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ARTICLE

Carbon nanotube/PTFE as a hybrid platform for lipase B from *Candida antarctica* in transformation of α-angelica lactone to alkyl levulinates

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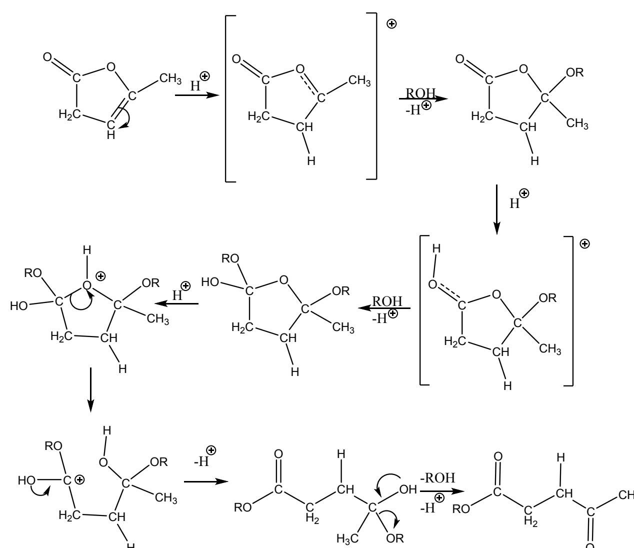
In this work the enzymatic method for the synthesis of alkyl levulinates from α-angelica lactone has been reported for the first time. Lipase B from *Candida antarctica* was immobilized *via* interfacial activation on the surface of hybrid support, consisting of commercially available multi-walled carbon nanotubes (MWCNTs) and polytetrafluoroethylene (PTFE). Among the biocatalysts with various content of PTFE in the support, the CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst with 22.5 wt. % CALB loading was determined as the most active one in model synthesis of the *n*-butyl levulinate in toluene. *n*-Butyl levulinate was obtained quantitively after 120 min of the reaction under the selected reaction conditions (2-fold molar excess of *n*-butanol, 0.150 g of biocatalyst per 1 mmol of α-angelica lactone, 20 °C). The yield of *n*-butyl levulinate was found as higher than in the presence of accurate amounts of sulfuric acid or Novozyme-435. Additionally, a unique stability of developed biocatalyst was presented in 6 reaction cycles at 20 °C. The biocatalyst remained stable in 3 reaction cycles at 60 °C as well. The essence of proposed approach lies in the possibility to overcome the equilibrium limitations occurring in the conventional Fisher esterification. Activity of the elaborated hybrid biocatalyst in the reactions non-specific for lipases is a clear proof for versality of the novel system.

Introduction

Environmental regulations forced the modification of a number of chemical processes toward circular economy with low waste production and low energy consumption, in which the challenge for both academia and industry is to generate economically sustainable consensus. Biomass valorisation to energy and value-added chemicals has become one of the major solutions for the replacement of fossil-based resources.[1]
Levulinic acid (LA) and levulinic acid esters (LAEs) as biomass-derived platform chemicals are applied as versatile building blocks in organic synthesis for the synthesis of pharmaceuticals, flavours and fragrances, agrochemicals, resins and coatings, plasticizers, solvents, fuel additives, and biofuels production.[1] LA can be produced from sugars, lignocellulosic biomass, and waste materials while its esters are obtained by direct esterification of LA, in the presence of acidic catalysts.[2] LAEs can be also produced directly from polysaccharides at high temperature (175 °C)[4] or furfuryl alcohol at 120–140 °C, in the presence of sulfonated materials,[4] or acidic ionic liquids.[5] The synthesis of LAEs is also possible by catalytic upgrading of α-angelica lactone (5 methyl-2(3*H*)-furanone, α-AL, Scheme 1).[1]



Scheme 1 Proposed mechanism for the synthesis of LAEs from α-AL in the presence of Brønsted acidic catalysts.[1]

α-AL is a natural compound, occurring in grapes, soybean and liquorice, used as raw material in various transformations to value added-compounds. The advantage of this method over a direct esterification of LA is the *one-pot* procedure enabling

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[d] † Electronic Supplementary Information (ESI) available: : [Supplemental figures, tables, schemes, GC, 1H, 13C NMR spectra, TGA, TEM/EDS images].]. See DOI: 10.1039/x0xx00000x

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the conversion of α-AL *via* multistep process to LAEs. The design of an active catalyst that can efficiently promote more than one reaction step for this transformation is crucial. Clearly, the α-AL-to-LAEs route is a promising one, but an efficient, selective, and recyclable catalyst is yet to be found. In the literature, only a few reports are available tackling this issue. In each case a Brønsted acidic catalyst, Amberlyst 36[6] or a range of recyclable choline-exchanged heteropolyacids[7] were used. 79–94%-Selectivity and full conversion of α-angelica lactone were reached at 75 °C. The main by-products included the unreacted pseudo-*n*-alkyl levulinate.[6,7] In fact, the promise of overcome the equilibrium which is formed also during the classical esterification process of LA was not successful.

Our recent studies showed that chloride-free Lewis acidic ionic liquids, containing trifloaluminate anions, $[Al(OTf)^{3+n}]^{n-}$, can be applied for the α-AL-to-LAEs route using homogeneous conditions what resulted in the full α-AL conversion and unprecedented selectivities to LAEs, reaching >99% (60 min at 60 °C for 0.1 mol % catalyst loading). Additionally, a highly selective and active heterogeneous catalyst obtained by supporting the trifloaluminate ionic liquid on multi-walled carbon nanotubes (MWCNTs) gave an easily recyclable system, with no leaching observed over six catalytic cycles.[8]

As it was presented above, only acidic catalysts were employed for the *one-pot* conversion of α-AL-to-LAEs. To meet all of the contemporary environmental and economic criteria it would be undeniably beneficial to eliminate the acidic catalyst. The use of enzymes as selective biocatalysts for processing biomass into value-added products received an increased attention during the last decade.[1] Lipase B from *Candida antarctica* (CALB) is the most frequently used enzyme for the esterification.[9-14] The immobilization of lipases on the hydrophobic supports is a powerful tool to improve stability, activity, specificity, selectivity or inhibition resistance.[15] Interfacial activation on hydrophobic and medium hydrophobic supports, at low ionic strength, has been reported to be a simple and efficient method to immobilize lipases on various supports,[15-23] *e.g.* carbon (nano)materials,[16-18] functionalized bentonites,[19] metal oxides,[19] modified and unmodified mesoporous silica,[20] modified magnetic microspheres,[21] resins,[22] sol-gel materials[23] or polymers and biopolymers.[23] However, the investigations toward mechanism of adsorption of lipases onto hydrophobic materials showed that, in the case of CALB only the small lid was present, which allowed adsorbing of enzyme onto oil drops or hydrophobic supports.[9,24-26]

In recent years, the importance of formation of hybrid supports for enzymes is growing fast.[27-29] The final synergy of properties deriving from more than one material could lead to supports of unique properties. And, if dedicated for the selected enzymes, could improve their activity, selectivity as well as stability in the organic environments. Up to date, in order to synthesize hybrid carriers, an approach based on combining organic and inorganic materials was applied, and the following constituents were used for this purpose: metal oxides, silica materials, polymers, ion-exchange resins and CNTs.[19]

Particularly the latter support could yield excellent activation and operational stability of numerous enzymes.[16-19,30] Its pro-activating nature was assigned to hydrophobic interactions working in a highly developed active surface area. Nevertheless, this beneficial behaviour, confirmed both in computations[31] and experiments, now seems to reach its limits due to nanotube defects and hydrophilic termini (crystallographic defects, oxygen functional groups, etc.). Hence, amalgamation of CNTs with an additional enzyme pro-activating component could conquer the so-far obtained promising catalytic characteristics. It was therefore a question whether combination of CNTs with highly both hydrophobic and oleophobic polytetrafluoroethylene (PTFE) particles, and the enzyme activated by hydrophobic interactions such as lipase, could produce a biocatalytic system for an industrially relevant process. This approach, constituting the central research hypothesis of this work, if successful, would deliver a new opening in the biocatalytic transformations exploiting lipases as the actual catalyst.

Hence here, taking the advantage of biocatalysis and environmental viability, we present the design, elaboration and proof-of-concept of novel, non-covalently conjugated biocatalyst based on lipase and the organic-inorganic hybrid support. Lipase B from *Candida antarctica* has been selected as the most appropriate biocatalyst for the model process due to its high activity in hydrolysis/esterification reactions, promiscuity, versality as well as stability.[9-26] The support was composed from MWCNTs and PTFE microspheres. The catalytic performance of this active and stable biocatalyst was demonstrated for the first time in the α-AL upgrade to LAEs.

Experimental

Materials and methods

All substances (alcohols, solvents, α-angelica lactone and *n*-decane) were purchased from Chemat, Poland. The aqueous solution of lipase B from *Candida antarctica* (activity 5,000 $U{\cdot}L{\cdot}kg^{-1}$), Novozyme-435 (commercially available biocatalyst consisting of CALB immobilized on Lewatit VP OC 1600, activity 40,000 $U{\cdot}L{\cdot}kg^{-1}$), and micro-size grains of polytetrafluoroethylene (PTFE) were purchased from Sigma-Aldrich. Industrial grade MWCNTs were purchased from Cheap Tubes Inc. (United States).

Analysis

GC-FID 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) (Tables S1-S2). ^{1}H NMR spectra were recorded at 600 MHz and ^{13}C NMR were recorded at 151 MHz (Varian system) (Schemes S1-S12).

The amount of PTFE in the supports and the lipase loading on the surface of the hybrid materials were determined by thermogravimetry analysis (TGA) using a Mettler Toledo TGA851e thermobalance. Samples of 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 (Figures S1-S19).

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Scanning electron microscopy (SEM) images were obtained with a Phenom Pro Desktop SEM instrument equipped with EDS detector (15 kV) (Figures S20-S24).

Transmission Electron Microscopy (TEM) images were obtained using a Tecnai F20 TWIN microscope (FEI Company, USA) equipped with a 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 (Figures S25-S26).

The determination of the contact angle was performed using GBX Digidrop apparatus. In order to measure the contact angle hysteresis, 6 µL distilled water droplet was settled on the surface of the pellet of samples, and next the advancing contact angle was measured. Then, 2 µL of water was sucked-off and the receding contact angle was determined. Both temperature (22 ± 2 °C) and humidity (50%) were kept at constant level. In order to monitor and record the bubble collisions with the investigated surfaces, the high-speed camera (SpeedCam 512+) was mounted horizontally to the pellet of samples.

Nitrogen adsorption/desorption isotherms for carbon materials were obtained corresponding to the literature.[32] The porous texture of the hybrid MWCNT-PTFE(0.01-20.00 wt.%) supports as well as CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst was characterized by nitrogen adsorption at 77 K in ASAP 2020 (Micromeritics) apparatus (p/p_0 from 10^{-7} to 0.98). The adsorption data were used to determine the total pore volume V_T (at p/p_0 = 0.96) and the specific surface area *via* BET model (S_{BET}).

The presence of lipase in the filtrate after 7 reaction cycles was determined *via* Lowry's method of protein detection (application of UV VIS spectroscopy) corresponding to the literature.[17] UV-VIS spectra were recorded on a Jasco V-650 spectrophotometer at 25 °C in aqueous solution and the absorbance (λ=670 nm) was measured.

Synthetic procedures

Synthesis of hybrid MWCNT-PTFE(0.01-20.00 wt.%) supports

Into a 300-mL beaker, deionized water (150 mL), industrial grade MWCNTs (5.00 g), non-ionic triblock copolymer of polyethylene glycol and polypropylene glycol Pluronic F-127 (0.05 g) as well as PTFE micro-size grains (0.05–1.00 g, 0.01–20.00 wt.% of MWCNTs) were introduced. The suspension was mixed using high shear laboratory mixer Silverson L5M-A for 30 min (4500 rpm), filtered off under the vacuum, next washed with 20 mL of deionized water and dried under reduced pressure (1 mbar, 30 °C, 24 h).

Immobilization of lipase B from *Candida antarctica* on the hybrid support MWCNT-PTFE(0.01-20.00 wt.%)

The immobilization step was carried out according to the literature.[16-18] Into a 100-mL round bottom flask the aqueous solution of lipase B from *Candida antarctica* (0.1–1.0 g), hybrid support (MWCNT-PTFE) (0.1 g) and deionized water (3 mL) were introduced. Immobilization step was carried out for 3 h at 20 °C in a thermostatic shaker (180 rpm). Next, the mixture was filtered off under the vacuum and washed with 20 mL of deionized water. Then the catalyst was dried for 3 days in a desiccator at 5 °C.

General procedure of the synthesis of alkyl levulinates

A CALB/MWCNT-PTFE(0.01-20.00 wt.%) biocatalyst (10-200 mg / 1 mmol of α-angelica lactone) or Novozyme 435 (150 mg / 1 mmol of α-angelica lactone) was introduced into the 10-mL round-bottom flask. Next, *n*-decane (20 wt. % of α-angelica lactone, internal standard), solvent (0-1 mL / 1 mmol of α-angelica lactone), α-angelica lactone (1.0 mmol, 0.098 g) and alcohol (1.0–16.4 mmol) were added. The reaction mixture was sealed with septum and mixed using thermostatic shaker (250 rpm) at 25 °C for 2-6 h. Periodically, during the reaction, 10 µL of samples (diluted with 1.5 mL of acetonitrile) were collected for monitoring the reaction progress using GC-FID analysis.

Purification of alkyl levulinates

After the reaction, biocatalyst was filtered off from the post-reaction mixture. Then, the esters of linear alcohols with boiling point lower than 113 °C (1 atm) were purified using the rotary evaporator (ethanol, *n*-propanol, *n*-butanol: 40 °C, 8 h, 60 mbar). The post-reaction mixture with *iso*-propyl levulinate was concentrated using the rotary evaporator (20 °C, 4 h, 10 mbar) and the crude product was additionally purified *via* column chromatography (ethyl acetate: cyclohexane 2:5, Al_2O_3 as stationary phase). The esters of alcohols with boiling point higher than 113 °C (1 atm) were purified after removing toluene using the rotary evaporator *via* vacuum distillation. The yields and purity of corresponding esters (confirmed *via* ^1H NMR, ^{13}C NMR and GC-FID analyses):

Ethyl levulinate: ^1H NMR (600 MHz, $CDCl_3$, TMS) δ/ppm: 1.26 (t, J=7.1 Hz, 3H), 2.20 (s, 3H), 2.57 (t, J = 6.6 Hz, 2H), 2.75 (t, J = 6.6 Hz, 2H), 4.13 (q, J = 7.1 Hz, 2H); ^{13}C NMR (151 MHz, $CDCl_3$, TMS) δ/ppm: 14.28, 28.15, 29.99, 38.08, 60.74, 172.86, 206.79 (yield 85%, purity >99%).

n-Propyl levulinate: ^1H NMR (600 MHz, $CDCl_3$, TMS) δ/ppm: 0.94 (t, J=7.4 Hz, 3H), 1.65 (dd, J=14.2, 6.9 Hz, 2H), 2.19 (s, 3H), 2.58 (t, J = 6.6 Hz, 2H), 2.75 (t, J = 6.6 Hz, 2H), 4.04 (t, J = 7.6 Hz, 2H); ^{13}C NMR (151 MHz, $CDCl_3$, TMS) δ/ppm: 10.43, 22.05, 28.11, 29.95, 38.08, 66.36, 172.92, 206.78 (yield 96%, purity >99%).

Isopropyl levulinate: ^1H NMR (600 MHz, $CDCl_3$, TMS) δ/ppm: 1.14 (d, J=6.3 Hz, 6H), 2.11 (s, 3H), 2.46 (t, J=6.6 Hz, 2H), 2.65 (t, J=6.7 Hz, 2H), 4.91 (hept, J=6.3 Hz, 1H); ^{13}C NMR (151 MHz, $CDCl_3$, TMS) δ/ppm: 21.69, 28.30, 29.80, 37.92, 67.91, 172.17, 206.68 (yield 94%, purity >99%).

n-Butyl levulinate: ^1H NMR (600 MHz, $CDCl_3$, TMS) δ/ppm: 0.90 (t, J=7.4 Hz, 3H), 1.34 (dd, J= 15.0, 7.5 Hz, 2H), 1.51-1.63 (m, 2H), 2.16 (s, 3H), 2.54 (t, J=6.6 Hz, 2H), 2.71 (t, J=6.6 Hz, 2H), 4.04 (t, J=6.7 Hz, 2H); ^{13}C NMR (151 MHz, $CDCl_3$, TMS) δ/ppm: 13.80, 19.22, 28.13, 29.98, 30.74, 38.09, 64.67, 172.94, 206.78 (yield 97%, purity >99%).

Isooctyl levulinate: ^1H NMR (600 MHz, DMSO, TMS) δ/ppm: 0.84 (dt, J=15.0, 7.1 Hz, 6H), 1.25 (dd, J=26.2, 5.0 Hz, 8H), 1.49 (dt, J=11.8, 5.9 Hz, 1H), 2.08 (s, 3H), 2.39-2.47 (m, 2H), 2.68 (t, J=6.4 Hz, 2H), 3.89 (dd, J=5.7 Hz, 4.4 Hz, 2H); ^{13}C NMR (151 MHz, DMSO, TMS) δ/ppm: 10.82, 13.92, 22.43, 23.22, 27.70, 28.35, 29.53, 29.80, 37.46, 38.17, 65.92, 172.29, 206.72 (yield 88%, purity 99%).

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n-Dodecyl levulinate: ^{1}H NMR (600 MHz, $CDCl_3$, TMS) δ/ppm: 0.77-0.89 (m, 3H), 1.25 (m, 18 H), 1.61 (m, 2H), 2.18 (m, 3H), 2.57 (dd, J=6.2, 3.1 Hz, 2H), 2.74 (dd, J=6.1, 3.0 Hz, 2H), 3.99-4.08 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$, TMS) δ/ppm: 14.25, 22.83, 26.03, 28.16, 28.73, 29.39, 29.48, 29.66, 29.71, 29.78, 30.02, 32.06, 38.12, 65.02, 172.95, 206.72 [yield 87%, purity 90% (*n*-dodecanol as contaminant)].

Recycling of the biocatalyst

In order to recycle the biocatalyst after the reaction (99% yield of *n*-butyl levulinate), the biocatalyst was filtered off, washed with 20 mL of cyclohexane, dried under vacuum (1 mbar) on the Schlenk line (12 h, 20 °C, 1 mbar) and the next cycle of the reaction was performed.

10 mg of recovered biocatalyst was analysed by TGA and the filtrates from all reaction cycles were collected and concentrated using rotary evaporator at 45 °C. Next, the residue was analysed according to Lowry's method in order to confirmed the lack of enzyme leaching to the reaction mixture. (ESI – chapter S3).

Results and discussion

We set out to combine recent advances in the designing of hybrid supports, in the search of a robust biocatalyst. CALB was chosen as the active phase to catalyse the conversion of α-AL to LAEs. First, the synthetic strategy for the new hybrid support for CALB using both hydrophobic and oleophobic PTFE microspheres and MWCNTs; next, the strategy for immobilization of CALB onto new materials was developed. PTFE was used for the first time as the material improving the efficiency of immobilization of CALB on the solid, carbon-based support.

Synthesis and characterization of hybrid supports and biocatalysts

In the first step, several hybrid supports were synthesised *via* physical adsorption with various PTFE amounts (0.01-20.00 wt.%) in the relation to MWCNTs. Pristine MWCNTs (Cheap Tubes™) as well as grains of PTFE, with diameters in a range of 6-9 µm, both commercially available, were used in order to choose the most active hybrid support. Synthesis of hybrid materials was carried out in aqueous suspension using high shear laboratory mixer Silverson L5M-A. The mixing of strongly hydrophobic materials was improved by the addition of surfactant: non-ionic triblock copolymer of polyethylene glycol and polypropylene glycol (Pluronic F-127) (1 wt.% in relation to MWCNTs; 5-100 wt.% in relation to the PTFE). In the next step, after washing off the surfactant from the surface of hybrid material, CALB was immobilized [CALB:MWCNT mass ratio (MR) 1:1-10:1] *via* physical adsorption. The amount of CALB adsorbed on the hybrid material using CALB:MWCNT MR=7.5 was presented in Table 1. PTFE, MWCNTs and hybrid materials as well as the resulted biocatalysts were characterised (Table 1). Combining MWCNTs with micronized PTFE grains in various mass ratios slightly affected specific surface area and pore volume. It has been shown that the influence of PTFE on the specific surface area of the hybrids was marginally noticeable. On the other hand, the amount of PTFE in the preparation step of the synthesis of support was found as the crucial factor with a significant influence on the CALB loading after immobilization as well as further activity of enzyme in the catalytic system. Decrease in CALB loading after addition of the higher amount of PTFE at the synthesis step might be caused by interfering interactions between both materials and formation of agglomerates of MWCNTs around the grains of polymer, and hence decreasing the accessibility of pores for enzyme. PTFE loading was determined using TGA. The determined values clearly correspond with the amount of PTFE applied at the step of synthesis of the support. However, for the higher initial PTFE content (10 and 20 wt.%), a significant loss of PTFE in the hybrid material could be caused by the insufficient amount of surfactant in relation to the PTFE (10% and 5 wt.%, respectively) at the synthesis step or partial desorption of PTFE to the aqueous phase during filtration. In addition, for given morphology of the films prepared from neat PTFE grains and MWCNTs, pellets of both materials were prepared, and the contact angle was established using GBX Digidrop Apparatus at constant temperature and humidity. The water contact angles equalled 115.3 and 71.1°, respectively. It suggests that the addition of PTFE increases hydrophobicity of the final support.

Table 1 The properties of the selected hybrid MWCNTs/PTFE materials.

Support	S_{BET} (m^2/g)[a]	Pore volume (cm^3/g)[a]	PTFE loading (wt.%)[b]	CALB loading (wt.%)[b]	Total activity (U·L)[c]	Specific activity (U·L·kg^{-1})[c]
PTFE, grains	1.6	0.004	n/a	0.7	58	167
CheapTubesTM MWCNTs	89.0	0.490	n/a	15.7	3,389	26,603
MWCNT-PTFE(0.01 wt.%)	83.1	0.204	0.06	16.0	4,889	39,111
MWCNT-PTFE(0.10 wt.%)	**80.3**	**0.211**	**0.13**	**22.5**	**5,550**	**62,438**
(biocatalyst based on CALB and MWCNT-PTFE(0.10 wt.%))	**(67.9)**	**(0.173)**	**(0.13)**			
MWCNT-PTFE(0.50 wt.%)	86.3	0.223	0.46	15.9	5,056	40,192
MWCNT-PTFE(1.00 wt.%)	84.2	0.222	0.89	13.3	4,778	31,772
MWCNT-PTFE(10.00 wt.%)	87.4	0.215	4.24	12.4	4,333	26,867
MWCNT-PTFE(20.00 wt.%)	77.1	0.208	10.56	11.7	4,889	28,600

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[a] Values determined *via* nitrogen sorption in 77K with S_{BET} analysis; [b] values determined *via* thermogravimetric analysis; [c] Total and specific activity of biocatalyst calculated after 180 min in the synthesis of *n*-butyl levulinate. *Reaction conditions*: α-AL 0.098 g (1 mmol), *n*-BuOH 0.149 g (2 mmol), toluene 1 mL / 1 mmol of α-AL, biocatalyst 0.050 g / 1 mmol of α-AL, 20 °C, 250 rpm.

In order to determine and confirm the presence of PTFE in the structure of hybrid support, transmission electron microscopy (TEM), scanning electron microscopy (SEM) as well as energy dispersive X-Ray spectroscopy (EDS) were applied. Theoretical content of fluorine atoms was calculated in relation to PTFE loading determined *via* TGA/DTG technique and compared with the average value based on data collected *via* EDS analysis of five different scanned locations in each sample. The obtained results confirmed the presence of PTFE in the hybrid supports (Fig. 1 and Fig. S20-S24). Additionally, the presence of sulphur and nitrogen after immobilization of CALB confirmed the presence of lipase in the structure of CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst.

In addition, dark and bright field TEM (Fig. 2 and Fig. S25-S26) was used in order to study the morphology of the support and biocatalyst in the nanoscale. Larger PTFE grains are clearly visible as non-porous, bulky microparticles in the dispersed network of MWCNTs, to which CALB phase is anchored.

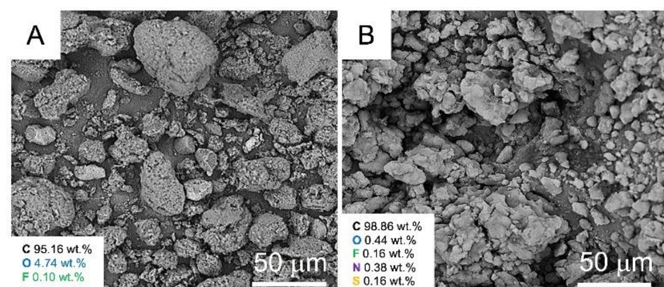


Fig. 1 SEM images of MWCNT-PTFE(0.10 wt.%) supported with EDS analysis (A) and MWCNT-PTFE(0.10 wt.%) supported with EDS analysis after immobilization of CALB (B).

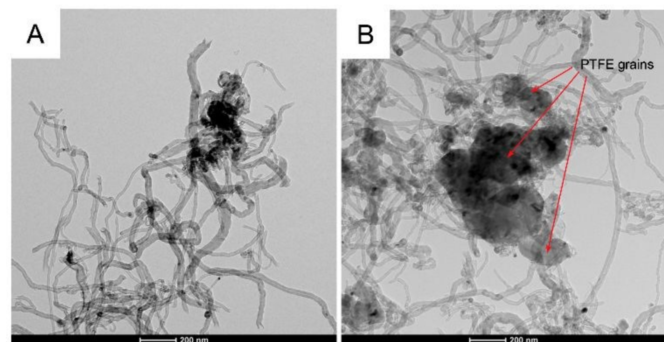
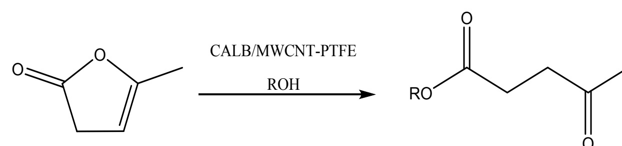


Fig. 2 TEM images of MWCNT-PTFE(0.10 wt.%) support (A) and CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst (B).

The synthesis of alkyl levulinates from α-angelica lactone in the presence of biocatalysts

The new biocatalysts were tested in the α-AL upgrade to LAEs (Scheme 2). In the preliminary screening, a wide range of biocatalysts with various content of PTFE and CALB were compared. The influence of the biocatalyst amount, the excess of alcohol and the kind of solvent were studied. The recycling strategy was demonstrated. Finally, because this reaction was for the first time catalysed with the enzyme, the mechanism was envisaged.



Scheme 2 Enzymatic synthesis of alkyl levulinates from α-angelica lactone; R=ethyl, *n*-propyl, isopropyl, *n*-butyl, isooctyl or *n*-dodecyl.

The influence of the composition of hybrid support on the biocatalyst activity

Using biocatalysts based on hybrid supports with various content of PTFE in this preliminary screening studies gave the opportunity to investigate the effect of PTFE on the biocatalyst activity. Synthesis of *n*-butyl levulinate (BLV) was chosen as a model reaction. The 2-molar excess of *n*-butanol (*n*-BuOH) and 0.050 g of biocatalyst per 1 mmol of α-AL were used, the reaction was carried out at 20 °C, in toluene as a solvent. The results were demonstrated as changes in the conversion of α-AL in time (Fig. 3) and as specific activity as well as total activity of enzyme after 180 min (Table 1).

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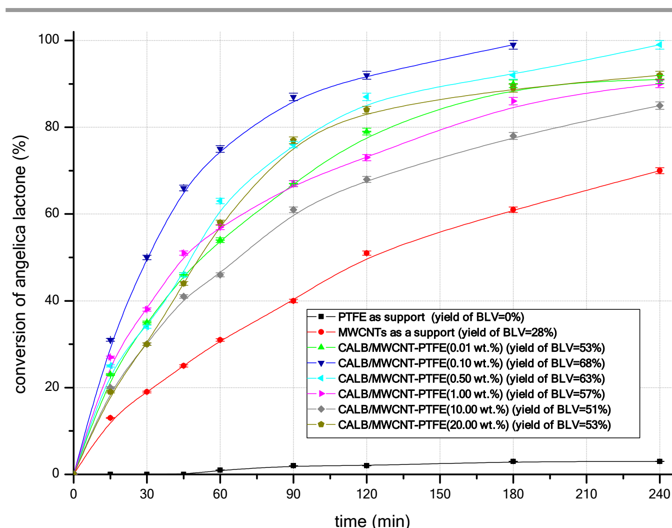

Fig. 3 The influence of PTFE content in the hybrid support on the activity of biocatalyst in the synthesis of *n*-butyl levulinate.

Reaction conditions: α-AL 0.098 g (1 mmol), *n*-BuOH 0.149 g (2 mmol), toluene 1 mL/ 1 mmol of α-AL, biocatalyst 0.050 g/ 1 mmol of α-AL, 20 °C, 250 rpm. All experiments were triplicated (standard deviation 1%). The yield of *n*-butyl levulinate at the end points is given in brackets.

The full conversion of α-AL after 180-240 min was obtained when the biocatalysts based on the hybrid supports with a 0.10 and 0.50 wt.% of PTFE were applied. Both biocatalysts are characterized by the highest amount of the lipase immobilized on the surface of hybrid carrier (15.9-22.5 wt.%). PTFE provided more efficient and homogeneous dispersion of the MWCNTs in the reaction mixture, which, in turn, increased the accessibility of support for enzyme and hence active centres for substrates. Overall, the enhanced activity might be also caused by increased hydrophobicity of the support.

Hildebrand parameters of water, MWCNTs and PTFE (б=97.95,[33] 20.40[34] and 13.60 ($J^{1/2} \cdot m^{3/2}$),[33] respectively) show that both solid materials possess relatively various solubility/dispersibility properties. It clearly demonstrates that, the cohesive energy density, and so the possibility of mixing two non-polar materials is diverse and addition of surfactant provides the better mass contact between components, especially in the presence of water at the synthesis step. Undoubtedly, PTFE exhibits the features of more hydrophobic substance in relation to the MWCNTs, which constitutes the proof for its role as a component improving hydrophobicity of the hybrid carrier. However, this phenomenon could be also a reason of drop in the activity for biocatalysts based on supports with higher amounts of PTFE. In fact, an excessively hydrophobic nature of the support could have negative effect on the activity of enzyme.[23] Additionally, increasing hydrophobicity might reduce the efficiency of diffusion of reagents towards the active centres of catalysts and hinder the contact between them and catalyst due to strong interactions between molecules of the support with relatively similar hydrophobicity, with exclusion of the more hydrophilic molecules from these interactions.[35]

For the comparison, biocatalysts constructed only from PTFE or MWCNTs and CALB were tested (0.7 wt.% of CALB and 15.7 wt.% of CALB, respectively). Biocatalysts based on PTFE were almost inactive while the CALB/MWCNTs was of lower activity then all CALB/MWCNTs-PTFE biocatalysts. In addition, the experiments without biocatalyst and in the presence of neat materials (MWCNT, PTFE) and surfactant (Pluronic F-127) revealed that the reaction did not proceed without CALB.

Essentially, the values of total and specific enzyme activity confirmed the highest activity of CALB/MWCNT-PTFE(0.10wt.%). The yields of *n*-butyl levulinate in these screening studies were moderate, and the unreacted pseudo-*n*-butyl levulinate was detected. The optimization of reaction conditions toward high yield of ester was demonstrated in the further studies. The influence of the following parameters: stirring speed, amount of CALB immobilized on the MWCNT-PTFE(0.10 wt.%) hybrid support, biocatalyst loading as well as α-angelica lactone: *n*-butanol molar ratio were determined (Fig. S27-S29 and Table S4).

The influence of the solvent

Since organic solvents can influence the structure of enzyme or deactivate the active centre, *via e.g.* interactions with heteroatoms, solvation effects, formation of hydrogen bonds, and consequently changes in the conformation of enzyme, the solvent selection is crucial.[34] Fig. 4 shows the formation of *n*-butyl levulinate in the processes carried out in various organic solvents.

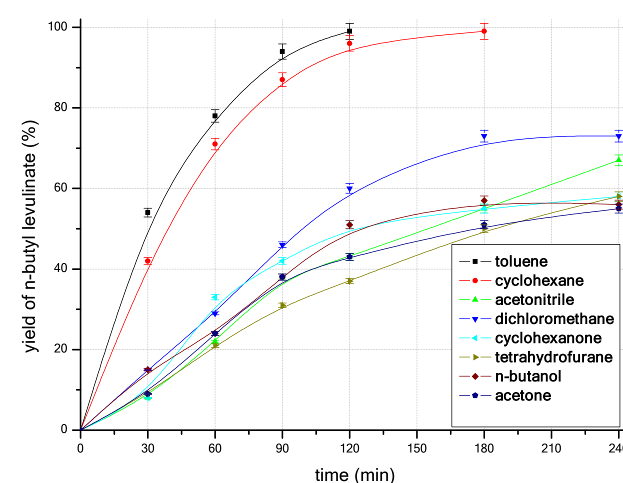

Fig. 4 The influence of type of the solvent on the reaction rate.

Reaction conditions: α-AL 0.098 g (1 mmol), *n*-BuOH 0.149 g (2 mmol), solvent 1 mL/ 1 mmol of α-AL, CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst 0.150 g/1 mmol of α-AL, 20 °C, 250 rpm. All experiments were triplicated (standard deviation 2%).

Despite the full conversion of α-AL after 60-120 min in all solvents, BLV yields and the selectivities toward BLV were varied (Fig. 4 and Table S5). As the most promising emerged non-polar, aprotic solvent – toluene, which application led to the selectivity >99% to BLV after only 120 min. Corresponding to the literature, non-polar solvents are expected to be the most suitable solvents for CALB.[36] Toluene provided slightly higher activity of enzyme than cyclohexane due to presence of π-

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electrons similar to the structure of MWCNTs providing better dispersion of biocatalyst, and similar to components of support Hildebrand parameter (18.9 $J^{1/2}\cdot m^{3/2}$).[33] On the other hand, application of more polar solvents leads to significant drop in reaction rate as well as selectivity to the BLV. Probably, the reason of this phenomenon lies in the presence of heteroatoms with lone electron pairs in the structure, which compete with substrates, decreasing the reaction rate. In addition, solvents more miscible with water are able to penetrate the water shell of enzyme, accelerating the destruction of the peptide bonds and structure of the protein. Polar solvents also can cause aggregation of macromolecules of CALB, which leads to decreasing of enzyme activity which in turn results in lowered selectivity. [9,37]

The comparison of acidic and enzymatic catalysts

In order to verify effectiveness of the proposed reaction system, the activity of CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst was compared with the most commonly used, commercially available, enzymatic as well as acidic catalysts. The following homogeneous and heterogeneous catalysts were taken into considerations: native CALB (activity 5,000 $U\cdot L\cdot kg^{-1}$), Novozyme-435 (commercially available and the most often used biocatalyst consisting of CALB immobilized on Lewatit VP OC 1600, activity 40,000 $U\cdot L\cdot kg^{-1}$), sulfuric acid as well as cationic ion-exchange resin Amberlyst 36 (Fig. 5). Heterogoneous (bio)catalysts were used in the same amount as CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst under the optimized reaction conditions (0.150 g/1 mmol of α-AL) due to unknown amount of CALB in Novozyme 435. In addition, different amount of solid in the reaction mixture might affect mass and heat transport. In contrast, native CALB and sulfuric acid were applied with the same amount (per weight) of active phase in the CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst.

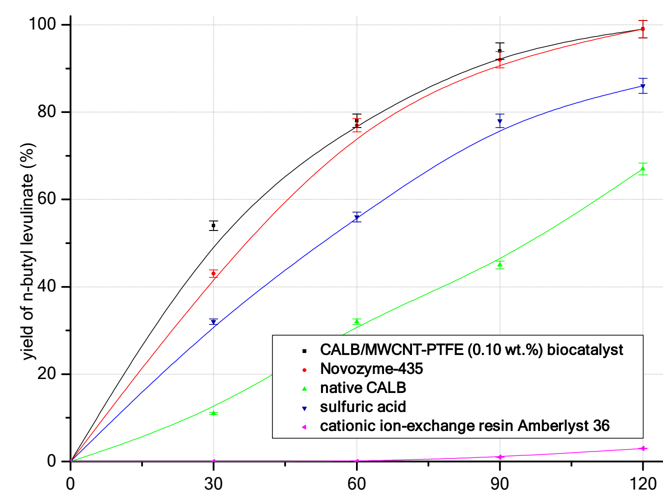

Fig. 5 The influence of the type of the catalyst on the yield of *n*-butyl levulinate.

Reaction conditions: α-AL 0.098 g (1 mmol), *n*-BuOH 0.149 g (2 mmol), toluene 1 mL/ 1 mmol of α-AL, heterogeneous catalyst (CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst, Novozyme-435 or Amberlyst 36) 0.150 g/1 mmol of α-AL or homogeneous catalyst (native CALB or sulfuric acid) 0.034 g (22.5 wt.% in relation to the total mass of heterogeneous catalyst), 20 °C, 250 rpm. All experiments were triplicated (standard deviation 2%).

The comparison of the selected catalysts has unambiguously revealed the unique activity of CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst, which allowed to achieve 99% yield of *n*-butyl levulinate after 120 min. It should be emphasized that the reaction rate was higher than in the presence of native CALB, which might be caused by destructive effect of environment of reaction as well as possibility to forming dimeric aggregates of lipase.[9] Despite the small lid, which partially closes the active centre,[24-25] the increase of activity of CALB after immobilization on the hydrophobic support was observed, the most likely due to interfacial activation or degradation of aggregates leading to enhanced homogeneity. [9,24] On the other hand, application of sulfuric acid – in the amounts comparable with the amount of the active phase in heterogeneous biocatalysts – leads to a lower reaction rate (85% yield of *n*-butyl levulinate after 120 min). Moreover, an Amberlyst 36 remained almost inactive under reaction conditions, probably due to low temperature of the reaction. Novozyme-435 was used in numerous recently described research with CALB as active phase of biocatalysts. Simplicity of immobilization, high activity, versatility and availability resulted in naming it as 'gold' and versatile lipase-based biocatalyst.[9] We decided to compare it with developed catalytic system. Indeed, the activity of Novozyme-435 was similar to CALB/MWCNT-PTFE(0.10 wt.%) in the model reaction, but the studies presented below has discovered the advantage in thermal stability of developed MWCNT/PTFE-based biocatalyst.

Recycling of biocatalyst

One of the most important features of heterogeneous catalysts is their ability of recycling (batch system) and stability (flow system). The recycling studies using CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst as well as for comparison with commercially available Novozyme-435 were performed (Fig. 6). The stability of CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst in simple esterification of levulinic acid was also demonstrated.

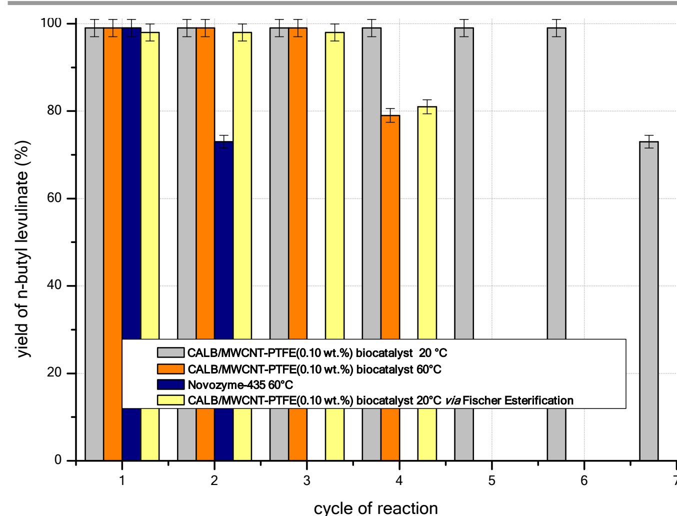

Fig. 6 The recycle studies.

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Reaction conditions: (grey) α-AL 0.098 g (1 mmol), *n*-BuOH 0.149 g (2 mmol), toluene 1 mL/ 1 mmol of α-AL, CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst 0.150 g/1 mmol of α-AL, reaction time 2h, 20 °C, 250 rpm. All experiments were triplicated (standard deviation 2%); (orange) α-AL 0.098 g (1 mmol), *n*-BuOH 0.149 g (2 mmol), toluene 1 mL/ 1 mmol of α-AL, CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst 0.150 g/1 mmol of α-AL, reaction time 30 min, 60 °C, 250 rpm. All experiments were triplicated (standard deviation 2%); (blue) α-AL 0.098 g (1 mmol), *n*-BuOH 0.149 g (2 mmol), toluene 1 mL/ 1 mmol of α-AL, Novozyme-435 biocatalyst 0.150 g/1 mmol of α AL, 30 min, 60 °C, 250 rpm. All experiments were triplicated (standard deviation 2%); (yellow) LA 0.117 g (1 mmol), *n*-BuOH 0.149 g (2 mmol), toluene 1 mL/1 mmol of LA, CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst 0.150 g/1 mmol of LA, reaction time 45 min, 20 °C, 250 rpm. All experiments were triplicated (standard deviation 2%).

CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst exhibited extremely high stability in the reaction environment expressed as a possibility to run 6 reaction cycles without any significant loss of CALB activity at 20 °C. Moreover, biocatalyst remained active even in the 3rd reaction cycle at 60 °C what contrasted with deactivation of Novozyme-435 after the first reaction cycle due to thermal as well as mechanical deterioration. Under these conditions, Lewatit (material used in Novozyme 435 as a support for CALB) can be dissolved in the reaction environment (due to presence of *n*-butanol), which leads to desorption of CALB and thermal degradation of its structure. In addition, Lewatit is a mechanically-fragile material – we have observed its complete destruction while carrying out the experiments.[9] Thus, high thermal and chemical stability as well as high mechanical performance of MWCNTs are most likely the key factors limiting the unique functionality of the elaborated and developed hybrid support. It is worth to underline that CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst enabled to carry out more reaction cycles without a significant loss of activity if α-AL was applied as the substrate in comparison with the conventional enzymatic esterification, which may occur as equilibrium or kinetically controlled process.[38] In the latter case the biocatalyst remained active only for 3 reaction cycles. This phenomenon is probably caused by a damaging effect of high concentration of levulinic acid on the active phase. However, presence of hydrophilic compounds (including water as a reaction by-product) might lead to generation of hydrophilic layer inside the biocatalyst and faster deactivation.[9] Nevertheless, TGA analysis and Lowry's method showed that the leaching of enzyme into the reaction mixture in both cases (Fig. S1) was below the detection limit, which suggests that deactivation of enzyme occurs principally due to chemical factors or conformational changes of the enzyme.[9,24] These aspects constitute a great advantage of the presented *one-pot* enzymatic α-AL-to-levulinates route due to possibility to overcome the equilibrium limitations as well as improving the life-time of enzyme in the reaction environment.

Substrate scope

As the next step, versality of the developed approach was confirmed *via* synthesis of a spectrum of levulinic acid esters. Table 2 shows the structures and yields of the obtained compounds.

Table 2 Synthesis of various esters in the presence of CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst.

Ester	Temp. (°C)	Time of reaction (h)	Yield (isolated yield) of ester (%)
ethyl levulinate	20	2	90 (85)
n-propyl levulinate	20	2	99 (96)
isopropyl levulinate	20	2	99 (94)
n-butyl levulinate	20	2	99 (97)
isooctyl levulinate	20	6	91 (88)
n-dodecanyl levulinate	20	2	94 (87*)

Reaction conditions: α-AL 0,098 g (1 mmol), alcohol 2 mmol, toluene 1 mL/1 mmol of α-AL, CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst 0.150 g/1 mmol of α-AL, 250 rpm. All experiments were triplicated (standard deviation 2%). *purity 90% (presence of *n*-dodecanol)

All the esters were obtained in high or excellent yields, which clearly showed the applicability of CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst for the synthesis of a wide spectrum of products and ability to overcome the limitations of the equilibrium state in the classical Fischer esterification.

Mechanistic considerations

Corresponding to the literature, lipases are enzymes naturally occurring in living organisms, playing a role of specific catalysts for hydrolysis of lipids. This is the reason of searching their potential applications in the reactions of formation or breaking the C-O bond in the ester group.[9] However, these enzymes are also able to catalyse non-specific reactions, *e.g.* formation or hydrolysis of amides, condensations toward formation of C-C bond or proton transfer reactions. These characteristics are known from the literature as enzyme promiscuity.[39,40] Therefore, the further considerations on the mechanism of enzymatic route of α-AL-to-levulinates were made. For this purpose, we focused on the aspects related to the structure of active centres of CALB as well as mechanism of the conventional acidic α-AL-to-levulinates route.

An active centre of lipase B from *Candida antarctica* is built by catalytic triade of amino acids: Ser105-His224-Asp187, existing in both open and closed forms.[41-43] It has been reported that the structure of active centre of CALB might contain an aqueous network which formed a hydrogen bonds with oxygen atoms in Ser105 hydroxyl groups as well as nitrogen atom from the aromatic ring of His224. This phenomenon could explain a lack of activity of lipases under totally anhydrous conditions.[41] Asp187 is connected with Glu188 *via* amide bonding in contrast to Ser105, which usually constitutes the *C*-terminus and participates in standard interactions with carbonyl group as a nucleophile, forming oxyanion hole.[44,45] In the open form of lipase, carboxyl group of Asp187 is fully deprotonated with a simultaneous, partial deprotonation of carboxyl groups of Glu188.[41] On the other hand, it is postulated that His224 starts

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the structure of helix exposing its side chain as a part of the active site.[46] On the other hand, Ser105 might be engaged in formation of hydrogen bonds with other components of the protein structure.[41-47]

Taking all above into consideration, we anticipate that, most likely, the moiety/ies responsible for the activation of α-AL in the enzymatic α-AL-to-levulinates route is one of the groups which might forms hydrogen bond with unsaturated C=C bond of lactone. Thus, this part most likely plays a role of acceptor of electrons from π-bonding of lactone, leading to changes in the electron density of double bond, then promotes a nucleophilic attack of -OH group from alcohol on the carbon atom with the deficit of electrons. Therefore, a dual role of CALB in *one-pot* synthesis of alkyl levulinates is suspected, confirming its versality and promiscuity. The experiments clearly revealed the need of using CALB as catalyst at both crucial steps. The first step covers nucleophilic attack of the alcohol on the carbon bonded with the methyl group after protonation of the C=C bond in the lactone as well as in the esterification stage. The second step, according to the typical mechanism in the presence of lipases, is based on the formation of tetrahedral intermediates with oxyanion holes, in which the hydroxy group of Ser105 is taking part.[48]

The proposed mechanism corresponds to the observed by-products as well as to the structure of the active centre, however, this issue requires further detailed studies. The other possible synthesis routes may be taken into consideration, *i.e.:* a) an earlier activation of C=O bond by Ser105 and His224 with the conventional enzymatic hydrolysis, further protonation of the C=C bond and obtaining levulinates after nucleophilic attack of alcohol; b) an earlier activation of alcohol *via* hydrogen bonds with His224 or Ser105, and further nucleophilic attack on the carbonyl carbon. The most important research challenges in this field are related to the determination which amino acid is forming a hydrogen bond in the starting point of this process as well as which reaction pathway is privileged and faster: formation of the oxyanion hole during hydrolysis or protonation of C=C double bond in the custom path.

Conclusions

In this work, efficient and environmentally friendly one-pot enzymatic conversion α-AL to alkyl levulinates has been developed. Simultaneously, an innovative and extremely stable hybrid support based on MWCNTs and PTFE dedicated for the adsorption of lipase B from *Candida antarctica* was designed. This amalgamation allowed enhancing the properties of both neat materials of the hybrid support toward increased stability and activity of the enzyme.

The research included characteristics and determination of the key parameters of the obtained hybrid materials as well as biocatalysts with further optimization studies in the model synthesis of *n*-butyl levulinate. The proposed approach allows to efficiently overcome the equilibrium limitations affecting the conventional reaction between acid and alcohol and allows to achieve very high yields of the corresponding esters (90-99%). Moreover, the biocatalytic system is outstanding in the light of extreme stability of the enzyme in the reactions proceeding under relatively harsh conditions for enzymes. It was possible to carry out 6 reaction cycles without any significant loss in activity of CALB at 20 °C. Essentially, higher activity of CALB/MWCNT-PTFE(0.10 wt.%) biocatalyst in comparison with the selected commercially available catalysts i.e. H_2SO_4 and Novozyme-435 has been undoubtedly proven.

To conclude, the proposed work extends the possibilities of using enzymes as catalysts for the chemical reactions, and also describes the construction of innovative, designable carriers. It is worth to highlight that this study is an encouraging extension of the possibilities of conducting the reactions non-specific for the enzymes as well.

Conflicts of interest

There are no conflicts to declare.

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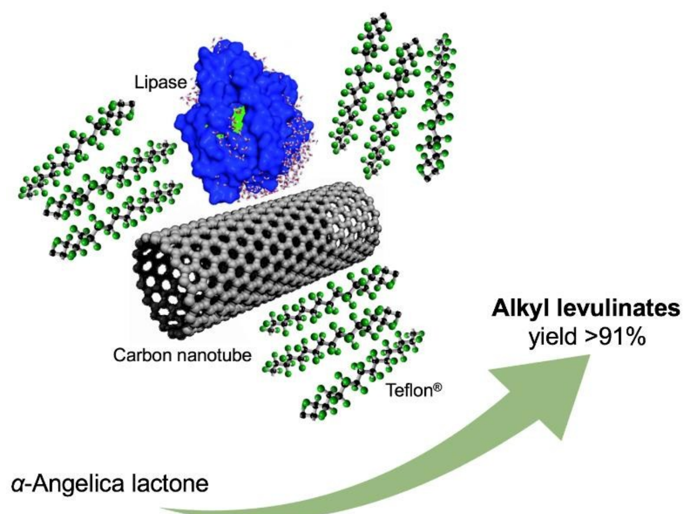

Highly active biocatalyst based on hybrid platform was designed for the conversion of α-angelica lactone to alkyl levulinates

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