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# Catalytic properties of membrane-bound *Mucor* lipase immobilized in a hydrophilic carrier

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**Abstract**

Membrane-bound *Mucor circinelloides* and *Mucor racemosus* lipases (mycelium treated with acetone and dried) were entrapped in cryogel beads obtained from polyvinyl alcohol (PVA) by a freezing–thawing method in two-phase system. Due to the porosity of PVA-cryogels, high molecular-weight substances can penetrate beads of the biocatalyst [1]. The biocatalyst was applied for various hydrolysis and synthesis reactions. The immobilization in cryoPVA-gel stabilized the enzyme [2] and ensured high mechanical and chemical stability of the biocatalyst, which could be used many times for *p*-nitrophenyl acetate (*p*-NPA), *p*-nitrophenyl palmitate (*p*-NPP) and triacyloglycerols hydrolysis. The biocatalyst can be also applied for esters synthesis in non-aqueous milieu, but before the reaction it has to be dried with acetone. The dynamics of four esters (butyl and propyl oleates, and caprylates) synthesis in petroleum ether was examined. The efficiency of butyl oleate synthesis was 99%. The yield of the ester synthesis varied within the initial 4 h of the reaction. The phenomenon resulted from the cyclic accumulation of the water released upon ester synthesis, in the hydrophilic PVA-gel, that favored either ester synthesis or hydrolysis. The lipases entrapped in the dried cryo-PVA beads showed enhanced thermal and operational stability during repeated uses in ester synthesis in the non-aqueous system.


*Keywords:* *Mucor*; Ester synthesis; Immobilized lipase; PVA-cryogel

## 1. Introduction

During the last decade, lipases (glycerol ester hydrolases, EC 3.1.1.3) have attracted the great interest of the chemical and pharmaceutical industries due to their usefulness in both hydrolysis and synthesis reactions. Lipases can be used industrially for: hydrolysis of oil and fats, synthesis of fatty acid esters as cosmetic ingredients or surfactants, production of intermediates for organic synthesis, and so on. Their stability upon storage and application in biotechnological processes can be improved by immobilization. The extent of stabilization depends on the enzyme structure, the immobilization method, and the type of support. Generally, the use of hydrophilic supports enhances the enzyme stability while hydrophobic matrixes appear to be disadvantageous [3]. Although lowering the water activity increased the stability of lipases, it also decreased significantly the activity of the enzyme. Thus, a delicate balance of the water in enzyme's micro-environment seems to be essential for high catalytic activity of immobilized enzymes. Numerous

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supports for the immobilization of lipases have been investigated [4]. The highly active membrane-bound lipases from *Mucor circinelloides* and *Mucor racemosus* can be immobilized in situ in the mycelium after its treatment with acetone [5]. An entrapment of thus obtained preparations—the source of lipases—could improve their mechanical and operational stability. In this respect, a promising carrier to obtain immobilized biocatalysts appeared to be the polyvinyl alcohol-cryogel that is prepared via the freezing–thawing method [1,2,6,7]. It is compatible with both water and organic solvents and keeps some amount of water molecules tightly attached even in dehydrative solvent [8], what is advantageous for enzyme stabilization.

In the presented work, the preparations of immobilized in PVA-cryogel *Mucor* lipases were tested in reactions of various substrates hydrolysis (*p*-nitrophenyl acetate (*p*-NPA), *p*-nitrophenyl palmitate (*p*-NPP), and sunflower oil) and esters synthesis (butyl and propyl oleates, and caprylates) in petroleum ether.

## 2. Materials and methods

### 2.1. Materials

*M. circinelloides* and *M. racemosus* strains were obtained from the Institute of Technical Biochemistry of the Technical University of Lodz microbial culture collection. *p*-Nitrophenyl acetate (*p*-NPA) and *p*-nitrophenyl palmitate (*p*-NPP), sodium alginate were purchased from Sigma Co. Polyvinyl alcohol ($M_w$ 72,000; degree of polymerization, 1600; degree of hydrolysis, DH = 97.5–99.5), Triton X-100 and polyethylene glycol (PEG) 1500, hexane and petroleum ether from Fluka Co. Oleic and caprylic acids were obtained from Merck, butanol, and propanol from BDH and molecular sieve (4 Å) from Supelco. Micro-granulated cellulose was purchased from Whatman Ltd. All others reagents were analytical grade.

### 2.2. Immobilization of Mucor mycelium

*M. circinelloides* and *M. racemosus* mycelium—obtained in 72 h culture (in a corn steep liquor-containing medium), washed three times with acetone and dried at room temperature—were immobilized in PVA-cryogel as was described earlier [1,2]. From 3 to 68 mg d.w. of mycelium in 1 g of the gel as well as additives of Triton X-100 (0.1%), cellulose Whatman (∼3%), PEG (3 and 8%), sodium alginate (0.24%) or treatment of the mycelium with 1% oleic acid (dissolved in hexane) for 20 h before the entrapment were used. Beads of the biocatalyst, wet or dried with acetone (approximately 2–3 mm and 1 mm in diameter, respectively) were applied in hydrolysis or synthesis reactions.

### 2.3. Determination of the lipase preparations activities

The hydrolytic activities ($A_H$) of lipase preparations towards *p*-NPA, *p*-NPP and sunflower oil were determined by standard methods. Details are described later (Section 3) in the figures and tables. The concentration of released *p*-NP was measured at $\lambda = 399$ nm. The amount of acid liberated during sunflower oil hydrolysis was determined by pH-metric titration up to pH 10. The specific hydrolytic activity of lipase was expressed in micromoles of *p*-NP (or micromoles of acid) released for 1 min by 1 g of non-immobilized or immobilized *Mucor* mycelium.

The reactions of ester synthesis were performed in petroleum ether milieu in closed vials (25 ml) at 50 °C, with shaking (200 rpm). Other details are described later in the figures and tables. The degree of ester formation was determined by acid titration with 0.05 M NaOH in 96% ethanol. One unit of synthetic activity ($A_S$) was defined as an amount of enzyme (lipase—containing dried mycelium) necessary to synthesize 1 μmol of ester for 1 min.

## 3. Results and discussion

A matrix of polyvinyl alcohol—highly porous hydrophilic gel—is gaining an increasing interest [7–10]. This synthetic polymer, which can be cheaply produced in industrial scale, dissolved in a hot water, forms stable macro-porous cryogel (pores up to 10 μm in diameter, in a wet form) at low temperature [11]. The PVA-cryogels appeared to be suitable for whole cells of microorganisms' immobilization [1,9,10,12–15]. The purpose of this work was the application of this carrier for enzyme entrapment—

*M. circinelloides* and *M. racemosus* mycelium-bound lipases (Section 2.2). Immobilized preparations were tested in hydrolysis and synthesis reactions, in water and organic solvent media (Section 2.3).

It was found, that such immobilization leads to obtaining the preparations with ~26–55% efficiencies, calculated as their hydrolytic activities against various substrates in comparison to specific activities of non-immobilized ("free") mycelium (Table 1). These preparations displayed the highest activity in sunflower oil (emulsion stabilized with 2% PVA) hydrolysis. To provide the efficient *p*-NPP hydrolysis, the biocatalyst has to be placed at the buffer–hexane interface. In this system, the substrate is dissolved in the organic phase, and the product (*p*-NP) diffuses into the buffer—the gel matrix functions as a stabilizer for this two-phase liquid system. On the contrary to the "free" lipase preparations (bound with mycelium), that are susceptible to abrasion, the preparations of PVA-entrapped mycelium can be used many times for ester bond hydrolysis, with virtually no decrease in their catalytic activity (Figs. 1 and 2). An enrichment of the cryogel with polyethylene glycol or sodium alginate enhanced the biocatalyst activity against sunflower oil and *p*-NPA (Table 2), as well as modified the bead porosity (as was revealed by the analysis of the microscopic images of the biocatalysts). Additionally, as was earlier shown [2], the entrapped *Mucor* lipase is characterized by higher thermal and storage stability, in comparison to the native enzyme.

Table 1
Hydrolytic ($A_H$) and synthetic ($A_S$) activities of lipase preparations

| | Membrane-bound lipase | Membrane-bound lipase immobilized in PVA-cryogel |
|---|---|---|
| Substrate $A_H$ ($\mu$mol (min g)$^{-1}$) | | |
| (1) *p*-NPA | 9.7 | 3.6 |
| (2) *p*-NPP | 17.2 | 9.5 |
| (3) Sunflower oil | 484 | 126 |
| Product $A_S$ ($\mu$mol (min g)$^{-1}$) | | |
| (4) Butyl oleate | 240 | 45 |
| (5) Propyl oleate | 460 | 95 |
| (6) Butyl caprylate | 559 | 66 |
| (7) Propyl caprylate | 497 | 32 |

Conditions of the reactions: (1) 1 $\mu$M *p*-NPA in 0.05 M Tris–HCl and 2% acetonitrile (2 ml); 50 mg of cell-bound lipase or 2 g of the immobilized biocatalyst (containing 3.5 mg of lipase per 1 g of the gel); pH 7.5, 25 °C; 10–15 min. (2) Biphasic system: 50 $\mu$M *p*-NPP in hexane saturated with water at 37 °C (1.3 ml) and 10 mM Tris–HCl buffer, pH 7.5 (1 ml); lipase preparation (~3 mg); 37 °C; 10 min. (3) Ten percent of sunflower oil emulsion in 2% PVA (10 ml), preparations of lipase (2 g of wet beads containing ~50 mg of membrane-bound lipase or 50 mg of free mycelium-bound lipase), pH 7; 45 °C; 200 rpm, 30 min. (4–7) 1-Butanol or 1-propanol (1 mmol); oleic or caprylic acid (1 mmol) in petroleum ether (5 ml); free (50 mg) or immobilized lipase (1 g of wet beads containing 51 mg of cell-bound lipase, and dried with acetone before the reaction); molecular sieve 4 Å (2 g); 50 °C; 200 rpm, 30 min.

The mycelium-bound lipases *Mucor* showed very high catalytic activity in synthesis of various esters. Apart from that, these enzymes are very stable in organic solvent solutions ($\log P = 2$–8). Because the PVA-cryogel is also stable in a milieu of organic solvents (alcohols, aldehydes, esters [11,16]) the obtained biocatalyst was tested in the synthesis of different esters. It has been found that water has to be removed from this hydrophilic carrier, before application of the beads for synthesis in organic solvents. Washing with acetone, which gives rise to a decline in dimensions and mass of the beads, appeared to be superior to other methods of water removal, such as washing with ethanol or drying at room temperature. *Mucor* lipases immobilized in the PVA-cryogel treated with acetone display about 20% of synthetic activity of the "free" mycelium (Table 1). Simultaneously, the beads may be used for a long time for the synthesis, in intensively agitated systems, contrary to the mechanically unstable mycelium.

Earlier studies on the dynamics of butyl and propyl oleate synthesis by the membrane-bound *Mucor* lipase in petroleum ether, revealed that during the initial 40 min of the reaction, an amount of ester in reaction mixture was several times enhanced and reduced (every 6–10 min, Fig. 3). After that period, the constant rate of the product synthesis was established. It is believed that water, which is the second product of ester synthesis reaction and remains attached to the *Mucor* mycelium, gives rise to a reverse reaction, namely the ester hydrolysis. This phenomenon was omitted when a molecular sieve (4 Å) or diethanol amine (DEtA) was added to the reaction mixture.

The similar and even stronger effect was observed when various esters synthesis was catalyzed by the cell-bound lipase entrapped in a hydrophilic PVA-cryogel—even in the presence of a molecular sieve in the reaction mixture (Fig. 4). The free acid concentration was either markedly decreased or in-

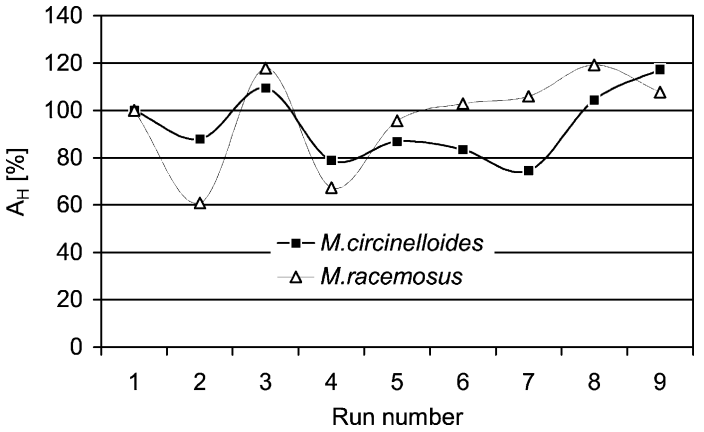


Fig. 1. Hydrolytic activity of *M. circinelloides* and *M. racemosus* lipases, immobilized in PVA-cryogel, as a function of a run number, in successive batch processes of *p*-NPA hydrolysis. Conditions: 1 μM *p*-NPA in 0.05 M Tris–HCl and 2% acetonitrile (2 ml); 2 g of immobilized biocatalyst (containing 3.5 mg of lipase per 1 g of the gel); pH 7.5; 25 °C.

creased (every 30–60 min of the reaction) within the initial 4 h of esterification. It is obvious that water, which is accumulated in the gel, brings about a spontaneous hydrolysis of the ester, which has not left the water-bearing beads of biocatalyst. Diffusion of the water from PVA-cryogel through the petroleum ether to the molecular sieve is markedly slower than in the case of the membrane-bound enzyme. The problem of necessity of selective removal of an excess of water from the biocatalyst was also observed when the microemulsion-based organogels (MBGs) were applied for an immobilization of *Chromobacterium viscosum* lipase [17]. To avoid the decline in synthetic activity of these preparations, the authors invented a method of water removal after every third run cycle. In our case, an application of a molecular sieve into the reaction mixture appeared to provide the high operational stability of the PVA-biocatalyst in ester synthesis. Its synthetic activity continued to gradually increase upon consecutive esterification cycles. Giacometti et al. [18] also described a protocols for the synthesis of acyloglycerols that involved

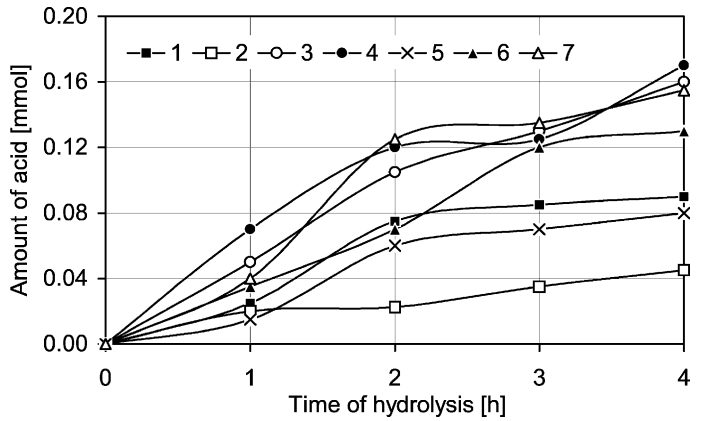


Fig. 2. Hydrolysis of sunflower oil with *M. circinelloides* lipase immobilized in PVA-cryogel in seven (1–7) successive batch hydrolysis processes. Conditions: 10% sunflower oil emulsion in 2% PVA (10 ml); 2 g of immobilized biocatalyst (containing 51 mg of lipase per 1 g of the gel); 45 °C; 200 rpm.

Table 2
Hydrolytic activities of various immobilized lipase preparations

| Preparation of lipase | $A_H$ ($\mu$kat g$^{-1}$)[a] | Yield of hydrolysis ($mmol_{acid}/g_{lipase}$) | $k_{cat}$[b] ($s^{-1}$) |
|---|---|---|---|
| Membrane-bound lipase (free) | 8.07 | 8.58 | – |
| Immobilized in PVA | 1.78 | 1.89 | 2.23 |
| Immobilized in PVA + 3% PEG | 1.98 | 2.23 | 2.19 |
| Immobilized in PVA + 8% PEG | 2.10 | 2.79 | 2.08 |
| Immobilized in PVA + cellulose Whatman | 1.75 | 1.75 | 2.22 |
| Immobilized in PVA + sodium alginate | 1.73 | 1.70 | 2.37 |
| Immobilized in PVA + 0.1% Triton X-100 + oleic acid[c] | 1.50 | 2.66 | 1.41 |

Conditions: ten percent of sunflower oil emulsion in 2% PVA (10 ml), preparations of lipase (2 g of wet beads containing ~50 mg of membrane-bound lipase or 50 mg free mycelium-bound lipase); pH 7; 45 °C; 200 rpm; 30 min.

[a] $\mu kat = \mu mol\, s^{-1}$.

[b] Values of $k_{cat}$ calculated using the program Encora (enzymatic conversion rate analysis) for *p*-NPA enzymatic hydrolysis ($S_0 = 1\,\mu M$ in 0.05 M Tris–HCl buffer pH 7.5 and 2% acetonitrile) which was automatically monitored ($\lambda = 399$ nm) with Beckman spectrophotometer once a minute.

[c] This preparation contained 51 mg of lipase per 1 g of the gel (~100 mg of lipase in the reaction medium).

immobilized lipase in solvent (*n*-hexane) system with 5 Å molecular sieve to extract water formed during the reaction. The conversion rate of esterification was favored by continuous adsorption of water produced during the esterification. Prolonged reaction gave rise to more than 95% efficiency of the esterification, e.g. in reaction of butyl alcohol with oleic acid (Table 3).

The *M. circinelloides* lipase concentration (it means an amount of the acetone dried mycelium in the cryogel beads) appeared to be the most important factor influencing the reaction of butanol and oleic acid. The experiments proved that to achieve the most active PVA-biocatalyst, the beads had to contain the maximum possible concentration of the *Mucor* mycelium, namely approximately 0.05 g per 1 g of the wet gel. Fig. 5 presents the interior of the biocatalyst, which contained 51 mg of the mycelium per 1 g of the bead. The large amount of the lipase-containing hydrophobic *Mucor* mycelium is believed to decrease the

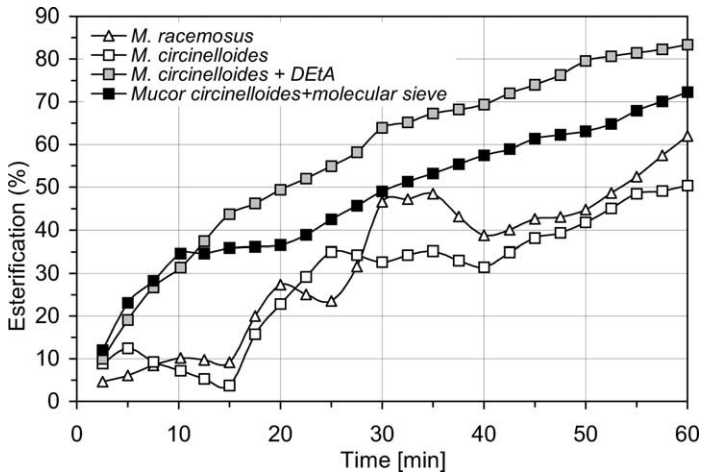


Fig. 3. The dynamics of butyl oleate synthesis by membrane-bound *Mucor* lipases. Conditions: 1-butanol (1 mmol); oleic acid (1 mmol); petroleum ether (5 ml); preparations of free (membrane-bound) lipases (0.05 g); DEtA (0.015 mmol) or molecular sieve 4 Å (2 g); 30 °C; 220 rpm.

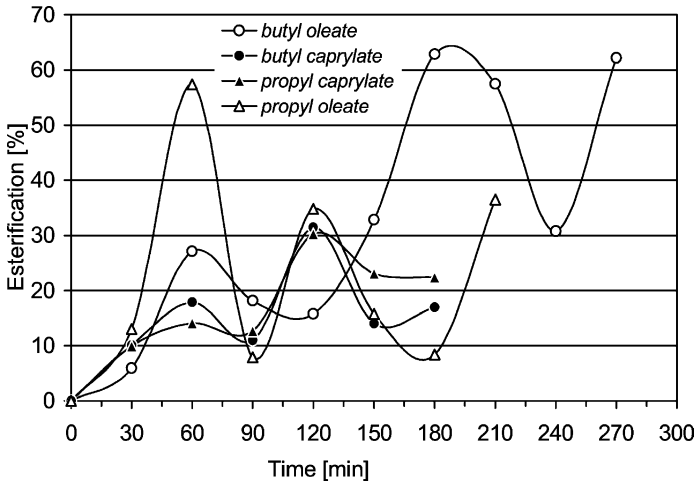


Fig. 4. The dynamics of various esters synthesis by *M. circinelloides* lipase immobilized in PVA-cryogel. Conditions: 1-butanol or 1-propanol (1 mmol); oleic or caprylic acid (1 mmol); petroleum ether (5 ml); preparation of immobilized lipase (1 g of wet beads containing 0.051 g of membrane-bound lipase, and dried with acetone before the reaction); molecular sieve 4 Å (2 g); 50 °C; 220 rpm.

biocatalyst's hydrophilic properties, which are characteristic of the PVA-gel. This is favorable for diffusion of hydrophobic substrates into the pores of the carrier. Treatment of *Mucor* mycelium with oleic acid in hexane and/or the presence of detergent (Triton X-100) in polyvinyl alcohol solution before the cryogel forming, also have a positive influence on synthetic activity of the biocatalyst. Some detergents, e.g. Triton X-100, applied at certain concentration were shown to activate lipases, including the membrane-bound *Mucor* lipases [19]. Hexane and oleic acid stabilize *Mucor* lipases [20], and probably, positively modify

Table 3
Influence of enzyme concentration and various additives on synthetic activity of immobilized lipase preparations

| Sample no. | Lipase concentration in the gel (mg/g) | Additive | Percentage of oleic acid esterification |
|---|---|---|---|
| (1) | 3.3 | – | 4.3 |
| | 8.8 | – | 10.2 |
| | 28.4 | – | 63.3 |
| | 68.0 | – | 74.2 |
| | 51.0 | 0.1% Triton X-100 | 83.1 |
| | 51.0 | 0.1% Triton X-100 + oleic acid | 80.8 |
| (2) | 28.4 | – | 82.0 |
| | 68.0 | – | 96.0 |
| | 51.0 | 0.1% Triton X-100 | 97.0 |
| | 51.0 | 0.1% Triton X-100 + oleic acid | 99.0 |
| | 25.6 | 3% PEG | 63.4 |
| | 22.1 | 8% PEG | 80.0 |
| | 29.4 | Cellulose Whatman | 81.1 |
| | 29.2 | Sodium alginate | 61.7 |

Conditions: 1-butanol (0.5 mmol); oleic (0.5 mmol); petroleum ether (2.5 ml); preparations of immobilized lipase (2 g of wet beads containing the membrane-bound lipase and dried with acetone before the reaction); molecular sieve 4 Å (2 g); 50 °C; 220 rpm; time: 20 h or 8 h. Sample (1): the first run of synthesis, 20 h of esterification; sample (2): the third run of synthesis, 8 h of esterification.

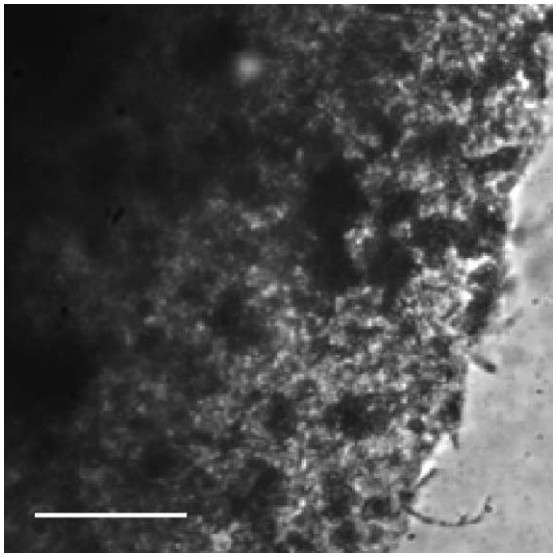

Fig. 5. Microscopic picture of the PVA-cryogel bead with mycelium-bound lipase *Mucor circinelloides*. The concentration of the mycelium 51 mg per 1 g of the gel (bar = 50 μm).

the micro-environment of the enzyme entrapped in hydrophilic PVA-gel. Furthermore, the treatment of mycelium with hexane removes some hydrophobic substances, which have a character of inhibitors, and which were detected in some *Mucor* mycelium [20].

Specific synthetic activity ($A_s$) of lipase-containing PVA-biocatalyst is also enhanced by PEG (8%) addition to the gel. It is not surprising, since preparations of lipases chemically modified with PEG to achieve better stability and solubility in organic solvents, were earlier reported [21,22].

Beads of the immobilized PVA-biocatalyst undergo activation throughout successive runs of butyl oleate synthesis (Table 3). It is impossible to explain this phenomenon at this stage of research, since it may result either from an influence of hydrophobic reaction milieu both on the mycelium bound enzyme (lipase activation [19,22–24], removal of some ballast substances from the mycelium [5]), and/or on the carrier (forming and purging of the channels, which provide diffusion, an increase in the hydrophobicity of the enzyme's micro-environment). An enhancement of activity of immobilized biocatalysts upon their exploitation has also been reported [2,17,21].

The *Mucor* lipases entrapped in polyvinyl alcohol cryogel retained their catalytic activity even after 1-year storage in the refrigerator or at room temperature.

## 4. Summary

Matrices prepared by the method of "cryostructurization" of polyvinyl alcohol aqueous solutions are excellent carriers for production of immobilized membrane-bound *Mucor* lipases preparations, which are stable and effective for a long time, both in hydrolysis and synthesis reactions. Neither organic solvents nor intensive agitation effect the cryogel up to 50 °C. To provide an efficient synthesis of esters in petroleum ether, the PVA biocatalyst, containing about 5% (w/w) of *M. circinelloides* mycelium, has to be dried with acetone before the reaction. The yield of the ester synthesis varies within the initial 4 h of the reaction. The phenomenon is believed to result from the cyclic accumulation of water, which is released during the ester synthesis, in the hydrophilic PVA-gel, which gives rise to hydrolysis of ester, occurring in beads of the biocatalyst.

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