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Functionalized Stober silica as a support in immobilization process of lipase from *Candida Rugosa*

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Abstract: Lipase from *Candida rugosa* was immobilized onto modified Stober silica. Modification was made with 3-(2,3-epoxypropoxy)propyltrimethoxysilane and glutaraldehyde. The immobilization process by covalent binding was performed for 1 and 24 h using different concentrations of enzyme solution. The obtained immobilized biocatalysts were subjected to physicochemical characteristics. The characteristics of functional groups (FTIR, ^{13}C CP MAS NMR), thermal stability (TG) and parameters of the porous structure (low temperature N_2 sorption) were determined. An elemental analysis was performed to determine the content of nitrogen, carbon and hydrogen. Using a Bradford method the immobilization yield (IY) and amount (P) of lipase loaded onto support were calculated. The obtained systems were also tested to evaluate their catalytic activity in hydrolysis reaction of *p*-nitrophenyl palmitate (*p*-NPP) to *p*-nitrophenol (*p*-NP). The results confirmed the effectiveness of immobilization process and high hydrolytic activity (2270 U/g) of immobilized biocatalysts.

Keywords: *Candida rugosa, functionalized Stober silica, immobilization process, hydrolytic activity*

Introduction

In recent years silica is very popular due to its specific properties, that is low density, large surface area, low coefficients of thermal expansion, mechanical and thermal stability and surface permeability. It can be obtained using numerous methods among which precipitation (Krysztafkiewicz et al. 1997; Wang et al., 1997; Jesionowski et al., 2002), emulsion route (Jesionowski et al., 2008b; 2010a; Esquena et al., 2001), sol-gel method (Stober et al., 1968; Ciriminna et al., 2013; De Bardi et al., 2014), hydrothermal (Melendez-Ortiz et al., 2013; Yu et al., 2015) as well as sonochemical process (Vetrivel et al., 2014; Hassan et al., 2010) can be mentioned. Colloidal silica particles with uniform size and shape have widespread applications as sintering aid of glass and ceramic materials, pharmaceutical binders, optical filters, chromatographic agents, sensing elements, catalysts, chemical and mechanical polishing materials etc. Due to its interesting properties and excellent chemical stability, nano-sized silica has

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variety of applications including photonics and biomedicines (Nandy et al., 2014). Because of the homogeneous morphology, well-developed surface area and presence of the reactive groups on the surface of SiO_2, it can be also used as a carrier in an immobilization process (Nguyen et al., 2014; Jesionowski et al., 2014, Zdarta et al., 2015).

Immobilization is defined as a group of methods which reduce either full or partial ability to move of individual atoms, molecules, whole substance or a biological material (for example enzymes), by using a solid support or bounding them inside a specific structure. The immobilization process generally causes an improvement of the properties of enzymes, although in some cases an impoverishment is observed upon immobilization (caused by distortion of the enzyme due to interaction with the support) (Netto et al., 2013). These alterations in enzyme properties are sometimes associated with changes in the enzyme structure. Immobilization of enzymes on different supports can alter their performance in many interesting processes, such as selective oxidation or hydrolysis, kinetic separation of racemic mixtures or kinetically controlled synthesis (Zdarta et al., 2015). To understand what is exactly happening with the enzyme, some facts should be analyzed. Firstly, after immobilization, the enzyme is completely suspended on the bearing surface what prevents either aggregation or other phenomena inactivation. In addition, multipoint covalent immobilization can cause a more rigid structure, less sensitive to conformational changes, whereby the enzyme activity under drastic conditions, may be higher than that of the free enzyme (Rodrigues et al., 2013).

Lipases (triacylglycerol ester hydrolases according to the International Union of Biochemistry, EC. 3.1.1.3) are one of the most popular enzymes with an important physiological significance and considerable industrial potential (Li et al., 2014). The main application of lipases is catalysis of the hydrolysis reaction of triacetin toward acetic acid and glycerol (Abd-Elhakeem et al., 2013). In addition, soluble enzymes are used as catalysts in the hydrolysis of insoluble compounds to more polar lipolysis products (Whitaker et al., 2003). The lipases show little ability to catalyze the hydrolysis of water soluble compounds. These reactions are very slow in the case of short-chain carboxylic acid esters (Netto et al., 2013). Lipases can be of plant, animal and microbial origin. However, only microbial lipases are of high interest due to their commercially significance (Zucca and Sanjust, 2014, Zdarta et al., 2016).

Modification of silica surface performed using different functional groups improves its ability towards enzymes immobilization. Amine ($–NH_2$), thiol (–SH), phenyl ($–C_6H_5$), vinyl (=C=C=), nitrile (=C=N–) and epoxide ($=CH–O–CH_2–$) groups are the most reactive and can be introduced onto SiO_2 surface. The presence of these groups enables immobilization via covalent binding (Chong and Zhao, 2004; Gaffney et al., 2012). Several literature reports have shown that carriers used in immobilization have been usually modified with either polymers or silane compounds, in most cases with amine groups in their structure (Jung et al., 2010; Jesionowski et al., 2014; Khoobi et al., 2014; Banjanc et al., 2016). The thiol groups (–SH) also exhibit strong

interactions with amine groups present in the enzyme structure. They are mostly arising from silanol compounds such as eg. 3-mercaptopropyltrimethoxsysilane (Yiu et al., 2001; Jang et al., 2006). An epoxy group (=CH–O–CH_2–) has also an equally strong interactions with the enzymes (Bernal et al., 2012; Adlercreutz, 2013; Faure et al., 2014; Yuce-Dursun et al., 2016). The Eupergit, acrylate-based material with epoxy groups, is often used as a carrier in the immobilization process. This support is very desirable for the industrial scale enzyme immobilization because it is commercially available worldwide, resistant to mechanical and chemical stresses and adaptable to a variety of configurations and specific processes carried out in reactors. Eupergit has been successfully used for lipase immobilization by several authors and the resulting biocatalyst has been found to have better stability than catalysts obtained applying other supports (Katchalski-Katazir and Kraemer, 2000; Knezevic et al., 2006).

In this paper the immobilization of lipase from *Candida rugosa* onto Stober silica (obtained via a modified sol-gel method) is presented. Hydroxyl groups present on the silica surface enabled the covalent attachment of 3-(2,3-epoxypropoxy)propyl-trimethoxysilane. In addition the surface of modified silica was crosslinked with glutaraldehyde. Besides the routine study (the immobilization yield IY, amount P of lipase and hydrolytic activity of immobilized lipase), spectroscopic (FTIR and ^{13}C CP MAS NMR), physicochemical and structural properties (TG/DTG, BET and elemental analysis) were also presented in this manuscript to confirm the effectiveness of immobilization process.

Experimental

Materials

Tetraethyl orthosilicate (TEOS), ethanol (EtOH) and 25% aqueous ammonia solution were purchased from Chempur (Gliwice, Poland). Glutaraldehyde (GA), 3-(2,3-epoxypropoxy)propyltrimethoxysilane (EPOTS), 10 mM sodium phosphate pH=7 (PBS), lipase from *Candida rugosa* (Lipase, CRL), *p*-nitrophenyl palmitate (*p*-NPP), *p*-nitrophenol (*p*-NP), 2-propanol, Triton X-100 and Arabic gum were purchased from Sigma Aldrich (Saint Louis, MO, USA).

Synthesis of Stober silica support and immobilization process

Stober silica was obtained using the modified sol-gel method, according to procedure described by Klapiszewski et al. (2014). The modifying compound (EPTOS) was introduced as a mixture consisting of ethanol and water (4:1 v/v). The modification process was carried out via the in situ method (during the synthesis of silica). In addition the surface of modified silica was crosslinked by glutaraldehyde solution (5%). Two kinds of supports were used in the immobilization process: silica modified with epoxy groups (SiO_2_EPTOS) and silica modified with epoxy groups additionally crosslinked with glutaraldehyde (SiO_2_EPTOS_GA).

The process of immobilization of lipase from *Candida rugosa* on prepared supports consisted with placing 500 mg of the support in a conical flask and adding a solution of the enzyme at different concentrations (1, 3 and 5 mg/cm^3), in a phosphate buffer at pH=7. The mixture was shaken using a shaking device for a specified time (1 and 24 h) at ambient temperature. The samples were then filtered under reduced pressure and dried at room temperature for 48 h. All the protein concentrations were determined via the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as a standard. The immobilization yield (IY) and amount (P) of lipase loaded onto the support were calculated using the equations:

$$IY = \frac{C_0}{C_1} \cdot 100\% \tag{1}$$

$$P = \frac{(C_0 - C_1) \cdot V}{m} \tag{2}$$

where C_0 and C_1 denote concentrations of enzyme (mg/cm^3) in the solution before and after adsorption, respectively, V is the volume of solution (cm^3), and m is the mass of the silica support (g).

Physicochemical evaluation

In order to identify the characteristic groups present on the surface of obtained products, the samples were subjected to the FTIR analysis using a Vertex 70 spectrophotometer (Bruker, Germany). The samples were studied in the form of KBr tablets, as KBr crystals are inactive in the IR range. The analysis was performed over a wavenumber range of 4000–450 cm^{-1}.

^{13}C CP MAS NMR measurement was carried out on a DSX spectrometer (Bruker, Germany). For determination of NMR spectra, a sample of approximately 100 mg was placed in a ZrO_2 rotator 4 mm in diameter, which enabled spinning of the sample. Centrifugation at the magic angle was performed at a spinning frequency of 8 kHz. The ^{13}C CP MAS NMR spectra were recorded at 100.63 MHz in a standard 4 mm MAS probe using single pulse excitation with high power proton decoupling (pulse repetition 10 s, spinning speed 8 kHz).

A thermogravimetric analyzer (TG, model Jupiter STA 449F3, made by Netzsch, Germany) was used to investigate the thermal decomposition behavior of the samples. Measurements were carried out under flowing nitrogen at a heating rate of 10 °C/min and a temperature range of 25–1000 °C with an initial sample weight of approximately 10 mg.

Additionally a low temperature N_2 sorption was performed. The surface area (A_{BET}), total pore volume (V_p) and mean pore diameter (S_p) were determined using an ASAP 2020 instrument (Micromeritics Instrument Co., USA). All samples were degassed at 120 °C for 4 h prior to measurement. The surface area was determined by

the multipoint BET (Brunauer-Emmett-Teller) method using the adsorption data as a function of relative pressure (p/p_0). The BJH (Barrett-Joyner-Halenda) algorithm was applied to determine the total pore volume and average pore diameter.

The elemental composition of the materials was established with the use of Vario El Cube instrument made by Elementar Anlaysensysteme GmbH (Germany) which gave the elemental content of carbon, nitrogen and hydrogen after high temperature combustion of the analyzed samples.

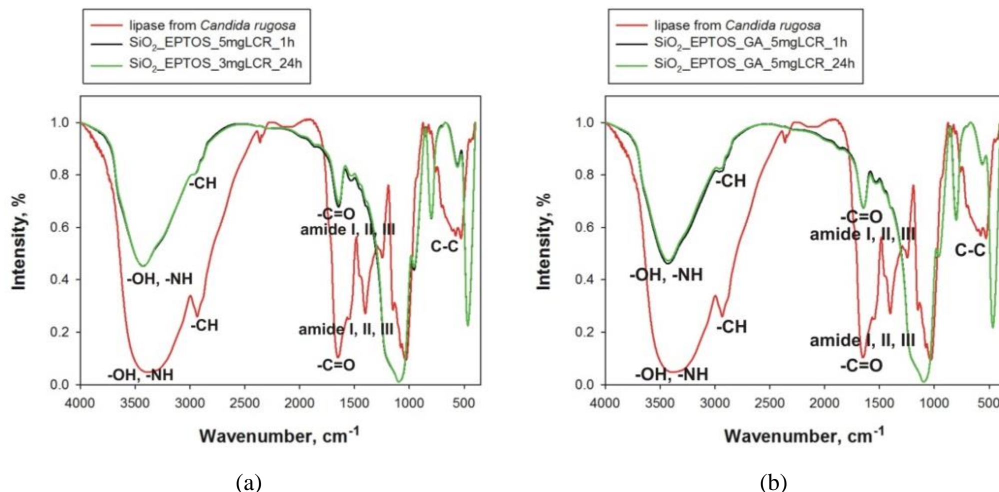
Enzyme assay

The hydrolytic activity of the immobilized enzymes was estimated according to methodology described by Matte et al. (2014). Briefly, the spectrophotometric measurements were based on the ability of enzymes to transform the *p*-NPP (*p*-nitrophenyl palmitate) into *p*-NP (*p*-nitrophenol). The release of the product was observed at 410 nm (using a JASCO 650 spectrophotometer, Japan). The enzymatic activity was measured in quartz cuvettes containing 3 to 5 mg of support with the immobilized lipase and 2.7 cm^3 of working solution. All reactions (performed in triplicate) were carried out with stirring (1000 rpm), at 30 °C and within 2 minutes. The activities of the immobilized lipase were calculated using *p*-NP standard calibration curve. One U/g of enzyme activity was defined as the release of 1 µmol of *p*-NP per one minute per 1 g of catalyst, under the measurement conditions.

Result and discussion

Spectroscopic analysis

Interpreting the FTIR spectra (Fig. 1) it was found that the most characteristic band for lipase, derived from stretching vibration of –CH group, occurred at a wavenumber of 2900 cm^{-1}. Moreover, strong and broad band derived from stretching vibration of –OH bond between 3200-3600 cm^{-1} masked the band corresponding to stretching vibration of –NH group that occurred in the same wavenumber range. The spectrum of the enzyme also contained signals at 1700 cm^{-1}, which corresponded to stretching vibrations of –C=O, as well as a wide band with a maximum at 1550 cm^{-1} assigned to bending vibrations of –NH groups (amide II). In addition, the signal arising from C–OH bending vibrations was visible at 1440 cm^{-1}. Below 1000 cm^{-1} there were several signals assigned to stretching vibrations of C–C bonds. The bands characteristic for enzyme were also noted in the spectra of obtained biocatalysts. The effective immobilization of lipase was confirmed with the band generated by –C–H symmetric stretching vibrations of CH_2 and CH_3 groups occurring at wavenumber 2900 cm^{-1} and signals at 1641, 1538, and 1256 cm^{-1} that were linked to the presence of bonds attributed to amide I-III, respectively (Wang et al., 1991; Vejayakumaran et al., 2008; Zdarta et al., 2015).



(a) (b)

Fig. 1. FTIR spectra of lipase and lipase immobilized onto (a) SiO_2_EPTOS and (b) SiO_2_EPTOS_GA supports

Thermal stability

The thermogravimetric analysis was performed in order to confirm the effectiveness of immobilization and thermal stability of obtained products. The thermogravimetric curve of lipase (Fig. 2) confirms its low thermal stability. The first mass loss of about 15% occurred at temperature range of 30-150 °C (accompanied by an exothermic peak at about 90 °C in DTG curve). This effect was related to desorption of physically bound water. The next mass loss appeared at 200-500 °C (accompanied by an exothermic peak at 320 °C) resulted from loss of the crystallization water. A smaller mass loss of about 5% taking place above 800 °C corresponded to the exothermic peak at 950 °C. It was probably caused by enzyme decomposition and CO_2 and H_2O formation (Hou et al., 2013; Motevalizadeh et al., 2013; Poppe et al., 2013). Thermogravimetric curves of samples after immobilization (Fig. 2) almost overlapped each other, which may indicate a similar thermal stability (similar amount of immobilized lipase). This was in agreement with data obtained applying the Bradford method. In case of epoxy SiO_2-based biocatalyst, the first mass loss of about 6% occurred even up to 180 °C (irrespective of the immobilization time). A total mass loss (ca. 30%) in obtained biocatalysts was smaller as compared to native enzyme. Similar changes were noted in the thermogravimetric curve of SiO_2_EPTOS_GA. Those data strictly confirmed an increase in thermal stability of immobilized enzyme.

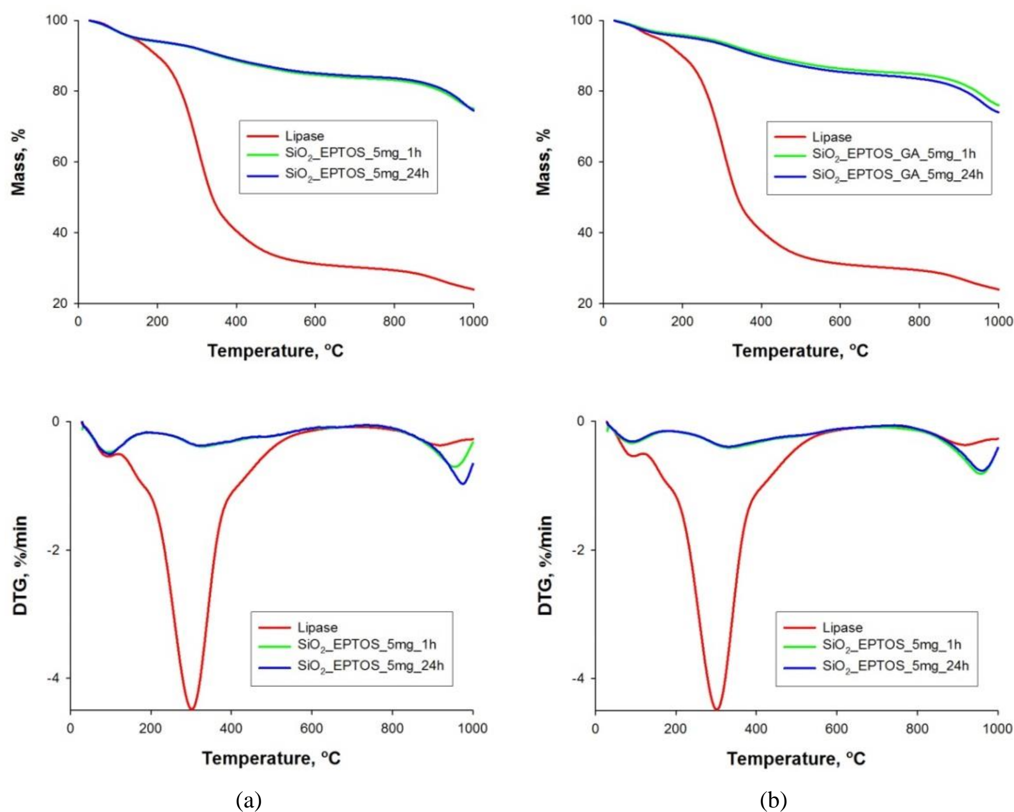


Fig. 2. TG/DTG curves of lipase and lipase immobilized onto (a) SiO_2_EPTOS and (b) SiO_2_EPTOS_GA supports

^{13}C CP MAS NMR spectroscopy

^{13}C CP MAS NMR spectra of lipase before and after immobilization onto functionalized silica support are presented in Fig. 3. The efficiency of immobilization was confirmed by the typical signals in the ^{13}C CP MAS NMR spectra of the obtained biocatalytic system. Two small peaks observed at 49 and 52 ppm (C6 and C5) and attributed to carbon atoms of the epoxy ring were noted. This indicates that most of the reactive organic groups of the epoxy ring retained under the reaction conditions (Kao et al., 2005). Another peaks at 0–20 ppm and 80 ppm (C1, C2 and C3-C4) were generated by carbon atoms in methyl groups (Veyayakumaran et al., 2008). Characteristic signal at chemical shift of 170 ppm, corresponding to the carbonyl bonds (Horchani et al., 2012) was observed in the spectrum of lipase. The same signal was also present in the spectrum of the sample after immobilization, which indirectly confirms the effectiveness of immobilization process.

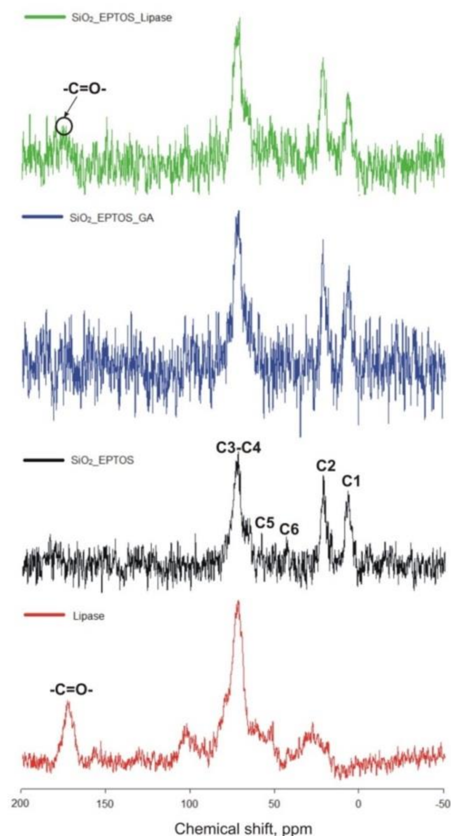


Fig. 3. ^{13}C CP MAS NMR spectra of free lipase, supports and obtained biocatalyst

Characteristics of porous structure of obtained biocatalyst

Nitrogen adsorption/desorption isotherms and pore size distributions of the supports, used in the immobilization process, are shown in Fig. 4 and Table 1. The isotherms were classified as type IV with the hysteresis loop type H3, which pointed to the mesoporous structure of modified silica. Thanks to the well-developed porous structure, modified silica can be classified as a potential support in the immobilization process.

Table 1. Parameters of the porous structure of obtained materials

Name of sample	Surface area, m^2/g	Total pore volume, cm^3/g	Mean pore diameter, nm
SiO_2_EPTOS	77.9	0.24	12.5
SiO_2_EPTOS_5mg_24h	34.6	0.03	3.0
SiO_2_EPTOS_GA	73.1	0.41	22.3
SiO_2_EPTOS_GA_5mg_24h	45.0	0.04	3.0

The modified supports were characterized with the relatively high surface area of about 77 m^2/g (SiO_2_EPTOS) and 73 m^2/g (SiO_2_EPTOS_GA) and large pores (12.5 nm and 22.3 nm, respectively). Immobilization of lipase onto modified silica caused a decrease in all parameters of the porous structure. This indicates that lipase probably filled the pores channels and blocked them (Vinu et al., 2004; Yu et al., 2012; Wang et al., 2014; Zhang et al., 2005). Such a significant change in the samples porosity proves the effectiveness of immobilization process.

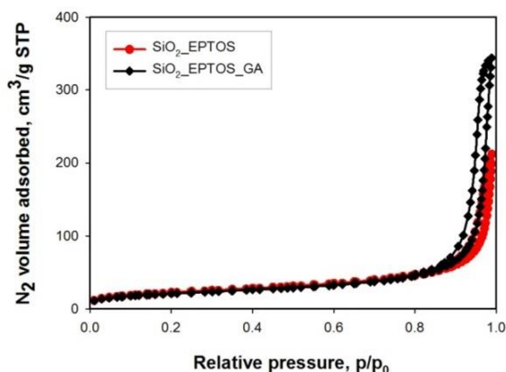


Fig. 4. Nitrogen adsorption/desorption isotherms of analyzed materials

Table 2. Elemental composition of the supports and obtained biocatalysts

Name of sample	Content, %		
	N	C	H
SiO_2_EPTOS	0.48	3.17	1.72
SiO_2_EPTOS_1mg_1h	0.53	2.33	1.62
SiO_2_EPTOS_5mg_1h	0.80	3.81	1.79
SiO_2_EPTOS_1mg_24h	0.51	2.72	1.61
SiO_2_EPTOS_5mg_24h	0.73	3.90	1.73
SiO_2_EPTOS_GA	0.32	2.31	1.32
SiO_2_EPTOS_GA_1mg_1h	0.50	2.89	1.39
SiO_2_EPTOS_GA_5mg_1h	0.79	4.13	1.53
SiO_2_EPTOS_GA_1mg_24h	0.49	2.81	1.38
SiO_2_EPTOS_GA_5mg_24h	0.77	4.86	1.60

Elemental analysis

Table 2 shows an elemental composition of the biocatalysts. Based on the result of elemental analysis it was found that the immobilization process was performed with relative high effectiveness (the amount of nitrogen, carbon and hydrogen in the samples after immobilization increased as compared to composition of initial support). The immobilization time and type of support used (similarly to immobilization yield

and the amount of enzyme loaded onto support) did not have a significant influence on a content of analyzed elements.

Performance of the immobilization process and hydrolytic activity

After a series of spectrophotometric measurements, the concentration of enzyme left in the solution after immobilization was read off the plot presenting absorbance vs. enzyme concentration dependence. Based on the known amount of support, volume and concentration of the initial enzyme solution, it was possible to calculate the amount of enzyme immobilized on the modified silica supports. The results are given in Table 3.

Table 3. The immobilization yield (IY) and the amount (P) of lipase from *Candida rugosa* loaded in support

Name of sample	IY, %	P, $mg_{enzyme}/g_{support}$
SiO_2_EPOTS_1mg_1h	64.8	64.8
SiO_2_EPOTS_3mg_1h	84.4	253.1
SiO_2_EPOTS_5mg_1h	89.8	449.2
SiO_2_EPOTS_1mg_24h	66.4	66.4
SiO_2_EPOTS_3mg_24h	86.3	258.9
SiO_2_EPOTS_5mg_24h	91.4	457.4
SiO_2_EPOTS_GA_1mg_1h	63.6	63.6
SiO_2_EPOTS_GA_3mg_1h	81.6	244.9
SiO_2_EPOTS_GA_5mg_1h	80.9	404.6
SiO_2_EPOTS_GA_1m_24h	66.0	66.0
SiO_2_EPOTS_GA_3mg_24h	85.0	255.1
SiO_2_EPOTS_GA_5mg_24h	89.9	449.6

Data presented in Table 3 prove that increasing concentration of enzyme caused an increase in the amount of enzyme (mg) adsorbed per 1 g of supports. Similar results were obtained during determination of the immobilization yield (IY). The highest immobilization yield, and thus the amount of adsorbed enzyme, were achieved applying its highest concentration. Interpreting the results it can be concluded that the time of immobilization had no significant impact on the efficiency of immobilization (only slight increase in amount of adsorbed enzyme was noted).

The hydrolytic activity tests were carried out in order to determine whether the immobilization process did not affect the enzymatic activity of biocatalyst. Enzymes immobilized onto silica-based supports were used in hydrolysis reaction of *p*-nitrophenyl palmitate to *p*-nitrophenol. Based on experimental data collected in Table 4 it was found that obtained biocatalysts were characterized with high hydrolytic activity. Lipase immobilized onto SiO_2_EPTOS support exhibited the highest hydrolytic activity (2270 U/g). It was furthermore noted that the enzymatic activity of

biocatalysts increased together with increasing concentration of enzyme used in immobilization process as well as immobilization time. The obtained activities proved the potential application of synthesized materials in different catalytic reactions.

Table 4. Hydrolytic activity

Name of sample	Hydrolytic activity, U/g
SiO_2_EPTOS_1mg_1h	1267
SiO_2_EPTOS_5mg_1h	1928
SiO_2_EPTOS_1mg_24h	1322
SiO_2_EPTOS_5mg_24h	2270
SiO_2_EPTOS_GA_1mg_1h	497
SiO_2_EPTOS_GA_5mg_1h	562
SiO_2_EPTOS_GA_1mg_24h	153
SiO_2_EPTOS_GA_5mg_24h	273

The same lipase immobilized on MSU-H (mesoporous silica with pore size of 13.3 nm) reported by Yu et al. (2015) in the amount of 48.2 mg $CRL/g_{support}$ gave the maximum hydrolytic activity of 3418 U/g. Similar results were obtained by Banjanc et al. (2016) who applied epoxy functionalized silica nanoparticles (GFNS) as a support in immobilization process of lipase. The immobilized enzyme had the highest activity of approximately 1495 U/g and was characterized with loading capacity of 294 mg/g. Zniszczol et al. (2016) immobilized lipase onto epoxy-functionalized mesoporous silica (f-SBA-15). The activity of immobilized samples was 101 U/g and the amount of enzyme per 1 g of support was estimated as 23 mg/g. It was found that biocatalysts obtained in presented research had quite similar activities (2270 U/g) and enzyme loadings (457 mg/g) as compared to the published so far materials.

Conclusions

The functionalized Stober silica was proved to be an effective support in immobilization of lipase from *Candida rugosa*. The immobilization process was performed with the efficiency of 90%, as evidenced by the results obtained applying the Bradford method. The spectroscopic analysis also confirmed the effectiveness of immobilization process. This was mainly evidenced by the presence of signals coming from the amide I-III bonds. The results of thermogravimetric analysis showed that the immobilized enzymes had better thermal stability as compared to native enzyme. The parameters of the porous structure significantly decreased after the immobilization process which resulted from enzyme loading onto the support surface as well in its pores. The effective immobilization of the enzyme was also confirmed by increasing content of N and C in the tested biocatalyst. All synthesized biocatalysts exhibited high catalytic activity and can work in different catalysis reactions. Based on the performed studies, it was found that the additional crosslinking of the modified silica

(performed with glutaraldehyde) did not increase the yield of immobilization process and enhanced the catalytic activity of enzyme. The obtained biocatalysts were characterized with high hydrolytic activity, even up to 2270 U/g.

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