_{NH₂}-PAL (2 M NH₃, pH 10.0, 15 bar) in the 30-80 °C temperature range. No significant loss of PAL activity was observed over 72 h in the microreactor up to 60 °C. Productivity of SwCNT_{NH₂}-PAL at 30 °C was significantly higher in the enantioselective ammonia addition performed in a packed-bed reactor operated in continuous-flow mode ($r_{\text{flow}} = 2.41 \text{ mmol min}^{-1} \text{ g}^{-1}$) than in the reaction performed in batch system ($r_{\text{batch}} = 1.38 \text{ mmol min}^{-1} \text{ g}^{-1}$).

Keywords

PAL, aminated SwCNT, covalent immobilization, continuous-flow system

1 Introduction

Biocatalysis is a resource efficient, economical, energy saving, and environment-friendly way to perform synthetic processes [1]. Since the biocatalyst is often dissolved in the reaction medium, separation and reuse of the catalyst may be difficult [2]. Immobilization of the biocatalysts offers a solution to enhance the properties of these catalysts for synthesis of fine chemicals and pharmaceuticals [3], for food modification or energy production [4]. Immobilization of the enzymes can overcome the major drawbacks of enzyme application, such as the lack of long-term operational stability and the difficulty of the enzyme recovery [5]. Enzyme immobilization can be achieved by binding to a solid support, cross-linking or entrapment [6]. Covalent coupling to a solid support is the most typical way of immobilizing enzymes [7].

The unique properties of carbon nanotubes attracted attention due to their potential applications [8]. The use of carbon nanotubes ranges from large scale structures in automobiles to nanometer scale electronics [9]. Carbon nanotubes are used primarily in composites [10], but also in tensile strength fibers and fire resistant materials [11]. Due to their unique structural, mechanical, electrical, electrochemical properties there is an increasing interest in their biological applications [12], mainly as nanocarriers for proteins aiming to develop efficient biosensors. Carbon nanotubes are widely used for the immobilization of biomacromolecules, exploiting their mechanical, thermal, electrical properties and general biocompatibility [13].

Ammonia-lyases, acting on C–N bonds, catalyze the formation of α,β -unsaturated bonds by elimination of ammonia from their substrates [14]. Several ammonia-lyases were applied in the stereoselective synthesis of enantiopure α -amino acids such as L-phenylalanine or (*S*)-2-chlorophenylalanine by addition of ammonia to (*E*)-cinnamic acid [15], or (*E*)-2-chlorocinnamic acid [16]. PAL can be also used in medicine, as enzyme replacement treating the phenylketonuria [17], or even in cancer treatment [18]. The synthetic potential of PAL has been utilized in production of PAL immobilized on carbon nanotubes [19] or on magnetic nanoparticles [20-21] which had been successfully used in microfluidic reactors

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for stereoselective biotransformation. Application of immobilized enzymes in miniaturized or microfluidic reactors for biotransformations offers multiple advantages such as higher efficiency of the process and improved operational stability of the biocatalysts [22, 23].

In our previous study [19], phenylalanine ammonia-lyase from *Petroselinum crispum* (*PcPAL*) was immobilized by covalent linkage onto carboxylated single-walled carbon nanotubes ($\text{SwCNT}_{\text{COOH}}$) to prepare *PcPAL* biocatalysts with improved properties for continuous-flow applications. In this study we performed the covalent immobilization of the same enzyme on aminated single walled carbon nanotubes ($\text{SwCNT}_{\text{NH}_2}$) with the aim to further improve the enzyme stability for the ammonia addition reaction. The $\text{SwCNT}_{\text{NH}_2}$ -PAL obtained in this way was tested as biocatalyst for stereoselective biotransformations, both in batch mode and in continuous-flow reactors.

2 Materials and Methods

2.1 Materials

2.1.1 Enzyme

Phenylalanine ammonia-lyase from parsley (*Petroselinum crispum*, *PcPAL*) was obtained after overexpression in *E. coli* and purification using a previously reported protocol [14].

2.1.2 Chemicals

Synthesis of the racemic 2-amino-3-(tiophen-2-yl)propionic acid (**1**) and 3-(tiophen-2-yl)acrylic acid (**2**) was performed starting from the corresponding aldehyde by known methods [24, 25]. Single-wall carbon nanotubes (SwCNT ; ID = 0.8-1.6 nm, OD 1-2 nm, Length = 5-30 μm) were purchased from Chengdu Organic Chemicals Co. Ltd (Chengdu, China). Glycerol diglycidyl ether (GDE), Bradford reagent for protein determination, tris(hydroxymethyl)-aminomethane (TRIS), sodium phosphate and $[\text{D}_4]$ -acetic acid were purchased from Sigma-Aldrich (St. Luis, MO, USA). Technical grade solvents such as methanol and dichloromethane were dried and/or freshly distilled prior to use.

2.1.3 Equipment

Transmission electron microscopy (TEM) was performed using an automatic H-7650 TEM, Hitachi (Tokyo, Japan) with accelerating voltage 40-120 kV, zoom 200x-600000x, by dipping a holey-carbon TEM grid into a suspension of the immobilized enzyme. Sample was imaged in the TEM operating at 120kV using an Olympus KeenView G2 camera.

Elemental analyses were carried out with a Vario Micro Cube analyzer, Elementer Analysensysteme GmbH (Langeselbold, Germany).

The quantitative protein assay based on Bradford method was performed on an Agilent 8453 UV-Vis spectrophotometer (Santa Clara, CA, USA).

The enzymatic reactions in batch mode were shaken in a Titramax 1000 instrument.

For ultrasonication a Transsonic 460/H ultrasonic bath, Elma Schmidbauer GmbH (Singen, Germany), operated at 100 W, 40 kHz was used.

The conversions for batch reactions were calculated using the $^1\text{H-NMR}$ spectra in $[\text{D}_4]$ -acetic acid as solvent at 25 $^\circ\text{C}$, recorded on Ascend 400 or Ascend 600 NMR spectrometers, Bruker (Karlsruhe, Germany) operating at 400 and 600 MHz, respectively [19].

The amination reaction of the single-walled carbon nanotubes was performed at 600 W power at 50 bar pressure, 300 $^\circ\text{C}$ for 1 h in a CEM Microwave-assisted Reactor, CEM Corporation (Matthews, USA).

SwCNTs were filtered on a PTFE membrane filter with 0.22 μm pore size, Membrane-solutions, Nantong Co., Ltd. (Sanghai, China).

2.2 Methods

2.2.1 Functionalization of SwCNT with amino groups

The functionalization of carbon nanotubes was carried out using a modified method by Mirzarakhmetova et al. [26]. The reaction mixture contained SwCNT (400 mg), urea (400 mg) and dimethyl formamide (DMF, 5 mL). The reaction was carried out in a microwave assisted reactor at 300 $^\circ\text{C}$ for 1 h. After the reaction the modified SwCNT was washed with DMF, methanol, CH_2Cl_2 , and dried. Elemental analysis data shown an $\sim 4.8\%$ NH_2 content for the aminated single walled carbon nanotubes ($\text{SwCNT}_{\text{NH}_2}$). According to TEM analysis, the geometry of the parent SwCNT (ID = 0.8-1.6 nm, OD 1-2 nm, Length = 5-30 μm) did not changed significantly by amine functionalization.

2.2.2 Covalent binding of *PcPAL* onto $\text{SwCNT}_{\text{NH}_2}$

$\text{SwCNT}_{\text{NH}_2}$ (20 mg) was incubated with a solution of glycerol diglycidyl ether (GDE, 32.4 mg, 0.2 mmol) in CH_2Cl_2 (5 mL) under shaking at 1350 rpm at room temperature overnight, with occasional sonication to avoid bundled SwCNT formation (Fig. 1, step *i*). The sample was filtered on a membrane filter and then washed with CH_2Cl_2 (3×1 mL). To the resulted modified $\text{SwCNT}_{\text{NH}_2}$, a solution of *PcPAL* (2.0 mg in 1 mL of 0.1 M Tris buffer, pH 8.8) was added and the mixture was shaken at room temperature at 1350 rpm, overnight (Fig. 1, step *ii*). The formed biocatalyst was filtered off on a membrane filter and washed with distilled water (3×10 mL). The amount of immobilized *PcPAL* was determined by comparison of the mass of *PcPAL* in the solution before the immobilization (2.0 mg in 1 mL solution) and after immobilization (0.06 mg, in the unified filtrates) by spectrophotometric assay using the Bradford method.

2.2.3 Ammonia elimination from **1** catalyzed by SwCNT_{NH₂}-PAL

Into the solution of racemic 2-amino-3-(thiophen-2-yl)propanoic acid (**1**, 4.7 μmol, 0.8 mg) in Tris buffer (1 mL, 0.1 M, pH 8.8) was added SwCNT_{NH₂}-PAL biocatalyst (6 mg).

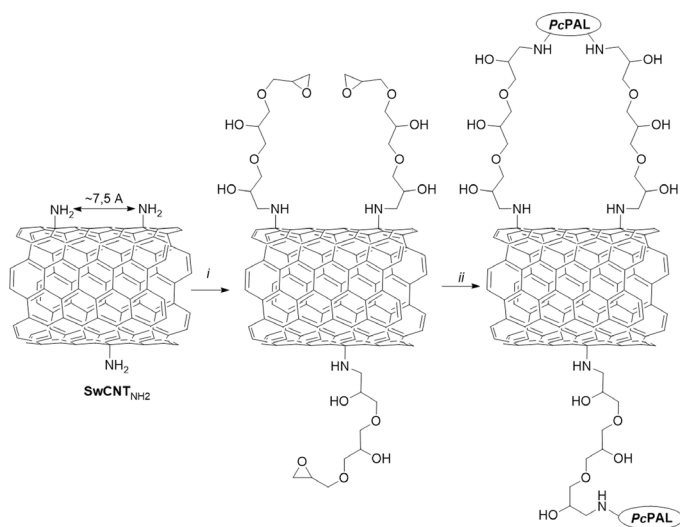


Fig. 1 Immobilization of PAL on SwCNT_{NH₂}: *i*) GDE in CH₂Cl₂; *ii*) PAL in Tris buffer (0.1 M Tris buffer, pH 8.8)

After performing the reaction at room temperature for 17 h under shaking (1250 rpm), the reaction mixture was filtered on a membrane filter and the filtrate was concentrated. ¹H-NMR spectra of the residue, after removal of the solvent, were recorded and used to calculate conversions as previously reported [19]. The recovered SwCNT_{NH₂}-PAL was washed with Tris buffer (1 mL, 0.1 M, pH 8.8) and after filtration it was reused under the same reaction conditions.

2.2.4 Ammonia addition to **2** with SwCNT_{NH₂}-PAL in batch mode

Into the solution of 3-(thiophen-2-yl)acrylic acid (**2**, 4.5 μmol, 0.7 mg) in ammonia solution (2 M, 1 mL, pH set to 10 with CO₂), the SwCNT_{NH₂}-PAL biocatalyst (6 mg) was added and the reaction was performed for 17 h at room temperature under shaking (1350 rpm). The reaction mixture was filtered and the filtrate was concentrated. ¹H-NMR spectra of the residue, after removal of the solvent, were recorded and used to calculate conversions as previously reported [19]. Before reuse under the same reaction conditions, the recovered SwCNT_{NH₂}-PAL was washed with Tris buffer (1 mL, 0.1 M, pH 8.8), conditioned by shaking for 2 h in phosphate buffer (1 mL, 0.1 M, pH = 6) and washed with Tris buffer (1 mL, 0.1 M, pH 8.8) again.

2.2.5 Ammonia addition to 3-(thiophen-2-yl)-acrylic acid **2** with SwCNT_{NH₂}-PAL in continuous-flow packed-bed microreactor

The continuous-flow experiments were performed in a thermostated SynBioCart microreactor (30 mm × 3 mm ID PTFE SynBioCart columns filled with 110 mg of SwCNT_{NH₂}-PAL in a PEEK SynBioCart column holder closed with PTFE membrane filter of 0.22 μm pore size) attached to the pump module of an Agilent LC 1150 HPLC system and a back-pressure regulator valve (VICI AG, JR-BPR1). Prior to perform the reaction, the microreactor was washed with 2 M ammonia solution (pH set to 10 with CO₂) at 0.5 mL min⁻¹ flow rate for 20 min.

The experiments were performed by pumping the solution of 3-(thiophen-2-yl)acrylic acid (**2**, 4.5 mM) in 2 M ammonia solution (pH set to 10 with CO₂) through the thermostated SwCNT_{NH₂}-PAL-filled column at various temperatures (25–80 °C) at a flow rate of 0.1 mL min⁻¹. Samples (10 μL) were withdrawn at every 10 min (after reaching the stationary state, usually 30 min) and analyzed by an UV spectrophotometer (added to 990 μL of 2 M ammonia solution, at 290 nm). The experiments at room temperature (25 °C) were conducted without backpressure regulation, the experiments between 30 and 80 °C were performed with backpressure (set to 15 bar).

All experiments were performed with the same SwCNT_{NH₂}-PAL-filled column in the temperature range 25–60 °C. Intermittently, the immobilized PAL-filled column was stored at 4 °C (after washing with distilled water, at 0.5 mL min⁻¹ for 30 min). In tests above 60 °C, new SwCNT_{NH₂}-PAL-filled columns were used at each temperature.

3 Results and Discussion

The aim of this study was to create covalently immobilized PAL biocatalyst with even higher activity and reusability in the ammonia addition reaction onto arylacrylates than was observed for SwCNT_{COOH}-PAL [19]. To achieve this goal SwCNT was functionalized first with amino groups to give a support (SwCNT_{NH₂}) suitable for direct bisepoxide-functionalization and subsequent covalent attachment of PcPAL (SwCNT_{NH₂}-PcPAL, see Fig. 1). According to our estimation assuming zig-zag conformation for the linkers, the resulted linker in SwCNT_{NH₂}-PcPAL was about 0.7 nm shorter than the previously applied linker in SwCNT_{COOH}-PAL [19]. Furthermore, this shorter linker was expected to be more stable because it did not contain hydrolysable amide bond. The catalytic properties of the resulted novel immobilized form of PcPAL (SwCNT_{NH₂}-PAL) were evaluated in the ammonia elimination from 2-amino-3-(thiophen-2-yl)propanoic acid **1** and in the ammonia addition onto 3-(thiophen-2-yl)acrylic acid **2** as test reactions (Fig. 3 and Table 1).

3.1 Covalent immobilization of *PcPAL* onto SwCNT_{NH₂}

The method applied for covalent attachment of *PcPAL* onto SwCNT_{COOH} [19] was simplified for the covalent immobilization of the enzyme onto functionalized SwCNT_{NH₂}. First, SwCNT_{NH₂} was activated by reaction with a large excess of glycerol diglycidyl ether (GDE), followed by attachment of *PcPAL* onto the surface of SwCNT_{NH₂} via the GDE-based linker (Fig. 1). Immobilization yield (97±1.5% of the *PcPAL* was bound to the bisepoxide-activated SwCNT_{NH₂} resulting in 88 µg protein/mg biocatalyst) as well as the stability of covalently immobilized enzyme (Fig. 2 showing TEM data before and after 12 cycles) were high and reproducible.

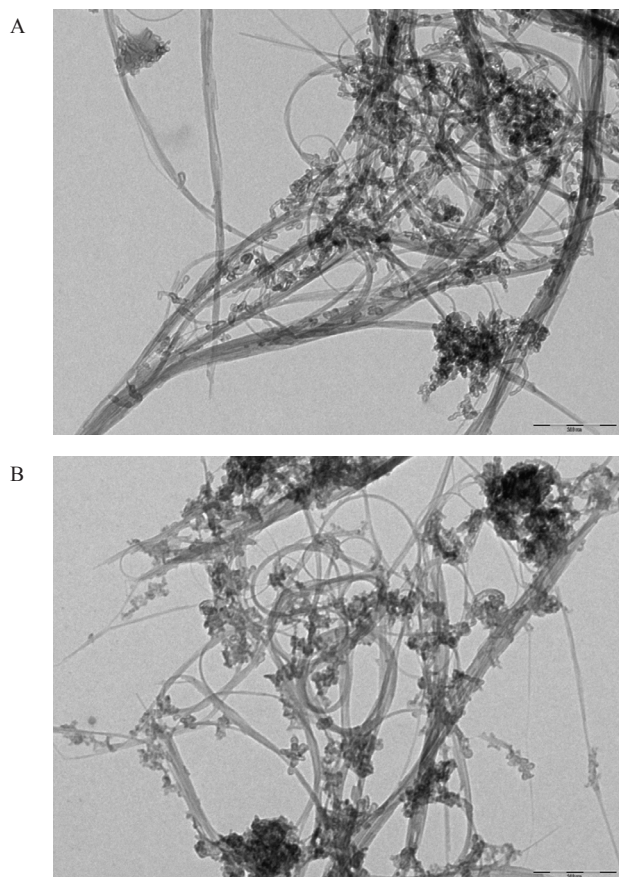


Fig. 2 TEM images of SwCNT_{NH₂}-PAL (A) before and (B) after 12 cycles of ammonia addition onto **2** (4.5 mM) in 2 M ammonia (pH 10.0). Length of the scale bars is 200 nm.

3.2 Biocatalytic behavior of SwCNT_{NH₂}-PAL in stereoselective biotransformations of 2-amino-3-(thiophen-2-yl) propanoic acid **1** and 3-(thiophen-2-yl)acrylic acid **2** in batch mode

The novel SwCNT_{NH₂}-PAL preparation was tested as biocatalyst at room temperature in the ammonia elimination from 2-amino-3-(thiophen-2-yl)propanoic acid **1** and in the ammonia addition to 3-(thiophen-2-yl)acrylic acid **2** performed in batch mode (Fig. 3).

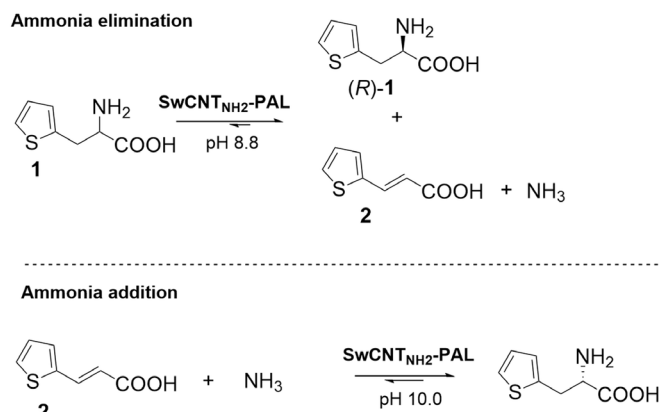


Fig. 3 SwCNT_{NH₂}-PAL for ammonia elimination from 2-amino-3-(thiophen-2-yl)propanoic acid **1** and ammonia addition to 3-(thiophen-2-yl)acrylic acid **2**

The kinetic resolution of the racemic **1** catalyzed by SwCNT_{NH₂}-PAL occurred with high conversions (close to the theoretically possible 50% conversion). Moreover, the biocatalyst retained most of its initial activity and could be reused several times (Table 1).

Table 1 Conversion of the reactions catalyzed by the immobilized *PcPAL* on aminated SwCNT_{NH₂} (25 °C, 17 h)

Run	Conversion (%)	
	Addition to 2	Elimination from 1
1	63.9	49.6
2	63.5	48.5
3	64.2	48.8
4	63.8	47.4
5	60.0	46.3
6	49.2	42.4
7	16.2	42.3

In the case of ammonia addition to **2** requiring much harsher conditions (6 M ammonia, pH 10) the new SwCNT_{NH₂}-PAL form was significantly more active (64.2% conversion, see Table 1) than SwCNT_{COOH}-PAL (36.9% conversion [19]). Moreover, SwCNT_{NH₂}-PAL was also much more durable, losing more than 80% of its original activity only after 6 cycles (Table 1), in contrast to SwCNT_{COOH}-PAL which inactivated completely after 3 cycles [19]. In ammonia solutions the enhanced durability of SwCNT_{NH₂}-PAL compared to SwCNT_{COOH}-PAL was expected because the linker of SwCNT_{NH₂}-PAL contained non-hydrolyzable ether and alkylamine bonds besides the C-C bonds. Contrarily, it was indicated that an amide bond – being present in the linker of SwCNT_{COOH}-PAL – could undergo hydrolysis under various conditions [27] or transamidation with other amines even under mild conditions by enzyme catalysis [28].

As it was known that ammonia addition onto arylacrylates can be performed at lower ammonia concentrations [19, 29], the

reaction was tested with **2** in 3 M and 2 M ammonia solutions as well. At the lower ammonia concentrations SwCNT_{NH₂}-PAL exhibited significantly higher stability (Fig. 4). In 2 M ammonia at pH 10, more than 82% of the initial activity was retained even after 12 cycles.

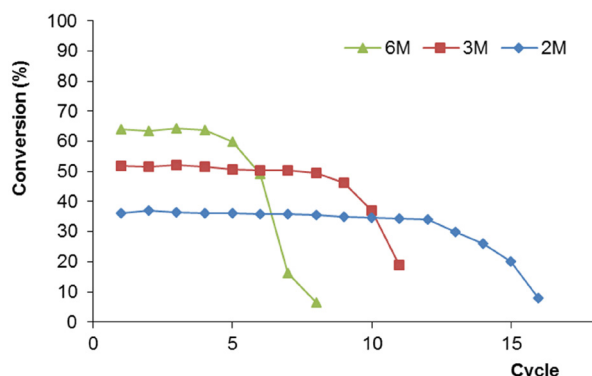


Fig. 4 Recyclability of SwCNT_{NH₂}-PAL in ammonia addition reaction to 3-(thiophen-2-yl)acrylic acid **2** (4.5 mM) in batch mode at various ammonia concentrations (2 M, 3 M and 6 M; pH 10.0; 25 °C, 17 h).

The unusual shape of the inactivation curves in recycling study with a plateau followed by rapid inactivation (Fig. 4) initiated a hypothesis. It could be assumed that at high concentration of ammonia, the small NH₃ molecules penetrated gradually into the protein and replaced the structural water. Further, it could be assumed that such replacements happened at first in the region close to the surface of the enzyme which resulted in non-detrimental changes in the *Pc*PAL structure. The rapid deactivation happened when deeper penetration of NH₃ molecules into the enzyme destroyed the active structure.

Thus, it could be assumed that penetration of ammonia into the surface layer of protein might be reversed by conditioning the enzyme in slightly acidic medium. This possibility was tested in the recycling of SwCNT_{NH₂}-PAL from 6 M ammonia medium used for ammonia addition to **2** (Fig. 5). Thus, in order to regain the activity and increase the durability of the immobilized *Pc*PAL the biocatalyst was washed and kept in phosphate buffer (pH = 6) for 2 h at room temperature under shaking at 1250 rpm when the enzyme's apparent deactivation was observed. Because the enzyme regained its initial activity for the ammonia addition reaction to **2** the initial hypothesis has been validated (Fig. 5).

Based on these results, a modified washing/reconditioning protocol was applied for recycling of SwCNT_{NH₂}-PAL from 6 M ammonia medium (pH =10). The modified protocol involved acidic reconditioning after each ammonia addition reaction as described above. In this way the enzyme preserved more than 90% of its initial activity even after 25 cycles (Fig. 6). This means at least five-fold durability enhancement [from five cycles (see Fig. 4) to at least 25 cycles (Fig. 6)].

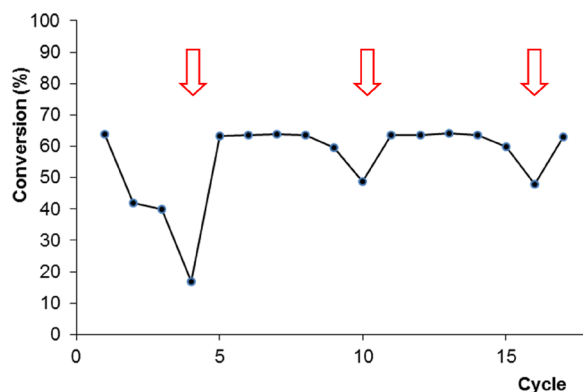


Fig. 5 Repeated ammonia addition reactions to 3-(thiophen-2-yl)acrylic acid **2** (4.5 mM, in 6 M ammonia; pH 10.0; 25 °C, 17 h) including washing of SwCNT_{NH₂}-PAL with phosphate buffer (pH 6) when the apparent inactivation is observed (indicated by the red arrows).

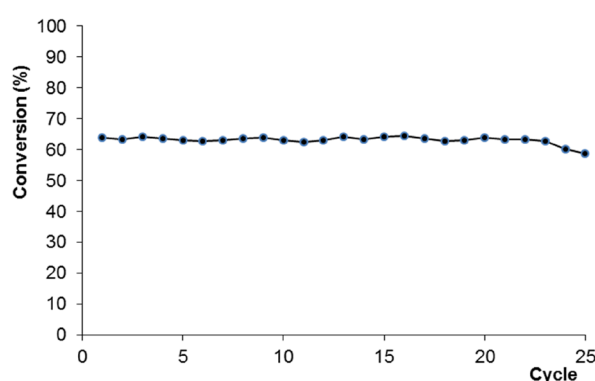


Fig. 6 Repeated ammonia addition reactions to 3-(thiophen-2-yl)acrylic acid **2** (4.5 mM, in 6 M ammonia; pH 10.0; 25 °C, 17 h), including washing of SwCNT_{NH₂}-PAL with phosphate buffer (pH 6) after each reaction

3.3 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

A further way of process intensification is the application of the biocatalysts in continuous-flow microreactors [30]. Application of continuous-flow microreactors can significantly enhance the operational stability of immobilized biocatalysts [23]. Thus, ammonia addition to **2** was studied in a biocompatible, SwCNT_{NH₂}-PAL-packed-bed PTFE microreactor column (SynBioCart) operated in continuous-flow mode (Fig. 7). The continuous-flow system comprised the biocatalyst-filled microreactor in the column thermostat of a HPLC system equipped with a back-pressure regulator.

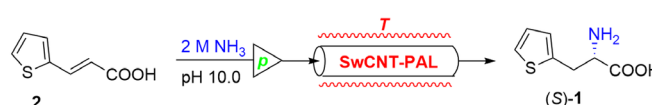


Fig. 7 Ammonia addition to 3-(thiophen-2-yl)acrylic acid **2** in a continuous-flow packed-bed microreactor

Our previous study with SwCNT_{COOH}-PAL-filled continuous-flow microreactor indicated that application of proper back-pressure (15 bar) was necessary to avoid apparent enzyme activity loss due to bubble formation [19]. Thus, in this study all experiments above room temperature were performed with back-pressure regulation (15 bar).

To evaluate the degree of activity enhancement of SwCNT_{NH₂}-PAL in the ammonia addition reaction to **2** the specific reaction rate of the biotransformation was determined at 30 °C in continuous-flow and in batch modes as defined by Eq. (1) and Eq. (2), respectively [30],

$$r_{flow} = \frac{[P] \times f}{m_e} \quad (1)$$

$$r_{batch} = \frac{n_p}{(t \times m_e)} \quad (2)$$

where r_{flow} : specific reaction rate in a continuous-flow reactor; $[P]$: product concentration ($\mu\text{mol mL}^{-1}$), f : flow rate (mL min^{-1}), m_e : mass of immobilized biocatalyst (g); and r_{batch} : specific reaction rate in a shake or stirred batch system, n_p : amount of the product (μmol), t : the reaction time (min).

The specific reaction rate is an applicable measure to compare the biocatalyst's productivity under various conditions. For a comparison between the productivity of an immobilized biocatalyst in a continuous-flow reaction and in the same reaction in batch mode, the specific reaction rates should be investigated at the same degree of conversions since the rate of product formation is not a linear function of conversions [30]. The conversion of 64% (being far enough from the >90% equilibrium conversion [24]) was chosen for comparison of the batch and of the continuous-flow mode ammonia addition reactions to **2** catalyzed by SwCNT_{NH₂}-PAL in 2 M ammonia (pH 10, at 30 °C). As expected, the specific reaction rate in the continuous-flow reactor ($r_{flow} = 2.63 \mu\text{mol min}^{-1} \text{g}^{-1}$) was significantly higher than that in the batch reaction ($r_{batch} = 1.38 \mu\text{mol min}^{-1} \text{g}^{-1}$). Notably, the specific reaction rate for SwCNT_{NH₂}-PAL in the continuous-flow reactor was also higher than for SwCNT_{NH₂}-PAL under similar conditions ($r_{flow} = 2.39 \mu\text{mol min}^{-1} \text{g}^{-1}$) [19].

The enhanced stability and higher specific activity of SwCNT_{NH₂}-PAL compared to the previously studied SwCNT_{COOH}-PALs [19] could be rationalized by analysis of the functional group density of the two carriers. From the weight proportion of NH₂ (4.8 w/w%) in SwCNT_{NH₂} an average distance of 0.75 nm could be calculated between the amino functions on the surface of SwCNT_{NH₂} (Fig. 1). Considering the elongated spheroid shape of homotetrameric PcPAL with axes of ~8.5 nm and ~17 nm, there is a high possibility of multipoint fixation of the enzyme onto the GDE-treated SwCNT_{NH₂}.

It is known that multipoint covalent attachment can stabilize the immobilized enzymes [31, 32]. Moreover, it was demonstrated that multipoint attachment could enhance the operational stability of immobilized enzymes in microfluidic reactor as well [33].

Thus, the six-fold lower surface density of the carboxylate functions could be calculated for the carboxylated carbon nanotube used as basic carrier for SwCNT_{COOH}-PALs [19], which results in much lower possibility of multipoint fixation as compared to SwCNT_{NH₂}-PAL in the present study. Moreover, the linkers are ~0.8 nm shorter in SwCNT_{NH₂}-PAL as the ones in SwCNT_{COOH}-PALs [19], and they do not contain hydrolysable functional groups like the amide bond within the linkers of SwCNT_{COOH}-PALs. This may also contribute to the enhanced durability of PcPAL in the novel SwCNT_{NH₂}-PAL biocatalyst.

The temperature dependence of the SwCNT_{NH₂}-PAL-catalyzed continuous-flow mode ammonia addition to **2** in 2 M ammonia (pH 10) in the 30-80 °C range was quite similar, to that of SwCNT_{COOH}-PAL [19]. A local minimum of the initial conversion was observed at 50 °C (Fig. 8) with significantly lower conversion than at 40 °C or even at 30 °C.

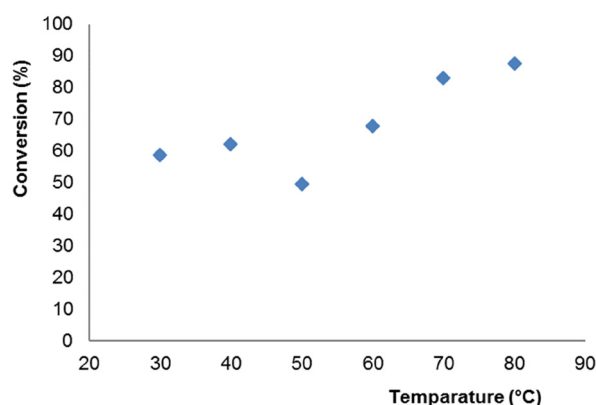


Fig. 8 Temperature effect on the ammonia addition reaction to **2** (4.5 mM) in a continuous-flow microreactor (in 2 M ammonia, pH 10, 30 °C)

The long term operational stability study of the SwCNT_{NH₂}-PAL was studied in the continuous-flow mode ammonia addition to **2** in 2 M ammonia (pH 10) in the 30-80 °C temperature range (Fig. 9). This study indicated that at 50 °C and 60 °C the activity of SwCNT_{NH₂}-PAL remained stable over 72 h and was higher than those obtained between 30 °C and 50 °C. Thus, the apparent decrease of the activity of SwCNT_{NH₂}-PAL at 50 °C cannot be attributed to irreversible thermal inactivation. At 70 °C and 80 °C however, while the initial activity of the biocatalyst increased with temperature, the enzyme inactivated rapidly and irreversibly.

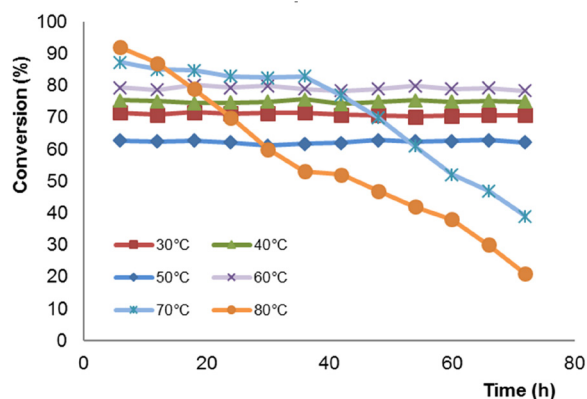


Fig. 9 Temperature effect on the long term stability in ammonia addition to 3-(thiophen-2-yl)acrylic acid **2** (4.5 mM, 2 M ammonia, pH 10, 30 °C)

4 Conclusions

In this study, covalent immobilization of phenylalanine ammonia-lyase from *Petroselinum crispum* (*PcPAL*) on amine-functionalized single-walled carbon nanotubes ($\text{SwCNT}_{\text{NH}_2}$) was achieved. The novel immobilized form of *PcPAL* ($\text{SwCNT}_{\text{NH}_2}$ -PAL) was applicable for the synthesis of (*R*)-2-amino-3-(thiophen-2-yl)propanoic acid (*R*)-**1** by kinetic resolution of the corresponding racemate as well as of its enantiomer (*S*)-**1** by enantioselective ammonia addition to the achiral 3-(thiophen-2-yl)acrylic acid **2**. By applying glycerol diglycidyl ether (GDE)-activated $\text{SwCNT}_{\text{NH}_2}$ as support material, the resulted $\text{SwCNT}_{\text{NH}_2}$ -PAL biocatalyst showed higher activity in the ammonia addition reaction to **2** than the previously reported *PcPAL* form immobilized on carboxy functionalized nanotubes ($\text{SwCNT}_{\text{NH}_2}$ -PALs) and could be reused more times. Further stability enhancement could be achieved by conditioning the immobilized PAL biocatalyst in a slightly acidic buffer (phosphate, pH 6) for several hours during recycling. In this way, $\text{SwCNT}_{\text{NH}_2}$ -PALs retained more than 90% of its initial activity even after 25 cycles. When tested in a packed-bed continuous flow microreactor, the $\text{SwCNT}_{\text{NH}_2}$ -PAL was stable in the ammonia addition reaction to **2**, with its initial activity being fully maintained for 72 h at 60 °C.

The main factors leading to the enhanced stability are the higher linker density in $\text{SwCNT}_{\text{NH}_2}$ -PAL as compared to $\text{SwCNT}_{\text{NH}_2}$ -PALs resulting in higher probability of multipoint enzyme fixation and the absence of hydrolysable functions in the linkers.

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