Immobilization of lipase by sol-gel method

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INTRODUCTION

Lipases (EC 3.1.1.3) are one of the most common enzymes. They are widely used in biotechnology because they can catalyze very different reactions (Salis A. 2005). In addition to typical, enantioselective hydrolysis, esteryfication and transesteryfication, they can also catalyze lactonic reactions, polymerization (optically active polymers), amide and peptide synthesis, and even such reactions as oxydaction (synthesis of peroxyacids) (Haliniarz E. 1996). There are some limitations with using enzymes in their free (native) form. Most of them can be solved by using immobilized enzymes instead of native. There are many methods of enzyme immobilization. One of them is the so called sol-gel process which has been under more attention for the last few years. In this method an enzyme is entrapped inside a three-dimensional matrix of metal oxides, most commonly silica. The gel matrix is formed from a sol mixture containing molecules of enzyme. Precursors for that process are usually derivatives of monosilicate acid $Si(OH)_4$ containing an alkyl group R. Alkoxysilanes $Si(OR)_4$ hydrolyse to silanes and then condense with each other or with alkoksysilanes to give siloxanes (≡Si-O-Si≡) (Khimich N. N. 2004). Quite often various alkyl-derivatives are used together with traditional alkoxysilanes. For example alkyl-trialkoxysilanes or dialkyl-dialkoksysilanes (Khimich N. N. 2004, Pierre A. C. 2004, Livage J. 1997, Douglas A. L. 2006). Traditionally the process is leaded in block which is dried and crushed (Reetz M.T. 1996, Soares C. M. F. 2004, Noureddini H. 2007). Then the particles can be classified depending on their sizes. In this work there are two modifications. The main one is an emulsification method that can help to avoid large size distribution of particles. The second one is the addition of magnetite (Fe_3O_4) to the precursor mixture. That gives magnetic properties to the particles which therefore can be simply separated from the products of catalytic reaction.

MATERIAL AND METHODS

Chemicals : Sol-gel precursors: tetramethoxysilane (TMOS, Fluka) and isobutyl(trimethoxy)silane (i-BTMS, Aldrich). Stabilizer: poly(vinyl alcohol) (PVA, Fluka). Catalyst of sol-gel process: sodium fluoride (NaF, Zaklady Azotowe Tarnow). Additive making separation of beads possible: magnetite (Fe_3O_4 powder < 5 μm, Sigma-Aldrich). Dispersion phase for emulsification method (1:1): hexane (Sigma-Aldrich) and rapeseed oil (from the local supermarket). Substrate for lipase: p-nitrophenyl acetate (pNPA, Sigma-Aldrich), product: p-nitrophenol (pNP, Sigma-Aldrich). Solvent for pNPA and pNP: 96% ethanol (POCh S.A.).

Enzyme : Lipase from *Candida rugosa* (Type VII, product number L1754, Sigma-Aldrich).

Immobilization of lipase : Lipase is usually immobilized by using 1M sodium fluoride solution and the gelation process lasts then from 15 to 120s (Reetz M. T. 1996, Soares C. M. F. 2004, Noureddini H. 2007). Previous research (Kmiecik J. 2007) with less concentrated solutions shows that the gelation time is prolonged without significant effect on lipase activity. Because of that

0.01M solution was used to make emulsification method possible. No less important is using magnetite which enables simple separation of biocatalyst from the solution and its secondary usage.

Example 1 : The solution of lipase (40mg/ml of 0.02M buffer phosphate, pH=7) was prepared and magnetite (25mg/ml) was suspended in it. Then 7.5ml of that mixture was put into the reaction vessel containing PVA (4% w/w in water, 1.5ml added) and sodium fluoride (0.01M, 0.75ml added). The mixture was homogenized for 15 seconds. Then 8ml of TMOS was added and again the whole mixture was homogenized for 1 minute. All content was replaced into the plastic vessel. The gelation process lasted about 12 minutes. The vessel was left to stand closed for 24 hours in 4ºC. After that it was opened and left for drying under the same conditions for further 7-10 days. Prepared xerogel was crushed in a mortar. The particles of biocatalyst were classified by sieving depending on their sizes. Fraction of sizes between 0.1mm and 0.325mm was examined for activity.

Example 2 : The procedure is similar to example 1 but instead of 8ml of TMOS 1.8ml and 6.2ml of i-BTMS was added. (The biocatalyst named i-BTMS/TMOS on Figures 1-4, because of the reaction mixture composition).

Example 3 : The procedure is exactly the same as example 1 till the second homogenization. After that the whole amount was gently poured into a vessel containing a dispersion phase (hexane: rapeseed oil, 1:1). The whole mixture was mixed on the magnetic stirrer for 20-25 minutes, filtrated and rinsed by hexane. The beads were left to stand in a closed container for 24 hours. Then the container was opened and biocatalyst left for drying under the same conditions for further 7-10 days. After that the beads were classified by sieving and fraction of sizes 0.1-0.325mm was examined for activity.

Example 4 : The procedure is exactly the same as example 3 but instead of 8ml TMOS 1.8ml and 6.2ml i-BTMS was added.

Activity of immobilized lipase : The activity was measured spectrophotometrically by enzymatic hydrolysis of pNPA to pNP (Salis A. 2005, Hara P. 2008). Standard curve was prepared for pNP. Appropriate amount of biocatalyst was placed in a vessel together with 10ml of phosphate buffer (0.02M, pH=7). The vessel was placed on the magnetic stirrer and thermostated to temperature of 37ºC. The reaction was started by addition of pNPA in ethanol solution. After 10 minutes the biocatalyst was separated from products of the reaction by using a simple device with magnet. The absorbance of solution was measured at 400nm wavelength by using spectrophotometer UV-VIS (Jasco V-530) and Spectra Manager program.

RESULTS AND DISCUSSION

Activity and secondary usage : 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 precision of analytic method was checked by measuring the activity of 4 samples of the same biocatalyst. The measurement error was lower than ±3.5% of real value. The activity of each biocatalyst sample was measured 10 times periodically. Each period was preceded by magnetic separation.

During 10 periodic measurements the activity of immobilized lipase in all examples was quite stable (Figure 1-2). That means the enzyme does not leak from particles of the gel. The initial activity rise in case of i-BTMS/TMOS biocatalyst (Figure 1) is probably caused by moisture effect.

All proteins of lipase are not reachable for substrate at the beginning. This is caused by slow diffusion of a substrate within each particle of biocatalyst.

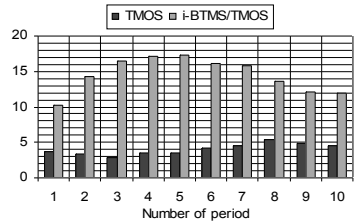

Figure 1: Activity of block polymerized biocatalysts (Examples 1-2)

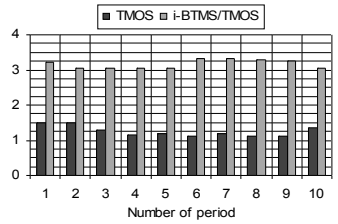

Figure 2: Activity of emulsion polymerized biocatalysts (Examples 3-4)

The differences between activities of TMOS and i-BTMS/TMOS biocatalysts are caused by two main factors. The most important is the influence of hydrophobic group. Matrix formed from TMOS is rather hydrophilic. It does not provide the most optimal environment for lipase's activity. That enzyme needs an interface for activation of its active centre (Haliniarz E. 1996). Using alkyl-derivative precursor such as i-BTMS together with traditional alkoxysilane - TMOS for the sol-gel process leads to more hydrophobic matrix (Pierre A. C. 2004) and increase of immobilized lipase activity. Isobutyl groups probably interact with an active centre of that enzyme and cause the most required conformation. Not less important are the pore sizes. Notable is that the monolith containing isobutyl groups bonding to some atoms of silicon did not shrink as much as the one without alkyl group (Soares C. M. F. 2004). There are also differences between activities of particles prepared by block and emulsion polymerization (compare Figure 3 and 4). Activity decrease of emulsion source beads may be caused by enzyme leakage from forming beads to dispersion phase during sol-gel process. It is possible that lipase has some affinity with rapeseed oil and using non-fatty organic liquid might help to avoid that process. The second possibility is that pore sizes at the surface of beads are narrower than inside and because of that a diffusion of substrate and products is slower. Nevertheless, using additive of magnetite for sol-gel process makes simple separation of biocatalyst beads possible. It is very important when we want to use our biocatalyst many times in batch processes.

Particles shape and size distribution : Pictures of biocatalysts (fractions of sizes 0.1-0.325mm) prepared by four methods described before are shown on Figures 3 and 4. Regular, spherical shape of emulsion polymerized biocatalysts (Figure 4) is seen at first sight. That form is preferable, because it is easier to separate spherical beads from the reaction products than irregular ones and to use it in further following batch catalytic reactions. Moreover emulsion method gives narrower distribution of particles' sizes which leads to more efficient usage of substrates.

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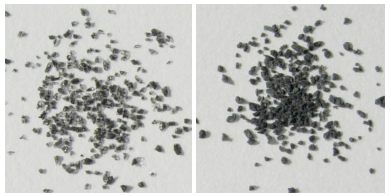

Figure 3: Irregular shape of block polymerized biocatalysts: TMOS (left) and i-BTMS/TMOS (right).

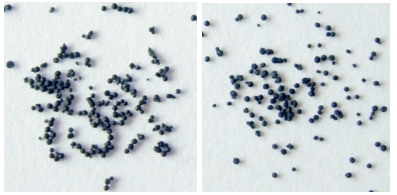

Figure 4: Spherical shape of emulsion polymerized biocatalysts: TMOS (, 4) i-BTMS/TMOS

CONCLUSIONS

Using less concentrated NaF solutions prolongs the gelation time and makes emulsification method possible. Addition of magnetite enables simple separation of the biocatalyst and its reusage. Lipase does not leak from particles of the gel and its activity during 10 periodic measurements is quite stable. Isobutyl(trimethoxy)silane used for the sol-gel process together with tetramethoxysilane (i-BTMS/TMOS) leads to more hydrophobic matrix and increases an immobilized lipase activity. Emulsion method gives nice spherical beads which size distribution is narrower than crushed block particles.

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