

Expression and characterization of lipase produced by *Mucor griseocyanus*

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ABSTRACT

Few papers have reported on *Mucor griseocyanus* as a good lipase producer. The purpose of this study was the extracellular production of lipase by *M. griseocyanus* strain 55.1.1 on different substrates to select the most appropriate substance. The carbon sources used were: olive oil, glycerol, coconut oil, sunflower seed oil, glucose, starch and sucrose. Results indicate that enzyme synthesis is possible in the presence of all substrates. That lipase was produced constitutively in the microorganism. Lipase activity was in the range of 0.04 to 0.1 IU/mL. It was found that the most suitable carbon source for enzyme production was found to be a combination of coconut oil and sucrose at 0.5% and 1.5% (W/V) respectively. Optimum pH and temperature for enzymatic extract were of pH 4 to 6 and 60 °C. Enzymatic extract was stable for 5 hours in neutral and weak acid media (pH 6) at a moderate temperature of between 20 and 40 °C.

Key words: *Mucor griseocyanus*, submerged cultures, lipases

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RESUMEN

Expresión y caracterización de lipasa producida por *Mucor griseocyanus*. En la literatura existen pocas publicaciones que reporten a la especie de *Mucor griseocyanus* como buena productora de lipasa. El objetivo principal de este trabajo es estudiar la producción extracelular de lipasa por la cepa 55.1.1 de *Mucor griseocyanus* sobre diferentes sustratos para seleccionar el más adecuado. Las fuentes de carbono evaluadas fueron: aceite de oliva, glicerol, aceite de coco, lipasa aceite de girasol, glucosa, almidón y sacarosa. Los resultados obtenidos mostraron que la enzima se sintetizó en presencia de todos los sustratos empleados como fuente de carbono. Por esta razón, se consideró que la producción de la lipasa por el microorganismo se realizó de forma constitutiva. Se obtuvieron valores de actividad lipasa entre 0.1 y 0.04 UI/mL. La fuente de carbono más adecuada para la producción de la enzima fue la combinación de aceite de coco y sacarosa al 0.5% y 1.5% (P/V) respectivamente. Las condiciones óptimas para el extracto enzimático se encontraron en un intervalo de pH de 4 a 6 y 60 °C de temperatura. El extracto enzimático resultó estable durante 5 horas en un medio neutro ligeramente acidulado y a moderadas temperaturas entre 20 y 40 °C.

Palabras claves: *Mucor griseocyanus*, lipasa

Introduction

The application of enzymes can be traced back to ancient civilizations. Today, about 4000 enzymes are known, of which 200 have had a wide commercial use. The majority of the industrial enzymes are of microbial origin. At least 75% of all enzymes (including lipases) are hydrolytic in action and are used in many fields including: scientific research, cosmetic applications, medical diagnostics and chemical analysis, as well as therapeutic applications and industrial catalysis.

Lipase (EC 3. 1. 1. 3) hydrolyzes triglycerides to fatty acid and glycerol, and under certain conditions, catalyses the reverse reaction forming glycerides from glycerol and fatty acids. Some lipases also catalyse both trans-esterification and enantio-selective hydrolysis reactions [1].

There is a growing interest in these enzymes due to their excellent catalytic properties [2]. They are therefore considered valuable catalysts for a wide range of industrial applications including: detergent additives, the preparation of dietetic food, to obtain bioactive molecules in the pharmaceutical industry, in chemical synthesis to obtain pure optical compounds [3], and the modification of fats and lipids by hydrolysis and esterification reactions [4]. Most industrial lipases

are derived from fungi and bacteria. The most widely employed fungi genera are *Aspergillus*, *Rhizopus*, *Penicillium* and *Mucor* [2].

The problems in the systematic and controlled use of enzymes are far from being solved [5, 6]. Both the structural composition of the enzyme and the processing conditions will determine the catalytic properties of the enzyme.

The information on new lipase producing microorganisms and their properties, is essential when using enzymes for the above purposes, and this should be the basic aim of all studies.

Lipases have been thoroughly studied due to their importance. Research in the past has been mainly focused on their Structural characterization, mechanism of action, kinetics, sequencing and cloning of lipase genes, and the general characterization of performance [2]. Yet, a relatively small number of papers have been dedicated to the development of large bioreactor systems for commercial use.

The present paper examines the results of a preliminary study on the production of lipase expressed by *Mucor griseocyanus* and the effect of different substrates on lipase production.

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Materials and methods

The microorganism and cultures

The *Mucor griseocyanus* 55.1.1 strain from the microbial culture collection of the Sugar Cane Derivatives Institute (ICIDCA) was used and the stock culture was grown on potato-dextrose agar at 4 °C. This microorganism was isolated from a lysine sample in 1985.

The mineral growth medium contained (g/L): NaH_2PO_4 (12), KH_2PO_4 (2), MgSO_4 heptahydrate (0.3), CaCl_2 (0.25), and $(\text{NH}_4)_2\text{SO}_4$ at 1 % (W/V) as the nitrogen source and olive oil at 2% (W/V) as the carbon source. The initial pH was adjusted to 6.

To study the effect of different substrates on lipase production, other carbon sources were used at 2% (W/V), they included: sunflower oil, coconut oil, starch, glycerol and glucose. Furthermore, we studied the effect of combinations of coconut oil-glucose on lipase synthesis according to the feeding scheme represented in Table 1. Later, in the best combination, the glucose was substituted by sucrose in the medium at the same concentration.

The medium was inoculated with 10^7 conidia transferred from the stock culture to 100 mL erlenmeyer flasks containing 20 mL of the sterile growth medium. The flasks were incubated at 30 °C in a shaker operating at 120 rpm for 8 days.

Fungal growth was measured in terms of the dry weight of the mycelia in g/L.

The fungal biomass was filtered under vacuum and later centrifuged at 12000 rpm for 5 min. The clarified supernatant was used as the source of enzymes.

Characterization of the Enzyme

Temperature

Lipase activity in the hydrolysis of p-nitrophenyl propionate (pNPP) was measured at temperatures ranging from 20 °C to 90 °C at pH 7. For thermostability, the enzymatic extract was incubated at 20, 30, 40, 50 and 60 °C for 24 hours at pH 7. After incubation, the enzyme was immediately cooled in an ice bath for 15 minutes and the residual activity was determined (taking the initial activity of the enzyme as 100%).

pH

The activity was measured at different pH in a range of 4 to 10 at 30 °C. To ensure pH stability, the enzymatic extract was incubated in a 0.05 M buffer solution at pH values from 4 to 10 (Sodium Acetate for pH 4 and 5, Sodium Phosphate for pH 6 and 7 and Sodium Carbonate for pH 8, 9 and 10) for 24 hours at 30 °C. Residual activity was then assayed.

Table 1 Combinations of olive oil and glucose concentrations for the production of *M. griseocyanus* lipase

Combination	Coconut oil % (W/V)	Glucose % (W/V)
1	0	2.0
2	0.5	1.5
3	1.0	1.0
4	1.5	0.5
5	2.0	0

Enzyme Activity Assay

Lipase activity was determined by measuring the increase in absorbance at 348 nm produced by the p-nitrophenol released in the hydrolysis of 0.4 mM p-nitrophenyl propionate (pNPP) at pH 7 and 37 °C; 0.1 mL of lipase solution was added to 2.5 mL of the substrate solution [6].

An activity unit was defined as the amount of enzyme required to hydrolyze 1 μmol of pNPP per minute under the conditions described.

Glucose Assay

Sugar concentration was determined in cultures media using the total reducing sugar assay with 3,5-Dinitrosalicylic acid [7].

Results and discussion

Growth kinetics and lipase expression demonstrated by *Mucor griseocyanus* on different substrates is outlined in Figure 1.

The growth of the microorganism was very distinct. The highest value, 20 g/L, was reached in coconut oil on the 8th day of fermentation, followed by sunflower oil 14 g/L, olive oil 10 g/L, and glucose 8 g/L. However, when starch and glycerol sources were added to the culture medium, the biomass was of 4 and 7 g/L, respectively. None of these substrates were considered suitable as carbon sources in the *M. griseocyanus* cultures since the microorganism grew less than in the other carbon sources. On the other hand, lipase synthesis was observed in all substrates. Lipase activity was of between 0.04 and 0.1 IU/mL. This is why the lipase production by *Mucor griseocyanus* was considered to be done in a constitutive manner. This finding leads to the possible substitution of olive oil by coconut oil in the medium.

Experimental results were used to analyze the influence of carbon sources in regard to the energy they supply and how this energy is used in the growth of the microorganism and the expression of metabolites. The biomass/substrate yield for each carbon source regardless the microorganism employed can be determined by applying the mass balance to the system. This assumes that all energy supplied is used only for growth.

According to values reported by Erickson L. et al [8], and taking into consideration the maximum energy

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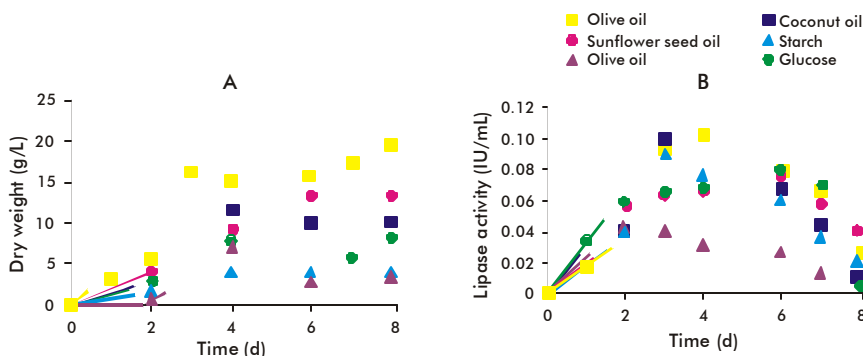


Figure 1. Production of lipase by *Mucor griseocyanus* cultures on different carbon sources added at 2% (W/V) and at 30 °C and 120 m^{-1} : (A) Microbial growth, (B) Lipase activity.

yield for growth as 0.7, the theoretical biomass/substrate yield ($Y_{x/s}$) can be calculated for some of the substrates studied. Since the concentration of each substrate in the medium was 20 g/L, the maximum possible growth of this microorganism can be estimated assuming a maximum value of biomass/substrate yield ($Y_{x/s}$) and 100% substrate conversion ($AS=S_0$), considering that the glucose was completely consumed on the second day. Table 2 outlines a comparison between the theoretical and true biomass/substrate yields for the substrates studied.

In all cases growth was less than the theoretical value. The difference between the theoretical and true growth values was more significant when starch was the carbon source. However, the highest efficiency for the glucose carbon source is approximately 71.4%. It can be assumed that the energy supplied by the starch was mainly used for enzyme synthesis. In the presence of starch, lipase activity levels were similar to those observed in cultures where coconut and olive oil were added. Olive oil, in particular, is a good inducer of lipase as indicated by many researchers [2, 9, 10]. In the other substrates, specifically glucose, the carbon source was more efficiently used for growth than for enzyme synthesis.

In terms of the contribution of the nitrogen source, 3 remarks can be made:

- Fungal microorganisms have about 30% (W/V) protein on a dry basis [11].

- Nitrogen represents 16% (W/V) of the proteins in the microorganism [11].

- Ammonia sulphate contains 21.2% (W/V) nitrogen.

Due to the presence of 10 g of ammonia sulphate in the culture medium, each liter of the culture contains 2.12 g of nitrogen. Assuming that all the nitrogen is transformed into protein, it is possible to obtain 13.25 g of protein and 44.17 g of the biomass.

Therefore, the theoretical production potential with 1% (W/V) of this nitrogen source is very different from those obtained in other cultures studied. This result can be attributed to two facts: first of all, the physiological and genetic characteristics of the microorganism in the consumption and efficient use of the nitrogen source employed in the culture medium; and second, the concentration of the nitrogen source in the medium, since it would not respond to what is considered an optimal concentration under appropriate conditions for microorganism growth.

In biotechnological processes, specifically in relation to fermentation, raw materials are an important part of the production cost of any protein. This cost has been estimated to be in the range of 30% to 80% [12], and is one of the main elements affecting the physiological state of microorganisms. This is why it is important to study the effect of different nutrients on the culture medium, especially carbon sources.

Table 2. Comparison of theoretical and true biomass/substrate yields

Substrate	$Y_{x/s}$ (th)	Growth(t) (g/L)	Growth(t) (g/L)	Efficiency (%)
Starch	0.63	12.6	3.5	27.8
Glycerol	0.63	12.6	7.0	55.6
Glucose	0.56	11.2	8.0	71.4

th): theoretical, (t): true

Table 3 shows the results obtained on the third day of fermentation where the best levels of lipase activity were reached when different combinations of coconut oil and glucose were added to the *M. griseocyanus* cultures to improve the medium from an economic stand point.

This microorganism expressed the lipase in all experimental runs. The second and fifth experiments showed the best results at about 0.1 IU/mL of lipase activity. These experiments mainly differ in growth rate in the medium. Similar values of lipase activity and productivity were achieved despite using a coconut oil concentration in the second experiment of 0.5% (W/V), which is 4 times smaller than that of the fifth experiment. This result is very interesting since it allows for the substitution of olive oil by coconut oil, including a reduction in the concentration of coconut oil in the culture medium, without affecting lipase expression.

The growth obtained on the third day of fermentation using coconut oil and glucose at 0.5 and 1.5% (W/V) respectively, is an intermediate value compared to the growth reported for each individual substrate (Figure 1a). This result is a good indication that the microorganism uses the oil source more efficiently for growth. Nevertheless, the behaviour of the lipase activity obtained in the combined medium was similar to the lipase activity observed in the medium containing coconut oil. The enzymatic activity decreased as of the third day for both media even when microbial growth continued. It can be assumed that the decline in enzymatic activity is attributable to a decrease in pH as a consequence of the fatty acids that are released through lipid hydrolysis. The pH of the culture medium on the third day and the last day of fermentation were of 2.23 and 1.43 respectively.

Figure 2 shows that when one half of the glucose supplied has been consumed (day one), it has already begun to produce the enzyme. This is again proof that the enzyme synthesis carried out by *M. griseocyanus* is done in a constitutive manner. This result contrasts with the results obtained with *A. niger* and *A. fumigatus* cultures, as previously reported [13]. However, it should be noted that similar results were reported for the *C. rugosa* strain when it was fermented in different concentrations of olive oil and glucose [14].

Figure 3 shows the same second experimental run but where the glucose added to the *M. griseocyanus* cultures was substituted by sucrose. The highest growth rate observed was 15 g/L, which was obtained during the third and fourth day of fermentation for the three carbon sources. The lipase activity obtained with

Table 3. *M. griseocyanus* cultures using coconut oil and glucose with different concentrations at 30 °C and 120 m³. Growth and lipase activity measured on the third day of fermentation. Productivity was calculated by dividing lipase activity by time

Experiment No.	Carbon source (% W/V)		Dry weight (g/L)	Lipase activity (IU/mL)	Productivity (IU/mL/d)
	Coconut oil	Glucose			
1	0.0	2.0	8.98	0.069	0.017
2	0.5	1.5	11.80	0.096	0.032
3	1.0	1.0	13.54	0.061	0.030
4	1.5	0.5	15.67	0.071	0.023
5	2.0	0.0	16.35	0.100	0.033

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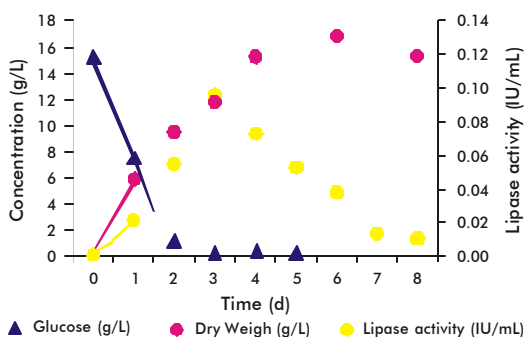


Figure 2. Lipase production and glucose consumption rate in *M. griseocyanus* cultures in the presence of coconut oil 0.5% - glucose 1.5% (W/V) at 30 °C and 120 m³.

sucrose combined with coconut oil was slightly higher than the lipase activity obtained when coconut oil was combined with glucose as the carbon source. Its value was 0.113 IU/mL. Hence, we concluded that it is possible to produce lipase by replacing the glucose by sucrose while reducing the coconut oil concentration from 2% to 0.5% (W/V). This result could help establish a more economically efficient process.

Figure 4 shows the effect of temperature and pH on the enzyme activity expressed by *Mucor griseocyanus*. The lipase of *M. griseocyanus* was more active at 60 °C. The activity drastically decreased when temperature was above 60 °C and it was practically lost at 70 °C. Most lipases were active between 25 and 40 °C [15, 16]. The *M. griseocyanus* lipase was found to be most active at a pH range of 4 to 6. This is the same range reported for a lipase expressed by *Rhizopus rhizopodiformis* [17]. There is very little research information on the production of lipase by *M. griseocyanus*.

Furthermore, this enzyme was very unstable at the different temperatures and pH values tested. It only retained about 80% of its initial activity at 20, 30 and 40 °C for 5 hours. Later the residual activity decreased considerably.

The stability profile of the enzyme with temperature shows a recovery of enzymatic activity after incubating for three hours at 30, 40 and 50 °C. It is known that the active site of lipases is protected by a flap that can activate the enzyme when exposed to certain conditions, [18-20]. In this particular case, it may be considered

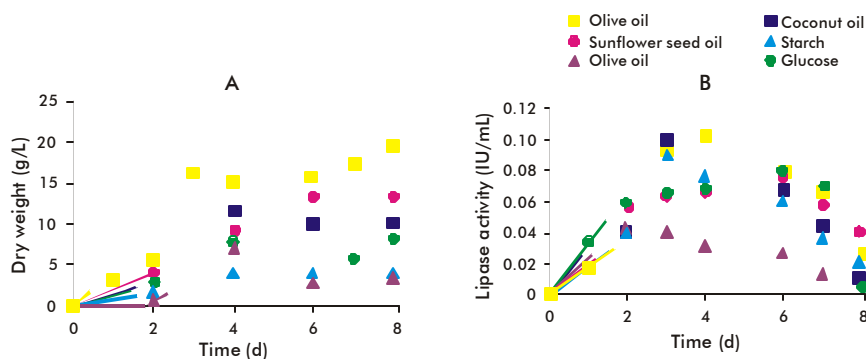


Figure 4. Behaviour of the enzymatic activity of *M. griseocyanus* lipase. (A) Effect of temperature at pH 7.0, (B) Effect of pH at 30 °C.

that the increase in temperature favoured the exposure of the active site of the lipase and its activation. This produces a better contact between the active site of the lipase and the substrate. The enzyme will be activated until the deactivation rate of the enzyme increases because of its denaturalization occurring when it is exposed to these temperatures. At 60 °C the recovery of enzymatic activity is less detectable since enzyme denaturalization occurs faster than the recovery obtained at other temperatures.

Results on the effect pH of on the stability of the enzyme are shown in Figure 5. Enzyme activity was more stable at pH 6 retaining almost 80% of its initial

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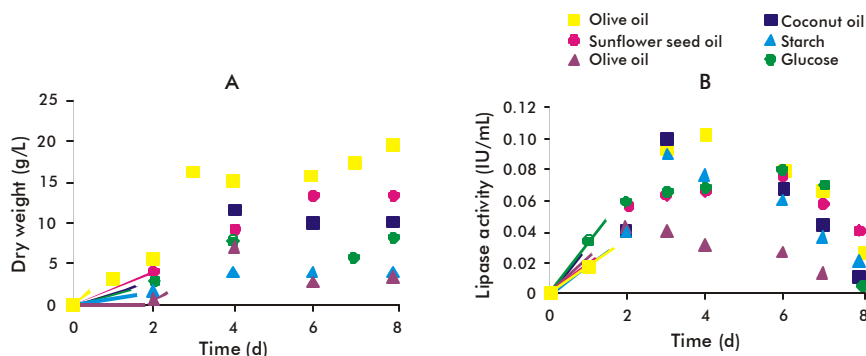


Figure 5. Stability profile of *M. griseocyanus* lipase according to temperature without including a buffer at pH 7 (A) and according to pH with a buffer solution at 30 °C (B).

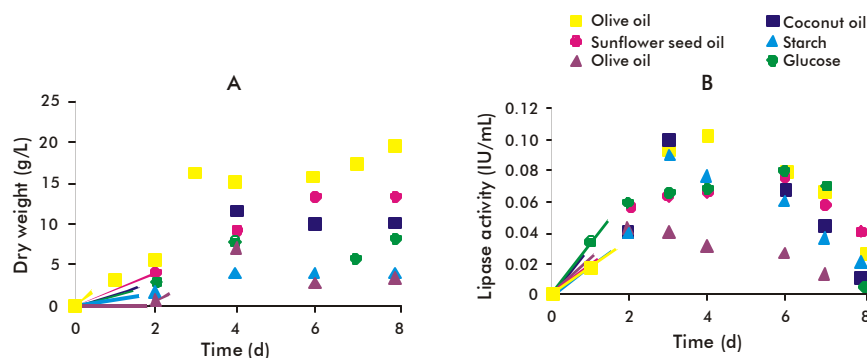


Figure 3. Microbial growth (A) and lipase activity (B) of *M. griseocyanus* when glucose is substituted by 1.5% sucrose (W/V) at 30 °C and 120 m³.

activity for 24 hours. Nevertheless, it rapidly became inactivated when it was incubated at pH 9 and 10 and lost about 30% and 40% of its initial activity within 30 minutes.

Conclusions

There is very little information on lipase expression by *Mucor griseocyanus* or its characteristics. The results obtained in this study demonstrate that the

synthesis of an extracellular lipase by the *Mucor griseocyanus* strain 55.1.1 is carried out in a constitutive manner, and the most suitable carbon source for enzyme production was a combination of coconut oil and sucrose at 0.5% and 1.5% (W/V) respectively.

At the same time this is a thermotolerant enzyme, which is an important property in the production of detergents. Future research should be focused on improving the stability of the enzyme.

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