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Lipase from a Brazilian Strain of *Penicillium Citrinum*

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ABSTRACT

A lipases (glycerol ester hydrolases E. C. 3.1.1.3) from a brazilian strain of *Penicillium citrinum* has been investigated. When the microorganism was cultured in the simple medium (1.0% olive oil and 0.5% yeast extract), using olive oil in as carbon source in the inocula, the enzyme extracted showed maximum activity (409 IU/mL). In addition, decrease of yeast extract concentration also reduces the lipase activity. Nevertheless, when yeast extract was replaced by ammonium sulfate, no activity was detected. Purification by precipitation with ammonium sulfate showed best activity in the 40–60% fraction. The optimum temperature for enzyme activity was found in the range of 34–37°C. However, after 30 min at 60°C, the enzyme was completely inactivated. The enzyme showed optimum at pH 8.0. The dried concentrated fraction (after dialysis and lyophilization) maintained its lipase activity at room temperature (28°C) for 8 mo. This result in lipase stability suggests an application of lipases from *P. citrinum* in detergents and other products that require a high stability at room temperature.

Index Entries: *Penicillium citrinum;* lipase; olive oil; detergents.

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INTRODUCTION

Lipases (glycerol ester hydrolases E. C. 3.1.1.3), a class of serine hydrolases not requiring cofactors, are widely distributed in animals, plants, and microorganisms (*1*). Microbial lipases are current receiving particular attention because of their actual and potential application in industry (detergents, oils and fats, dairy, and pharmaceutical industries). Most recent interest centers on use of lipases (particulary in an immobilized form) to catalyze, in low-water environments, esterification and transesterification reactions.

The main uses of industrial fungal lipases are as additives to washing detergents and in the food industry (*2*), such as cheese ripening, flavor production, and preparation of cocoa butter substitutes. Lipases have also been used for tanning, sewage treatment, and in the cosmetic industries. Lipases have also been used in transesterification reaction of triglycerides as a good alternative for chemical processes. The main advantages of a biotechnological process are the possibility of obtaining a specific product, lower process temperature, and avoiding the acid catalysts (*3*).

The production of lipases by *Penicillium s.p.* has been studied by various researchers. Lipases of *P. caseicolum* were investigated by Alhir et al. (*4*) and Sztajer et al. (*5*) investigated the production, purification, and physicochemical properties of lipases from *P. simplissicimum*. A wild strain of *Penicillium citrinum*, isolated from soil, has been investigated as a new lipase source by Malizewska and Mastalerz (*6*), Sztajer and Malizewska (*7*), and Sztajer et al. (*5*). They used a rich medium composed of starch, peptone, yeast extract, mineral salts, and rapeseed oil as inducer. According to Malizewska and Mastalerz (*6*), olive oil and Tween 80 could stimulate and the lauric acid inhibit the lipase production.

Industrial enzymes are generally commodity products, produced on a large scale, requiring the use of cheap, bulk nutrient components (*9*). Since nutrient costs represent up to 50% of production costs, the search for appropriate medium components (and inducers in the case of lipases) is continuous. This article discusses optimization of production and partial purification of lipase from a wild Northeast Brazilian strain of *Penicillium citrinum*.

MATERIAL AND METHODS

Microorganism

Penicillium citrinum investigated in this article was isolated as an olive oil contaminant. The culture was maintained on potato dextrose agar plates.

Growth Conditions

P. citrinum was cultured in the following media:

Glucose medium: 1.0% glucose, 0.5% yeast extract, pH 6.0 (initial);
Olive oil medium: 1.0% olive oil, 0.5% yeast extract, pH 6.0 (initial);
Rich medium: according to Sztajer and Malizewska (*7*), consisting of 2.0% starch, 0.5% $(NH_4)_2SO_4$ 0.025% $MgSO_4 \cdot 7\ H_2O$, 0.5% $CaCO_3$, 0.05% K_2HPO_4, 0.1% yeast extract, 5.0% peptone, and 1.0% olive oil, pH 7.2 (initial).

Growth curve experiments, started with a 10.0% (v/v) inoculum, were cultured for 48 h at 28°C, with orbital shaking (100 rpm). Samples were removed at time intervals, and the supernatants were used to measure lipolytic activity, protease activity, and protein concentration.

The effect of olive oil concentration on lipases activity was investigated using simple medium containing 0.5% yeast extract plus olive oil in the following concentration: 0.2, 0.4, 0.6, and 1.0%. Growth curve experiments started with cells grown in 0.2% glucose and 0.5% yeast extract (glucose inoculum) or in the same medium as that of the growth curve (olive oil inoculum). Both inocula were cultured under the same above conditions.

The effect of yeast extract concentration was determined with 1.0% olive oil plus 0.1, 0.2, and 0.5% yeast extract or replacement of yeast extract with 0.755% $(NH_4)_2SO_4$. This value corresponds to 16.0% nitrogen, based on total biomass (*9*), according to the estimated amount of nitrogen obtained when *P. citrinum* was cultured in 1.0% olive oil and 0.5% yeast extract. The experiments started with 10.0% inoculum cultured as before.

BIOMASS DETERMINATION

Biomass was determined by reproducing the above experiments under batch conditions. Cells were separated by filtration washed exhaustively, and dried at 80°C overnight to constant weight. In this case, fermentation was performed in Erlenmeyer flasks containing 100 mL of medium, inoculated with spore suspensions containing 10^7 spores/mL.

PREPARATION OF CRUDE EXTRACT

The crude enzyme used in the investigation of physicochemical properties of the lipases from *P. citrinum* was isolated as described below: Cells were grown in medium consisting of 0.5% yeast extract and 1.0%

olive oil, in Erlenmeyer flasks at 28°C, with shaking (100 rpm). Fermentation was interrupted after 4–6 d of culture, and the mycelium was separated from the supernatant by filtration. Solid ammonium sulfate was added to the supernatant to 80% saturation and left overnight with gentle stirring at 4°C. The resultant precipitate was collected by centrifugation (13,000*g*, 30 min) and resuspended in 0.05*M* phosphate buffer, pH 7.0. The soluble crude extract was dialyzed against the same buffer (6 h, two changes of buffer) and lyophilized.

ANALYTICAL METHODS

Lipase Assay

Two methods were employed for determining lipase activity. The titrimetric method used purified olive oil (*10*) in a Radiometer pH-Stat. The emulsion was prepared with Arabic gum (3.0%) and olive oil (3.0%), under shaking for 15 min at 150 rpm (*11*). The free fatty acids (FFAs) liberated by enzymatic hydrolysis were titrated with 0.025*M* NaOH. Units of activity were defined as the amount of enzyme that liberates 1 μmol of titratable FFA/min, under standard conditions (37°C, pH 8.0). The other method used to determine lipase activity was the spectrophotometric method (*12*), based on the hydrolysis of *p*-nitrophenylpalmitate (pNPP) into *p*-nitrophenol (pNP) and palmitate. Liberation of pNP at 37°C was detected at 410 nm (in a Cary spectrophotometer). One unit was defined as the cleavage of 1 μmol/min pNPP, pH 8.0 (0.05*M* phosphate buffer), 37°C. The molar absorption coefficient of pNPP(1.32×10^4 $M^{-1} \cdot cm^{-1}$) (*14*) was used to calculate enzyme activity. Protein was determined according to Lowry et al. (*15*) using bovine serum albumin as the standard. The method of Ginther (*16*) was used to follow the protease production by *P. citrinum* during growth. The determination was carried out at 37°C, pH 7.2, using azocasein solution as substrate. One unit was defined as the amount of the enzyme that produces a difference of absorbance at 440 nm of 1.0 U/h and mL of the sample. To determine the type of protease produced by *P. citrinum*, samples of the crude extract were incubated in the presence of EDTA (ethylinediamintetraacetic acid, a metaloprotease inhibitor) and PMSF (Phenylmethylsulonyl fluoride, a serine protease inhibitor) for 30 min at 37°C. After this, residual protease activity was determined.

Effect of pH on Lipase Activity

For determination of optimum pH of the enzyme, the reaction mixture buffer of the pNPP assay was varied over the pH range 5.0–9.0. The buffers used were citrate-phosphate (pH 5.0–7.0, 0.05*M*), phosphate (pH 7.0–8.0, 0.05*M*), and Tris-HCl (pH 8.0–9.0, 0.05*M*).

Effect of Temperature on Lipase Activity

For the determination of optimum temperature, the reaction mixture of pNPP method was incubated over a range of 20 to 62°C, using 0.05*M* phosphate buffer pH 8.0.

Thermal Stability of Lipase

For determination of thermal stability, the enzyme was pre-incubated over a range of temperature from 25 to 60°C. The time of incubation of the samples varied from 30 to 120 min. After incubation, samples were submitted to determination of lipase activity, using the pNPP method, at 37°C.

Stability at Different pH Values

The enzyme was preincubated at 37°C in buffers of different pH values (pH 6.0–9.0, 0.05*M* buffers as above) for 1 h. Lipase activity of the sample was measured at 37°C, using 0.05*M* phosphate buffer, pH 8.0, by the pNPP method.

Effect of Substrate Concentration on Lipase Activity

Both methods of lipase activity determination (pNPP and titrimetric) were used to investigate the influence of substrate concentration in the reaction medium. Substrate was varied from 0.0 to 1.0 m*M* of pNPP, and from 0.0 to 2.4 g% of purified olive oil, respectively. For these experiments, we used the best conditions obtained in the experiments above (pH 8.0 and 37°C).

RESULTS AND DISCUSSION

Effect of Medium Composition on Lipase Production:

The wild strain of *P. citrinum* isolated from olive oil showed the best lipase activity (409 IU/L) when it was cultured for 9 d (late log phase) in the simple medium (1.0% olive oil, 0.5% yeast extract). The activity obtained was three times higher than that obtained in the rich medium (151 IU/L), after 24 h (late log phase). However, after 15 d, the culture grown on the rich medium showed the highest enzyme activity (605 IU/L). There was no detectable lipase activity when *P. citrinum* was cultured in glucose medium, suggesting that it needs an inducer (Fig. 1A). At the maximal activity, the initial pH changed from 6.0 to 7.5 in simple medium, from 7.2 to 6.9 in rich medium, and from 5.7 to 6.2 in glucose medium. In all the studied media, the pH reached 7.8 at the late stationary phase (Fig. 1B). According to Sztajer and Malizewska (7), the best condition for

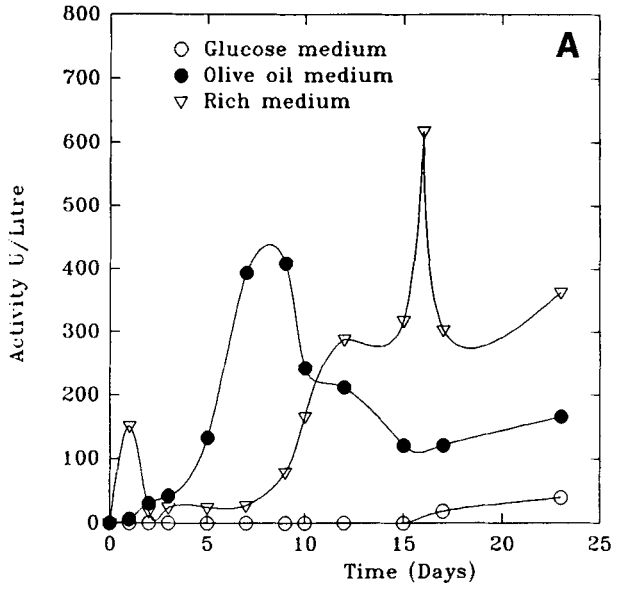

Fig. 1A. Effect of medium composition on lipase production by *P. citrinum*.

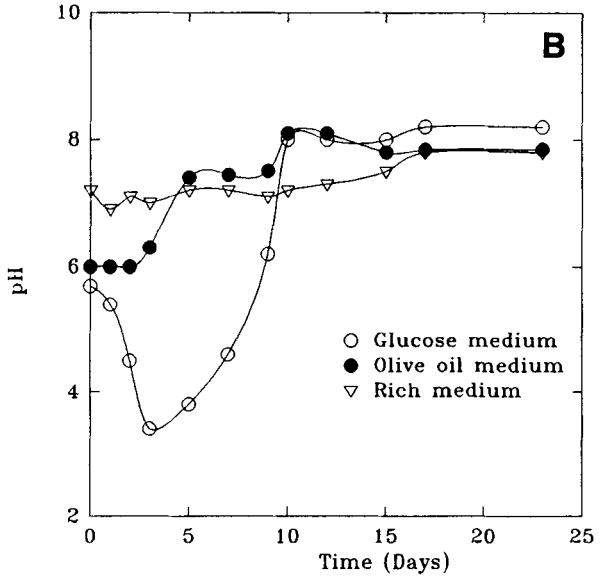

Fig. 1B. pH medium profile on the growth of *P. citrinum* using different growth media.

lipase activity yield (1015 IU/L) was obtained when the wild strain of *P. citrinum* isolated from soil was cultured in the rich medium containing 5.0% peptone as nitrogen source and 2.0% starch as carbon source, after 48 h, at 22°C and under shaking (150 rpm). Maximal lipase activity corresponded with a pH of 7.2.

Table 1
Effect of Olive Oil Concentration
and Nitrogen Sources on Lipase Production from *Pencillium Citrinum*

	C-source medium olive oil, %	Time to max. activity length, d	Maximum activities U/L	U/mg P
Olive oil-grown cells	0.2	14	205	0.419
	0.4	14	323	0.577
	0.6	14	810	2.080
	1.0	5	2880	3.200
Glucose-grown cells	0.2	11	333	0.368
	0.4	14	242	0.258
	0.6	14	379	0.289
	1.0	14	212	0.097

The comparison between specific lipase activities obtained in olive oil medium and in rich medium has shown that in the olive oil medium, the extracellular protein production was more specific for lipases: the specific activity in olive oil medium was 259 U/g protein, and it was reached after 7 d of culture. In the rich medium, the specific activity obtained was 3.9 U/g protein after 16 d of culture, showing that the majority of extracellular proteins produced by the microorganism in this medium are not lipases. Because of these results (high lipase production and relatively low cost of the medium), it was decided to investigate lipase production by *P. citrinum* further by improving the olive oil medium.

According to Haas et al. (*17*), the use of triglycerides as a carbon source has caused turbidity and difficulty of enzyme purification owing to the unhydrolyzed triglycerides. Therefore, they studied *Rhizopus delemar* lipases cultured in a medium containing amino acids, trace elements, and biotin in a basal salt medium. The use of glycerol as carbon source resulted in enzyme levels comparable to those obtained with triglycerides. However, the olive oil medium is simpler than that used by Haas et al. (*17*). No turbidity owing to the presence of olive oil was noticed. In addition, good protein recovery was obtained from the broth supernatant.

Effect of Olive Oil and Yeast Extract Concentration on Lipase Production

In order to find the best conditions for enzyme production in olive oil medium, it was decided to investigate the influence of the amounts of the carbon and nitrogen sources on lipase production. A survey on the effect of olive oil concentration in the simple medium (Table 1) showed that

using glucose instead of olive oil as grown inocula, the maximal lipase activity, in 0.2, 0.4, 0.6, and 1.0% olive oil, reached 333 U/L (after 11 d); 242 U/L (after 14 d); 379 U/L (after 14 d), and 212 U/L (after 17 d), respectively (Fig. 2A). However, when those activities were related to the extracellular protein concentration, the lipase activity using 0.2% olive oil (0.368 U/mg proteins) was about threefold higher than in medium containing 1.0% olive oil (0.097 U/mg proteins).

Using olive oil inocula, the maximal activity in olive oil medium was proportional to olive oil concentration in the medium reached using 0.2, 0.4, 0.6, and 1.0%, 205 U/L (after 14 d); 323 U/L (after 14 d); 810 U/L (after 14 d), and 2880 U/L (after 5 d), respectively. In the medium with 1.0% olive oil, the maximal activity (2880 U/L) was about eightfold higher than that obtained earlier with 0.2% olive oil (333 U/L) (Fig. 2B). These results could be explained by the free fatty acid accumulation, resulting from unbalance between production (by lipase activity on olive oil) and microbial consumption owing to growth. As described by Malizewska and Mastalerz (*6*), lipase production can be inhibited by fatty acids. They found that 0.1% olive oil stimulated by sixfold the extracellular lipase activity in the medium (*7*), whereas lauric acid addition caused inhibition of lipase production.

Figures 3A and 3B show that lipase production is related to biomass: when the biomass was in the maximum, lipase activity was also high. Based on the protein values from growth curves using 0.5% yeast extract, it was shown that this *P. citrinum* strain used only 0.2% yeast extract in the log phase. Therefore, the possibility of obtaining the same biomass and lipase production with only yeast extract was investigated. The results of these experiments demonstrated that after 7 d, the biomass obtained with 0.5% yeast extract (9.9 g/L dry wt) was higher than that obtained with 0.2% yeast extract (7.57 g/L dry wt). The biomass was 30-fold higher than that obtained with ammonium sulfate (0.329 g/L dry wt) after 15 d. According to Sztajer and Malizewska (*7*), ammonium sulfate did not stimulate lipase production when they used rich medium containing 2.0% starch as the carbon source, mineral salts, 0.1% yeast extract, and rapeseed oil as inducer. Further decrease of yeast extract concentration reduced the maximal lipase activity from 2880 U/L (0.5%) to 621 U/L (0.2%). There was no lipase activity when the yeast extract was replaced by ammonium sulfate (0.2 or 0.75%) (Table 1).

The sharp biomass reduction and lack of lipase production with ammonium sulfate could be the result of lack of some mineral salts present in yeast extract that are necessary for growth of the fungi, such as KH_2PO_4, NaCl, $MgSO_4$, Zn^{2+}, and Fe^{2+} (*18*). These results confirm that the simple medium containing 1.0% olive oil and 0.5% yeast extract was the best for lipase production by the wild *P. citrinum* investigated in the present work.

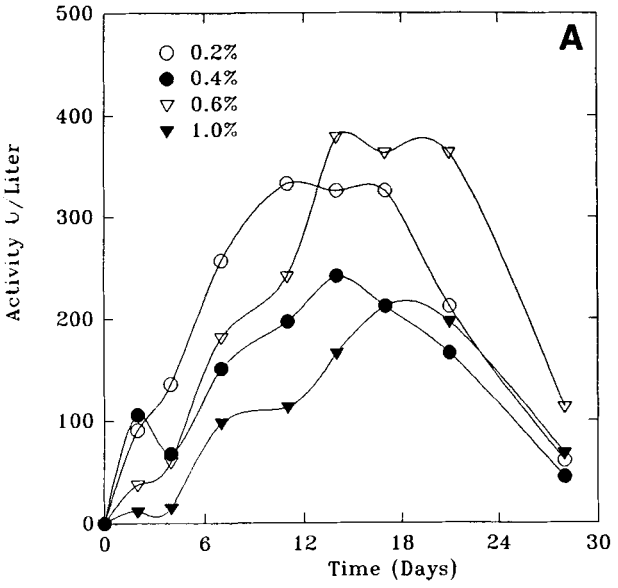

Fig. 2A. Effect of olive oil concentration and the preinocula on lipase production (activity) from *P. citrinum*-grown inoculum on glucose. Media were composed of 0.5% yeast extract and variable amounts of olive oil: 0.2, 0.4, 0.6, and 1.0%.

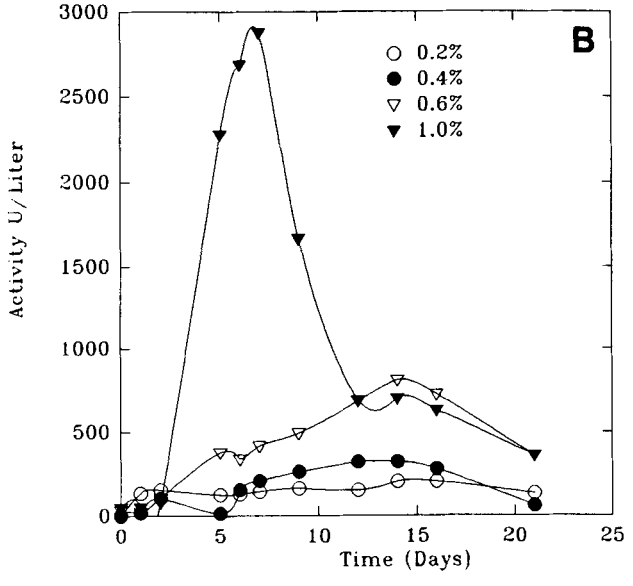

Fig. 2B. Effect of olive oil concentration and the preinocula on lipase production (activity) from *P. citrinum*-grown inoculum on olive oil. Media were composed of 0.5% yeast extract and variable amounts of olive oil: 0.2, 0.4, 0.6, and 1.0%.

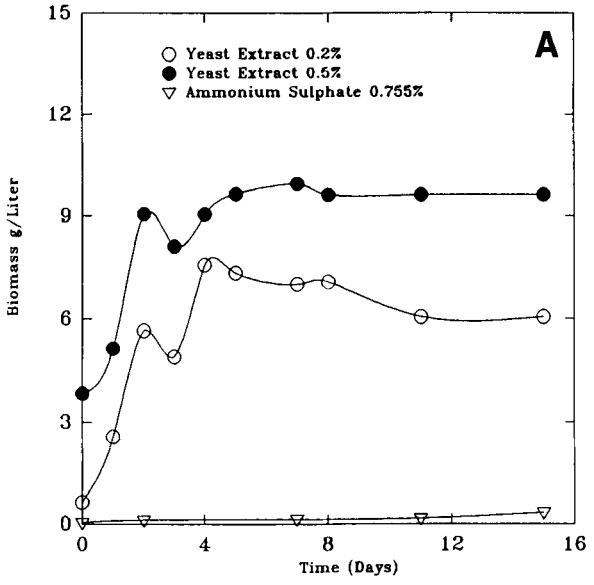

Fig. 3A. Effect of nitrogen sources on biomass production by *P. citrinum*. Media were composed of ammonium sulfate (0.755%) and different yeast extract concentrations: 0.2 and 0.5%.

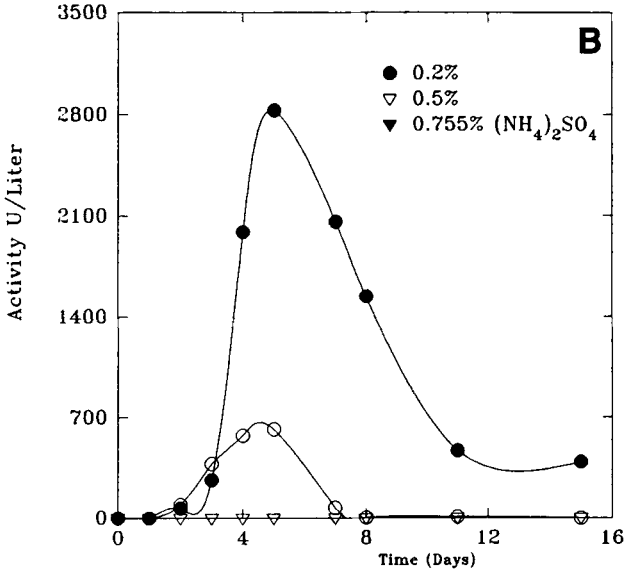

Fig. 3B. Effect of nitrogen sources on lipase production by *P. citrinum*. Media were composed of ammonium sulfate (0.755%) and different yeast extract concentrations: 0.2 and 0.5%.

Studies on protease production in olive oil medium (Figs. 4A and 4B) have shown that the production of this enzyme was proportional to olive oil concentration, reaching the maximum activity 3460 U/L at the same culture as the maximum for lipase activity (medium containing 1.0% olive oil). The protease production was higher in the medium containing 0.5%

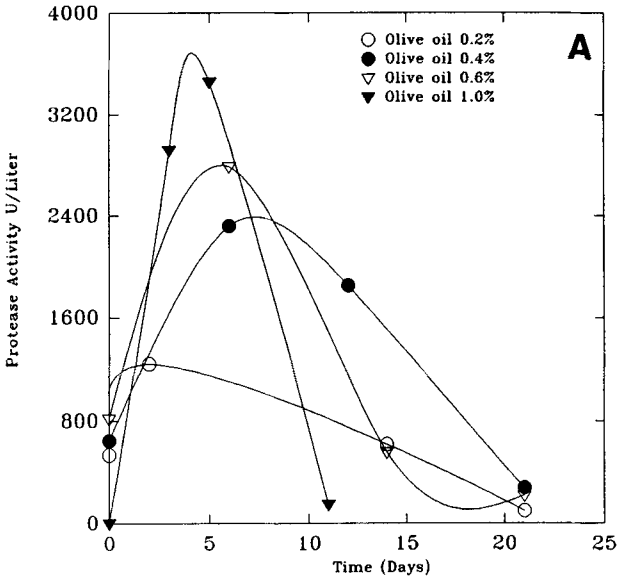

Fig. 4A. Effect of olive oil concentration on protease activity from *P. citrinum* grown on olive oil. Media were composed of 0.5% yeast extract and variable amounts of olive oil: 0.2, 0.4, 0.6, and 1.0%.

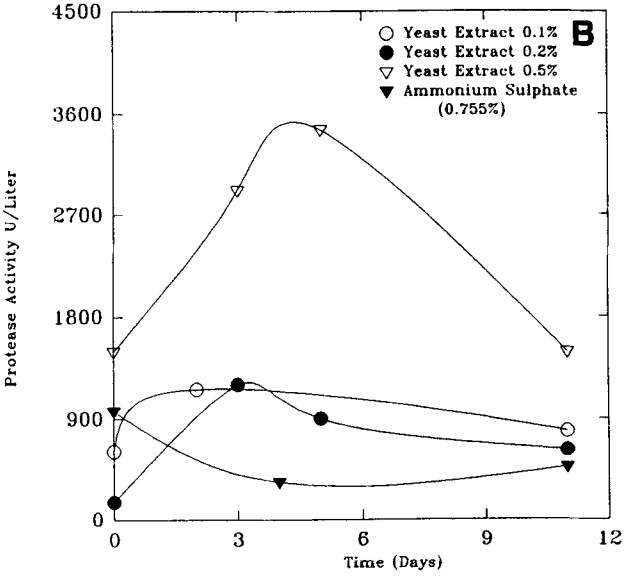

Fig. 4B. Effect of nitrogen sources on protease activity from *P. citrinum*. Media were composed of ammonium sulfate (0.755%) and different amounts of yeast extract: 0.1, 0.2, and 0.5%.

yeast extract than that obtained in low-yeast extract concentration. There was no protease production when the yeast extract was replaced by ammonium sulfate. Experiments with protease inhibitors (PMSF and EDTA) revealed that *P. citrinum* produces a serine protease. PMSF inhibited protease activity by 91.24%. No inhibition effect was observed by

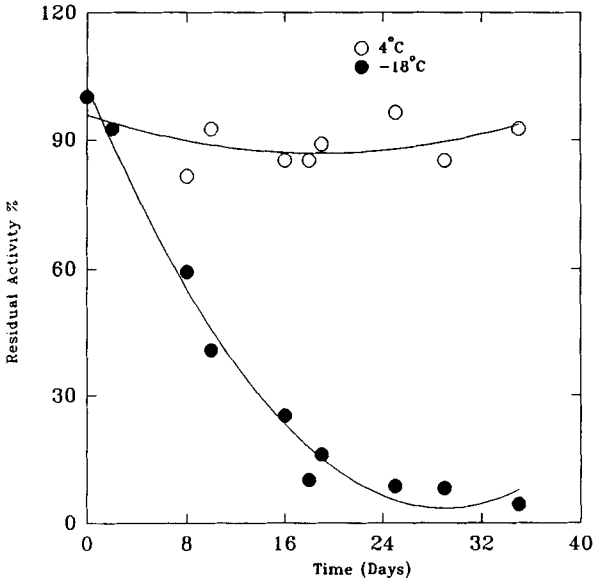

Fig. 5. Effect of temperature on lipase activity stored at 4 and −18°C for 35 d.

addition of EDTA to the crude extract. In spite of the presence of proteases in the culture supernatant, high lipase activity remained, even after 35 d of storage at 4°C, without adding any protease inhibitor (Fig. 5).

PHYSICOCHEMICAL CHARACTERISTICS OF LIPASES FROM *P. CITRINUM*

Concentration of the Crude Extract

Table 2 shows the results of previous experiments in concentration and/or partial purification. The addition of narrow band cuts of ammonium sulfate to the broth supernatant has shown that the highest specific activity was obtained in the 40–60% fraction. In this fraction, the enzyme was purified 11-fold. However, taking into account the best total activity recovery (71.2%) was obtained with a wide band cut (0–80%) of ammonium sulfate, and the fact that lipases could consist of a group of isoenzymes, which might be lost using selective cuts, the wide band cut as the condition for concentration of the crude extract was chosen. The dried concentrated fraction (after dialysis and lyophilization) maintained its lipase activity (at −20°C) (data not shown) and even at room temperature (28°C) for 8 mo (Fig. 6) with 95% of its initial activity. The excellent stability of this lipase suggests that *P. citrinum* may have application in detergents and other products that require a long-term storage stability of the enzyme at room temperature.

Table 2
Partial Purification of Crude Extract
from *Penicillium Citrinum* via Ammonium Sulfate Fractionation

Fractions	Specific activity, U/mgP	Purification factor	Activity recuperation, %
Crude extract	0.92	1.00	100.00
Narrow band cuts			
0–20	0.70	0.76	0.30
20–40	0.68	0.73	0.29
40–60	10.48	11.38	10.30
60–80	7.95	8.64	43.15
80–100	2.13	2.31	3.50
Wide-band cuts			
0–50	6.12	3.60	8.82
0–60	4.62	2.70	14.26
0–70	7.05	4.14	47.88
0–80	7.74	4.50	71.20
0–100	4.91	2.88	65.77

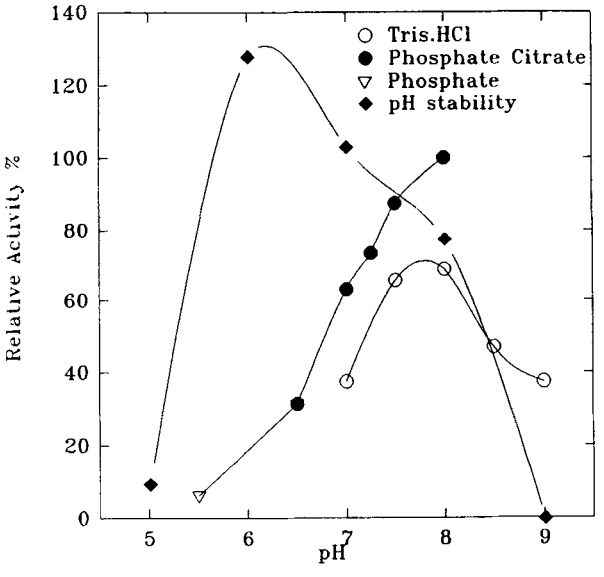

Fig. 6. Effect of pH on activity and stability (samples were incubated for 1h at 37°C at the specified pH value) of lipase using pNPP as substrate at 37°C with the following buffers: 5.0–7.0, 0.05*M* citrate-phosphate; pH 7.0–8.0, 0.05*M* phosphate, and pH 8.0–9.0, 0.05*M* Tris-HCl.

The Effect of pH on Activity and Stability of Lipase

The effect of pH on lipase activity is shown in Fig. 6. The optimum for lipase activity was at pH 8.0. This result is not in agreement with those found by Malizewska and Mastalerz (*6*) for another strain of *P. citrinum* (pH optimum 7.2). Most microbial lipases have their optimum activity in a range of pH 7.0–9.0. Figure 6 also shows the stability of lipase when incubated against various pH values. The enzyme was stable over a pH range 6.0–7.0. The best pH value to maintain enzyme activity was 6.0. At higher pH values, lipase activity decreases. At pH 8.0, 71% of the relative activity remained, whereas at pH 9.0, the enzyme was completely inactivated.

The Effect of Temperature on Activity and Stability

The optimum temperature for enzyme activity of lipases from *P. citrinum* was found in the range of 34–37°C. Because the relative activity of the enzyme was high even at 40°C (88%), for practical applications, a broad range of temperature can also be used (30–40°C). These results are also not in agreement with those of Malizewska and Mastalerz (*6*). They found the maximum lipase activity at 30°C.

The lipase from *P. citrinum* was not thermostable. Nevertheless, at temperature below 45°C, maximum lipase activity was maintained for at least 2 h. Results from shorter incubation times (30 and 60 min.) showed only minor differences in enzyme activity. The enzyme was completely inactivated at 60°C after 30 min of incubation.

Comparing the shape of the curves for optimum pH activity and that for pH stability, it can be seen that they are not coincident, as the curves obtained by Malizewska and Mastalerz (*6*). In their case, the enzyme was still active at 60°C, stable in the pH range 5.0–7.0, and at pH 9.0, 60% of activity remained. These differences could be justified by the methodology used in this work. The time and temperature of incubation used in our experiments were higher than that those used by the other investigators (1 h, 37°C in comparison to 10 min, 4°C).

The Influence of Substrate Concentration on Lipase Activity

Lipase activity is not governed by substrate concentration, but by the interfacial area after substrate available, as proposed by Sarda and Desnuelle (*19*) and Benzonana and Desnuelle (*20*). Figure 7 shows that using pNPP as substrate, the enzyme showed a Michaelis-Menten behavior, with an apparent K_m of 4.72×10^{-1} m*M* (SE 8.86×10^{-2}). Results obtained by the titrimetric method did not show this kind of behavior (data not shown). It was determined that at maximum substrate concentrations, for both methods, the specific activity was nearly the same (11.0 U/mgP for pNPP and 11.27 U/mgP for titrimetric method). This suggests

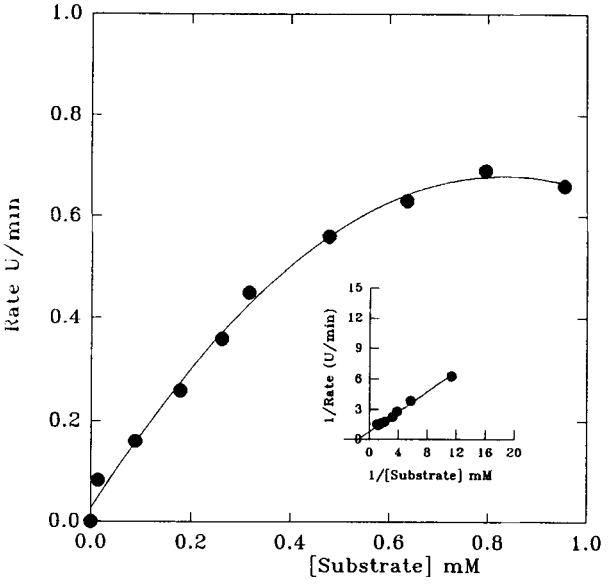

Fig. 7. Michaelis and Menten curve for lipase using pNPP as substrate.

that the pNPP method is as useful as the titrimetric method for investigating lipases from *P. citrinum*. The pNPP method has several advantages over the titrimetric method: it is more sensitive, more reliable, and faster.

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