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Simplified kinetics and thermodynamics of geraniol acetylation by lyophilized cells of *Aspergillus oryzae*

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Abstract

Kinetics and thermodynamics of geranyl acetate production by direct geraniol acetylation with lyophilized cells of Aspergillus oryzae were studied in n-heptane and compared with those of ethanol acetylation. Batch tests were performed varying the starting substrates equimolar level from 25 to 150 mM, the cell concentration from 5.0 to 30 g l^{-1}, and the temperature from 30 to 95°C. The progressive increase in the starting product formation rate observed with increasing temperature up to 80°C and the successive fall beyond this value confirmed the occurrence of reversible biocatalyst inactivation. The simplified Arrhenius model was used to estimate the apparent activation enthalpies of both the acetylation of geraniol ($\Delta H^{\#} = 35$ kJ mol^{-1}) and the reversible inactivation of the biocatalyst ($\Delta H^{\#}_{i} = 150$ kJ mol^{-1}). The thermodynamic parameters of the irreversible enzyme denaturation were also estimated by residual activity tests performed on lyophilized cells previously exposed in the solvent at different temperatures for variable times ($\Delta H^{\#}_{d} = 28$ kJ mol^{-1}; $\Delta S^{\#}_{d} = -0.28$ kJ mol^{-1} K^{-1}). These results on the whole suggest that the reversible inactivation and the irreversible denaturation of mycelium-bound carboxylesterases are thwarted by increases either in the hydrophobicity or in the molecular weight of the alcoholic substrate.

Keywords: Aspergillus oryzae; Carboxylesterase; Geraniol acetylation; Geranyl acetate; Kinetics; Thermodynamics

1. Introduction

Terpene alcohols and their esters are among the most important components of essential oils. Because of their flavor and fragrance [1], they are widely used in the food, cosmetic and pharmaceutical industries. Although terpene esters, like geranyl acetate, are nowadays produced by chemical synthesis, their organoleptic characteristics are superior when isolated from natural sources [2]. To overwhelm the problems associated to the extraction of terpene esters, several authors proposed and studied their lipase-catalyzed productions, which can be considered "natural" under certain conditions [3]. Many attempts were made to produce terpene esters by direct acylation of terpene alcohols [4–10] or by transesterification [7,11–13] using immobilized lipases from different sources, but only in a few cases the results appeared interesting for future industrial application.

Geranyl and citronellyl acetates were produced with excellent yields (up to 97–98%) using triacetin or acetic anhydryde as acyl donors by transesterifications catalyzed by commercial lipases of *Candida antarctica* or *Pseudomonas sp.* [14,15]. Excellent yields were also obtained by the same authors for the synthesis of other terpene esters by varying the reaction conditions, the catalyst and the acyl donor. Direct condensation between alcohol and free acid is usually more difficult to obtain. It is an equilibrium which leads to the formation of water, therefore it is unfavored by an increase in water activity in the organic medium [16]. Moreover, direct esterification is not only hampered by water formation, but also by the strong inhibition exerted by the free acid on the lipase activity, especially when acetic acid is employed [8,10,14,17,18]. For this reason, only a few studies were published on this subject in the past [5,7–9,19] and the yields obtained were rarely satisfactory [10]. Therefore, the discovery of new microbial lipases and esterases suited for this application is very attractive.

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Fungal lipases are generally secreted as extracellular enzymes, although evidences exist regarding notable mycelium-bound activity which can be directly exploited by using whole cells [20–24]. In addition, the use of whole cells as biocatalyst can protect the enzymes even better than an immobilization matrix does. Lyophilized mycelium of a newly isolated strain of *Aspergillus oryzae*, which showed an outstanding ability to promote direct acetylation, was employed to point out the factors influencing the production of cell-bound carboxylesterases [24]. Previous work demonstrated that its esterification activity was strongly inhibited by acetic acid [21], whereas the effect of geraniol was nearly negligible [23].

The less developed research-field of non-aqueous enzymology appears to be that dealing with kinetics and thermodynamics either of the enzyme-catalyzed process or the thermal biocatalyst inactivation, which are essential to clarify the reaction mechanisms and to optimize these processes.

No systematic kinetic and thermodynamic study was performed up to now on the activity and thermal stability of mycelium-bound lipases in organic solvents, probably because of the complexity of the network of reactions. Nevertheless, a preliminary study was done on the ethanol acetylation in *n*-heptane by the same catalytic system, using the simplified Arrhenius model [25]. This approach is now extended to the acetylation of geraniol, which has been selected as representative of a family of alcohols whose long hydrophobic chain can be responsible for notable steric hindrance occurrence during the transition state formation. The experimental data of starting specific rate of geranyl acetate formation, collected under different conditions of starting substrates level, biocatalyst concentration and temperature, were utilized to investigate the kinetics and thermodynamics of this biotransformation.

2. Materials and methods

2.1. Microorganism

The cells of *Aspergillus oryzae* MIM were routinely maintained on malt extract (8 g l^{-1}, agar 15 g l^{-1}, pH 5.5), cultured in 500 ml Erlenmeyer flasks containing 100 ml of medium, and incubated for 48 h at 28°C on a reciprocal shaker (100 spm). The mold was grown on media containing Difco yeast extract 1 g l^{-1}, $(NH_4)_2SO_4$ 5 g l^{-1}, K_2HPO_4 1 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.2 g l^{-1}, pH 5.8, previously additioned with Tween 80 as carbon source. Suspensions of spores were used as inoculum.

The biocatalyst was prepared by a lyophilization protocol that proved optimal for acetylation reactions [21,23], likely because it was able to ensure the minimum content of internal water necessary for the enzyme activity. The mycelium was submitted to five successive vacuum filtrations alternated to four washings with 1:10,000 Tween 80:water solution, *n*-hexane, 100 mM phosphate buffer at pH 7.0 and deionized water, respectively. The cells were then re-suspended in deionized water, homogenized for 5 min (Silverson L2R, Silverson Machines LTD, Waterside, UK), frozen at –20°C and finally lyophilized (Alfa Criosec, Milano, Italy) at a plate temperature of 25°C.

2.2. Bioesterification tests

Geraniol acetylation was performed in 10 ml screw capped test tubes by suspending lyophilized mycelium in *n*-heptane (5 ml) and then adding geraniol and acetic acid up to the selected equimolar levels. Esterification tests were carried out in triplicate magnetically stirring the reaction mixtures under the selected operative conditions. To avoid any possible influence of water content on the kinetic and thermodynamic parameters, the study was performed utilizing the initial product formation rates, collected under conditions of negligible water formation (start of the reaction).

2.3. Analytical methods

Geraniol and geranyl acetate concentrations were determined by gas-chromatographic analysis on a Carlo Erba Fractovap GC equipped with a hydrogen flame ionization detector. The column (3 × 2000 mm) was packed with Carbowax 1540 (10% on Chromosorb 80–100 mesh). The injector temperature was 200°C. Oven temperatures ranged from 80 to 150°C. Samples (0.25 ml) were taken at intervals and added to an equal volume of an internal standard solution (1-octanol) in *n*-heptane.

2.4. Denaturation tests

The kinetics of biocatalyst irreversible denaturation were studied through experimental determinations of the lipasic residual activity. Amounts of lyophilized *A. oryzae* cells corresponding to 20 ± 0.1 g l^{-1} were added to 5 ml of *n*-heptane in 4 different test tubes for each tested temperature (35, 50, 65, and 80°C). Acetic acid and geraniol were daily added into a different test tube up to 35 ± 0.2 mM. The starting product formation rates obtained with cells previously exposed at each temperature in the solvent for variable times (1, 3, 5, and 7 days) were expressed as fractions of that obtained with fresh cells (activity coefficient, ψ) and used for estimation of the kinetic and thermodynamic parameters of the enzyme denaturation.

3. Theory

3.1. Bioesterification mechanism and kinetics

Considering the reversible character of the reactions catalyzed by carboxylesterases, a ping-pong mechanism was recently proposed by the authors for ethanol acetylation in

organic solvent [25], which is opposite to that demonstrated by Jencks for esters hydrolysis in water [26]. This general model, which may be extended, in principle, to any acylation reaction [27], implies the preliminary formation of an enzyme-acid complex, which is attacked by a serine hydroxyl group onto the acid carbonyl, giving a tetrahedral intermediate whose collapse is responsible for the release of water and a stable acyl-enzyme intermediate. The following nucleophilic attack by alcohol leads to the formation of a new tetrahedral enzyme-product complex that deacylates releasing free enzyme and ester (for symbols see appended Nomenclature):

$$\begin{array}{ccccc} & & \overset{k_1}{\rightarrow} & & \\ \mathrm{E} + \mathrm{A} & & \underset{k_{-1}}{\leftarrow} & \mathrm{E_A} + \mathrm{W} & \\ & & & + & \\ & & & \mathrm{B} & \\ & & k_{-2}\uparrow & & \\ & & & \downarrow k_2 & \\ & & \overset{k_3}{\leftarrow} & & \\ \mathrm{E} + \mathrm{P} & & \underset{k_{-3}}{\rightarrow} & \mathrm{E_P} & \end{array}$$

Since at the start of the transformation both substrates (A and B) are present nearly at their starting concentrations, whereas geranyl acetate and water levels are negligible, both the first and the third reactions can be considered irreversible. Assuming steady-state conditions for the different enzyme forms and considering that the inactive fraction of the enzyme is negligible at the start of the reaction, the starting product formation rate, v_o, can be described, under conditions of equimolar levels of both substrates, as those investigated in this study, by the Michaelis-Menten-type equation:

$$v_o = \frac{k_{cat} S_o}{K_m + S_o} \quad (1)$$

where K_m is a composite term involving the individual Michaelis constants for both substrates

As will be demonstrated later, this equation is valid for geraniol acetylation only for $S_o \leq 75$ mM, while a substrate inhibition is evident at higher starting substrate levels. For this reason, it was used at $S_o \leq 75$ mM to estimate, by direct fitting of v_o against S_o, the values of k_{cat} and K_m at 80°C and $X_o = 20 \pm 0.1$ g l^{-1}.

3.2. Reversible thermal inactivation

As recently done for ethanol acetylation [25], it was supposed that mycelium-bound carboxylesterase is subject to an instantaneous equilibrium between the completely active form, E, and a less active form, E_I, K_i being the related equilibrium constant. This equilibrium, whose position could depend on the structures of substrates and product, is progressively shifted to the formation of E_I with increasing temperature, thus resulting in the typical bell-shaped curve of enzyme activity.

It is likely that the thermal inactivation of mycelium-bound carboxylesterase observed in this study is the result of the solvent molecules interacting with the "membrane-lipase" system, which reversibly produces a conformational change (unfolding) disturbing the active conformation of the enzyme.

3.3. Irreversible denaturation

Another important phenomenon is the progressive irreversible inactivation (denaturation) of the biocatalyst, depending either on the operating time or the temperature, which could lead to an aggregated stable (inactive) protein, as it occurs for suspended enzymes [28]. It is possible that the reversible partial enzyme unfolding in organic solvent could be responsible for the exposure of a progressively growing number of hydrophobic groups to solvent molecules. This should be especially true for lipases, whose stronger link with the lipidic membrane would be possible through a rearrangement, which could irreversibly trap the enzyme molecules in an aggregated stable (inactive) form. This situation can be described by a first unfolding equilibrium followed by a second irreversible denaturation step, K_i and k_d being the equilibrium constant of partial enzyme unfolding and the first-order rate constant of denaturation, respectively.

To separate these effects, the reversible inactivation was studied in this work under conditions of negligible irreversible denaturation, that is at the starting phase of the transformation. In addition, if the latter irreversible stage of denaturation is much slower than the former, E and E_I can be considered in equilibrium and the overall denaturation process can kinetically be described by first-order kinetics. So, the first-order denaturation constant, k_d, can be estimated at different temperatures from the slopes of the straight lines obtained plotting the experimental data of $\ln\psi$ versus time.

3.4. Thermodynamics

As well known, the enzyme activity increases with temperature up to a maximum value, according to the Arrhenius-type equation [29]:

$$v_o = a \exp(-\Delta H^{\#}/RT) \quad (2)$$

So, the apparent activation enthalpy of the enzymatic reaction, $\Delta H\#$, can be estimated from the slope of the straight line obtained plotting $\ln v_o$ versus the reciprocal temperature.

At temperature higher than the optimum, on the contrary, the reversible thermal inactivation of the enzyme takes place at higher rate than the acetylation and an opposite

Table 1
Effect of biocatalyst concentration on the kinetic parameters of geraniol acetylation by lyophilized cells of *A. oryzae*. S_o = 50 ± 0.3 mM; T = 50°C

X_o (g l^{-1})	5.0	10	20	30
v_o (mmol g^{-1} h^{-1})	0.095	0.19	0.39	0.41
P_f (mM)	34.1	46.9	48.0	48.5

behavior is observed. Also this decrease of product formation rate with temperature can be approximated by an Arrhenius-type equation, by which an analogous activation parameter can be estimated. An empiric approach was proposed by Sizer, according to which the activation enthalpy of enzyme inactivation, $\Delta H^{\#}_{i}$, would be given by the sum of this parameter and the activation enthalpy of the reaction [30].

The thermodynamic parameters of the irreversible denaturation of the enzyme were estimated, on the other hand, by plotting the logarithm of the apparent first-order rate constant of denaturation, k_d, versus $1/T$, according to the Arrhenius-type equation [29]:

$$k_d = b \exp(-\Delta H^{\#}_d/RT) \quad (3)$$

where $\Delta H^{\#}_{d}$ is the activation enthalpy of the enzyme denaturation.

The activation entropy of the enzyme denaturation, $\Delta S^{\#}_{d}$, was calculated as the difference between the related activation Gibbs free enthalpy, estimated from k_d by the Eyring relation [31], and $\Delta H^{\#}_{d}$.

Notwithstanding the greater complexity of the above network of reactions, it was supposed that the limiting step of the bioesterification follows a simplified scheme, which can be described by the Arrhenius model. For this reason, all thermodynamic parameters estimated in this study are only "apparent" and can provide a rough overall thermodynamic picture of this biotransformation.

4. Results and discussion

4.1. Effect of biocatalyst concentration

Four batch tests were carried out at 50°C in *n*-heptane, using 50 ± 0.3 mM starting substrate concentration and variable biocatalyst concentration (namely 5.0, 10, 20 and 30 g l^{-1}), in order to study the effect of this parameter on the kinetics of geraniol acetylation.

The results of these tests, listed in Table 1, show an excellent average biotransformation yield on starting substrate (0.89 mol^{-1}), especially for $X_o \geq 10$ g l^{-1} (0.96 mol^{-1}), and a starting product formation rate, v_o, which linearly increased with the biocatalyst concentration up to 20 g l^{-1}. At $X_o \geq 20$ g l^{-1} v_o achieved a maximum value of about 0.40 mmol g^{-1} h^{-1}. As suggested later on by residual activity tests at different X_o values, such a behavior could be the result of a protective effect on the enzyme activity of the other constituents of the whole cell preparation. Comparison with the results of previous work [25] demonstrates that geraniol acetylation was about 8 times slower than that of ethanol at low biocatalyst concentration (5.0 g l^{-1}), but progressively accelerated reaching nearly the same rate at X_o = 30 g l^{-1}.

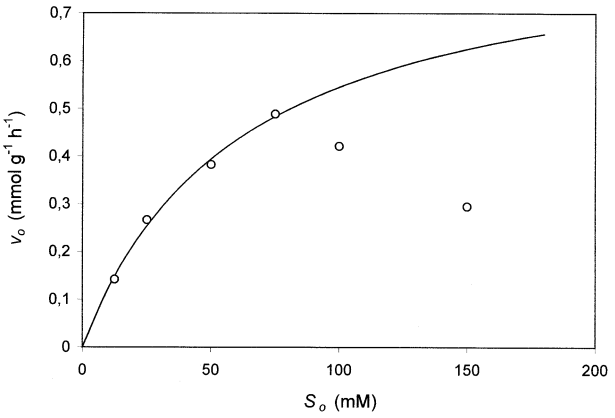

Fig. 1. Dependence of starting geranyl acetate formation rate, v_o, on starting substrate equimolar concentration, S_o. T = 80°C; X_o = 20 ± 0.1 g l^{-1}.

4.2. Effect of starting substrate concentration

Additional tests were carried out at 80°C and X_o = 20 ± 0.1 g l^{-1}, varying the starting equimolar concentration of both substrates (geraniol and acetic acid) from 12.5 and 150 mM, in order to verify whether also this bioacetylation follows the proposed mechanism kinetically described by the Michaelis–Menten-type equation [1] as well as to estimate the related kinetic parameters.

The experimental data of product concentration versus time were used to calculate the starting product formation rates at different S_o values. An apparent Michaelis constant, K_m, of 62 mM and a k_{cat} value of 0.88 mmol g^{-1} h^{-1} were estimated by direct fitting of v_o against S_o for $S_o \leq 75$ mM (Fig. 1). These values are about 50 and 38% less, respectively, than those estimated for ethanol acetylation at 55°C. Considering the quite large errors of such kinds of estimates and that these parameters act in opposite ways on the starting productivity, it is easy to conclude that ethanol and geraniol are acetylated with comparable rates at their respective optimum temperatures. The very high K_m values calculated for both ethanol and geraniol acetylations, which are consistent with the well-known remarkable increase observed for this parameter in organic solvents, could be ascribed, as suggested by Kasche et al. for similar enzymatic systems [32], to a competitive inhibition between solvent and substrate for binding at the active site.

This kinetic study demonstrates that, at relatively low starting substrates concentration ($S_o \leq 75$ mM), geraniol acetylation by mycelium-bound carboxylesterase of *A.*

Table 2
Influence of temperature on starting geranyl acetate formation rate, v_o, and product yield on starting substrate, $Y_{P/So}$. Biocatalyst = lyophilized cells of *A. oryzae*. $X_o = 20 \pm 0.1$ g l^{-1}. $S_o = 50 \pm 0.3$ mM

T(°C)	v_o (mmol g^{-1} h^{-1})	$Y_{P/So}$ (mol mol^{-1})
30	0.11	0.97
40	0.22	0.97
50	0.43	0.97
60	0.52	0.97
70	0.65	0.97
80	0.85	0.95
85	0.58	0.83
90	0.30	0.72
95	0.18	0.59

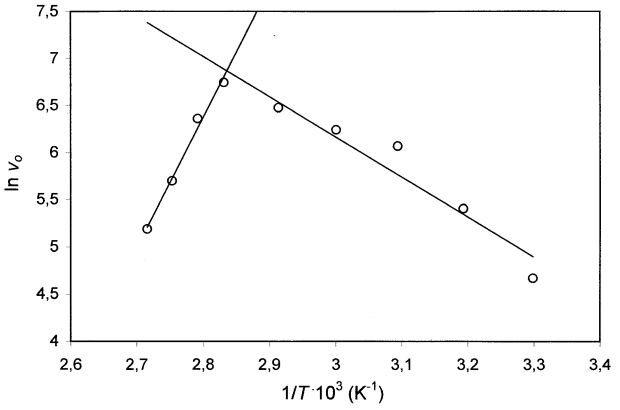

Fig. 2. Arrhenius plots for the estimation of the thermodynamic parameters of both a) geraniol acetylation by mycelium-bound carboxylesterase of *A. oryzae* and b) reversible inactivation of the biocatalyst. $S_o = 50 \pm 0.3$ mM; $X_o = 20 \pm 0.1$ g l^{-1}.

oryzae can theoretically be handled, likewise that catalyzed by free enzymes, with the Michaelis–Menten theory. At higher S_o values, on the contrary, Equation [1] is not able to rigorously describe the acetylation of geraniol because of the occurrence of an evident substrate inhibition phenomenon, likely due to acetic acid. In fact, the strong inhibiting role of acetic acid on the system under consideration was previously demonstrated varying independently the concentrations of both substrates [21,23].

4.3. Effect of temperature

The final part of the work was addressed to the thermodynamic study of geraniol acetylation by *A. oryzae* mycelium-bound carboxylesterase. As the results of Table 2 show, v_o progressively increased with temperature up to 80°C, which confirms the thermoresistance of our biocatalyst. Comparison of this threshold value with those observed for covalently immobilized lipases [33] suggests not only an enzyme protection against thermal inactivation exerted by its natural confinement within the cell but also its likely localization in the cell membrane [24]. In addition, the lower temperature threshold previously determined for ethanol acetylation (55°C) [25] demonstrates that the thermal stability of this carboxylesterase could be significantly influenced by the structures of substrates and products. Finally, the reversible nature of thermal inactivation of biocatalyst used for very short times (less than 0.5 h) was demonstrated by the nearly negligible loss of activity (less than 2%) observed when it was re-used up to 3–4 times.

The main apparent thermodynamic parameters of both geraniol acetylation and biocatalyst reversible inactivation were estimated with satisfactory correlation ($0.94 < r^2 < 0.99$) with the Arrhenius model from the plots of Fig. 2. These values, listed Table 3, confirm that the phenomenon responsible for reversible thermal inactivation requires for the transition state formation higher activation energy than the enzymatic reaction. Besides, since the activation enthalpies of ethanol and geraniol acetylations are nearly coincident, the transition state of the limiting step should scarcely be influenced by steric hindrance. Considering the much bulkier structure of geraniol with respect to ethanol, such a situation appears to be consistent only with the formation of a transition state not involving geraniol, which would be possible, according to the proposed mechanism, only if the enzyme acetylation would be the limiting step. Finally, the activation enthalpy of reversible inactivation is much higher when associated to the acetylation of geraniol (150 kJ mol^{-1}) rather than of ethanol (63 kJ mol^{-1}), which is in agreement with the well-known greater inactivation of enzymes in organic solvents by shorter chain alcohols [34,35].

As far as the biocatalyst denaturation is concerned, the first-order rate constant of enzyme denaturation, k_d, estimated from Fig. 3 as previously described, progressively increased with temperature, showing the typical one-step decay of the first-order denaturation pattern, like that observed for other lipases [33]. The values of k_d calculated in this study are 40–60% less than those previously presented for ethanol acetylation [25], pointing out that biocatalyst activity is lost more slowly when used for geranyl acetate rather than for ethyl acetate production. From the thermodynamic viewpoint (Table 3), such a stability enhancement is made explicit by a constancy of $\Delta S^{\#}_{d}$ (-0.29 and −0.28 kJ

Table 3
Apparent thermodynamic parameters of geraniol and ethanol acetylations by lyophilized cells of *A. oryzae*, estimated by the Arrhenius model under different conditions. Reference temperature: 50°C

	Alcohol acetylation	Reversible inactivation	Irreversible denaturation
a) Ethyl acetate production			
$\Delta H^{\#}$ (kJ mol^{-1})	31	63	22
$\Delta S^{\#}$ (kJ mol^{-1} K^{-1})	—	—	−0.29
r^2	0.98	0.96	0.99
b) Geranyl acetate production			
$\Delta H^{\#}$ (kJ mol^{-1})	35	150	28
$\Delta S^{\#}$ (kJ mol^{-1} K^{-1})	—	—	−0.28
r^2	0.94	0.99	0.99

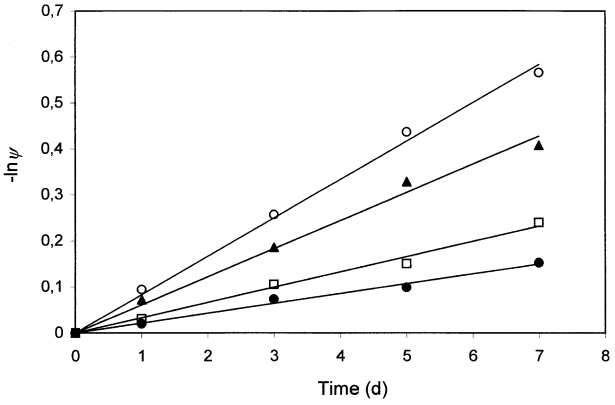

Fig. 3. Semi-log plots of irreversible denaturation of mycelium-bound carboxylesterase of *A. oryzae*. S_o = 35 ± 0.2 mM; X_o = 20 ± 0.1 g l^{-1}; *T* (°C): 35 (●); 50 (□); 65 (▲); 80 (○).

mol^{-1} K^{-1} for ethanol and geraniol acetylations, respectively) and a valuable increase in $\Delta H^{\#}_d$ (22 and 28 kJ mol^{-1}, respectively). These results on the whole suggest that also the irreversible denaturation of this system could be thwarted by increases either in the hydrophobicity or in the molecular weight of the alcoholic substrate.

A comparative analysis of these thermodynamic parameters qualitatively confirms the situation previously observed for ethanol acetylation. Because of the very low $\Delta H^{\#}_d$ value, the phenomenon of irreversible denaturation could act at all temperatures, once the energy barrier necessary for geraniol acetylation would be overcome. At low temperature, it would take place simultaneously with the enzymatic transformation, even in the presence of negligible reversible inactivation, while at high temperature all three phenomena would take place at the same time. However, despite of its low activation enthalpy, the irreversible denaturation is a very slow process, showing an activity half-life at 80°C (about 200 h) which is from one to three orders of magnitude longer than those of other lipases either in aqueous or in organic media [31,33,36]. This result is due to $\Delta S^{\#}_d$ values of our carboxylesterase which are much lower than those estimated by the above authors (-0.092 and −0.19 kJ mol^{-1} K^{-1}).

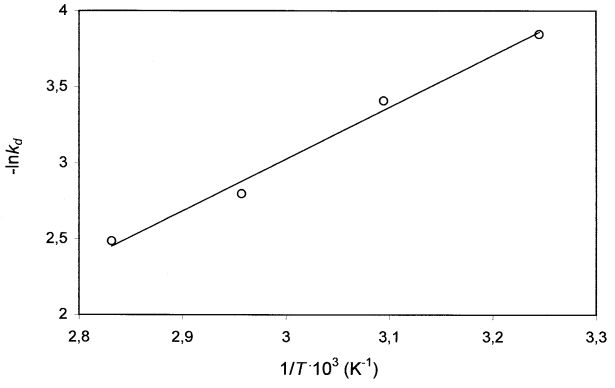

Fig. 4. Arrhenius plot for the estimation of the thermodynamic parameters of irreversible denaturation of mycelium-bound carboxylesterase of *A. oryzae*.

Finally, to shed light on the nature of the linear increase in ν_o with X_o using geraniol, two additional tests of biocatalyst stability were performed at 80°C using either lower (10 g l^{-1}) or higher (30 g l^{-1}) biocatalyst concentrations. Although the residual activity decreased or increased, respectively, by about 6 and 8%, this variation is sufficiently higher than the standard deviation (less than 1%) as to suggest the occurrence of a protective effect on the enzyme activity of the other constituents of the whole cell preparation.

5. Conclusions

A kinetic and thermodynamic study was performed on the geraniol acetylation by mycelium-bound carboxylesterase of *Aspergillus oryzae*, varying temperature, starting substrate level and biocatalyst concentration. The results of these tests showed:

- a progressive increase in the specific geranyl acetate formation rate with increasing X_o, possibly due to a protective effect on the enzyme activity;
- a dependency of ν_o on starting substrate concentration, in accordance with Michaelis-Menten-type kinetics for $S_o \leq 75$ mM (K_m = 62 mM; k_{cat} = 0.88 mmol g^{-1} h^{-1});
- an increase in ν_o with temperature up to an optimum (80°C) and a decrease beyond this threshold, which are consistent with the typical thermal patterns of most biosystems.
- The kinetic results of experiments on both geraniol acetylation and residual activity performed at variable temperature confirmed the occurrence of two different enzyme inactivation phenomena:
- a temperature-dependent reversible inactivation, possibly due to the interaction of the “membrane-lipase” system with the solvent molecules;
- a first-order irreversible denaturation of the biocatalyst, whose rate grew with both time and temperature, likely due to trapping of the enzyme within the mycelium.
- The apparent thermodynamic parameters of all these phenomena were estimated to shed light on the geranyl acetate formation mechanism. Compared with purified enzymes, lyophilized mycelium can be used in organic media with more favorable kinetics of biocatalyst inactivation. In addition, its thermal stability, resulting from combination of reversible and irreversible inactivations, appears to increase with both the alcohol hydrophobicity and molecular weight.
- The next attempt will deal with a more thorough kinetic study taking into account the individual affinities and inhibition constants of both substrates.

Nomenclature

a	Constant defined in Eq. (2), mmol g^{-1} h^{-1}
A	Acetic acid
b	Constant defined in Eq. (3), h^{-1}
B	Geraniol
E	Free enzyme
E_A	Complex of the enzyme with acetic acid
E_I	Less active form of the enzyme
E_P	Complex of the enzyme with geranyl acetate
k	Kinetic rate constant, h^{-1} or mM^{-1}
k_{cat}	Kinetic constant defined in Eq. (1), mmol g^{-1} h^{-1}
Ki	Equilibrium constant of enzyme unfolding, dimensionless
K_m	Apparent Michaelis constant, mM
P	Geranyl acetate
P_f	Geranyl acetate concentration at the equilibrium, mM
R	Ideal gas constant, kJ mol^{-1} K^{-1}
r^2	Determination coefficient, dimensionless
S	Substrate concentration, mM
t	Time, h or d
T	Temperature, °C or K
W	Water
X	Biomass concentration, g L^{-1}
$Y_{P/So}$	Product yield on starting substrate, mol mol^{-1}

Subscripts

d	Values referred to the biocatalyst irreversible denaturation
i	Values referred to the biocatalyst reversible inactivation
o	Starting value
1,2,3	Values referred to the forward reactions of the geranyl acetate formation scheme
-1,-2,-3	Values referred to the reverse reactions of the geranyl acetate formation scheme

Greek symbols

$\Delta H^{\#}$	Activation enthalpy, kJ mol^{-1}
$\Delta S^{\#}$	Activation entropy, kJ mol^{-1} K^{-1}
ν	Specific product formation rate, mmol g^{-1} h^{-1}
ψ	Activity coefficient, dimensionless

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