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# Ethanol acetylation by mycelium-bound carboxylesterase of *Aspergillus oryzae*: estimation of thermodynamic parameters and integral productivity

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**Summary**

The results collected at different temperatures for ethanol acetylation by cell-bound carboxylesterase from lyophilized cells of *Aspergillus oryzae* have been used to investigate the kinetics and thermodynamics of this esterification in *n*-heptane. The occurrence of reversible unfolding followed by irreversible denaturation of the enzyme has been proposed to explain the increase in the starting rate of ethyl acetate formation with temperature observed up to 55 °C and the consequent fall beyond this threshold. The Arrhenius model has been used to estimate the apparent activation enthalpies of both the acetylation reaction ($\Delta H^{\neq} = 29$–$33$ kJ mol$^{-1}$) and reversible enzyme unfolding ($\Delta H_u^{\neq} = 56$–$63$ kJ mol$^{-1}$). The results of residual activity tests performed with cells previously exposed at different temperatures for variable times enabled us also to estimate the first-order rate constant of irreversible denaturation ($2.40 \times 10^{-3}\,h^{-1} < k_d < 8.11 \times 10^{-3}\,h^{-1}$) as well as the related thermodynamic parameters ($\Delta H_d^{\neq} = 22\,kJ\,mol^{-1}$; $\Delta S_d^{\neq} = -0.29\,kJ\,mol^{-1}\,K^{-1}$). This last phenomenon proved particularly slow for the system under consideration, probably because the biocatalyst link to the mycelium was able to improve its thermostability. In view of future continuous application, the effects of operating time, starting substrate concentration and temperature on the theoretical integral productivity of a fixed-bed column filled with this biocatalyst have been investigated.

## Introduction

Direct acylations of alcohols in organic media are equilibria which lead to the formation of water in addition to the desired ester, therefore they are not favoured by an increase in water activity (Svensson *et al.* 1994). A further problem is the strong inhibition exerted by the free acid on the carboxylesterase activity, especially when acetic acid is employed (Langrand *et al.* 1990; Claon & Akoh 1993, 1994; Castro *et al.* 1997; Bourggarros *et al.* 1998). For this reason, we searched for new microbial lipases and esterases, such as the one described here which is associated with the mycelium of a newly isolated strain of *Aspergillus oryzae* (Molinari *et al.* 1996, 2000; Converti *et al.* 2002).

The less developed research-field of non-aqueous enzymology is, in our opinion, that dealing with the kinetic and thermodynamic characterization of biocatalysts as well as their heat inactivation. Several studies have demonstrated that lipase-catalysed transesterifications, ester hydrolysis and alcohol acylations in organic media proceed via a ping-pong acyl–enzyme mechanism (Zaks & Klibanov 1985), similar to that taking place in water, which follows Michaelis–Menten-type kinetics with the formation of tetrahedral transition states. Most studies have been devoted to the effects of water and solvent hydrophobicity on the enzyme activity (Laane *et al.* 1987), the estimation of rate constants of the single steps (Chatterjee & Russell 1992) and the transition state stabilization (Xu *et al.* 1994). $K_m$ values larger than in aqueous media have been observed for many enzymes in organic solvents; in particular, it seemed to increase for lipases with the substrate partition coefficient (Secundo *et al.* 1992).

Although the general mechanisms of suspended enzyme thermoinactivation in water and in organic solvents are now definitively explained (Volkin *et al.* 1991), only a few systematic kinetic and thermodynamic studies have been performed on both the activity and thermal stability of enzymes in organic solvents (Baptista *et al.* 2000). Because of the complexity of the mycelium-bound system used in this work, it has been studied by a simplified thermodynamic approach, leaving to future efforts a more rigorous analysis. Ethyl

acetate has been selected as reference product because of its small size, in order to minimize interference due to steric hindrance in the transition state with thermodynamic parameters. In addition, it is an interesting flavour and fragrance component in the food and cosmetic industry, the biotechnological production of which would be useful.

## Materials and methods

Chemicals, mycelium and biocatalyst preparations, procedures for ethanol acetylation and residual activity tests, and analytical methods have previously been described (Molinari *et al*. 2000; Converti *et al*. 2002).

## Theory

### *Mechanism and kinetics of ethanol acetylation*

A ping-pong mechanism can be proposed for ethanol acetylation in organic solvent, which is opposite to that observed for ester hydrolysis in water (Yang & Russell 1996). It involves the formation of a non-covalent enzyme–acid complex, which is transformed into a tetrahedral intermediate by the attack of a serine hydroxyl group onto the acid carbonyl. This intermediate collapses, releasing water and a stable acyl–enzyme intermediate. The subsequent nucleophilic attack of alcohol results in the formation of a new tetrahedral enzyme–product complex which deacylates to release the free enzyme and the ester. For ethanol acetylation, this mechanism can be written in the simplified form:

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_A + W \tag{1}$$

$$E_A + B \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_P \tag{2}$$

$$E_P \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P \tag{3}$$

where $E$ is the active free enzyme, $A$ the acetic acid, $B$ the ethanol, $P$ the ethyl acetate, $E_A$ and $E_P$ the complexes of the enzyme with acetic acid and ethyl acetate respectively, $W$ the water and $k_j$ the kinetic rate constants of the single steps.

At the start of the reaction, the concentrations of both substrates remain practically unchanged at their zero time values ($A_0$ and $B_0$) whereas those of both products $P$ and $W$ can be neglected, so the reactions (1) and (3) can be considered irreversible. Moreover, assuming steady-state conditions for the enzyme forms $E_A$, $E_P$ and $E$, one can write:

$$k_1 E A_0 - k_2 E_A B_0 + k_{-2} E_P = 0 \tag{4}$$

$$k_2 E_A B_0 - k_{-2} E_P - k_3 E_P = 0 \tag{5}$$

where $A_0$ and $B_0$ are the starting concentrations of the substrates.

Considering that the inactive fraction of the enzyme is negligible at the start of the reaction, the material balance for the enzyme is:

$$E_A + E_P + E = E_{t0} \tag{6}$$

where $E_{t0}$ is the starting concentration of the fresh enzyme.

From Equations (4) to (6) we obtain:

$$E_A = \frac{E_{t0}}{1 + \frac{k_2}{k_{-2}+k_3} B_0 + \frac{k_3}{k_1}\frac{k_2}{k_{-2}+k_3}\frac{B_0}{A_0}} \tag{7}$$

$$E_P = \frac{E_{t0}\frac{k_2}{k_{-2}+k_3} B_0}{1 + \frac{k_2}{k_{-2}+k_3} B_0 + \frac{k_3}{k_1}\frac{k_2}{k_{-2}+k_3}\frac{B_0}{A_0}} \tag{8}$$

$$E = \frac{E_{t0}\frac{k_3}{k_1}\frac{k_2}{k_{-2}+k_3}\frac{B_0}{A_0}}{1 + \frac{k_2}{k_{-2}+k_3} B_0 + \frac{k_3}{k_1}\frac{k_2}{k_{-2}+k_3}\frac{B_0}{A_0}} \tag{9}$$

The starting product formation rate, $v_0$, is defined as:

$$v_0 = k_3 E_P \tag{10}$$

Substituting Equation (8) into Equation (10), we obtain:

$$v_0 = \frac{k_3 E_{t0}}{1 + \frac{k_3}{k_1}\frac{1}{A_0} + \frac{k_{-2}+k_3}{k_2}\frac{1}{B_0}} \tag{11}$$

Under conditions of equimolar levels of both substrates as those investigated in this study ($S_0 = A_0 = B_0$), Equation (11) simplifies to the Michelis–Menten type equation:

$$v_0 = \frac{k_{cat} S_0}{K_m + S_0} \tag{12}$$

where

$$k_{cat} = k_3 E_{t0} \tag{13}$$

and

$$K_m = \frac{k_3}{k_1} + \frac{k_{-2} + k_3}{k_2} \tag{14}$$

The constants $K_m$ and $k_{cat}$ were previously estimated for this system ($K_m = 121.4$ mmol $l^{-1}$; $k_{cat} = 28.3$ mmol $l^{-1}$ $h^{-1}$) by tests performed at 50 °C with high activity mycelium at different starting substrate concentrations (Converti *et al*. 2002). Since the apparent Michaelis constant, $K_m$, is very large in *n*-heptane, probably due to diffusion limitations of substrates and products through the media, it cannot be neglected in Equation (12), as it was possible to make for other enzymatic systems (Converti & Del Borghi 1997, 1998).

*Kinetics of biocatalyst inactivation*

As previously explained (Converti *et al.* 2002), the classic two-step model proposed by Volkin *et al.* (1991) can be used to describe the overall phenomenon of thermal enzyme inactivation. First, the interaction of solvent molecules with the system 'membrane-lipase' would reversibly produce a conformational change (unfolding) disturbing the active conformation of the enzyme. The resulting less active (unfolded) conformation of the enzyme, $E_u$, would be subsequently subject to an irreversible step, leading to an aggregated stable (inactive) protein, $E_d$:

$$E \overset{K_u}{\rightleftharpoons} E_u \overset{k_d}{\rightarrow} E_d \tag{15}$$

where $K_u$ is the equilibrium constant of partial enzyme unfolding and $k_d$ the first-order rate constant of irreversible thermal denaturation.

Despite the simplicity of this model, it seemed to be of general validity and to hold true for different proteins (Baptista *et al.* 2000), including enzymes in organic media (Converti *et al.* 2002). If the latter irreversible stage is much slower than the former, $E$ and $E_u$ can be considered to be in equilibrium, thus the overall process can kinetically be described by first-order kinetics:

$$v_d = k_d E_t \tag{16}$$

where $E_t$ is the total concentration of the active enzyme, following a thermal treatment of duration $t$.

The first-order rate constant of irreversible denaturation, $k_d$, was previously estimated at different temperatures by residual activity tests from the experimental data of the activity coefficient (Converti *et al.* 2002), defined as the ratio of total active enzyme concentration at time $t$ to that at the beginning of the thermal treatment ($\psi = E_u/E_{t0}$) (Mozhaev 1993).

*Thermodynamics of ethanol acetylation and biocatalyst inactivation*

In all bioprocesses, the product formation rate progressively increases with temperature up to a threshold value beyond which it quickly decreases. Plotting $v_0$ according to Arrhenius in a semilog plot vs. the reciprocal temperature, this behaviour can be approximated by two straight lines.

For enzymatic reactions involving only one substrate, implying the formation of binary transition states whose rupture leading to the product is the limiting step, the activity increase consequent to a temperature increase up to an optimal value, $T_{opt}$, can be described by the Arrhenius-type equation (Roels 1983):

$$v_0 = a \exp(-\Delta H^{\neq}/RT) \quad T < T_{opt} \tag{17}$$

where $\Delta H^{\neq}$ is the activation enthalpy, $R$ the ideal gas constant, $T$ the absolute temperature and $a$ a parameter depending on the activation entropy and the biocatalyst concentration. The activation enthalpy of the enzymatic reaction has been estimated from the slope of this straight line.

At temperatures higher than the optimum, on the contrary, the reversible thermal inactivation of the enzyme, which is negligible at $T < T_{opt}$ and grows with temperature, can prevail over the former effect and an opposite behaviour is observed. Also this decrease of product formation rate can be approximated by an Arrhenius-type equation, by which another activation parameter ($\Delta H^{\neq\prime}$) can be estimated. According to Sizer (1944), the activation enthalpy of enzyme inactivation, $\Delta H_u^{\neq}$, can approximately be estimated as the sum of this parameter and the activation enthalpy of the reaction it catalyses:

$$\Delta H_u^{\neq} = \Delta H^{\neq} + |\Delta H^{\neq\prime} \tag{18}$$

The activation enthalpy of the irreversible denaturation of the enzyme, $\Delta H_d^{\neq}$, has been estimated by plotting the logarithm of the apparent first-order rate constant of denaturation, $k_d$, versus $1/T$, according to the Arrhenius-type equation (Roels 1983):

$$k_d = b \exp(-\Delta H_d^{\neq}/RT) \tag{19}$$

where $b$ is parameter related to the activation entropy of this phenomenon.

The activation energies, $\Delta E^{\neq}$, Gibbs free energies, $\Delta G^{\neq}$, and entropies, $\Delta S^{\neq}$, have been estimated for each phenomenon by standard methods (Aiba *et al.* 1973).

Notwithstanding the complexity of the network of reactions described by Equations (1)–(3), such an approach can be applied to the bioprocess under consideration provided that the limiting step of the overall transformation follows a simplified scheme as that consistent with the Arrhenius model. For this reason, it should be stressed that all thermodynamic parameters estimated in this study are only 'apparent', because they refer to a more complex reality, and should then be used, only for comparative purposes, to get a rough picture of the esterification thermodynamics.

*Integral productivity*

If the irreversible enzyme denaturation is taken into consideration, the enzyme activity becomes a function of the operating time. So, the integral productivity up to a given time, $P$, could be estimated, for a continuous process employing enzymes with $K_m$ negligible with respect to $S_0$, by integrating the product of the starting forward reaction rate to the activity coefficient, $\psi$ (Roels 1983):

$$P(t) = \int k_3 E_{t0} \psi \, dt = \int k_{cat} \exp(-k_d t) \, dt = \frac{k_{cat}}{k_d}[1 - \exp(-k_d t)] \tag{20}$$

A different situation takes place with enzymatic systems, like that used in this study, with large $K_m$ values, for which $v_0$ can be significantly lower than $k_{cat}$ and the expression of the starting productivity, as defined in Equation (12), must be used in Equation (20) instead of $k_{cat}$. Making reference to 1 g of lyophilized mycelium, the specific integral productivity can be defined as:

$$P/X_0 = \frac{v_0}{X_0 k_d}[1 - \exp(-k_d t)] \quad (21)$$

Further parameters useful for kinetic comparison that, contrary to integral specific productivity, are independent of the time, are the values it achieves in a continuous process up to infinity of time:

$$P_\infty/X_0 = \frac{v_0}{X_0 k_d} \quad (21')$$

and up to activity half-life:

$$P_{1/2}/X_0 = \frac{v_0}{2X_0 k_d} \quad (21'')$$

## Results and discussion

### *Effect of temperature on mycelium-bound carboxylesterase activity*

This part of the work deals with the thermodynamics of ethanol acetylation by lyophilized cells of *A. oryzae* prepared using different carbon sources, according to the desired carboxylesterase activity (Tween 80 or glucose plus olive oil for high- or low-activity cells, respectively). For this purpose, we have used the starting rates of product formation collected from batch experiments carried out at given starting levels of biomass ($X_0 = 20$ g l$^{-1}$) and substrates ($S_0 = 50$ and 100 mmol l$^{-1}$), and varying the temperature from 30 to 80 °C (Converti *et al.* 2002).

The results of Table 1 show that $v_0$ progressively increased with temperature up to 55 °C and decreased above this value, with no appreciable dependence on the starting substrate level. On the other hand, the positive influence of Tween 80 on the biocatalyst activity, has recently been ascribed to its capacity of ensuring an optimal final macrostructure of the system 'membrane-lipase', that carries to the maximum number of active lid-opened lipase molecules, and suggested that the enzyme could be a membrane-bound catalyst located in the external zone of the membrane (Converti *et al.* 2002). By comparison with other enzymatic systems (Aiba *et al.* 1973; Adams & Brawley 1981; Dring & Fox 1983; Fox & Stepaniak 1983; Roels 1983), such a relatively high temperature threshold put in evidence the thermoresistance of the enzymatic system under consideration. In particular, comparison with covalently immobilized lipases (Moreno *et al.* 1997) suggests the existence of a sort of enzyme protection against thermal inactivation exerted by its natural link to the mycelium (Molinari *et al.* 2000).

The above two-step behaviour of biosystems, with respect to traditional chemical processes, was clearly explained for both fermentations and enzymatic systems (Converti *et al.* 1996; Converti & Del Borghi 1997; Arni *et al.* 1999): the formation of product via the activated state is coupled to the phenomenon of reversible inactivation of the biocatalyst described by the former step of Equation (15), which kinetically acts in the opposite direction, reducing the number of active sites, and whose relative significance grows with temperature.

As the values of Table 2 show, the initial substrate concentration did not appreciably influence the thermodynamic parameters. In fact, the apparent activation enthalpies of the acetylation reaction and the thermal unfolding of the most active biocatalyst only varied

*Table 1.* Influence of temperature on $v_0$ and $Y_P/S_0$ of ethanol acetylation by lyophilized cells of *A. oryzae*.

| Type of culture | Low activity | | High activity | | | |
|---|---|---|---|---|---|---|
| $S_0$ (mmol l$^{-1}$) | 100 | | 100 | | 50 | |
| | $v_0$ (mmol l$^{-1}$ h$^{-1}$) | $Y_P/S_0$ (mol mol$^{-1}$) | $v_0$ (mmol l$^{-1}$ h$^{-1}$) | $Y_P/S_0$ (mol mol$^{-1}$) | $v_0$ (mmol l$^{-1}$ h$^{-1}$) | $Y_P/S_0$ (mol mol$^{-1}$) |
| $T$ (°C) | | | | | | |
| 30 | 3.31 | 0.45 | 6.10 | 0.42 | 2.94 | 0.48 |
| 35 | 3.98 | 0.50 | 7.78 | 0.52 | 3.98 | 0.55 |
| 40 | 5.28 | 0.53 | 10.02 | 0.61 | 4.77 | 0.70 |
| 45 | 6.54 | 0.60 | 11.95 | 0.68 | 5.95 | 0.72 |
| 50 | 7.51 | 0.75 | 13.81 | 0.75 | 6.98 | 0.81 |
| 55 | 8.59 | 0.82 | 14.22 | 0.79 | 7.51 | 0.83 |
| 60 | 7.76 | 0.79 | 12.99 | 0.74 | 7.01 | 0.75 |
| 65 | 6.88 | 0.75 | 12.03 | 0.71 | 6.19 | 0.72 |
| 70 | 5.79 | 0.62 | 10.45 | 0.68 | 5.20 | 0.67 |
| 75 | 4.98 | 0.55 | 8.46 | 0.60 | 4.20 | 0.61 |
| 80 | 3.95 | 0.41 | 7.15 | 0.48 | 3.30 | 0.56 |

$X_0 = 20$ g l$^{-1}$.

*Table 2.* Apparent thermodynamic parameters of ethanol acetylation by lyophilized cells of *A. oryzae*, estimated by the Arrhenius model under different conditions.

| | Acetylation reaction | Reversible unfolding |
|---|---|---|
| (a) Low activity cells, $S_0 = 100$ mmol $l^{-1}$ | | |
| $\Delta E^{\neq}$ (kJ $mol^{-1}$) | 35.3 | 64.9 |
| $\Delta H^{\neq}$ (kJ $mol^{-1}$) | 32.6 | 62.2 |
| $r^2$ | 0.987 | 0.976 |
| (b) High activity cells, $S_0 = 100$ mmol $l^{-1}$ | | |
| $\Delta E^{\neq}$ (kJ $mol^{-1}$) | 31.8 | 58.5 |
| $\Delta H^{\neq}$ (kJ $mol^{-1}$) | 29.1 | 55.8 |
| $r^2$ | 0.959 | 0.959 |
| (c) High activity cells, $S_0 = 50$ mmol $l^{-1}$ | | |
| $\Delta E^{\neq}$ (kJ $mol^{-1}$) | 34.0 | 65.9 |
| $\Delta H^{\neq}$ (kJ $mol^{-1}$) | 31.3 | 63.2 |
| $r^2$ | 0.976 | 0.956 |

Reference temperature: 50 °C.

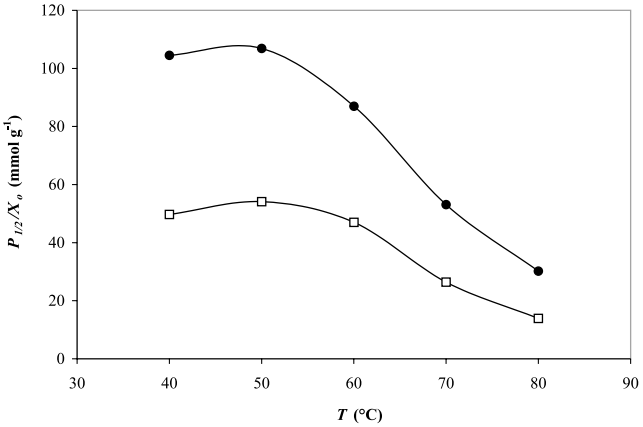


*Figure 1.* Temperature dependence of the activity half-life, $t_{1/2}$ (●), and the rate constant of denaturation, $k_d$ (□), of mycelium-bound carboxylesterase from *A. oryzae*.

between 29 and 31 kJ $mol^{-1}$ and between 56 and 63 kJ $mol^{-1}$, respectively. This result disagrees with the observations made for other immobilized enzymatic systems, such as glucose isomerase or other lipases, that proved to be protected by substrate (Chen & Wu 1987; Converti & Del Borghi 1997, 1998; Palazzi & Converti 1999) or carbohydrates (Sánchez-Montero *et al.* 1991; Moreno *et al.* 1997) against thermal inactivation. On the other hand, the little influence exerted by the carboxylesterase activity level seems to support at qualitative level the above indication of external enzyme localization. These results on the whole suggest that a more rigorous thermodynamic approach could be carried out utilizing all the experimental data of starting product formation rate obtained with both high- and low-activity cells, simply normalizing the kinetic results to the unit enzymatic activity.

A kinetic study on irreversible enzyme denaturation has recently been performed by residual activity tests at different temperatures (Converti *et al.* 2002), assuming that the latter irreversible step of Equation (15) is much slower that the former equilibrium and that the overall enzyme activity decay could be reasonably described by first-order kinetics. The values of the first-order rate constant of enzyme denaturation, $k_d$, are plotted in Figure 1 against the temperature together with those of the enzyme activity half-life. Nearly coincident results were obtained at $S_0 = 50$ and 100 mmol $l^{-1}$, which means that, like the reversible unfolding, the irreversible denaturation is also not appreciably influenced by starting substrate concentration.

Like native and immobilized purified lipases (Moreno *et al.* 1997), our mycelium-bound carboxylesterase followed the typical decay of a first-order denaturation pattern, consistent with $k_d$ exponential growth with temperature. The increased enzyme stability offered by its natural immobilization to the mycelium is also demonstrated in Figure 1 by the particularly high values of its activity half-life, $t_{1/2}$, if compared with those calculated for native pure lipases (0.28–0.5 h at 50 °C) or for commercial lipases immobilized onto different supports in aqueous medium (5–15 h at 50 °C) (Moreno *et al.* 1997) or in organic solvents (3–16 h at 40 °C) (Arcos *et al.* 2001) and with those reported for lyophilized pure lipase (12 h at 100 °C) in heptanol/tributyrin (Zaks & Klibanov 1984).

On the analogy of the apparent activation parameters of the acetylation reaction and the reversible enzyme unfolding, those of irreversible denaturation (Table 3) have been estimated by Equations (19)–(22). The general validity of this approach is demonstrated by its success in the estimation of inactivation thermodynamic parameters of different enzymatic systems, like immobilized glucose isomerase (Chen & Wu 1987; Converti & Del Borghi 1997), invertase (Queiroz *et al.* 1996) and other lipases (Owusu *et al.* 1992).

A comparative analysis of the thermodynamic parameters gathered in Tables 2 and 3 suggests a situation for the system under consideration which is completely different from that reported in the literature for other lipases in aqueous media, thus confirming the importance of the solvent in the mechanism of enzyme inactivation. The three-step behaviour observed for heat inactivation of suspended psychrotroph lipases (Adams & Brawley 1981; Dring & Fox 1983; Fox & Stepaniak 1983; Owusu *et al.* 1992) was indicative of different rate-limiting reactions at low and high temperatures. The enzyme unfolding, recognized by Owusu *et al.* (1992) as the phenomenon responsible for lipase inactivation at low temperatures (40–60 °C), showed activation enthal-

*Table 3.* Thermodynamic parameters of irreversible denaturation of *A. oryzae* mycelium-bound carboxylesterase.

| | |
|---|---|
| $\Delta E_d^{\neq}$ (kJ $mol^{-1}$) | 24.8 |
| $\Delta H_d^{\neq}$ (kJ $mol^{-1}$) | 22.1 |
| $\Delta G_d^{\neq}$ (kJ $mol^{-1}$) | 116.8 |
| $\Delta S_d^{\neq}$ (kJ $mol^{-1}K^{-1}$) | −0.293 |
| $r^2$ | 0.988 |

Reference temperature: 50 °C.

pies (170–221 kJ $mol^{-1}$) which were three to four times higher than the apparent values estimated in this study for both reversible inactivation (56–63 kJ $mol^{-1}$) and irreversible denaturation (22 kJ $mol^{-1}$). On the other hand, the activation enthalpies (44–78 kJ $mol^{-1}$) estimated by the same authors at high temperatures (100–140 °C), at which the irreversible denaturation due to aggregation was the limiting step, were comparable with those estimated in this study for reversible unfolding and those reported in the literature (about 60 kJ $mol^{-1}$) for ordinary chemical reactions (Owusu *et al.* 1992). Besides, the activation entropy values estimated by the same authors for irreversible denaturation have the same negative sign as that estimated in this work for the same phenomenon (−0.29 kJ $mol^{-1}$ $K^{-1}$).

The peculiar activity pattern of our mycelium-bound carboxylesterase can be explained by comparing the apparent values of the activation enthalpy estimated in this study for reversible unfolding (Table 2) and irreversible denaturation (Table 3). Since $\Delta H_d^{\neq}$ is significantly lower than the corresponding parameters estimated for both the reversible unfolding ($\Delta H_u^{\neq}$) and even the acetylation reaction ($\Delta H^{\neq}$), it is likely that the phenomenon of irreversible denaturation could act at all temperatures, once the energy barrier necessary for the transformation had been overcome. At low temperature, it would occur together with the esterification even in the presence of negligible reversible inactivation (characterized by a higher energy barrier), while at high temperature all three phenomena would quickly take place at the same time.

From this 'scenario' one can envisage our carboxylesterase, externally linked to the mycelium, finding itself in its natural environment in a less favourable situation than when isolated and suspended in organic solvent. In reality, this is not true because the thermodynamic state of a system is always given by a combination of enthalpic and entropic factors. In fact, despite a lower activation enthalpy of irreversible denaturation, $k_d$ of our system in organic solvent at 90 °C ($8.11 \times 10^{-3}$ $h^{-1}$) was about 40 and 100 times less than those one can estimate from the results obtained by Owusu *et al.* (1992) for partially purified and crude psychrotroph lipases in aqueous medium.

This result is due to a $\Delta S_d^{\neq}$ value of our system (−0.29 kJ $mol^{-1}$ $K^{-1}$) which is remarkably lower than those estimated by the above authors (−0.092 and −0.186 kJ $mol^{-1}$ $K^{-1}$), thus suggesting completely different denaturation mechanisms: the enzyme link to the mycelium could be responsible for the formation of a transition state more rigid than that experienced during the aggregation of enzyme molecules in water. These conclusions would be consistent with the very low $k_d$ value calculated for our system at 50 °C ($3.23 \times 10^{-3}$ $h^{-1}$), which is two to three orders of magnitude less than for artificially immobilized ($0.12 < k_d < 0.13$ $h^{-1}$) and native lipases in water ($1.17 < k_d < 2.86$ $h^{-1}$) (Moreno *et al.* 1997) and corresponds to an activity half-life 10 to 900-fold higher.

In conclusion, it is possible that linkage to the mycelium could have imposed on our carboxylesterase a conformation which would be optimal for catalysis, but quite different from the lowest-energy conformation in water. Although this situation appears to be, at first sight, unfavourable from the thermodynamic point of view (less $\Delta H_d^{\neq}$), the favourable entropic factor ($\Delta S_d^{\neq} \ll 0$) could be responsible for the particularly high thermoresistance of this enzyme and then could be able to protect it against a rapid loss of activity.

*Integral productivity estimation*

In view of possible continuous application of this biotransformation, it is interesting to study the behaviours of the integral specific productivities up to a given time, $P/X_0$, and up to the activity half-life, $P_{1/2}/X_0$, defined in Equations (21) and (21″), respectively. It should be stressed, before examining these trends, that both these parameters provide the amounts of ethyl acetate which would theoretically be produced by 1 g of biocatalyst under continuous feeding conditions at $S_0$, like those existing in a continuous immobilized enzyme reactor. On the contrary, this approach, which takes into account the time productivity decay due to thermal inactivation, cannot apply as such to a batch process, for which the time-decreasing actual productivity, $v$, should be used in Equation (21) instead of its starting value, $v_0$.

The characteristics of Equation (21) are illustrated in Figure 2, where the integral specific productivities estimated at 50 °C for two different substrate levels ($S_0 = 50$ and 100 mmol $l^{-1}$) are plotted against time in a semi-log plot. These results demonstrate that $P/X_0$ depends not only on the operating time but, as foreseen by Equation (12), also on the substrate concentration. Therefore, larger amounts of ethyl acetate would be produced at the higher starting substrate concentration (curves 2 and 3). The satisfactory agreement between dashed and continuous curves, which refer to integral productivities estimated using the experimental values of $v_0$ and those calculated by Equation (12) respectively, confirms the validity of the kinetic model earlier proposed. A further confirmation comes from the large variations between curves 1 and 2, the former referring to the integral productivity which would be theoretically obtained in the case $K_m$ being negligible with respect to $S_0$, that is using $k_{cat}$ in Equation (20) instead of $v_0$ in Equation (21) for this calculation. It should also be noticed that all curves grow linearly with the same rate, as a consequence of the fact that the reaction rate constant of denaturation, $k_d$, is not a function of the starting substrate level. Finally, the long duration of this linear increase in all cases (about 13 days) is in accordance with the very low $k_d$ values as well as the excellent thermostability of our mycelium-bound carboxylesterase.

Even more interesting is the effect of temperature on the integral specific productivity, which is illustrated in Figure 3 for $S_0 = 50$ mmol $l^{-1}$. As can be seen for

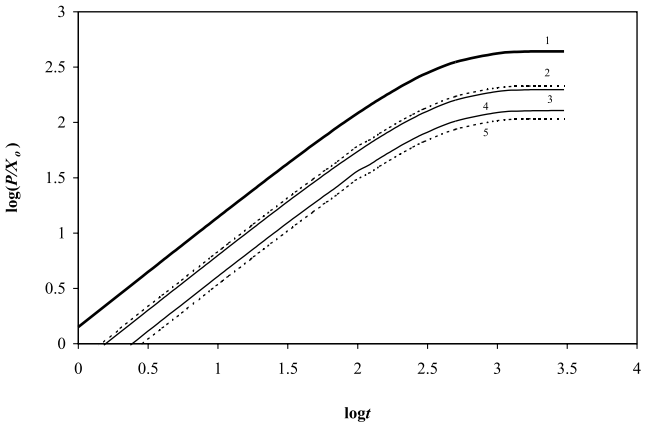


*Figure 2*. Specific integral productivity estimated at 50 °C under different conditions for continuous ethanol acetylation with mycelium-bound carboxylesterase of *A. oryzae*. (1) Estimation made using $k_{cat}$ in Equation (20). Dashed and continuous curves refer to estimations made using in Equation (21) experimental $v_0$ values and $v_0$ values calculated by Equation (12), respectively. $S_0 = 100$ mmol $l^{-1}$: (2) and (3). $S_0 = 50$ mmol $l^{-1}$: (4) and (5).

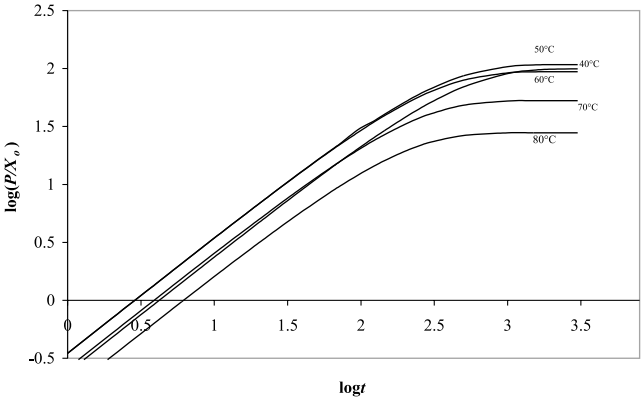


*Figure 3*. Effect of temperature on the specific integral productivity estimated for continuous ethanol acetylation with mycelium-bound carboxylesterase from *A. oryzae*. $S_0 = 50$ mmol $l^{-1}$.

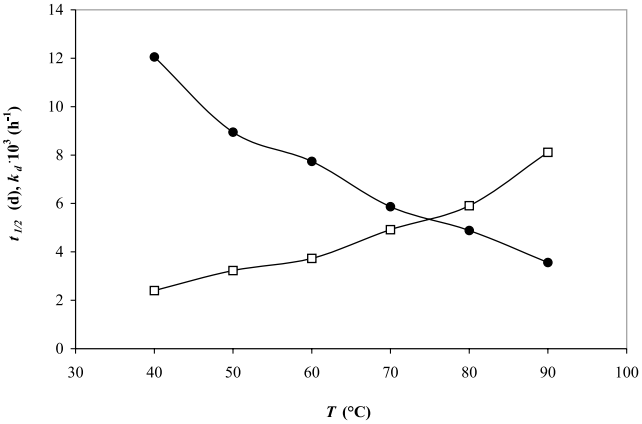


*Figure 4*. Temperature dependence of the specific productivity up to activity half-life, $P_{1/2}/X_0$, of mycelium-bound carboxylesterase from *A. oryzae*. (■) $S_0 = 100$ mmol $l^{-1}$; (○) $S_0 = 50$ mmol $l^{-1}$.

temperatures higher than the optimum ($T > 55$ °C), the highest productivity would always be obtained at the lowest temperature, independently of the operating time. This behaviour could be a direct consequence of the fact that the apparent activation enthalpy of the reversible unfolding, which seems to control the start of the esterification at $T > T_{opt}$, is higher than that of the acetylation reaction. In contrast, the decrease of $P_\infty/X_0$ with the temperature (see final values of the curves in Figure 3) is due to a corresponding increase in $k_d$, which reduces the total number of completely active molecules of the enzyme. The $P/X_0$ values estimated at 40 °C, which are lower than those at 50 °C over the whole time range investigated, are likely due to a lower significance of reversible unfolding of the enzyme with respect to the temperature-dependent activity increase described by the Arrhenius model. These effects are more evident from the values of the integral productivity up to activity half-life, $P_{1/2}/X_0$, calculated at different temperatures for both $S_0 = 50$ and 100 mmol $l^{-1}$ (Figure 4).

These results can be compared with those reported by Roels (1983) for the integral productivity of glucose isomerase, an enzyme which has a much higher value of $\Delta H_d^{\neq}$ (235.2 kJ $mol^{-1}$). Because of the negligible reversible inactivation even at relatively high temperatures, the starting rate of product formation was in that case an increasing function of temperature over the whole $T$ range of practical significance. Since $k_d$ also increased with $T$, the combination of these opposite effects led to the highest productivity at the highest temperature for short operating times and to a reverse situation for long operating times. This behaviour, ascribed by Roels (1983) to an activation enthalpy of the irreversible denaturation which was higher than that of the enzymatic reaction, is quite different from that of our mycelium-bound carboxylesterase, which is characterized by a reverse thermodynamic situation.

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