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Exceptionally active and reusable nanobiocatalyst comprising lipase non-covalently immobilized on multi-wall carbon nanotubes for the synthesis of diester plasticizers

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ABSTRACT

A new method for the synthesis of dicarboxylic acid esters in the presence of a new heterogeneous nanobiocatalyst consisting of *Candida antarctica* lipase B immobilized on multi-walled carbon nanotubes (MWCNTs) has been developed. Selection and characterization of the support for *Candida antarctica* lipase B (CALB) was initially performed. Pristine and modified MWCNTs of different geometries, morphologies and surface/core modifications were tested as a lipase carrier and determining that the CheapTubes™ MWCNTs nanobiocatalyst with a 15.7 wt.% CALB loading was the most active nanobiocatalyst. The model reaction of succinic acid with *n*-butanol was carried out with a 4-molar excess of alcohol and 150 mg of nanobiocatalyst per 1 mmol of succinic acid at 45 °C in cyclohexane. The di-*n*-butyl succinate was obtained with 95% yield after 3 h. The activities of the new nanobiocatalysts were compared with the benchmark Novozyme-435 and other acidic catalysts. Recycling studies demonstrated the possibility of utilizing the most active MWCNTs-lipase biocatalyst six times without any significant loss of activity. The main advantage of this study is the superior activity of the new nanobiocatalyst, which resulted in a significant reduction of reaction times as compared to those reported in the literature.

1. Introduction

Plasticizers are used extensively to provide flexibility and ease of processing of polymers, to improve elasticity and durability, mostly for poly(vinyl chloride) which may contain up to 50% by weight of plasticizers [1,2]. As phthalates have been subjected to stricter regulations, alternative non-toxic plasticizers were used instead [2]. Among them, esters of dicarboxylic acids, such as adipates, terephthalates, and succinates [3] have been utilized. The direct Fischer reaction between dicarboxylic acid and alcohol causes problems associated with overcoming the reaction equilibrium; water elimination is the primary hurdle to overcome. In addition, esterification processes are carried out under harsh conditions with relatively high-energy demands and significant costs which include efficient and selective catalysts. The most often used Lewis or Brønsted acidic catalysts can cause corrosion. Increasingly stringent environmental regulations of industrial processes with regard to waste disposal have compelled the modification of a number of fine chemical processes, including esterification.

Another issue is the demand of biodegradable, benign and non-petroleum-based plasticizers with properties similar to or better than phthalates, which can ensure *inter alia* biobased succinic acid esters. Dimethyl succinate, diethyl succinate and di-*n*-butyl succinate can be obtained in the presence of montmorillonite clay catalysts, acidic ion-exchange resins (Amberlyst-15, Amberlyst XN-1010) or Nafion NR50 [4,5]. However, the important achievement in the synthesis of dialkyl esters was the development of a heterogeneous, non-acidic catalyst in the form of an enzyme immobilized on the solid carrier [4]. To this aim, the benchmark Novozyme-435, the lipase B from *Candida antarctica* (CALB) immobilized on acrylic resin was used. Enzymatic esterification processes are characterized by mild reaction conditions, low energy consumption, high chemo-, regio- and enantioselectivities, all of which prevent the formation of significant amounts of waste [9]. Enzymatic pathways using Novozyme-435 for the synthesis of dioctyl succinate [5], diethyl succinate [4] and dioleoyl succinate [5] under mild reaction conditions have been described. It is worth noting that there is no data concerning biocatalyst recyclability for ester syntheses from

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dicarboxylic acids in the literature.

On the other hand, the application of enzymes generates potential problems such as possible mechanical disintegration of the enzyme structure, low stability of native lipases in organic environments and sensitivity for pH and temperature changes [6]. As a consequence, the stability of lipase may decrease and recycling of the biocatalyst could be troublesome or economically impractical. Those difficulties are even more striking for the development of continuous processes [7]. As we have previously verified, Novozyme-435 was not promising for recycling [8] and could be destroyed by mechanical damage. This resin swells upon a contact with organic solvent and the enzyme loses its activity. These problems could be overcome *via* immobilization of enzyme on a more mechanically stable solid support. Depending on the support nature, the final features of the immobilized enzyme may be different. Therefore, the selection of the support may be a critical step in the design of a enzymatic biocatalyst [9].

Under favorable conditions, lipases can be immobilized *via* the hydrophobic surrounding of their active center, fixing them in an open conformation. Interfacial activation on hydrophobic supports at low ionic strength has been reported to be a simple and efficient method to immobilize lipases on various supports [8–11]. The immobilization of lipase on solid supports may additionally improve enzyme rigidity, stability and integrity of the lipase-support hybrid [12,13].

Recently, nanoscale materials have opened opportunities in the field of nanobiocatalysis [14–16]. Carbon nanomaterials have been described as versatile supports for enzyme immobilization due to their small size, large surface area, mechanical and thermal stability and other unique properties. The simple combination of a nanoscale support and an enzyme has led to a much higher enzyme loading and, more importantly, increased enzyme stability [16]. The economy of processes utilizing carbon nanotubes (CNTs) has become increasingly favorable as the prices of industrial-grade MWCNTs (multi-walled carbon nanotubes) have achieved the level from 100$ per 1 kg (Nanocyl NC7000™ MWCNTs) to 200$ per 1 kg (Cheap Tubes™ MWCNTs). In our previous work, nanobiocatalysts from lipase non-covalently immobilized on MWCNTs for the Baeyer-Villiger synthesis of lactones have been demonstrated [8]. Consequently, we deemed it important to test its potential as a catalytic system in esterification – considered as an exemplary enzyme-catalyzed reaction of a major importance.

Herein, we present the design of an alternative, highly stable and active nanobiocatalyst based on *Candida antarctica* lipase B anchored onto carbon nanomaterials as a catalytic system for the synthesis of plasticizers based on aliphatic diesters (dicarboxylates) with the potential to become industrially relevant processes. Our work seeks to realize the challenge of environmentally and economically sustainable processes yielding safer and environmentally friendly industrial-scale polymer additives.

2. Experimental

2.1. Materials and methods

Solvents, alcohols, aqueous solutions of *Candida antarctica* Lipase B and decane were purchased from Sigma-Aldrich; all acids were purchased from Chemat, Poland. Industrial grade MWCNTs were purchased from Cheap Tubes Inc. (United States), Nanocyl NC7000™ MWCNTs were purchased from Nanocyl (Belgium); active carbon and graphite were commercial materials obtained from Avantor Performance Materials (Poland) and the United Quantum Factory (Poland), respectively.

GC analyses were performed using a SHIMADZU GC-2010 Plus equipped with a Zebron ZB-5MSi column (30 m × 0.32 mm × 0.25 μm film). ^{1}H NMR spectra were recorded at 600 MHz and ^{13}C NMR at 150 MHz (Varian system).

Lipase loadings on the surface of the carbon materials were determined by thermogravimetry (TGA) using a Mettler Toledo TGA851e thermobalance. Samples (approximately 10 mg) were heated from 25 °C to 800 °C at a rate of 10 °C/min in standard 70 μL Al_2O_3 crucibles under a dynamic nitrogen flow of 60 mL/min.

Transmission Electron Microscopy (TEM) images were obtained using a Tecnai F20 TWIN microscope (FEI Company, USA) equipped with field emission gun, operating at an acceleration voltage of 200 kV. Images were recorded on the Eagle 4k HS camera (FEI Company, USA) and processed with TIA software (FEI Company, USA). The samples were prepared on a copper grid with holey carbon film. Scanning electron microscopy (SEM) images were obtained with a Phenom Pro Desktop SEM instrument at accelerating voltage 15 kV and energy-dispersive X-ray spectroscopy (EDX) used detector BSD full.

Nitrogen adsorption/desorption isotherms for carbon materials were obtained using a Micrometrics ASAP 2420 M instrument at −196 °C to calculate their specific surface areas (SBET) and pore volumes. The pore size was obtained using the Barrett–Joyner–Halenda (BJH) method with the Kruk–Jaroniec–Sayari correction. Prior to the experiments, samples of supports were outgassed at 200 °C and 1.33×10^{-3} Pa for 5 h. The sample of the (nano)biocatalyst was outgassed at 140 °C and 1.33×10^{-3} Pa for 5 h.

The presence of lipase in the filtrate after six reaction cycles was determined using Lowry's UV-VIS method of protein detection. UV-VIS spectra (λ = 670 nm) were recorded on a Jasco V-650 spectrophotometer at room temperature in aqueous solution.

2.2. Synthetic procedures

2.2.1. Synthesis of pristine MWCNTs, N-MWCNTs, Fe-MWCNTs, ultra-long curly MWCNTs (ulc-MWCNTs) and helical CNTs (h-CNTs)

Pristine MWCNTs, high Fe-content MWCNTs (Fe-MWCNTs) and ulc-MWCNTs [17] were synthesized *via* catalytic chemical vapor deposition (c-CVD) using a slightly modified protocol [18]. N-MWCNTs were grown according a previously described protocol [19]. h-CNTs were synthesized also *via* c-CVD using addition of copper microflakes (1–10 wt.%) to a ferrocene/toluene feedstock as the catalyst growth under otherwise unchanged conditions.

2.2.2. Immobilization of the Candida antarctica lipase B on nanocarbon solid supports

The immobilization step was carried out according to a previously published procedure [8]. A *Candida antarctica* lipase B (0.25 g – 1 g), nanocarbon solid support (0.1 g) and demineralized water (3 mL) were introduced into a 100 mL round bottom flask. The immobilization step was carried out for 3 h at 20 °C in a thermostatic shaker (180 rpm). After that, the mixture was filtered under vacuum and washed with 20 mL of demineralized water. The catalyst was then dried for 3 d in a desiccator at 5 °C.

2.2.3. General esterification procedure

A (nano)biocatalyst (10–200 mg/l mmol of dicarboxylic acid) was introduced into a 10 mL round-bottom flask. Next, decane (20 wt. % per acid, internal standard), solvent (0–2 mL/L mmol of acid), dicarboxylic acid (1.0 mmol) and alcohol (2.0–32.8 mmol) were successively added. The reaction mixture was then inserted into the thermostatic shaker (250 rpm) at 25–45 °C and the reaction was carried out for 2–24 h. During the reaction, 10 μl of the samples (diluted with acetonitrile) were periodically collected to monitor the reaction progress by GC-FID. After the completion of the reaction, the (nano)biocatalyst was filtered and washed with 20 mL of cyclohexane. The filtrate was concentrated using a rotary evaporator (7 mbar, 110 °C, 6 h for di-*n*-butyl esters and 5 mbar, 135 °C, 8 h for 2-ethylhexanol esters) to remove cyclohexane and alcohols. The esters were purified by column chromatography using Al_2O_3 as the stationary phase and CH_2Cl_2 as the eluent. NMR spectra are available in Supplementary Information (Figs S9-S24).

2.2.4. Recycling of the catalyst

In experiments where the catalytic system was recycled, the (nano) biocatalyst was filtered, washed with 20 mL of cyclohexane, dried under vacuum on a Schlenk line (1 mbar, RT, 4 h) and used for the next catalytic cycle.

General procedure of Lowry's method for protein detection was described in Supplementary Information.

3. Results and discussions

3.1. Selection and characterization of the support for Candida antarctica lipase B (CALB)

Several carbon (nano)supports were selected for the preparation of the heterogeneous (nano)biocatalysts. The following pristine CNTs of different geometries, morphologies and surface/core modifications were used: commercially available Nanocyl NC7000™ and CheapTubes™ MWCNTs as well as modified ('bamboo'-like nitrogen-doped and iron-encapsulated, Fe-MWCNTs, MWCNTs, ultra-long curly MWCNTs (ulc-MWCNTs) and helical CNTs (h-CNTs) prepared in our laboratory according to unchanged or modified procedures described elsewhere [17,18]. The newly tested nanocarbon support h-CNTs were applied. This material was characterized by a helical periodic morphology with a helix pitch of *ca.* 500 nm and a non-linear change of handedness every 5–8 units. Total length of the h-CNTs was greater than 5 μm (Supplementary Information, Fig. S1: SEM and TEM images of representative h-CNTs). Hence, the additional rationale behind the selection of h-CNTs as the support was a supposition that the grooves, apart from the generation of new active surface areas, could serve as sites of enhanced hydrophobic interactions.

Active carbon and graphite were selected as reference carbon supports due to their dramatically differing morphologies. Importantly, for c-CVD-derived CNTs, their essential characteristic is the presence of encapsulated ferromagnetic iron-based nanoparticles which are typically irremovable under lower technological and biotechnological regimes. The use of a magnetic field, obviously, particularly for iron-rich Fe-MWCNTs, can help with easy removal of the nanobiocatalysts from the post-reaction mixtures, which could be a significant advantage in light of the process economy. Table 1 presents the characterization of supports and the CALB loadings they are bound to. CALB was immobilized using a physical adsorption technique according to a literature procedure with modifications concerning the optimization solvent amounts used [14,15]. For the (nano)biocatalysts shown in Table 1, the mass ratio of CALB:MWCNTs (MR) was fixed at 7.5:1, found as the optimal ratio from our previous work [8]. After immobilization, protein loading was determined by TGA according to a previously reported methodology (Supplementary Information, Figs S25-S38) [8]. As expected, the protein loadings correlated with specific surface area (S_{BET}) of the (nano)supports with commercially available active carbon as the highest capacity one.

Table 2
CALB loading for various MR for CALB-CheapTubes™ MWCNTs nanobiocatalysts.

CALB:CheapTubes™ MWCNTs mass ratio (MR)	CALB loading, wt.%
1 : 1	4.1
2.5 : 1	8.9
3.75 : 1	11.7
5 : 1	14.7
7.5 : 1	15.7
10 : 1	15.7

Next, to check the influence of the MR (from 1:1 to 10:1) on CALB loading CheapTubes™, MWCNTs were chosen as the carrier (Table 2). The rationale behind this selection was twofold: use of this system was beneficial from an activity point-of-view (confirmed further) and strictly economic. As shown, higher CALB loadings required higher amounts of protein in the immobilization step (higher MR). Protein loading was found in the range of 4.1–15.7 wt.%. Importantly, additional MR increases did not influence the CALB loading showing; this revealed a maximum value of 15.7 wt.%. In order to study the nanoscale morphology of the optimal hybrid catalytic system, TEM analyses were performed (Fig. 1). Additionaly, to confirm the presence of lipase on the surface of CALB-CheapTubes™ MWCNTs nanobiocatalyst the SEM- EDX analysis was added (Supplementary Information Figs S2-S3). The detection of oxygen and sulphur can provide evidence of the occurrence of protein.

These studies revealed that the principle location of the enzyme was the outer non-fully graphitized MWCNT shell while the nanotube coverage was inhomogeneous and formed 'islands' along the nanotube of 20–60 nm in length. Although a small fraction of nanotubes was open-ended, the tip location of the enzyme cannot be fully excluded; though its probability is negligible due to the high aspect ratio of the nanotubes. Furthermore, to track changes in the active surface area and pore volumes, additional S_{BET} analyses were carried out. As expected, after immobilization, S_{BET} (55 m^2/g) and pore volumes (0.36 cm^3/g) were reduced by 40% and 27%, respectively.

3.2. Screening of the (nano)biocatalysts esterification activity

Screening of the (nano)biocatalytic activity was performed using the model esterification of succinic acid with *n*-butanol (molar ratio 1:4) in cyclohexane (Supplementary Information, Scheme S1). The reactions were carried out in a thermostatic shaker at 45 °C (250 rpm) in the absence of diffusion limitation since the same reaction rate was observed for the reaction carried out with 200 rpm (Supplementary Information, Fig. S4: The influence of the rate of stirring on esterification reaction rate). In each case, 0.150 g of the final (nano)biocatalyst (MR = 7.5:1) per 1 mmol of succinic acid was applied (Fig. 2). The yield of the ester was determined using GC analysis.

Table 1
Characterization of (nano)carbon-based supports and (nano)biocatalysts.

Support*	S_{BET}, m^2/g	Pore volume, cm^3/g	Length, μm	Outer diameter, nm	Inner diameter, nm	CALB loading, wt.%
CheapTubes™ MWCNTs	89	0.49	10-30	20-40	5-10	15.7
Nanocyl NC7000™ MWCNTs	253	1.06	1.5	9.5	7	19.8
Fe-MWCNTs	46	0.14	1000	40-60	20-40	9.5
N-MWCNTs	39	0.15	40	5-64	4-40	8.8
ulc-MWCNTs	108	0.27	2000	60	10	14.3
h-CNTs	24	0.10	> 5	400-500	N/A	15.8
MWCNTs	34	0.11	130-370	35-85	15-40	10.3
Active carbon	1158	0.43	N/A	N/A	N/A	22.7
Graphite	3	0.01	N/A	N/A	N/A	3.8

* Commercially available MWCNTs (multi-walled carbon nanotubes): CheapTubes™ MWCNTs (Cheap Tubes Inc., United States) and Nanocyl NC7000™ (Nanocyl, Belgium); prepared and modified in our laboratory: MWCNTs (pristine, synthesized *via* c-CVD), Fe-MWCNTs (iron-encapsulated); N-MWCNTs (nitrogen-doped), ulc-MWCNTs (ultra-long curly MWCNTs) and h-CNTs (helical CNTs).

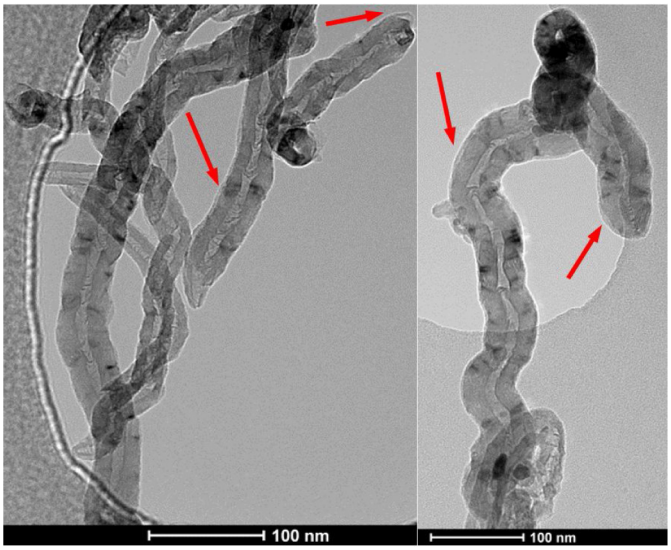


Fig. 1. Exemplary TEM images of CALB-CheapTubes™ MWCNTs nanobiocatalysts; arrows indicate unevenly distributed coating by the enzyme.

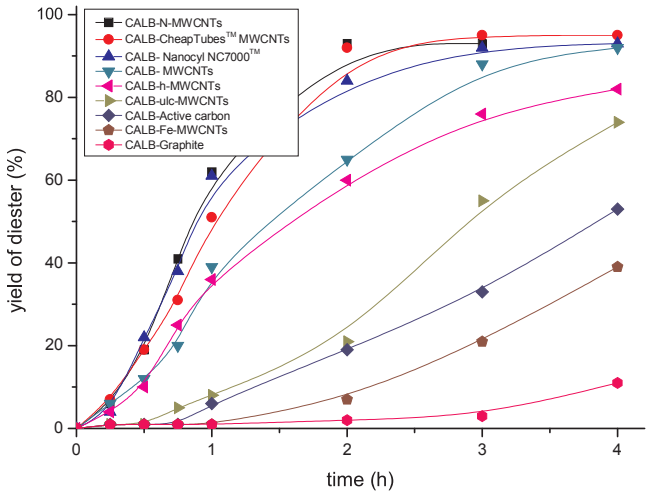


Fig. 2. The activities of CALB immobilized on various (nano)carbon supports in esterification; all experiments were performed in triplicate and the results for each of the three measurements differed by less than 1%. *Reaction conditions*: succinic acid 0.118 g (1 mmol), *n*-butanol 0.297 g (4 mmol), cyclohexane 2 mL, (nano)biocatalyst (MR 7.5:1; 150 mg), 45 °C, 250 rpm.

The most active catalysts were (nano)biocatalysts based on N-MWCNTs, Nanocyl NC7000™ MWCNTs and CheapTubes™ MWCNTs. Less active were (nano)biocatalysts based on ulc-MWCNTs and MWCNTs as supports. All (nano)catalysts were characterized with CALB loadings from 8.8 to 19.8 wt.%. It must be emphasized that high yields of di-*n*-butyl succinate were obtained, up to 95% with 100% selectivity (Supplementary Information, Table 1) is the key achievement here as compared with other literature methods [4,5]. The reaction carried out in the presence of Novozyme-435 and a 42 M excess of succinic acid in phosphate buffer gave 68% of diester after 24 h at 50 °C (Supplementary Information, Table S1) with comparison of the methods described in the literature) [5].

Interestingly, the CALB-Active carbon with the highest surface area support (1158 m^2/g, Table 1) and high lipase loading did not 'translate' into high catalytic activity. The reason for this can be explained by the generally lower accessibility of succinic acid and/or different enzyme conformations, (including non-active ones) for this substrate. The crowded agglomerations of lipase could also explain the activity decline [20]. CALB-Graphite with a low active surface and CALB loading had the lowest activity.

Nevertheless, it is difficult to correlate only the BET specific surface of carbon nanocarriers determined by adsorption of the nitrogen molecules in the gas-solid system with the catalytic activity of the immobilized enzyme. It should be emphasized that the overall catalytic activity of the adsorbed protein macromolecules, which may be additionally agglomerated *via* various interactions, strongly depends on the local conditions - the number and type of solvent molecules, statistically variable carbon support surface characterized by the number and size of grooves, roughness, number and type of functional groups, co-adsorbed water and air. Hence, the access of substrates to the enzyme active sites may vary.

Unfortunately, CALB-Fe-MWCNTs with higher encapsulated iron contents did not result in higher catalytic activity, while the activity of MWCNTs after modification with nitrogen did not vary significantly as compared to the non-modified MWCNTs. The CALB macromolecules, when immobilized on the surface of MWCNTs, most likely maintained the flexibility of lipase in their activated conformation. Taking the above into account, one could expect that the CALB-CheapTubes™ MWCNTs to emerge as the most promising candidate for further studies, from both an economical and activity point-of-view; this observation was, in fact, confirmed.

3.3. The influence of the catalyst loading for the esterification reaction

In preliminary studies, for the commercial CheapTubes™ MWCNTs support, the CALB loading was studied as a function of the amount of protein used for the immobilization (MR). This influence was determined using the same catalyst mass (0.050 g) per 1 mmol of acid in each test (Supplementary Information, Fig. S5: The influence of the CALB mass ratio to CheapTubesTM MWCNTs (MR) on the activity of nanobiocatalyst in esterification reaction). For this experiment, a lower amount of the catalytic system (50 mg instead of 150 mg used in item 3.2) was used to better differentiate the activity after prolonged reaction times (12 h) for different catalyst loadings. The overall activity of the catalytic system dropped as the CALB loading decreased (from 4.1–15.7 wt.%). The difference in reaction kinetics between MRs 10:1 and 7.5:1 was nearly negligible as the amount of CALB loading was identical (15.7). Therefore, the catalyst synthesized at MR 7.5:1 was chosen for further studies.

Finally, the influence of the catalyst amount for CALB-CheapTubes™ MWCNTs (MR 7.5:1) from 0.010- 0.200 g on the reaction course was studied (Supplementary Information, Fig. S6: The influence of the CALB-CheapTubes™ MWCNTs (MR 7.5:1) amount on the esterification reaction rate). The fastest reaction was observed in the presence of 0.150 g of the nanobiocatalyst. Once again, higher amounts of catalyst (0.200 g) slowed the reaction and consequently led to poor conversion. Moreover, volume of the catalytic system was large; this, in turn, led to a higher reaction mixture viscosity. Hence, poor mass transfer and the total accessibility of the substrate to the active sites were limited.

3.4. Key parameters affecting the reaction

At this stage, while determining whether the reaction temperature could be decreased while maintaining optimum kinetics and catalyst structure, a set of experiments at four various temperatures was performed (Fig. 3).

The nanobiocatalyst was stable for one reaction cycle, even at 45 °C, producing the diester in high yield after 4 h. A slightly lower yield of the diester was obtained at 60 °C in what could be the result of slow enzyme deactivation as the temperature increased. Lower temperatures, *i.e.* 25 and 35 °C caused a significant reduction of the reaction rate.

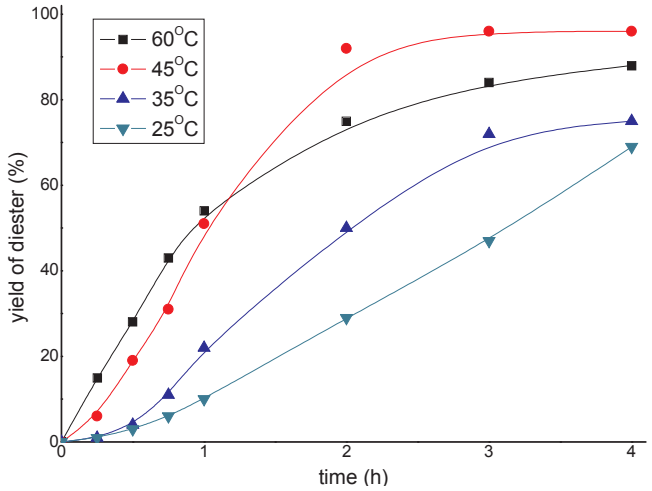


Fig. 3. Influence of temperature on esterification reaction rate; all experiments were performed in triplicate and all three measurements differed by less than 1%. *Reaction conditions*: succinic acid (0.118 g, 1 mmol), *n*-butanol (0.297 g, 4 mmol), cyclohexane 2 mL, CALB-CheapTubes™ MWCNTs (MR 7.5:1; 150 mg), 250 rpm.

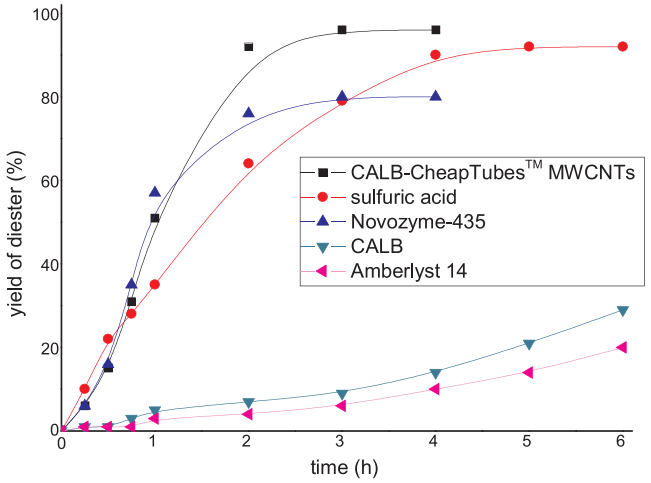


Fig. 4. Comparison of the selected acidic and enzymatic catalysts; all experiments were performed in triplicate and the results of all three measurements differed by less than 1%. *Reaction conditions*: succinic acid (0.118 g, 1 mmol), *n*-butanol (0.297 g, 4 mmol), cyclohexane 2 mL, Novozyme-435, Amberlyst 14 and CALB-CheapTubes™ MWCNTs (0.150 g), sulfuric acid and CALB (0.024 g), 45 °C, 250 rpm.

3.5. Screening of solvents

The choice of organic solvents in enzymatic catalysis is crucial. Usually, it is not possible to establish a general rule on the behavior of activity and stability of lipase in solvents. The high activity of lipases in non-polar solvents (toluene, *n*-hexane, *n*-decane) is attributed to the fact that non-polar organic solvents do not strip off the water layer from the surface of the enzyme [21]. The conformational stability of CALB in non-polar solvents was confirmed by molecular dynamics and quantum mechanics/molecular mechanics simulations [22]. The interaction of a polar solvent and active site leading to lower activity was postulated.

Experiments with polar and non-polar solvents (cyclohexane, toluene, nitrobenzene, *n*-butanol, acetone, acetonitrile, THF) showed that higher yields of the diester product were achieved when non-polar and aprotic solvents were applied (Supplementary Information, Fig. S7: The influence of the solvent on esterification reaction rate). Cyclohexane and toluene were favorable solvents for the model reaction and yields above 92% yield of di-*n*-butyl succinate after 2 h were obtained. Due to its lower boiling point, cyclohexane was used for additional further studies. Nevertheless, hydrophobic solvents provided excellent dispersion of the preferable nanobiocatalytic system as immiscible with water surrounding lipase in the reaction mixture. It resulted in long-lasting lipase activity and reaction system stability. Further, for the reaction performed in nitrobenzene, a 68% yield of di-*n*-butyl succinate was achieved after 2 h. This was a better result than for other polar solvents used, all of which resulted in yields of less than 20% for di-*n*-butyl succinate for the same reaction time. Additionally, THF, acetone and acetonitrile might interact with active sites of the enzyme *via* hydrogen bonding.

3.6. Influence of excess of alcohol substrate on the reaction rate

In esterification, an excess of one of the reagents, most often alcohol, is typically used to shift the reaction equilibrium towards product formation. At the same time, lowering the alcohol excess is beneficial from economic and sustainability standpoints (smaller plant size, less energy to recover and to recycle alcohol). Therefore, succinic acid:*n*-butanol molar ratios from 1:2 to 1:12 were investigated to find the chemical (yield) and economical (process efficiency) optimum (Supplementary Information, Fig. S8: The influence of the succinic acid molar ratio to *n*-butanol (SA:BU) on the esterification reaction rate). As expected, the reaction rate slowed as the alcohol:acid MR increased. For an extremely high concentration of *n*-butanol (2 mL, used as the solvent), the reaction was very slow and confirmed the high sensitivity of the nanobiocatalyst to protic and polar solvents and unwanted changes of enzyme conformations with possible denaturation. Indeed, a two-fold molar excess of alcohol (SA:BU 1:4) was found as the most efficient combination.

3.7. Enzymatic versus acidic catalysts

The activity of the new nanobiocatalyst CALB-CheapTubes™ MWCNTs was compared with the benchmark Novozyme-435 and native form of CALB as well as with the acidic catalysts Amberlyst 14 and sulfuric acid (Fig. 4). Novozyme-435, Amberlyst 14 and CALB-CheapTubes™ MWCNTs (0.150 g per 1 mmol of succinic acid) were used while sulfuric acid and native CALB were used with the same amounts (0.024 g) as the amount of protein which was immobilized on the surface of CheapTubes™ MWCNTs.

The commercial biocatalyst Novozyme-435 was less active than CALB-CheapTubes™ MWCNTs while the reaction for the former plateaued at an 80% yield of the diester. The native form of CALB was, as suspected, of low activity and illustrated the evident improvements achieved using MWCNTs as a support for lipase. As a homogenous catalyst, sulfuric acid was also very active. However, it is worth noting that the activity of sulfuric acid was on the same level as heterogeneous CALB immobilized on the MWCNTs (both used in the same amounts). This comparison proves the superactivity of the developed nanobiocatalytic system. Additionally, for H_2SO_4, the same yield of diester was achieved in approximately twice the time. The conclusive finding of this study is that this new method for the synthesis of esters has a great industrial application potential.

3.8. Recycling studies

Recycling of the CALB-CheapTubes™ MWCNTs was studied over six consecutive runs at 45 °C (Fig. 5). After each run, the catalyst was filtered, washed with cyclohexane and dried under vacuum for 4 h at room temperature. Only a slight mass loss of the catalyst was observed after each run (approximately 2%). Yields of di-*n*-butyl succinate declined slightly only after the 6th run. However, at 60 °C, adverse effects on yields were noted due to faster deactivation of the nanobiocatalyst. Nevertheless, even if one considers a decent stability of the new

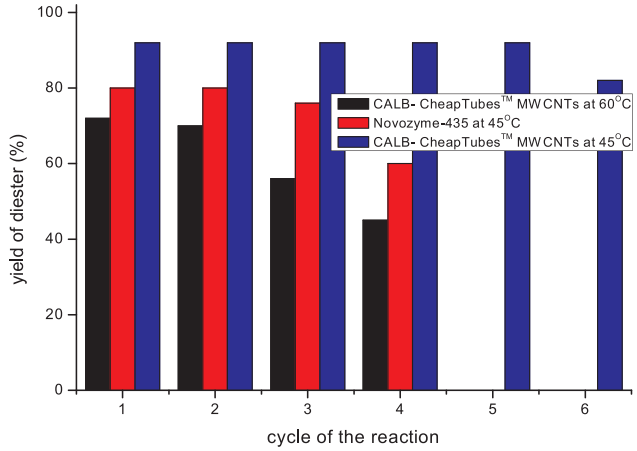


Fig. 5. Recycling of CALB-CheapTubes™ MWCNTs as compared with Novozyme-435; all experiments were per-formed in triplicate and all three measurements differed by less than 1%. *Reaction conditions*: succinic acid (0.118 g, 1 mmol), *n*-butanol (0.297 g, 4 mmol), solvent 2 mL, biocatalyst (150 mg), 250 rpm, reaction time 2 h.

catalyst, its scarce deactivation after the 6th cycle appears to be a substantial advantage. Transferring the obtained results into the continuous flow system could significantly prolong the life of the catalyst, but such experiments are beyond the scope of this paper.

Further, to verify whether the enzyme slowly detached and was washed out or simply lost its activity, the catalyst after the 6th run was analyzed using TGA but this time the calculation of the amount of CALB was impractical. The signals from other substances adsorbed on the MWCNT surface (likely the ester and acid) strongly interfered with the signals derived from the CALB. The efforts undertaken to efficiently wash the catalyst (*e.g.*, sonication) were not effective, and the signals from CALB in TGA still overlapped (Supplementary Information, Fig. S38).

To check if a sluggish leaching of the catalyst from the solid surface happened during recycling, an experiment with a fast catalyst filtration was performed. Catalyst filtration after 0.5 h of the first reaction cycle at 45 °C demonstrated no reaction in the filtrate after removal of the catalyst. The slight deactivation after several cycles probably was not an effect of proteins leaching from the nanobiocatalyst. To further confirm this observation, in the reaction mixture after the 6th cycle, the total level of protein was analyzed using colorimetric techniques (Lowry protein assay). Indeed, no proteins were detected and suggests that CALB adsorbed onto MWCNTs had slowly lost its activity under the applied reaction conditions.

The application of benchmark Novozyme-435 led to lower diester yields and the stability of biocatalyst was poor in subsequent cycles. The use of Novozyme-435 highlighted the problems with filtering the biocatalyst after the reaction, cussed by resin swelling.

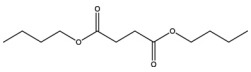
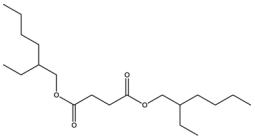
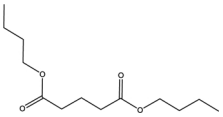
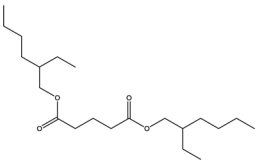
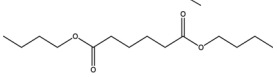
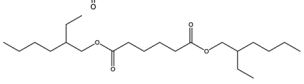
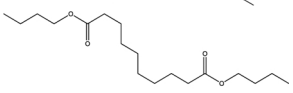
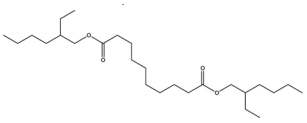
3.9. Substrate scope

Finally, in order to test the robustness of the new catalytic system, the esterification reactions of *n*-butanol and 2-ethylhexanol with dicarboxylic acids: succinic, glutaric, adipic and sebacic were carried out (Table 3). It was demonstrated that the catalyst CALB-CheapTubes™ MWCNTs was active for all reactant combinations and the diesters were formed with high to excellent isolated yields (57–95%).

4. Conclusions

The environmentally benign enzymatic process for the production of esters from dicarboxylic acids (succinic, glutaric, adipic and sebacic) proposed herein utilize a heterogeneous nanobiobiocatalyst consisting

Table 3
Substrate scope.

Ester	Time, h	Yield, %
	3	95 (92)
	24	73(70)
	4	94 (91)
	24	72 (68)
	6	94 (90)
	24	69 (66)
	6	85 (80)
	24	61 (57)

Reaction conditions: dicarboxylic acid 1 mmol, alcohol 4 mmol, cyclohexane 2 mL, CALB-CheapTubes™ MWCNTs (MR 7.5:1; 150 mg), 45 °C, 250 rpm, yields from GC analysis (isolated yields).

of *Candida antarctica* lipase B immobilized on the surface of MWCNTs. Among these studies, several carbon (nano)supports were tested as a lipase carrier and established nanobiocatalysts as a stable and highly enzyme-loaded system. The highly active and commercially available CheapTubes™ MWCNTs were designated for further works.

The optimization studies concerning the influence of substrate concentrations, solvent selection and temperature were carried out. In the chosen reaction conditions, the studied esters of dicarboxylic acids were readily synthesized with high yields (57–95%) under mild conditions in reasonable reaction times. Finally, operational stability tests showed no significant activity loss over 6 consecutive use cycles and recovery of CALB-CheapTubes™ MWCNTs at 45 °C. However, the activities of the studied nanobiocatalysts can be inversely proportional to its stability, *i.e.*, increasing temperature may accelerate the esterification reaction; beyond a certain temperature, the enzyme becomes inactive.

Generally, comparing methods proposed in the literature, there were clear improvements using MWCNTs as a support for lipase. The reaction times were significantly shortened as well as improved stability and surprisingly long for proteins. The recycling of the Novozyme-435 benchmark, which exhibited lower activity, was not very effective. The activity of the conventional acidic catalyst sulfuric acid was comparable, while the activity of Amberlyst 14 was lower.

In summary, this work proves the enhanced activity of nanobiocatalysts and presented a new method for the synthesis of esters of dicarboxylic acids which may find future application in the *fine-chemical* industry.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.apcata.2019.01.030.

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