Research Paper

Mycelium-bound lipase production from *Aspergillus niger* MYA 135, and its potential applications for the transesterification of ethanol

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The potential biotecnological applications of both constitutive and inducible lipase sources from *Aspergillus niger* MYA 135 were evaluated. To this end, the effect of environmental conditions on mycelium-bound lipase production from this strain was studied, when cultured either in the absence or presence of 2% olive oil. It was previously reported that mycelium-bound lipase from *Aspergillus niger* MYA 135 possess high stability in reaction mixtures containing ethanol; which could be especially important for their use in biodiesel synthesis. In this connection, the performance of the lipase sources produced in the transesterification of ethanol using p-nitrophenyl palmitate as acyl donor was also explored.

Under our assay conditions, hydrolytic and synthetic activity of the mycelia produced in the absence or presence of olive oil were not highly correlated. While the hydrolytic activity was strongly increased by the addition of lipid to the culture medium, the best performance in the transesterification reactions of ethanol were associated with mycelia produced in absence of olive oil. Interestingly, the supplementation of the culture medium with Fe^{+3} increased the transesterification activity by 71%, as compared to the activity previously reported for this strain. Therefore, the constitutive lipase sources from *Aspergillus niger* MYA 135 are considered to be promising for industrial biodiesel-fuel production.

Keywords: *Aspergillus niger* / Mycelium-bound lipase / Hydrolytic activity / Transesterification activity / Biodiesel synthesis

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Introduction

The demand for industrial enzymes, particularly those of microbial origin, is ever increasing, because of their potential applications in a wide variety of biotechnological processes. Currently, enzyme-mediated reactions are attractive alternatives to more expensive chemical methods. For these reasons, lipases (E.C. 3.1.1.3) have emerged as one of the leading types of biocatalysts, with proven potential for contributing to the multibillion dollar, underexploited lipid technology bio-industry, and have been used for in-situ lipid metabolism as well as ex-situ, multifaceted industrial applications.

Microbial lipases have attracted great interest from the chemical and pharmaceutical industries due to their potential applications in both hydrolysis and synthesis reactions [1]. A clear example is the development of the use of lipases for the synthesis of some compounds by esterification and transesterification mechanisms [2, 3]. Considering that the current energy resources in our region, petroleum (41%) and natural gas (49%) are essentially non-renewable, biodiesel synthesis has attracted great interest in recent years [4–6]. These steres, which are obtained from vegetable oils by transesterication with methanol or ethanol, can be synthesized by chemical or enzymatic methods. However, research into improving biodiesel production processes has focused on the use of heterogeneous catalysts, fa-

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cilitating the separation stage of the catalyst from the products formed.

Romero *et al.* [7] reported on the high stability of mycelium-bound lipase from Aspergillus niger MYA 135 in reaction mixtures containing ethanol, which could make them especially usefull for the lipase-catalized biodiesel synthesis [8]. In addition, we found that extracellular lipases production from this strain was strongly affected by the initial culture conditions [9].

In the present work, the effect of environmental conditions on mycelium-bound lipase production from *A. niger* MYA 135 was studied, when cultured either in the absence or presence of olive oil. In order to evaluate potential applications in biodiesel synthesis, the performance of the lipase sources produced in the transesterification of ethanol 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 study. The strain was maintained by monthly transfers to glucose-potato agar slant tubes, which were incubated at 30 °C and stored at 4 °C.

Culture conditions and enzymatic production

The basic culture medium (BM) contained (g/l): sucrose, 10.0; KH_2PO_4, 1.0; NH_4NO_3, 2.0; $MgSO_4$, 0.2; and $CuSO_4$, 0.06. The mycelium-bound lipase production in BM with an initial pH of 5.0 and at 30 °C was considered as control or reference. The influence of agitation, pH, temperature, ions, etc., on this production was examined by dose-response experiments. Then, the most influential environmental factors such as initial pH (2.0–8.0), the growth temperature (25–37 °C) and the presence of 0.5 g/l Ca^{+2} or 1.0 g/l Fe^{+3} ions in the BM were chosen to conduct this work. The Ca^{+2} and Fe^{+3} ions were added under form of chlorides to the MB before inoculation. The cultures were carried out in 500 ml conical flasks, containing 100 ml of BM on an orbital shaker (200 rpm). Culture flasks were inoculated at a final concentration of 10^5 conidia/ml. Each set of culture conditions were assayed both in the absence (constitutive production) and presence (inducible production) of 2% olive oil.

Enzyme determination

The mycelium-bound lipase production was determined on the basis of their hydrolytic activity in aqueous medium. Hydrolytic activity was measured as follows: about 0.010 g of wet mycelium was added to 1 ml of 100 mM phosphate buffer (pH 7.0) containing 2 mM of *p*NPP, 0.1% (*mass/volume*) gum Arabic, and 0.4% (*mass/volume*) Triton X-100 [10]. The reaction mixture was shaken at 37 °C, and the absorbancy of the supernatant containing *p*-nitrophenol (*p*NP) was determined at 405 nm. One unit of enzyme activity was defined as the amount of biocatalyst that released 1 μmol of *p*NP per minute. The molar extinction coefficient of *p*NP under this assay condition was 0.0230 μM^{-1} cm^{-1}.

The mycelia were harvested at the time of maximum production by filtration and centrifugation, and subjected to transesterification assays of ethanol with *p*NPP as acyl donor. As a small amount of water molecules is essential to obtain a sufficient enzyme conformational flexibility [11], the solvent as well as the biocatalysts were not dried before use. In previous experiments, no significant correlation was found between the amount of biocatalyst water in reaction mixtures (from 0.11 to 1.47%) and the synthetic activity (r = –0.066, P = 0.629). Thus, enzymatic transesterification was carried out as follows: to 800 μl of *n*-hexane, 100 μl of 20 mM *p*NPP dissolved in acetone, 100 μl of ethanol and about 0.010 g of wet mycelium were added [7]. The reaction mixture was shaken for 1 h at 37 °C, and the absorbancy of the supernatant containing *p*NP was determined at 405 nm. In *n*-hexane, the *p*NP was extracted with 1 ml of 0.25 M Na_2CO_3 before measurement. Hydrolytic activity in *n*-hexane was carried out similarly [12], but without the addition of ethanol. In this way, the reaction mixtures without the alcohol served as a hydrolysis control in organic medium. In the absence of cells no reaction was observed. One unit of transesterification activity was defined as the amount of biocatalyst that released 1 μmol of pNP per minute. The molar extinction coeffi-cient of pNP under this assay condition was 0.0205 μM^{-1} cm^{-1}. Specific activity was expressed as milliunits per gram of dry weight (mU/g). 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).

Qualitative analysis of the reaction product using thin layer chromatography (TLC) was carried out on silica gel 60 using chloroform/methanol/acetic acid (50:25:8 *volume/volume*) as developing solvents. Spots were viewed in iodine vapor.

Biomass determination

Biomass concentration was determined at the end of each cultivation period by drying washed mycelia at 105 °C until arriving at a constant weight.

Statistical analysis

Statistical analysis was performed using Infostat (version 2004) and Minitab (version 14) software for Windows. Results are presented as the mean ± standard deviation. Statistical significance values for the means were evaluated using one-way analysis of variance. Subsequent comparisons were performed using Tukey's post-hoc test. Differences were accepted as significant when $P < 0.05$. Associations between variables were assessed using Pearson's correlation coefficient.

Results

Time course of mycelium-bound lipase production from *A. niger*

The time course of mycelium-bound lipase production from *A. niger* MYA 135 was evaluated based on hydrolytic activity. As depicted in Fig. 1 a constitutive level of hydrolytic activity was detected in each set of assayed culture conditions. However, in the media supplemented with 2% olive oil (Fig. 2) the specific activity was greatly increased compared to their counterparts without oil.

Concerning the production kinetics, the constitutive hydrolytic activity gradually increased over time, reaching their highest value after 48 hours of cultivation. This value did not then changed significantly during 72 h ($P > 0.05$). In contrast, inducible hydrolytic activity changed drastically during the course of cultivation, reaching their highest value after 4 d of incubation with olive oil. The effect of modifications to the initial pH, growth temperature, and the presence of Ca^{+2} or Fe^{+3} in the BM on both constitutive and inducible production, is described next:

Effect of initial pH of the culture medium

Both constitutive and inducible hydrolytic activities were detected across the pH range tested. However, the kinetics and patterns of production were completely different. As shown in Figure 1A, the highest constitutive production was detected at initial pH levels of 4.0 and 8.0. In contrast, the mycelia grown at initial pH of 2.0 and 6.0 showed the lowest production levels. The effect of initial pH of the BM on inducible production was, however, completely different (Fig. 2A). After 4 days of cultivation in the presence of olive oil, the highest enzymatic production was detected at initial pH levels of 3.0 and 7.0; where specific activity increased by 57% and 38%, respectively, compared to the control medium with pH 5.0. In contrast, the mycelia grown at initial pH levels of 2.0, 4.0, and 6.0, showed an

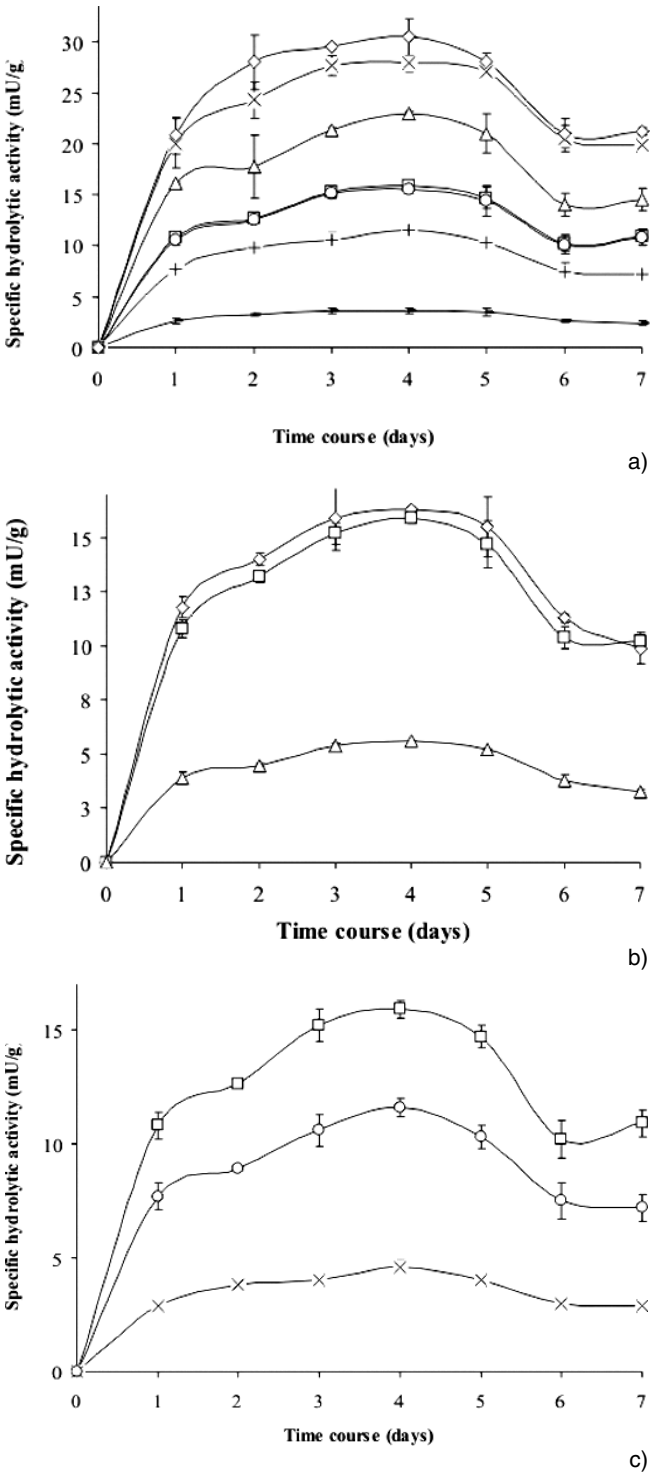

Figure 1. Time course of mycelium-bound lipases production from *A. niger* MYA 135, cultured in the BM without olive oil. (a) At different initial pH values: pH 2.0 (+); pH 3.0 (Δ); pH 4.0 (x); pH 5.0 (□); pH 6.0 (–); pH 7.0 (○); pH 8.0 (◇). (b) At different growth temperatures: 25 °C (◇); 30 °C (□); 37 °C (△). (c) In absence of Ca^{+2} and Fe^{+3} (□); supplemented with Ca^{+2} (x) or Fe^{+3} (○). Error bars represent the standard deviation calculated from three independent experiments.

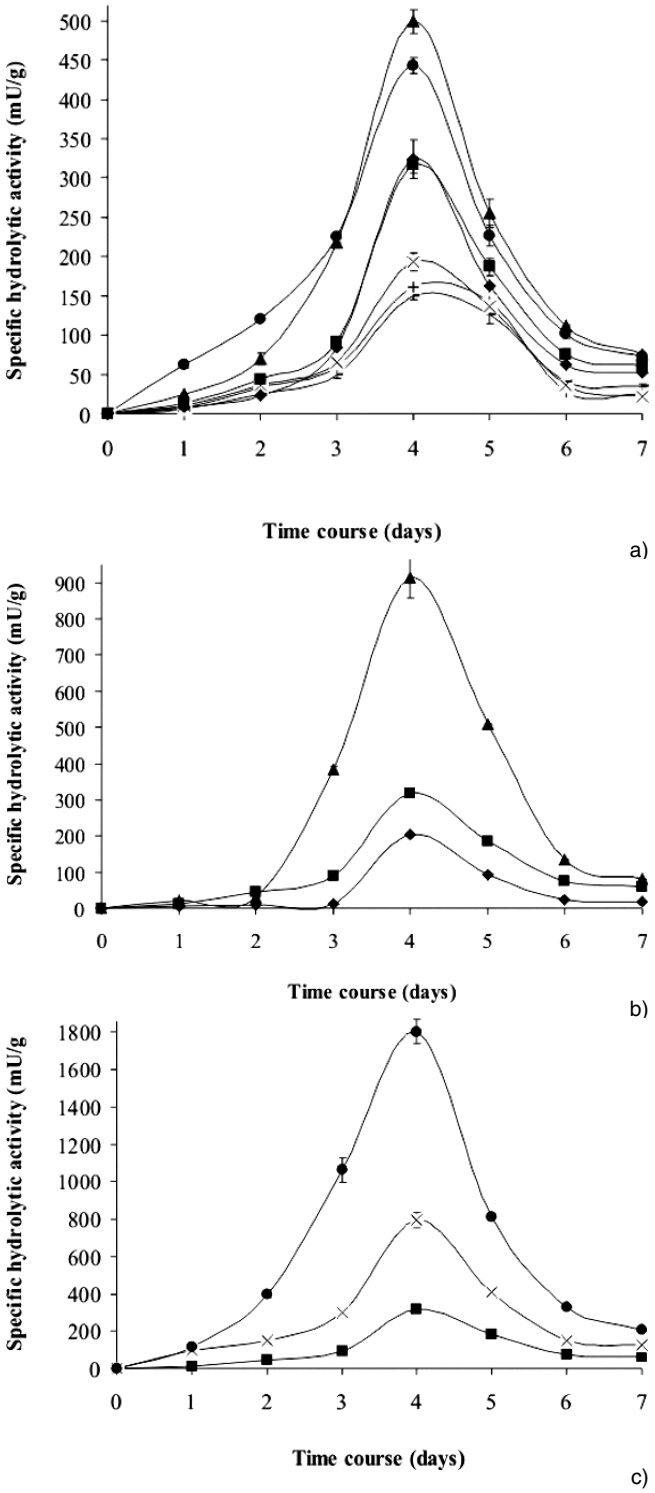

Figure 2. Time course of mycelium-bound lipases production from *A. niger* MYA 135, cultured in the BM with 2% olive oil. (a) At different initial pH values: pH 2.0 (+); pH 3.0 (▲); pH 4.0 (×); pH 5.0 (■); pH 6.0 (–); pH 7.0 (●); pH 8.0 (◆). (b) At different growth temperatures: 25 °C (◆); 30 °C (■); 37 °C (▲). (c) In absence of Ca^{+2} and Fe^{+3} (■); supplemented with Ca^{+2} (x) or Fe^{+3} (●). Error bars represent the standard deviation calculated from three independent experiments.

opposite catalytic response; and enzymatic production was drastically reduced.

Effect of the growth temperature

Both constitutive and inducible hydrolytic activities were detected across the temperature range assayed. However, while the increase in the growth temperature from 30 to 37 °C decreased the constitutive production (Fig. 1B), the inducible production behaved differently. In this case, after 4 d of cultivation at 37 °C with olive oil, the specific hydrolytic activity increased by 345% over mycelia grown at 25 °C, and by 188% over mycelia grown at 30 °C (Fig. 2B).

Effect of adding Ca^{+2} and Fe^{+3} ions to the culture medium

Finally, the effects of Ca^{+2} and Fe^{+3} in the BM on both constitutive and inducible mycelium-bound lipase production was determined. While supplementation with either ion decreased the constitutive production (Fig. 1C), after 4 d of cultivation with olive oil the hydrolytic activity of the mycelia grown in presence of Ca^{+2} or Fe^{+3} increased by 150% and 469% respectively, as compared to the control (Fig. 2C).

Biocatalyst performance of the mycelium-bound lipase sources from *A. niger* in trasesterification of ethanol

Fig. 3 shows the trasesterification profiles of ethanol with *p*NPP using the lipase sources produced in both the absence (*open bars*) and the presence of 2% olive oil (*filled bars*). Both constitutive and inducible synthetic activities were detected across the pH range of growth. For the enzymatic sources produced in the absence of olive oil, the highest transesterification activity was associated with the mycelium grown at pH 3.0 (43.8 ± 3.2 mU/g). However, in the presence of olive oil the best performance in the transesterification of ethanol was associated with the mycelia grown at initial pH levels of 5.0 (15.9 ± 1.5 mU/g) and 8.0 (14.7 ± 1.3 mU/g).

In regard to growth temperature, both constitutive and inducible transesterification activity was detected across the range of temperature assayed; however, the profiles were different. For the constitutive sources the highest transesterification activity was associated with the mycelium grown at 37 °C (46.0 ± 4.0 mU/g). In contrast, the inducible lipase sources showed an opposite catalytic response. In this case, the best performance in the synthetic reaction was observed with the mycelium grown at 25 °C (16.7 ± 0.3 mU/g).

Concerning the supplementation of the BM with Ca^{+2} or Fe^{+3} ions, the behavior of constitutive and inducible

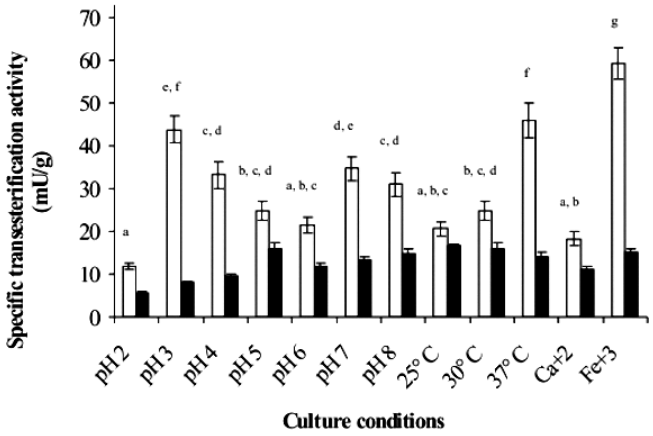

Figure 3. Transesterification in *m*-hexane of ethanol with *p*-nitrophenyl palmitate using lipase sources from *A. niger* MYA 135, produced in the BM either without olive oil (*open bars*) or supplemented with 2% olive oil (*filled bars*). Error bars represent the standard deviation calculated from three independent experiments. Bars with different letter are significantly different ($P < 0.05$).

lipase sources was also different. For the enzymatic sources produced in the absence of olive oil, the best performance in the transesterification of ethanol was associated with the mycelium grown in the presence of Fe^{+3} (59.3 ± 3.8 mU/g), where the specific activity increased by 71%, as compared to the activity previously reported for this strain [7]. However, supplementation of the BM with this ion was not favorable for inducible activity. Transesterification activity did not improve for either constitutive or inducible lipase sources when the mycelium was grown in the presence of Ca^{+2}.

The amount of water in the reaction mixture determines the direction of lipase-catalyzed reaction. In agreement with reports about hydrolytic activity in water and *n*-heptane of commercial lipase preparations [13], no significant hydrolytic activity was detected in *n*-hexane under our assay conditions. In addition, comparison of TLC profiles of hydrolytic and synthetic reactions in *n*-hexane, only showed a new spot (R_f = 0.70) in synthetic reactions, which could correspond to the ethyl palmitate production (data not shown).

To evaluate the relationship between hydrolytic and synthetic activity of the mycelia produced in the absence or presence of olive oil, correlation studies were performed. Finally, association between constitutive and inducible activity in terms of hydrolysis or synthesis was also determined (Table 1).

Effects of the culture conditions on biomass concentration from *A. niger*

Previously, we reported on the effect of environmental factors in the biomass concentration from *A. niger* MYA 135, when cultured in the presence of 2% olive oil [9].

Table 1. Pearson's correlation between mycelium-bound lipases activities from *A. niger* MYA 135.

Specific lipase activity	Inducible hydrolytic	Constitutive synthetic
Constitutive hydrolytic	r = 0.208; P = 0.516	r = 0.576; P = 0.143
Inducible synthetic	r = 0.287; P = 0.391	r = –0.338; P = 0.309

In the present work, fungal growth in the media without olive oil is also described (Fig. 4). After 7 days of cultivation in absence of the lipidic substrate the final biomass concentration decreased between 52 and 59%, as compared to their counterparts with oil. This suggests the use of olive oil as carbon source.

Discussion

In previous studies we found that extracellular lipases production from *A. niger* MYA 135 was strongly affected by the initial culture conditions [9]. However, naturally immobilized catalysts such as cell-bound microbial lipases have advantages over their extracellular counterparts, and have recently been receiving increased attention [14, 15]. Such systems are potentially cost-effective because the biomass can be directly utilized; eliminating the complex procedures of enzyme isolation, purification, and immobilization. Related to this, we examined the influence of environmental factors on mycelium-bound lipase production from *A. niger* MYA 135, including agitation, pH, temperature, and addition of different ions, through dose-response experiments (data not shown). Then, the factors found to have the most

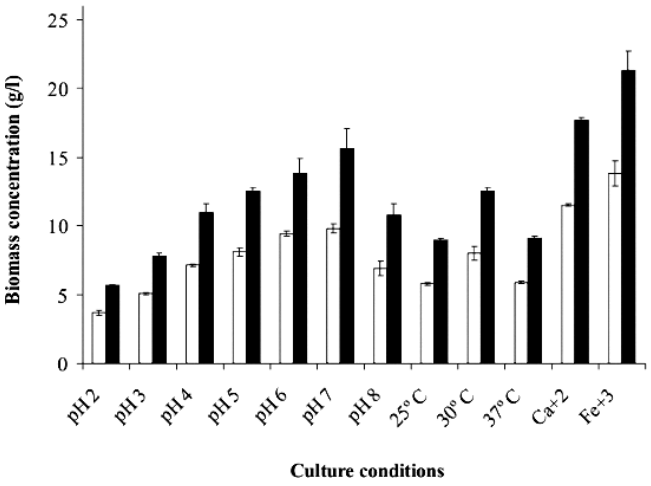

Figure 4. Biomass concentration from *A. niger* MYA 135 produced in the BM either without olive oil (*open bars*) or supplemented with 2% olive oil (*filled bars*). Error bars represent the standard deviation calculated from three independent experiments.

influence on this production were chosen to conduct this work.

Time course of lipase production was determined on the basis of hydrolytic activity in aqueous medium, and different responses by the constitutive and inducible lipases were observed. It was also noted that the production kinetics of the two enzymatic sources differed. While the constitutive production gradually increased over time, the inducible lipase sources changed drastically during the course of cultivation. Thus, in the presence of olive oil the mycelium-bound lipase production patterns from this strain were similar to those previously reported for their extracellular counterparts.

The incubation temperature is a variable that most affects the production of microbial enzymes [16–18]. In our particular case, the increase in the growth temperature had a positive effect on the inducible lipase production; which promotes their application in most industrial processes [19]. However, it is important to consider that the temperature could be a limiting factor in industrial fermentations because of the higher production costs. Consequently, it would be useful to implement a cost-benefit balance to determine optimal working conditions. Concerning the influence of metal ions, the addition of Ca^{+2} or Fe^{+3} to the BM significantly increased the inducible mycelium-bound lipase production from *A. niger* MYA 135. Although the presence of certain ions in the culture medium can either stimulates or inhibites microbial lipase production, usually Ca^{2+} have a positive effect [20–22]. On the other hand, the role of Fe^{+3} on extracellular lipase production by *Pseudomonas fluorescens* B52 has been widely studied. It has been reported that initial concentration in the medium is critical [23]. However, most of the studies about Fe^{+3} concern the influence of this ion on the lipase activity [24, 25], and there is a lack of knowledge about the role on the enzyme synthesis.

Although most studies were performed based on hydrolytic activity of the lipases, nearly all of the mycelium-bound lipases are used in organic solvents to catalyze synthesis reactions, because of their exceptional stability in this context [26]. Related to this, lipase-catalized biodiesel synthesis has attracted great interest in recent years. The most common route to biodiesel synthesis is through transesterification of vegetable oils with methanol or ethanol, using the action of a homogeneous catalyst, usually alkali metal hydroxides. However, the biggest problem when using homogeneous catalysis in industrial applications is the need for separation of the catalyst from the products formed during the same phase.

It was observed that the mycelium-bound lipase of *A. niger* MYA 135 possess high stability in reaction mixtures containing ethanol; which could be especially important for their use in biodiesel synthesis. In view this, in the present article the performance of the lipase sources produced in the transesterification of ethanol with *p*NPP is also described.

Although some whole-cell lipases exhibit only one type of catalytic ability, under certain conditions lipases can catalyze reactions in both directions. However, an enzyme's synthetic activity in organic solvents do not necessarily correspond with hydrolytic activity in aqueous solutions [27]. Under our assay conditions, hydrolytic and synthetic activity of the mycelia produced in the absence or presence of olive oil were not highly correlated. Nor was correlation found between constitutive and inducible activity in terms of hydrolysis or synthesis. In fact, Romero *et al.* [7] reported that constitutive and inducible lipase sources displayed different catalytic properties, probably due to different composition in isoenzymes.

In our particular case, while the hydrolytic activity was strongly increased by the addition of olive oil to the BM, the best performance in the transesterification reactions of ethanol were associated with mycelia produced in absence of lipid. In fact, it was found that Rhizopus chinensis whole-cell lipases with hydrolytic and synthetic activity have different fermentation characteristics [28, 29]. Recently, Teng *et al.* [30] also reported on whole-cell lipase production by *Rhizopus chinensis* in submerged fermentation, observing that two catalytical characteristics (hydrolytic and synthetic activity) of lipases were affected differently by the addition of lipids.

In conclusion, the lipase's catalytic ability in terms of hydrolysis or synthesis can be substantially improved not only by modification of the reaction mixture conditions [31], but also by varying the conditions of the biocatalyst's production. Based upon our results, it is proposed that constitutive lipase sources from *A. niger* MYA 135 could be potential candidates for use in future biodiesel production. Therefore, one of our next objectives is to design an enzyme reactor for biodiesel synthesis, using the mycelium-bound lipase activity from this strain.

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References

[1] Joseph, B., Ramtek, P.W., Thomas, G., 2008. Cold active microbial lipases: Some hot issues and recent developments. Biotechnol. Adv., **26**, 457–470.

[2] Antczak, T., Patura, J., Szczesna-Antczak, M., Hiler, D., Bielecki, S., 2004. Sugar ester synthesis by a mycelium-bound Mucor circinelloides lipase in a micro-reactor equipped with water activity sensor. J. Mol. Cat. B: Enzym., **29**, 155–161.

[3] Thakar, A., Madamwar, D., 2005. Enhanced ethyl butyrate production by surfactant coated lipase immobilized on silica. Process Biochem., **40**, 3263–3266.

[4] Park, E.Y., Sato, M., Kojima, S., 2008. Lipase-catalyzed biodiesel production from waste activated bleaching earth as raw material in a pilot plant. Bioresour. Technol., **99**, 3130–3135.

[5] Shah, S., Gupta, M.N., 2007. Lipase catalyzed preparation of biodiesel from Jatropha oil in a solvent-free system. Process Biochem., **42**, 409–414.

[6] Ranganathan, S.V., Narasimhan, S.L., Muthukumar, K., 2007. An overview of enzymatic production of biodiesel. Bioresour. Technol., DOI: 10.1016/jbiortech.2007.04.060.

[7] Romero, C.M., Baigorí, M.D., Pera, L.M., 2007. Catalytic properties of mycelium-bound lipases from *Aspergillus niger* MYA 135. Appl. Microbiol. Biotechnol., **76**, 861–866.

[8] Bernardes, O.L., Bevilaqua, J.V., Leal, M.C.M.R., Freire, D.M.G., Langone, M.A.P., 2007. Biodiesel fuel production by the transesterification reaction of soybean oil using immobilized lipase. Appl. Biochem. Biotechnol., **136**, 105–114.

[9] Colin, V.L., Baigorí, M.D., Pera, L.M., 2010. Effect of environmental conditions on extracellular lipases production and fungal morphology from *Aspergillus niger* MYA 135. J. Basic Microbiol., **50**, 52–58.

[10] Winkler, U., Stuckman, M., 1979. Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exo-lipase by *Serratia marescens*. J. Bacteriol., **138**, 663–670.

[11] Ye, P., Xu, Z.K., Wang, Z.G., Wu, J., Deng, H.T., Seta, P., 2005. Comparison of hydrolytic activities in aqueous and organic media for lipases immobilized on poly(acrylonitrile-*co*-maleic acid) ultrafiltration hollow fiber membrane. J. Mol. Catal. B: Enzym., **32**, 115–121.

[12] Pencreaćh, G., Baratti, J.C., 1996. Hydrolysis of *p*-nitrophenyl palmitate in *n*-heptane by the *Pseudomonas cepacia* lipase: A simple test for the determination of lipase activity in organic media. Enzyme Microb. Technol., **18**, 417–422.

[13] Pencreaćh, G., Baratti, J.C., 2001. Comparison of hydrolytic activity in water and heptane for thirty-two commercial lipase preparations. Enzyme Microb. Technol., **28**, 473–479.

[14] Tamalampudi, S., Talukder, M., Hama, S., Tanino, T., Suzuki, Y., Kondo, A., Fukuda, H., 2007. Development of recombinant *Aspergillus oryzae* whole-cell biocatalyst expressing lipase-encoding gene from *Candida antarctica*. Appl. Microbiol. Biotechnol., **75**, 387–395.

[15] Hama, S., Tamalampudi, S., Suzuki, Y., Yoshida, A., Fukuda, H., Kondo, A., 2008. Preparation and comparative characterization of immobilized *Aspergillus oryzae* expressing *Fusarium heterosporum* lipase for enzymatic biodiesel production. Appl. Microbiol. Biotechnol., **81**, 637–645.

[16] En-Shyh, L., Chee-Chan, W., Shu-Chiao, S., 2006. Cultivating conditions influence lipase production by the edible Basidiomycete *Antrodia cinnamomea* in submerged culture. Enzyme Microb. Technol., **39**, 98–102.

[17] Snajdr, J., Baldrian, P., 2007. Temperature affects the production, activity and stability of ligninolytic enzymes in *Pleurotus ostreatus* and *Trametes versicolor*. Folia Microbiol., **52**, 498–502.

[18] Swain, M.R., Kar, S., Ray, R.C., 2009. Exo-polygalacturonase production by *Bacillus subtilis* CM5 in solid state fermentation using cassava bagasse. Braz. J. Microbiol., DOI: 10.1590/S1517-83822009000300028.

[19] Hasan F., Ali, S.A., Hameed, A., 2006. Industrial applications of microbial lipases. Enzyme Microb. Technol., **39**, 235–251.

[20] Sharma, R., Chioti, Y., Banerjee, U.C., 2001. Production, purification, characterization and applications of lipases. Biotech. Adv., **19**, 627–662.

[21] Shariff, F.M., Leow, T.C., Mukred, A.D., Salleh, A.B. *et al.*, 2007. Production of L2 lipase by *Bacillus* sp. strain L2: nutritional and physical factors. J. Basic. Microbiol., **47**, 406–412.

[22] Wolski, E., Menusi, E., Mazutti, M., Toniazzo, G. *et al.*, 2008. Response surface methodology for optimization of lipase production by an immobilized newly isolated *Penicillium* sp. Ind. Eng. Chem. Res., **47**, 9651–9657.

[23] McKellar, R.C., Shamsuzzaman, K., San Jose, C., Cholette, H., 1987. Influence of iron (III) and pyoverdine on extracellular proteinase and lipase production by *Pseudomonas fluorescens* B52. Arch. Microbiol., **147**, 225–230.

[24] Abbas, H., Hiol, A., Deyris, V., Comeau, L., 2002. Isolation and characterization of an extracellular lipase from *Mucor* sp. strain isolated from palm fruit. Enzyme Microb. Technol., **31**, 968–975.

[25] Dharmendra, S.D., Friaa, J.M., Henehan, G.T.M., 2009. Influence of cultivation conditions on the production of a thermostable extracellular lipase from *Amycolatopsis mediterranei* DSM 43304. J. Ind. Microbiol., **37**, 1–17.

[26] Zanotto, S.P., Romano, I.P., Lisboa, L.U.S., Duvoisin, J.S., Martins, M.K., Lima, F.A., Silva, S.F., Albuquerque, P.M., 2009. Potential application in biocatalysis of mycelium-bound lipases from Amazonian fungi. J. Braz. Chem. Soc., DOI: 10.1590/S0103-50532009000600008.

[27] Sandoval, G., Marty, A., 2007. Screening methods for synthetic activity of lipases. Enzyme Microb. Technol., **40**, 390–393.

[28] Yan, X.Y., Xu, Y., 2005. Synthetic activity of *Rhyzopus chinensis* lipase in organic media. Industrial Microbial. China, **35**, 24–28.

[29] Ruan, Z.H., Wang, D., Xu, Y., 2007. Purification and characterization of lipase from *Rhyzopus chinensis*. Microbiol., **34**, 13–18.

[30] Teng, Y., Xu, Y., Wang, D., 2009. Production and reguation of different lipases activvities from *Rhizopus chilensis* in submerged fermentation by lipids. J. Mol. Catal. B: Enzym., **57**, 292–298.

[31] Shah, S., Sharma, S., Gupta, M., 2004. Biodiesel Preparation by Lipase-Catalyzed Transesterification of *Jatropha* Oil. Energy Fuels., **18**, 154–159.

