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Lipase from a Brazilian Strain *Penicillium citrinum* Cultured in a Simple and Inexpensive Medium

Heat-Denaturation, Kinetics, and pH Stability

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ABSTRACT

This work is a study of lipase production by a Brazilian strain of *Penicillium citrinum* using an inexpensive and simple medium without organic nitrogen sources and of some important industrial properties, including thermostability in relation to ionic strength. The maximal lipase activity (1585 U/L) was obtained when *Penicillium citrinum* was cultured on 0.75% ammonium sulfate complemented with minerals salts instead of yeast extract. Although this activity was about 55% lower than that produced in medium with yeast extract (2850 U/L), the specific activity (7.8 U/mg proteins) was higher than that obtained with the yeast extract (4.9 U/mg proteins). The morphology of fungus changed totally, with yeast extract there are smooth, solid, and spherical pellets whereas on ammonium sulfate there are small "hairy" pellets uniformly suspended in the medium. The effect of ferrous (Fe^{++}) ions was carried out using medium MA with and without Fe^{++} ions. Lipase production by *Penicillium citrinum* in medium MA requires Fe^{++} ions, the absence of

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which caused a decreased of about 50% in the specific activity (3.5 U/mg proteins). The utilization of commercial, locally available oils as carbon sources, such as soybean oil (236 U/L) and corn oil (74 U/L) resulted in lower activity compared to olive oil, showing that lipase production by *Penicillium citrinum* is specifically induced by olive oil. Potassium concentration in the medium can effects the production of lipase (1 m*M* (1585 U/L), 10 m*M* (1290 U/L), and 30 m*M* (1238 U/L), 50 m*M* (195 U/L), and 100 m*M* (2 U/L). The crude culture filtered was susceptable to thermal deactivation. It was stable at pH 6.0, but was not stable at the optimum pH (8.0–8.5) at 50 m*M*. At the low ionic concentration (1–25 m*M*) this lipase was stable at low pH (3.5–4.0). The activation energy was 22.4 ± 2.2 Kcal. mol^1.

Index Entries: *Penicillium citrinum;* lipase; heat-denaturation; stabilitil-ity studies.

INTRODUCTION

Lipases are widely distributed in nature. They have considerable biotechnological potential for the general hydrolysis and synthesis of esters, with different specificities. In addition, lipases are active in both aqueous and nonaqeous solvents systems *(1)*. These enzymes are very important industrially and have been produced more abundantly from fungi than other sources. Fungal lipases have been used mainly as additives to washing detergents and in the food industry *(2)*. Several fungi have been studied as lipase sources in different culture media, which sometimes makes enzyme recovery difficult *(3–5)*. Industrial enzymes are, in most instances, commodity products and raw materials that generally account for 25–50% of the total cost of production *(6)*. On the other hand, several factors, such as medium composition and dissolved oxygen affect, pellet formation and consequently rheological properties and productivity *(7)*. A balance needs to be found between production conditions and enzyme yield. *Penicillium citrinum* has been studied as a new lipase source, using media containing starch, peptone, mineral salts, and rapeseed oil as inducer *(4)*, or using olive oil and yeast extract *(8)*. It has been reported that lipase production from *Penicillium citrinum* can be enhanced by olive oil, oleic acid, or Tween-80 and inhibited by lauric acid. It has also been described that addition of salts, such as, NaCl, KCl, $FeSO_4$, $FeCl_2$, $MgSO_4$, and $CaCl_2$ to the reaction mixture, at the beginning of incubation, can enhance or reduce the enzyme activity *(3)*. Published work suggests that there are multiple forms of commercial *Candida rugosa* lipase produced under different culture conditions. These purified enzymes display quite different physicochemical properties *(1)*. The present work is a study of lipase production by *Penicillium citrinum* using inexpensive and simple medium without organic nitrogen sources and of

some important industrial properties, including thermostability in relation to ionic strength.

MATERIALS AND METHODS

Microorganism

Brazilian strain of *Penicillium citrinum* isolated from olive oil according to Pimentel et al. *(8)*.

Growth Conditions

Penicillium citrinum was cultured in the following media: MY: 1.0% olive oil, 0.5% yeast extract, at an initial pH of 6.5. MA: 1.0% olive oil, 0.75% ammonium sulfate, 0.015% KH_2PO_4, 0.015% NaCl, 0.11% $MgSO_4$, 1.5 mg/L $ZnSO_4$, 0.1 mg/L $FeSO_4$ according to Cox and Thomas *(9)*, at an initial pH of 4.5.

Culture Conditions

Cultures were carried out in Erlenmeyer flasks (2 L, working capacity of 1 L) with 10% inoculum, incubated at 28°C and shaken (100 rpm). At times intervals, samples were withdrawn asceptically from the growth medium and then fitrated in order to get the sample without cells (used in the kinetics studies) as described in Pimentel et al. *(8)*.

Lipase Activity

Two methods were applied for determining lipase activity. The titrimetric one used purified olive oil in a Radiometer pH-stat, the system was as described in Pimentel et al. *(8)*. The second one was the spectrophotometric method based on the hydrolysis of *p*-nitrophenylpalmitate (pNPP) into *p*-nitrophenol (pNP) and palmitate. The pNP liberation at 37°C was detected at 410 nm. The molar extinction coefficient for pNP was $1.32 \times 10^4/M/\text{cm}$ *(10)*. One activity unit was defined as the cleavage of 1 μmol of pNPP per min at pH 8.0 (50 m*M* Tris-HCl buffer) and 37°C. One unit of activity by pNPP methods was equal to one activity unit by Colorimetric Method for determination of free fatty acids *(11)*. Protein concentration by Lowry Method *(12)* was measure using BSA as standard. Medium turbidity was determined at 650 nm. The purity of the crude enzyme was observed by SDS-polyacrylamide gel electrophoresis *(13)*.

pH Effect 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 3.5–9.0. The buffers used were sodium citrate (pH 3.5–6.0, 50 m*M*) and Tris-HCl (pH 7.0–9.0, 50 m*M*).

Temperature Effect on Lipase Activity

The optimum temperature was determined using the reaction mixture of pNPP method incubated over a range from 25 to 60°C, in 50 m*M* Tris-HCl buffer pH 8.0.

Heat Denaturation Conditions

Heat denaturation studies were carried out following methodology described by Ferrer et al. *(22)* for lignin peroxidase. Aliquots of the crude culture filtrate were incubated in a water bath at the desired temperature and periodically shaken, at the final culture pH (2.6). At the specific time, samples were removed from the incubated enzyme. After 15 min at the room temperature, lipase activity was determined by pNPP method at standard conditions.

pH Stability Conditions

Studies on pH stability were carried out following the methodology described by Ferrer et al. *(22)* for lignin peroxidase. Aliquots of crude culture filtrate were incubated in 50 m*M* (final concentration) buffer at specific pH in the proportion 1:1 v/v. Sodium citrate buffer to 3.5–6.0 and Tris-HCl to 7.0–9.0. At the specific time, samples were removed to measure lipase activity by pNPP method at standard conditions.

RESULTS AND DISCUSSION

The maximal lipase activity (1585 U/L) was obtained when *Penicillium citrinum* was cultured on 0.75% ammonium sulfate complemented with minerals salts instead of yeast extract. Although this activity was about 55% lower than that produced in medium with yeast extract (2850 U/L), the specific activity (7.8 U/mg proteins) was higher than that obtained with the yeast extract (4.9 U/mg proteins). Lipase produced using ammonium sulfate as nitrogen source is thus obtained at higher initial specific activity (Fig. 1). The purity of this preparation compared with that produced in the medium using yeast extract instead of ammonium sulfate was confirmed via electrophoresis. It showed only two bands in which the sample grown with yeast extract was observed on 5 bands.

The pH of the medium varied from 4.5 to 2.3, being equal to 2.4 at the maximal activity. Experiments adjusting the pH to 6.5 with NaOH or potassium phosphate buffer, did not show extracellular lipase activity, although the fungus grew well. This result suggested the need for low pH for a better production rate and for the release of the enzyme (Fig. 1). *Penicillium citrinum* lipases has been produced at pH 7.2 *(4)* and 7.5 *(8)*, using peptone or yeast extract as nitrogen sources.

The morphology of the fungus changed totally, with yeast extract there are smooth, solid, and spherical pellets, whereas on ammonium sulfate there are small "hairy" pellets uniformly suspended in the medium.

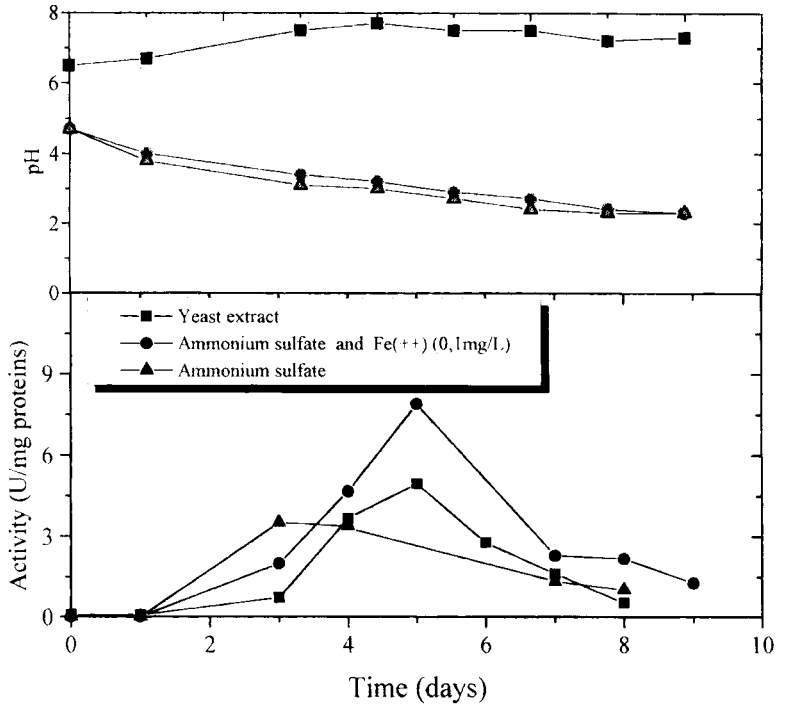

Fig. 1. Effect of yeast extract or ammonium sulfate (with and without iron) as nitrogen sources on lipase activity and pH during the growth of *P. citrinum*. The microorganism, was cultured on 0.75% ammonium sulfate complemented with minerals salts instead yeast extract with $FeSO_4$. Cultures were carried out in Erlenmeyer flasks with 10% inoculum, incubated at 28°C and shaken (100 rpm).

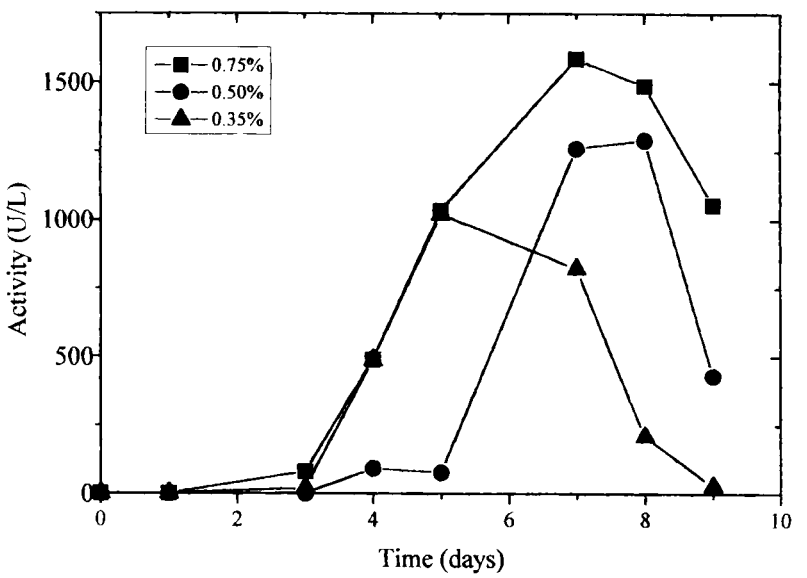

Fig. 2. Effect of ammonium sulfate concentration on lipase production by *P. citrinum*. Using medium **MA**. The *P. citrinum*, was cultured on 0.75, 0.50, 0.35% ammonium sulfate complemented with minerals salts instead yeast extract. Cultures were carried out in Erlenmeyer flasks with 10% inoculum, incubated at 28°C and shaken (100 rpm).

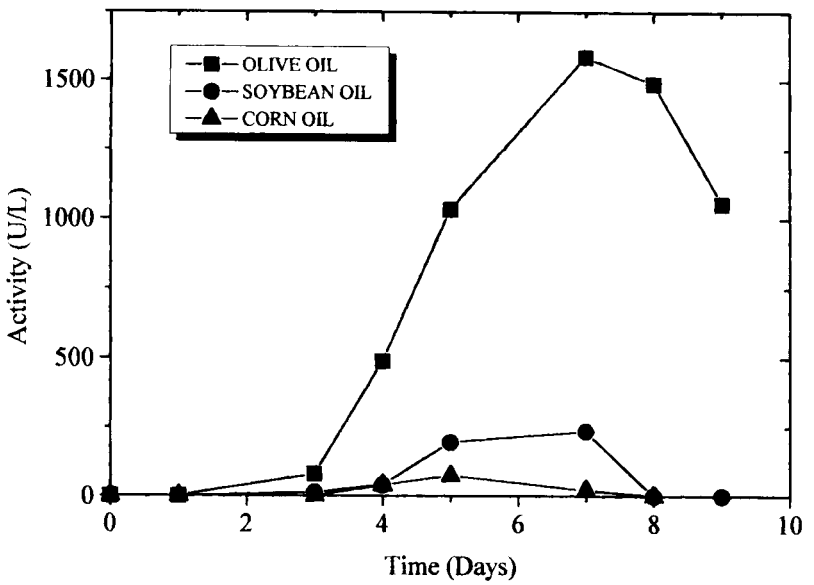

Fig. 3. Effect of oils as carbon source on lipase production by *P. citrinum*, using medium MA. The *P. citrinum*, was cultured on 1.0% of olive, soybean, and corn oil. Cultures were carried out in Erlenmeyer flasks with 10% inoculum, incubated at 28°C and shaken (100 rpm).

When the medium pH was adjusted with 1*M* NaOH solution at pH 6.5, the pellets presented the same characteristics when on yeast extract, but using buffer pH 6.5, there is no change. Reported work has suggested that pH is an important parameter for fungus morphology *(17)*.

Further decrease on lipase production was observed with a reduction on ammonium sulfate concentration using medium MA, from 1585U/L (0.75%) to 1290 U/L (0.5%) and 1020 U/L (0.35%) (Fig. 2). According to Pimentel et al. *(8)*, there was no activity when *Penicillium citrinum* was cultured on medium containing olive oil and ammonium sulfate without mineral salts. Nitrogen sources such as urea, inorganic nitrogen [$(NH_4)_2SO_4$ and $NaNO_3$] were not suitable for lipase production in *Aspergillus oryzae (14)*.

The effect of ferrous (Fe^{++}) ions was carried out using medium MA with and without Fe^{++} ions. Lipase production by *Penicillium citrinum* in medium MA requires Fe^{++} ions, the absence of which caused a decrease of about 50% in the specific activity (3.5 U/mg proteins). This result is not in agreement with that data reported for *Pseudomonas fluorenscens* and *Aspergillus niger* lipase, which was inhibited by the Fe^{++} ions *(15,16)*. However, according to Maliszewska and Mastalerz *(3)*, the addition of $FeSO_4$ to reaction mixture at the beginning of incubation, caused inhibition on lipase activity from *Penicillium citrinum*.

The utilization of commercial, locally available oils as carbon sources, such as soybean oil (236 U/L) and corn oil (74 U/L), resulted in lower activity compared to olive oil, showing that lipase production by *Penicillium citrinum* is specifically induced by olive oil (Fig. 3). It has been reported that

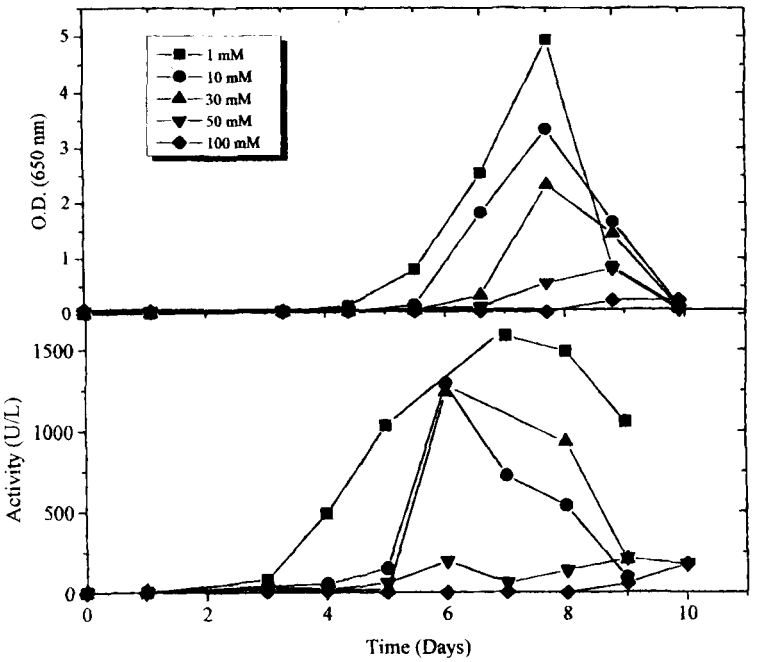

Fig. 4. Medium turbidity (650 nm) and lipase activity production by *P. citrinum* on different KH_2PO_4 concentration, using medium **MA** with 1, 10, 30, 50, or 100 m*M* of KH_2PO_4 at pH 4.5. Cultures were carried out in Erlenmeyer flasks with 10% inoculum, incubated at 28°C and shaken (100 rpm).

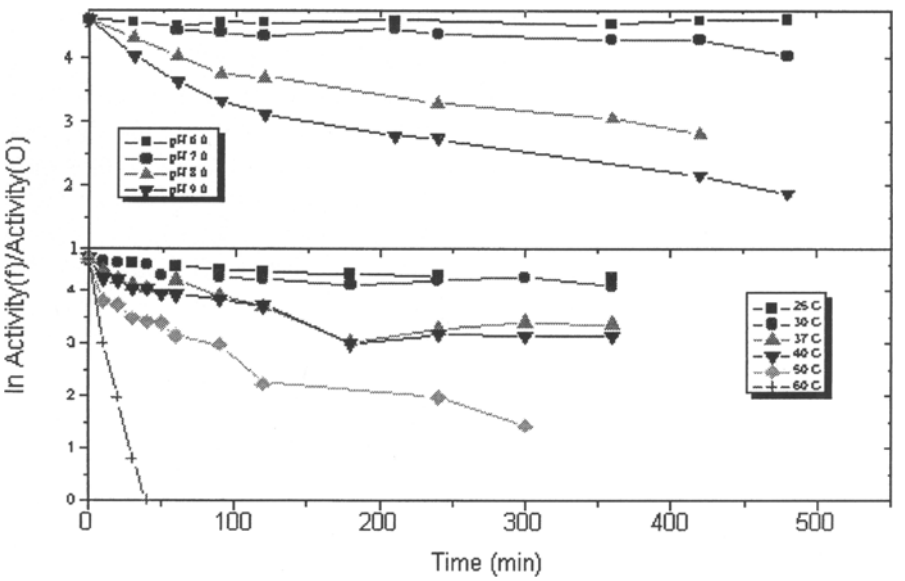

Fig. 5. Heat and denturation of *P. citrinum* lipase. Aliquots of the crude culture filtrate were incubated in a water bath at the desired temperature and periodically shaken or were incubated in 50 m*M* (final concentration) buffer at specific pH in the proportion 1:1 v/v. Sodium citrate buffer to 3.5–6.0 and Tris-Hcl to 7.0–9.0.

the rapeseed oil (high level of triolein) was the best inducer of *Penicillium citrinum* lipases production *(4)*. According to Maliszewska and Mastalerz *(3)*, olive oil, oleic acid, and Tween-80 enhanced lipase production whereas lauric acid caused inhibition. Chang et al. *(1)* showed that in *Candida rugosa*, Tween-80 and Tween-20 promoted a increase of lipase productivity of 2.9 and 5.7 fold, respectively *(1)*. Lipase production by *Aspergillus oryzae* was induced by soybean oil, triolein, and oleic acid, although this lipase was constitutive, since it can be produced without lipids *(14)*.

The Fig. 4 shows the effect of potassium concentration, using medium MA, on lipase production at pH 4.5. The maximal production was obtained on 1 m*M* (1585 U/L), 10 m*M* (1290 U/L) and 30 m*M* (1238 U/L) of potassium. There was little lipase production at the high potassium concentration 50 m*M* (195 U/L) and 100 m*M* (2 U/L). One should keep in mind that although these elements are essential for life, an excess will normally result in toxic side effects and, in some instances, cell death. In larger concentrations, mineral ions may compete for other coordination sites that normally bind a different metal, and so the biological activity of that site may be lost *(21)*. This can explain the lower lipase activity when microorganisms were grown in higher potassium concentration media. However, this higher potassium concentration can inhibit the cell's growth. But, there was no effect when potassium was added to the reaction mixture, at concentrations of up to 1*M* *(3)*. Furthermore, it has been reported that lipase production by *Aspergillus niger* was high with the addition of small amounts of KH_2PO_4, using NH_4NO_3 as nitrogen source. When no KH_2PO_4 was added, peptone gave better results than NH_4NO_3 for lipase yield *(16)*.

The medium turbidity was high at low potassium concentration (1, 10, and 30 m*M*) (Fig. 4). There was no detectable turbidity in the medium with yeast extract *(8)*. The maximal enzyme production was at high turbidity, suggesting that the by-product production can affect enzyme excretion to the medium.

The optimum pH for lipase activity was in a range of pH 8.0–8.5. The optimum pH for lipases from *Penicillium citrinum* on yeast extract is similar to the one from *Rhizopus delemar (8,18)*. Figure 5 shows the enzyme stability at different pH values. The lipase produced on ammonium sulfate was stable at pH 6.0 (50 m*M*), but it was not stable at the optimum pH 8.0–8.5 in the same concentration (50 m*M*). At pH below 6.0, the stability was not good, although the pH of culture medium at the maximal activity was 2.6, because it was observed that the ionic concentration of buffer was 50 times higher than in the medium (1 m*M*). The lipase produced on ammonium sulfate was unstable at ionic concentration above 25 m*M*. This can be observed in Table 1, where the first decay constant (K_{D1}) is related to pH values.

The optimum temperature for lipase activity from *Penicillium citrinum* was 37°C. Similar value was obtained for lipase produced on yeast extract *(8)*. According to Maliszewska and Mastalerz *(3)*, the optimum tempera-

Table 1
Decay Constant and Half-Lives for pH and Temperature of *P. citrinum* Lipase

pH	$Kd.min^{-1}$	$t_{1/2}$(min)
6	0.0016±0.000038	433.12
7	0.00079±0.000180	316.44
8	0.0096±0.000028	72.26
9	0.012±0.001300	56.80
Temperature (°C)		
25	0.00206±0.0002	336.40
30	0.0034±0.0002	203.82
37	0.017±0.0017	40.34
40	0.019±0.0009	35.76
50	0.038±0.0045	17.99
60	0.1141±0.0075	6.07

ture for *Penicillium citrinum* lipase was found at 30°C, the same value was found for enzyme from *Rhizopus delemar (18)*. The heat and pH denaturation kinetics is complex, according to Henley *(19)*, because of the presence of more than one phase in the denaturation curve. But it is an important parameter for enzymes, which can be used in large scale production, such as lipase. The first decay constant for lipase of *Penicillium citrinum* was obtained from the first linear part (K_{D1}) of the denaturation curves (Fig. 5). In Table 1 are shown these constant for temperature, where their values increased as temperature goes up, indicating that some structural change resulted from the exposure to different temperatures.

Half-lives were obtained from the first decay constant, ranging from 5.6 h at 25°C to 0.1 h at 60°C and 7 h with pH 6.0 to 1 h at pH 9,0 (Table 1). The relationship between decay constant and temperature shows that these results are not in agreement with those for lipase produced in yeast extract *(8)* in which the maximum activity was maintained for at least 2 h at temperature below 45°C, but are in agreement with the stability at 60°C. *Penicillium citrinum* lipase is stable compared to *Rhizopus delemar* lipase, which is reported as unstable above 30°C after 15 min *(18)*. Others lipases, such as from *Candida rugosa*, have been reported for different thermostabilities because of the presence of multiple forms of lipase caused by culture conditions *(1)*. It has also been reported that the kinetics of deactivation of lipase from *Candida rugosa* is also complex, especially because at lower temperature a biphasic model could be fitted, but a transition phase appered at higher temperature *(20)*.

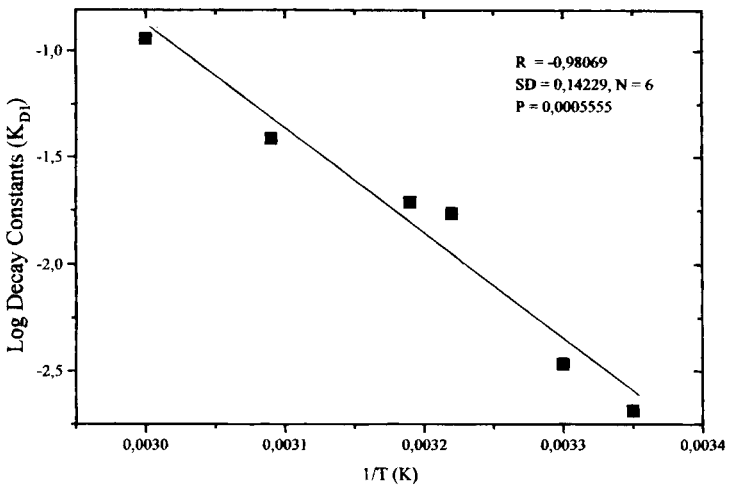

Fig. 6. Arrhenius plot of the first decay constant of heat denaturation for lipase from *P. citrinum*.

An Arrhenius plot of the first decay constant is shown in Fig. 6. Under the assay conditions, the activation energy of thermal deactivation was 22.4 ± 2.2 Kcal/mol.

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