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# Immobilization of Lipase from *Aspergillus carbonarius* on Polyethylene Terephthalate Beads.

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**ABSTRACT**

Polyethylene terephthalate was tested as a carrier for *Aspergillus carbonarius* NRRL 369 lipase immobilization. Surface modification of poly (ethylene terephthalate) beads was performed in order to produce functional groups that bind covalently to the enzyme. The properties of the immobilized enzyme were assayed and compared with the free one. As a consequence of enzyme immobilization, some characteristics were altered. The pH tolerance, thermal and storage stability of the immobilized lipase were significantly improved. The immobilized lipase onto modified PET beads with chitosan retained over 70% of its initial hydrolytic activity and 90 % of its transesterification activity after 5 cycles. Therefore, the polyethylene terephthalate beads are a promising solid support media for lipase immobilization, and the immobilized preparations could have broad biocatalytic applications.

**Keywords:** lipase, immobilization, enzyme modification, poly (ethylene terephthalate), *Aspergillus carbonarius*

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## INTRODUCTION

Lipases (triacylglycerol ester hydrolase, EC 3.1.1.3) catalyze hydrolysis of triacylglycerol into glycerol and fatty acids and in organic medium they catalyze esterification and transesterification reactions [1]. Lipase enzymes have various industrial applications in food, pharmaceutical, and chemical industries.

The development of simple protocols for immobilization and stabilization of industrial enzymes is of significant meaning for their application in different fields of industry [2]. Efficacious immobilization of enzymes is important from technological point of view. Immobilization often stabilizes enzymes structure, allowing their applications at harsh environmental conditions of pH, temperature and organic solvents, and enables their uses in nonaqueous media [3,4]. Immobilization also allows a reuse of the enzymes for many reaction cycles.

Lipases have been immobilized with different methods including adsorption and covalent attachment, cross-linking, adsorption followed by cross-linking, and physical entrapment. To fully exploit the technical and economical advantages of lipases, it is recommended that they be used in an immobilized state to reduce the cost and the poor stability of the soluble form. Immobilization also facilitates the separation of products, enhances lipase properties such as thermal stability and activity, and provides more flexibility with enzyme/substrate contact by using various reactor configurations [5].

Different carriers have been used for lipase immobilization. Polyethylene terephthalate (PET) is a polymer considered to be appropriate for enzyme immobilization for its various forms–granules, films, membranes, fibers, non-woven and woven textiles [6]. PET has some disadvantages for enzyme immobilization as most of the synthetic polymers. It is relatively inert and hydrophobic without functional groups able to take part in immobilization. In some situations, it is an unsuitable material to use due to its low surface energy, leading to poor wettability and poor adhesion which makes coating or immobilization of enzymes difficult [7]. To overcome this drawback chemical modifications have been attempted to alter the surface properties of the material. Many physical and chemical methods for surface modification of PET have been used [8]. Often they include chemical breaking of ester bonds, by reaction of PET with low molecular weight substances containing hydroxyl, carboxyl or amine groups. As a result the hydrophility of the polymer is increased and corresponding functional groups are incorporated onto the surface [9]. Functional groups created during modification processes can serve as anchor sites for covalent immobilization of enzymes.

The aim of the research is investigation of different methods for chemical modification of PET beads for covalent immobilization of *Aspergillus carbonarius* lipase and determination of the biochemical characteristics of the immobilized enzyme.

## MATERIALS AND METHODS

### Materials

The strain *A. carbonarius*s NRRL 369 was from ARS Culture Collection and lipase was produced as described previously [10]. Ethylenediamine (EDA), p-nitrophenyl palmitate (p-NPP), TBO (toluidine blue) were purchased from Sigma. All other chemicals were of analytical grade.

### Preparation and modification of PET support for enzyme immobilization

### Modification of PET beads with EDA and glutaraldehyde

The PET beads were treated with certain concentrations of aqueous EDA at 80°C for 30 min. The treated beads were extensively washed out with ethanol and bidistilled water and dried at room temperature.

Concentration of the amine groups formed on PET surface was determined by reaction with bromophenol blue (BPB) [11]. The beads were immersed in 25 ml of BPB solution in DMF (0.1mg/ml) for 30 min, and rinsed a few times with ethanol to remove unbound dye. The sample was then treated with 20% (v/v) solution of piperidine in DMF and the absorbance of the solution obtained was measured at 605 nm.

Cross-linking of PET beads with glutaraldehyde was performed by immersion the beads in glutaraldehyde solution (1-10% v/v) for 10-60 min.

**Modification of PET beads with chitosan**

PET beads were incubated in an aqueous solution of 1-15 mol/l NaOH at 70°C for 30 min. The beads were taken out and washed with 1M HCl to rinse off NaOH. After sequentially washing with alcohol and water the beads were dried at 30°C.

The concentration of carboxyl groups was determined using a colorimetric method [9]. The PET beads were incubated in 10 ml 0.05M TBO (pH10) at room temperature for 12 h, rinsed with 0.1 mM NaOH to remove unbound TBO, and immersed in 4 ml 50% (v/v) acetic acid for 10 minutes to desorb the bound TBO. The concentration of TBO in acetic acid was determined by measuring the absorbance at 633 nm. The concentration of COOH groups was obtained by assuming that 1 mol COOH groups coupled to 1 mol TBO using a standard calibration curve.

Coating of PET beads with chitosan was performed with chitosan solution (0.1-2.5%) in 2 ml of 1% acetic acid. The beads were incubated for 30 min to adsorb a layer of chitosan and rinsed with distillated water [12]. The concentration of amine groups was determined using the method with BPB described above.

**Lipase immobilization**

The PET beads (0.5g) were immersed into lipase solution at 25°C with shaking at 255 rpm. After the immobilization process, enzyme loaded PET beads were removed from the solution and dried for 24 h at room temperature. The amount of the immobilized lipase was determined by the lipase activity difference of the initial and final lipase solutions. To achieve a reliable result, each test was repeated three times at least. Immobilization activity was expressed as follows: Immobilization Efficiency (IE) (%) = (I/A-B) X 100, where A - Added enzyme (U), B - Unbound enzyme (U), I - Immobilized enzyme (U).

**Lipase characteristics**

**Optimal temperature and pH of immobilized and free lipase**

The optimal pH of the immobilized and free lipase was studied in pH range of 4.0-9.0 at 35°C. Acetate buffer was used for pH 4.0-5.0, phosphate buffer for pH 6.0-8.0, and Tris-HCl buffer for pH 9.0. The effect of temperature was investigated in the range of 25-50°C at pH 6.0.

**Thermal and pH stability of immobilized and free lipase**

The pH stability of immobilized and free lipase was studied at pH 4.0 (acetate buffer); 6.0; 8.0 (phosphate buffer), and the thermal stability was investigated at 40°C; 50°C and 60°C for 120 min. Periodically, samples were withdrawn and their residual activities were assayed. The initial activity was taken as 100%.

**Effect of organic solvents on immobilized and free lipase**

The effect of organic solvents (methanol, ethanol, DMSO, DMF, 2-propanol and Hexane) on lipase activity was analyzed by using 10 and 20 % (v/v) solvent concentration in the reaction mixture.

**Reusability and storage stability of immobilized lipase**

The reusability of immobilized lipase was studied in aqueous and organic medium by repeated batch experiments. After each reaction run, the immobilized enzyme preparation was removed from the reaction mixture and washed out with buffer to remove any residual substrate.

The storage effect on lipase activity was evaluated by measuring the residual activities after dry storage at 4°C for 90 days.

**Kinetic parameters determination**

Kinetic parameters Km and Vmax of hydrolytic reaction for free and immobilized enzyme were determined with p-nitrophenyl palmitate as substrate in concentrations of 0.1-1.0 mM by the method of Lineweaver –Burk [13].

**Lipase activity assay**

**Hydrolytic activity**

Lipase activity was measured by spectrophotometric method [14] with modification. p-Nitrophenyl palmitate (p-NPP) buffered with 50 mM phosphate buffer with pH 6 was used as substrate. The reaction mixture, containing 2.4 ml 0.8 mM substrate and 0.1 ml of enzyme solution, was incubated for 15 min at 35°C. Then 1.0 ml saturated solution of plumbous acetate was added to stop the reaction. After centrifugation absorbance was measured at 410 nm. One unit (U) of lipase activity was defined as the amount of lipase which formed 1.0 µmol of p-nitropfenol per minute under the experimental conditions.

**Transterification activity**

p-NPP and butanol were used as substrates for determination of transesterification activity [15]. The immobilized enzyme was suspended in 1 mL p-NPP (15 mM in n-hexane), 50 µL butanol were added, and the reaction was performed at 25°C for 4 h under agitation (200 rpm). 100 µl of the reaction mixture were mixed with ethanol. p-Nitrophenol released, extracted from the alkaline aqueous phase, was measured at 310 nm against a blank (without enzyme). A calibration curve of p-nitrophenol in ethanol was prepared in order to obtain quantitative results.

## RESULTS AND DISCUSSION

**Modification of PET beads with EDA and glutaraldehyde**

The first approach used for modification of the support included two successively steps for insertion of a spacer arm. At the first step the PET beads were modified with EDA in order to introduce amino groups on PET surface, that can serve as an anchor sites for covalent immobilization of enzymes. At the second step glutharaldehide was used as a spacer for covalent immobilization [16].

The main problem is to find the proper parameters of carrier modification, which gain active surface and do not cause high degradation of the mechanical properties of the support. The results for modification of PET beads by aminolysis are presented in Table 1.

**Table 1: Modification parameters of aminolysis of PET beads**

| EDA, % | $NH_2$ groups, nmol/g |
|---|---|
| 5 | 2.18 |
| 10 | 2.37 |
| 15 | 2.59 |
| 20 | 2.76 |
| 50 | 170.26 |
| 100 | 926.47 |

* modification was performed at 80°C for 30 min

Different modification conditions led to release of different concentration of amine groups. The effect of amine groups on lipase immobilization was investigated (Fig 1).

It was found that immobilized lipase displayed highest activities at low density of amine groups. After reaching certain amine group concentration (2.59 nmol/g), a decrease of immobilization level was observed.

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The binding of more functional groups probably caused the enzyme molecule to change its shape in such a way that the active site become less effective, which reduced the productivity of the enzyme.

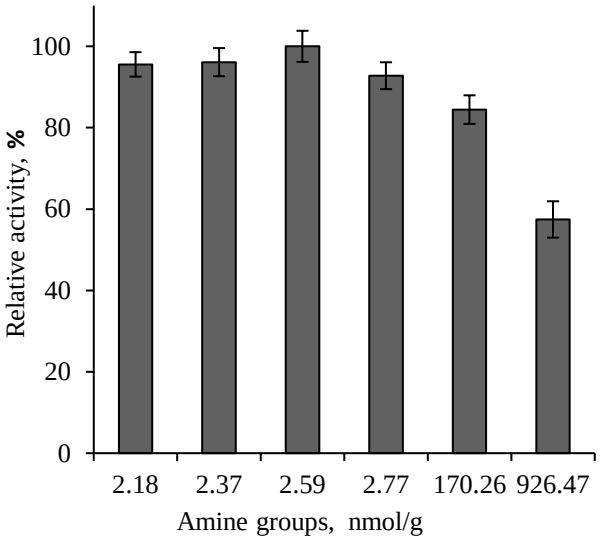
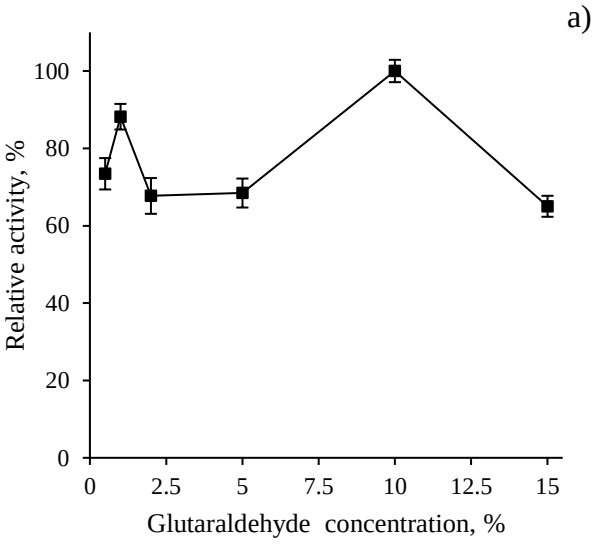



**Fig. 1. Influence of surface amine groups concentration of modified PET beads on immobilized lipase activity**

After modification of PET beads by aminolysis with 15 % EDA, at 80°C for 30 min the second step of modification was performed. The amino groups of PET surface were activated with glutharaldehide as a cross-linking reagent. The effect of glutaraldehyde concentration and cross-linking time on the immobilized enzyme activity is presented in Fig.2 a,b.



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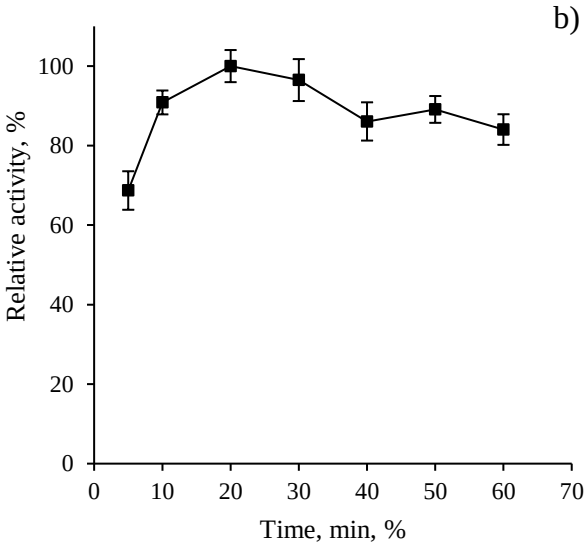


**Fig. 2. Effect of glutaraldehyde concentration (a) and glutaraldehyde incubation time (b) on immobilized lipase activity**

Fig. 2a shows that glutaraldehyde concentration of 10% was most appropriate for cross-linking.

At low glutaraldehyde concentration, the immobilized enzyme were insufficiently cross-linked and released unbound enzyme in the aqueous medium. At higher concentration, the enzyme was excessively cross-linked, resulting in a decline in enzyme flexibility and inactivation.

The cross-linking time of 20 min was enough for effective immobilization of lipase. A shorter period led to decrease in enzyme activity (Fig. 2b).

**Modification of PET beads with chitosan**

The second strategy for modification of the carrier included alkaline hydrolysis of PET beads and subsequent coating with chitosan.

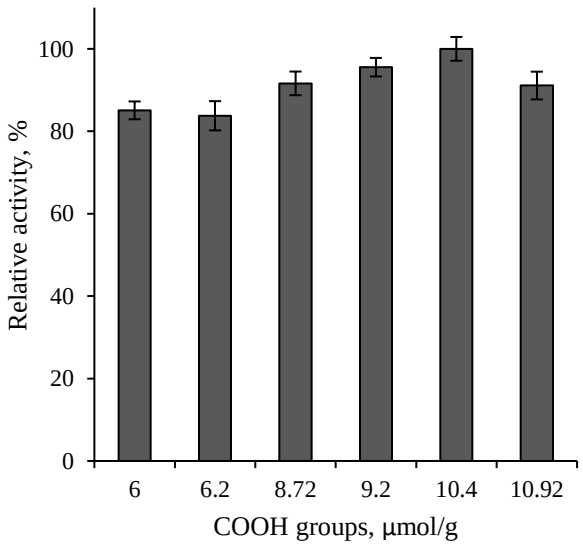


**Fig. 3. Influence of surface COOH groups concentration of modified PET beads on immobilized lipase activitiy**

As a result of the alkaline hydrolysis, carboxyl groups were formed on PET surface. The influence of NaOH concentration on the formation of carboxyl groups is presented in Table 2. As can be seen, the surface active carboxyl groups concentration depended on the amount of NaOH used.

**Table 2: Modification parameters of hydrolysis of PET beads**

| NaOH, mol/l | COOH groups, µmol/g |
|---|---|
| 1.00 | 6.00 |
| 2.00 | 6.20 |
| 4.00 | 8.72 |
| 6.00 | 9.20 |
| 8.00 | 10.40 |
| 10.00 | 10.92 |
| 15.00** | 4.50 |

*modification was performed at 70°C for 30 min
**the sample began to destroy during modification

The PET beads with different surface concentration of carboxylic groups were tested as supports for immobilization of lipase (Fig.3). The concentration of carboxylic groups did not affect significantly the activity of immobilized enzyme. The following experiments were performed using PET beads modified with 10 M NaOH at 70°C for 30 min.

The carboxylic groups produced during alkali hydrolysis were used for coating of PET beads with chitosan, which provided free amine groups for immobilization of lipase. Fig. 4 showed the influence of chitosan concentration on the amount of the free amine groups on the surface of coated PET beads. The free amine groups increased with increasing of chitosan concentration. When chitosan concentration was low, all amine groups probably were bound to carboxylic groups of the hydrolysed PET surface. Increasing of chitosan concentration could lead to multilayer formation onto support, which is observed from the large amount of free amine groups detected onto PET surface.

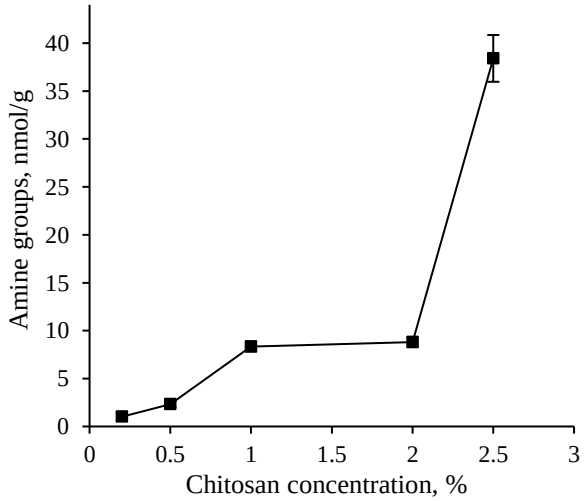


**Fig. 4. Concentration of amine groups on the surface of chitosan modified PET beads**

The effect of chitosan concentration and incubation time on immobilized lipase activity was evaluated (Fig.5 a,b). Because of the high viscosity of the chitosan solution, experiments were carried out at highest concentration of 2.5%. The results showed that the immobilized lipase activity and immobilization efficiency were maximum in the presence of 0.5 % (w/v) chitosan.

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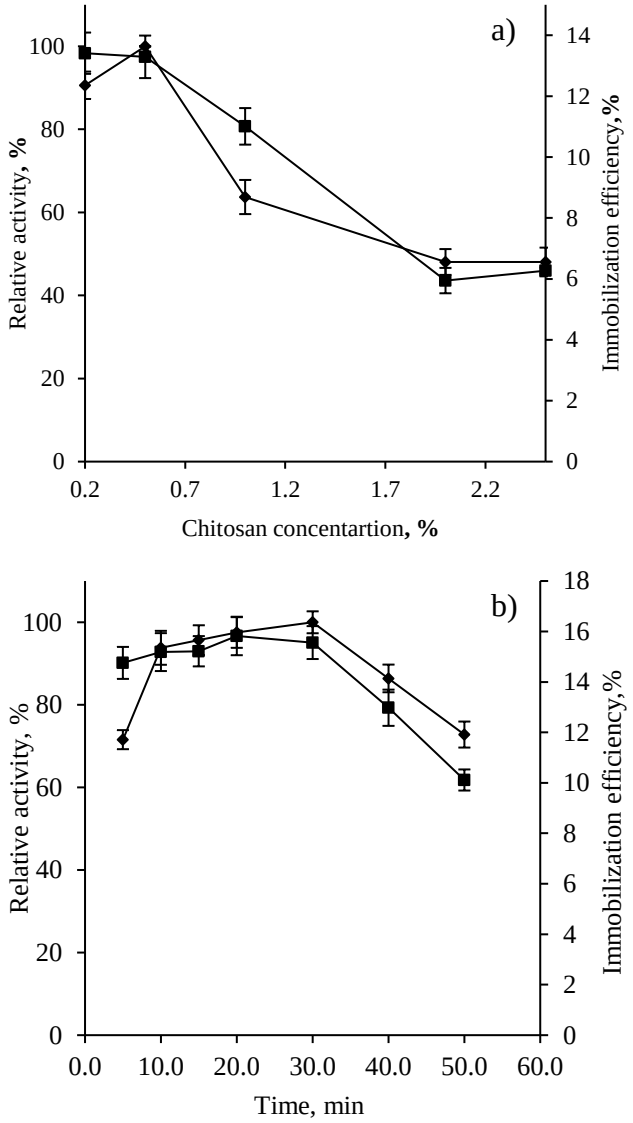


**Fig. 5. Effect of chitosan concentration (a) and chitosan incubation time (b) on immobilized lipase activity (♦) and immobilization efficiency (■)**

The higher concentration of chitosan led to decrease in enzyme activity. The immobilization efficiency and the enzyme activity were significantly affected by the incubation time in chitosan solution. The immobilization efficiency increased as the reaction proceeded for up to 30 min. When duration time was too long the immobilized enzyme configuration may change due to excessive cross-linking, causing denaturation of the enzyme protein.

**Characteristics of immobilized and free lipase**

The main biochemical characteristics of lipase enzyme immobilized on modified PET beads with EDA and glutaraldehyde (PET-EDA-GA beads) and the enzyme immobilized on modified PET beads with chitosan (PET-chitosan beads) were investigated.

**Thermal and pH optimum of immobilized and free lipase**

After immobilization, considerable changes in temperature optimum of immobilized enzyme preparations were observed (Fig. 6). Both immobilized enzymes showed significant activity at 25-50°C, and the free enzyme had a clear thermal optimum at 40°C. Several factors may be the reason for this result, including

three-dimensional structure changes that possibly occur during the immobilization procedure. These properties of the immobilized lipase significantly increase the potential for their application in different areas.

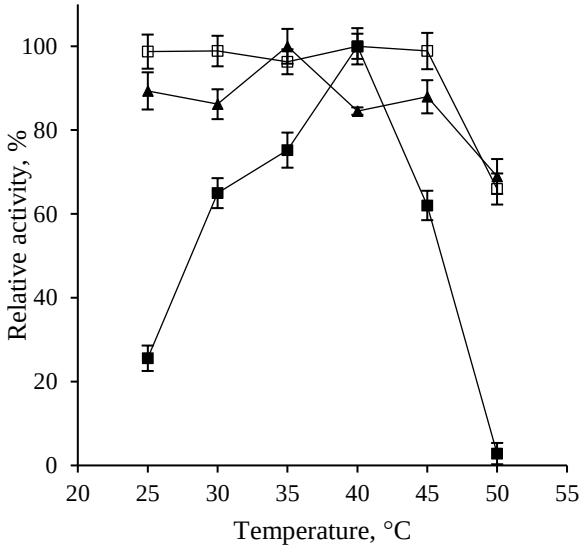


**Fig. 6. Effect of temperature on enzyme activity of free lipase (■), immobilized lipase on PET-EDA-GA beads (▲) and immobilized lipase on PET-chitosan beads (□)**

Fig. 7 showed that lipase immobilization did not significantly change the sensitivity of enzyme to pH of the medium. Both preparations showed maximum activity at pH 6.0. In alkali media the free and immobilized lipase were not active.

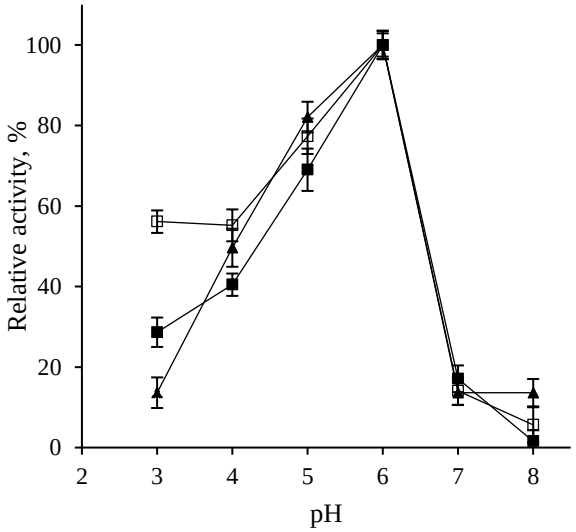


**Fig. 7. Effect of pH reaction media on enzyme activity of free lipase (■), immobilized lipase on PET-EDA-GA beads (▲), and immobilized lipase on PET-chitosan beads (□)**

**Thermal and pH stability of immobilized and free lipase**

The results for the thermal stability of immobilized and free lipase are shown in Fig.8. The highest enzyme stability was achieved for lipase immobilized on PET-chitosan beads. It retained 96 % of its initial activity at 60°C, whereas the free lipase retained 84% of its activity, and lipase immobilized on PET-EDA-GA beads showed 55 % activity.

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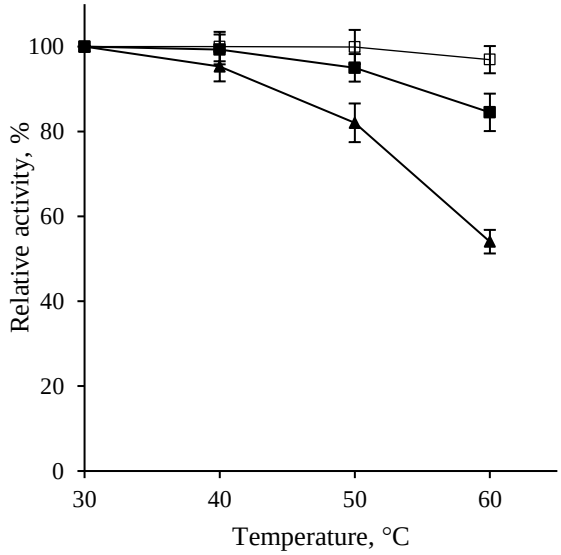
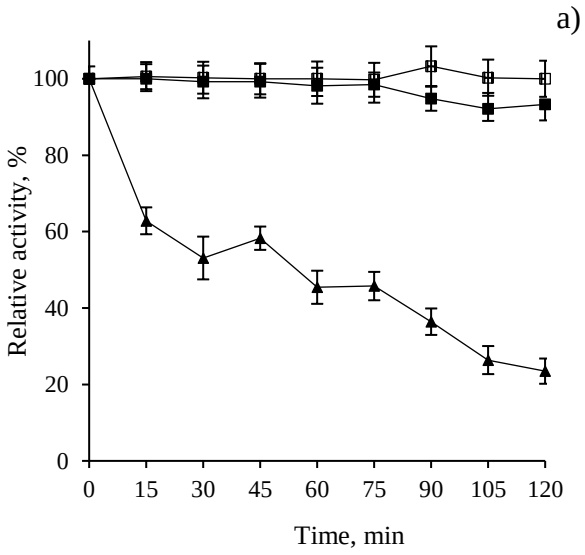



**Fig. 8. Thermal stability of free lipase (■), immobilized lipase on PET-EDA-GA beads (▲), and immobilized lipase on PET-chitosan beads (□) at pH 4.0 for 1 h**

The immobilized lipase preparations differed significantly in their pH stability (Fig. 9 a,b). The enzyme immobilized on PET-chitosan beads showed higher stability. At pH 6 it retained about 100 % of its activity for 120 min, which is comparable with the free enzyme (Fig. 9a). At alkali pH of the medium (pH 8) free and immobilized lipase on PET-chitosan beads displayed about 50 % of its activity for 30 min, whereas the enzyme immobilized on PET-EDA-GA beads were inactivated at the same conditions for 30 min (Fig. 9b).



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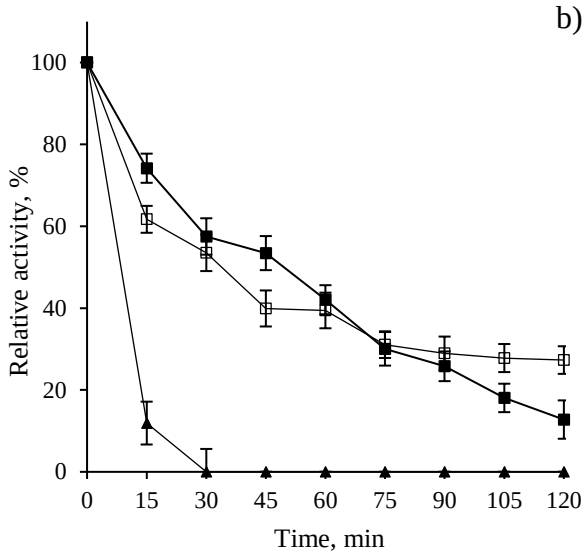


**Fig. 9. pH stability of free lipase (■), immobilized lipase on PET-EDA-GA beads (▲), and immobilized lipase on PET-chitosan beads (□): a) pH 6.0 and 40°C; b) pH 8.0 and 40°C**

**Effect of organic solvents on the activity of immobilized and free lipase**

One objective of lipase immobilization is the possibility to perform heterogeneous catalysis of esterification and transesterification reactions in organic medium. Therefore the effect of organic solvents on lipase activity is crucial for the possibility of conducting this type of reactions [17]. In Table 3 the effect of organic solvents on immobilized and free lipase is presented.

**Table 3: Effect of organic solvents on immobilized and free lipase activity**

| Solvent | Solvent concentration, % | Relative activity, % | | |
|---|---|---|---|---|
| | | Free enzyme | Lipase immobilized on PET-chitosan beads | Lipase immobilized on PET-EDA-GA beads |
| Control | 0 | 100.00 | 100.00 | 100.00 |
| Methanol | 10 | 296.11 | 143.02 | 150.68 |
| | 20 | 273.28 | 117.91 | 82.29 |
| Ethanol | 10 | 95.23 | 123.04 | 82.96 |
| | 20 | 10.16 | 59.71 | 61.71 |
| 2-Propanol | 10 | 73.93 | 83.29 | 79.20 |
| | 20 | 270.21 | 94.21 | 14.13 |
| DMSO | 10 | 125.39 | 116.49 | 66.80 |
| | 20 | 161.49 | 35.03 | 32.50 |
| DMF | 10 | 136.47 | 171.08 | 121.03 |
| | 20 | 53.45 | 78.82 | 52.19 |
| Hexan | 10 | 111.25 | 101.09 | 108.85 |
| | 20 | 401.76 | 111.25 | 152.45 |

The free enzyme was significantly activated in the presence of methanol, DMSO, 20 % propanol, 20 % hexan, and it was inhibited by ethanol, 10 % propanol, and 20 % DMF. As a result of the immobilization of the enzyme and the substantial reduction of the flexibility of the immobilized protein molecule, the influence of the organic solvents was restricted. The immobilized enzymes were affected to a lesser extent with respect to their activation and inhibition by organic solvents. The immobilized lipase showed high activity in the presence of methanol, hexan, 10 % DMF and ethanol. Therefore these solvents may be used as medium for carrying out esterification and transesterification reactions.

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**Reusability and storage stability of immobilized lipase**

The reusability of immobilized lipase in aqueous and organic medium is presented on Fig. 10. In aqueous medium lipase immobilized on PET-chitosan beads retained over 74% of its hydrolytic activity after being used five times. The residual activity of lipase immobilized on PET-EDA-GA after third cycle was 22.8%. It can be concluded that *Aspergillus carbonarius* NRRL 369 lipase showed higher reusability when immobilized on PET modified with chitosan, due to the presence of stronger interactions between the enzyme and the carrier. The residual transesterification activity of both immobilized preparations in hexan medium was about 90% after 5 cycles.

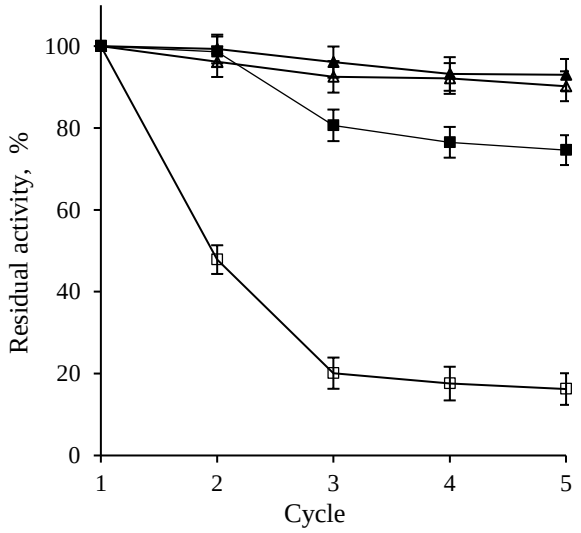


**Fig. 10. Reusability of immobilized lipase in aqueous media (PET-EDA-GA beads (□), PET-chitosan beads (■)) and organic media (PET-EDA-GA beads (Δ), PET-chitosan beads (▲))**

The investigation of the storage stability of the immobilized enzyme preparations showed that they retained about 80% of their activity after 90 days 4°C (Fig. 11).

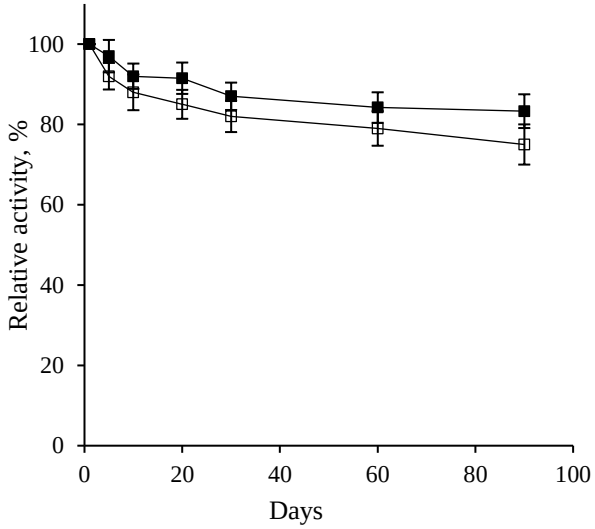


**Fig. 11. Storage stability of lipase immobilized on PET-EDA-GA beads (□), and PET-chitosan beads (■)**

**Kinetic parameters of immobilized and free lipase**

Vmax and Km values of the hydrolytic reaction for free lipase were determined to be 0.036 µmol.min$^{-1}$.ml$^{-1}$ and 1.24 mM. After immobilization the values changed to 0.083 µmol.min$^{-1}$.ml$^{-1}$ and 1.58 mM for PET-chitosan beads, and 0.030 µmol.min$^{-1}$.ml$^{-1}$ and 1.59 mM for PET-EDA-GA beads. The increase in Km after

immobilization signifies the lower accessibility of substrate to active site. Immobilization process gives opportunity to perform the enzyme reaction with higher substrate concentration and to speed up the reaction rate.

## CONCLUSION

Lipase produced by *Aspergillus carbonarius* NRRL 369 was immobilized by covalent binding on modified PET beads. It was proven that the methods used for PET beads modification led to formation of surface amino and carboxylic groups, which can serve as anchor sites for covalent enzyme immobilization. The immobilization of lipase on modified PET beads significantly changed the biochemical characteristics of the enzyme. Lipase immobilized on PET-EDA-GA beads and on PET-chitosan beads showed over 80 % relative activity at 25-50°C, and the free enzyme displayed only 25 % activity at 25°C, and it was almost inactivated at 50°C. The immobilized enzyme on PET-chitosan beads showed significant thermal and pH stability in slightly acidic pH range, which is comparable to the stability of the free enzyme. This is useful in expanding the fields of application of the immobilized enzyme preparation. The immobilized enzyme on PET-chitosan retained significant activity at repeated use. It displayed over 70 % of its hydrolytic activity and 90 % of its transesterification activity after 5 cycles. The high reusability of the obtained immobilized enzyme preparations in hexan medium allows their use in the catalysis of esterification and transesterification reactions.

## REFERENCES

[1] Wang XY, Jiang XP, Li Y, Zeng S, Zhang YW, Int J Biol Macromol 2015; 75: 44-50.
[2] Mateo C, Grazu V, Palomo JM, Lopez-Gallego F, Fernandez-Lafuente R, Guisan JM. Nat Protoc 2007; 2(5): 1022-1033.
[3] Homaei AA, Sariri R, Vianello F, Stevanato R. J Chem Biol 2013; 6: 185-205.
[4] Mohamad NR, Marzuki NHC, Buang NA, Huyop F, Wahab RA. Biotechnol Biotechnol Equip 2015; 29(2): 205-220.
[5] Silva JA, Macedo GP, Rodrigues DS, Giordano RLC, Goncalves LRB. Biochem Eng J 2012; 60: 16-24.
[6] Yigitoglu M, Temoc Z. 2010. J Mol Catal B: Enzym 2010; 66: 130-135.
[7] Temoçin Z. J Biomater Sci Polym Ed 2015; 24(14): 1618-1635.
[8] Kanelli M, Vasilakos S, Nikolaivits E, Ladas S, Christakopoulos P, Topakas E. Process Biochem 2015; 50: 1885-1892.
[9] Irena G, Jolanta B, Karolina Z. Appl Surf Sci 2009; 255: 8293-8298.
[10] Dobrev G, Zhekova B, Dobreva V, Strinska H, Doykina P, Krastanov A. Biocatal Agric Biotechnol 2015; 4: 77-82.
[11] Liu Y, He T, Gao C. Colloids Surf B: Biointerfaces 2005; 46: 117-126.
[12] Jovanović-Malinovska R, Cvetkovska M, Kuzmanova S, Tsvetanov C, Winkelhausen E. Maced J Chem Eng 2010; 29(2): 169-179.
[13] Price NC. Biochem Edu 1985; 13(2).
[14] Kaushik R, Saran S, Isar J, Saxena RK. J Mol Catal B: Enzym 2006; 40: 121-126.
[15] Fu X, Zheng J, Ying X, Yan H, Wang Z. Chinese J Catal 2014; 35: 553-559.
[16] Nimni ME, Cheung D, Strates B, Kodama M, Sheikh K. 1987. J Biomed Mater Res 1987; 21: 741-771.
[17] Kumar A, Dhar K, Kanwar SS, Arora PK. Biol Proced Online 2016; 18(2).