O9-2 **Comparison of properties of immobilized lipase biocatalysts**

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INTRODUCTION AND OBJECTIVES

Usage of native enzymes leads to many complications with their separation. Replacing them with immobilized ones solves most of the problems. In that case lipase is entrapped in pores of sol-gel derived glasses, created via hydrolysis and policondensation of alkoxysilanes (Reetz 1996, Soares 2004, Noureddini 2007). The most popular precursor of the process is tetramethoxysilane. However, nowadays many other alkyl-derivatives are in use (Khimich 2004, Livage 1997, Douglas 2006). Traditionally the process is leaded in block which is dried and crushed (Reetz 1996, Soares 2004, Noureddini 2007, Kmiecik 2007). The main objective of this study is to obtain spherical biocatalyst beads in one operation (emulsification) and examine its properties comparing with traditional block method.

MATERIAL AND METHODS

Materials

Tetramethoxysilane (TMOS, Fluka) – precursor, lipase from *Candida rugosa* (Type VII, Sigma-Aldrich) – enzyme for immobilization, poly(vinyl alcohol) (PVA, Fluka) – stabilizer, sodium fluoride (NaF, POCh S.A.) – process catalyst, magnetite (Fe_3O_4 powder<5µm, Sigma-Aldrich) – magnetic additive for separation method, mixture of n-heptane (POCH S.A.) and liquid paraffin (POCh S.A.) – dispersion phase, p-nitrophenyl acetate (pNPA, Sigma-Aldrich) – lipase substrate, p-nitrophenol (pNP, Sigma-Aldrich) – lipase product, 96% ethanol (POCh S.A.) – solvent for pNPA and pNP.

Methods

It is widely accepted that sodium fluoride 1M solution causes immediately combined hydrolysis and policondensation of TMOS (Reetz 1996, Soares 2004, Noureddini 2007). Our previous research (Kmiecik 2007) showed how strictly the reaction rate depends on the concentration of sodium fluoride in solution. Using less concentrated NaF prolongs reaction. Duration time of emulsification method requires only 2 minutes; obviously it involves adequate concentration of catalyst. Moreover, using magnetite enables simple separation of biocatalyst and its reusage.

In the block method, the lipase solution in concentration of 53mg/ml in 0.02M buffer phosphate at pH=7 was prepared and appropriate amount of magnetite was added. Then 1.5ml of 4% PVA in water solution and 0.75ml of sodium fluoride solution were added to buffered lipase. The whole amount was mixed for 15 seconds. In the next step 8ml of TMOS was added. The mixture was mixed for the further 40 seconds and placed into the closed plastic vessel for 24 hours ageing in 4°C. After that the vessel was opened and left drying for 7 days. Then the received block of xerogel was dispersed in a mortar.

The block policondensation was replaced by emulsification operation method. After 40 seconds of mixing the whole amount was placed in to the mixture of n-heptane and liquid paraffin. Suspension was mixed for the further 5 minutes to give nice spherical beads which then were filtrated and rinsed 3 times by n-heptane and 3 times by phosphate buffer. Drying was the same way as in block's procedure and the same fraction was taken for further analysis.

Model reaction of hydrolysis of pNPA to pNP (Salis 2005, Hara 2008) was used for activity analysis. The method was adjusted to the measurement requirement. 50µl of 0.04M ethanol solution of pNPA was added into 10ml of phosphate buffer (pH=7), containing the appropriate amount of biocatalyst and being mixed and thermostated in 37°C. The reaction duration time was 5 minutes. Operation was accomplished by magnetic separation of biocatalyst beads. The solution absorbance at 400nm wavelength was immediately measured by using spectrophotometer UV-VIS (Jasco V-530) and Spectra Manager program.

RESULTS AND DISCUSSION

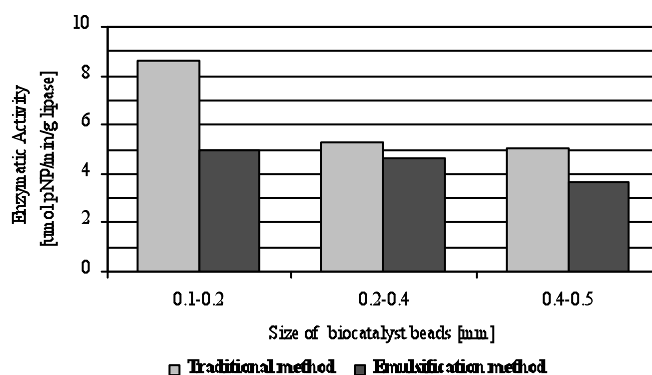

Figure 1: Influence of beads' sizes on biocatalyst activity

The activity of biocatalyst is given as amount of µmol of released pNP per one gram of lipase in biocatalyst, per minute [µmol pNP/min/g lipase]. The measurement error is lower than 3.5% of real value.

Figure 1 shows parallel increase of the activity with decrease of the beads sizes. That is obviously caused by the diffusion effect. High activity increase of 0.1-0.2mm beads, prepared with the block method, is probably caused by irregular beads shape and domination of dust in fraction. For further analysis beads of sizes 0.2-0.4mm were taken.

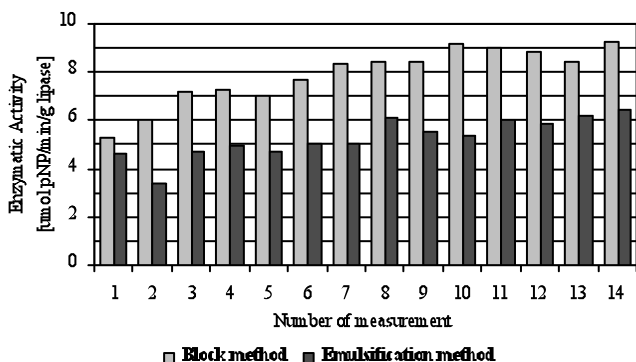

Figure 2 : Activity of biocatalysts in the 14th following bath reactions

The activity of biocatalysts increases in the 14th following batch reactions (Figure 2). That is probably caused by the moisture effect. Reagents need a time to reach all protein in beads. The activity decreased while biocatalyst was prepared by the emulsification method, in opposite to the traditional one. That was caused by the surface/volume ratio, which is the lowest for sphere. Moreover, the advantage of the spherical beads is better mechanical resistance.

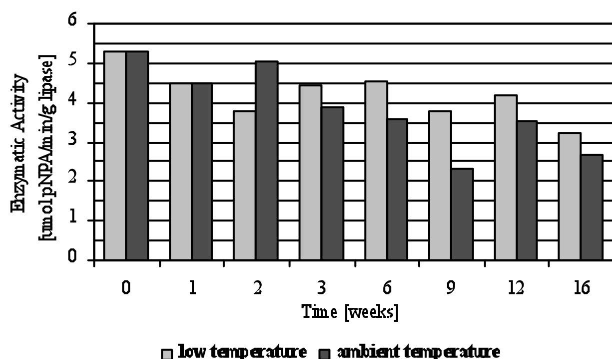

Figure 3 : Activity of stored biocatalyst prepared by block method

The fraction size 0.2-0.4mm was divided in two portions, which were stored in ambient and low temperature (4°C). The activity was measured periodically during the time of storage. Results are shown in Figure 3 and 4. The activity decreased more significantly during the time of storage of emulsification prepared biocatalyst. Beads with irregular surface showed up more activity fluctuation among each sample.

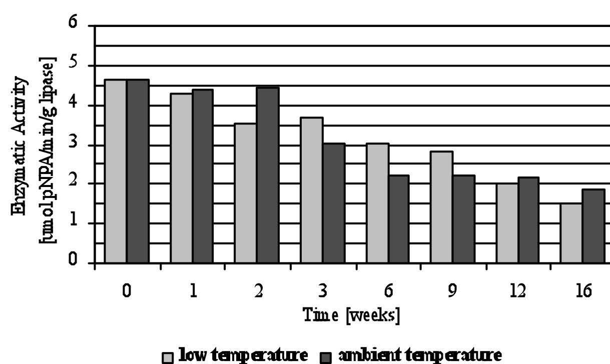

Figure 4 : Activity of stored biocatalyst prepared by emulsification method

CONCLUSIONS

Results presented in this study proved that an experimental emulsification method leads to spherical beads, which are more mechanical resistant and have more stable activity than in the block method. In both methods there is a directly proportional dependence between activity and beads sizes, caused by the diffusion effect. The smaller beads show higher activity whereas the bigger ones can be more easily separated and reused. The 14th following bath reactions of the same biocatalyst sample showed that enzyme is immobilized well and does not leak from the biocatalyst's beads. Unfortunately, the biocatalyst needs more time to achieve the stable activity in the following bath reactions. Both methods lead to the product, which can be stored in low and ambient temperature.

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