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Biocatalysis in Organic Media by Cutinase Immobilized in Reversed Micelles and Zeolites

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1. Introduction

Lipases have been successfully used in the hydrolysis of triglycerides, for the production of free fatty acids. However, in a low-water environment, the reverse of hydrolysis, namely esterification and transesterification reactions, have been performed. Lipases are effective biocatalysts when acting at an interface between an insoluble substrate (triglycerides) and an aqueous phase wherein the enzyme is soluble, which renders lipases quite suitable for the applications in non-conventional media [1].

These reactions can be carried out in reversed micellar media, where the water, necessary for the reaction, is stored inside the micelles, while the enzyme acts on the lipidic interface separating the organic and aqueous phase. When used on a support matrix, the activity and quantity of the water retained by the immobilization support are very important factors for the enzyme activity in non-aqueous solvents. Taking into account these characteristics, it is envisaged that it is also worthwhile to use zeolites as lipase immobilization supports. Zeolites are known to be capable of storing water in their intracrystalline void space, and it could be easily envisaged the use of the external surface of the zeolitic material as the interface between the aqueous phase, contained within the zeolite's framework, and the organic medium with the substrate.

In the present study, a *Fusarium solani pisi* recombinant cutinase displaying lipolytic activity was encapsulated in reversed micelles and adsorbed onto zeolites. Cutinases are a group of hydrolytic enzymes

capable of degrading the insoluble lipid polyester matrix, *i.e.*, cutin, which covers plant surfaces. They belong to the class of serine esterases containing the classic catalytic triad serine, histidine, and a carboxy group [2], and are able to hydrolyze a wide variety of synthetic esters and triglycerides [2][3]. Cutinase, however, differs from classical lipases in that no flap exists in its structure, and no measurable interfacial activation around the critical micellar concentration (CMC) of the triglyceride substrate is observed [3].

This work describes the use of microencapsulated cutinase and, adsorbed on zeolites, in hydrolytic and transesterification (alcoholysis) reactions.

2. Microencapsulated Cutinase in Reversed Micelles

Reversed micelles are a suitable medium to promote biocatalysis in organic media. The solubilization of an aqueous enzyme in the water pool of reversed micelles allows the retention of catalytic activity due to the shell formed by the surfactant molecules, which protects the enzyme against denaturation by the organic solvent. The amount of water solubilized is usually defined by the parameter W_o ($W_o = [H_2O]/[surfactant]$). Such enzymatic systems create a microenvironment that provides a particular reaction medium, the protein molecule being in an optimal localization corresponding to its nature. These systems provide an interfacial area of *ca.* 100 m²ml⁻¹, which is much higher than that of other conventional biphasic biocatalytic systems [4].

One of the most used and well characterized surfactant is sodium di(2-ethylhexyl) sulfosuccinate (AOT) which was applied to the microencapsulation of cutinase. Isooctane was chosen as the organic solvent due to its suitability to perform enzyme microencapsulation in AOT-reversed micelles.

2.1. Transesterification Activity of Microencapsulated Cutinase

Cutinase solubilized in AOT-reversed micelles was used to catalyze the transesterification reaction of butyl acetate with hexanol. The optimization of the reaction conditions followed an experimental planning based on a 2⁵⁻¹ fractional factorial design, expanded further to a central composite design (CCD) according to *Box et al.* [5] and *Barker* [6]. Some relevant parameters for the cutinase activity such as pH, concentrations of surfactant, hexanol, and butyl acetate, and the amount of water were studied. The effects of each factor were calculated and the interaction effects were determined. It was found that an increase of acetate concentration enhances proportionally the reaction rate in the conditions studied although hexanol shows an optimum. In addition, several interactions were detected, the most important was between hexanol and AOT concentrations. As the *Fig.* shows, when a low concentration of hexanol was used, the increase of the AOT concentration reduces the reaction rate, although for hexanol concentrations of *ca.* 600 mM the transesterification was enhanced with AOT. The molar ratio water to surfactant, W_o , influenced greatly the activity and showed an optimum range between 5 and 8.

2.2. Cutinase Stability in AOT-Reversed Micelles

In the range of temperatures studied, the highest enzyme activity was obtained at 40°, which was quite different from those previously obtained in the hydrolysis and esterification reactions catalyzed by cutinase in AOT-reversed micelles [7][8]. The studies carried out with cutinase microencapsulated in AOT-reversed micelles in the absence of hexanol [7] showed an optimum at 18° and a total inactivation of the biocatalyst at 35°. At the experimental conditions of W_o 5, pH 9.6, and a temperature of 25° the half-life time displayed by cutinase was *ca.* 5 h [7]. This fast decrease on stability is probably due to the toxicity of AOT for cutinase [9], as in aqueous solution, it is active at temperatures up to 85° [2].

The stability of cutinase in AOT-reversed micelles and in the presence of 400 mM hexanol (close to the optimum activity) was tested at 25° and 40°. At 25°, cutinase was very stable (90% of initial activity after 11 d of incubation), and the enzyme displayed a half-life time of 3 d at 40°. These results confirm the stabilization effects of hexanol on the cutinase microencapsulated in AOT-reversed micelles [8].

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3. Adsorption of Cutinase on Zeolites

The well-known general features of zeolites arise from their negatively charged aluminosilicate crystalline structures. They consist of a three-dimensional arrangement of SiO_4 and AlO_4 tetrahedra linked to each other by a shared O-atom. The resulting framework leads to the generation of an intracrystalline porous network with a pore size of molecular dimensions and to the presence of compensating cations within the framework itself. These features have been responsible for the extensive applications of these materials in catalysis, separation processes, and ion-exchange. The possibility of creating and regulating acid-base, hydrophobic, and selective adsorption properties within a wide range of values constitutes an important factor in the widespread use of zeolites. Another important aspect is the possibility of synthesizing zeolitic structures that range from the hydrophobic character to a highly hydrophilic behavior, making them suitable for processes involving both aqueous and organic media.

In the present study, cutinase was immobilized by adsorption on different zeolites [10]. The immobilized enzyme was tested on the hydrolysis of tricapylin in organic medium. The effect of the framework composition, expressed by the Si/Al ratio, on the enzyme activity was evaluated. The activity and stability results obtained with cutinase adsorbed on NaY zeolite were compared with those obtained with cutinase adsorbed onto a polyamide (Accurel PA6) support.

3.1. Selection of Zeolites as Cutinase Supports

Cutinase adsorbed on different types of zeolites showed a wide range of activity values, depending on the type of zeolite that was used (Table). It can be seen that the coupling yields are usually rather high, indicating that the enzyme has very good affinity towards the zeolite's surface. The specific activity, however, changes widely with the zeolite support, being the best results obtained with the NaY zeolite.

3.2. Stability of Cutinase Adsorbed on NaY Zeolite

Cutinase immobilized on each support was stored in isooctane at 30°, and its hydrolytic activity was periodically evaluated. Cutinase adsorbed on the zeolite presented the highest stability, with no loss of initial activity after 45 d, while the cutinase immobilized on PA 6 displayed a half-life time of 6.5 d. The higher stability presented by the enzyme immo-

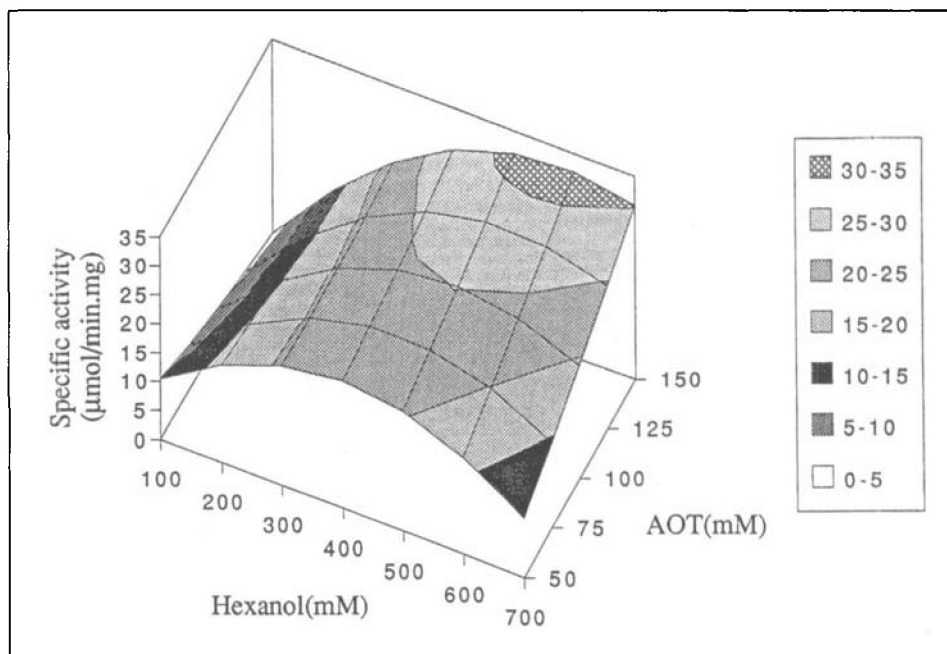


Figure. Effect of surfactant and hexanol concentrations on the specific transesterification activity of microencapsulated cutinase. Experimental conditions: 50 mM carbonate buffer pH 9, $T = 25^\circ$, [enzyme]: 0.3 mg/ml, 400 mM butyl acetate and 1.08% (v/v_{total}) H_2O .

Table. Effect of Zeolite Type on the Coupling Yield for Cutinase Immobilization and Activity for the Hydrolysis of Tricaprylin

Zeolite	Framework Si/Al	Activity [U/g support]	Specific Activity [$\mu\text{mol}/\text{min mg}$]	Coupling Yield [%]
NaA	1	13.5	0.74	73
NaX	1.5	20.3	1.08	75
NaY	2.4	91.7	5.41	69
NaUSY	4.5	39.5	2.14	74
NaDY	6.0	77.0	4.03	76

bilized on the NaY zeolite may be linked to the characteristics of this support, which has a strong interaction with water. This remains within the enzyme preparation in the case of the zeolite, while with the polyamide support some decrease in the hydration of cutinase can occur during the incubation with isooctane.

- [1] E. Rogalska, S. Ransac, R. Verger, *J. Biol. Chem.* **1990**, 265, 20271.
- [2] M. Lauwereys, P. de Geus, J. de Meutter, P. Stanssens, G. Matthyssens, in 'Lipase-Structure, Mechanism and Genetic Engineering', Vol. 16, Eds. L. Alberghina, R.D. Schmid, and R. Verger, VCH, Weinheim, 1991, p. 243.
- [3] C. Martinez, P. de Geus, M. Lauwereys, G. Matthyssens, C. Cambillau, *Nature* **1992**, 365, 615.

- [4] C. Laane, R. Hillhorst, V. Veeger, in 'Methods in Enzymology', Vol. 136, Ed. K. Mosbach, Academic Press, New York, 1987, p. 216.
- [5] G.E.P. Box, W.G. Hunter, J.S. Hunter, 'Statistics for Experimenters', John Wiley & Sons, New York, 1985.
- [6] T.B. Barker, 'Quality by Experimental Design', Marcel Dekker Inc., New York, 1985.
- [7] E.P. Melo, M.R. Aires-Barros, J.M.S. Cabral, *Appl. Biochem. Biotechnol.* **1994**, 49, 45.
- [8] M.J. Sebastiao, J.M.S. Cabral, M.R. Aires-Barros, *Biotechnol. Bioeng.* **1993**, 42, 326.
- [9] E.P. Melo, S.M.B. Costa, J.M.S. Cabral, *Photochem. Photobiol.* **1996**, 63, 169.
- [10] A.P.V. Goncalves, J.M. Lopes, F. Lemos, F.R. Ribeiro, D.M.F. Prazeres, J.M.S. Cabral, M.R. Aires-Barros, *J. Mol. Catal. B: Enzymatic* **1996**, 1, 53.