PRODUCTION OF EXTRACELLULAR LIPASE BY THE PHYTOPATHOGENIC FUNGUS *FUSARIUM SOLANI* FS1

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ABSTRACT

A Brazilian strain of *Fusarium solani* was tested for extracellular lipase production in peptone-olive oil medium. The fungus produced 10,500 U.L^{-1} of lipase after 72 hours of cultivation at 25ºC in shake-flask at 120 rpm in a medium containing 3% (w/v) peptone plus 0.5% (v/v) olive oil. Glucose (1% w/v) was found to inhibit the inductive effect of olive oil. Peptone concentrations below 3% (w/v) resulted in a reduced lipase production while increased olive oil concentration (above 0.5%) did not further stimulate lipase production. The optimum lipase activity was achieved at pH 8.6 and 30ºC and a good enzyme stability (80% activity retention) was observed at pH ranging from 7.6 to 8.6, and the activity rapidly dropped at temperatures above 50ºC. Lipase activity was stimulated by the addition of n-hexane to the culture medium supernatants, in contrast to incubation with water-soluble solvents.

Key words: Enzyme kinetics, ester hydrolysis, extracellular lipase, *Fusarium solani*

INTRODUCTION

Lipases (triacylglycerol acylhydrolases E. C. 3.1.1.3) are enzymes that hydrolyse fatty acyl ester bonds of acylglycerols at the interface between oil and water (2). In organic medium, lipases are able to catalyse reactions of synthesis and transesterification of glycerides and phosphoglycerides, as well as a variety of non-glyceride, ester bonds (2,9,18,22). Lipases have been extensively studied because of their actual and potential applications in the detergent, oil and food industries. Recently, various strategies in the pharmaceutical and chemical industries have used lipases in the synthesis of optically pure drugs and agrochemicals that are more effective and produce fewer side effects compared with their racemates (11,22). Fungi are preferable lipase sources because fungal enzymes are usually excreted extracellularly, facilitating extraction from fermentation media. The selectivity of lipases has been exploited in industry in the synthesis of chiral compounds as well as in the resolution of racemic mixtures (19). The production of lipase by several *Fusarium oxysporum* strains has been studied in terms of enzyme production, protein properties and purification (7,8,11). The aim of this work was to describe the production of an extracellular lipase by a F*usarium solani* FS1 isolated from *Carica papaya* fruit.

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MATERIALS AND METHODS

Microorganism: *Fusarium solani* FS1 was obtained from the culture collection of the Plant Health Department of the Federal Rural University of Pernambuco. The cultures were maintained at 4ºC on potato dextrose agar plates.

Growth conditions: *Fusarium solani* FS1 was batch cultivated in a basal medium (BM) pH 6.0 consisting of (g.L^{-1} of deionized water): $MgSO_4$.7 H_2O, 0.5; KH_2PO_4, 1; $NaNO_3$, 3; peptone, 30. It was supplemented with different carbon sources (glucose, olive oil and peptone). Pre-inoculum consisted of 10^5 spores incubated in 10 ml of BM in a rotary shaker (120 rpm) for 2 days at 28°C. After this time, a 500 ml flask containing 90 ml of BM was inoculated with the pre-inoculum and cultivated as above. Aliquots of 10 ml of this culture were withdrawn at different periods of incubation and centrifuged. The supernatants were used to measure lipase activity, protease activity and pH. The pellet was washed in water, filtered and dried in pre-weighed filters for biomass determination. Peptone and oil concentrations varied as shown in results. The results were calculated from three independent cultures. Each measurement was carried out in triplicate.

Enzyme assay: Lipase activities were assayed in the enzyme-containing supernatants with p-nitrophenyl palmitate (pNPP) as substrate determined spectrophotometrically at 410nm (23). One lipase unit (U) is defined as the amount of enzyme that liberated 1 µmol p-nitrophenol per minute. Protease in the supernatant was measured according to Ginther (5) using azocasein as substrate. One unit of protease activity is defined as the amount of enzyme that produces na increase in absorbance of 1.0 in 1h at 440 nm.

Effect of pH and temperature toward pNPP: The pH values of the reaction mixtures were varied from 5.4 to 7.8 (in 50 mM tris-maleate) and 8.0 to 8.8 (in 50 mM tris-HCl) and lipase activity was measured at 25ºC. To determine the pH stability, lipase-containing supernatant was diluted 1:1 in 100 mM of the buffers described above and incubated for one hour at 25ºC before assayed. Lipase activities were assayed in reaction mixtures of pNPP containing tris-HCl pH 8.0 at a range of temperatures from 15°C to 60°C to determine optimum lipase activity. For thermal stability, lipase-containing supernatants were pre-incubated for 60 minutes over a range of temperature from 25°C to 50°C in tris-HCl pH 8.0 buffer. After this time, the mixtures were left for 10 minutes at room temperature and lipase activity were measured using the standard procedure.

Effect of organic solvents: The supernatant was diluted with organic solvents in the concentrations of 0 to 50% (v/v). After 1 hour of incubation with constant shaking at 25ºC, lipase activity was measured in the aqueous phase as described above.

RESULTS AND DISCUSSION

Effect of the medium composition on lipase activity

The activity of extracellular lipase of *F. solani* FS1 cultures was evaluated after 96 hours of incubation in medium containing 3% (w/v) peptone alone and supplemented with different carbon sources (Table 1). Highest lipase activity of 9,500 U.L^{-1} was observed in the medium containing 1% olive oil. This represents an 8-fold lipase induction

Table 1. Effect of the carbon source on production of lipase activity by FS1 after 96h cultivation.

| Additional carbon source [1% (w/v)]	Maximum lipase activity (U.L^{-1})	Maximum mycelial dry weight (g.L^{-1})	Maximum lipase activity/maximum mycelial dry weight	Lipase Induction factor [a]
			Specific lipase activity (U.g^{-1})	
_	1240	6.2	200	1
Glucose	3800	6.7	567	3
Olive oil	9500	15.3	620	7.7
Gluc. + olive oil	4000	10.7	374	3.2

[a] Induction rate: ratio of specific activities with and without additional carbon source.

in relation to the basal medium (BM). The presence of 1% glucose, although stimulating a 3-fold lipase activity in relation to BM, abolished the enzyme induction produced by 1% olive oil. The results presented in Table 1 indicate a higher biomass production in 1% olive oil medium (15.3 g.L^{-1}) in relation to glucose or glucose/olive oil medium (6.7 and 10.7 g.L^{-1}, respectively). Our data shows similar lipase activities (in terms of Units per mg biomass) for both glucose and olive oil supplements. However, the presence of both supplements depresses lipase induction. Rapp (14) showed this effect with *Fusarium oxysporum* when mixtures of glucose and olive oil (both at 1%) resulted in a peak of biomass at approx 100h while lipase peaked at 220h. The effect of different concentrations of olive oil and peptone on lipase production was evaluated after 72 hours of cultivation (Fig. 1). At a peptone concentration of 3% (w/v), the highest lipase activity of 10,500 U.L^{-1} was obtained in the media containing 0.5% (v/v) olive oil. Lower (0.25%) and higher (1%) oil concentrations decreased lipase production to 8,300 U.L^{-1} and 7,800 U.L^{-1}, respectively (Fig. 1A). Decreases in lipase production with increases in inducer concentrations have been noted (11, 3) for *Aspergillus oryzae (*soyabean oil) and *Candida rugosa* (olive oil). However, others have reported (17, 13) increased lipase production with increasing inducer concentrations for *Penicillium citrinum* (olive oil) and *Candida rugosa* (oleic acid). Pokorny *et al.* (12) showed that the addition of olive oil in concentrations between 0.5% and 2% increased the lipase production by *Aspergillus niger* to a maximum at 72h. The addition of olive oil to the basal medium at a concentration of 0.2% increased lipase activity by *Torulopsis ernobii*, but amounts of olive oil greater than 0.8 % gave smaller increases (24). These authors suggest that the role of lipids in augmenting lipase production appears to be related to growth. These observation reinforce the constitutive and the biomass-linked lipase production by FS1 strain. Higher oil concentrations could be affecting the aeration rate of the culture and promoting a delay in mycelial growth and lipase production.

Further evidences for that biomass-linked lipase production are showed in the Fig. 1B, where a decrease in peptone concentration to half (1.5% w/v) promoted 2.5-times decrease in lipase activity after 72 hours of cultivation in BM containing 0.5% olive oil. Peptone concentration of 0.5% (w/v) almost abolished lipase activity (Fig. 1B). This peptone-dependence was demonstrated for the lipase production by *F. oxysporum* f. sp. *vasinfectum* (14).

Batch fermentations were carried out in basal medium supplemented with 0.5% (v/v) olive oil and 3% (w/v) peptone for lipase production by FS1 strain (Fig. 2). A low lipase activity was observed until 48 hours of cultivation, at the moment that the culture entered into stationary phase of growth. However, it increased exponentially up to 96h to a value of 9,500 U.L^{-1}. The production of lipase is accompanied by an increase in the supernatant pH up to value of 8.3. Such pH increase was also observed during lipase production by *Aspergillus oryzae* (11). In spite of the media containing peptone, the FS1 strain produced low quantity of protease during the period of fermentation, that reached maximal activity at 72 hours of cultivation (500 U.L^{-1}) and decreased thereafter (Fig. 2). Low protease activity should facilitate downstream process for lipase recovering (6).
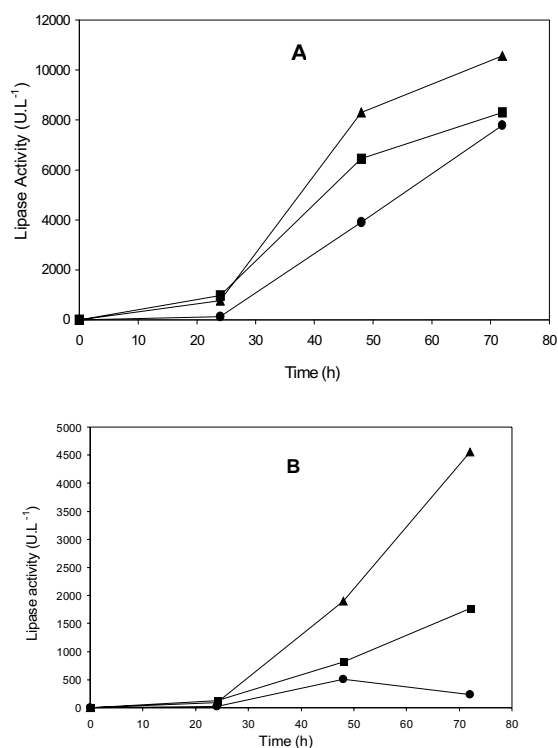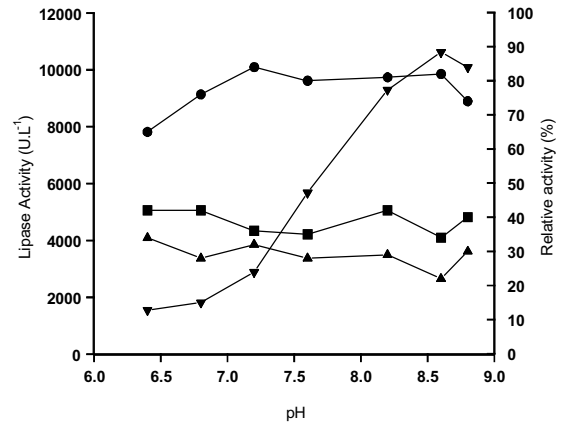

Figure 1. Effect of the olive oil and peptone concentration on lipase production by FS1 strain.**(A)** Medium containing 3% (w/v) peptone was supplemented with olive oil at concentrations (v/v) of 0.25% (■), 0.5% (▲) and 1% (●); **(B)** Medium containing 0.5% (v/v) olive oil was prepared with peptone at concentrations (w/v) of 0.5% (●), 1.5% (■) and 3% (●). Values are means of three experiments.
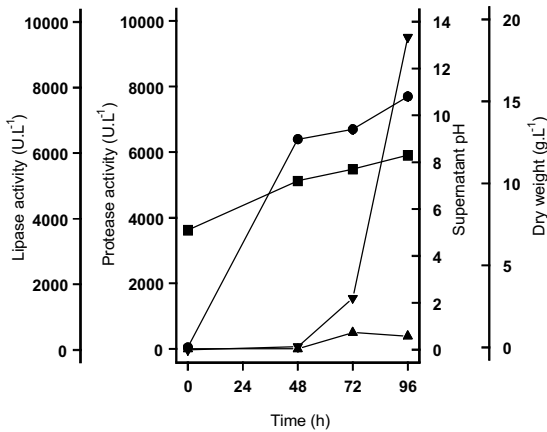
Figure 2. Batch fermentation of the FS1 strain in the medium containing 3% (w/v) peptone and 0.5% (v/v) olive oil (120 rpm at 28ºC). Values of lipase activity, (▼) mycelial dry weight (●), supernatant pH (■) and protease activity (▲) were taken in different period of cultivation. Values are means of three experiments.
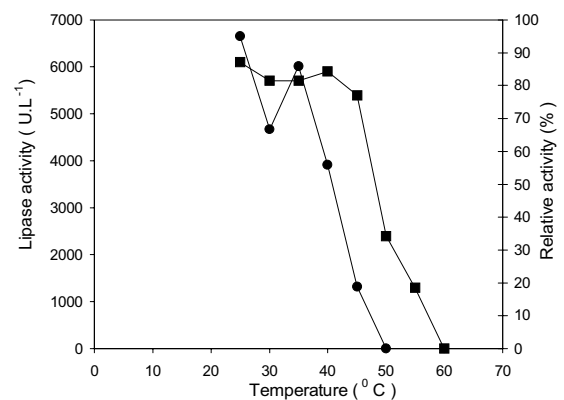

Figure 3. Effect of the pH on FS1 lipase activity and stability. Lipase activity was measured in reaction mixtures prepared with buffers at different pH values (▼) after previous incubation of the enzyme for one (●), two (■) and four (▲) hours (relative activity)at 25ºC in the same buffers. Buffers were 50 mM tris-maleate (pH 5.4 to 7.8) and 50 mM tris-HCl (pH 8.0 to 8.8).

Effect of the pH and temperature on the lipase activity of *Fusarium solani* FS1 and stability

The lipase activity was evaluated at different pH values at 25°C using pNPP as substrate (Fig. 3). The optimum activity for pNPP hydrolysis of 10,620 U.L^{-1} was reached at pH 8.6 in 50 mM tris-HCl buffer and pH values below 8.0 decreased lipase activity. Extracellular lipase of *F. oxysporum* f. sp. *line* and *F. oxysporum* f. sp. *vasinfectum* showed optimum activity at pH 7.0 (in 25 mM Tris-HCl buffer, 37ºC) and at pH 5.8 (in 0.06 M sodium phosphate, 35ºC), respectively (8,14). Lipase mantained around 80% its initial activity when incubated for one hour at pH values between 7.2 and 8.6, with an decrease in lipase activity at pH beyond this range. The enzyme was more stable at pH 7.2 in tris-maleate buffer, despite its optimum activity at pH8.6 in tris-HCl buffer (Fig. 3). The incubations of the enzyme for 2h and 4h at 25^{0}C in the pH range of 6.4 to 8.8 decreased lipase activity for the values around 40% and 30%, respectively (Fig. 3). No lipase activity was observed after 24h incubation at any pH value tested. This decrease in enzyme stability should be due to some denaturation effect, as well as to the presence of protease in the samples. Extracellular lipase of *F. oxysporum* f. sp. *vasinfectum* showed stability over the pH range of 4.0 to 10 during 1h incubation at 30ºC (14). On the other hand, others strains of *F. oxysporum* showed stability only at alkaline pH (15; 8).

Lipase presented two peaks of optimum temperature at 25°C and 45°C (Fig. 4). This behaviour of lipase activity from *Fusarium solani* FS1 may be indicative of the presence of isoenzymes. A considerable decrease in the lipolytic activity was observed for highest temperatures, reaching zero activity at 60ºC. Maximal stability of the enzyme was observed in the temperature range of 25ºC to 30ºC after its thermal exposure for 1h (Fig. 4). Incubations above 40°C promoted inactivation of this enzyme. The lipase produced by *F. oxysporum* f. sp. *line* and *F. oxysporum* f. sp. *vasinfectum* showed optimum activity at 42ºC and 45ºC, respectively


Figure 4. Effect of the temperature on FS1 lipase activity and thermal stability. Lipase activity was measured in 50 mM tris-HCl pH 8.0 over a range of temperature from 25ºC to 60ºC (■) and after previous incubation of the supernatant mixtures for one hour in the temperatures indicated (●).

(8,14). Moreover, the lipases produced by both of the strains above were more thermostable than the FS1 lipase. The optimun temperatures were 25°C for *A. oryzae* (16), 37°C for *C. cylindrace*a (16), and 40°C for *Geotrichum candidum* (21).

Stability of the FS1 lipase in organic solvents

Experiments presented in Fig. 5 demonstrate that 1-pentanol completely abolished lipase activity at 10% (v/v), while acetone and n-propanol maintained 30% of activity under this condition. At 20% (v/v), the last two solvents completely inhibited lipase activity. In contrast, n-hexane increased lipase activity to 170% of its initial activity at 40% (v/v) solvent concentration (Fig. 5). A similar result was demonstrated when *F. Oxysporum* f. sp. *vasinfectum* lipase was incubated with n-hexane (14). On the other hand, lipase from *Bacillus sp* was inhibited in the presence of n-hexane (20). This effect has been discussed by Rapp (14) as an increase of the reaction mixture emulsification by n-hexane, an water-immiscible solvent, promoting the best contact between enzyme and substrate. The activity of lipase depends on the quality of an oil-water interface (4)
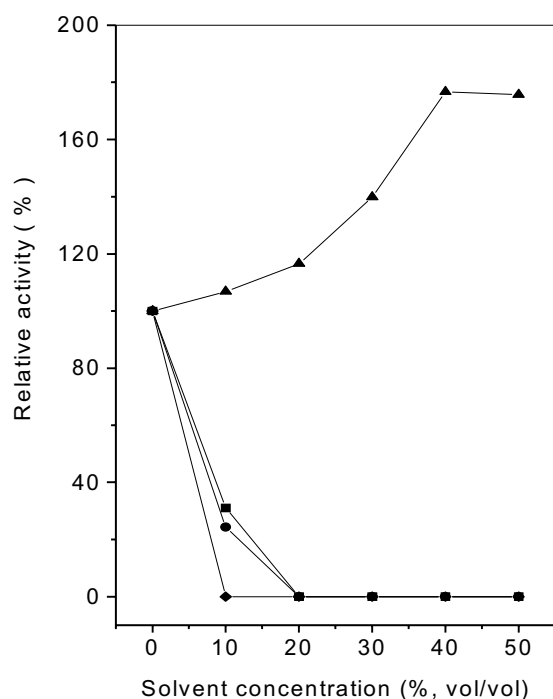
and water-miscible organic solvents generally cause protein denaturation (1). In studies on the use of lipase for transesterification and synthesis of esters, the reactions have previously been carried out in media containing water-immiscible organic solvents and a small amount water, due to the fact that enzymes are less susceptible to denaturation in such systems (20). Since lipase from *Fusarium solani* FS1 has been shown to be stimulated by the addition of n-hexane, the enzyme might be useful for transesterification and ester synthesis. Experiments in flavour synthesis using FS1 lipase has been initiated in our laboratory with very promising results.

RESUMO

Produção de lipase extracelular pelo fungo fitopatogênico *Fusarium solani* FS1

Uma estirpe brasileira de *Fusarium solani* foi testada para produção de lipase em meio contendo peptona e óleo de oliva. O fungo produziu 10.500U.L^{-1} de lipase após 72h de incubação a 25°C e 120 rpm em meio contendo 3% (p/v) de peptona e 0,5% (v/v) de óleo de oliva. A glicose (1% w/v) parece inibir o efeito estimulador do óleo de oliva. Concentrações de peptona abaixo de 3% (w/v) reduziram a produção de lipase enquanto que o aumento na concentração de óleo de oliva acima de 0,5% (v/v) não estimulou a produção da enzima. Ótima atividade lipásica foi alcançada em pH 8,6 e 30°C e uma boa estabilidade enzimática foi observada em pH variando de 7,6 a 8,6, mas a atividade caiu rapidamente à temperaturas acima de 50°C. A atividade lipásica foi estimulada pela adição de n-hexano à mistura de incubação, em contraste com a incubação em solventes orgânicos solúveis em água.

Palavras-chave: cinética enzimática, hidrólise de ésteres, lipase extracelular, *Fusarium solani*, pNPP.

Figure 5. Effect of the organic solvent on activity of the FS1 lipase. The enzyme was incubated for one hour at 25°C with constant with different concentrations of acetone (■), n-propanol (●), n-hexane (▲),1-pentanol (♦).

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