

## Catalytic Properties of Lipase Extracts from *Aspergillus niger*

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Received: November 3, 2005

Accepted: March 5, 2006

### Summary

Screening of lipolytic strains using Rhodamine-B/olive oil plate technique allowed the selection of *Aspergillus niger* MYA 135. Lipase production in submerged culture containing 2 % olive oil was enhanced by more than 50 % compared to basal cultural conditions. Optimal catalytic conditions for olive oil-induced lipase were pH=6.5 and 30–35 °C. These values were shifted to the acid region (4.0–6.5) and 35–37 °C when lipase extract was produced under basal conditions. Slight changes of the residual lipase activity against the pH were found. However, preincubation at either 37 or 40 °C caused an increase in the olive oil-inducible lipolytic activity. On the contrary, lipase residual activity decreases in the 30–55 °C range when it was produced in basal medium. Lipolytic extracts were almost not deactivated in presence of 50 % water-miscible organic solvents. However, water-immiscible aliphatic solvents reduced the lipase activity between 20 and 80 %.

*Key words:* lipase, *Aspergillus niger*, substrate specificity, solvent tolerance, thermoresistance, enzyme stability, lipase screening

### Introduction

Extracellular lipases have been proven efficient and selective biocatalysts in many relevant industrial applications from biosensors, chemicals, pharmaceuticals, pesticides, foods, leather, and cosmetics to detergents (1). In 2000, enzyme market was one of the top in the biotechnology field and it was estimated at 1.6 billion US dollars (2).

Lipases are triacylglycerol acylhydrolases (E.C. 3.1.1.3) able to catalyze many reactions on ester bonds with preference on water-insoluble substrates. One of the unique properties of lipases is the ability of interfacial catalysis, in which those biocatalysts become more active in presence of a substrate partially soluble in aqueous environments. Also, lipases are able to catalyze ester synthesis and transesterification in organic media containing minute water concentration (1).

Lipases are produced by many microorganisms either alone or together with other members of the hydrolases family, like esterases. Among microorganisms, fungi are widely recognized as preferable lipase sources because they generally produce extracellular enzymes, which facilitates the enzyme recovery from the fermentation broth. *Aspergillus niger* is one of the most important microorganisms used in biotechnology. It has already been in use for decades to produce many extracellular enzymes that are considered GRAS (Generally Regarded As Safe) by the FDA (Food and Drug Administration of the United States of America) (3).

Extensive work about lipases has previously been published, ranging from industrial applications, production, and immobilization to biocatalytic properties of pure enzymes (4–10). Lipases possess a wide range of catalytic properties which are mostly strain-dependent. They have frequently been used in the form of a crude extract

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for synthesis of chiral building blocks and enantiomeric compounds. So, catalytical properties such as specificity, enantioselectivity, and operational parameters like thermostability, and optimum pH, among others are relevant because they define the enzyme application range and type of process.

The aim of the present work is to study the biocatalytic properties and stability of lipase extracts produced by *Aspergillus niger* MYA 135 cultured in either presence or absence of olive oil.

## Materials and Methods

### *Microorganism and maintenance*

*Aspergillus niger* ATCC MYA-135, 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 pH was adjusted to 7.0 with NaOH.

### *Lipolytic activity on Rhodamine-B/olive oil agar plates*

Agarized fermentation medium was supplemented with both 2.0 % olive oil and 0.001 % Rhodamine B according to the method described by Kouker and Jaeger (11). Culture plates were incubated at 30 °C and examined for 4 days. Lipolytic activity was monitored by irradiation at 350 nm.

### *Enzyme production*

Fermentation was carried out at 30 °C in 500-mL shaken flasks (250 rpm) containing 100 mL of fermentation medium inoculated with about 10<sup>5</sup> spores/mL from a stock culture. After 24 h of incubation, the culture was transferred to another 500-mL shaken flask containing either 50 mL of 3 % (by volume) olive oil or distilled water and was further incubated for 4 days under the same conditions. The mould developed a pelleted form of growth. The supernatant obtained by filtration was used as source of enzyme.

### *Enzyme determination*

Lipase activity was measured spectrophotometrically at 405 nm with *p*-nitrophenyl palmitate (*p*NPP) as substrate at 37 °C in 100 mM phosphate buffer (pH=7.0), 0.1 % (by mass per volume) gum arabic and 0.4 % (by mass per volume) Triton X-100 according to the method by Winkler and Stuckman (12). One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute. The molar absorptivity of *p*-nitrophenol under the assay conditions was found to be 0.0103 L/(μmol·cm). Specific activity was expressed as mU per μg of protein.

Protease activity was measured spectrophotometrically at 420 nm according to Secades and Guijarro (13) using azocasein as substrate.

### *Protein determination*

To 500 μL of sample, 500 μL of Coomassie Blue G-250 reagent was added. After the mixture was incubated for 10 min at room temperature the protein concentration was estimated at 595 nm using BSA (fraction V) as standard (14).

### *Gel electrophoresis*

Proteins were separated by native-PAGE (15) using 10 % (by mass per volume) polyacrylamide gel. Lipase and esterase activities were detected using 1.3 mM of α-naphthyl derivatives of acetate (C2), propionate (C3), butyrate (C4), caproate (C6), caprylate (C8), caprate (C10), laurate (C12), myristate (C14), palmitate (C16) or stearate (C18), as substrate. Released naphthol was coupled with 1 mM Fast Blue to give a coloured product. Reactions were carried out at 37 °C in shaken plates containing 100 mM phosphate buffer (pH=7.0).

### *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 2.0–8.0, using the following 100 mM buffers: KCl-HCl (pH=2.0), citrate-phosphate (pH=3.0 and 4.0) and phosphate (from pH=6.0 to 8.0).

Stability assay was done by incubating crude extract 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=3.0, 4.0, 5.0 and 6.0; phosphate, pH=7.0; and borate-HCl pH=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.

Enzyme solution was also preincubated in 100 mM phosphate buffer (pH=7.0) for 1 h at different temperatures covering the range of 30–55 °C. The remaining enzyme activity was then determined and compared with the control without incubation.

### *Stability assays in water-miscible solvents*

Lipase-containing culture supernatant was diluted in the ratio of 1:1 with each organic solvent tested and incubated for 1 h at 37 °C. Residual activity was then quantified.

### *Stability assays in water-immiscible solvents*

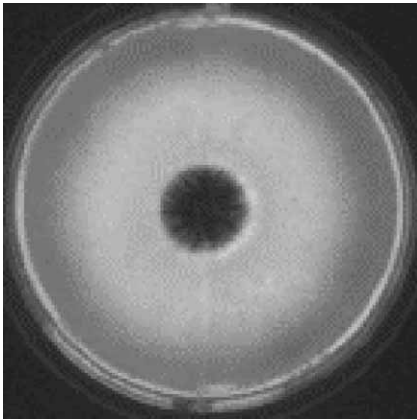
To 500 μL of enzyme solution, 500 μL of the organic solvent was added. The biphasic system was incubated in a shaken tube (60 rpm) for 1 h at 37 °C. The aqueous phase was sampled and residual activity was then determined and compared with the control without solvent.

## Results and Discussion

### *Screening of lipase producing microorganisms*

In preliminary experiments, a range of filamentous fungi were screened for lipolytic activity (data not shown). It was found that *A. niger* MYA 135 has the highest lipo-

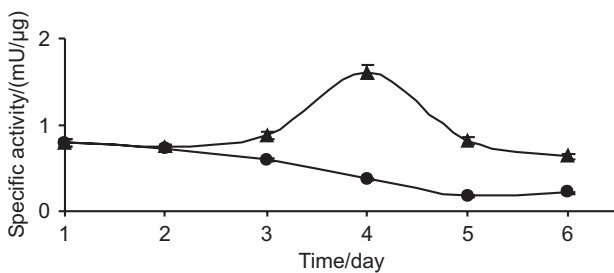
lytic activity according to the fluorescent diameter surrounding the colonies growing on Rhodamine-B/olive oil agar plates (Fig. 1).



**Fig. 1.** Lipolytic activity of *A. niger* MYA 135 after 84 h of growth on Rhodamine-B/olive oil agar plate

#### Time course of lipase production

As shown in Fig. 2, a constitutive level of lipolytic activity was detected. After 4 days of incubation in medium supplemented with 2 % olive oil, the specific lipase activity was increased by 51 %. Similar enhanced production pattern using both free and immobilized cells was reported for a lipase activity from *A. niger* ANT 90 obtained by mutagenesis (5).

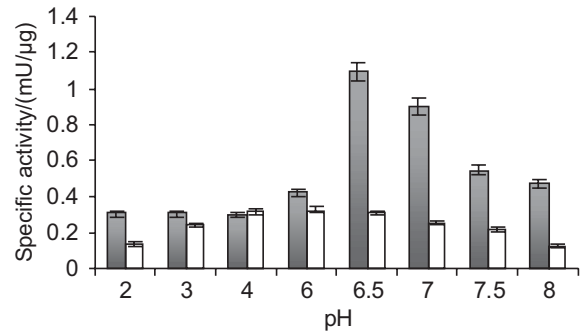


**Fig. 2.** Time course of specific lipase activity during fermentation of *A. niger* MYA 135 using a medium either without olive oil (●) or supplemented with 2 % olive oil (▲). Error bars represent the standard deviation calculated from at least three independent experiments

Interestingly, under our experimental conditions, no protease activity was detected when cells were grown either with or without the addition of olive oil.

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

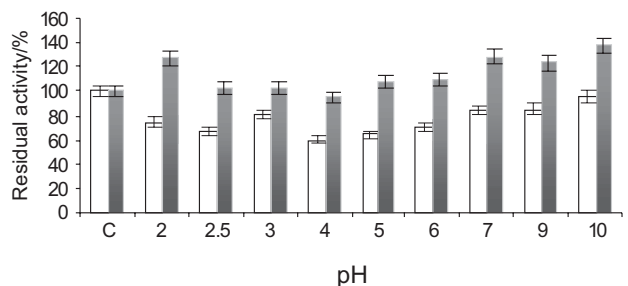
The lipase crude extract produced in presence of olive oil was active within the pH range tested (2.0–8.0). However, the activity occurred mainly in the near-neutral region with a maximum at pH=6.5 (Fig. 3). This value was almost similar to that reported for lipase activity from *Aspergillus niger* MTCC 2594 (6), but different from that found for *Aspergillus niger* NCIM 1207 (7) and for *Aspergillus carneus* (8), which showed maximum ac-



**Fig. 3.** Effect of pH on specific lipase activity from *A. niger* MYA 135 using a medium either without olive oil (□) or supplemented with 2 % olive oil (■). Error bars represent the standard deviation calculated from at least three independent experiments

tivity at pH=2.5 and 9.0, respectively. On the other hand, constitutive lipase showed an optimal activity in the pH range of 4.0–6.5 (Fig. 3). Interestingly, both lipolytic extracts have a similar specific activity in the pH range of 3.0–6.0; while in the near-basic pH region the olive oil induced specific lipase increases abruptly.

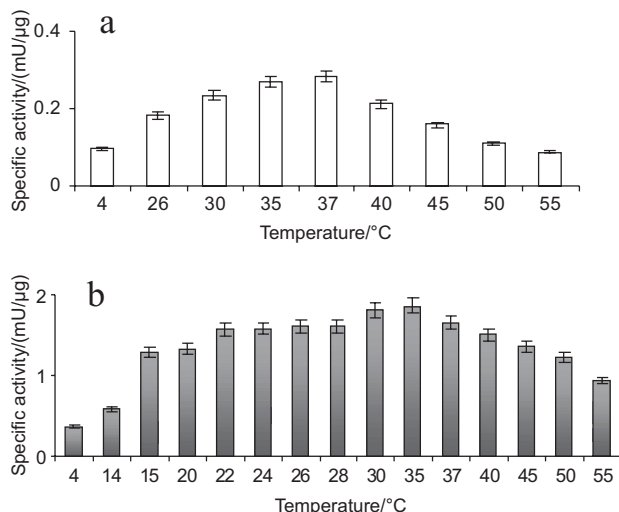
The pH stability pattern of the lipolytic extract produced in the presence of olive oil also differs from that produced without the addition of olive oil. In the first case, the lipase activity remained stable in the pH range of 2.0–10.0 during preincubation for 1 h at 37 °C, with residual activities in general above 100 % (Fig. 4). In the second case, incubation at the same pH values caused a decrease in the activity, compared to the activity of the crude extract without incubation. These results show a different pH stability behaviour of both lipolytic extract activities.



**Fig. 4.** Effect of pH on residual specific lipase activity from *A. niger* MYA 135 using a medium either without olive oil (□) or supplemented with 2 % olive oil (■). Remaining activity was compared with the control (C) without incubation. Error bars represent the standard deviation calculated from at least three independent experiments

#### 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 maximum lipolytic activity was shifted from 35–37 (Fig. 5a) to 30–35 °C (Fig. 5b), respectively. Coincidentally, the optimal temperature of the olive oil-induced lipase extract fits to that reported for lipase from *Rhi-*

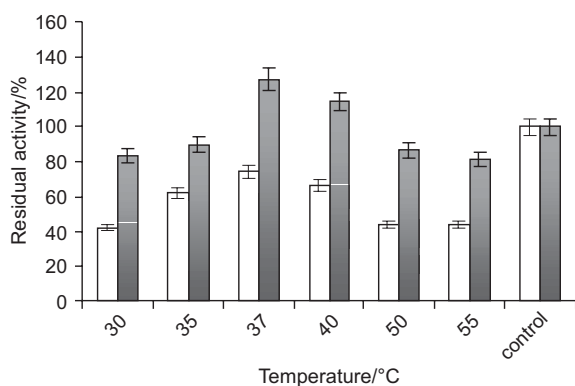


**Fig. 5.** Effect of temperature on specific lipase activity from *A. niger* MYA 135 using a medium either without olive oil (a) or supplemented with 2 % olive oil (b). Error bars represent the standard deviation calculated from at least three independent experiments

*zopus oryzae* (9), which showed maximum activity at 30 °C. In relation to constitutive lipase activity, its optimum temperature was similar to the lipase from *A. niger* MTCC 2594 (6) and *A. carneus* (8), which both showed maximum activity at 37 °C.

As the lipolytic extract obtained in presence of olive oil retains 70 % of its activity at 20 °C for more than one hour, it makes this activity suitable for the manufacturing of tea, where reactions are carried out at temperatures from 15 to 20 °C and pH range of 5.0–7.0 (4).

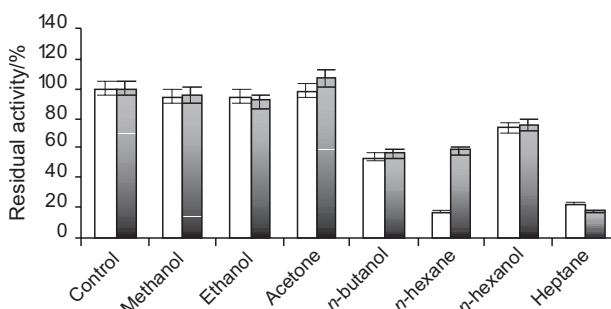
The lipase crude extract produced in non-supplemented medium retained from 42 to 74 % of its activity after heating for 1 h in the tested temperature range (Fig. 6), while the preincubation of olive oil-induced lipase at either 37 or 40 °C caused an increase of the initial activity of 27 and 14 %, respectively.



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

### 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 solvents (10). In this work, both constitutive and olive oil-induced lipolytic activities showed high stability in the presence of water miscible organic solvents, since they retained almost 100 % of their activities after exposure for 1 h at 37 °C in 50 % assayed hydrophilic solvents (Fig. 7). Similar stability profile was found for the lipase produced by the extremophilic microorganism *Bacillus thermoleovorans* CCR11 (16).



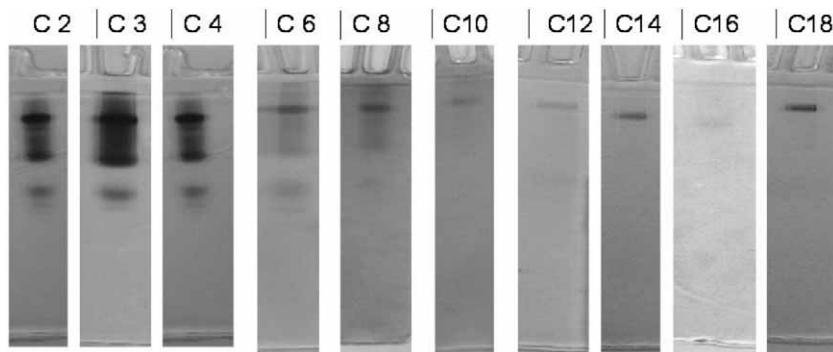
**Fig. 7.** Effect of organic solvents on residual specific lipase activity from *A. niger* MYA 135 using a medium either without olive oil (□) or supplemented with 2 % olive oil (■). Remaining activity was compared with the control without incubation. Error bars represent the standard deviation calculated from at least three independent experiments

### Stability assays in water-immiscible solvents

In these assays both inactivation by the interface (interfacial toxicity) and inactivation by dissolved organic solvent (molecular toxicity) were analyzed. All solvents tested caused a decrease in enzymatic activity. However, both constitutive and olive oil-induced lipolytic extracts retained around 80 and 60 % of their activities after incubation for 1 h at 37 °C with *n*-butanol and *n*-hexanol, respectively. Interestingly, the olive oil induced lipase was more stable in presence of *n*-hexane than the basal one (Fig. 7).

### Substrate affinity patterns

Supernatant of *A. niger* MYA 135 cultured in mineral medium supplemented with olive oil was analyzed by PAGE under non-denaturing conditions. The number and similarity of enzyme activities that use  $\alpha$ -naphthyl derivatives as substrate were compared. As shown in Fig. 8, the *in situ* gel assay provides an efficient method for differentiation of lipases from esterases. Post-electrophoretic detection of the lipolytic extract with  $\alpha$ -naphthyl derivatives of caprate, laurate, myristate, palmitate or stearate revealed only one coloured band (top band), while in the presence of  $\alpha$ -naphthyl derivatives of acetate, propionate or butyrate at least another three bands were observed.



**Fig. 8.** Native polyacrylamide gel stained with  $\alpha$ -naphthyl derivates and Fast Blue showing lipase and esterase activities from *A. niger* MYA 135. Chain length of acyl group: acetate (C2), propionate (C3), butyrate (C4), caproate (C6), caprylate (C8), caprate (C10), laurate (C12), myristate (C14), palmitate (C16) and stearate (C18)

Based on the hydrolases classification and according to these results the supernatant contains one lipase activity and at least three esterase activities.

Under our experimental conditions, basal activity bands from non-supplemented medium were not properly detected.

### Concluding Remarks

Extracellular lipolytic extracts of *A. niger* MYA 135 produced in mineral medium with or without olive oil supplementation displayed different catalytic properties. This finding could be interpreted in terms of microbial physiology since lipase isoenzymes have also been reported in *Aspergillus terreus* (17). Another possible explanation for the enhanced lipase performance in the presence of olive oil could be interfacial activation mediated by the interaction of solvent-oil-protein which stabilizes the biocatalyst structure (18). Also, a third choice is the combination of two previous hypotheses. Studies in our laboratory are pursuing to find out the factors which determine the increase of lipase activities in those different cultural conditions.

The effect of organic solvents on lipase extracts can be differentiated in two groups. In organic water-miscible solvents both lipolytic extracts retain almost 100 % of their enzyme activities after preincubation during 1 h, while in the presence of water-immiscible solvents different stability degree was observed. In this case, all the solvents tested caused a decrease in residual enzymatic activity. In both solvent type cases, no correlation between solvent hydrophobicity ( $\log P$ ) (19) and lipase activity can be established.

Besides, it is important to remark that under both cultural conditions lipolytic extracellular extracts were active in a broad range of pH and temperature. In addition, the presence of proteolytic activity in both extracts, which is one of the most relevant detrimental factors for extracellular enzymes, was not detected in *A. niger* MYA 135. The extracellular extracts were also tolerant to high organic solvent concentrations, which justifies their potential application in non-aqueous biocatalysis conversions.

The results presented in this work suggest that by manipulating the cultural conditions of *A. niger* MYA

135 it is possible to produce lipase extracts with different enzymatic properties, which would allow them to be used in diverse industrial processes.

### Acknowledgements

The present work was supported by grant PIP 2052/00 CONICET and CABBIO 2000/12.

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