ARTICLE HTTP://DX.DOI.ORG/10.5504/BBEQ.2013.0010 PB

EXCELLENT STABILITY AND SYNTHETIC ACTIVITY OF LIPASE FROM *B. STEAROTHERMOPHILUS* MC7 IMMOBILIZED ON TIN DIOXIDE IN ENVIRONMENTALLY FRIENDLY MEDIUM

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ABSTRACT

A thermostable lipase from B. strearothermophilus MC7 was stabilized via immobilization on nanostructured tin dioxide (nanoSnO$_2$-MC7). The synthetic activity of nanoSnO$_2$-MC7 in two ionic liquids containing chloride anions was assessed. In the presence of 3-methyl-1-octyl imidazolium chloride, the stearyl stearate production proceeded at a much higher reaction rate than in solvent-free medium and 95 % conversion of the substrates was achieved for 5 h at 65 °C. Our results showed that it also had a beneficial effect on the operational and thermal stability of the immobilized lipase MC7. In this green solvent, the biocatalyst was almost fully active after eight synthetic cycles and up to 60 % of the initial activity of nanoSnO$_2$-MC7 lipase was preserved after long heating at 75 ºC.

Biotechnol. & Biotechnol. Eq. 2013, **27**(6), 4317-4322

Keywords: *Bacillus stearothermophilus* MC7 lipase, immobilization, wax-ester synthesis, ionic liquids, solvent-free system

Introduction

Lipases (triacylglycerol acylhydrolases, EC. 3.1.1.3) are enzymes that catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids at the interface between insoluble hydrophobic substrates and water. In non-aqueous environment they can catalyze the reverse reaction, esterification. Recently, researchers have been focusing on novel nonspecific applications of lipases in organic synthesis such as: carbon–carbon bond formation, carbon–heteroatom and heteroatom–heteroatom bond formation, oxidative processes, synthesis of heterocycles, etc. (22). After amylases, they are the second most applied enzymes in food, pharmaceutical, detergent and other industries (18).

To date, more than hundred lipases from the *Bacillus* genus have been isolated and preliminarily characterized; over 15 ones were discovered in the last year, amongst which some with exceptional heat-, solvent-, and pH-stability (1, 4, 9, 38). Furthermore, the majority of the known thermostable lipases have been obtained from *Bacillus* strains (15). Their potential applications are of great interest, especially for large-scale productions in which high temperatures are required. Higher temperatures ensure better homogenization of the reaction mixture, higher rate of conversion of the substrates, lower risk of microbial contamination, and minimize the utilization of organic solvents (2).

The advantages of biocatalysts over conventional catalysts are many: performing the reaction under mild reaction conditions (low energy consumption), controlled product formation, and rapid termination of the reaction. However, the price of biocatalysts remains uncompetitive to that of the inorganic catalysts. Enzyme applications are also limited by their lower stability in the harsh reaction conditions (high temperatures, pressures, solvents). Hence, much effort has been directed to enzyme stabilization and utilization in multiple runs, respectively to enzyme immobilization as one of the most effective approaches.

Still, most of the lipases from *Bacillus* genus have not been fully explored, and only a few of them have been analyzed in terms of their behavior upon immobilization and stability in several consecutive reaction cycles (9, 23, 40).

There is no generally universal procedure for immobilization applicable for all enzymes. The selection of a suitable carrier and method of immobilization should be applied with great care because the support may cause structural changes to the enzyme and, as a result, alter the specificity of the biocatalyst (30). There are several methods for enzyme immobilization, each having both advantages and disadvantages (16). Covalent bonding and cross-linking ensures tight fixation of the enzyme to the support. It prevents formation of molecule aggregates and enzyme leaching in the aqueous media. Protein molecules become less flexible and are preserved from denaturation and unfolding. Another widely applied method is enzyme entrapment or encapsulation into organic polymers or sol-gels. The protein molecule is protected from the environment and mechanical shearing. However, the drawbacks of the method are low protein loading and serious mass transfer limitations, especially when it comes to large substrates. Physical adsrorption is a cheap one-step method of immobilization that does not require expensive and hazardous reagents and is friendly to the enzyme activity. The protein molecules are

bonded to the carrier via hydrophobic, hydrophilic or ionic interactions. In an aqueous environment, the enzymes are prone to leaching and, in multiple cycles, a fast drop in activity can be observed which can be a serious disadvantage. The method is best suited for lipases when they are applied in a solvent-free or organic medium where no desorption is likely to occur. Much higher protein loading is usually achieved by physical adsorption than by covalent bonding or entrapment.

Inorganic materials, due to their structural stability, environmental acceptability, resistance to microbial attack and organic solvents, and because of their low cost, are preferred in comparison to polymeric ones. Materials on the basis of silica (SBA, MCM, KIT) are the most examined and applied inorganic lipase carriers. There are many techniques that allow functionalization or synthesis of particles with tunable pore size which makes them applicable for various bioadsorptions (17, 25).

Recently, the focus of attention has shifted toward the use of nanosized materials as enzyme supports. The small sizes of the particles provide larger surface area and thus ensure higher enzyme loading per unit carrier. The large lipase molecules are usually adsorbed on the surface of the particle and the access of the substrate to the enzyme active centre is facilitated (27). Our earlier studies on nanostructured tin dioxide (nanoSnO_2) have revealed its potential as a lipase carrier. The enzyme from *Candida rugosa* immobilized on nanosized SnO_2 exhibited eight-fold higher activity than those immobilized on polypropylene (8). At the same time, the lipase from *Rhizopus delemar* has shown higher affinity towards two tin dioxide materials in comparison to silica (10).

In this paper we report the stabilization of a thermostable lipase from *B. stearothermophilus* MC7 via immobilization on nanosized tin dioxide (nanoSnO_2-MC7). Due to its broad specificity and relatively good thermal stability (10, 21), we considered that the enzyme could be applied in the synthesis of stearyl stearate, a wax ester extensively used as an emulsifier, emollient and drug carrier (39, 45). We examined the effectiveness of nanoSnO_2-MC7 in stearyl stearate synthesis in both an organic medium and an environmentally-friendly medium (two ionic liquids and a non-solvent system). Recently, medium engineering has attracted much attention as an approach to improve enzyme activity and stability and as an alternative to conventional solvents. We assessed the operational stability of the biocatalyst in eight consecutive reaction cycles.

Materials and Methods

Chemicals

The lipase from *B. stearothermophilus* MC7 (lipase MC7) was produced as described by Kambourova et al. (21). The enzyme stock solution contains protein concentration of 4 mg·mL^{-1} (total activity of 1 400 U·mL^{-1}, specific activity of 350 000 U·g^{-1} protein). The following chemicals were purchased from Fluka (Germany): 4-nitrophenyl palmitate (98 % purity), 1-octadecanol (95 % purity), stearic acid (97 % purity) and PEG1500. The nanostructured tin dioxide (nanoSnO_2), with a surface area of 170 m^2·g^{-1}, a total pore volume of 0.13 cm^3·g^{-1} and pore size distribution with a maximum at 3.1 nm, was synthesized as previously described (8). The ionic liquids: 3-methyl-1-octyl imidazolium chloride [OMIM][Cl] (95 % purity) and methyl trioctyl ammonium chloride [TOMA][Cl] (90 % purity) were purchased from Merck (Germany).

Immobilization of lipase MC7

Lipase MC7 solution (0.5 mL) was gently stirred with 20 mg nanostructured tin dioxide for 2 h at room temperature. Then, the mixture was kept for 12 h at 4 °C, then filtered, and dried at room temperature. The amount of protein immobilized onto the carrier was estimated as the difference in the protein concentration of the lipase solution before and after immobilization, according to Lowry et al. (26).

Hydrolytic activity and stability

The activity of the free and the immobilized lipase was estimated using a spectrophotometrical test with 4-nitrophenyl palmitate as a substrate (46). One lipase unit (U) was defined as the amount of the enzyme required to liberate 1 μmol of fatty acid per 1 min under the assay conditions.

The thermal stability of nanoSnO_2-MC7 was determined by measuring the residual hydrolytic activity after pre-incubation for 60 min in 50 mmol·L^{-1} sodium phosphate buffer (pH 7.5) at different temperatures. The residual activity was defined as the ratio between the activity after treatment and the initial activity.

The stability of nanoSnO_2-MC7 in organic solvents was tested as follows: 10 mg of nanoSnO_2-MC7 (or 100 μL of free lipase MC7, 4 mg·mL^{-1} protein) were incubated for 1 h with 1 mL of various water miscible or immiscible solvents (alcohols, ethers, hydrocarbons) at room temperature. The activity of the non-treated free and immobilized lipase MC7 was taken for 100 %. Residual activity (%) was calculated in respect to the activity of the non-treated enzymes.

Synthesis of stearyl stearate catalyzed by lipase MC7

Stearic acid (142 mg, 0.5 mmol) and 1-octadecanol (135 mg, 0.5 mmol) were incubated for 30 min at definite temperature (60 °C to 75 ºC), and then 20 mg of nanoSnO_2-MC7 was added. The reaction mixture was magnetically stirred at 200 r·min^{-1} for 5 h at the reaction temperature. To shift the equilibrium toward the synthetic reaction, the water separated during the process was removed using a flow of nitrogen. Samples of 20 mg were periodically withdrawn from the reaction mixture, purified and analyzed. In the experiments in which the effect of the media was determined, 100 μL of ionic liquid (or 5 mg of PEG 1500) was added.

To assess the multiple usages of the biocatalysts, the immobilized lipase was separated from the reaction mixture after each cycle, washed with 0.5 mL of hexane and added to a fresh mixture of substrates.

Isolation and analysis of stearyl stearate

Stearyl stearate was isolated by solid phase extraction on silica gel G 60 eluted with hexane/diethyl ether/acetic acid (20:5:0.1 by vol). Samples were analyzed by GC equipped with an HP-1 MS column (30 m × 0.25 mm; 0.25 μm) and a flame ionization detector. The column was maintained initially at 165 ºC for 10 min and then the temperature was raised to 310 ºC at 5 ºC·min^{-1}. The temperature of the injector was 260 ºC and that of the detector was 320 ºC. As an internal standardm, n-octyl octanoate was used. The retention time for stearyl stearate was 37.5 min.

Statistical analysis

All experiments were done in triplicate: the value reported refers to means. Standard deviation was determined using Microsoft Excel.

Results and Discussion

Immobilization of *B. stearothermophilus* MC7 lipase

The thermostable lipase from *Bacillus stearothermophilus* MC7 was physically adsorbed on the nanostructured tin dioxide. As proposed by other researchers, upon immobilization, hydrophilic and hydrophobic interactions between the enzyme and the carrier, both negatively charged, are more likely to occur than electrostatic ones (3). We estimated that for nanoSnO$_2$, the optimal protein loading was 15.3 mg·g^{-1} carrier (**Fig. 1**). A further increase of the amount of loaded enzyme led to a decrease in the activity of the nanoSnO$_2$-MC7 preparations. We could ascribe this to multilayer deposition or aggregation of lipase molecules, which hinders the access of the substrate to the active site of the enzyme.

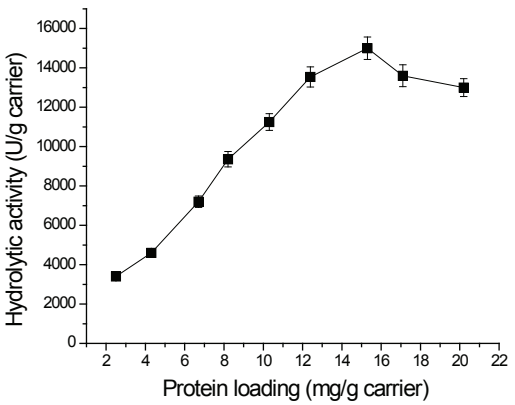

Fig. 1. Effect of the amount of loaded protein on the activity of nanoSnO$_2$-MC7.

Many organic and inorganic carriers have proved to be suitable supports for various lipases. For example, the adsorption of lipase from *Humicula lanuginosa* on polypropylene exceeds 120 mg·g^{-1}, while those for lipases from *Rhizopus oryzae* and *Candida rugosa* on modified silica are 80 mg·g^{-1} and 343.6 mg·g^{-1}, respectively (6, 32, 37). Polylactide nanoparticles reveal a remarkable adsorption capacity in respect to the lipase from *Candida rugosa*, 590 mg·g^{-1} (27).

In contrast, within the lipases isolated from *Bacillus* strains, a typical amount of the immobilized protein is 0.2 mg·g^{-1} to 2.7 mg·g^{-1} (19, 29). With the studied carrier (nanoSnO$_2$), we obtained adsorption for lipase MC7 comparable with that of polypropylene and polyurethane (11) which is five-fold higher than that for other *Bacillus* lipases.

Thermal stability of nanoSnO$_2$-MC7

Heat-stable lipases are indispensible catalysts in the reactions of hydrolysis or modifications of solid fats with a high melting point. In order to assess only the effect of nanoSnO$_2$ on lipase MC7 thermal stability, the assay was conducted in absence of compounds which are known to stabilize the enzyme, i.e. substrates, glycerol, polyethylene glycol (14, 21). As expected, the immobilization of lipase MC7 on tin dioxide improved the thermal stability of lipase MC7 (**Fig. 2**). We observed a sharp decline in the activity of free lipase MC7 at temperatures above 55 ºC. At the same time, nanoSnO$_2$-MC7 was fully active even at 60 °C. Lipase MC7 immobilized on tin dioxide retained at least 60 % of its activity after being incubated at 75 °C for 60 min. Probably, due to the adsorption of lipase MC7 on nanoSnO$_2$, the protein flexibility is reduced and the rate of the irreversible unfolding due to heating is decreased.

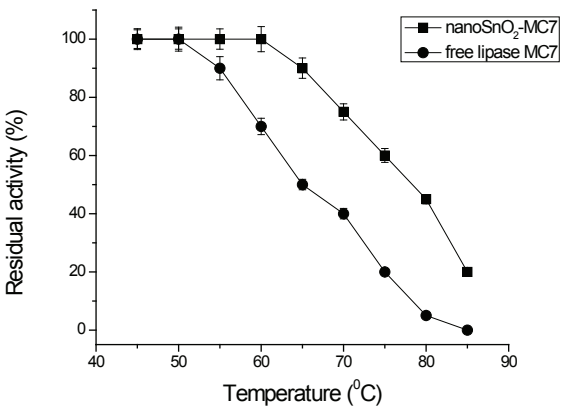

Fig. 2. Effect of immobilization on thermal stability of lipase MC7.

Stability of nanoSnO$_2$-MC7 in organic solvents

The high stability of enzymes in various organic solvents increases the possibility of their industrial application. Hydrophobic solvents are good solvents for lipase natural substrates (fats and oils) and are used for partitioning of reagents and products in different phases. In many lipase-catalyzed reactions, alcohols are either reagent (esterification reactions) or product (hydrolysis reactions). Many authors have shown that water miscible solvents have a detrimental effect on lipases. A structural feature of protein molecules is the presence of a layer of water molecules tightly bound to the enzyme surface whose role is to support its active conformation. Polar solvents tend to extract this thin water layer and distort

the enzyme conformation, resulting in lower activities. The results from stability studies of lipase MC7, free and adsorbed on tin dioxide, with a large number of water miscible and water-immiscible solvents are presented in **Fig. 3**. NanoSnO$_2$-MC7 samples exhibited a remarkable stability in 20 % to 50 % v/v conc. of alcohols, tetrahydrofuran and ethyl acetate. The enhanced stability of the immobilized nanoSnO$_2$ lipase MC7 in the presence of polar solvents can be attributed to additional stabilization of the native protein conformation. Probably, the carrier preserves the enzyme microenvironment and thus prevents the loss of the of structural water layer. The exposure to *i*-octane and *n*-hexadecane even had a stimulatory effect on the hydrolytic activity of nanoSnO$_2$-MC7. Hydrophobic solvents usually prevent enzyme molecules from aggregation or may induce structural changes in lipases, which results in enhancement of activity. They are also able to modify the water–oil interface or increase the substrate solubility, i.e. substrate concentration. We observed an enhanced stability of the nanoSnO$_2$-MC7 in comparison to the free enzyme in all studied solvents, which can expand its application.

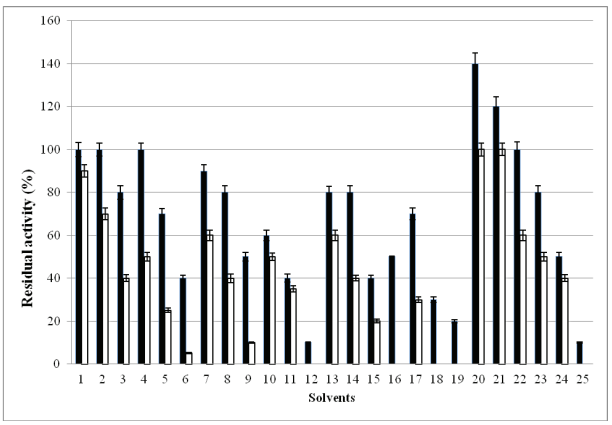

Fig. 3. Stability of nanoSnO$_2$-MC7 in organic solvents. NanoSnO$_2$-MC7 (■); free lipase MC7 (□). Solvents: **1:** methanol (20 % v/v); **2:** methanol (50 % v/v); **3:** methanol (100 %); **4:** ethanol (20 % v/v); **5:** ethanol (50 % v/v); **6:** ethanol (100 %); **7:** propanol (20 % v/v); **8:** propanol (50 % v/v); **9:** propanol (100 %); **10:** acetone (20 % v/v); **11:** acetone (50 % v/v); **12:** acetone (100 %); **13:** tetrahydrofuran (20 % v/v); **14:** tetrahydrofuran (50 % v/v); **15:** tetrahydrofuran (100 %); **16:** toluene; **17:** ethyl acetate; **18:** methylene chloride; **19:** diethyl ether; **20:** *i*-octane; **21:** *n*-hexadecane; **22:** hexane; **23:** dimethyl sulfoxide (20 % v/v); **24:** dimethyl sulfoxide (50 % v/v); **25:** dimethyl sulfoxide (100 %). [a]The activity of the non-treated counterpart is taken to be 100 %. Residual activity (%) was calculated in respect to the activity of the non-treated enzymes.

Synthetic activity of nanoSnO$_2$-MC7 in various reaction media

The optimal temperature for stearyl stearate synthesis catalyzed by nanoSnO$_2$-MC7 was 65 ºC (**Fig. 4**). In solvent-free medium, we obtained 75 % yield of the target wax-ester for 5 h. We found that lipase from *B. stearothermophilus* MC7 carried out the synthesis of long-chain esters twice more effectively than immobilized lipases from *C. antarctica*, *R. niveurs* and *A. niger* (7). The synthetic activity of nanoSnO$_2$-MC7 was comparable to those described for immobilized lipases from *R. miehei* and *C. rugosa* (12, 34). The further increase of the reaction temperature with 5 ºC led to reduction in the yield by half when using nanoSnO$_2$-MC7 as a catalyst. The immobilization of nanostructured tin dioxide partially protected lipase MC7 from thermal denaturation. The biocatalyst preserved up to 50 % of its synthetic activity after 5 h heating at 70 ºC in a solvent-free system only in the presence of the substrates.

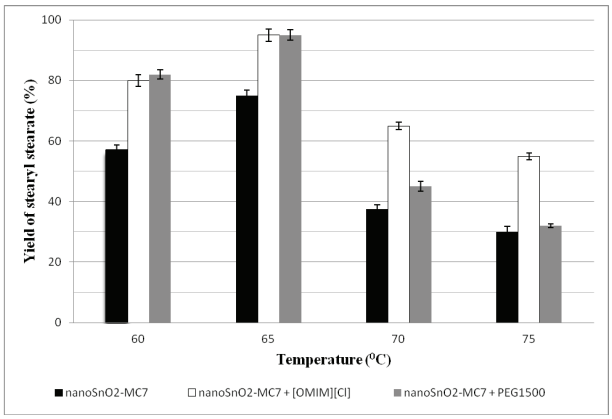

Fig. 4. Effect of the reaction temperature and additives on stearyl stearate synthesis catalyzed by nanoSnO$_2$-MC7.

Organic solvents with various hydrophobicity (logP from 1.3 to 8.8) have been tested as reaction media for stearyl stearate synthesis (24). In a water-restricted microenvironment, nanoSnO$_2$-MC7 exhibited remarkable stability and activity in solvents with logP higher than 3.5 (**Table 1**). *N*-heptane and *i*-octane accelerated the stearyl stearate synthesis. The same enzyme activation in nonpolar solvents has been previously observed for lipase-MC7-catalyzed acidolysis of tripalmitin with oleic acid (13).

TABLE 1

Effect of the solvent on the catalytic activity of nanoSnO$_2$-MC7 in the esterification of stearic acid with stearyl alcohol

Solvent	logP [24]	Yield of stearyl stearate (%)
No solvent		75.0±2.6
n-Hexadecane	8.8	67.4±2.7
n-Dodecane	6.6	70.2±2.8
n-Decane	5.5	74.8±3.1
i-Octane	4.5	86.2±3.4
n-Heptane	4.0	84.3±3.3
n-Hexane	3.5	70.5±2.9
Toluene	2.5	40.0±1.6
Tetrahydrofuran	0.5	10.0±0.4
Dimethyl sulfoxide	-1.3	2.1±0.1

In recent years, ionic liquids have been extensively studied and used as green solvents in organic synthesis. They are not volatile and show good chemical and thermal stability (47). Imidazolium salts are the most frequently applied ionic liquids as media in lipase-catalyzed reactions. In many cases, it is observed that the imidazolium hexafluorophosphates,

tetraborates, bis[(trifluoromethyl)sulfonyl]amides or trifluoromethylsulfonates improve the performance of the immobilized biocatalysts (28, 31). There are several papers on increased enantioselectivity and activity of lipases from *C. rugosa*, *C. antarctica* and *Ps. cepacia* in hydrophobic ionic liquids (20, 41, 43, 44).

The effect of the ionic liquids containing chloride anion on the lipase activity has not been extensively studied yet. We tested the effect of 3-methyl-1-octyl imidazolium chloride [OMIM][Cl] and methyl trioctyl ammonium chloride [TOMA][Cl] on *B. stearothermophilus* MC7 lipase activity. As expected, the imidazolium derivative had a beneficial effect on the nanoSnO_2-MC7 activity (**Fig. 4**). The reaction proceeded at a higher rate and almost a complete conversion of substrates was achieved for 5 h at 65 °C. This made the performance of the biocatalyst 1.3-fold more effective than that in the solvent-free system. The effect of the medium in this case was comparable to the effect of the activation of nanoSnO_2-MC7 by the non-ionic surfactant PEG1500. Both stearic acid and stearic alcohol were easily soluble in [OMIM][Cl] at the reaction temperature, which ensures better homogenization and facilitates mass transfer. This ionic liquid (E_T^N = 0.549), similarly to the other imidazolium salts, is found in the polarity range of dipolar hydrogen-bond donor solvents such as alcohols (33). As seen in **Fig. 4**, [OMIM][Cl] had a beneficial effect on MC7 thermal stability. In its presence, the enzyme preserved about 60 % of its initial activity after 5 h heating at 75 °C.

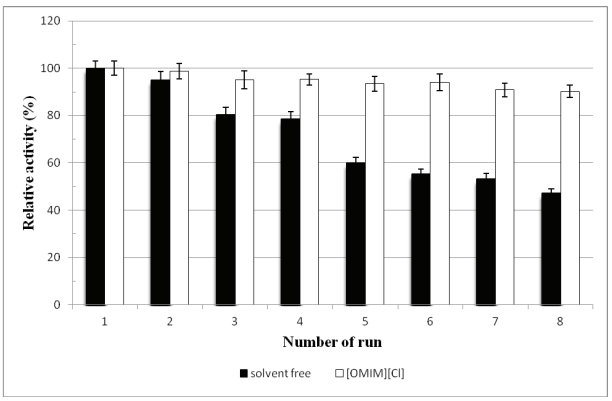

Fig. 5. Multiple use of nanoSnO_2-MC7 in stearyl stearate synthesis.

There are insufficient data on the lipase behavior in tetraalkylammonium salts and their effect on the efficiency of the biocatalysts varies within the enzyme species as well as the type of the reaction. The most studied cationic surfactant is cetyltrimetyl ammonium bromide (CTAB). It is applied in enzyme purification and preparation of revised micellar systems which can be hosts for lipases. The opposite effect of trialcylamonium liquids on different lipases can be illustrated on the example of CTAB. Low concentrations of CTAB increased *C. rugosa* lipase synthetic activity by 2.5-fold compared to the silica immobilized enzyme (42). It also recovered the activity of *T. lanuginosus* lipase fully inactivated by saturated solutions of guanidine (35). In contrast, this surfactant strongly inhibited lipases from *R. miehei* and *B. cereus* C7 (5, 36). This should imply that the results on the effect of ionic liquids obtained with one enzyme cannot be assigned directly to another one.

In our study we found that [TOMA][Cl] completely inactivated nanoSnO_2-MC7 lipase when added to the reaction mixture. Its normalized solvent polarity (E_T^N) is 0.414, which is in the range of dipolar non-hydrogen-bond donor solvents, such as dimethylformamide (DMF) and dimethyl sulfoxide (33). The result is in agreement with the very poor synthetic activity of nanoSnO_2-MC7 determined in dimethyl sulfoxide (below 2 % yield of stearyl stearate).

Multiple use of nanoSnO_2-MC7

The ability of reuse in several consequent cycles is a major advantage of immobilized enzymes over their free counterparts. The immobilized lipase MC7 was easily separated from the reaction media containing imidazolium chloride due to its lower viscosity. As expected, it ensured a higher operational stability of the biocatalyst (**Fig. 5**). Our results showed that nanoSnO_2-MC7 applied in the synthesis of stearyl stearate in a solvent-free system kept 47 % of its activity at the eighth run, while in the presence of [OMIM][Cl] it retained 90 % activity.

Conclusions

The lipase from *Bacillus stearothermophilus* MC7 was stabilized via physical adsorbtion on nanosized tin dioxide. The biocatalyst exhibited excellent synthetic activity and operational stability in the green solvent [OMIM][Cl]. Such ILs can partially unfold the tight polysaccharide molecule of the cellulose and thus make it accessible to the enzyme. This implies new potential application of nanoSnO_2-MC7 such as synthesis of cellulose esters, which are valuable for chemical industry functional biopolymers. On the other hand, the stability of nanoSnO_2-MC7 in short-chain alcohols suggests its applicability in biodiesel production as well.

Acknowledgements

The study was supported by the National Science Fund of Bulgaria (project DMU 02/20) and Ministry of Education, Youth and Science of Bulgaria (Project "Science and business" BG051PO001-3.3.05-0001).

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