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Enantioselective properties of *Fusarium solani pisi* cutinase on transesterification of acyclic diols: activity and stability evaluation

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Abstract

The regio and enantioselectivity of a recombinant cutinase from *Fusarium solani pisi* was tested on three racemic and one prochiral phenylalkanediols via irreversible transesterification with vinyl acetate. The optimization of the reaction conditions involved the screening of different organic solvents as well as the variation of the substrate concentrations. Thus, the enzymatic activity was checked by measuring initial reaction rates, overall yields, and enantiomeric excess of the reaction products. Only the smaller molecules were recognized by the enzyme, and a denaturing effect of the acyl donor was observed. Nevertheless, a stabilising effect on the enzyme caused by a pre-incubation with the diol was also noted.

Keywords: Cutinase; Transesterification; Phenylalkanediols; Alcohol-stabilising effect; Substrate recognition

1. Introduction

Cutinase is an extracellular enzyme excreted by phytopathogenic fungi which is responsible for the in vivo hydrolysis of cutin, a hydroxy fatty acid polymer that covers and protects plants against pathogenic microorganisms [1–4]. When used in vitro, this enzyme behaves like a lipase, being able to catalyse the hydrolysis of both water-soluble esters and long-chain triglycerides [5–9], as well as the reversal of hydrolytic reactions [10–14]. The X-ray structure of native cutinase from *Fusarium solani pisi* [15] revealed its parallel $\forall/\exists$ fold, and the classical serine-hydrolase catalytic triad Ser120, His188, Asp175. Unlike lipases, the catalytic site of cutinase is not protected by an amphipathic helicoidal loop (or lid), and the oxyanion hole, normally formed upon substrate binding in lipases, is preformed in cutinase [16,17]; these two facts could be responsible for the absence of interfacial activation detected in cutinase catalytic behaviour [15]. This intermediate behaviour, between lipolytic and esterolytic enzymes, has made cutinase

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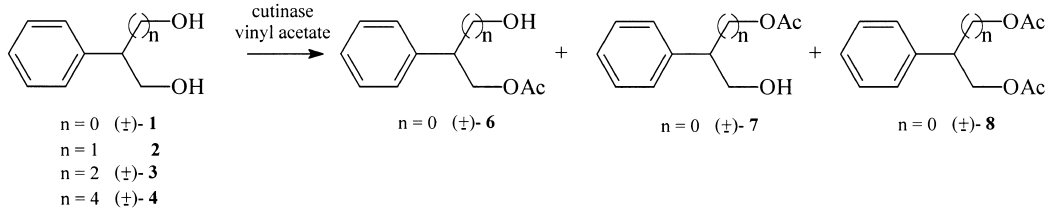

Fig. 1. Cutinase-catalysed resolution of diols **1**–**4**.

the object of many studies about the different aspects of its catalytic performance, such as substrate binding [18–20], hydrophobicity mapping by xenon and krypton binding [21], unfolding modelling [22], protein engineering of the enzymatic surface [23] or study of the effects of amphipaths on the enzymatic activity [9].

Despite the high thermostability of this enzyme [6], there is still little information dealing with its use as enantioselective catalyst for biotransformations [15,18,24]. The cutinase specificity towards small substrates (esters, fatty acids, and alcohols) has been described [12,18,20,24], and it is also known that the alcohol used as substrate in acyl-transfer processes (esterifications or transesterifications) stabilises cutinase encapsulated in AOT-reversed micelles [7,11,13]. Thus, in this work, we study the transesterification in the organic media of three racemic and one prochiral acyclic phenylalkanediols (Fig. 1) with vinyl acetate in order to test the enzymatic ability for the stereodiscrimination of these substrates, as well as to prove if the alcohol-stabilising effect is produced in the absence of any micellar system.

2. Experimental

2.1. Materials

Recombinant cutinase from *F. solani pisi* was obtained and purified according to a previously published method [25]. The racemic alcohol (±)-**1** and all the reagents and solvents used were purchased from Aldrich Chemical, Alcobendas, Spain. The prochiral diol **2**, as well as the racemic diols (±)-**3** and (±)-**4**, were prepared as described elsewhere [26].

2.2. Previous study of the reaction conditions

In order to establish the adequate conditions for the transesterification of the diols **1**–**4**, a preliminary study of the influence of the quantity of biocatalyst, acylating agent/substrate molar ratio and logP of organic solvent on the reaction performance was made. The standard reaction conditions were 25°C, 10 ml of solvent, and 0.2 M of substrate, with magnetic stirring. In all cases, the influence of those parameters was evaluated measuring the initial rate in the biotransformation of diol **1** (standard substrate).

2.3. Enzyme-catalysed transesterifications of diols *1*–*4*

A solution of diols **1**–**4** (0.2 M) and vinyl acetate (1.6 M) in dichloromethane (10 ml) was stirred at 25°C with cutinase (5 mg). Then, aliquots of 0.1 ml were taken from the solution (at different times) and added to 0.9 ml of an 80/20 *n*-hexane/isopropanol mixture; after microfiltration, they were analyzed by HPLC. The spectrophotometrical quantification (8 = 254 nm) of product concentration and the enantiomeric excess of the products were calculated using a standard method. Analysis conditions, as well as the establishment of the absolute configuration of the reaction products were described elsewhere [26].

2.4. Incubation test

Three flasks containing 5 ml of dichloromethane and 2.5 mg of cutinase were incubated into an orbital shaker (150 rpm) at 25°C during 24, 96, and 192 h,

respectively. Once finished, the incubation time, the substrate (diol **58 1**, 0.2 M) and the vinyl acetate (1.6 M) were added to each flask, beginning the transesterification reaction, in the experimental conditions previously described.

Similarly, three flasks containing 5 ml of dichloromethane, diol **1** (0.2 M), and 2.5 mg of cutinase were incubated during the same time periods. The reaction began after the addition of vinyl acetate (1.6 M) to each flask. Finally, three flasks containing 5 ml of dichloromethane, vinyl acetate (1.6 M), and 2.5 mg of enzyme were incubated in the same conditions, beginning the reactions after the addition of the substrate (0.2 M).

In all cases, throughout the reaction time, aliquots were taken and analyzed by HPLC to monitor the substrate transformation. The reaction was followed during 72 h.

3. Results

3.1. Study of the reaction conditions

Fig. 2 shows the results obtained in the transesterification of diol **1** with vinyl acetate when using 2.5, 5, and 10 mg of biocatalyst, respectively. As can be noticed from Table 1, an increase in the quantity of biocatalyst added to the reaction medium led to a lower specific enzymatic activity. Nevertheless, considering the evolution of the process, this higher specific activity obtained with the lower enzyme amount, did not lead to an optimal substrate transformation (only 44.1% yield in 96 h). On the other hand, in the assays using 5 and 10 mg of the biocatalyst, the specific activity ratio was 5/1, while the kinetic evolution of the process was very similar (around 70% of yield in 96 h). Taking into account these results, we decided to employ 5 mg of the biocatalyst throughout all the assays.

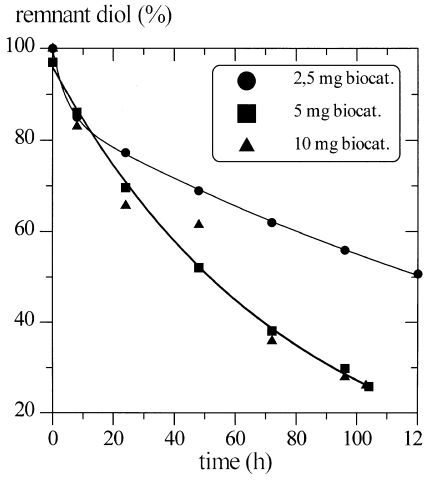

Fig. 2. Influence of the quantity of biocatalyst in the cutinase-catalysed transesterification of diol **1**.

Table 1
Influence of the amount of biocatalyst in the cutinase-catalysed transesterification of diol **1**

lx10256 Biocatalyst (mg)	Initial rate ($M\ h^{-1}$)	Specific activity ($M\ h^{-1}\ mg$)	Yield (%)[a]
2.5	0.0071	0.003	44.1
5	0.0045	0.001	70.2
10	0.0024	0.0002	87.21

[a] Calculated for a reaction time = 96 h.

In order to study the influence of the vinyl acetate/diol **1**058 molar ratio, were carried out in different reactions varying the concentration of the acylating agent (0.8, 1.6, and 3.2 M, molar ratios of 4/1, 8/1, and 16/1, respectively) The results obtained are shown in Fig. 3. As can be seen, the initial rate of the process was similar in all cases, being slightly higher when a molar ratio 8/1 was used (Table 2). A decrease of the vinyl acetate concentration to 0.8 M (molar ratio 4/1) implies a considerable diminution in the reaction yield, which may suggest that a smaller concentration of vinyl acetate in the reaction medium may suppose a restriction factor for the evolution of the process. On the other hand, the reactions in which the molar ratios were 8/1 and 16/1 showed a similar behaviour. Taking into account the described possibility of a denaturation of the biocatalyst because of the acetaldehyde liberated to the reaction medium when vinylic esters are employed as acylating agents, [27,28] it was decided that a molar ratio of 8/1 be used as a balance between the kinetic evolution of the process and the stability of the biocatalyst.

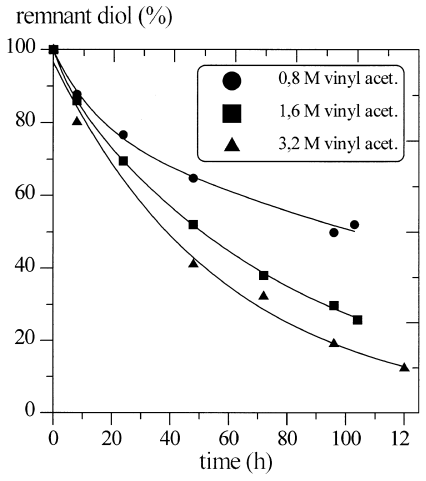

Fig. 3. Influence of the acylating agent/substrate molar ratio in the cutinase-catalysed transesterification of diol **1**.

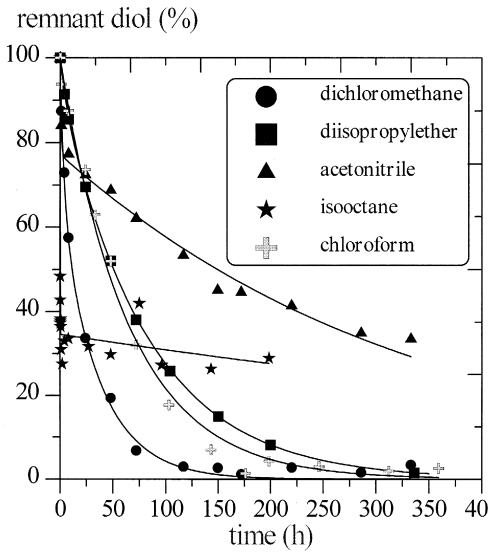

Fig. 4. Influence of the solvent in the cutinase-catalysed transesterification of diol **1**.

Finally, the influence in the reaction of the type of solvent used was studied (Fig. 4). Table 3 shows the solvents tested and the initial rates obtained in each case. It can be observed that the nature of solvent used does exert a clear influence on the evolution of the process. In terms of initial rates, the better results were obtained when isooctane was employed (logP $= 4.5$) while the initial rate decreased drastically when acetonitrile was used (logP $= -0.33$). These results agree with the described rules [29,30] which reports that lipases show low activity in solvents with logP values smaller than 2, moderate activity in solvents with logP from 2 to 4, and high activity in solvents with logP higher than 4. The rest of the solvents employed (diisopropylether, chloroform, and dichloromethane) possess similar values of logP (1.9, 1.97, and 1.4, respectively). Nevertheless, although the results obtained with diisopropylether and chloroform were very similar, the reaction rate was significantly higher when dichloromethane was employed (Table 3). This experimental result can be rationalised if another solvent feature is taken into account, the dielectric constant (ϵ). Considering that the standard substrate (diol **1**) is a polar compound, a solvent with a higher dielectric constant would help the solubilisation of the substrate in the medium, therefore, favouring its access towards the biocatalyst and its transformation. This consideration would explain the better results obtained when dichloromethane ($, = 8.9$) vs. diisopropylether and chloroform, was used as solvent. On the other hand, as can

Table 2
Influence of the acylating agent/diol molar ratio on the cutinase-catalysed transesterification of diol **1**

Molar ratio[a]	Initial rate (M h^{-1})	Yield (%)[b]
4/1	0.0034	50.12
8/1	0.0045	70.2
16/1	0.0032	80.9

[a] Molar ratio, acylating agent/diol **1**.
[b] Calculated for a reaction time = 96 h.

Table 3
Influence of the nature of solvent in the cutinase-catalysed transesterification of diol **1**

llx8401 Solvent	logP	ϵ	Initial rate (M h^{-1})	Yield (%)[a]
Isooctane	4.5	2.09	2.23	73.7
Chloroform	1.97	4.8	0.0031	93
Diisopropylether	1.9	3.8	0.0062	85
Dichlorometane	1.4	8.9	0.019	97.3
Acetonitrile	−0.33	36.6	0.054	54.9

[a] Calculated at reaction time = 150 h.

be observed from Fig. 3, when isooctane was employed, in spite of the high initial rate observed, a maximum limited yield of 80% was obtained, and the reaction did not progress after this value was reached. When this solvent was used, we also observed that part of the substrate remained adhered to the flask walls; thus, we suppose that, in this case, the yield is limited by the fraction of substrate which is solubilised by the solvent, which would be related with its low dielectric constant, 2.09. Therefore, we decided to employ dichoromethane as the standard solvent for the transesterification of all diols (**1**–**4**).

*3.2. Enzyme-catalysed transesterifications of diols **1**–**4***

Once the adequate reaction conditions were determined in the precedent study, we proceeded to assay the cutinase performance in the transesterification of diols **1**–**4**. No reaction was detected in any case when diols **2**–**4** were used as substrates. The progress curves for the transesterification of diol **1** are showed in Fig. 5A. For a better visual following the reaction course, in Fig. 5B and C, the consumption of diol **1** and the formation of monoacetates **6** and **7** are

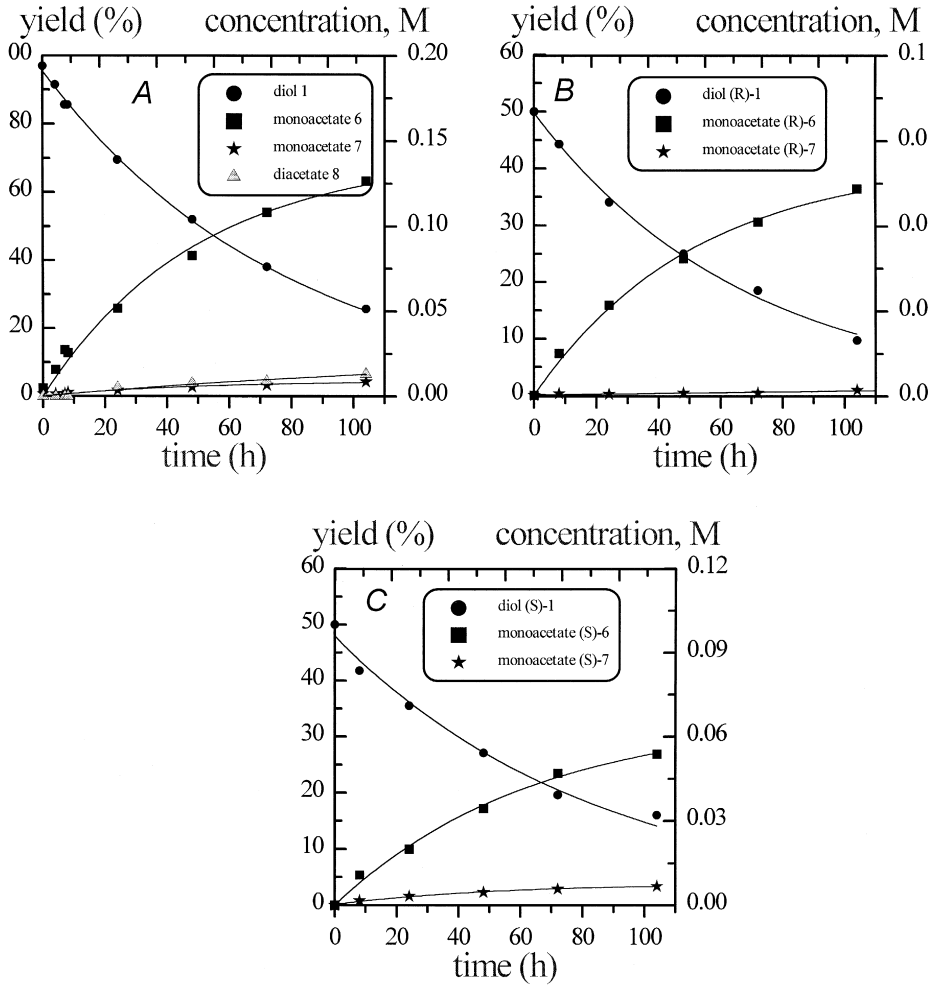

Fig. 5. Progress curve of the cutinase-catalysed transesterification of diol **1**. (A) Consumption of **1** and formation of **6**, **7**, and **8**. (B) Consumption of R-**1** and formation of R**8**-**6** and R-**7**. (C) Consumption of S-**1** and formation of S-**6** and S-**7**.

displayed as two separate curves in each case, corresponding to the single enantiomers, *R* (Fig. 5B) and *S* (Fig. 5C).

As can be observed, the acylation took place mainly on the primary hydroxy group, yielding the major monoacetate **6**, with a slightly *R*-stereopreference. The acylation in the secondary hydroxy group was observed as well, rendering the minor monoacetate **7** (*S*-enriched). Traces of diacetate **8**, produced by a second acylation on the free-OH group of the monoacetates **6** and/or **7** were also detected.

In order to quantify the behaviour of cutinase in the kinetic resolution of diol **1**, we decided to assume the mathematical model proposed by Kroutil et al. [31] for sequential two-step asymmetrization/kinetic resolutions, which is summarised in Fig. 6A. In this scheme, S is a prochiral (or meso) substrate, P and Q are the enantiomeric products obtained in the asymmetrization step, while R is the prochiral (or meso) product obtained through the enzymatic kinetic resolution of P3 and Q. The selectivity of this type of reactions has been described to be solely governed by the so-called selectivity factor (∀), which is equal to the ratio of the apparent first-order constants k_1 and k_2, while E_2 (the ratio of k_3 and k_4) is the enantioselectivity ratio of the kinetic resolution. The

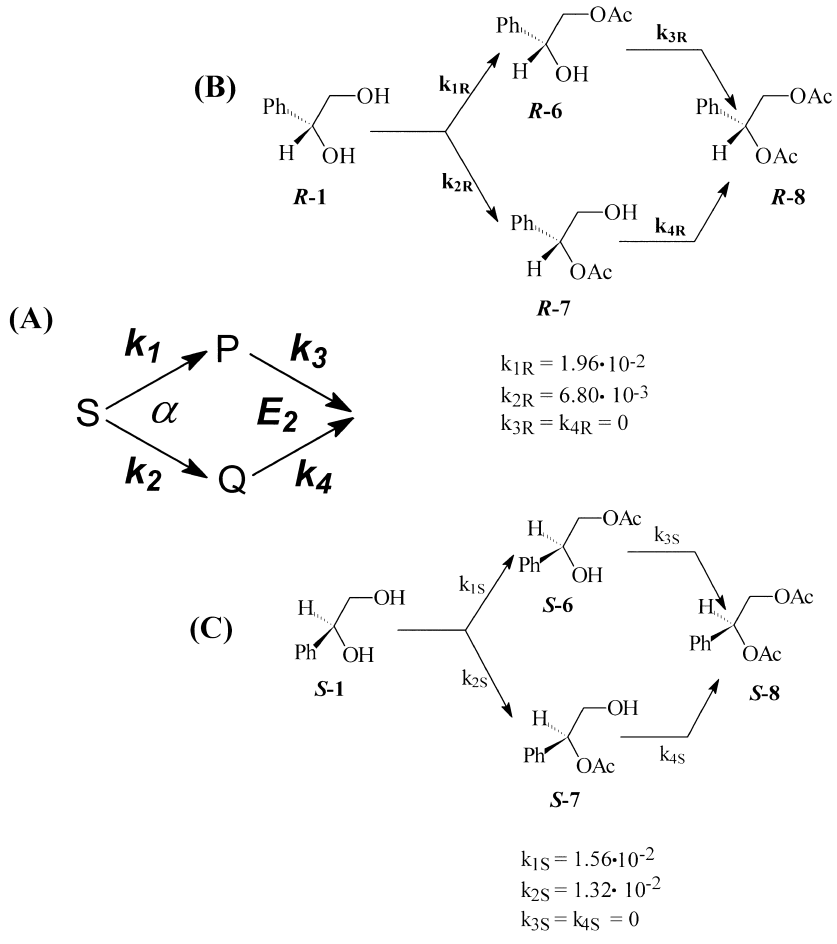

Fig. 6. (A) Model of Kroutil et al. [31] for two-step asymmetrization/kinetic resolution. (B) Schemes for the kinetic resolution of **1**. Consumption of R-**1** and formation of R-**6**, R-**7**, and R-**8**. (C) Consumption of S-**1** and formation of S-**6**, S-**7,** and S-**8**.

kinetics of the overall process are defined by the following equations:

$$S = S_0 e^{-(k_1+k_2)t} \quad (1)$$

$$P = S_0 \frac{k_1}{k_3 - (k_1 + k_2)} \left[e^{-(k_1+k_2)t} - e^{-k_3 t}\right] \quad (2)$$

$$Q = S_0 \frac{k_2}{k_4 - (k_1 + k_2)} \left[e^{-(k_1+k_2)t} - e^{-k_4 t}\right] \quad (3)$$

$$R = S_0 - S - P - Q. \quad (4)$$

This model can be used to fit the curves obtained for the resolution of diol **1033 1**, a racemic compound. In this way, some similar constants can be obtained, but now with different meanings, which can be envisaged as follows (taking schemes from Fig. 6A and C as reference).

$E_{1R} = 1.25 = (k_{1R}/k_{1S})$ and $E_{2R} = 0.51 = (k_{2R}/k_{2S})$ represents the enantioselectivity ratio for the formation of the major and minor monoacetates (acetoxy group further or closer from the stereocentre, respectively).

$E_{DIAC} = 0 = (k_{3S} + k_{4S})/(k_{3R}57 + k_{4R})$ would quantify the enantioselectivity of the diacetate, because it describes the ratio between the two pathways leading to it. In this case, due to the fact that

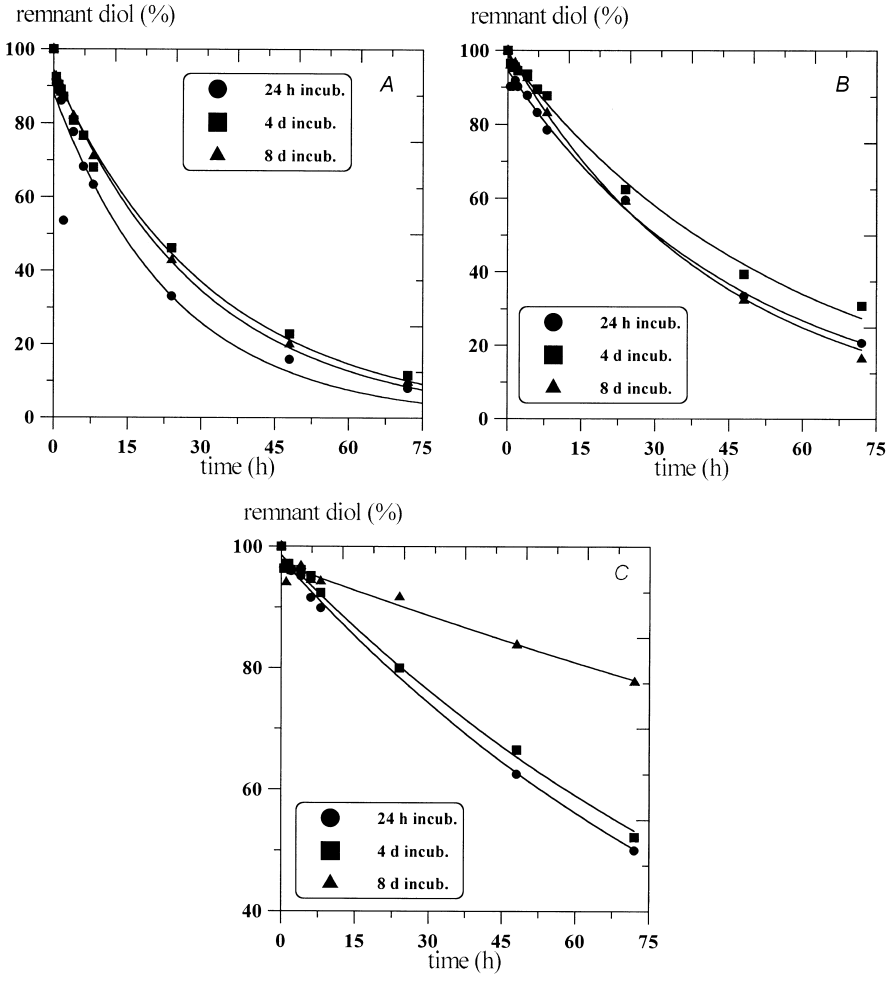

Fig. 7. (A) Influence on the cutinase stability of the pre-incubation with diol **1**. (B) Pre-incubation with dichloromethane. (C) Pre-incubation with vinyl acetate.

only traces of diacetate **8** were obtained, $k_{3R} = k_{3S} = k_{4R} = k_{4S} = 0$.

$rr = 1.76 = (k_{1R} + k_{1S})/(k_{2R} + k_{2S})$ stands for the regioselectivity ratio, as a quantification of the overall ratio between the formation of the major and minor monoacetates.

The values of the constants for the resolution of **1** by cutinase are shown in Fig. 6B and C. It can be observed that the first acylation renders mainly the monoacetate **6** ($k_{1R} + k_{1S} \gg k_{2R} + k_{2S}$), which indicates a pronounced regioselectivity in the acylation on the primary hydroxyl group (rr = 1.76), with an *R*-stereopreference ($k_{1R} > k_{1S}$). This regioselection in the acylation of diols possessing both primary and secondary hydroxyl groups has been frequently described [32–34].

As we stated before (see Introduction), there is still little information about the alcohol binding site in the cutinase structure. Fontes et al. [24] modelled the cutinase enantiorecognition of racemic 1-phenylethanol, which resembles the secondary hydroxy group of our substrate **1**. These authors reported an excellent enzymatic resolution of racemic 1-phenylethanol via transesterification with vinyl butyrate, observing a marked *R*-stereopreference. By means of molecular modelling, this fact was explained because of the better complementary of the *R*-isomer and the enzymatic active site, through an initial interaction of the aromatic moiety of the substrate with a hydrophobic area limited by the residues Tyr 119, Val 184 and Leu 189. Fixing this planar phenyl group, for the *R*-isomer, a stabilising interaction betweeen Tyr 119 and the methyl group of the substrate was observed. Comparing 1-phenylethanol with 1-phenyl-1,2-ethanodiol (diol **1**), if we assume, after Fontes et al. [24], that the recognition of the phenyl group is the key step for the alcohol binding, the acylation of the primary alcohol of **1** would take place, although with a much smaller stereoselectivity. Thus, the secondary hydroxy group of **1** would occupy the same position of methyl group in 1-phenylethanol, therefore losing the stabilising interaction with Tyr 119, what would be the cause for the low enantioselectivity observed with **1**.

On the other hand, the acylation in the secondary hydroxyl group of **1** is very limited (yield lower than 10%). This could be due to a steric and/or electronic hindrance caused by the substitution of a methyl group by a hydroxymethyl one in such position.

3.3. Stability of cutinase in the reaction conditions

Fig. 7A,B, and C shows the results obtained in the incubation test. Once calculated, the initial rate in the transformation of diol **1** in each case (Table 4), we can observe that higher rates were obtained for those reactions initiated after a pre-incubation of the enzyme with a solution of the diol **1** in dichloromethane (standard solvent). In these reactions, independently of the incubation time, the values of the initial rates, as well as the evolution of yield with the reaction time, were very similar (Fig. 7A).

When the enzyme was pre-incubated solely in the solvent (Table 4, entries 2–4), the initial rates measured in all cases were similar to that obtained in the non-pre-incubated reaction. We did not observe any effect of dichloromethane on the enzyme stability. Nevertheless, the initial rates obtained when the enzyme was pre-incubated in a solution of vinyl acetate in dichloromethane (Table 4, entries 5–7), were considerably smaller than that obtained in the non-pre-incubated reaction (entry 1). Moreover, the reaction rate decreases with the pre-incubation time. This fact confirms that cutinase stability was seriously affected by the presence of vinyl acetate in the

Table 4
Initial rates obtained in the incubation test

lx10256 Entry	Incubation medium	Incubation time (h)	Initial rate ($M\ h^{-1}$)
1	No incubation	–	0.0045
2	Dichloromethane	24	0.004
3	Dichloromethane	96	0.0035
4	Dichloromethane	192	0.005
5	Dichloromethane + vinyl acetate	24	0.002
6	Dichloromethane + vinyl acetate	96	0.0017
7	Dichloromethane + vinyl acetate	192	0.0006
8	Dichloromethane + diol **1**	24	0.0072
9	Dichloromethane + diol **1**	96	0.0060
10	Dichloromethane + diol **1**	192	0.0063

Table 5
Results obtained in the reactions previously incubated in the presence of diol **1**

Entry	Incubation time (h)	k_{1R}	k_{2R}	k_{1S}	k_{2S}	E_{1R}	E_{2R}	rr
l	Not incubated	1.96×10^{-2}	6.8×10^{-3}	1.32×10^{-2}		1.25	0.51	1.76
2	24	8.97×10^{-2}	1.35×10^{-2}	4.31×10^{-2}	6.98×10^{-2}	2.08	0.19	1.6
3	96	5.33×10^{-2}	1.42×10^{-2}	3.68×10^{-2}	4.44×10^{-2}	1.44	0.32	1.54
4	192	6.0×10^{-2}	1.49×10^{-2}	3.37×10^{-2}	1.88×10^{-2}	1.78	0.79	2.78

reaction medium, which could be caused either by the acylation of external nucleophylic residues of the protein by the vinylic ester or by the formation of imines through a condensation of the acetaldehyde liberated from the vinyl acetate, as described in literature [27,28], with any of the six lysine residues of the enzyme, which are relatively highly exposed to the solvent [23]. Any of these modifications would lead to an alteration of the enzymatic structure, with the concomitant lost of activity.

On the other hand, the higher rates observed when the cutinase was previously incubated in the presence of diol **1** (Table 4, entries 8–10) indicate that the substrate would not only operate as protecting agent limiting the denaturing effect of other reactives (like dichloromethane and vinyl acetate), but also the pre-incubation of the enzyme with the substrate seems to increase its biotransformation rate. Thus, once the enzyme accepts the substrate in its active center, the pre-incubation would facilitate the «fitting» of the enzyme structure to adapt to the substrate («bioimprinting»), favouring its transformation. These results would confirm the observed stabilisation of the cutinase by hexanol in an AOT-reverse micellar system, [7,11,13] proving that this protection effect may be provided by alcohols with different structural features and would be related to the interaction of the alcohol with the protein molecule, regardless of the state of the enzyme in the system (native or encapsulated).

Analyzing the cutinase regio and stereoselectivity, using the kinetic model described by Borreguero et al. [35], in the reactions in which the enzyme was previously incubated in the presence of diol **1**, we obtained the constants and parameters summarised in Table 5. From these data, it can be inferred that an increase of stereoselectivity in the acylation of the primary hydroxy group was obtained with a pre-incubation of the enzyme in the presence of the substrate, although without a correlation with the incubation time. In this sense, the best result was obtained with an incubation of 24 h ($E_{1R} = 2.08$). Similarly, the stereoselectivity in the acylation of the secondary hydroxy group was increased in the pre-incubated reactions (except for a pre-incubation of 192 h), obtaining the best enantiorecognition ($E_{2R} = 0.79$) in the reaction pre-incubated during 24 h. Moreover, the regioselectivity showed by cutinase for the acylation in the primary hydroxy group was also influenced by the pre-incubation with the substrate, observing that, in this case, the higher regioselectivity was obtained for an incubation time of 192 h (rr = 2.78).

Thus, the pre-incubation of cutinase in the presence of diol **1** not only increased the biotransformation rate of this substrate but the stereoselectivity and regioselectivity in its acylation, evidencing that a change in the recognition pattern of the enzyme must be induced by the previous contact with this substrate.

Acknowledgements

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