Research Paper

Effect of environmental conditions on extracellular lipases production and fungal morphology from *Aspergillus niger* MYA 135

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Under the current assay conditions, lipase production in mineral medium was only detected in the presence of vegetable oils, reaching the highest specific activity with olive oil. In this way, effect of different environmental conditions on fungal morphology and olive oil-induced extracellular lipases production from *Aspergillus niger* MYA 135 was studied. It was observed that addition of 1.0 g l^{-1} $FeCl_3$ to the medium encouraged filamentous growth and increased the specific activity 6.6 fold after 4 days of incubation compared to the control. However, major novelty of this study was the satisfactory production of an acidic lipase at initial pH 3 of the culture medium (1.74 ± 0.06 mU μg^{-1}), since its potencial applications in food and pharmaceutical industry are highly promising.

Keywords: *Aspergillus niger* / Environmental conditions / Lipase production / Fungal morphology

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Introduction

Lipases (E.C. 3.1.1.3) are enzymes that hydrolyse triglyceride ester bonds at the oil-water interface. These enzymes are also able to catalyze ester synthesis and transesterification in organic media containing minute water concentrations [1]. Lipases constitute the most important group of biocatalysts for biotechnological applications, and in recent years, they have been successfully used in the synthesis of biopolymers and biodiesel as well as in production of enantiopure pharmaceuticals, agrochemicals and flavour compounds. Therefore, optimization of industrially relevant lipase production has led to the research of different culture strategies [2, 3].

The use and preparation of chiral pharmaceutical molecules is one of the most relevant goals in pharmaceutical science. In this connection, potencial applications of acidic lipases are very promising since, an improvement in the enantioselectivity has been reported when these enzymes are used in an acidic environment [4]. However, active lipases from microbial organisms at highly acidic pH are not often detected.

Filamentous fungi are widely used as lipase sources because of extracellular enzyme secretion, which facilitates enzyme recovery from the fermentation broth. In this connection, *Aspergillus niger* is one of the most important microorganisms used in biotechnology and lipid requirement is essential to increase the lipase production.

Although a large number of factors determines the yield in submerged cultures, morphological growth forms have a significant effect on the fermentation broth rheology, so a particular fungal morphology may be preferred to obtain maximal performance [5, 6]. Considering that environmental factors not only define the physiology but also growth and morphology of filamentous fungi, the aim of the present work was carried out an initial analysis of the effect of environmental conditions on extracellular lipases production and fungal morphology from *Aspergillus niger* MYA 135. In this connection, our search focused primarily on the production of acidic lipases.

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Materials and methods

Microorganism and fermentation medium

Aspergillus niger ATCC MYA 135, from our own culture collection, was used throughout this study. Culture flasks were inoculated at a final concentration of 10^5 conidia ml^{-1}. The basic culture medium (BM) contained ($g\ l^{-1}$): sucrose,10.0; KH_2PO_4 1.0; NH_4NO_3 2.0; $MgSO_4$ 0.2; $CuSO_4$ 0.06.

Optimization of parameters for inducible lipase production

Cultures were carried out in 50 ml conical flasks containing 10 ml of BM on an orbital shaker at 200 rpm. Cultures in BM incubated at 30 °C and with initial pH 5, were used as controls. For lipase production the 24 h fermentation cultures were supplemented with different vegetable oils (olive, sunflower, corn, soya and grape) and incubated for 4 d, after which the effect of the selected oil concentration was tested. Selected oil was then used as a sole carbon source or by adding it to BM at the start, after 24 or 48 h of fermentation.

Oil-induced lipase production according to environmental conditions

Fermentations were carried out in 500 ml conical flasks on a orbital shaker at 200 rpm, containing 100 ml of each medium. Modifications of the culture conditions were assayed either by changing the initial pH of the medium, the incubation temperature or by addition of 0.5 $g\ l^{-1}$ $CaCl_2$ or 1.0 $g\ l^{-1}$ $FeCl_3$ to the medium. According to our previous studies, after 48 h of fermentation the cultures were supplemented with 2% olive oil and time course of olive oil-induced lipase production during 6 days was monitored.

Lipase determination

Lipase activity was measured spectrophotometrically with *p*-nitrophenyl palmitate (*p*NPP) as substrate, according to the method described by Winkler and Stuckman [7]. One unit of enzyme activity (U) was defined as the amount of enzyme that released 1 μmol of *p*-nitro phenol per min. Lipase production was expressed as specific activity (mU per μg of protein).

Protein determination

500 μl of Coomassie Blue G-250 reagent was added to 500 μl of sample. 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 [8].

Gel electrophoresis

Proteins were separated by native-PAGE [9] using a 10% (by mass per volume) polyacrylamide gel. Lipase and esterase activities were detected using 1.3 mM of α-naphthyl derivatives of acetate (C2) or stearate (C18) as substrate. Released naphthol was bound 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).

Characterization of morphological patterns

Morphological patterns and pellet diameters (mm) were analysed using a Nikon SMZ 1000 stereo microscope. Pellets diameters were calculated using a pre-calibration set.

Biomass determination

Biomass was determined at the end of each incubation period by drying washed mycelia at 105 °C until 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 of 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 and discussion

Effect of vegetable oils on lipase production and biomass

Lipase production by *Aspergillus* seems to be constitutive and independent of addition of lipid substrates to the fermentation medium [10]. However, under the current assay conditions, extracellular lipases production was only detected in the presence of vegetable oils (Table 1). Concerning mycelial growth, the addition of oils to BM showed a 3.3-fold increase in biomass, although this raise was not significant according to the nature of the oil assayed ($F = 3.51$; $df = 4$; $P = 0.100$) (Table 1).

Effect of natural fats on stimulation of lipase production in fungi has been previously reported [11]. In this connection, among the various fatty acids reported to induce lipase secretion by *Candida rugosa*, oleic acid was the best substrate [12]. In addition, northern blotting hybridization experiments showed that during batch

Table 1. Extracellular lipase production from *A. niger* MYA 135, after 4 d of incubation in basic medium supplemented with 2% vegetable oils.

Basic medium	Oleic acid (%)	Specific lipase activity (mU μg^{-1})	Biomass (g l^{-1})
Without oil[a]	–	–	3.3 ± 0.1[a]
Grape	17.3	0.03 ± 0.01[a]	10.5 ± 0.2[b]
Soya	22.0	0.12 ± 0.01[a, b]	10.6 ± 0.2[b]
Corn	24.1	0.13 ± 0.01[b]	10.5 ± 0.0[b]
Sunflower	31.5	0.75 ± 0.01[c]	10.1 ± 0.1[b]
Olive	75.0	1.46 ± 0.05[d]	10.9 ± 0.2[b]

[a] Control. Data are presented as mean value ± standard deviation calculated from at least two independent experiments. Values with different letters (a–d) are significantly different ($P < 0.05$).

growth of *C. rugosa* in rich medium containing oleic acid, the lipase specific mRNA reached its highest level during the late exponential growth phase [13].

Under our experimental conditions, the highest lipase production was observed with olive oil after 4 d of incubation, suggesting a positive correlation between specific lipase activity and oleic acid contents of the oils assayed ($r = 0.957$; $P = 0.01$) (Table 1). In subsequent assays, olive oil only was used.

Effect of concentration of olive oil on lipase production and biomass

The increase in oil concentration demonstrated a progressive increase in biomass, showing maximum growth (18.4 ± 0.4 g l^{-1}) at an olive oil concentration of 3.5% (Table 2). Like us, Gulati *et al.* (1999) [14] and Mahadik *et al.* (2004) [15] also reported an increase in biomass from *A. terreus* and *A. niger* NCIM 1207, respectively, with increasing oil concentration, suggesting the use of lipidic substrate as carbon source.

Table 2. Extracellular lipase production from *A. niger* MYA 135, after 4 d of incubation in basic medium supplemented with different concentration of olive oil.

Olive oil Concentration (%)	Specific lipase activity (mU μg^{-1})	Biomass (g l^{-1})
0.0[a]	–	3.2 ± 0.1[a]
0.5	0.20 ± 0.01[b]	5.7 ± 0.2[b]
1.0	0.45 ± 0.01[c]	7.2 ± 1.0[b]
1.5	0.66 ± 0.04[d]	11.0 ± 0.0[c]
2.0	0.86 ± 0.03[e]	12.3 ± 0.2[c, d]
2.5	0.72 ± 0.05[d]	13.5 ± 0.2[d]
3.0	0.13 ± 0.01[a, b]	17.9 ± 0.1[e]
3.5	0.06 ± 0.01[b]	18.4 ± 0.4[e]

[a] Control. Data are presented as mean value ± standard deviation calculated from at least two independent experiments. Values with different letters (a–e) are significantly different ($P < 0.05$).

Table 3. Extracellular lipase production from *A. niger* MYA 135, after 4 days of incubation in basic medium supplemented with 2% olive oil as sole carbon source or as inducer.

Culture conditions	Specific lipase activity (mU μg^{-1})	Biomass (g l^{-1})
BM[a]	–	3.3 ± 0.2[a]
BM+olive oil as only carbon source	0.10 ± 0.01[a]	3.2 ± 0.1[a]
BM+olive oil added at 0 h	0.15 ± 0.00[a]	10.5 ± 0.1[b]
BM+olive oil added after 24 h	2.03 ± 0.02[b]	10.7 ± 0.1[b]
BM+olive oil added after 48 h	2.45 ± 0.07[c]	10.9 ± 0.1[b]

[a] Control. Data are presented as mean value ± standard deviation calculated from at least two independent experiments.Values with different letters (a–c) are significantly different ($P < 0.05$).

Concerning the lipase production, the highest specific activity was detected using 2% olive oil (Table 2), after which it showed a decline. This response also is in agreement with previous studies [14], suggesting that an excess of lipidic substrate causes a negative effect on lipase synthesis.

Effect of olive oil as carbon source or as inducer on lipase production and biomass

Under our experimental conditions, extracellular lipase from *A. niger* MYA 135 only was detected in the presence of oils. The highest enzymatic activity was obtained using olive oil as inducer, added after 48 h of incubation to BM (Table 3). Biomass was not significantly modified by using sucrose or olive oil as carbon source ($F = 0.54$; $df = 1$; $P = 0.539$), although highest growth was obtained in BM supplemented with olive oil, regardless the time of addition (Table 3).

Similarly, previous studies with *Aspergillus* strains have shown that extracellular lipase production and biomass were higher in mineral medium containing olive oil compared with other media [16]. However, sugars have both positive and negative effects on lipase production. Indeed, it has become clear that not all microorganisms respond similarly, even though they belong to filamentous fungi, because the response of each organism is dependent to its physiological properties.

Effect of the initial culture pH on lipase production

After 4 d incubation in medium supplemented with olive oil the extracellular lipase production increased 1,6 and 1,4 fold in media with initial pH 3.0 and 7,0 respectively, compared to the lipase activity in control media with pH 5 (Fig. 1A).

Extracellular lipase production by *A. niger* MYA 135 at initial pH 7.0 of the basic medium, has been reported

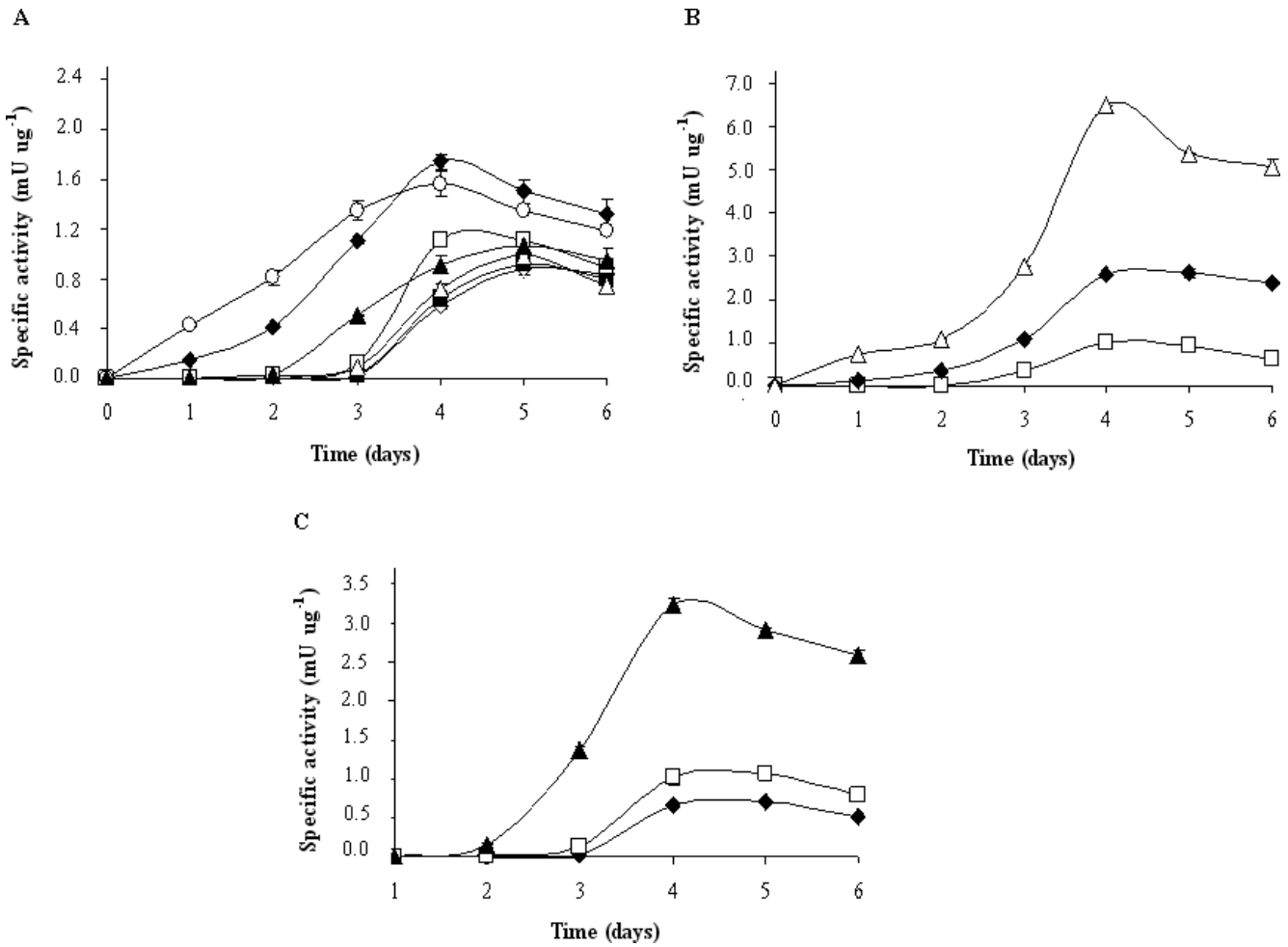

Figure 1. Time course of specific lipase activity from *A. niger* MYA 135, after the addition of 2% olive oil to the basic medium. [b]Control with olive oil. (A) At different initial pH values: pH 2 (◊); pH 3 (♦); pH 4 (■); pH 5 (□)[b]; pH 6 (Δ); pH 7 (○); pH 8 (▲). (B) With and without supplementation of metal ions: without metallic ions (□)[b]; 0.5 g l^{-1} $CaCl_2$ (♦); 1.0 g l^{-1} $FeCl_3$ (Δ). (C) Incubation at different temperatures: 25 °C (♦); 30 °C (□)[b]; 37 °C (▲). Error bars represent the standard deviation calculated from at least two independent experiments.

previously [17], while active lipases from microbial organisms at highly acidic pH are not often detected.

In previous studies, UV and nitrous acid derived mutants of *A. niger* were isolated in a media containing bile salts [18]. These authors observed that although nitrous acid mutants exhibited increased efficiency of lipase production compared with UV mutants, this lipase was active at pH 6 and not at extremely acidic pH.

Mahadik *et al.* [19] reported the hyper-production of acidic lipase by *A. niger* NCIM 1207 in solid state fermentation. However, the solid state fermentation has some limitations such as the choice of microorganisms capable of growth under reduced moisture conditions, controlling and monitoring of parameters such as temperature, pH, humidity and air flow. In view of this, optimization studies on lipase production by one mutant of *A. niger* capable of producing enhanced levels of acidic lipase in submerged culture was carried out [15].

Interest in acidic lipases is mainly due to their potential applications in the pharmaceutical industry. In fact, immobilized *Candida antarctica* lipase, commercially available and one of the most commonly used enzymes, has significantly shown to improve enantioselectivity when used in a highly acidic environment compared to a normal pH of 7.0 for hydrolysis of a ketoprofen ethyl ester at 45 °C [4]. This improved enantioselectivity was ascribed to the conformational change of the enzyme in the highly acidic environment. In view of this, satisfactory lipase production by *A. niger* MYA 135 at highly acidic pH is very promising, and future studies on the catalytic properties would be necessary to develop its full potential.

Effect of the metallic ions on lipase production

Addition of both 0.5 g l^{-1} $CaCl_2$ and 1.0 g l^{-1} $FeCl_3$ to BM, substantially increased extracellular lipase production from *A. niger* MYA 135 (Fig. 1B). Thus, after 4 d of incubation in medium supplemented with olive oil, the specific lipase activity increased 2.6 and 6.6 fold, respectively, compared to control.

The exocytosis of protein in eukaryotes occurs through *"regulated secretion"* pathways controlled by Ca^{++} or Mg^{++} ions concentration. In view of this, the increase in extracellular lipase production observed in the presence of $CaCl_2$ may be attributed to control of Ca^{++} ions on protein secretion. In this connection, Hoshino *et al.* [20] reported that lipase production and release is calcium-regulated in *Fusarium oxysporum.*

The role of iron (III) on regulation of extracellular lipase production has been widely studied, and the concentration is usually critical. Although in *Pseudomona fluorescens* iron seems to regulate extracellular lipase production, the involvement of pyoverdine in this process is not clear. However, free iron (not chelated by pyoverdine) was found responsible for repression of enzyme synthesis [21]. Although trace elements, particularly metals, are frequently necessary enzyme components, under our experimental conditions the highest lipase production from *A. niger* MYA 135 was observed by addition of 1.0 g l^{-1} $FeCl_3$ to BM.

Effect of the incubation temperature on lipase production

The incubation temperature is a variable that most affects enzyme production, and in this case an increase in temperature within the range assayed (25–37 °C) had a positive effect on the lipase production (Fig. 1C). Thus, after 4 d of incubation in medium supplemented with olive oil the specific activity at 37 °C increased 4.8 and 3.2 fold, compared to the activity observed at 25 and 30 °C, respectively. Similarly, Gulati *et al.* [14] reported maximum lipase production for *A. terreus* at 37 °C. However, this increase in incubation tempera-

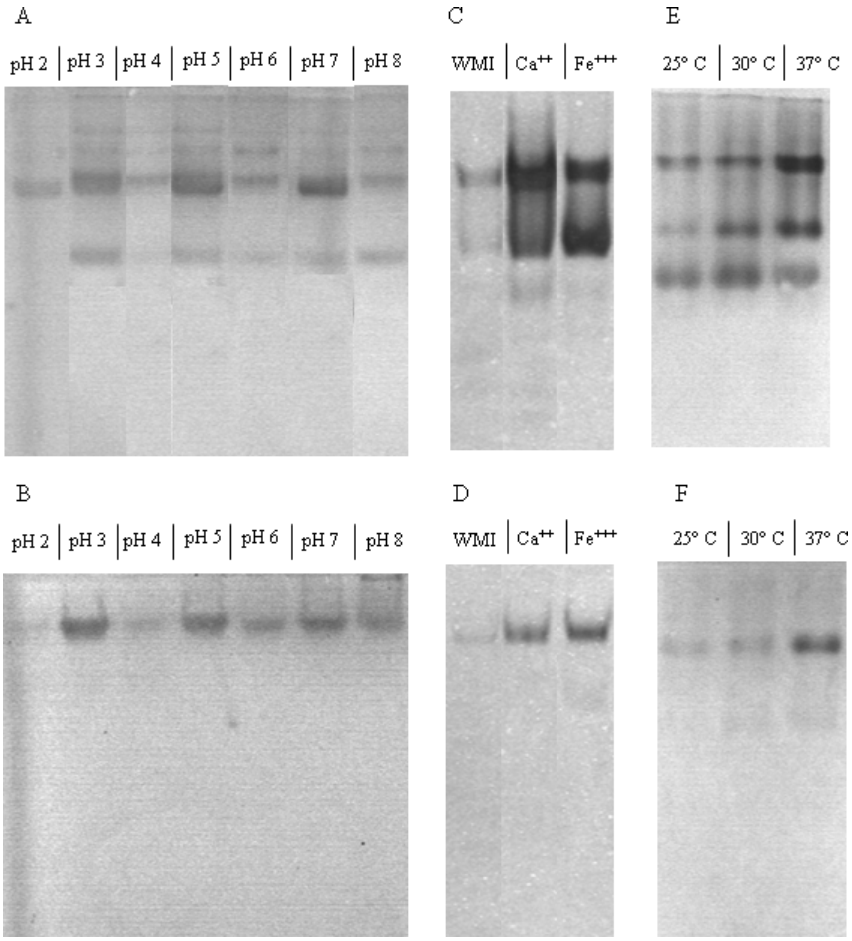

Figure 2. Native polyacrylamide gels of supernatants of *A. niger* MYA 135 after 4 d of fermentation, stained with Fast Blue and either α-naphtyl acetate (C2) for esterase activity (A, B and C) or α-naphtyl stearate (C18) for lipase activity (D, E and F).

ture could be a limiting factor in industrial fermentations because of the higher production costs. It would be desirable to conduct a cost-benefit balance to determine optimum working conditions.

Activity patterns in polyacrylamide gels

Non-denaturing PAGE was carried out to compare the number and similarity of enzyme activities that used α-naphtyl acetate or α-naphtyl stearate as substrate. While post-electrophoretic detection of lipolytic activity with α-naphtyl acetate revealed at least two bands (Fig. 2A, B and C), α-naphtyl stearate showed only one band (top) or eventually two coloured bands after addition of $FeCl_3$ or after incubation at 37 °C (Fig. 2 D, E and F).

According to the hydrolase activity, under the experimental conditions the supernatants contained one or two lipase enzymes and at least two esterase enzymes. Besides, the band intensity observed was consistent with the specific lipase activity so the in situ gel assay proved to be an efficient method, not only for differentiation of lipase from esterase activity but also for physiological characterization of cultures with respect to the enzyme production.

Biomass production and fungal morphology according to environmental conditions

Biomass from *A. niger* MYA 135 was significantly modified by the environmental conditions ($F = 70.9$; $df = 11$; $P < 0.001$) (Fig. 3). Concerning the influence of initial pH of the culture medium, optimal growth was observed at initial pH 7.0. On the other hand, in BM supplemented with $CaCl_2$ or $FeCl_3$ the biomass increased with 42 and 70%, respectively, compared to the mycelial yield obtained under standard culture conditions. Moreover, at 25 °C and 37 °C the biomass decreased with 27 and 28%, compared to growth at 30 °C.

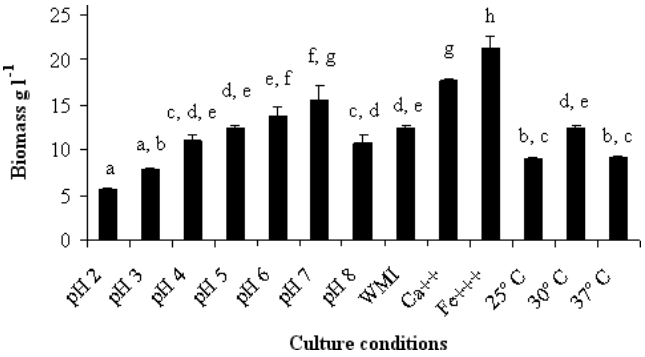

Figure 3. Biomass production during submerged incubation of *A. niger* MYA 135 under different environmental conditions. (WMI) BM without metallic ions. Error bars represent the standard deviation, calculated from at least two independent experiments. Bars with different letter (a–h) are significantly different ($P < 0.05$).

Modifications in the culture conditions showed different growth forms. In this connection, under standard culture conditions, pellets with a approximate diameter of 1.0 mm were obtained. Addition of $FeCl_3$ to BM developed filamentous growth; however, small pellets with a diameter less than 0.5 mm were observed after addition of $CaCl_2$ to the medium. Concerning the influence of initial pH of the culture medium on fungal morphology, a filamentous growth was observed at pH 2.0, while at the other conditions pelleted growth was developed. Thus, an increase in the initial pH progressively increased pellet size. Conversely, an increase in incubation temperature showed a decrease in the diameter of mycelial structures.

Filamentous fungi exhibit different growth morphologies in submerged culture varing from compact pellets to dispersed mycelila which strongly affect the overall cell performance [22]. Thus, while filamentous growth of *Aspergillus niger* is preferred for pectic enzyme production [23], the pelleted form is preferred for citric acid production [24]. In our particular case, the highest extracellular lipase production from *A. niger* MYA 135 in BM was associated with filamentous growth, enhanced by addition of $FeCl_3$ to the medium. However, at initial pH 2 of the culture medium, associated also with filamentous growth, the specific lipase activity substancially decreased. A satisfactory enzymatic production was also observed under certain culture conditions stimulating pelleted growth. Consequently, it would be necessary to conduct a further studies on the relationship between the lipase production and morphological growth forms, before any valid conclusions might be drawn.

Concluding remarks

Under the conditions assayed, extracellular lipases from *A. niger* ATCC MYA 135 only was detected in the presence of lipidic substrate, suggesting the inducible nature of these enzymes. In addition, the nature of lipidic substrate in lipase production seems a key factor.

Highest lipase production was observed by addition of $FeCl_3$ to the medium resulting in filamentous growth. However, interesting lipase activity was also observed under certain culture conditions resulting in pelleted growth. It is important to remark the satisfactory production of acidic lipase at initial pH 3 of the culture medium, due to its potential use in the food and pharmaceutical industry.

In conclusion, it is possible to produce lipase extracts after manipulation of the cultural conditions, which

would allow their use in diverse industrial applications. Consequently, it would be useful to implement a cost-benefit balance to determine optimal working conditions and the most appropriate fungal morphology.

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