

# Catalytic properties of mycelium-bound lipases from *Aspergillus niger* MYA 135

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**Abstract** A constitutive level of a mycelium-bound lipolytic activity from *Aspergillus niger* MYA 135 was strongly increased by 97% in medium supplemented with 2% olive oil. The constitutive lipase showed an optimal activity in the pH range of 3.0–6.5, while the mycelium-bound lipase activity produced in the presence of olive oil had two pH optima at pH 4 and 7. Interestingly, both lipolytic sources were cold-active showing high catalytic activities in the temperature range of 4–8°C. These mycelium-bound lipase activities were also very stable in reaction mixtures containing methanol and ethanol. In fact, the constitutive lipase maintained almost 100% of its activity after exposure by 1 h at 37°C in ethanol. A simple methodology to evaluate suitable transesterification activities in organic solvents was also reported.

**Keywords** *Aspergillus niger* · Mycelium-bound lipase · Solvent tolerance · Enzyme stability · Hydrolytic activity · Transesterification

## Introduction

Lipases (EC 3.1.1.3) have attracted the great interest of chemical and pharmaceutical industries due to their usefulness in both hydrolysis and synthesis reactions (Jaeger and Eggert 2002). The study of enzyme activity and stability is an important aspect to consider in biotechnological process, as this information helps to optimize the economic profitability of the biocatalyst reaction.

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The use of naturally bound lipases is potentially cost effective because the biomass can be directly utilized. This allows the elimination of complex procedures of enzyme isolation, purification, and immobilization, which often results in loss of its activity. 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 matrix used for enzyme immobilization.

Mycelium-bound lipase from *Aspergillus orizae* has been used to synthesize phenylacetate (Converti et al. 2005). Liew et al. (2001) reported transesterification activity of a mycelium-bound lipase from *Rhizomucor miehei* on palm kernel olein:anhydrous milk fat blends.

Previously, we reported extracellular lipolytic extracts from *Aspergillus niger* MYA 135 that were very stable in the presence of 50% water-miscible organic solvents. It was also suggested that by manipulating the culture conditions, it is possible to produce lipase extracts with different enzymatic properties (Pera et al. 2006).

The aim of the present work is to study the biocatalytic properties and stability of mycelium-bound lipase activities produced by *A. niger* MYA 135 cultured in either presence or absence of olive oil. The performance of these lipase sources in transesterification of different alcohols using *p*-nitrophenyl palmitate (*p*NPP) as acyl donor was also explored.

## Materials and methods

### Microorganism and maintenance

*Aspergillus niger* ATCC MYA 135, formerly *A. niger* 419 from our own culture collection, was used throughout this work. It was maintained by monthly transfers to glucose-

potato agar slant tubes, incubated at 30°C and stored at 4°C.

#### Fermentation medium

The fermentation medium comprised (in g/l): sucrose, 10.0; KH<sub>2</sub>PO<sub>4</sub>, 1.0; NH<sub>4</sub>NO<sub>3</sub>, 2.0; MgSO<sub>4</sub>·7H<sub>2</sub>O, 2.0; CuSO<sub>4</sub>, 0.06. The initial pH was adjusted to 7.0 with NaOH.

#### Enzyme production

Fermentation was carried out at 30°C in 500-ml shake flasks (250 rpm) containing 100 ml of fermentation medium. Culture flasks were inoculated with 10 ml of a conidial suspension (about 10<sup>6</sup> conidia/ml) from a stock culture. After 24 h of incubation, the culture was transferred to another 500-ml shake flask containing either 50 ml 3% (v/v) olive oil or distilled water and was further incubated for 4 days under the same conditions. The mould developed a pelleted form of growth. Final biomass concentrations expressed as dry weight (g/l) in medium deprived of olive oil and in medium supplemented with 2% olive oil were 10.5±1.3 and 16.2±0.8, respectively. The mycelium was collected and washed with acetone by filtration at 4°C for 3 min at 6000 g. These cells were used as source of enzyme.

#### Enzyme determination

As a small amount of water molecules is essential to obtain a sufficient enzyme conformational flexibility (Bone 1987; Zaks and Klibanov 1988), the solvent as well as the biocatalysts were not dried before use. In our experiments, no significant correlation between the amount of biocatalyst water in reaction mixtures (from 0.11 to 1.47%) and the transesterification activity was found ( $r=-0.066$ ,  $P=0.629$ ).

Hydrolytic activity in either aqueous or organic media was measured with *p*NPP as substrate. In aqueous solvents, about 0.010 g of wet mycelium was added to 1 ml 100 mM phosphate buffer (pH 7.0) containing 2 mM *p*NPP, 0.1% (w/v) gum Arabic and 0.4% (w/v) Triton X-100 (Winkler and Stuckman 1979). The molar extinction coefficient of *p*-nitrophenol (*p*NP) under this assay condition was found to be 0.0103  $\mu\text{M}^{-1} \text{cm}^{-1}$ . In organic solvents, about 0.010 g of wet mycelium was added to 1 ml of *n*-hexane containing 2 mM *p*NPP (Pencreac'h and Baratti 1996). The reaction mixture was shaken (150 rpm) at 37°C. The absorbance of the supernatant containing *p*NP was determined at 405 nm. In *n*-hexane, the *p*NP was extracted with 1 ml 0.25 M Na<sub>2</sub>CO<sub>3</sub> before measurement. The molar extinction coefficient of *p*NP under this assay condition was found to be 0.0205  $\mu\text{M}^{-1} \text{cm}^{-1}$ . One unit of enzyme activity was defined as the amount of biocatalyst that released 1  $\mu\text{mol}$  of *p*NP per minute.

Enzymatic transesterification was carried out as follows: to 800  $\mu\text{l}$  of *n*-hexane, 100  $\mu\text{l}$  of 20 mM *p*NPP, dissolved in either *n*-hexane or acetone, 100  $\mu\text{l}$  of each alcohol, and about 0.010 g of wet mycelium were added. The reaction mixture was shaken (150 rpm) for 1 h at 37°C, and the supernatant containing *p*NP was measured as above. A reaction mixture without alcohol served as a hydrolysis control. In absence of cells, no reaction was observed. One unit of transesterification activity was defined as the amount of biocatalyst that released 1  $\mu\text{mol}$  of *p*NP per minute.

Qualitative analysis of alkyl-palmitates by thin layer chromatography (TLC) was carried out on silica gel 60 using chloroform as developing solvent. Spots were visualized in iodine vapor.

Specific activity was expressed as milliunits per gram of dry weight. Calibration curves were generated with wet and dry mycelia developed in media either without olive oil ( $R^2=0.973$ ) or with 2% olive oil ( $R^2=0.982$ ).

#### Effect of pH on activity and stability

The effect of pH on the enzyme activity was tested at 37°C in the pH range of 2.0–9.0, using the following 100-mM buffers: KCl–HCl (pH 2.0), citrate–phosphate (pH 3.0 and 4.0), phosphate (from pH 6.0 to 8.0), and Borate–HCl pH 9.0.

Stability assay was done by incubating about 0.010 g of wet mycelium at 37°C for 1 h in 100 mM buffers of different pH values (KCl–HCl, pH 2.0; glycine–HCl, pH 2.5; citrate–phosphate, pH values 3.0, 4.0, 5.0, and 6.0; phosphate, pH 7.0; and borate–HCl, pH values 9.0 and 10.0). Residual activity was then calculated considering the enzyme activity at time zero as 100%.

#### Effect of temperature on activity and stability

Measurements of enzyme activity were carried out in standard reaction mixture at different temperatures covering the range of 4–55°C.

Mycelium-bound enzyme was also preincubated in 100 mM phosphate buffer (pH 7.0) for 1 h at different temperatures covering the range of 4–55°C. The remaining enzyme activity was then determined and compared with the control without preincubation.

#### Stability assays in organic solvents

To 1 ml of each organic solvent, about 0.010 g of wet mycelium was added. The system was incubated in a shake tube (150 rpm) for 1 h at 37°C. The mycelium-bound enzyme was collected by filtration, and the residual activity was then quantified.

## Statistical analysis

Data were analyzed using analysis of variance (ANOVA). All results are presented as the mean $\pm$ SD. Differences were accepted as significant when  $p<0.05$ .

Associations between variables were assessed by using Pearson's correlation coefficient.

## Results

### Time course of mycelium-bound lipases production

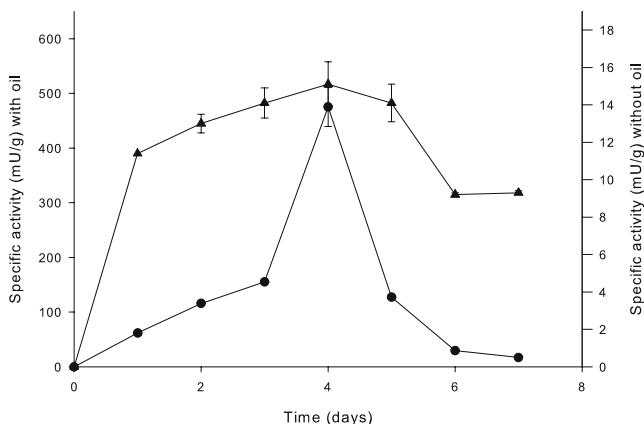
As depicted in Fig. 1, a constitutive level of a mycelium-bound lipolytic activity was detected. The highest specific activity was observed after 2 days of incubation, and it was not significantly modified during 72 h ( $F=1.98$ ;  $df=3$ ;  $P=0.258$ ).

After 4 days of incubation in medium supplemented with 2% olive oil, the specific lipase activity was strongly increased by 97%.

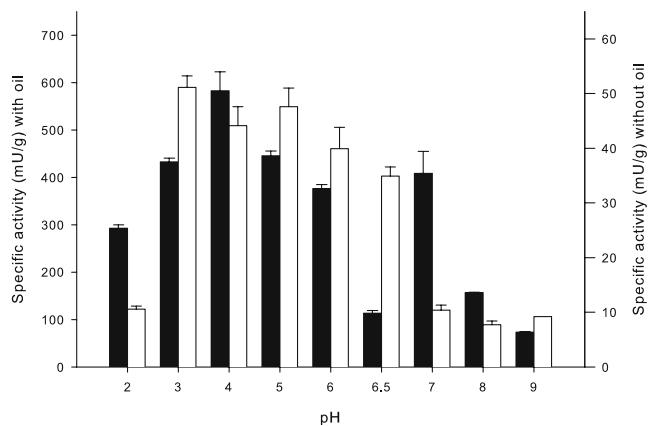
### Effect of pH on lipolytic activity and pH stability

Both the constitutive and the olive oil-induced mycelium-bound lipase activities were active within the pH range tested (2.0–9.0). The constitutive lipase showed an optimal activity in the pH range of 3.0–6.5 (Fig. 2). On the other hand, the mycelium-bound lipase produced in the presence of olive oil showed two pH optima at pH 4 and 7 (Fig. 2).

The pH stability behavior of the constitutive mycelium-bound lipase was also different from that obtained with the olive oil-induced one. In the first case, a significant



**Fig. 1** Time course of mycelium-bound specific lipase activity during fermentation of *A. niger* MYA 135 using a medium either deprived of olive oil (filled triangle) or supplemented with 2% olive oil (filled circle). Error bars represent the standard deviation calculated from at least three independent experiments

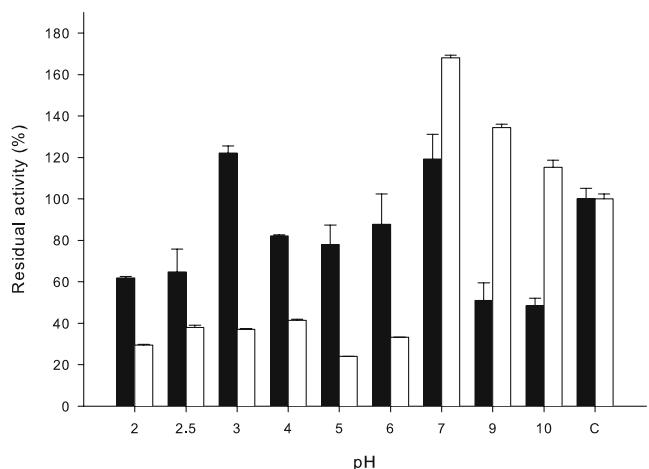


**Fig. 2** Effect of pH on mycelium-bound specific lipase activity from *A. niger* MYA 135 using a medium either deprived of olive oil (open bars) or supplemented with 2% olive oil (filled bars). Error bars represent the standard deviation calculated from at least three independent experiments

decrease in the lipolytic activity after preincubation under acid and near-neutral pH conditions was observed. However, treatments at pH values from 7 to 10 give residual activities above 100%. In the second case, more than 47% of the initial activity was recovered. Interestingly, preincubation at pH values of 3 and 7 caused an increase of around 20% of the initial activity (Fig. 3).

### Effect of temperature on lipase activity and stability

Both constitutive and olive oil-induced activities were active within the temperature range assayed (4–55°C). However, the mycelium-bound lipase produced in the presence of olive oil showed optimal activity in the temperature range of 8–35°C ( $F=4.46$ ,  $df=3$ ,  $P=0.091$ ),



**Fig. 3** Effect of pH on residual mycelium-bound specific lipase activity from *A. niger* MYA 135 using a medium either deprived of olive oil (open bars) or supplemented with 2% olive oil (filled bars). Remaining activity was compared with the control (C) without incubation. Error bars represent the standard deviation calculated from at least three independent experiments

while the constitutive lipase activity displayed two maxima, one at 4–8°C ( $F=0.12$ ,  $df=1$ ,  $P=0.763$ ) and another at 35–45°C ( $F=5.83$ ,  $df=3$ ,  $P=0.060$ ; Fig. 4).

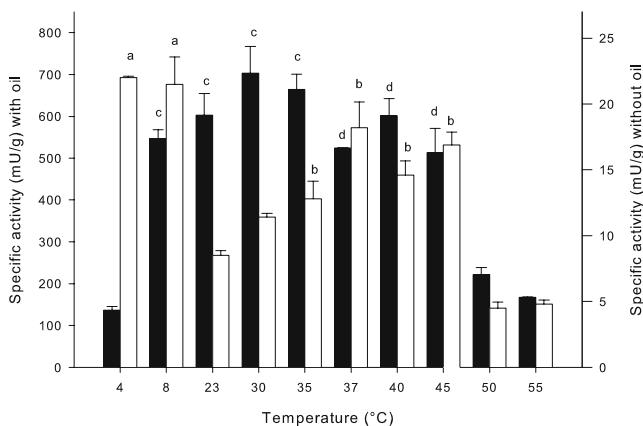
Thermal stability of both lipase activity sources was also different. The mycelium-bound lipase produced in non-supplemented medium shows residual activities above 100% after treatment for 1 h at temperatures from 25 to 40°C. On the contrary, the lipase activity obtained in the presence of olive oil retains from 14 to 58% of its activity after preincubation at temperature values in the range of 4–55°C (Fig. 5).

#### Stability assays in water-miscible solvents

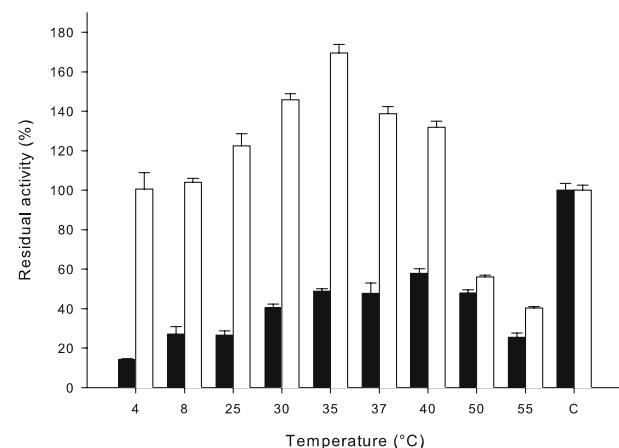
Lipases are diverse in their sensitivity to solvents, but there is a general agreement that polar water-miscible solvents are more destabilizing than water-immiscible ones (Nawani et al. 1998). In this work, olive oil-induced lipolytic activity showed high stability in the presence of water-miscible organic solvents such as methanol, ethanol, propanol, and acetone, as it retained about 70% of its activity after exposure by 1 h at 37°C (Fig. 6). In fact, the constitutive lipase maintained almost 100% of its activity in the presence of ethanol under the same conditions.

#### Stability assays in water-immiscible solvents

All solvents tested (butanol, hexanol, *n*-hexane, and heptane) caused a decrease in enzymatic activity. However, both constitutive and olive oil-induced lipase retained around 45% of their activities after incubation for 1 h at 37°C in *n*-hexane (Fig. 6). This similar behavior makes the *n*-hexane ( $\log P=3.5$ ) a chosen solvent for biotransformation reactions described in this article.



**Fig. 4** Effect of temperature on mycelium-bound specific lipase activity from *A. niger* MYA 135 using a medium either deprived of olive oil (open bars) or supplemented with 2% olive oil (filled bars). Error bars represent the standard deviation calculated from at least three independent experiments. Bars with the same letter are not significantly different ( $p<0.05$ )

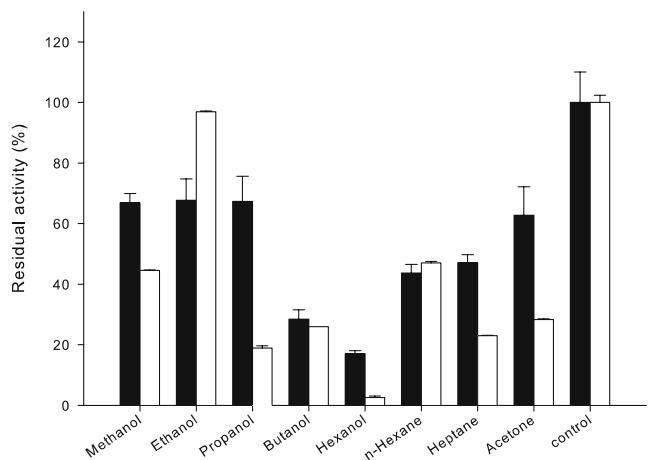


**Fig. 5** Effect of temperature on mycelium-bound residual specific lipase activity from *A. niger* MYA 135 using a medium either deprived of olive oil (open bars) or supplemented with 2% olive oil (filled bars). Remaining activity was compared with the control (C) without incubation. Error bars represent the standard deviation calculated from at least three independent experiments

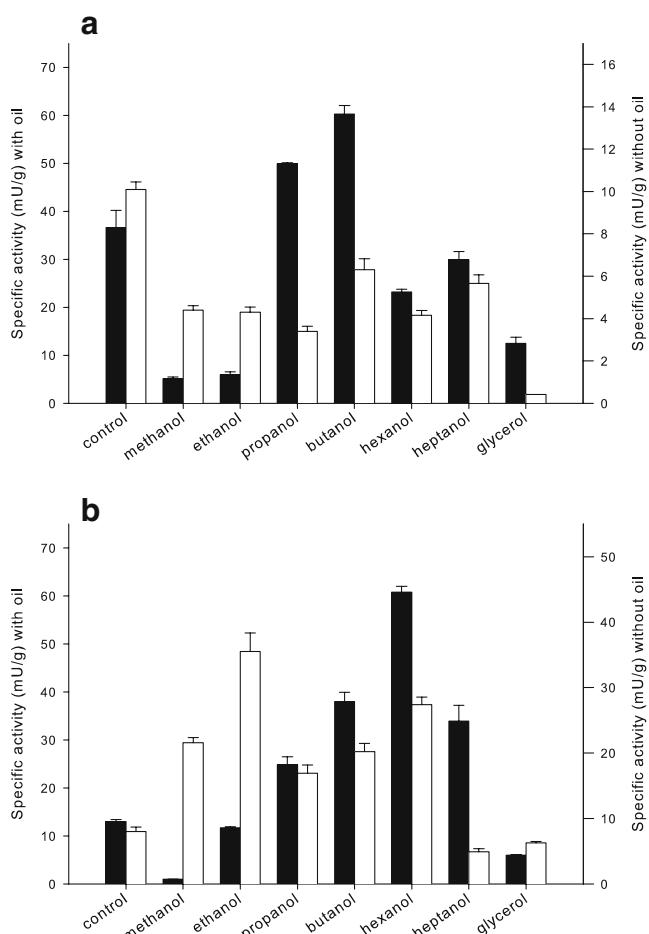
#### Transesterifications of different alcohols with *p*-nitrophenyl palmitate as a screening tool

It is generally accepted that lipase-catalyzed hydrolytic activity does not usually correlate with its esterification or transesterification activity (Pencreac'h and Baratti 2001). Thus, the performance of mycelium-bound lipases in transesterification of different alcohols in *n*-hexane using *p*NPP as acyl donor was explored (Fig. 7).

In our initial experiments, we found that transesterification activity considerably varied depending on the way of *p*NPP preparation, showing consequently different biocatalytic performance. In general, transesterification activities in reaction mixtures containing *p*NPP directly dissolved in



**Fig. 6** Effect of organic solvents on mycelium-bound residual specific lipase activity from *A. niger* MYA 135 using a medium either deprived of olive oil (open bars) or supplemented with 2% olive oil (filled bars). Remaining activity was compared with the control without incubation. Error bars represent the standard deviation calculated from at least three independent experiments



**Fig. 7** Transesterification in *n*-hexane of different alcohols with *p*-nitrophenyl palmitate directly dissolved in *n*-hexane (**a**) or dissolved in acetone (**b**) using mycelium-bound lipase from *A. niger* MYA 135 cultured in medium either deprived of olive oil (open bars) or supplemented with 2% olive oil (filled bars). Reaction mixture without alcohol served as hydrolysis control. Error bars represent the standard deviation calculated from at least three independent experiments

*n*-hexane were drastically decreased (Fig. 7a) compared with those developed in the presence of acetone *p*NPP solution (Fig. 7b). However, under the first condition, the olive oil-induced lipase activity displays high specificity towards propanol and butanol showing transesterification activities of  $49.9 \pm 0.23$  and  $60.3 \pm 1.77$  mU/g, respectively. The effect of acetone *p*NPP solution on transesterification activity using either the basal lipase source or the induced one was interesting. In the first case, maximal activities in reaction mixtures containing ethanol ( $35.5 \pm 2.85$  mU/g) and hexanol ( $27.4 \pm 1.18$  mU/g) were detected. While in the second case, a relation between the alcohol chain length and the transesterification activity was observed. In Fig. 7b is shown the maximum specific activity obtained in the presence of hexanol ( $60.8 \pm 1.21$  mU/g).

Finally, comparison of TLC profiles of hydrolytic and synthetic reactions shows new spots that could correspond to the alkyl palmitate production (Table 1).

**Table 1** *R*<sub>f</sub> values corresponding to alkyl palmitates produced in *n*-hexane reaction mixtures containing about 0.01 g of wet mycelium, 2 mM *p*NPP, and the assayed alcohol

Alkyl palmitate	<i>R</i> <sub>f</sub>
Methy palmitate	0.15
Ethyl palmitate	0.14
Propyl palmitate	0.17
Butyl palmitate	0.13
Hexyl palmitate	0.19
Heptyl palmitate	0.20

## Discussion

To evaluate the potential application of mycelium-bound lipase activities from *A. niger* ATCC MYA 135 in biotechnology, their catalytic properties as well as their stabilities were assessed.

As depicted in Fig. 1, both constitutive and induced lipolytic activities, produced in mineral medium either without or supplemented with olive oil, were detected. The naturally bound lipase production pattern obtained in the presence of 2% olive oil was similar to that previously reported for an extracellular lipase activity from this strain. In that study, after 4 days of incubation under the same conditions, the specific lipase activity was increased by 51% (Pera et al. 2006).

Interestingly, both mycelium-bound lipolytic sources display different catalytic properties, probably due to different composition in isoenzymes. For *Candida rugosa* lipase isoenzymes, it has been reported that each isoenzyme shows different catalytical selectivity and/or stereobias towards different substrates in organic media (De la Casa et al. 2006). They are also secreted in different ratios/concentrations depending on the fermentation conditions (Domínguez de María et al. 2006). Lipolytic enzymes LipA and LipB secreted by *Bacillus subtilis* have also been reported. They are differentially expressed, depending on the composition of the growth medium. LipA is produced in both rich and minimal medium, whereas LipB is present only in rich medium. In connection with their biochemical properties, both lipolytic enzymes display different substrate specificities (Eggert et al. 2001). These conclusions suggest the feasibility of producing new crude lipase preparations with totally different biocatalytical applications simply by controlling the fermentation parameters.

The constitutive mycelium-bound lipase exhibits an optimum activity at pH range of 3.0–6.5, which is similar to that previously described for an extracellular basal lipase produced by the same strain (Pera et al. 2006). While the induced mycelium-bound lipase shows two pH optima at pH 4 and 7, this behavior is different from that found for the corresponding extracellular lipase activity, which displays a low specific activity in the acid pH region (Pera et al. 2006).

Optimal pH values of 2.5 and 6.0 as well as 5.5 and 10.0 have been reported for *Penicillium roqueforti* (Lamberet and Menassa 1983) and *Aspergillus terreus* (Yadav et al. 1998), respectively. In connection to pH stability, constitutive mycelium-bound lipase treatments at pH values from 7 to 10 give residual activities above 100%. Similar pattern was reported for a lipase from *Bacillus thermoleovorans* CCR11, which showed an increase in its residual activity after preincubation at pH 7 during 26 h (Castro-Ochoa et al. 2005).

Both lipolytic sources were active within the temperature range assayed (4–55°C). The mycelium-bound lipase produced in nonsupplemented medium shows residual activities equal or above 100% after treatment for 1 h at temperatures from 8 to 40°C. To our knowledge, this wide reactivity of lipase activities produced by a filamentous fungus was not found elsewhere. On the other hand, the cold-active behavior of both lipolytic sources makes these activities useful as a catalyst for organic syntheses and for bioremediation processes at low temperatures (Gerday et al. 2000; Kojima et al. 2006).

Especially interesting is the enzyme stability towards hydrophilic solvents. This property could be used in lipase-catalyzed biodiesel production where the methanol or ethanol tolerance is a key factor (Fukuda et al. 2001; Nie et al. 2006).

Besides, it has shown a simple methodology to evaluate suitable transesterification activities in organic solvents. This procedure could also be used as a screening tool. Furthermore, by changing the alcohol chain length, it is possible to explore the biocatalyst performance of each lipase source.

In the light of these findings, combining this cost-effective whole-cell biocatalyst with its promising properties can be expected to be useful industrial applications.

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