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Immobilization of *Yarrowia lipolytica* Lipase—a Comparison of Stability of Physical Adsorption and Covalent Attachment Techniques

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Abstract Lipase immobilization offers unique advantages in terms of better process control, enhanced stability, predictable decay rates and improved economics. This work evaluated the immobilization of a highly active *Yarrowia lipolytica* lipase (YLL) by physical adsorption and covalent attachment. The enzyme was adsorbed on octyl–agarose and octadecyl–sepabeads supports by hydrophobic adsorption at low ionic strength and on MANAE–agarose support by ionic adsorption. CNBr–agarose was used as support for the covalent attachment immobilization. Immobilization yields of 71, 90 and 97% were obtained when *Y. lipolytica* lipase was immobilized into octyl–agarose, octadecyl–sepabeads and MANAE–agarose, respectively. However, the activity retention was lower (34% for octyl–agarose, 50% for octadecyl–sepabeads and 61% for MANAE–agarose), indicating that the immobilized lipase lost activity during immobilization procedures. Furthermore, immobilization by covalent attachment led to complete enzyme inactivation. Thermal deactivation was studied at a temperature range from 25 to 45°C and pH varying from 5.0 to 9.0 and revealed that the hydrophobic adsorption on octadecyl–sepabeads produced an appreciable stabilization of the biocatalyst. The octadecyl–sepabeads biocatalyst was almost tenfold more stable than free lipase, and its thermal deactivation profile was also modified. On the other hand, the

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Y. lipolytica lipase immobilized on octyl–agarose and MANAE–agarose supports presented low stability, even less than the free enzyme.

Keywords Immobilized lipase · *Y. lipolytica* · Biocatalysis · Glutaraldehyde · Hydrophobic supports

Introduction

Microbial lipases (acylglycerol acylhydrolase, EC 3.1.1.3) have been employed in a broad range of applications such as treatment of wastewater with high oil and grease content [1], synthesis of low chain fatty esters as component for cosmetics and surfactants and in production of pharmaceuticals intermediates [2, 3]. Over the past few years, attractive processes have appeared using enzymes as biocatalysts for the synthesis of fine chemicals [4]. Furthermore, biocatalysis has been considered as the most efficient way of producing chiral drugs. However, one of the most important drawbacks of the use of lipases for asymmetric synthesis is the poor water solubility of the majority of organic compounds, while most enzymes present very low stability and activity in organic media. One of the best enzymatic approaches to carry out the production of enantioenriched or enantiopure compounds is based on the application of lipases in organic medium [2, 3, 5]. In this way, microbial lipases are effective biocatalysts due to high-substrate specific activity, good stability in organic solvents, stereoselectivity and also produce low impact on the environment. In fact, the use of lipases in organic solvents has already proved to be an excellent methodology for the preparation of chiral drugs [2, 6].

Reports on immobilization of enzymes first appeared in the 1960s [7]. Since then, immobilized enzymes have been widely used in the processing of a variety of products and in the synthesis of chiral products [6, 8]. The advantages of immobilized over soluble enzymes arise from their enhanced stability and ease of separation from the reaction media, leading to significant savings in enzyme consumption [3]. Lipase immobilization offers important and unique advantages in terms of better process control when the reactions are processed in nonaqueous media. Furthermore, immobilized preparations are frequently more stable than free enzyme and can be easily recycled, which is of crucial importance in industrial processes. Enzyme immobilization can also avoid deleterious effects caused by organic solvents such as enzyme denaturation and agglomeration [1, 9]. The improvement on enzyme stability by immobilization cannot be easily predicted as it is dependent on several factors and even the explanation for the observed stabilization may be diverse [10–14].

Immobilization methods range from binding to prefabricated carrier materials to packaging in enzyme crystals or powders. Adsorption is the most usual methodology for lipase immobilization because it presents minor deleterious effects on the enzyme activity, and it is a low-cost method [3]. Polymeric resins and materials containing hydrophobic groups are often described as good supports for lipase immobilization. Hydrophobic adsorption on porous supports in which the inner shell is covered with thick layer of hydrophobic compounds causes the interfacial activation phenomenon. It has been stressed in the literature that most lipases present a ‘lid’ controlling the access to the active site [15, 16]. Thus, in the hydrophobic adsorption, the enzyme recognizes those supports as a lipid/water interface. As a consequence, it leads to conformation changes exposing the catalytic residues toward the solvent making the binding site accessible to the substrate. The hyperactivation phenomenon is usually observed as a consequence of this method of immobilization which provides stabilization of the active open form of the enzyme [17, 18].

In this context, the present work evaluates the behavior of a highly active *Yarrowia lipolytica* lipase immobilized by two different techniques such as adsorption on hydrophobic (octyl–agarose, octadecyl–sepabeads) and hydrophilic supports (MANAE–agarose) with covalent attachment using cyanogens–bromide–agarose (CNBr–agarose) and cross-linking of YLL immobilized on MANAE–agarose with glutaraldehyde.

Materials and Methods

Materials

Lipase from *Y. lipolytica* was obtained from Centre Wallon de Biologie Industrielle, Faculté Universitaire des Sciences Agronomiques, Gembloux, Belgium. Octyl–agarose, CNBr–agarose and agarose 4BCL were obtained from Hispanagar S.A. (Burgos, Spain). Octadecyl–sepabeads was donated by Resindion S.R.L. Mitsubishi Chemical Co. (Milan, Italy). *p*-Nitrophenyl-butyrate (*p*NPB), Triton X-100 and Cetyl-trimetyl-ammonium bromide (CTAB) were obtained from Sigma Chemical Co. (St. Louis, USA). All other chemicals were of analytical or chromatographic grade.

Methods

Protein Determination

The amount of protein in all samples was determined as described by Lowry et al. [19] using bovine serum albumin as standard.

Yarrowia lipolytica Lipase

Lipase from *Yarrowia lipolytica* was produced in a 2,000-l fermentor containing (*w*/*v*) 1% of glucose, 3% of whey powder, 0.8% of ammonium sulphate, 1% of corn steep syrup and 0.5% of olive oil. After 30 h of fermentation, the culture broth was centrifuged, and the supernatant was dried by lyophilization [20]. The lyophilized powder was than diluted in sodium phosphate buffer 5 mM, pH 7.0 in a final protein concentration of 0.11 mg/ml.

Enzyme Activity

Yarrowia lipolytica lipase (YLL) activity was performed by continuously measuring the increase in the absorbance in 348 nm produced by the release of *p*-nitro-phenol in the hydrolysis 0.4 mM *p*-nitrophenyl-butyrate (*p*NPB) in 25 mM sodium phosphate buffer pH 7 and 28°C. The reaction was initialized by addition of 0.2 ml of lipase suspension to 2.5 ml of substrate solution. One international unit (IU) of *p*NPB was defined as the amount of immobilized YLL necessary to hydrolyze 1 μmol of *p*NPB per minute in assay conditions [21].

Immobilization of Yarrowia lipolytica Lipase

Immobilization on Hydrophobic Supports Standard preparations of octyl–agarose or octadecyl–sepabeads (hydrophobic supports) consisted first in exhaustively washing the supports with distilled water. After that, 1 g of the support was suspended in 10 ml of YLL solution in sodium phosphate buffer 5 mM pH 7, and the mixture was shaken at 25°C and

250 rpm for 2 h, washed with distilled water and stored at 4°C. Periodically, samples of the suspensions and the supernatants were withdrawn, and hydrolytic activity was measured using *p*NPB as substrate as described earlier.

Immobilization on Hydrophilic Supports

By ionic adsorption Amino-glyoxyl–agarose (MANAE–agarose), used as hydrophilic support, was prepared as described by Fernandez-Lafuente et al. [22]. Standard immobilization procedure consisted of the addition of 1 g of the support to 10 ml of enzymatic solution containing Triton X-100 0.1% in phosphate buffer 5 mM pH 7. The mixture was shaken at 25°C and 250 rpm for 2 h, washed with distilled water and stored at 4°C. Periodically, samples of the suspensions and the supernatants were withdrawn and enzymatic activity was measured using *p*NPB as substrate as described above.

By Covalent Attachment

With CNBr–Agarose One gram of CNBr–agarose support was added to 10 ml of YLL solution containing 0.1% (*v*/*v*) Triton X-100. The suspension was stirred (250 rpm) at 4°C for 20 min. After that, it was firstly washed with bicarbonate buffer 0.1 M pH 8.3 and then the suspension was gently stirred for 2 h with 0.1 M Tris–HCl pH 8, filtered and stored at 4°C. Immobilization process was followed by measuring the hydrolytic activity using *p*NPB as substrate.

MANAE–Agarose Cross-linked with glutaraldehyde One gram of the YLL immobilized onto MANAE–agarose was incubated with 1% (*v*/*v*) glutaraldehyde solution in sodium phosphate buffer 5 mM pH 7 and 25°C for 1 h, under mild stirring. After that, the suspension was washed with 5 mM phosphate buffer and then filtered and stored at 4°C. The samples of the suspensions were withdrawn, and YLL activity was measured using *p*NPB as substrate as described above.

Parameters of Immobilization

The yield of the immobilization (η) and the activity retention (R) were calculated according Eqs. 1 and 2.

$$\eta(\%) = \frac{U_A - U_E}{U_A} \times 100 \tag{1}$$

$$R(\%) = \frac{U_H}{U_A - U_E} \times 100 \tag{2}$$

where:

U_A added units or units of activity offered for immobilization
U_E output units or units of activity in the solution after immobilized procedure
U_H immobilized units or units of immobilized enzyme.

Kinetic Parameters of Thermal Inactivation

The thermal inactivation assays of soluble YLL and immobilized preparations on octyl–agarose, octadecyl–sepabeads and MANAE–agarose were carried out incubating the same amount of lipase in three different buffers at 45°C: 25 mM sodium acetate pH 5, 25 mM sodium phosphate pH 7 and 25 mM sodium carbonate pH 9. Periodically, residual enzyme activity was estimated by hydrolysis of *p*NPB. Soluble enzyme was submitted to the same conditions.

The half-life ($t_{1/2}$) time was calculated according to Eqs. 3 and 4:

$$E = E_0 \exp(-kt) \tag{3}$$

$$t_{1/2} = \frac{\ln 2}{k} \tag{4}$$

Where

E enzyme specific activity for a reaction in a time=t(U/g);
E_0 enzyme specific activity for t=0 (U/g);
k observed deactivation rate constant;

Results and Discussion

Immobilization of YLL through adsorption process even on hydrophobic or ionic supports was quite fast. In both cases, the immobilization procedures were completed after 50 min of contact time between the support and enzyme, resulting in immobilization yield up to 97% when *Y. lipolytica* lipase was immobilized in MANAE–agarose. Immobilization yield was slightly lower for enzyme immobilization on octadecyl–sepabeads (90%) and achieved 71% when octyl–agarose was used. The activity retention values were lower for every preparation (34% for octyl–agarose, 50% for octadecyl–sepabeads and 61% for MANAE–agarose), indicating that the immobilized lipase lost activity during immobilization procedures.

Among the two hydrophobic supports, octadecyl–sepabeads presented the best result concerning YLL immobilized activity (15.5 U/g), as its activity was over twice higher than YLL immobilized onto octyl–agarose (7.0 U/g). The other immobilization parameters calculated (immobilization yield and activity retention) were also better for octadecyl–sepabeads (Table 1). These results were probably related with the chemical nature of hydrophobic supports. When a very hydrophobic support like octadecyl–sepabeads is used, there might be an increment of the affinity between the support and the substrate that

Table 1 Immobilization yield (η) and activity retention (R) of *Y. lipolytica* lipase on different supports and hydrolytic activity of the immobilized enzyme.

YLL preparation	Hydrolytic activity ($U_H/g_{support}$)	η (%)	R (%)
Octyl–agarose	7.0	71	34
Octadecyl–sepabeads	15.5	90	59
MANAE–agarose	17.0	97	61
MANAE–agarose–glutaraldehyde	0	97	0
BrCN–agarose	0.6	99	2

promote an increase in substrate concentration in the microenvironment of the enzyme leading to higher enzyme activity. Petkar et al. [23], studying immobilization of lipases on different hydrophobic supports, demonstrated that hydrolytic activity was higher when immobilized in very hydrophobic supports as octadecyl–sepabeads.

On the other hand, the ionic adsorption of *Y. lipolytica* lipase on MANAE–agarose showed activity retention, immobilization yield and immobilized activity of 61%, 97% and 17 U/g, respectively (Table 1). The use of ionic adsorption has been very successfully used for lipase immobilization [24]. Although, the above results are better than the YLL lipase immobilized by adsorption in polypropylene support [25], they showed that these immobilization procedures did not lead to lipase hyperactivation phenomenon, as it was described for lipases of different sources [17, 18]. Hyperactivation of the lipase when immobilized on very hydrophobic octadecyl–sepabeads derivative probably occurs because it occurred with the "open structure" of the lipase that is also much more active than the corresponding "closed" structure even when undergoing multipoint covalent immobilization. In our study, this phenomenon was not observed probably due to the absence of the lid in this *Y. lipolytica* lipase [26].

Although immobilization of YLL onto BrCN–agarose and MANAE–agarose–glutaraldehyde led to higher immobilization yield, the resulted immobilized biocatalysts had almost null activity (Table 1). The immobilization of YLL onto MANAE–agarose support by CNBr-mediated covalent interaction or glutaraldehyde cross-linking with resin amino groups has probably led to inactivation of the enzyme. Covalent interaction is followed by the formation of a Schiff base between aldehyde groups and Lys residues placed on the enzyme surface. This sort of interaction is likely to result in undesired mobility restriction of the enzyme or even displacement of the active site which may inactivate the YLL lipase [3].

Thermal stability study was performed for soluble YLL and immobilized onto octyl–agarose, octadecyl–sepabeads and MANAE–agarose (Figs. 1 and 2). Previous results (not shown) with immobilized and soluble YLL showed that those preparations maintained 100% activity when incubated at pH 5 during 24 h at 25°C and 3 h at 37°C. However, at 50°C, they were very unstable losing 80% enzyme activity in less than 2 h. Based on these results, stability curves were carried out at 45°C at three different values of pH: 5, 7 and 9 as described in the Materials and Methods section. Figures 1 and 2 showed that octadecyl–sepabeads preparations for both pH 5 and 7 presented an expressively higher stability than the soluble enzyme.

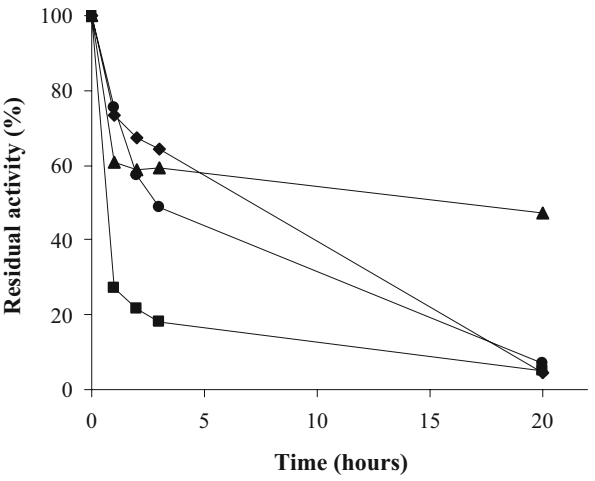

Fig. 1 Thermal stability of the different immobilized preparations and soluble YLL. Experiments were carried out in sodium acetate buffer 25 mM pH 5 and 45°C. Soluble YLL (*filled diamonds*) and immobilized on (*filled squares*) octyl–agarose, (*filled triangles*) octadecyl–sepabeads, (*filled circles*) MANAE–agarose

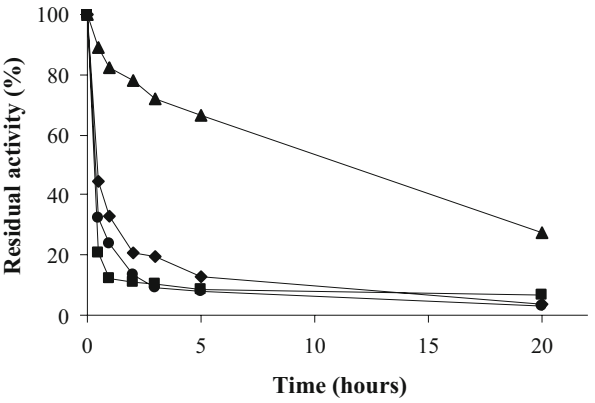

Fig. 2 Thermal stability of the different immobilized preparations and soluble YLL. The inactivation courses were carried out in 25 mM sodium phosphate buffer pH 7 and 45°C. YLL immobilized on (*filled squares*) octyl–agarose, (*filled triangles*) octadecyl–sepabeads, (*filled circles*) MANAE–agarose and (*filled diamonds*) soluble

However, for those experiments carried out at pH 9, all immobilized preparations and the soluble enzyme showed a very slight stability losing activity in less than 1 h.

Table 2 shows half-life time and inactivation coefficient for YLL soluble and immobilized on different supports. The enzyme immobilized on MANAE–agarose support presented lower stability than the soluble enzyme, perhaps because the immobilized derivative has been prepared in the presence of detergent to ensure the enzyme desegregation could be monomers, while soluble enzyme as dimers [27, 28]. Random immobilization may not really improve enzyme rigidity; even in some cases, the enzyme stability may decrease after immobilization [10–14], e.g., if the support is able to establish undesired interactions with the enzyme.

The YLL lipase immobilized onto octadecyl–sepabeads was the most stable one in all conditions, presenting half-life ten times higher than the soluble preparation at pH 7.0. Palomo et al. [8] demonstrated that the interfacial adsorption on a hydrophobic resin (octadecyl–sepabeads) was the best immobilization technique for *B. thermocatenulatus* lipase. This immobilized preparation, retaining 100% of the initial activity at high temperature, is even better than the covalent immobilization by multipoint interaction on glyoxyl support, where the lipase retained only 80% activity.

Wilson et al. [27] working with lipase QL from *Alcaligens* sp immobilized in different supports also found that for octadecyl–agarose preparation, the stability enhanced about 20 times when compared with animated supports or multipoint covalent immobilization.

Table 2 Kinetic (k and $t_{1/2}$) parameters of thermal inactivation of the soluble *Y. lipolytica* and immobilized onto different supports.

YLL preparation	$t_{1/2}$ (h)	k (h^{-1})	Experimental conditions
Soluble	4.7	0.15	
Octyl–agarose	1.4	0.48	pH 5
Octadecyl–sepabeads	26.6	0.03	45°C
MANAE–agarose	2.9	0.24	
Soluble	0.9	0.72	
Octyl–agarose	0.7	1.06	pH 7
Octadecyl–sepabeads	10.5	0.06	45°C
MANAE–agarose	0.7	0.93	

Conclusion

This work shows that lipase from *Y. lipolytica* does not undergo hyperactivation phenomenon that is largely observed for lipases from other sources when immobilized in hydrophobic supports [12, 13]. However, immobilization by physical adsorption showed to be the key for the immobilization process and especially in very high hydrophobic supports that are by far, the best way to get an optimal compromise between activity and stability. Our results also showed that YLL lost activity when immobilized by multipoint covalent attachment.

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