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Immobilization of a Recombinant Cutinase by Entrapment and by Covalent Binding

Kinetic and Stability Studies

A. P. V. GONÇALVES, J. M. S. CABRAL, AND M. R. AIRES-BARROS*

Laboratório de Engenharia Bioquimica, Centro de Engenharia Biológica, Instituto Superor Técnico, Av. Rovisco Pais, 1000 Lisboa, Portugal

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ABSTRACT

Fusarium solani pisi recombinant cutinase, immobilized by entrapment in calcium alginate and by covalent binding on porous silica, was used to catalyze the hydrolysis of tricaprylin. The influence of relevant parameters on the catalytic activity such as pH, temperature, and the substrate concentration were studied. Cutinase immobilized by entrapment presented a Michaelis-Menten kinetics for tricaprylin concentrations up to 200 m*M*. At higher concentrations of substrate, inhibition was observed. For covalent binding immobilization, diffusional limitations were observed at low substrate concentrations and substrate inhibition occurred for concentrations higher than 150 m*M*. The stability of immobilized cutinase was also evaluated. The enzyme immobilized by entrapment showed a high stability, in contrast to the immobilization on porous silica.

Index Entries: Recombinant cutinase; gel entrapment; covalent binding; hydrolysis; kinetic; stability.

INTRODUCTION

The use of organic solvents as reaction media can improve the productivity of the biotransformation of hydrophobic substrates like triglycerides. However, when an organic solvent is introduced in the medium

*Author to whom all correspondence and reprint requests should be addressed.

containing the enzyme, the intramolecular interactions between the enzyme molecules may be changed, and the enzyme may become inactive. Another problem is poor solubility or poor dispersibility of enzymes in organic solvents (*1*), which may lead to apparent low or no enzyme activity.

Enzyme immobilization seems to be a good approach to solve these problems (*2*), as often a higher enzyme stability is obtained due to the interaction of the enzyme with the support, leading to a more rigid enzyme conformation. Furthermore, the hydrophilic supports entrap water in the microenvironment of the enzyme (*3*), which is essential for enzyme stabilization and activity, and immobilized enzymes may be reused and operated in continuous reactors.

In this paper two different methods of immobilization using hydrophilic matrices are studied: entrapment in calcium alginate gel and covalent binding on porous silica. The model enzyme is a recombinant cutinase from the fungus *Fusarium solani pisi*, overproduced in *Escherichia coli* (*4*). The cutinase immobilized by the two methods was used to catalyze the hydrolysis of tricaprylin. Some relevant parameters that influence the catalytic activity of the enzyme such as pH, temperature, water content in the immobilized enzyme, and substrate concentration are studied. The effect of agitation rate and of support particle size on the activity of the enzyme covalently bound to porous silica are also investigated. Cutinase stability is evaluated in the presence of iso-octane and also with several additives. The deactivation parameters are calculated using a model of series-type mechanism of Henley and Sadana (*5*).

MATERIALS AND METHODS

Enzyme

Fusarium solani pisi was produced and purified in our laboratory from *Escherichia coli* recombinant strain, which was a kind gift of Corvas International (Gent, Belgium). Cutinase production and purification was done according to methods previously published (*4*). This enzyme has a molecular weight of 22,000 Da and an isoelectric point of 7.8.

Chemicals

Sodium alginate of *Laminaria hyperborea* was purchased from BDH. Porous silica with a mean pore size of 1250 A and a particle size between 100 and 200 μm was from Prolabo. γ-Aminopropiltrietoxisilane (98% purity) was from Aldrich (Milwaukee, MI). Glutaraldehyde (25%, w/v) and glycerol (87%, w/v) were from Merck (Rahway, NJ). Tricaprylin (97% purity) was purchased from Fluka (Buchs, Switzerland). Caprylic acid (99% purity) was obtained from Sigma (St. Louis, MO). All salts and organic solvents were analytical reagent grade.

IMMOBILIZATION OF CUTINASE

Entrapment in Calcium Alginate

Entrapment of cutinase in calcium alginate (*6*) was carried out by adding a mixture of aqueous sodium alginate (1.5%, w/v, 2 mL) and cutinase solutions (5 mg in 60 μL buffer) dropwise into a solution of calcium chloride (0.1*M*, 4 mL) containing the same concentration of cutinase. All the solutions were prepared in tris-maleate buffer (50 m*M*, pH 8.0). The gelling took 15 min and the gel beads were carefully dewatered between two sheets of filter paper before use.

Covalent Binding on Porous Silica

Support activation was carried out by the silanization method (*7*) by adding 25 mL of a 1% (v/v) solution of γ-aminopropiltrietoxisilane in acetone to 1 g of porous silica. The mixture was evaporated until dryness and heated at 155°C overnight. Glutaraldehyde was used as a bifunctional reagent to link the ε-amino groups of the enzyme to the amino groups of the activated silica, by adding 2.5 mL of a 0.625% (v/v) solution of glutaraldehyde in phosphate buffer (50 m*M*, pH 8.0) to 0.1 g of activated silica. The reaction was allowed to continue for at least 60 min. After exhaustively washing the support with distilled water, the immobilization reaction was carried out by adding 1 mL of enzyme solution (5 mg), in phosphate buffer with 1% (v/v) glycerol to 0.1 g of support. The coupling reaction took 2 h. The immobilized enzyme was washed with buffer for 15 min before use.

Water Determination

The water content of the immobilized cutinase was determined using a Mettler DL18 Karl Fisher Titrator.

Protein Assay

The amount of immobilized protein was determined by a modified Folin assay (*8*).

Cutinase Activity Assay

The enzymatic activity was assayed by the hydrolysis of tricaprylin, following the formation of caprylic acid by the method of Lowry and Tinsley (*9*). Reactions were started by the addition of 10 mL of a solution of tricaprylin (100 m*M*, unless otherwise stated) in iso-octane to flasks containing the immobilized enzyme. The reactions were carried out in an orbital stirrer at 300 rpm and 30°C. Samples (0.5 mL) were taken every 5 min during 25 min and every 15 min during 75 min for cutinase immobilized by covalent binding and by entrapment, respectively. The samples

Table 1
Hydrolytic Activity and Coupling Efficiency of Immobilized Cutinase[a]

Immobilization method	Activity, μmole/min·g of support	Specific activity, μmole/min·mg	Coupling yield, %
Entrapment	0.633	0.254	50
Covalent binding	39.3	3.93	26

[a]([tricaprylin] = 100 m*M*, at 30°C, and pH 8.0).

were added to test tubes containing 2 mL of toluene. A copper (II) acetate-pyridine aqueous solution (0.5 mL) was added and the mixture was immediately vortex mixed for 1 min. After centrifugation (4000 rpm, 10 min) the absorbance of the organic phase was measured at 715 nm. A blank was prepared taking a sample at zero time and using the same procedure as described above.

Cutinase Stability Assay

The immobilized cutinase was incubated at 30°C, without stirring, in flasks containing 5 mL of iso-octane. The cutinase thermal stability was evaluated by determining the initial reaction rate for the hydrolysis of tricaprylin (100 m*M*) at different incubation times. The deactivation parameters were obtained using the Henley and Sadana series-type mechanism (*5*). The application of the Henley and Sadana model was done using a non-linear regression procedure with the Marquadt method of interactive convergence.

RESULTS AND DISCUSSION

Enzyme Immobilization Efficiency

The immobilization efficiency and hydrolytic activity of the immobilized cutinase preparations are shown in Table 1. Cutinase covalently bound to porous silica displays a catalytic activity 60-fold higher than the entrapped enzyme. However, its coupling efficiency is only half of that observed with the calcium alginate entrapped cutinase.

The immobilization methods (entrapment and covalent binding) are quite different concerning the water content of the immobilized enzyme preparations. After immobilization the amount of water is 1.65% (v/v) for cutinase covalently bound to silica and 9.60% (v/v) for cutinase entrapped in calcium alginate gel, relative to the reaction medium volumes.

The activities displayed by immobilized cutinase (Table 1) are very low when compared with those observed in aqueous solutions. Lauwereys

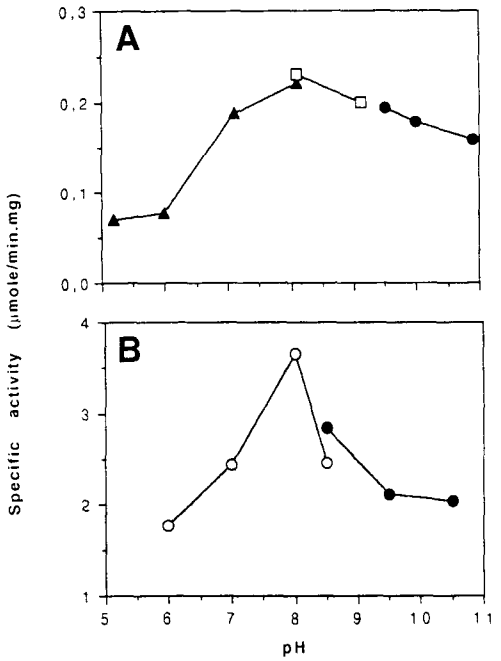

Fig. 1. Effect of pH on the catalytic activity of immobilized cutinase by entrapment in calcium alginate gel (**A**) and covalently bound to silica (**B**) for the hydrolysis of tricaprylin (100 m*M*) in iso-octane, at 30°C. (A) (▲) tris-maleate buffer; (□) tris-HCl buffer; (●) sodium carbonate buffer. (B) (○) sodium phosphate buffer; (●) sodium carbonate buffer.

et al. (*4*) obtained 4000 µmole/min.mg of cutinase for tributyrin hydrolysis and 800 µmole/min.mg of cutinase for triolein hydrolysis. Melo et al. (*10*) obtained 30 µmole/min.mg of cutinase for the hydrolysis of triolein in micelles of AOT-isooctane.

The lower activities obtained with the immobilized cutinase, for the hydrolytic reactions in iso-octane, may be due to a toxic effect of the solvent on the enzyme, as no activity was detected with the free enzyme, in iso-octane. It may also be caused by the substrates' and/or products' diffusional limitations across the immobilization matrix.

Effect of pH

The effect of immobilization pH on the catalytic activity of immobilized cutinase for the hydrolysis of tricaprylin (100 m*M*) at 30°C was evaluated (Fig. 1A and B). The maximum activities were obtained at pH 8.0, for both methods. This result is slightly different from the optimum pH, 8.5, obtained for the hydrolysis of tributyrin with free enzyme (*4*). This difference is probably due to partitioning pH effects in the microenvironment of the immobilized enzyme preparations.

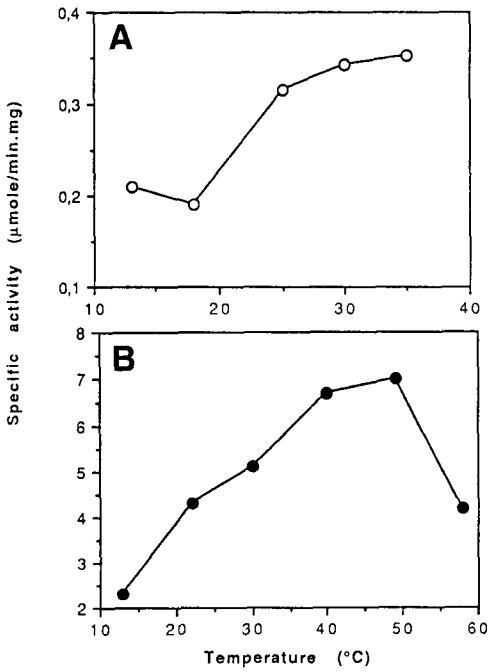

Fig. 2. Effect of temperature on the catalytic activity of immobilized cutinase: (**A**) (○) by entrapment in calcium alginate gel, and (**B**) (●) covalently bound to silica, for the hydrolysis of tricaprylin (100 m*M*) in iso-octane, at pH 8.0.

The selected pH for further studies was 8.0, in sodium phosphate buffer for cutinase covalently bound to silica, and in tris-maleate buffer for cutinase immobilized by entrapment in calcium alginate.

Effect of Temperature

The temperature-activity profiles of immobilized cutinase at pH 8.0 are shown in Fig. 2A and B. Optimum temperatures were obtained at 35 and 50°C for cutinase entrapped in calcium alginate and covalently bound to porous silica, respectively.

The catalytic activity at high temperatures observed with cutinase covalently bound to silica may be related to the lower mobility of the enzyme molecules. These molecules are covalently bound to a solid matrix and in a system with a low amount of water. Both factors are known to contribute to increase the enzyme rigidity and thermostability (*11*).

Immobilization by entrapment, at temperatures higher than 35°C, lead to the collapse of calcium alginate gel beads.

The activation energy of the hydrolysis of tricaprylin was calculated from an Arrheniun plot. The values obtained were 6.65 kcal/mole and 4.32 kcal/mole for cutinase covalently bound to porous silica and entrapped in calcium alginate, respectively. The values of activation energy imply

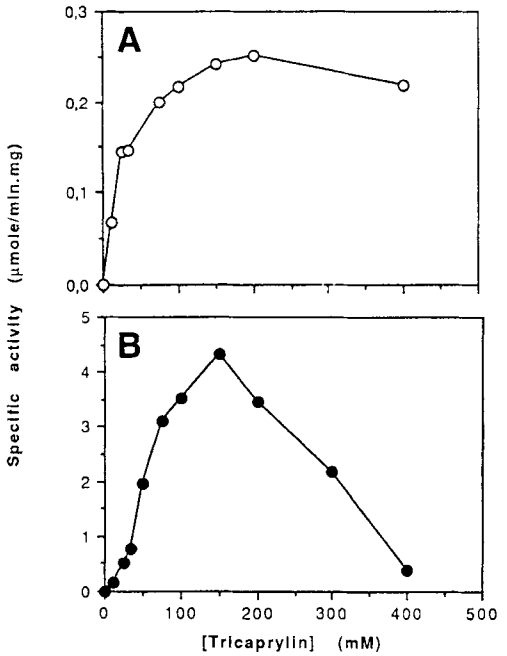

Fig. 3. Effect of tricaprylin concentration on the catalytic activity of immobilized cutinase: (**A**) (○) by entrapment in calcium alginate gel, and (**B**) (●) covalently bound to silica at pH 8.0 and 30°C.

that the enthalpy of the transition state or activated complex in covalently bound cutinase is higher than in the entrapped enzyme. This suggests that a larger amount of energy is necessary for the formation of the activated complex in the cutinase immobilized by covalent binding.

Effect of Substrate Concentration

The effect of tricaprylin concentration on the activity of immobilized cutinase preparations at pH 8.0 and 30°C was also evaluated. The results are shown in Fig. 3A and B.

Entrapped cutinase presents a Michaelis-Menten kinetics for substrate concentrations below 200 m*M*. Substrate inhibition was observed at 400 m*M*. The apparent kinetic constants of the Michaelis-Menten equation were determined using a computer iterative method: $k_{cat,app}$ = 6.51 min^{-1} and $K_{m,app}$ = 35.6 m*M*. The apparent specificity constant (k_{cat}/K_m) was also calculated, k_{cat}/K_m = 0.183 min^{-1} M^{-1}.

For covalent binding immobilization, diffusional limitations were present at low substrate concentrations (12.5, 25, and 35 m*M*) and substrate inhibition was observed for concentrations higher than 150 m*M*.

In order to reduce external diffusional resistances, the catalytic activity of covalently immobilized cutinase was determined at agitation rates of 300, 370, and 415 rpm, at 30°C, using tricaprylin concentrations of 12.5

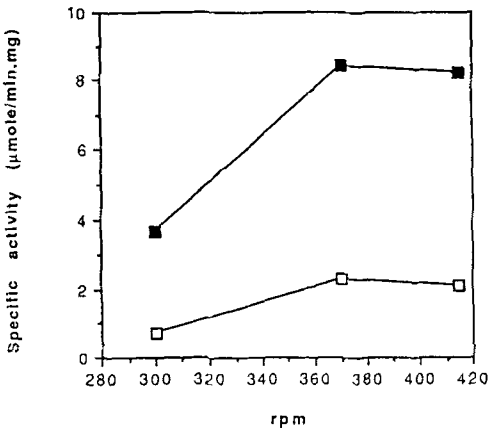

Fig. 4. Effect of agitation rate on the activity of covalently bound cutinase, for tricaprylin hydrolysis at concentrations of (□) 12.5 m*M* and (■) 100 m*M*, pH 8.0 and 30°C.

m*M* and 100 m*M* (Fig. 4). Above 370 rpm, and for both tricaprylin concentrations, the cutinase activity becomes independent of the agitation rate. Therefore, a slightly higher agitation rate (380 rpm) was chosen to be used in the kinetic studies.

The internal diffusional resistances can be lowered by increasing tricaprylin concentration or decreasing silica particle size. A strong inhibition of the cutinase was observed at high tricaprylin concentrations. Alternatively, the effect of silica particle size on the internal diffusional resistances was analyzed. The support was separated in four groups using the following sizes of sieves: 90, 125, 177, and 200 µm. The activity of the immobilized enzyme was evaluated, at pH 8.0, 30°C, 380 rpm, at different tricaprylin concentrations, using the four particle sizes. The results obtained are shown at Fig. 5. Inhibition of the enzymatic activity was observed at high substrate concentrations, above 75 or 100 m*M*. depending on silica particle sizes.

Table 2 shows the cutinase specific activity and the amount of covalently bound protein at pH 8.0, 30°C, 380 rpm and 75 m*M* tricaprylin, using different particle sizes. Decreasing particle size led to a decrease of the cutinase specific activity, probably because the enzyme is more exposed to the organic solvent (iso-octane).

The second order rate constant of the reaction, which can be considered as the apparent specificity constant (*12*), was also determined for the different particle size. The results presented in Fig. 6 show that the apparent specificity constant is independent of particle size up to 108 µm and after that increases proportionally with the increase of particle size. The intrinsic value of the specificity constant is then obtained when the reaction is not diffusional limited (at the minimum value of the specificity constant). The intrinsic specificity constant for cutinase immobilized in organic media was calculated from Fig. 6 being 1.3 10^3 M^{-1} min^{-1}. The different

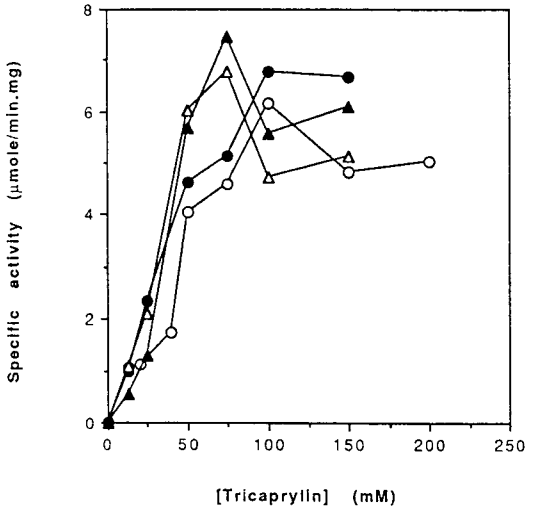

Fig. 5. Effect of tricaprylin concentration on the activity of covalently bound cutinase, at pH 8.0, 30°C, and 380 rpm, using the following silica particle sizes (average values): (○) – 45 μm; (●) – 108 μm; (△) – 151 μm; (▲) – 189 μm.

Table 2
Effect of Silica Particle Size on the Activity of Immobilized Cutinase[a]

Particle size, μm	Specific activity, μmole/min·mg of enzyme	Bound protein, mg protein/g of support
45	4.58	16.0
108	5.14	12.2
151	6.79	11.9
189	7.46	12.2

[a]([tricaprylin] = 75 mM, pH 8.0, 30°C, and 380 rpm).

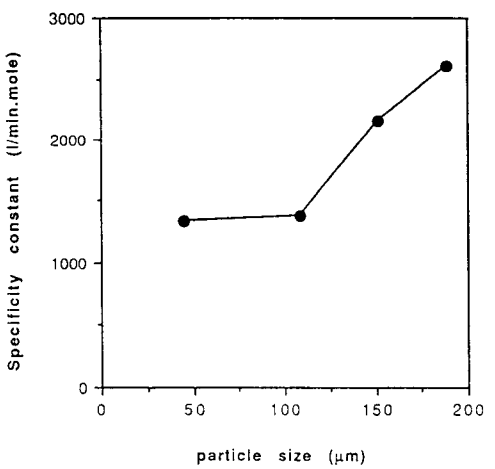

Fig. 6. Effect of silica particle size on the apparent specificity constant for the hydrolysis of tricaprylin catalyzed by covalently bound cutinase at pH 8.0, 30°C, and with an agitation rate of 380 rpm.

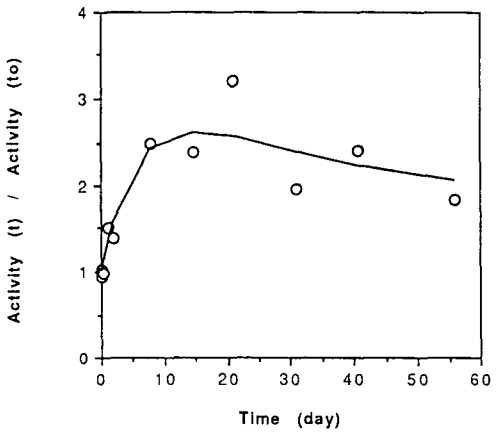

Fig. 7. Stability studies of cutinase immobilized by entrapment in calcium alginate gel, after incubation in iso-octane at 30°C. Experimental points and curve obtained with the Henley and Sadana model (k_1 = 0.092 day^{-1}, k_2 = 0.092 day^{-1} α_1 = 4.70, α_2 = 2.00).

behavior obtained in organic media when compared with immobilized enzymes in aqueous solution, where the specificity constant increases with decreasing particle size until being independent of it, is probably due to a toxic effect of the solvent on the enzyme.

Cutinase Stability

The thermal stability of immobilized cutinase in iso-octane at 30°C was investigated. The enzyme immobilized by entrapment showed a high stability. The catalytic activity increases during the first ten days of incubation, probably because the gel porosity increases faciliting the flux of substrate, and after 56 d the activity of the immobilized enzyme is 1.84-fold higher than the initial activity.

The Henley and Sadana series-type mechanism was applied to the results and the calculated parameters are shown in Fig. 7. The ratios of specific activities indicated that the cutinase is converted in a more active enzymatic form (E_1 = 3.2 E) and then there is a transition to a third enzymatic form (E_2 = 1.84 E). The first transition is faster than the second one (k_1 = 4.4 k_2). The higher activity observed after the first week of incubation should be a consequence of the reorganization of the alginate gel structure, leading to a better transport process of substrate and/or products.

Immobilization by covalent binding on porous silica lead to very low stabilities, with a half-life of 24 min (Fig. 8), as the enzyme is in direct contact with the solvent and not protected by a gel layer as in calcium alginate entrapment.

The cutinase stability was also evaluated after incubation with glycerol at 30°C, for 45 h, and the half-life obtained was 1 h 30 min (Fig. 8),

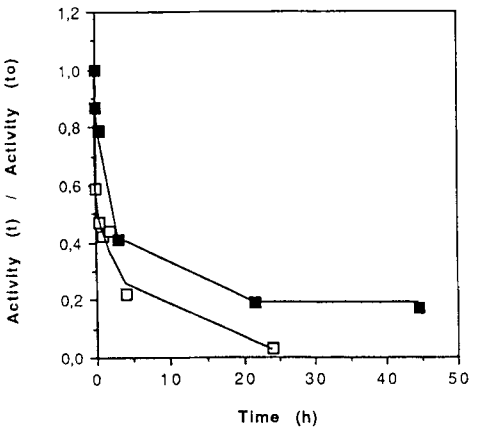

Fig. 8. Deactivation profile of cutinase covalently bound to silica, after incubation in iso-octane (■) with and (□) without glycerol at 30°C. Experimental points and curve obtained with the application of the Henley and Sadana model. (■): $k_1 = 1925h^{-1}$, $k_2 = 0.369\ h^{-1}$, $\alpha_1 = 0.887$, $\alpha_2 = 0.187$); (□): ($k_1 = 23.2\ h^{-1}$, $k_2 = 0.189\ h^{-1}$, $\alpha_1 = 0.525$, $\alpha_2 = 0.021$.

3-fold higher than without glycerol. Organic molecules like polyhydric alcohols, interact strongly with water molecules, reducing the water activity and promoting hydrophobic interactions within the polypeptide chain, thereby increasing thermal stability of enzymes (*13,14*).

CONCLUSIONS

Fusarium solani pisi recombinant cutinase was immobilized by entrapment in calcium alginate gel and by covalent binding on porous silica, and both systems were able to hydrolyze tricaprylin.

Cutinase immobilized by covalent binding presented a catalytic activity 60-fold higher than cutinase immobilized by gel entrapment. Maximal activities were obtained at pH 8.0 and 35 and 50°C for cutinase entrapped in calcium alginate and covalently bound to silica, respectively.

Cutinase immobilized by entrapment presented a Michaelis-Menten kinetics relative to tricaprylin concentrations, while a first-order kinetics was observed for cutinase immobilized by covalent binding. For both methods inhibition of enzymatic activity occurred at high substrate concentrations.

Cutinase immobilized by covalent binding on porous silica showed poor enzyme stability in iso-octane, with a half-life of 24 min. Glycerol improved the enzyme half-life to a value three-fold higher. However cutinase immobilized by entrapment in calcium alginate presented high stability, with no deactivation after 56 d of incubation in iso-octane.

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