New Organic – inorganic Hybrid Materials for Enzymes Immobilization

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The purpose of this study is the preparation and characterization of organic-inorganic hybrid materials used for enzyme immobilization on silica-based support by cross-linking method. The cross-linking agent used was glutaraldehyde and the enzyme used for immobilization was lipase. Organic-inorganic hybrid materials were obtained by the sol-gel process in the presence of different surfactant systems. A nanosized silica-based support was synthesized and characterized by dynamic light scattering and scanning microscopy analysis. The modifications in the chemical composition of the organic-inorganic hybrid materials before and after lipase immobilization were analyzed by infrared spectroscopy. The amount of immobilized lipase (Lowry method) and the enzymatic activity were determined.

Keywords: silica-based support, enzyme immobilization, lipase, enzymatic activity

Enzymes and other biologically active cells can be immobilized on a variety of the insoluble polymeric supports with retention of the biologic activity often with increase of their thermal and operational stability [1-3]. A broad variety of immobilization techniques have been applied to immobilize enzymes, including adsorption to solid supports, covalent attachment and polymer entrapment. The formation of covalent bonds provides a powerful link between the enzymes and its carrier support and therefore has been the most studied method [4].

Silica based hybrid supports obtained in the presence of surfactants have been successfully used for enzyme immobilization due to their good mechanical, chemical and thermal resistance [5]. Enzyme immobilization often requires support activation, the presence of coupling agents and special conditions of reaction. In case of silica based support, the activation step involves formation of free Si-OH groups which are responsible for linkage of enzymes through different coupling agents like glutaraldehyde [6].

Lipase (classified as triacylglycerol ester hydrolase, EC 3.1.1.3) is one of the body most important digestive enzymes; it splits fat, which is then utilized to nourish the skin cells, protect the body against bruises and blows, and ward off the entrance of infectious virus cells and allergic conditions, being reported to have antimicrobial properties. Lipase has proven to be a versatile and efficient biocatalyst used in a variety of reactions, such as: enantio- and regio-selective hydrolysis (esterification, transesterification and aminolysis) [7-12]. Lipase may be used in the synthesis of food additives and flavor esters (including ethyl butyrate - pineapple-bananalike flavor, isoamyl acetate, and isobutyl acetate).

The objective of this study is to immobilize lipase on a silica-based support using the cross-linking method. Glutaraldehyde was used as cross-linker. Organic-inorganic hybrid materials were obtained by the sol-gel process. The modifications in the chemical composition of the organic-inorganic hybrid materials before and after immobilization were analyzed by DLS, FT-IR and SEM measurements. The amount of immobilized lipase was determined by Lowry method and enzymatic activity by base titration.

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Experimental part

Equipment

The resulting silica-based support (denoted **S1** and **S2**) was investigated before and after lipase immobilization using the following techniques:

- fourier transform infrared spectroscopy (FTIR). Infrared spectroscopy (IR) measurements were performed on a Vertex 70 instrument (Brucker) with Fourier transformation (FTIR), equipped with ATR module based on diamond crystal. The spectra were recorded over the wavenumber range of 400–4000cm^{-1} with a resolution of 2 cm^{-1} for all materials;
- scanning electron microscopy (SEM). SEM images were recorded on a HITACHI S2600N instrument with an EDS probe. Before imaging, all samples were covered with a thin gold layer;
- X-ray diffraction analysis was performed using a Shimadzu XRD 6000 diffractometer at room temperature. In all the cases, Cu K_α radiation from a Cu X-ray tube (run at 15mA and 30 kV) was used. The samples were scanned in the Bragg angle, 2θ range of 10 – 80 degrees;
- the UV-Vis measurement were recorded at the room temperature on a Jasco V560 in diffuse reflectance technique;
- size distribution and polydispersity index measurement of the synthesised hybrid silica materials were evaluated by the dynamic light scattering (DLS) technique on a Zetasizer Nano ZS (Malvern Instruments Ltd., U.K.), at a scattering angle of 90 and 25°C. All samples were diluted with deionised water to an adequate scattering intensity prior to the measurement. For each sample, the hydrodynamic radius was measured in triplicate. The particle size analysis data were evaluated using intensity distribution.

Materials

3-(trimethoxysilyl)-propylamine (H_2N-$(CH_2)_3$–$Si(OCH_3)_3$), tetraethoxysilane (TEOS, $Si(OC_2H_5)_2$), glutaraldehyde (HOC-$(CH_2)_3$-COH), Triton X-100 ($C_{14}H_{22}O(C_2H_4O)_n$), Tween 80 (Polysorbate 80), D-glucose, lipase (from Aspergilius Niger, 184 U/g), were analytical

grade and used as received. Commercially available solvents: acetone and ethanol were used without further purification.

Synthesis of silica-based support, ***S1***

The surfactant mixture (Triton 100 X - 1 mL, 0.00165 moles and Tween 80 - 2 mL, 0.00165 moles) was solubilized into a mixture H_2O: EtOH and heated to 40^{0}C. Then TEOS (11mL, 0.05 moles) was added under continuous magnetic stirring and the *p*H was adjusted to 3 with mineral acid (HNO_3). The reaction mixture was then heated to 60-65^{0}C, for 2 h, followed by another hour consisting of magnetic stirring only. The resulting product was allowed to evaporate for 72 h in an oven, at 40^{0}C. A calcinations step followed at 600^{0}C, for 2 h (at a heating rate of 2 degrees/min), ensuring the complete surfactant removal. The final product (denoted support **S1**) was recovered.

Synthesis of silica-based support, ***S2***

The surfactant mixture D-glucose (0.2973 g, 0.00165 moles) and Tween 80 (2 mL, 0.00165 moles), was solubilized into a mixture H_2O: EtOH and heated to 40 ^{0}C. TEOS (11 mL, 0.05 moles) addition was performed under continuous magnetic stirring and the *p*H was adjusted to 3 with mineral acid (HNO_3). The reaction mixture was then heated to 60-65^{0}C, for 2 h, followed by another hour consisting of magnetic stirring only. The resulting product was allowed to evaporate for 72 h in an oven, at 40^{0}C. A calcinations step followed at 600 ^{0}C, for 2 h (at a heating rate of 2 degrees/min), ensuring the complete surfactant removal. The final product (denoted support **S2**) was recovered.

Lipase immobilization

Immobilization of lipase was performed as described in the literature [13]: 0.5 g of dry silica gel was mixed with 0.15g 3-(trimethoxysilyl)-propylamine (H_2N-$(CH_2)_3$–$Si(OCH_3)_3$) in 20 mL acetone and kept under constant mixing at 50^{o}C for 2 h. The treated silica-based supports were then filtered, washed with water and dried at 60 ^{o}C for 2 h.

The obtained silica-based supports (**S1, S2**) were suspended in 0.05 M phosphate buffer (*p*H 7). Modified glutaraldehyde (glutaraldehyde was modified in aqueous solution at 64-66 ^{0}C for 20 min, in order to reduce its toxicity) was added to the silica suspension, stirred at 20^{0}C for 2 h and filtered. The activated silica-based supports were washed with 0.05 M phosphate buffer (*p*H 7) and resuspended in the same buffer.

Lipase immobilization to the activated silica was performed by adding 2 mL (1mg/mL) lipase solution to the suspension and stirring at 20^{0}C for 2 h. The final product was filtered, washed with water and resuspended in 0.05 M phosphate buffer (*p*H 7). The product was set aside for storage at 4 ^{0}C.

Determination of the amount of immobilized lipase

The amount of immobilized lipase on silica based support (**S1, S2**) was estimated using standard method proposed by Lowry [14], using BSA as standard protein and readings at 600 nm. The amount of immobilized lipase was computed based on the concentration of the lipase solution before and after immobilization.

Determination of lipase activity

Hydrolytic activity of free lipase and immobilized lipase was determined by carrying out hydrolysis of olive oil. The reaction was carried out at 40°C and *p*H 8. The hydrolysis reaction was initiated by adding free lipase or immobilized lipase and was ceased by 5 mL acetone addition.

The reaction mixture was a 5 mL olive oil emulsion consisting of olive oil, gum acacia, sodium benzoate and 5 mL phosphate buffer 0.1M. The amount of free fatty acid released during hydrolysis was estimated by titration with NaOH 0.025N.

The activity of free lipase was estimated as IU/mL of enzymatic solution. One IU is the amount of enzymes that liberates 1mmole free fatty acid from olive oil per minute at *p*H 8 and 40°C.

Results and discussions

The two silica based supports **(S1, S2)** were compositional and morphologycal characterized.

DLS measurements

The particle size is an important factor which influences both the immobilization process and the enzymatic activity of an enzyme. Being a surface process, it is mainly influenced by pore size, being inverse proportional with the support specific area.

Analyzing the particles size distribution of the two sample **(S1, S2)** one may observe the different nature of the two samples. From DLS curves it can be observed that sample **S1** presents an individual peak with the average size of 2404 nm (100%), while sample **S2** presents a bimodal distribution of particles. Even there is a verey narow size distribution in case of synthesized hybrid silica support **S1**, with a polidispersity index of 0.073, the support **S2** presents a better size distribution, with two peaks whose average sizes are 154.9 nm (62.1%) and 2.58 nm (37.9%).

Based on DLS curve, the ratio between the two kind of particles is 32:68.

The different particle size distributions were consistent with the different nature of surfactants used. The results clearly show that when Triton X-100/Tween 80 (1:1 molar) was used, the particle size well exceeds 1000 nm; these surfactants are typically non-ionic surfactants. As D-glucose replaces Triton X-100 (in the second surfactant system, equimolar D-glucose/Tween 80), the particle size decreases considerably; D-glucose is recognized in the literature as being a so-called “non-surfactant”, however, its use is employed in surfactant-based systems due to the OH- groups contained. The network created by H-bonds between the surfactants micelles enhances the nucleation mechanism and this explains the very small peak around 1-2 nm that was observed for sample **S2**.

SEM

The obtained samples were analyzed by scanning electron microscopy (SEM) in order to determine their morphology. The silica based supports (**S1, S2**) have irregular shapes after immobilization. The particle size of **S1** is higher than that of **S2** this being related to the different surfactant systems used.

For sample **S1** the particle size reach 100 μm while in the case of sample **S2** they won’t exceed 50 μm. At higher magnification the surface of **S2** seems to contain more enzyme than sample **S1** and this observation is also supported by FTIR data.

FT-IR spectra

FTIR analysis was used to characterize the silica-based support **(S1, S2)** before and after immobilization.

Considerable modification of the IR spectra can be identified due to immobilization. One may identify in the spectra the new characteristic absorption bands of lipase

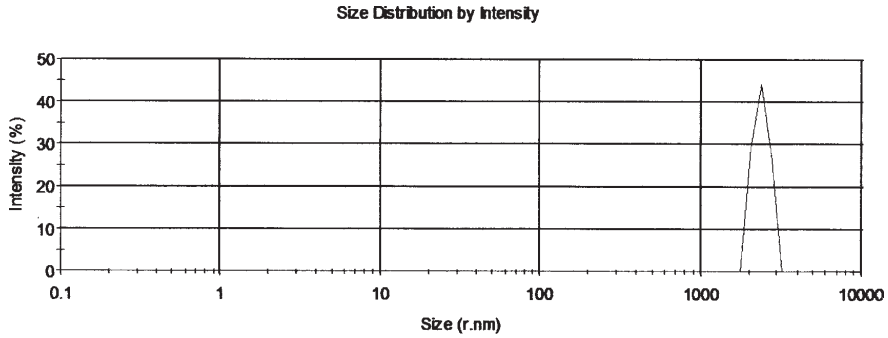

Fig. 1. Particles size distribution of the synthesized silica based support S1 (DLS)

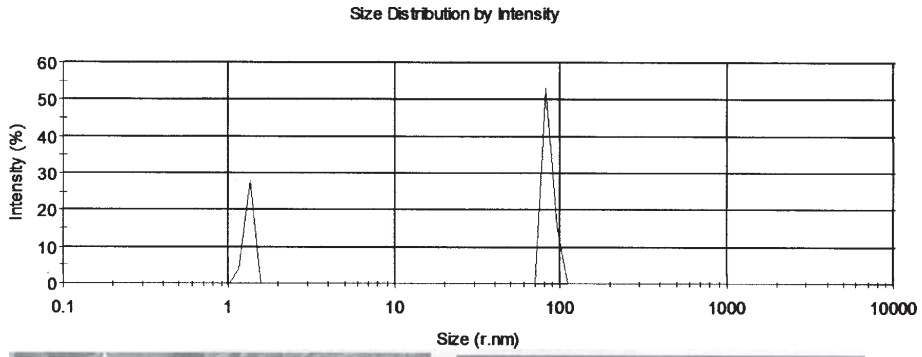

Fig. 2. Particles size distribution of the synthesized silica based support S2 (DLS)

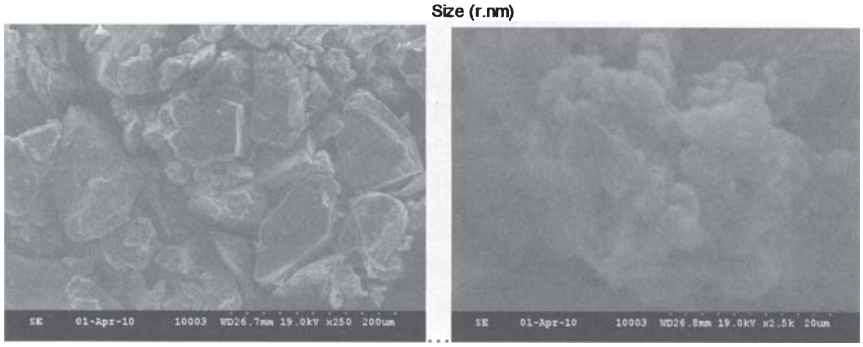

Fig. 3. SEM images of sample S1 before and after lipase immobilization

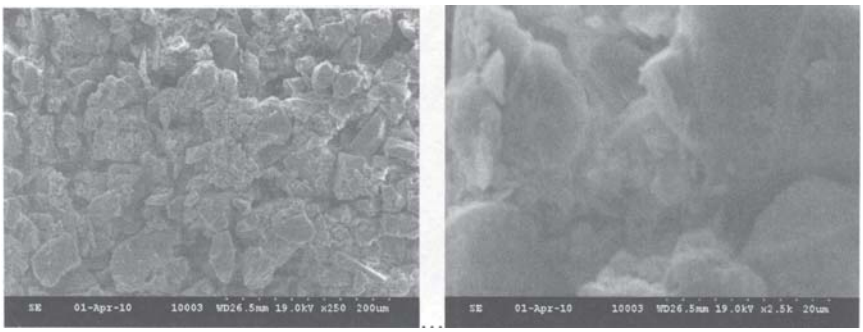

Fig. 4. SEM images of sample S2 before and after lipase immobilization

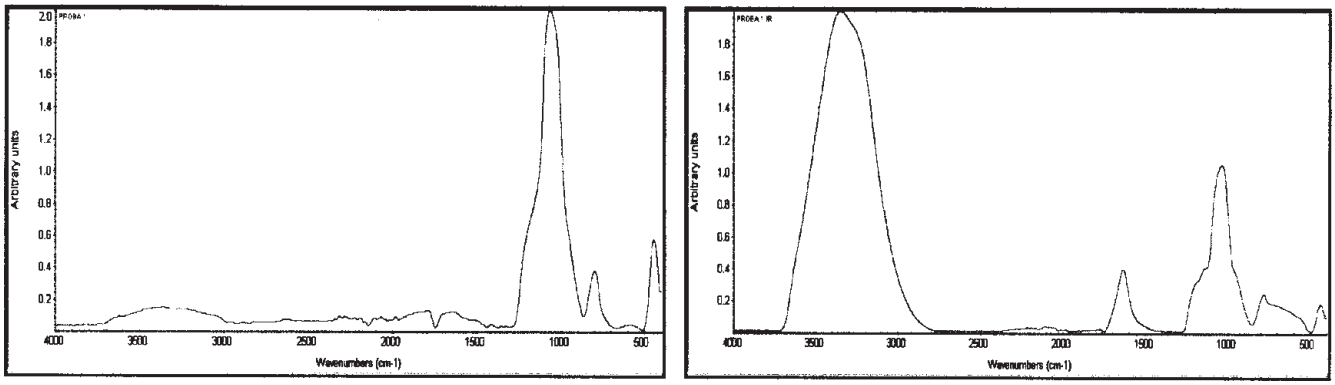

Fig. 5. IR spectra of sample S1 before and after lipase immobilization

at 1638 cm^{-1} and also some shifted bands corresponding to the support (1036 and 785 cm^{-1}). The broad bands from 3000-3700 cm^{-1} are strongly influenced by immobilization; their intensity increases considerably after immobilization and this is characteristic to both free water and enzyme.

XRD

The silica based support **(S1, S2)** has a quasi-amorphous character even after surfactants removal at 600 ^{0}C. As the XRD patterns before and after immobilization are similar in both cases, one may conclude that enzyme

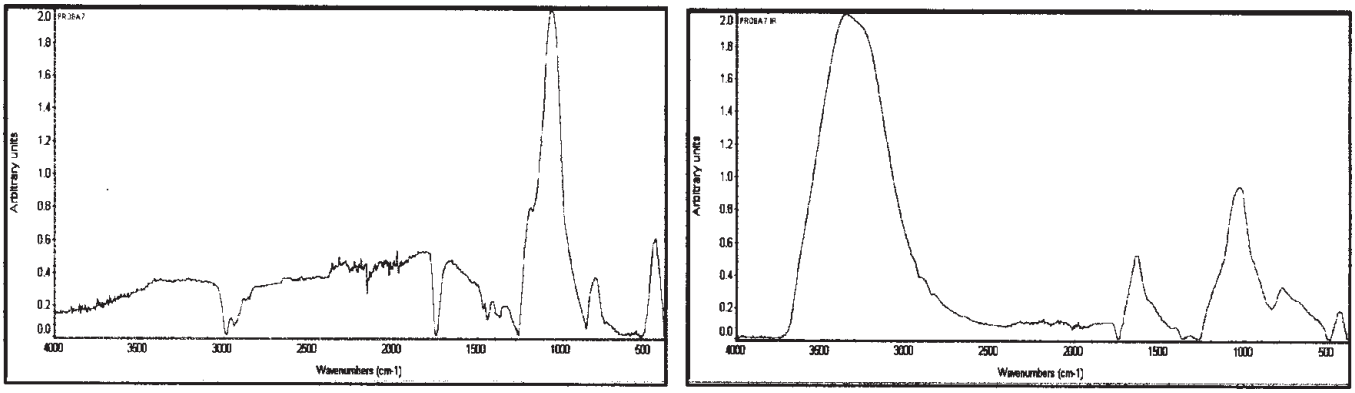

Fig. 6. IR spectra of sample S2 before and after lipase immobilization

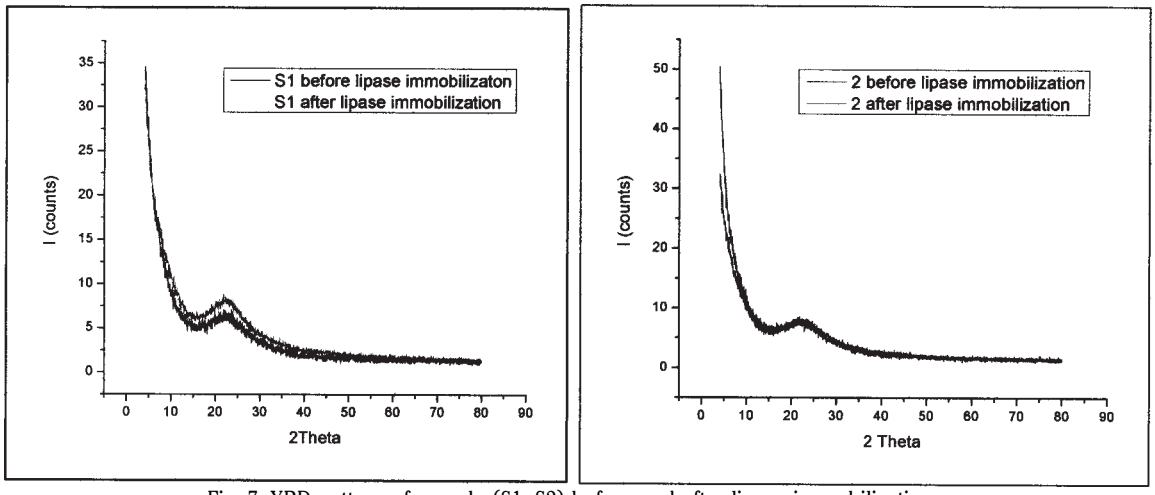

Fig. 7. XRD pattern of sample (S1, S2) before and after lipase immobilization

Sample	S1		S2		Ratio S1:S2
Intensity of peaks	1638	1030	1638	1030	-
Arbitrary unities	0,4002	1,0296	0,5714	1,0286	
Relative intensity	0,3891		0,5555		0,7

Table 1
FTIR DATA FOR SAMPLES S1 AND S2

Sample	Quantity of immobilized enzime	V(NaOH used for titration) (mL)	Enzymatic activity (µmol fatty acid/ h* mg)	Relative activity[b]
S1	1,2886 mg/0.5g support	1,9	54,67	4.95
S2	1,9830 mg/0.5g support	3	56.1	5.08
Ratio S1:S2	0,6499	0,6333	0.9745	0.9744

[b] Spec. activity of commercial ("free") lipase aspergilius niger : 11,04 µmol . h^{-1} . mg^{-1}

Table 2
DETERMINATION OF ENZYME ACTIVITY IN S1 AND S2

immobilization has no significant influence over the XRD patterns of the samples. However, the crystallinity of the sample **S1** is enhanced by enzyme, as the wide angle Bragg diffraction shows a higher peak for the enzyme-immobilized support as compared to the enzyme-free support.

Determination of immobilized lipase

The quantity of immobilized enzyme is strongly influenced by the support. As such, the amount of immobilized lipase is 1.2886 mg enzyme/0.5g support **S1** and 1.9830 mg enzyme/0.5g support **S2.**

The quantity of the entrapped enzyme can be estimated by FTIR based on the ratio of the main peaks of lipase (1638 cm^{-1}) and silica network (~1030 cm^{-1}) (table1) [15].

Analyzing the relative intensity ratio of peaks 1638:1030 we can estimate the ratio of the immobilized enzyme on sample **S1** and **S2**. Based on IR measurements we conclude that ~ 40% more lipase is immobilized on sample **S2** than on sample **S1**, while by Lowry method (table 2) the excess of lipase immobilized on sample **S2** is ~ 33% higher than that immobilized on sample **S1**.

The enzymatic activity of samples **S1** and **S2** can be easily estimated based on the required NaOH volume used for titration of the released free fatty acid (table 2).

Because the ratio between the quantity of immobilized lipase in **S1** and **S2** is almost equal to that between the two values of NaOH required for titration of samples **S1** and **S2** we can say that the activity of enzymes is not influenced by the support used. Absorption data obtained from UV-Vis measurements at 600 nm allow determination of lipase enzymatic activity, which is slightly higher than that of the corresponding free enzyme.

Conclusions

Two new silica-based materials were reported and tested as support for lipase immobilization. The two supports were morphological and compositional

characterized. Using D-glucose/Tween 80 system instead of TritonX-100/Tween 80 leads to smaller silica-based particles. The amount of immobilized lipase is higher on support **S2**. Lipase immobilization leads higher enzymatic activity of sample **S1** and **S2**, the activity of **S2** being slightly higher than **S1**.

Advances in molecular biology should lead to a more cost-effective production process, one that targets enzymes that are more specific, yet less expensive. Immobilization techniques such as the one proposed in this article increase the effectiveness of lipases, by the higher number of succesive uses before its enzymatic activity would decrease.

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