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A Stable Lipase from *Candida lipolytica*

Cultivation Conditions and Crude Enzyme Characteristics

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ABSTRACT

Although lipases have been intensively studied, some aspects of enzyme production like substrate uptake, catabolite repression, and enzyme stability under long storage periods are seldom discussed in the literature. This work deals with the production of lipase by a new selected strain of *Candida lipolytica*. Concerning nutrition, it was observed that inorganic nitrogen sources were not as effective as peptone, and that oleic acid or triacylglycerides (TAG) were essential carbon sources. Repression by glucose and stimulation by oleic acid and long chain TAG (triolein and olive oil) were observed. Extracellular lipase activity was only observed at high levels at late stationary phase, whereas intracellular lipase levels were constant and almost undetectable during the cultivation period, suggesting that the produced enzyme was attached to the cell wall, mainly at the beginning of cultivation. The crude lipase produced by this yeast strain shows the following optima conditions: pH 8.0–10.0, temperature of 55°C. Moreover, this preparation maintains its full activity for at least 370 d at 5°C.

Index Entries: *Candida lipolytica; Candida;* lipase; lipase production; lipolytic activity.

INTRODUCTION

There is a growing interest in microbial lipases (acylglycerol hydrolases, E.C.3.1.1.3), because of their substrate specificity, which can be exploited for fine organic synthesis and related applications *(1–5)*.

Although lipase utilization, mainly for chiral compound synthesis, is a field of growing interest and investigation, lipase production and char-

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acterization are not well studied from a biochemical and physiological point of view.

Yeasts are interesting lipase producers because they have short generation times. Consequently, the cultivation of yeasts may be performed in shorter periods of time, using simple control procedures. Additionally, many yeasts have a generally recognized as safe (GRAS) status. These characteristics make yeasts good candidates to be genetically manipulated aiming at the overexpression of heterologous proteins.

Production of lipases by yeasts was reviewed *(6)* with most published works on the *Candida* species. Few yeast lipases are commercially available, however, there is an increasing interest in these enzymes. Commercialization will require a better understanding of the production process as well as novel and well-characterized lipases. Many aspects of the lipase production process are not well understood and questions related to substrate consumption and induction or repression are not elucidated.

The aim of this work was to study the production of lipase by a new *C. lipolytica* strain, to determine the best conditions for enzyme production by this strain. This work attempts to address issues concerning intra and extracellular lipase levels and substrate (olive oil) consumption during cultivation and its relationship with lipolytic activity.

MATERIALS AND METHODS

Materials

Triolein, oleic acid, tributyrin, and azocasein were obtained from Sigma Chemical (St Louis, MO). Peptone and yeast extract were obtained from Difco (Detroit, MI). Acid alumina was obtained from BDH Chemicals (UK) and all the other analytical-grade materials are from Riede-de Haen (Germany).

Microorganism

A wild type strain of *Candida lipolytica* was selected from an estuary in the vicinity of Rio de Janeiro, Brazil *(7)*.

Media Composition (all w/v):

SPC (sucrose 2%, bac. peptone 0.64%), TBS (the same as SPC plus tributyrin 1%), TBT (bac. peptone 0.64%, tributyrin 1%), OO (olive oil 1%, bac. peptone 0.64%), SA (olive oil 1%, ammonium sulfate 0.28%), SAT (the same as SA buffered with KH_2PO_4/K_2HPO_4, pH 6.0), U (olive oil 1%, urea 0.14%), UT (the same as U, buffered as SAT), G (glucose 2%, bac. peptone 0.64%), GOO (the same as G plus olive oil 1%), TO (triolein 1%, bac peptone 0.64%), AO (oleic acid 0.96%, bac. peptone 0.64%), OL (glycerol 0.1%, bac. peptone 0.64%). All the media were supplemented with yeast extract (0.1%) and the pH was adjusted to pH 6.0.

Inoculum and Cultivation Conditions

Incubations were carried out in 2000-mL Erlenmeyer flasks, containing 400 mL of culture medium, in a rotary shaker (160 rpm) at 29°C. A mass of cells leading to an initial concentration of 0.5 mg dry weight cell per mL (mg d.w.mL^{-1}) was used as inoculum.

Olive Oil Purification

To remove free fatty acids and other impurities from the commercial olive oil, a purification procedure was performed as follows: olive oil was mixed with a solvent mixture (1:1 v/v) containing petroleum ether and ethyl ether (10:1 v/v), and added to a column (2.4 cm diameter; 21 cm height) containing acid alumina. The solvent from the eluate was evaporated under mild stirring at room temperature. Purified olive oil was used in further experiments.

Analytical Methods

Cell growth was followed by optical density measurements at 570 nm and those values were converted to mg d.w.mL^{-1} using a standard curve relating those variables. Glucose concentration in the cultivation medium was determined at selected time intervals by using the classical 3,5 dinitrosalicilic acid (DNS) method *(8)* (olive oil does not interfere with the assay). Sucrose was determined by Anthrone method *(9)*. Olive oil content was determined according to Frings and Dunn *(10)* after three extractions with the same volume of chloroform, as established by previous experiments carried out at our laboratory.

Enzymatic Activity Assays

Lipolytic activity was determined by three different procedures, as follows:

1. Spectrophotometric method—0.1 mL of the cultivation supernatant was added to a solution of 0.504 m*M* *p*-nitrophenyl laurate (*p*-NPL) in 50 m*M* phosphate buffer, pH 7.0. This mixture was incubated at 37°C in a Shimadzu (Mod UV2201) spectrophotometer cuvet. The production of *p*-nitrophenol was automatically monitored at 410 nm during the linear period of product accumulation *(11)*. One unit (U) of lipase activity was defined as the amount of enzyme that produces 1 μmol of product per minute.
2. Diffusion method—hydrolysis halo diameter was measured in a solid medium containing olive oil or babassu oil according to Sztajer and Maliszewska (*12*). Lipase activity was expressed as the ratio between halo diameter and supernatant volume (mm:mL^{-1}) after plate incubation at 37°C for 45 h when the maximum diameter was reached.

3. Titrimetric method—this protocol was developed in our laboratory using olive oil as substrate. Cultivation supernatant (3 mL) was added to reaction flasks containing 17 mL of olive oil (5%)-arabic gum (5%) emulsion in phosphate buffer pH 7.0. Reactions were conducted at 37°C for 10 min and stopped by the addition of acetone:ethanol (1:1 v/v). The free fatty acids produced were titrated with 0.05 N NaOH. One unit (U) of lipolytic activity was defined as the am.ount of enzyme that produces 1 μmol of product per minute under the assay conditions.

Invertase was measured in a specific experiment incubating 3 mg d.w. cell with 1 mL of sucrose (100 mg mL^{-1}) and 0.5 mL of 50 m*M* acetate buffer pH 5.0 for 2 min at 30°C. Reducing sugars were measured according to Nelson *(13)*.

Protease activity was determined according to Charney and Tomarelli *(14)*.

Cell-Free Extract Preparation

Cells (50 mg mL^{-1}), washed twice with distilled water, were resuspended in 0.5 mL of 0.1*M* MOPS buffer, pH 7.0, with 2.0 m*M* EDTA and 5 m*M* β-mercaptoethanol. Disruption was obtained using glass beads as previously described *(15)*.

Enzyme Stability Assays

Lipase stability experiments were carried out at different temperatures and pH values. In each case the supernatant of centrifuged samples (7000*g* for 30 min), namely, crude preparation, was incubated at the selected conditions and its activity was measured at different time intervals by the spectrophotometric or the titrimetric method as indicated in each case.

RESULTS AND DISCUSSION

Lipolytic Activity Measurement

To select the best method to quantify lipase production by *C. lipolytica*, experiments comparing the three methods were performed and their results are presented in Fig. 1. Similar lipase activity profiles were obtained, independent of the method used, supporting our choice of using the spectrophotometric method to quantify *C. lipolytica* lipase activity. Data from Fig. 1 lead to the following coefficients for the correlation between the spectrophotometric and the other two methods (diffusion and titrimetric, respectively): 0.946 and 0.970.

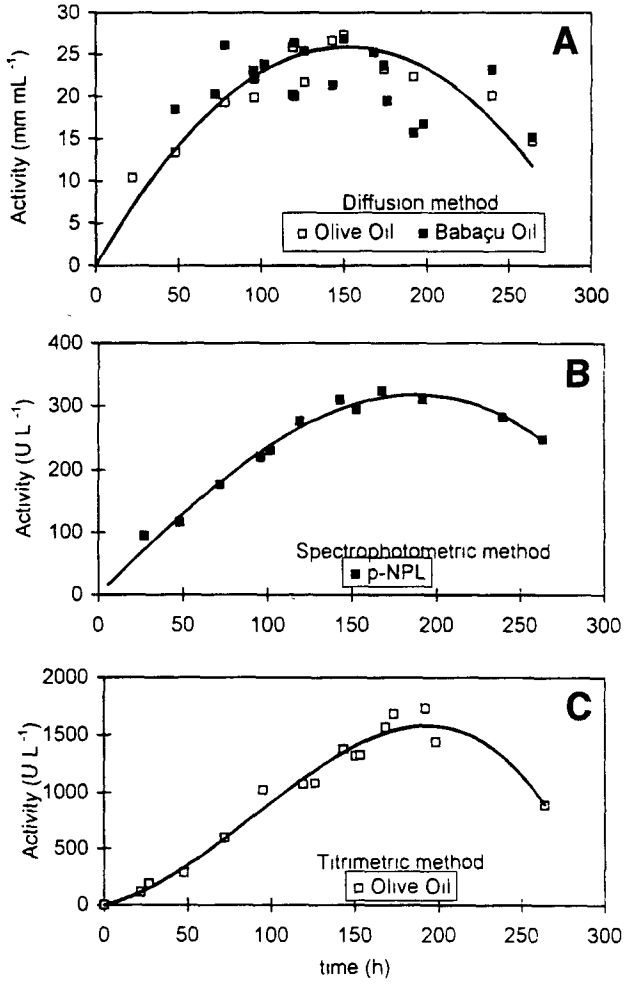

Fig. 1. Extracellular lipase activity measured simultaneously by three different methods during *C. lipolytica* cultivation in OO medium in a rotary shaker (160 rpm, 29°C). Lipolytic activity assay conditions are described in Materials and Methods.

Nutritional Conditions and Catabolite Repression

Some *Candida* species are usually cultivated in media containing a carbohydrate, a nitrogen source and an inducer molecule, generally, a triacylglyceride. In this work, several carbon and nitrogen sources were tested to produce lipase.

Results from Table 1 indicate that there is a correlation between maximum lipolytic activity and biomass concentration increase (ΔX) during cultivation (r = 0.991). However, no correlation was found between the specific growth rate (μ) and lipase activity. Glucose (G medium), but not sucrose (SPC medium), was effective for lipase production in the absence of TAG. This is explained by the absence of invertase activity in these cells. Invertase measurement in short-term

Table 1
Effect of Carbon Sources on Lipase Production

Main component	Code	ΔX ($mg.mL^{-1}$)[a]	μ (h^{-1})	Maximum activity ($U.L^{-1}$)	Volumetric productivity ($U.L^{-1}.h^{-1}$)
Carbohydrate	SPC	1.4	0.14	n.d.	—
	G	9.4	0.23	970	10.8
TAG	TBT	4.6	0.09	100	0.7
	OO	20.3	0.23	2700	17.7
	TO	20.0	0.42	3000	19.7
TAG +	TBS	5.6	0.09	60	0.4
Carbohydrate	GOO	19.1	—	3200[b]	8.0
Others	AO	24.5	0.51	3800	25.0
	OL	4.5	0.32	80	0.5

[a]Cell concentration increase expressed as dry weight per volume (ΔX is the difference between maximum and initial biomass concentrations.

[b]Only reached 200 h after in comparison with other maximum values.

n.d. not detectable.

C. lipolytica cells were cultivated at 29°C in a rotary shaker at 160 rpm, on peptone and different carbon sources. Lipolytic activity was measured by the spectrophotometric method.

experiments with baker's yeast and *C. lipolytica* showed activity values of 11 and 0 μmol reducing sugars mL^{-1}, respectively. Residual sucrose after growth phase was 17 and 100% for baker's yeast and *C. lipolytica* cultivations, respectively. Another evidence that sucrose is not being consumed is the identical specific growth rate (0.09 h^{-1}) obtained in TBS and TBT media.

Although GOO medium lead to a high lipase level, maximum activity was attained late in comparison with medium OO, as illustrated by the results of volumetric productivity (Table 1) and long-term cultivation experiments (activity and specific activity are shown in Table 2). These results indicate that lipase from *C. lipolytica* undergoes glucose repression and that derepression does not depend on inducer presence. This fact is supported by the following:

1. Glucose was identically consumed in G and GOO media, indicating preferential glucose uptake (Fig. 2);
2. High extracellular lipase levels were only later detected in GOO media (Table 2);
3. Intracellular lipase levels were almost the same in G, GOO, and OO media without any internal accumulation (as illustrated in Fig. 3C for OO medium).

Table 2
Effect of Glucose on Extracellular Lipase Production

Time (h)	G		GOO		OO	
	Specific activity ($U.g^{-1}$)[a]	Activity ($U.mL^{-1}$)	Specific activity ($U.g^{-1}$)[a]	Activity ($U.mL^{-1}$)	Specific activity ($U.g^{-1}$)[a]	Activity ($U.mL^{-1}$)
26	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
74	9	90	2	40	7	60
113	22	260	1	30	51	760
139	6	80	2	50	42	510
186	3	30	10	220	100	1240
210	n.d.	n.d	2	30	18	230
214	n.d.	n.d	n.d	n.d.	5	40
336	—	—	17	340	—	—
410	—	—	53	860	—	—
432	—	—	22	360	—	—

[a]Specific activity expressed as units per cell dry weight.
n.d. not detectable.
Time course activity results of three parallel cultivations carried out at 29°C in shaken flasks (160 rpm), on peptone and different carbon sources: glucose (G), glucose with olive oil (GOO) or olive oil (OO). Lipolytic activity was measured by the spectrophothometric method.

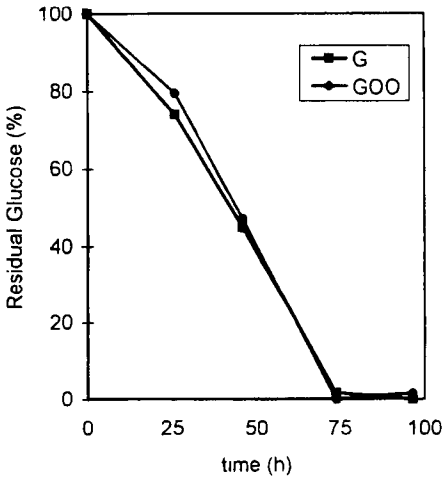

Fig. 2. Glucose consumption in presence (GOO media) or absence (G media) of olive oil. Experiments were carried out in parallel at 29°C and 160 rpm. Glucose concentration was measured by the DNS method.

It is important to note that previously reported results on glucose effect on lipase production are controversial. Muderhwa et al. *(16)* reported that lipase production by *Candida deformans* was repressed by

Table 3
Effect of Nitrogen Sources on Lipase Production

Code	ΔX ($mg.mL^{-1}$)[a]	μ (h^{-1})	Maximum activity ($U.L^{-1}$)	Volumetric productivity ($U.L^{-1}.h^{-1}$)	pH[b,c]
OO	20.3	0.23	2700	17.7	5.8
U	7.1	—	60	1.3	3.0
UT	7.0	0.23	150	3.1	6.1
SA	10.1	0.10	20	0.1	4.0
SAT	5.0	0.17	130	0.6	5.2

[a]Cell concentration increase expressed as dry weight per volume (ΔX is the difference between maximum and initial biomass concentrations.

[b]Measured at the time of maximum lipolytic activity.

[c]Initial pH=6.0.

C. lipolytica cells were cultivated at 29°C in a rotary shaker at 160 rpm, on olive oil and different nitrogen sources. Lipolytic activity was measured by the spectrophotometric method. OO medium contains peptone as nitrogen source.

glucose. On the other hand, no repression was observed with *Hansenula anomala (17).*

As indicated in Table 1, pronounced lipase volumetric productivity was only observed when long chain TAG or oleic acid were used as carbon sources with peptone on absence of sucrose (OO, TO, and AO media). Glycerol and a short chain TAG like tributyrin were not effective (OL and TBT media, respectively).

The stimulation effect of unsaturated fatty acids on microbial lipase production has been reported *(18–20)*, however, its biochemical basis has not been elucidated. Our results show that oleic acid has an important role on the stimulation of lipase production by *C. lipolytica*, as this fatty acid led to the highest level of lipolytic activity, when compared with other carbon sources (Table 1).

Nitrogen effect on lipase production by *Candida* is a feature that is not much investigated. The results of cultivation experiments performed with different nitrogen sources are shown in Table 3. A pH decrease was always observed when urea (U medium) and ammonium sulfate (SA medium) were used in unbuffered media. When these media were buffered (UT and SAT media), lipase activity was increased by a factor of 2.5 and 6.5, respectively. It is worth nothing that although a nonbuffered media was used, pH was constant (5.8) during the cultivation period in OO medium.

The overall results shown in Tables 1 and 3 indicate that oleic acid and peptone were the best carbon and nitrogen sources, respectively, for lipase production by *C. lipolytica*. Considering that oleic acid and triolein are very expensive in comparison with the other tested nutrients, and that olive oil (a low-cost and available carbon source) leads to an appreciable lipase level, OO medium was used in further experiments.

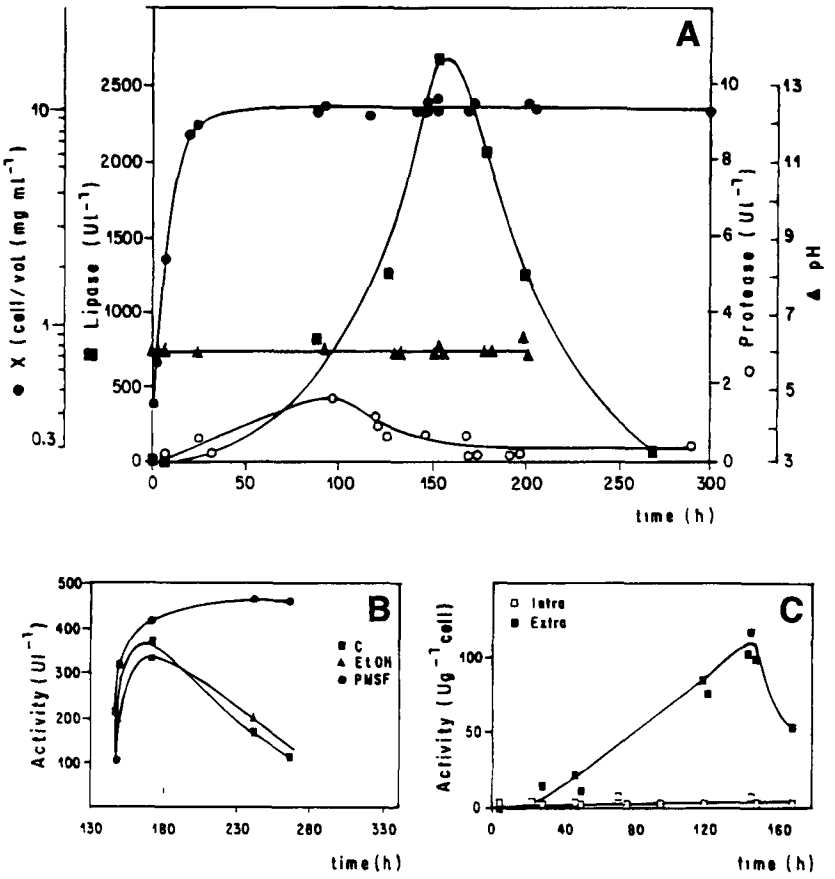

Fig. 3. Lipase production by *C. lipolytica* in OO medium. Cultivations were carried out in shaken-flasks at 29°C and 160 rpm. Lipolytic activity was measured by the spectrophotometric method. **(A)** Extracellular lipase and protease production, cell growth, and pH profile. **(B)** Extracellular lipase variation in OO medium (C-control experiment), OO medium with a serine protease inhibitor (PMSF-40 μg mL^{-1}) and OO medium with PMSF solvent (EtOH-ethanol). PMSF and/or ethanol were added after 142 hours of cultivation. **(C)** Intra- and extracellular lipolytic activity profiles (for comparison lipase levels were expressed as specific activity).

Enzyme Localization

A typical profile of cell concentration, pH, lipolytic, and proteolytic activities for the cultivation of the yeast in OO medium is shown in Fig. 3A. Maximum extracellular activity was only reached in the late stationary growth phase.

A sharp decrease on lipolytic activity occurs after 150 h of cultivation. In order to verify if this decay was caused by protease, a cultivation experiment was carried out by adding a serine protease inhibitor, phenylmethylsulphonyl fluoride (PMSF), to the culture medium. Figure 3B shows that the maximum level of lipase activity was maintained for an additional

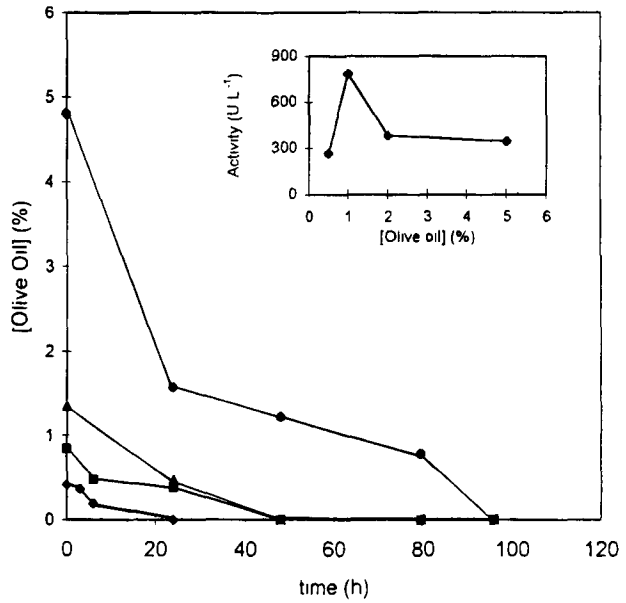

Fig. 4. Lipids content variation during cultivation of *C. lipolytica* in OO medium in a rotary shaker (160 rpm, 29°C). Olive oil initial concentration varied from 0.5 to 5% (w/v). Insert presents maximum extracellular lipolytic activity attained in each case, measured by the spectrophotometric method.

period of time (100 h) in comparison with the control experiment. Ethanol used as PMSF solvent had no effect on lipase production as also illustrated.

Intracellular lipase values for G and GOO media were similar to those presented in Fig. 3C (OO medium). A low intracellular lipase level was found during cultivation and no enzyme accumulation was observed within the cell. When lipolytic activity determinations (titrimetric method) were performed using no centrifuged cultivation broth samples (cells plus liquid medium) taken during the culture exponential growth phase (medium OO), a significant lipase activity value was obtained (2860 U L^{-1}). As neither intracellular nor extracellular lipase levels were detected at this cultivation time, our results suggest that lipase was cell-bounded. Emulsified hydrophobic substrates may attach to the cell wall retaining the enzyme in a supramolecular structure, containing lipolysaccharides, as observed by Kappeli et al *(21)*.

Substrate Consumption

Most of the published papers related to lipase production do not present profiles of oil exhaustion during cultivation, which might be a result of the difficulties in performing oil or lipids determinations in cultivation media. In this work, an attempt was made to quantify oil content, using chloroform to extract lipids from the supernatant. Oil was determined as described in the Materials and Methods section and typical results are shown in Fig. 4. This substrate practically vanished after 24 to

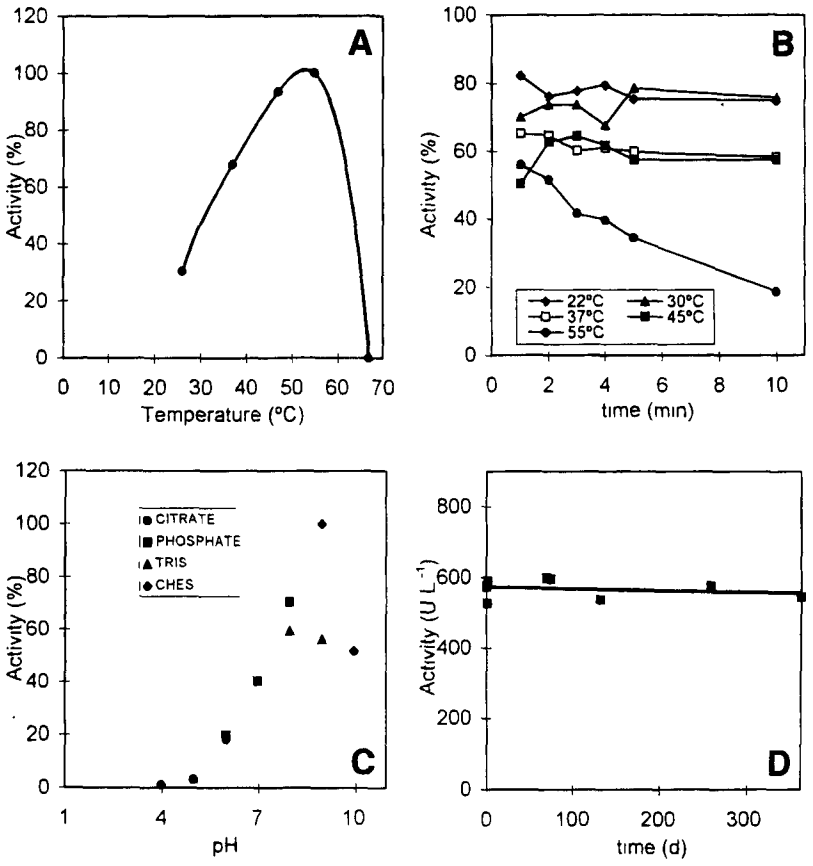

Fig. 5. Crude lipase preparation characteristics. Experiments were carried out with supernatants from *C. lipolytica* cultivations in OO medium. **(A)** Effect of temperature on extracellular lipolytic activity (spectrophotometric method). **(B)** Effect of temperature on extracellular lipase stability (spectrophotometric method). **(C)** Effect of pH on extracellular lipolytic activity (titrimetric method). **(D)** Long-term storage stability of the crude lipase preparation at 5°C (spectrophotometric method).

96 h of cultivation, depending on its initial concentration. The highest lipolytic activity was attained when 1% of olive oil was used.

Crude Enzyme Preparation Characteristics

After the selection of best conditions for lipase production by *C. lipolytica*, experiments were carried out to characterize the crude enzyme preparation.

The results concerning the effect of temperature on lipase activity are shown in Fig. 5A. The maximum activity was obtained at 55°C, but at this temperature the activity was quickly lost (Fig. 5B). However, the crude preparation was stable at 45°C and at lower temperatures (37, 30, and 22°C) as illustrated in Fig. 5B. Experiments carried out with different buffers (Fig. 5C) show that higher activities were observed in the pH range

of 8.0 to 10.0. Figure 5D illustrates the enzyme stability profile. A singular high stability of the crude lipase preparation was found when it was stored at 5°C. The enzyme remained 100% stable for at least 370 d without any additives.

Comparison of stability results with published data is very difficult because of the scarcity of information, as well as the lack of definition of stability indicators (full stability value, percentage of loss as a function of time, etc.). To our knowledge this is the first time that long-term stability assays were presented with crude preparations showing such a promising result—that the enzyme could be stored for more than 1 yr without any activity loss.

In conclusion, our results indicate that the selected *C. lipolytica* strain produces lipase at appreciable levels when cultivated in media containing oleic acid or long chain TAG and peptone. Our data also suggest that lipase is produced since the beginning of the cultivation, but it remains cell bounded and is only significantly released to the medium at late stationary phase when the carbon source (olive oil) is practically exhausted. Besides the crude enzyme's interesting characteristics (optimum activity at high pH levels and high stability for long periods of time), which are suitable for future applications, this work also quantifies TAG content in the culture medium and relates substrate consumption with lipolytic activity, a feature that is not emphasized in the current literature.

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REFERENCES

1. Chen, P. Y., Wu, S. H., and Wang, K. T. (1993), *Biotechnol. Lett.* **15,** 181–184.
2. Pozo, M. and Gotor, V. (1993), *Tetrahedron* **49,** 10,725.
3. Mustranta, A., Forssell, P., and Poutanen, K. (1993), *Enz. .Microbiol. Technol.* **15,** 133–139.
4. Cernia, E., Delfini, M., Mgrii, A. D., and Palocci, C. (1994), *Cell. Mol. Biol.* **40,** 193–199.
5. Nakano, H., Kiki, Y., Ando, K., Kawashima, Y., Kitahata, S., Tominaga, Y., and Takenishi, S. (1994), *J. Ferm. Bioeng.* **78,** 70–73.
6. Hadeball, W. (1991), *Acta Biotechnol.* **11,** 159–167.
7. Hagler, A. N. and Mendonça-Hagler, L. C. (1981), *Appl. Environ. Microbiol.* **41,** 173–178.
8. Sumner, J. B. (1924), *J. Biol. Chem.* **62,** 287–290.
9. Brin, M. (1966), in *Methods in Enzymology*, vol 9, Colowick, S. P. and Kaplan N. O., eds., Academic Press, New York, NY, pp. 506–514.
10. Frings, C. S. and Dunn, R. T. (1970), *Am. J. Clin. Pathol.* **53,** 89–91.
11. Wills, E. D. (1965), *Adv. Lipid Res.* **3,** 197–240.
12. Sztajer, H. and Maliszewska, I. (1988), *Enz. Microbiol. Tecnol.* **10,** 492–497.
13. Nelson N. (1944), *J. Biol. Chem.* **153,** 357.
14. Charney, J. and Tomarelli, R. M. (1947), *J. Biol. Chem.* **171,** 501–505.
15. Panek, A. C., Araujo, P. S., Moura Neto, V., and Panek, A. D. (1987), *Curr. Genet.* **11,** 459–465.

16. Muderhwa, J. M., Ratomahenina, R., Pina, M., Graille, J., and Galzy, P. (1985), *J. Am. Oil. Chem. Soc.* **62,** 1031–1036.
17. Banerjee, M., Sengupta, I., and Majumdar, S. K. (1985), *J. Food Sci. Technol.* **22,** 137–139.
18. Gomi, K., Ota, Y., and Minoda, Y. (1984), *Agric. Biol. Chem.* **48,** 1061–1062.
19. Del Rio, J. L., Serra, P., Valero, F., Poch, M., and Solà, C. (1990), *Biotechnol. Lett.* **12,** 835–838.
20. Ohnishi, K., Yoshida, Y., and Sekiguchi, J. (1994), *J. Ferment. Bioeng.* **77,** 490–495.
21. Kappeli, O. and Flechter, M. (1977), *J. Bacteriol.* **131,** 917–923.