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Mycelium-bound carboxylesterase from *Aspergillus oryzae*: an efficient catalyst for acetylation in organic solvent

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Abstract

Dry mycelium of a strain of *Aspergillus oryzae* efficiently catalyzed the esterification between free acetic acid and primary alcohols (geraniol and ethanol) in organic solvent. The growth conditions to obtain high activity of mycelium-bound enzymes were firstly evaluated. A medium containing Tween 80 as carbon source furnished mycelium with the highest activity in the hydrolysis of α-naphthyl esters (α-N-acetate, butyrate, caprylate). Dry mycelium was employed to select suited conditions for an efficient acetylation of ethanol and geraniol in heptane. Maximum productions were obtained using 30 g l^{-1} of lyophilized cells: 12.4 g l^{-1} of geranyl acetate were produced at 80°C starting from 75 mM geraniol and acetic acid (84% molar conversion) and 4.1 g l^{-1} of ethyl acetate at 50°C from 50 mM ethanol and acetic acid (94% molar conversion) after 24 h. The stability of the mycelium-bound carboxylesterases are notable since only 10–30% loss of activity was observed after 14 days at temperatures between 30 and 50°C. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Aspergillus oryzae; Biotransformation; Esterification; Acetylation; Lipase; Esterase; Organic solvent

1. Introduction

Only few examples are reported of lipase-catalysed acylation with free acetic acid able to furnish good yields of acetate ester [4, 5]. Direct acetylation of alcohols is difficult to obtain by enzymatic catalysis, since lipase activity is often inhibited by the occurrence of the free acid [6,8]. The discovery of new microbial lipases and esterases suited for this application is, therefore, very attractive. Fungal lipases show often interesting properties as biocatalysts; they are generally secreted as extracellular enzymes, although evidences exist regarding notable mycelium-bound activity which can be directly exploited by using whole cells [1–7] [11–33]. Lipases with broad substrate specificity have been found in the culture filtrates of several species of *Aspergillus* [2], but also mycelium-bound lipase from a strain of *Aspergillus flavus* showed interesting substrate specificity, being able to catalyse the acidolysis of several vegetable oils [9,10].

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In this work a newly isolated strain of *Aspergillus oryzae* was employed to investigate the production of cell-bound carboxylesterases and their performances for acetylation of geraniol and ethanol in organic solvent. The substrates were chosen since the importance of their acetate esters as flavor and fragrance components in the food and cosmetic industry.

2. Materials and methods

2.1. Materials

Chemicals were all from Fluka Chimica, Milano (Italy), except Tween 80 from Merck, Milano (Italy).

2.1.1. Microorganism

Aspergillus oryzae MIM was used throughout this study and routinely maintained on malt extract (8 g $l^{-1,}$ agar 15 g $l^{-1,}$ pH 5.5). Identification was carried out at CBS (Centraal Bureau voor Schimmelcultures, Baarn, Holland). To obtain cells for biocatalytic activity tests, the microorganisms were cultured in 500 ml Erlenmeyer flasks containing 100 ml of

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medium and incubated for 48 h at 28°C on a reciprocal shaker (100 spm). The mold was grown on media containing a basal medium (**BM**: Difco yeast extract 1 g l^{-1}, $(NH_4)_2SO_4$ 5 g l^{-1}, K_2HPO_4 1 g l^{-1}, $MgSO_4.7H_2O$ 0.2 g l^{-1}, pH 5.8) or soytone (0.5%) added with carbon sources (Tween 80, glucose, olive oil). Suspensions of spores at different concentrations were used as inoculum. The microorganisms were also cultured in 10 L stirred tank reactor containing 5 L of medium at 28°C, 200 rpm and aeration 1 vvm.

2.1.2. Hydrolytic assays

Esterase activity was assayed using α-naphthylacetate (α-NA), α-naphthylbutyrate (α-NB), α-naphthylcaprylate (α-NC) and α-naphthylpalmitate (α-NP) as substrates, and measuring (560 nm) the absorbance relative to the chromophores originated by reaction of the hydrolyzed substrate with Fast Garnet GBS salt. Hydrolysis were separately assayed on the filtered mycelium and on the filtrated broth. Cells grown on different media in submerged cultures were harvested by filtration at 4°C; the filtrate was directly employed for hydrolysis, while the mycelium was washed with phosphate buffer and homogenized at 4°C to obtain a suspension suited for the test. The solutions of the naphthyl derivatives were prepared by adding the substrates (0.42 mM) in 4 ml of ethanol to 96 ml of buffer solution (0.1 M Tris-HCl, pH 7.0) containing arabic gum (10 mg) and dioctyl sulfosuccinate sodium salt (AOT, 200 mg). The reaction mixtures were obtained by adding 1.5 ml of the substrate solution to 1 ml of the enzymatic fraction in 0.1 M Tris-HCl, pH 7. The enzymatic activity was expressed as unit (U) which is the amount of enzyme contained in 1 ml of filtered broth or in the mycelium filtered from 1 ml of the broth which catalysed the transformation of 1 μmole of substrate in 1 min at 45°C.

2.1.3. Acetylation with lyophilized mycelium

Initial transformations were performed with dry solvent and with solvent not treated before the use, showing no significant differences; therefore, all the sets of experiments were carried out using solvents not dried before the use. Ester synthesis was carried out in 10 ml screw capped test tubes. A typical procedure is reported: 150 mg of lyophilised mycelium is suspended in 5 ml of *n*-heptane and left under magnetic stirring for 15 min and then the alcohol and acetic acid are added. Different concentrations of dry mycelium and substrates were used in our experiments. The reaction mixtures were magnetically stirred at different temperatures. Experiments were also performed in the presence of activated 4Å molecular sieves to check the effect of water removal, but also in this case no significant differences were observed. The initial rates were expressed as μmol of product formed in one hour per gram of biocatalyst. Results are the means of three replicates.

2.1.4. Analytical methods

Alcohol and ester concentrations of the biotransformations in organic solvent were determined by gas-chromatographic (GC) 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.

3. Results

3.1. Influence of the growth conditions on carboxylesterase activity

The hydrolytic activity of extracellular and cell-bound carboxylesterases of *Aspergillus oryzae* MIM grown on different media was checked using α-naphthyl esters (α-N-acetate, butyrate, caprylate and palmitate) as substrates. Media containing glucose and/or substrates frequently used

Table 1
Effects of medium composition on lipolytic activity (U 10^2) and growth of *Aspergillus oryzae* MIM. The enzymatic activity was expressed as unit (U) defined as the amount of enzyme contained in 1 ml of filtered broth or in the mycelium filtered from 1 ml of the broth which catalyzed the transformation of 1 μmole of substrate in 1 minute at 45°C

Medium	Acetate		Butyrate		Caprylate		Palmitate		Dry weight (g/L)
	Mycelium	Extra	Mycelium	Extra	Mycelium	Extra	Mycelium	Extra	
Glucose 0.5% + BM	0.9	1.6	9.5	1.0	6.5	<0.1	0.4	<0.1	1.7
Glucose 2.0% + BM	0.7	1.3	9.0	1.0	4.7	0.3	0.3	0.1	2.5
Glucose 0.5% + soytone	1.0	1.5	11.5	1.1	6.2	0.3	0.4	0.1	2.2
Tween 80 0.5% + BM	7.5	1.5	74.0	12.5	68.4	7.6	0.6	1.9	1.4
Tween 80 2.0% + BM	4.9	0.6	18.9	0.6	6.5	<0.1	<0.1	<0.1	3.2
Tween 80 0.5% + soytone	7.0	1.5	62.0	2.1	19.2	4.8	0.3	1.8	2.4
Olive oil 0.5% + BM	2.1	0.4	50.1	4.9	3.3	0.2	4.3	0.3	3.1
Olive oil 2.0% + BM	0.5	0.5	1.5	1.5	1.8	0.2	<0.1	<0.1	5.3
Tween 80 0.5% + glucose 2.0% + soytone	1.1	1.4	48.1	7.9	3.4	8.5	0.5	4.5	3.8

Table 2
Formation of geranyl acetate catalyzed by *Aspergillus oryzae* grown on different media. Molar conversion after 24 h

Medium	Initial rate (μmol g^{-1} h^{-1})	Molar conversion (%)
Tween 80 0.5% + BM	300	97
Tween 80 2.0% + BM	<10	10
Tween 80 0.5% + soytone	130	97
Olive oil 0.5% + BM	25	25
Olive oil 2.0% + BM	10	15
Glucose 0.5% + BM	0	0
Glucose 2.0% + BM	0	0
Glucose 2.0% + soytone	<10	10
Tween 80 0.5% + glucose 2.0% + soytone	105	97

as inducers for lipases (Tween 80, olive oil) were employed for the growth. Soytone and a basal medium (BM) were used to provide nitrogen, phosphate and mineral salts. The cell-bound and extracellular activity are reported in Table 1.

The use of 0.5% Tween 80 strongly enhanced the overall lipolytic activity, which was mostly bound to the mycelium. Concentration of Tween 80 was crucial: a strong induction of the hydrolytic activity was generally observed when it was used at 0,5%, while at higher concentration the mold showed weak overall activity. The use of glucose as carbon source resulted in poor hydrolytic activity. In all the cases the highest overall activity was observed on medium chain acyl groups (butyrate and caprylate), while the activity on the palmitate ester was quite low and often mostly found in the filtered broth.

It was also evaluated if the mycelium-bound lipase/esterase activity could be exploited to catalyse ester formation in organic solvent. Geranyl acetate synthesis was chosen as current reaction. Mycelium grown for 48 h was filtered, washed, lyophilized and suspended (30 g l^{-1}) in *n*-heptane; the transformation was carried out under agitation using 50 mM substrate concentration at 50°C (Table 2).

The medium composed by 0.5% Tween 80 and BM furnished mycelium with the highest specific activity, confirming the trend observed in hydrolytic reactions and it was, therefore, used for further experiments.

Aspergillus oryzae MIM was grown in a stirred tank reactor using different spore concentrations ranging from 1.6×10^4 to 2×10^6 (spores ml^{-1}) and harvested at different times. Mycelium were lyophilised and used for geranyl acetate formation in heptane (Fig. 1)

Lyophilized mycelium from pellets obtained using the lowest spore concentration and after 72 h showed the highest activity and was used for the optimization of ethanol and geraniol acetylation.

3.2. Effect of biocatalyst concentration on ethanol and geraniol acetylation

The conditions for obtaining high rates and molar conversions of geranylacetate and ethylacetate synthesis catalysed by dry mycelium of *Aspergillus oryzae* MIM starting from the free acid and alcohol were evaluated. Biotransformations were carried out at various biocatalyst concentration by suspending different amounts of dry mycelium in *n*-heptane using 50 mM substrate concentration at 50°C. Biocatalyst concentration between 20 and 30 g l^{-1} gave the best results as shown in Fig. 2.

3.3. Effect of temperature on ethanol and geraniol acetylation

The synthesis of the two acetate esters was performed by resuspending dry mycelium of *Aspergillus oryzae* (30 g l^{-1}) in *n*-heptane using 50 mM substrate concentration at different temperatures. The maximum temperature evaluated was 70°C when ethanol was used as substrate.

The acetylation of geraniol was very fast at 80°C, being almost complete after 4 h, while for ethylacetate formation a sharp decrease of the molar conversions were observed

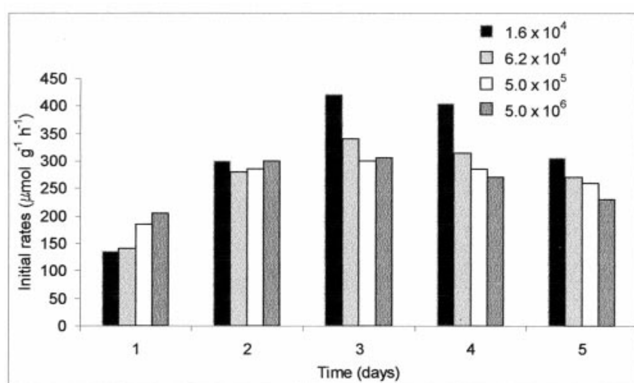

Fig. 1. Geranylacetate formation in heptane catalysed by *Aspergillus oryzae* obtained using different spore concentrations. Reaction conditions: initial alcohol and acetic acid concentration 50 mM in heptane containing 30 g l^{-1} of dry mycelium at 50°C.

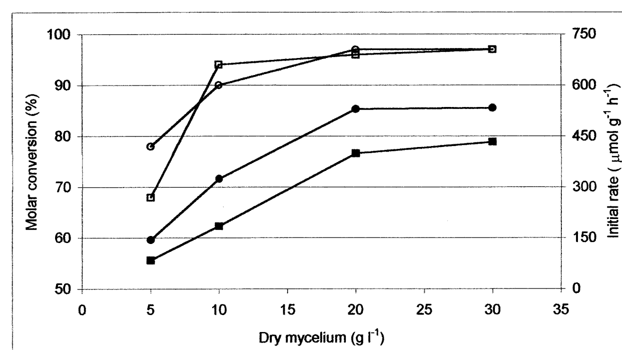

Fig. 2. Effect of the concentrations of dry mycelium of *Aspergillus oryzae* on acetate ester production. Initial rates: ■, geranylacetate; ●, ethylacetate. Molar conversions after 24 h: □, geranylacetate; ❍, ethylacetate. Reaction conditions: initial alcohol and acetic acid concentration 50 mM in heptane at 50°C.

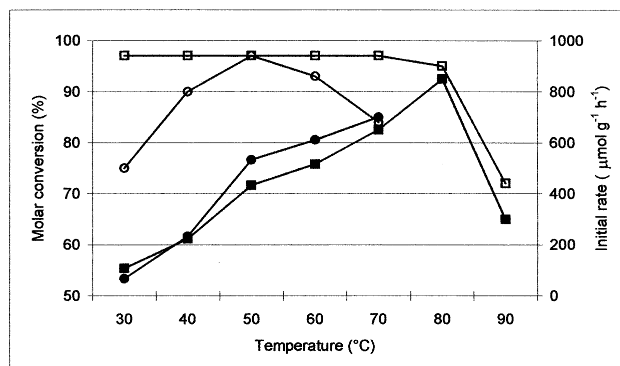

Fig. 3. Effect of temperature on acetate ester production catalysed by dry mycelium of *Aspergillus oryzae.* Initial rates: ■, geranylacetate; ●, ethylacetate. Molar conversions after 24 h: □, geranylacetate; ❍, ethylacetate. Reaction conditions: initial alcohol and acetic acid concentration 50 mM in heptane containing 30 g l^{-1} of biocatalyst.

above 50°C, the highest rate being observed at 70°C. Therefore, further experiments were carried out at 50°C for ethylacetate and 80°C for geranylacetate synthesis where high molar conversions were observed with still high initial rates.

3.4. *Effect of substrate concentration on ethanol and geraniol acetylation*

The influence of substrate concentration on acetylation reaction carried out with free acetic acid is very important, since several studies reported negative effect exerted by free acetic acid toward enzymatic activity [8] [6]. Experiments were carried out in equimolar concentrations of the substrates ranging from 25 to 150 mM.

The highest rates and yields of geranylacetate synthesis were achieved between 50–75 mM substrate concentration. Lower yields were always observed for ethylacetate formation, although the reaction still occurred with good rates even at 150 mM. This situation may be due to an incomplete shift of the equilibrium toward ester formation or to the toxic effect of ethanol after prolonged contact with the microorganism.

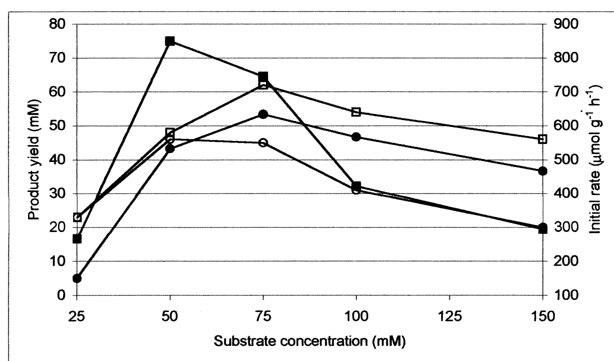

Fig. 4. Effect of substrate concentration on acetate ester production catalysed by dry mycelium of *Aspergillus oryzae.* Initial rates: ■, geranylacetate; ●, ethylacetate. Molar conversions after 24 h: □, geranylacetate; ❍, ethylacetate. Biotransformations carried out in heptane containing 30 g l^{-1} of biocatalyst. Ethylacetate synthesis was carried out at 50°C, geranylacetate synthesis at 80°C.

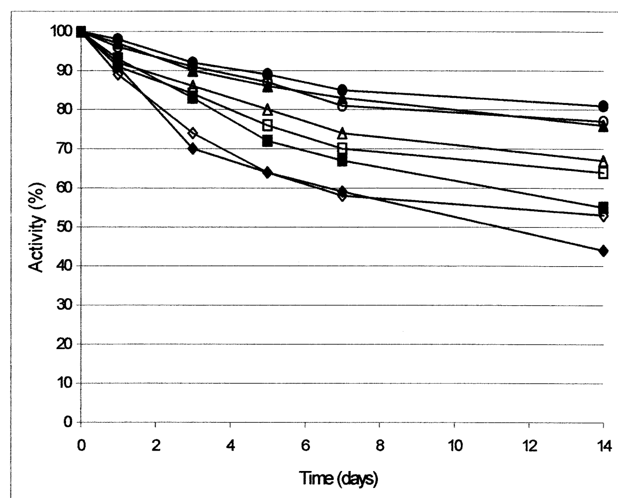

Fig. 5. Temperature stability of lyophilized mycelium of *Aspergillus oryzae* in heptane at 35°C (●, geranylacetate; ❍, ethylacetate), at 50°C (▲, geranylacetate; △, ethylacetate), at 65°C ■, geranylacetate; □, ethylacetate) and 80°C (◆, geranylacetate; Δ, ethylacetate),

The thermal stability of the lyophilized mycelia maintained in heptane at various temperatures was evaluated by checking their ability to catalyse geraniol (full symbols) and ethanol (hollow symbols) acetylation. Experiments were performed under standard conditions (35 mM, 50°C). The residual activity is expressed as transformation rates after 1 h (Fig. 5).

Mycelia showed fairly good stability with only 10–30% loss of activity after 14 days when stored at temperatures between 30 and 50°C; it is noteworthy that even at 80°C, still 40–55% of the original activity could be observed.

4. Discussion

Although a number of commercial lipases or esterases are now available for selective synthesis of esters, few problems are still hampering their employment on preparative scale, such as the difficulty to achieve high yields in direct esterification using free acids (especially when acetic acid is used), the need to control water activity and the need for enzymes with long-term stability in the reaction conditions. Most of the lipase-catalysed production of esters have been carried out by transesterification to avoid free acid toxicity and water formation. Only recently Claon and Akoh found that immobilized lipases from *Candida antarctica* promotes highly effective direct esterification of geraniol and citronellol with acetic acid [4,5]. Cell-bound enzymes may be potentially useful for these applications but their purification can be costly and sometimes they are relatively inactive outside the cell environment or partially hydrolysed

by other enzymes during procedures to fractionate cells. The activity of enzymes still bound to the microbial cell can be directly exploited for esterification in organic solvent by employing dry whole cells. In this work we have selected a mold able to catalyse direct acetylation of ethanol and geraniol furnishing good yields in batch biotranformations and showing fairly long-term stability in organic solvent when used as lyophilized mycelium. The microorganism was identified as a strain of *Aspergillus oryzae.* Species belonging to this genus have been already known to have mycelium-bound lipases with interesting features. The hydrolytic enzymes were assayed using α-naphthyl esters (α-N-acetate, butyrate, caprylate and palmitate) as substrates; extracellular and cell-bound activities were checked, showing that the use of Tween 80 as carbon source induced a high lipolytic activity toward esters of medium chain acid mostly associated with the mycelium. These enzymes can reverse their hydrolytic behavior in media with low water content thus promoting ester formation: the same medium resulted to be apt to produce mycelium able to catalyse geranylacetate formation starting from 50 mM substrate concentration. The synthesis of geranyl acetate catalysed by dry mycelium of *Rhizopus* in heptane has been already reported [11,13], but *Aspergillus oryzae* MIM gave much higher rates of ester synthesis than previously obtained. The production of geranyl and ethyl acetate was optimized by studying the best reaction conditions. Maximum productions were obtained using 30 g l^{-1} of lyophilized cells after 24 h: 12.4 g l^{-1} of geranyl acetate were produced at 80°C starting from 75 mM geraniol and acetic acid (84% molar conversion) and 4.1 g l^{-1} of ethyl acetate at 50°C from 50 mM ethanol and acetic acid (94% molar conversion). It must be stressed that only 30 g l^{-1} of lyophilized mycelium were used which means that the specific activity of the enzymes involved in the transformation is very high.

Water content is often a crucial problem for shifting the equilibria of enzyme-mediated esterifications [14]. In our experiments it was observed that solvent dried prior the use or removal of the water formed during the reactions did not significantly affect the transformation. This is an interesting feature of this biotransformation since in many enzyme-catalysed direct esterification the use of systems for water withdrawal is mandatory to achieve satisfactory yields. The reasons why direct esterification can be so efficiently catalysed by dry mycelia of molds [11–13] is puzzling. The water already present or released during the acylation may partition to microbial sites other than those involved in the enzymatic esterification, thus avoiding a shift of the equilibrium toward hydrolytic reactions.

The enzymes involved show a notable thermostability since only above 80°C they showed strong decrease of their activity. Mycelium had also high long-term thermostability when contacted with heptane. Still 40–50% of the original activity was observed after two weeks at 80°C, indicating that continuous processes may be feasible. The maximum yield for ethylacetate synthesis was observed at 50°C; this can be due to the fact that at higher temperatures ethanol and ethylacetate are highly volatile.

It is noteworthy that *Aspergillus oryzae* has been chosen as recombinant host system to clone and express high levels of secreted lipases [3], while in this work the production of mycelium-bound enzymes with high specific activity from the same species have been obtained by simply choosing suited growth conditions.

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