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# Bio-synthesis and hydrolysis of ethyl phenylacetate and ethyl 2-phenylpropionate in organic solvent by lyophilized mycelia

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*To select the best biocatalysts for ethanol acylations with phenylacetic and 2-phenylpropionic acids, lyophilized mycelia of* Aspergillus oryzae *CBS 10207,* A. oryzae *MIM,* Rhizopus oryzae *CBS 11207,* R. oryzae *CBS 39134,* R. oryzae *CBS 26028 and* R. oryzae *CBS 32847 were tested in this study. The carboxylesterase activities of* A. oryzae *MIM and* R. oryzae *11207, which revealed to be the best biocatalysts, were investigated either in 0.1 M phosphate buffer or in n-heptane to catalyze the hydrolysis or the synthesis of ethyl esters of these acids, respectively.* A. oryzae *proved more effective than* R. oryzae*, probably due to more favorable microenvironment conditions and thermodynamic scenario. The results in terms of product formation and substrate consumption versus time were used to estimate the maximum conversion yields, the equilibrium constants and the times needed to reach half maximum conversion, thus providing sufficient information about these equilibria.*

**Uniterms**
- Esterification
- Phenylacetic acid
- 2-Phenylpropionic acid
- Organic solvent
- Carboxylesterase

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## INTRODUCTION

Esterifications can be performed enzymatically using lipases or esterases in organic solvents, under conditions suitable to address their catalytic activity towards the synthesis (Sih *et al.*, 1996). The esterification capacity is usually favored in low-polarity solvents, although some enzymes, i.e. lipases from *Candida antarctica*, exhibited good activity even in polar solvents (Yang, Russell, 1996).

Acylations catalyzed by hydrolases in organic solvent are by now numerous and some of them are also used in the pharmaceutical industry (Furui *et al.*, 1996). Among these, the 2-arylpropionic acids, an important class of anti-inflammatory non-steroidal drugs, show their pharmacological activity mainly in the (*S*)-enantiomer (Stahly, Starret, 1997).

A direct enzymatic method to obtain the resolution of acid racemic mixtures is the enantioselective esterification. Enzymatic esterifications of arylpropionic acids are above all obtained by interesterification as for 2-phenylpropionic acid (Yang, Russell, 1996). Conversely, since the commercial enzymes are unsuitable to carry out the direct esterification

owing to thermodynamic constraints, only few examples are reported of lipase-catalyzed acylations with acetic acid able to furnish good yields of acetates.

Direct acetylation of alcohols is difficult to obtain by enzymatic catalysis, since lipase activity is often inhibited by the free acid (Castro *et al.*, 1997). The discovery of new microbial lipases and esterases suited for this application is, therefore, very attractive. Fungal lipases often show 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 lyophilized mycelium (Bell *et al.*, 1978; Gancet, Guignard, 1986; Molinari *et al.*, 1998, 2000).

Lyophilized cells can also be used in organic solvents, which would allow exploiting directly cell-bound lipases/esterases. Furthermore, the cell structure may act as natural matrix able to protect the enzymes from the possible negative action of external agents, providing an effect analogous to that exerted by common matrixes used for enzyme immobilization. Mycelium-bound lipase from a strain of *Aspergillus flavus* showed interesting substrate specificity, being able to catalyze the acidolysis of several vegetable oils (Berka *et al.*, 1992). Using lyophilized mycelium of *Rhizopus* sp. and *Aspergillus* sp., several esters were synthesized in organic solvent (Molinari *et al.*, 1998, 2000; Converti *et al.*, 2002, 2005; Gandolfi *et al.*, 2001; Romano *et al.*, 2005b). Recently, such a technology has proven effective in stereoselective transformations, among which the resolution of (*R, S*)-2-phenylpropionic acid (Gandolfi *et al.*, 2001) and (*R, S*)-1,2-*O*-isopropylideneglycerol (Romano *et al.*, 2006) by esterification and the enantioselective hydrolysis of esters of these compounds of pharmaceutical concern (Molinari *et al.*, 2004, 2005; Romano *et al.*, 2005a). However, no comprehensive study has been performed up to now on these equilibria.

Based on these premises, the capabilities of lyophilized mycelia of different strains of *Aspergillus oryzae* and *Rhizopus oryzae* to catalyze esterifications of phenylacetic acid and the hydrolysis of ethyl phenylacetate and racemic ethyl 2-phenylpropionate were investigated in *n*-heptane and phosphate buffer, respectively, in order to get information on these equilibria.

## MATERIAL AND METHODS

### Biocatalyst preparation and biotransformation conditions

Cells of *A. oryzae* CBS 10207, *A. oryzae* MIM (strain isolated but yet not definitely classified, belonging to the collection of the Department of Food and Microbiological Science and Technology, University of Milan), *R. oryzae* CBS 11207, *R. oryzae* CBS 39134, *R. oryzae* CBS 26028 and *R. oryzae* CBS 32847 were maintained on malt extract (8 g $L^{-1}$, agar 15 g $L^{-1}$, pH 5.5), cultivated in 500 mL-Erlenmeyer flasks containing 100 mL of medium and incubated for 48 h at 28°C on a reciprocal shaker (100 rpm). The liquid media contained a basic medium (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) supplemented with Tween 80 (0.5%). Suspensions of spores ($1.6 \cdot 10^4$) were used as inoculum. Mycelia grown for 48 h in submerged cultures were harvested by filtration at 4°C, washed with phosphate buffer (pH 7.0, 0.1 M) and lyophilized. Ester synthesis was carried out in 10 mL-screw capped test tubes by suspending lyophilized mycelium in *n*-heptane (5 mL) and then adding ethanol and the acid. The hydrolysis of esters was investigated in 0.1 M phosphate buffer. The reaction mixtures were magnetically stirred at 50ºC.

### Analytical procedures

After sample (0.5 mL) centrifugation, 200 µL of the supernatant were added to an equal volume of a $CHCl_3$ solution containing 2-phenyl-1-propanol as internal standard. Molar conversions were followed using a Fractovap G1 gas chromatograph (Carlo Erba, Milan, Italy) equipped with a hydrogen flame ionization detector; the column temperature was kept at 180°C. The column (3x2000 mm) was packed with Carbowax 20 M (10% 100/120 mesh, Supelcoport, Sigma-Aldrich, Milan, Italy).

## RESULTS AND DISCUSSION

### Selection of the biocatalysts

To select the best biocatalysts, lyophilized mycelia of *A. oryzae* CBS 10207, *A. oryzae* MIM, *R. oryzae* CBS 11207, *R. oryzae* CBS 39134, *R. oryzae* CBS 26028 and *R. oryzae* CBS 32847 were tested for their ability to acylate ethanol with phenylacetic acid (PAA) in *n*-heptane. The results of these bioconversions, carried out at 50°C using an equimolar level ($S_o$ = 86 mM) of PAA and ethanol, are illustrated in Figure 1 in terms of molar conversion versus time. These microorganisms exhibited, in this order, conversion yields of phenylacetic acid to ethyl phenylacetate (EPA) of 0.35, 0.50, 0.22, 0.20, 0.27, 0,23 after 72 h and 0.45, 0.56, 0.37, 0.35, 0.40, 0.37 after 144 h, respectively. These results demonstrate that the equilibrium position and the activity of the carboxylesterases are both species-dependent and that the lyophilized mycelia of *A. oryzae* MIM and *R. oryzae* 11207 are the most effective biocatalysts for the selected acylation,

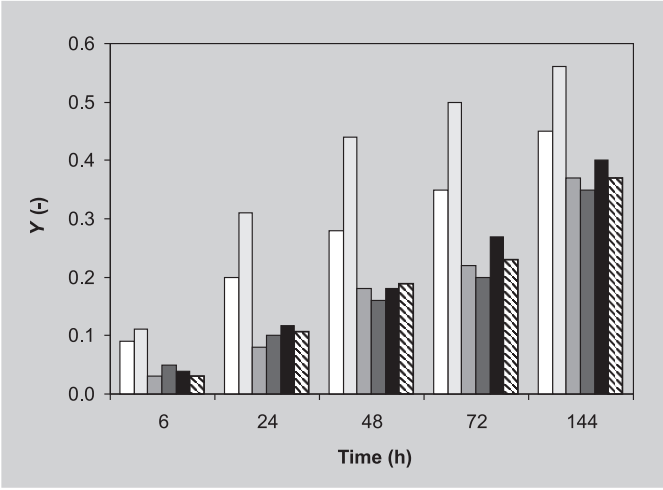


**FIGURE 1** – Molar conversion versus time of ethanol acylation with phenylacetic acid using different lyophilized mycelia as biocatalysts. (□) *A. oryzae* CBS 10207; (▫) *A. oryzae* MIM; (▪) *R. oryzae* CBS 11207; (▪) *R. oryzae* CBS 39134; (■) *R. oryzae* CBS 26028; (▨) *R. oryzae* CBS 32847. $S_o$ = 86 mM; $X_o$ = 30 $g_X$ $L^{-1}$; $T$ = 50°C.

being able to convert more than 50% and 40% of substrates into EPA at equilibrium, respectively.

There is no report in the literature, to the best of our knowledge, on the use of mycelium-bound carboxylesterases to catalyze the esterification of PAA. Nevertheless, *A. oryzae* MIM and *R. oryzae* 11207 were successfully applied to the esterification of 2-phenylpropionic acid with different primary alcohols (ethanol, 1-propanol, 1-butanol, 1-pentanol and 1-hexanol) in *n*-heptane at 50 °C, furnishing after 3 days yields in the ranges 0.15-0.31 and 0.12-0.29, respectively (Gandolfi *et al.*, 2001). An overall comparison of these results shows that the conversion yield increased with the alcohol molecular weight and decreased with the complexity of the acid structure, likely because of thermodynamic factors and increasing steric hindrance, respectively.

The observed behaviors of the molar yield show that, at the start of every runs, this parameter quickly increased with time and then slowly approached constant maximum equilibrium values ($Y^{max}$) according to the equation:

$$Y = \frac{Y^{max} t}{t_{1/2} + t} \qquad (1)$$

where $t_{1/2}$ is the time required to reach the half value of $Y^{max}$.

The values of $Y^{max}$ and $t_{1/2}$ have been estimated from the slopes and the intercepts on the ordinate axis of the straight lines obtained for each biosystem by linearization of this equation. To this purpose the reciprocal conversion yield ($1/Y$) has been plotted in Figure 2 versus the reciprocal time ($1/t$). The values of these parameters as well as those of the apparent equilibrium constant ($K_e$) estimated from those of $Y^{max}$ are summarized in Table I. It is noteworthy that *A. oryzae* MIM allowed ensuring, at the same time, very high values of the esterification yield ($Y^{max}$ = 0.732) and the equilibrium constant ($K_e$ = 7.5) as well as a quite low $t_{1/2}$ value (33.9 h), both being fundamental requisites to perform a good catalytic performance.

These results on the whole demonstrate that *A. oryzae* MIM and *R. oryzae* CBS 11207 were the best biocatalysts among those tested in this study; therefore, they were selected for the subsequent investigation on the hydrolytic activity of mycelium-bound carboxylesterases.

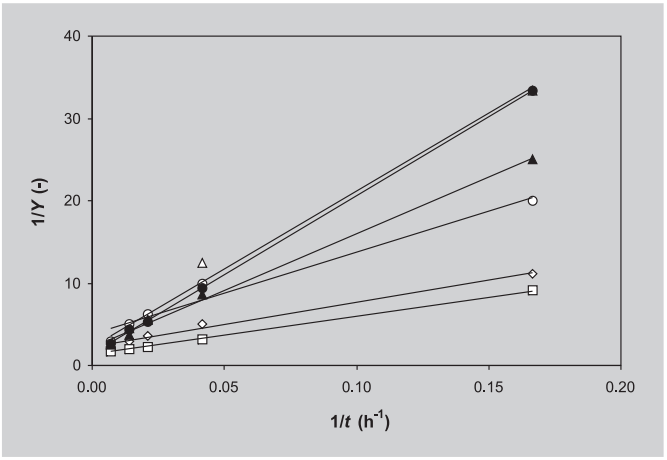


**FIGURE 2** – Reciprocal molar yield versus reciprocal time of ethanol acylation with phenylacetic acid using different lyophilized mycelia as biocatalysts. (△) *A. oryzae* CBS 10207; (□) *A. oryzae* MIM; (◇) *R. oryzae* CBS 11207; (○) *R. oryzae* CBS 39134; (▲) *R. oryzae* CBS 26028; (●) *R. oryzae* CBS 32847. $S_o$ = 86 mM; $X_o$ = 30 $g_X$ $L^{-1}$; $T$ = 50 °C.

**TABLE I** - Equilibrium results of ethanol acylation with phenylacetic acid in *n*-heptane by lyophilized mycelia of different fungi. $S_o$ = 86 mM; $X_o$ = 30 $g_X$ $L^{-1}$; $T$ = 50 °C

| Parameter | $Y^{max}$ (-) | $t_{1/2}$ (h) | $K_e$ (-) |
|---|---|---|---|
| *A. oryzae* | | | |
| MIM | 0.732 | 33.9 | 7.5 |
| CBS 10207 | 0.533 | 100 | 1.3 |
| *R. oryzae* | | | |
| CBS 11207 | 0.436 | 23.7 | 0.60 |
| CBS 39134 | 0.272 | 27.5 | 0.14 |
| CBS 26028 | 0.469 | 64.5 | 0.78 |
| CBS 32847 | 0.790 | 149 | 14 |

### Hydrolysis of ethyl phenylacetate and racemic ethyl 2-phenylpropionate

The hydrolysis of both ethyl phenylacetate and racemic ethyl 2-phenylpropionate (EPP) was then investigated using only the most two efficient biocatalysts, namely *A. oryzae* MIM and *R. oryzae* CBS 11207. It should be noticed that the hydrolysis reaction was in both cases much quicker than the acylation, the former biocatalyst exhibiting yields for EPA and EPP hydrolysis of 0.97 and 0.94 after 24 h, and the latter 0.56 and 0.51 after the same time. Moreover, *A. oryzae* MIM exhibited after only 1 h yields of EPA and EPP hydrolysis of 0.91 and 0.43, thus proving by far the more effective of the two biocatalysts to perform the hydrolysis of both esters.

These results can be compared with those reported in the literature for the enantioselective hydrolysis of (*R, S*)-isopropylidene glycerol acetate with whole cells of different strains of *Kluyveromyces marxianus*, which provided hydrolysis yields of 0.37-0.57 after 3 h (Molinari *et al.*, 2004), and of different esters of the same optically-active alcohol by thermally-treated cells of *Bacillus coagulans*, that exhibited after 2-24 h yields (0.10-0.63) decreasing with the complexity of the acid structure (Romano *et al.*, 2005a). Once more, the slower hydrolysis in this case can be ascribed to a series of different factors, among which the different biocatalysts as well as the different structures and molecular sizes of the esters employed.

In order to model the hydrolysis reaction, the reciprocal molar yields of hydrolysis of EPA and racemic EPP by lyophilized cells of either biocatalyst have been plotted in Figure 3 versus the reciprocal time. These data were then utilized to estimate, for the selected four biosystems, the values of the apparent constants of hydrolysis ($K_h$) without considering water participation to the equilibrium (Table II). To this purpose, we have followed the same approach as that used in the previous section to estimate the same parameters for the acylation reactions.

Consistently with the results of previous work where *A. oryzae* mycelium exhibited much higher starting hydrolysis rates with respect to *R. oryzae* (Romano *et al.*, 2005b), the time required to reach one half of $Y^{max}$ for EPA hydrolysis was with the former biocatalyst ($t_{1/2}$ = 0.058 h) two order of magnitude less than with the latter ($t_{1/2}$ = 5.43 h), while it was ($t_{1/2}$ = 1.29 h) only about 4 times less than for EPP hydrolysis ($t_{1/2}$ = 5.36 h). As a consequence, the hydrolysis equilibrium constant calculated for the former catalyst was always much higher than for the latter. These results also suggest that the steric hindrance could be a factor of greater significance for *A. oryzae* rather than for *R. oryzae* carboxylesterase.

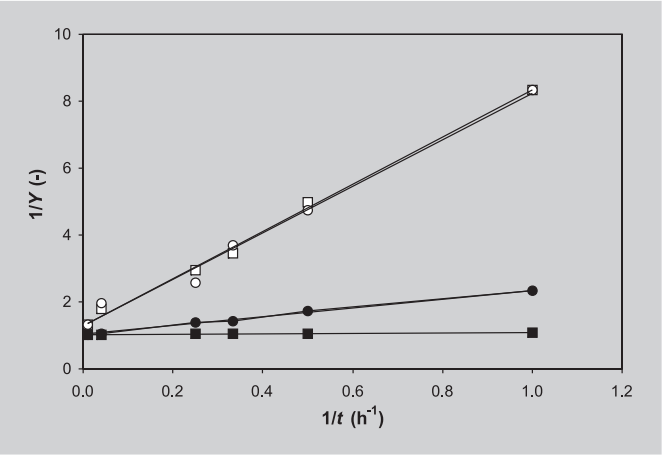


**FIGURE 3** – Reciprocal molar yield versus reciprocal time of the hydrolysis of ethyl phenylacetate (EPA) and racemic ethyl 2-phenylpropionate (EPP) using fungal lyophilized cells as biocatalysts. *A. oryzae* MIM: (■) EPA; (●) EPP. *R. oryzae* CBS 11207: (□) EPA; (○) EPP. $S_o$ = 23 mM; $X_o$ = 30 $g_X$ $L^{-1}$; $T$ = 50 °C; pH = 7.0.

**Table II -** Main equilibrium results of ethyl phenylacetate (EPA) and racemic ethyl 2-phenylpropionate (EPP) hydrolysis in 0.1 M phosphate buffer by lyophilized mycelia of *A. oryzae* MIM and *R. oryzae* CBS 11207. $S_o$ = 23 mM; $X_o$ = 30 $g_X$ $L^{-1}$; $T$ = 50 °C; pH = 7.0

| Microorganism | *A. oryzae* | | *R. oryzae* | |
|---|---|---|---|---|
| Ester | EPA | EPP | EPA | EPP |
| $Y^{max}$ | 0.976 | 0.976 | 0.771 | 0.770 |
| $t_{1/2}$ (h) | 0.058 | 1.29 | 5.43 | 5.36 |
| $K_h$ (-) | 0.91 | 0.90 | 0.060 | 0.059 |

The use of such biocatalysts seemed to significantly influence the hydrolysis equilibrium, thereby suggesting some unexpected, differentiated effect on reactants and products stabilization. The higher slope of the straight line obtained for EPP hydrolysis with respect to EPA hydrolysis by *A. oryzae*, responsible for a $t_{1/2}$ value almost 20 times higher, can be ascribed, as previously suggested by Romano *et al.* (2005b), to the steric hindrance in the former ester associated to the presence of the α-methyl group. On the contrary, this phenomenon appeared to be negligible with *R. oryzae* as a biocatalyst. Therefore, the steric control of esters hydrolysis by mycelium-bound carboxyl-esterases may become significant only at very high hydrolysis rates,

as it occur with *A. oryzae* mycelium, unless completely different mechanisms are implied.

The results of this work demonstrated that:

- lyophilized mycelia of *A. oryzae* MIM and *R. oryzae* CBS 11207, among others, are effective biocatalysts for the acylation of alcohols with phenylacetic and 2-phenylpropionic acids;
- the former biocatalyst exhibited the best performances, ensuring a maximum esterification yield at equilibrium of $Y^{max} = 0.732$, a time to reach half this value of $t_{1/2} = 33.9$ h and an apparent esterification equilibrium constant of $K_e = 7.5$, while the latter yielded $Y^{max} = 0.436$, $t_{1/2} = 23.7$ h and $K_e = 0.60$, respectively;
- *A. oryzae* MIM showed also quicker and effective hydrolysis either of EPA ($Y^{max} = 0.976$; $t_{1/2} = 0.058$ h; $K_h = 0.91$) or EPP ($Y^{max} = 0.976$; $t_{1/2} = 1.29$ h; $K_h = 0.90$) with respect to *R. oryzae* CBS 11207;
- dry mycelia of *A. oryzae* MIM and *R. oryzae* CBS 11207 could be exploited as enantioselective biocatalysts for the production of enantiomerically enriched esters in organic solvents, for the resolution of racemic mixtures, and to obtain chiral building blocks usable in the synthesis of compounds of concern for the pharmaceutical industry.

## ACKNOWLEDGMENTS

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## RESUMO

**Biossíntese e hidrólise de fenilacetato de etila e 2-fenilpropionato de etila em solvente orgânico por meio de micélios liofilizados**

*Micélios liofilizados de* Aspergillus oryzae *CBS 10207,* A. oryzae *MIM,* Rhizopus oryzae *CBS 11207,* R. oryzae *CBS 39134,* R. oryzae *CBS 26028 e* R. oryzae *CBS 32847 foram testados neste estudo com vista à seleção do melhor biocatalisador para efetuar a acilação de etanol com ácidos fenilacético e 2-fenilpropiônico. As atividades carboxilesterásicas de* A. oryzae *MIM e* R. oryzae *11207, que resultaram ser os melhores biocatalisadores, foram investigadas tanto em tampão fosfato 0,1 M como em* n*-heptano para catalisar a hidrólise ou a síntese dos ésteres etílicos destes ácidos*. A. oryzae *pareceu ser mais eficaz que* R. oryzae*, provavelmente devido a condições micro-ambientais e a um cenário termodinâmico mais favoráveis. Os resultados obtidos em termos de formação do produto e consumo dos substratos em função do tempo foram usados para a estimativa dos rendimentos de conversão máximos, as constantes de equilíbrio e os tempos necessários para alcançar metade da conversão máxima, fornecendo desta forma suficientes informações sobre esses equilíbrios.*

*UNITERMOS: Esterificação. Ácido fenilacético. Ácido 2-fenilpropiônico. Solvente orgânico. Carboxilesterase*

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