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

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 matrices 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-isopropylidenglycerol (Romano et al., 2006) by esterification and the enantiomeric 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.

**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 = 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,
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* CBS 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*\(_{\text{max}}\)) according to the equation:

\[
Y = \frac{Y_{\text{max}} t}{t_{1/2} + t}
\]

(1)

where *t*\(_{1/2}\) is the time required to reach the half value of *Y*\(_{\text{max}}\).

The values of *Y*\(_{\text{max}}\) 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*\(_{\text{max}}\) 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*\(_{\text{max}}\) = 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 1](image1.png)  
**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 L\(^{-1}\); *T* = 50 °C.

![Figure 2](image2.png)  
**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; (〇) *R. oryzae* CBS 39134; (▲) *R. oryzae* CBS 26028; (●) *R. oryzae* CBS 32847. *S*\(_{o}\) = 86 mM; *X*\(_{o}\) = 30 g L\(^{-1}\); *T* = 50 °C.

**TABLE 1** - 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 L\(^{-1}\); *T* = 50 °C

<table>
<thead>
<tr>
<th>Parameter</th>
<th><em>Y</em>(_{\text{max}}) (-)</th>
<th><em>t</em>(_{1/2}) (h)</th>
<th><em>K</em>(_{e}) (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. oryzae</em> MIM</td>
<td>0.732</td>
<td>33.9</td>
<td>7.5</td>
</tr>
<tr>
<td>CBS 10207</td>
<td>0.533</td>
<td>100</td>
<td>1.3</td>
</tr>
<tr>
<td><em>R. oryzae</em> CBS 11207</td>
<td>0.436</td>
<td>23.7</td>
<td>0.60</td>
</tr>
<tr>
<td>CBS 39134</td>
<td>0.272</td>
<td>27.5</td>
<td>0.14</td>
</tr>
<tr>
<td>CBS 26028</td>
<td>0.469</td>
<td>64.5</td>
<td>0.78</td>
</tr>
<tr>
<td>CBS 32847</td>
<td>0.790</td>
<td>149</td>
<td>14</td>
</tr>
</tbody>
</table>
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.

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 $\alpha$-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.

![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 L$^{-1}$; $T = 50$ °C; pH = 7.0.](image)

<table>
<thead>
<tr>
<th>Microorganism</th>
<th><em>A. oryzae</em></th>
<th><em>R. oryzae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ester</td>
<td>EPA</td>
<td>EPP</td>
</tr>
<tr>
<td>$Y_{max}$</td>
<td>0.976</td>
<td>0.976</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>0.058</td>
<td>1.29</td>
</tr>
<tr>
<td>$K_h$ (-)</td>
<td>0.91</td>
<td>0.90</td>
</tr>
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Bio-synthesis and hydrolysis of ethyl phenylacetate and ethyl 2-phenylpropionate 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 $Y_{\text{max}} = 0.732$, a time to reach half this value of $t_{\frac{1}{2}} = 33.9$ h and an apparent esterification equilibrium constant of $K_e = 7.5$, while the latter yielded $Y_{\text{max}} = 0.436$, $t_{\frac{1}{2}} = 23.7$ h and $K_e = 0.60$, respectively;
- *A. oryzae* MIM showed also quicker and effective hydrolysis either of EPA ($Y_{\text{max}} = 0.976$; $t_{\frac{1}{2}} = 0.058$ h; $K_h = 0.91$) or EPP ($Y_{\text{max}} = 0.976$; $t_{\frac{1}{2}} = 1.29$ 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.

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RESUMO

Biossíntese e hidrólise de fenilacetato de etila e 2-fenilpropiônico 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-fenilpropionico. As atividades carboxilesterásicas de *A. oryzae* MIM e *R. oryzae* I1207, 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 eétlicos destes ácidos. *A. oryzae* pareceu ser mais eficaz que *R. oryzae*, provavelmente devido a condições micróbiambientes e a um cenário termodinâmico mais favorável-veis. 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-fenilpropionico. Solvente orgânico. Carboxilesterase

REFERENCES


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