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IMMOBILIZATION OF LIPASE BY PHYSICAL ADSORPTION ON POLYETHYLENE TEREPHTHALATE BEADS

Hristina N. Panajotova[1], Valentina T. Dobreva[2], Hristina N. Strinska[1], Boriana Y. Zhekova[1] and Georgi T. Dobrev[1,*]

[1] Department of Biochemistry and Molecular Biology, University of Food Technologies, Bulgaria

[2] Department of Engineering Ecology, University of Food Technologies, Bulgaria

ABSTRACT— *An extracellular lipase of Aspergillus carbonarius was immobilized by adsorption onto a polyethylene terephthalate support. The optimum conditions for immobilization of lipase were studied. The optimum concentration of enzyme solution for immobilization was 35 kU/g of support, at which 2.28% immobilization efficiency was achieved. The optimum incubation time for immobilization of the enzyme was 4 hours. The optimum pH for immobilization was pH 5. The immobilized lipase retained 93% of its initial activity after 5 cycles of transesterification reaction in n-hexane medium.*

Keywords: adsoption, *Aspergillus carbonarius*, immobilization, lipase, polyethylene terephthalate

1. INTRODUCTUON

The steadily growing interest in lipases over the last two decades stems from their biotechnological versatility and the ability of these enzymes to catalyze a broad spectrum of bioconversion reactions with tremendous potential in various areas such as in food technology, biomedical sciences and chemical industry. Many of these applications are performed with immobilized lipases. Immobilized lipases have been successfully used as catalysts for synthesis of esters, which are applied in food industry as flavoring agents and in oleochemical industry for production of biodiesel or modified fats (Aravindan et al., 2007).

The esterification and transesterification reactions are performed in non-aqueous organic medium (Adlercreutz, 2013). In the presence of organic solvents many enzymes are easily denatured and inactivated. Several physical and chemical methods, such as immobilization, modification and entrapment for stabilizing enzymes in the presence of organic solvents may be used (Kumar et al., 2016). The immobilization not only improves the stability of the biocatalyst but also provides its repeated use and easy separation from the reaction media (Minovska et al., 2005).

The problem of selecting the support material for lipase immobilization and the proper technique is very important and therefore the pursuit for suitable materials has not yet ceased. The major methods for enzyme immobilization are adsorption, covalent coupling, cross-linking and entrapment. The physical immobilization by adsorption of enzymes has some advantages regarding covalent immobilization: the support may be reused after inactivation, saving costs of support expenses and disposal, especially when it is not biodegradable, the support groups are stable for long time periods even under non-controlled temperatures, and immobilization protocols are very simple (Virgen-Otiz et al., 2017). The main problem of physical adsorption is the risk of enzyme desorption during operation. However, there are cases when lipases have been

*Corresponding author at Department of Biochemistry and Molecular Biology, University of Food Technologies, 26 Maritza Blvd., 4002 Plovdiv, Bulgaria, e-mail: g_dobrev2002@yahoo.fr

adsorbed on hydrophobic supports via interfacial activation (Manoel et al., 2015) and the enzyme stability is improved significantly (Santos et al., 2015). Many lipases bind strongly to hydrophobic surface where they undergo interfacial activation (Zisis et al., 2015).

Synthetic polymers are widely used as supports for enzyme immobilization. Polyethylene terephthalate (PET) is a polymer considered to be appropriate for lipase immobilization for its various forms, durability, easy handling, and low cost.

The aim of the paper is optimization of some conditions for lipase immobilization by physical adsorption on PET beads.

2. MATERIALS AND METHODS

Enzyme

Lipase used for immobilization was produced in submerged cultivation of *Aspergillus carbonarius* NRRL 369 (Dobrev et al., 2015).

Immobilization conditions

Optimum conditions for immobilization were determined by changing the conditions individually. The optimization experiments were made with unmodified PET beads. The effect of incubation time, enzyme concentration, pH, and the amount of support was studied and hydrolytic lipase activity was determined by the method described below.

- *Effect of incubation time on lipase immobilization*

To determine the optimal incubation time for maximum activity of immobilized enzyme 0.5 g PET beads and 2.5 ml lipase solution (28 kU) were incubated at 25°C for 1-5 hours.

- *Effect of enzyme concentration on lipase immobilization*

To determine the effect of lipase concentration on the activity of the immobilized enzyme, 16-120 kU lipase and 0.5 g PET beads were used. They were incubated at 25°C for 4 hours.

- *Effect of pH on lipase immobilization*

The effect of pH on the activity of the immobilized lipase was studied in pH range from 3.0 to 9.0 (50 mM phosphate buffer) at 25°C for 4 hours.

- *Effect of support amount on lipase immobilization*

Immobilization was studied with various support amounts (0.2-1.0 g) in 28 kU lipase solution for 4 h adsorption at 25 °C.

- *Reusability and storage stability of immobilized lipase*

The operational stability was tested by repeated batch experiments. The reuses were evaluated in organic (n-hexane) and aqueous media by determination of transesterification and hydrolytic activity of immobilized lipase. After each reaction run, immobilized beads were removed and washed with buffer to remove any residual substrate, then reintroduced to fresh reaction medium.

The storage effect on the activity was evaluated by measuring the residual activities of PET beads in a batch operation mode after dry storage at 4°C for 90 days.

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Enzyme activity assay

- Hydrolytic activity

Lipase activity was measured by spectrophotometric method with modification (Kaushik et al., 2006). 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 (1 kU=1000 U).

- Transterification activity

p-NPP and butanol were used as substrates for determination of transesterification activity (Fu et al., 2014). 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. The conversion was calculated based on a calibration curve. One unit enzyme activity was the amount of lipase liberating 1 µmol of pNP per min at these conditions.

3. RESULTS AND DISCUSSION

Effect of incubation time on lipase immobilization

The duration of enzyme immobilization affects the activity of immobilized catalyst. Immobilization time from 2 to 5 h was investigated and the hydrolytic activity of lipase immobilized on PET beads was measured (Fig. 1).

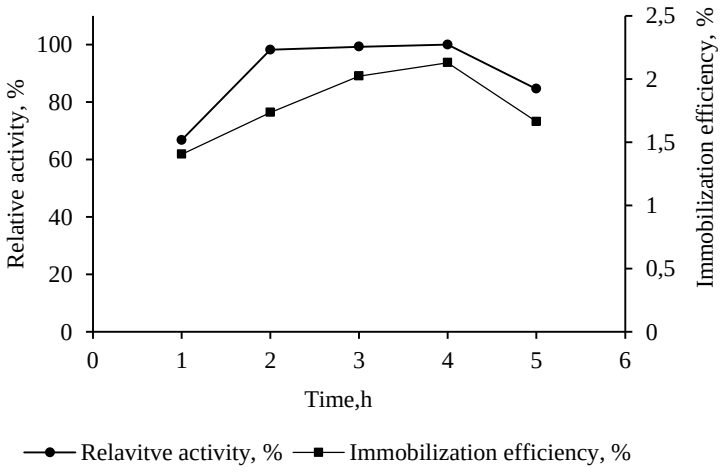

Fig.1 Effect of incubation time on lipase immobilization

It was found that at 4 h the activity of the immobilized enzyme reached its maximum. Longer immobilization time led to decline in relative activity. There are two possible reasons for decrease

of lipase activity: long term agglomeration of lipase on PET beads or limited active surface of the support, which may limit the access of the substrate. Thus, 4 h of lipase immobilization time was used throughout our studies.

Effect of enzyme concentration on lipase immobilization

In Table 1 the effect of enzyme concentration on lipase immobilization is shown. The enzyme concentration was varied from 16 to 120 kU lipase. When low enzyme concentration was used the immobilization efficiency and immobilization yield were higher. The maximum immobilization efficiency (2.25 %) was achieved when 28 kU enzyme concentration was used. With increasing the amount of lipase, activity yield and immobilization efficiency started to decrease, due to increase in unbound enzyme. At high enzyme loading (above 59 kU) the efficiency of immobilization declined as a consequence of the saturation of the support which leads to a loss in the capacity to bind protein.

Table 1. Effect of enzyme concentration on immobilized lipase activity

Added units, kU (A)	Unbound units, kU (B)	Active immobilized units, kU (I)	Immobilization yield, % (A-B)/A*100	Activity yield, % I/A*100	η, %
16.10	13.07	6.71	18.82	41.61	2.21
28.00	22.87	11.57	18.32	41.32	2.25
59.00	37.24	10.97	36.88	18.59	0.50
90.10	42.48	13.74	52.85	15.25	0.28
120.00	37.77	16.18	68.53	13.48	0.19

* η immobilization efficiency % = (Activity yield %/ Immobilization yield %)

Effect of pH on lipase immobilization

pH is one of the factors that influence the binding of lipase to the support due to change in ionization of the system components. The effect of pH on immobilization is presented on Fig. 2.

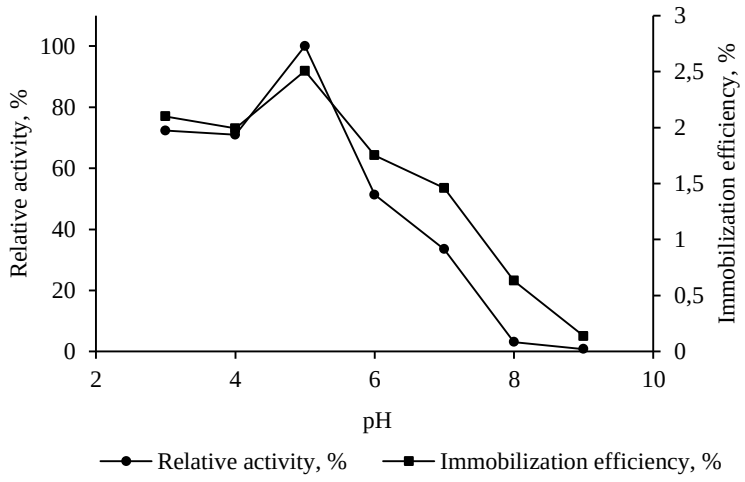

Fig.2 Effect of pH reaction media on lipase immobilization

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The results showed that an acidic pH range from 3.0 to 5.0 was optimal for immobilization. At pH higher than 5.0 the relative activity and immobilization efficiency rapidly decreased with increase of pH up to 9.0. At pH 5.0 the enzymatic polarity might be weakened, which could enhance lipase binding onto PET surface. Thus, pH 5.0 was selected for the following experiments.

Effect of amount of support on lipase immobilization

The relationship between the amount of the support and lipase immobilization is shown in Fig.3. The hydrolytic activity of the immobilized enzyme was linearly proportional to the amount of the support up to 0.6 g. At higher support amount than 0.8 g the relative activity of immobilized enzyme stayed constant, probably because all available lipase has been taken off by the support. Maximum immobilization efficiency of 2.28 % was achieved at enzyme loading of 35 kU/g of support.

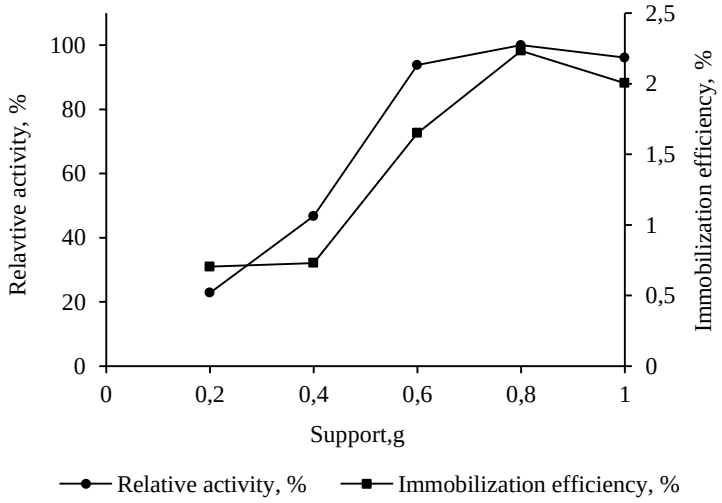

Fig.3 Effect of amount of the support on lipase immobilization

Reusability and storage stability and of immobilized lipase

In order to decrease the cost, enzymes must be reused while maintaining a high level of activity. In present study, the immobilized enzyme was reused up to 5 recycles in organic media (n-hexane) for transesterification reaction, retaining more than 90 % relative activity (Fig. 4).

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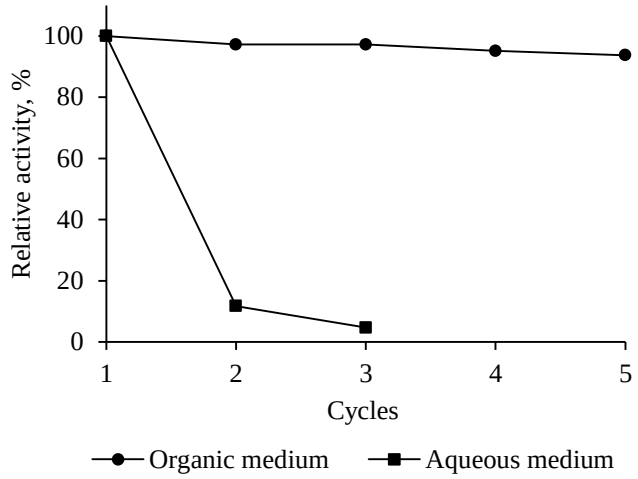

Fig.4 Effect of reuse on the activity of immobilized lipase

In aqueous media the immobilized lipase lost its hydrolytic activity after third cycle, which is probably due to the easy desorption of the enzyme from the carrier. However, repeated experiments using the same catalyst in organic transesterification reaction showed better stability. The immobilized lipase retained 93 % of its initial activity after 5 cycles. Organic solvents improve the solubility of substrates and thus, increase the initial rate of the reaction and enables catalysis of specific reactions occurring under these conditions. For industrial purposes, repeated interesterification using the same immobilized biocatalyst is of great importance.

In addition, the immobilized lipase on PET beads was kept dry at 4°C for 90 days, and its hydrolytic activity was tested periodically to examine the storage stability. The immobilized lipase retained more than 80 % of its hydrolytic activity after 90 days of the storage period.

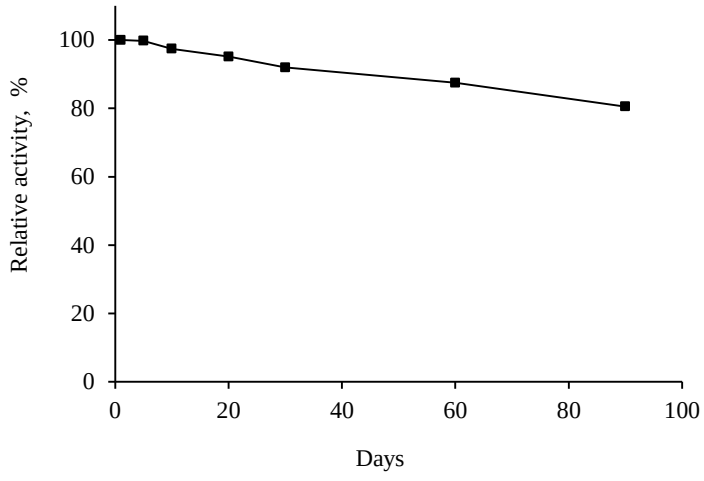

Fig.5 Storage stability of lipase immobilized on PET beads

The immobilized lipase was characterized by good storage stability, which can make the immobilized enzyme advantageous and allows long-term use.

Conclusion

Successful immobilization of lipase from *Aspergillus carbonarius* was carried out by physical adsorption on polyethylene terephthalate beads. The pH at which the adsorption is conducted is important since ionic interactions are crucial in such immobilization. The study of the influence of pH media showed that the maximum immobilization efficiency was observed at acidic pH 5.0. The maximum enzyme load of PET beads was 35 kU/g at the optimum conditions. The immobilized lipase in organic media (n-hexane) retained 93% of its initial transesterification activity after 5 cycles. It indicates that the immobilized lipase on hydrophobic polyethylene terephthalate beads has potential for industrial application.

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