# Production of lipase by *Beauveria bassiana* on broth supplemented with oily residue

## Produção de lipases por *Beauveria bassiana* em meio suplementado com resíduos oleosos

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**ABSTRACT**

The entomopathogenic fungus *Beauveria bassiana* CG432 produced an extracellular lipase on the 7th day of fermentation in Alves broth containing 0.25% Triton X-100 and Tween 80 surfactants, and 3.0% olive oil at 28°C and 200rpm. Optimal temperature and pH conditions for lipase activity were found to be range from 40 to 60°C and pH 7.5 and 8.0 using 50 mM Tris-HCl buffer, verified by a hydrolysis *p*-nitrophenol palmitate assay. Under these conditions, lipase was 50% stable for approximately 10h, however it was stable for up to 10 days (-18°C), refrigerated (4°C) or kept at room temperature (25°C).

**Keywords:** *Beauveria bassiana;* lipase production, vegetable oil, oily residue, deodorizing

**RESUMO**

O fungo entomopatogênico *Beauveria bassiana* CG432 produziu uma lipase extracelular no sétimo dia de cultivo em meio líquido de Alves contendo 0,25% dos surfactantes Triton X-100 e Tween 80 e 3,0% de óleo de oliva e 200 rpm. A temperatura e o pH ótimos de atividade na faixa de temperatura na faixa de 40 a 60ºC, e pH entre 7,5 e 8,0 em tampão TRIS-HCl 5mM utilizando a reação de hidrólise do substrato palmitato de p- nitrofenol. Sob tais condições, a lipase permaneceu ativa em 50% de sua atividade total durante 10 horas, e foi estável por mais de 10 dias quando armazenada sob congelamento (-18C, refrigeração (4ºC) e a 25C.

**Palavras-chave:** *Beauveria bassiana;* produção de lipase, óleos vegetais, resíduos oleosos, desodorização

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## INTRODUCTION

Lipases (EC.3.1.1.3, triacilglicerol acilhidrolases) have been used to catalyze the hydrolysis of triacylglycerides to glycerol, mono, diacylglicerides and free fatty acids. Thus, they have been used as an important industrial biotechnological agent (Sharma et al., 2001), like in treatment of wastewater with high oil and grease content (Cammarota and Freire, 2006) or to degumming refine of vegetable oils (Yang et al., 2006). Microbial enzymes are more useful than enzymes derived from plants or animals because of the great variety of catalytic activities available, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media (Hasan et al, 2006).

Lipases have been produced by submerged and state solid fermentation using many vegetable oils (Jones and Porter, 1998), using oil-related substrates (Wang et al., 2008) like neem oil (Gulati, 2005) and residue from the babassu oil industry (Gutarra et al., 2007). Research on newer substrate and producer microorganisms is very important to development of lipase applications.

Crude vegetable oils are constitute of triglycerides 95% and other lipids classes as phospholipids, free fatty acids, steroids, tocopherols, carotenóides, hydrocarbons and waxes. The last industrial refine stage removes some volatile compounds of oils by deodorization using steam stripping at 2 to 8 mmHg pressure, at temperature from 220 to 250$^{o}$C to obtain desirable sensorial characteristics of flavor for use of the refined oils on food (O'Brien, 1998). However, each of 10 liters of deodorized oil is collected 1 liter of an oily residue (OR) and it is constitute of high free fatty acids, triacylglycerol, remain phospholipids of degumming crude oil and tocopherols (SARIKAYA, 1994; HOFFMAN, 1989). ORs have been used as raw material to tocopherols extration, but (HUI, 1996).

Entomopathogenic fungus *B. bassiana* produced high lipases concentration on supplemented media with vegetable oils (HEGEDUS AND KHACHATOURIANS, 1988). Proteases, chitinases and lipases are produced by these fungi have been focus on biological control of plagues, since hydrolysis of exoskeletum during the fungal infection process (ALVES, 1998, TANADA, 1993)

The objective of this work was to produce and to investigate some activity properties of extracellular lipases produced by *B. bassiana* CG432 on Alves broth supplemented with oily residue from agroindustrial edible vegetable oils refine process.

## MATERIAL AND METHODS

### Fungal and inoculum ativation

*Beauveria bassiana* strain CG432 isolated from dead insects (Hemiptera: Membracidae) in Rio Grande do Norte State, Brazil was maintained on potato dextrose agar (PDA) and subcultured during 10 days on a complex solid medium optimized by Alves (1998) containing % (w/v): 1.0g anydrous glucose, 0.5g yeast extract, 0.158g $NaNO_3$, 0.105g $Na_2HPO_4.7H_2Og$, 0.1 KCl, 0.06g $MgSO_4.7H_2O$, 0.036g $KH_2PO_4$, and 2g agar. Suspensions of fresh conidia (water and 0.01% Triton X-100) were used to obtain $10^6$ conidia $ml^{-1}$on all cultivations.

### Grown conditions on vegetable oils and oily residues (OR)

Submerged cultivations were performed in 25mL (Erlemeyer flaks 250ml) of the liquid medium described above, but without agar (Alves broth). Triplicate of cultivations were supplemented with 3% olive, canola, cottonseed, soybean or sunflower refined oils; crude cottonseed oil; and oily residues (OR) from soybean or canola refinery. The ORs were pretreated with 10% of silica until neutral pH. Broths were autoclavated (1 atm, 121C during 15 min) and after cooling they were added of 0.1% $CaCl_2$ and 0.25%

Triton X-100 or Tween 80 surfactants autoclaved separately. All cultures were agitated vigorously and incubated at 28°C and 200rpm (shaker). Triplicate of controls without surfactants and sterile controls (without inoculum) were performed parallel. At five day, the cultivations were interrupted by filtration through paper filter Whatman No.1 and centrifugation at 8000x*g* for 20 min, both at 4°C. The supernatants or lipase extracts (LE) were dialyzed against 5mM phosphate buffer at pH 7 using 12kDa cut off membranes (Sigma) and assayed for lipase activity. The biomass collected on paper filter was washed three times using 100ml of distillated water and dried at 80°C until a constant weight by gravimetric method.

**Grown curve on OR from canola**

The growth curve of *B. bassiana* CG432 and time course of lipase production were obtained by cultivation like described above but using only OR from canola and Triton X-100 during eight days. At each 24 hours a triplicate cultivations were interrupted and the LEs were assayed for lipase activity, pH and analytical determinations.

The biomass collected on paper filter was washed three times using 100ml of distillated water and dried at 80°C until a constant weight by gravimetric method.

**Lipase assay**

The substrate *p*-nitrophenyl palmitate (*p*-NPP, Sigma) was used for most of the lipase activity determination LIMA et al., 2004), but with a 100mM TRIS-HCl pH 8.5 buffer containing 0,4% Triton X-100 at 50°C for 2 min. Lipase activity was confirmed by the titrimetric method (WINKLER; STUCKMANN, 1979) only on stability tests. One Unit of lipase activity was 1µM pNP $ml^{-1}$ $min.^{-1}$ and 1µmol free fatty acid $ml^{-1}$ $12h^{-1}$ liberated, respectively.

**Analytical determinations**

Protein, reducing sugar and lipids contents were determined by Bradford (1976), Miller (1959) and Frings and Dunn (1980) methods, respectively.

**Optimum temperature and pH determination of lipase**

LE obtained on the $7^{th}$ day of cultivation supplemented with 3% OR from canola oil and 0.25% Triton X-100 were six time concentrated and diafiltered by ultrafiltration (Stirred cell Sigma) using polyethersulfone cut off 100kDa membrane (Millipore), since lipases can form molecular mass aggregates greater than 80kDa on salt solutions (CASTRO-OCHOA, 1985). This was called ultra filtrate lipase fraction (UFLE) and was assayed for the hydrolysis of *p*-NPP from 20 to 60°C using the following pH buffers: sodium acetate (3.5 to 5.5), sodium phosphate (6.0 to 7.5), TRIS-HCl (8.0 to 9.0) and sodium phosphate NaOH (9.5 to 12.0) at 55°C.

**Lipase stability in storage and at optimal activity conditions**

UFLE as described above was assayed by the *p*-NPP emulsion hydrolysis method after having been stored at room temperature (25±5°C), under refrigeration (5±2°C) and frozen (-18°C) for 10 days. When preincubated at optimal temperature and pH conditions (55°C and TRIS-HCl 50mM buffer pH 8.0), it was assayed by *p*-NPP emulsion hydrolysis and titrimetric methods for 6 hours.

**Effect of NaCl concentration on lipase activity**

UFLE as described above was assayed by the *p*-NPP emulsion hydrolysis method at 55°C and pH 8.0 using TRIS-HCl buffer containing 0.1, 0.2, 0.4, 1.0 and 2M of NaCl.

## RESULTS AND DISCUSSION

### Lipase production on oils and oily residues (OR)

Lipase production by *B. bassiana* CG432 was greater on fermentations supplemented with refined olive and canola oils than others oils (Table 1). These results can be related with the specific action of lipases on triglycerides formed of free fatty acids with long chain and one insatured like oleic acid (C18:1) that constitute around 80.3 and 60.9 % of olive and canola oil, and only 18.6, 23.3 and 18.7% of cottonseed, soybean and sunflower, respectively (O´BRIEN, 1998). Hegedus and Khachatourians (1988) obtained higher lípase production by *B. bassiana* cultured on media containing oleic acid than the saturated free fatty acids palmitic and estearic acids.

OR from canola pretreatment with neutral alumina showed to be a good substrate to production of lipases by *B bassiana*, since the lipase activity was 168.93 U $ml^{-1}$, or 72.63% of the greatest activity produced on olive oil (Table 1). This result sustains the discussion about the positive effect of the unsaturated free fatty acids on lipase production, because this OR is a concentrate of free fatty acids stripped during the deodorizing of from canola oil (O´BRIEN, 1998). These results differ of some works where any free fatty acids were inhibitor factors, but agree with others that obtained stimulation effects of those with at least one unsaturation on the long chain carbon (18:0) to produce lipases. A despite of lipases require no cofactors, $Ca^{2+}$ ions help an important role because they form $Ca^{2+}$-fatty acids complex to avoid change on interfacial pH (BROCKERHOFF; JENSEN, 1974).

The use of surfactant Triton X-100 increased lipase production when compared with fermentations added of Tween 80 or without surfactants, but not increased the biomass production on fermentations supplemented with crude oils or ORs (Table 1). Silva et al. (2005) had demonstrated the greatest lipase activities when SDS and Tween 80 were added after 50h of the inoculation with *M. anisopliae,* probably related with increase permeability of cell membranes and consecutive greater secretion of lipase and to avoid toxics effects on the germination or growth of fungus.

Table 1 Production of lipases by *Beauveria bassiana* CG432 in Alves broth, 0.1% $CaCl_2$, 0.25% surfactants and supplemented with 3% vegetable oils and oily residues pretreated with neutral alumina (OR).

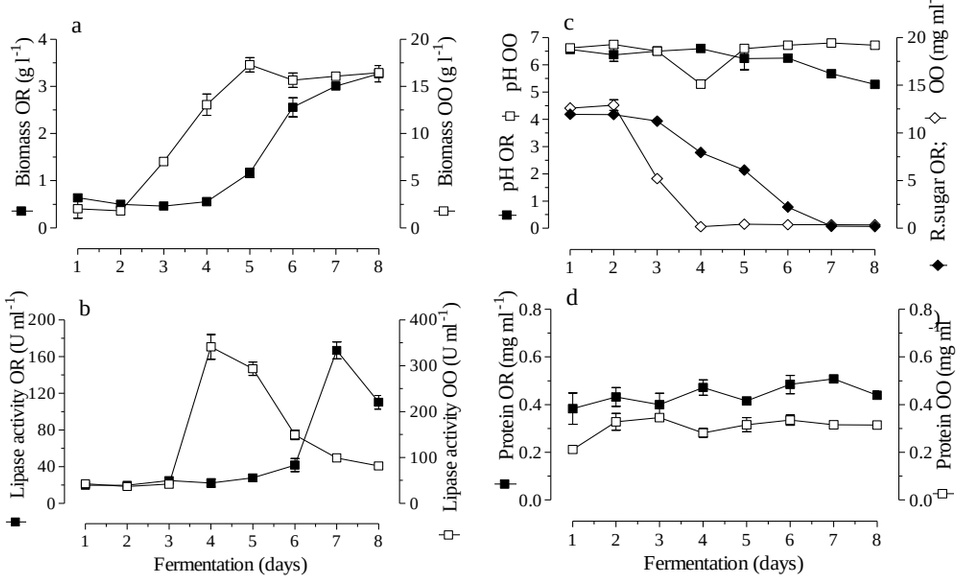
| Vogel supplementation | Lipase activity[a] (U $ml^{-1}$)[b] | | | Biomass (g $25ml^{-1}$) | |
|---|---|---|---|---|---|
| | Without surfactant | Triton X-100 | Tween 80 | Triton X-100 | Tween 80 |
| Olive oil | 7.52 | 229.11 | 84.44 | 0.423 | 0.180 |
| Canola oil | 9.86 | 232.60 | 75.30 | 0.920 | 0.236 |
| Cottonseed oil | 5.60 | 130.94 | 52.40 | 0.645 | 0.247 |
| Soybean oil | 3.16 | 120.45 | ND | 1.067 | 0.323 |
| Sunflower oil | 0.17 | 81.33 | 38.70 | 0.687 | 0.199 |
| Crude cottonseed oil | ND[c] | 14.02 | ND | 0.170 | 0.170 |
| OR from canola oil | 3.66 | 168.93 | 27.03 | 0.195 | 0.180 |
| OR from soybeam oil | 0.62 | 18.16 | ND | 0.130 | 0.124 |

[a]Means of triplicate of cultivations and [b]assay lipase by hydrolysis *p*-NPP method U $ml^{-1}$ was defined as µg *p*-nitrophenol; All cultures were inoculated with $10^6$conidia $ml^{-1}$ and performed at 28°C, at 200rpm during 5 days; [c]ND not detected

### Lipase production, fungal growth and lipase characteristics

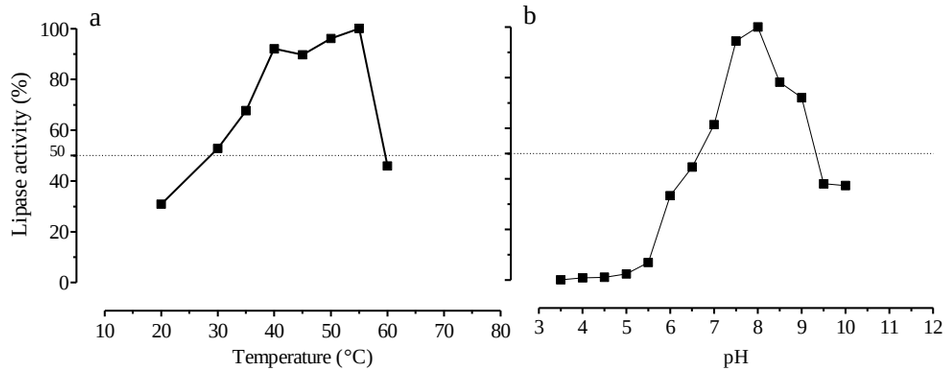
*B bassiana* CG432 cultured on Alves broth added of $CaCl_2$ 0.1% and olive oil 3% showed higher biomass production (Figure 1a), maximum lipase production (Figure 1b) and the reducing sugar was exausted (Figure 1c) on $5^{th}$ and $7^{th}$ days of the fermentations supplemented with olive oil (OO) and oily residue from canola (OO), respectively. On these days, the variations of pH reduced to 5.5 and 5.0 (Figure 1c), but the protein content was relatively constant during both fermentation time (Figure 1d). These results demonstrated the initial use of the disposable nutrient (glucose) to fungal growth followed of lipase production to use the lipids sources added at the fermentations.

**Figure 1** Growth curve (a), time course of lipase production (b), pH variations and reducing sugar concentration (c), and protein concentration (d) in fermentation by *Beauveria bassiana* CG432 in Alves broth supplemented with 3% refined olive oil (OO) (□ open symbols) or oily residue (■ closed symbols) from industrial process deodorization (OR) of vegetable oils refinery. Alves broth was added of $CaCl_2$ 0.1%, inoculated with $10^6$ conidia $ml^{-1}$ and was performed at 28°C at 200rpm.




The optimal activities of lipases produced by OR from canola supplementation were found at range temperature from 40 to 60°C, and 7.5 and 8.0 pH (Figure 2), like the majority of other lipases, and they were stable during 10 hours and 10 days when incubated at the optimum conditions (Figure 3). The optimal temperature and pH conditions were very similar than a lipase produced by the same strain *B. bassiana* CG 432 on olive oil, but they were presented shorter stability characteristics at storage and optimum conditions than the same work (CRUZ et al., 2009).

**Figure 2** Characteristics of ultra filtrate lipase extract produced by *Beauveria bassiana* CG432. (a) Incubation at 10 to 65°C, (b) pH sodium acetate at pH 3.5 to 6.0, sodium phosphate at pH 6.0 to 7.5, TRIS-HCl at pH 7.5 to 9.0, and sodium phosphate NaOH at pH 9.0 to 10.0.(c) Stability of lipases incubated in frozen at -20°C (●), room temperature at 25°C (o) and refrigerated at 4°C (▲) over 60 days; at optimal activity conditions at 45°C and pH 8.5 assayed for *p*-NPP emulsion hydrolysis (□) and titrimetric method (■) by during 60 hours.

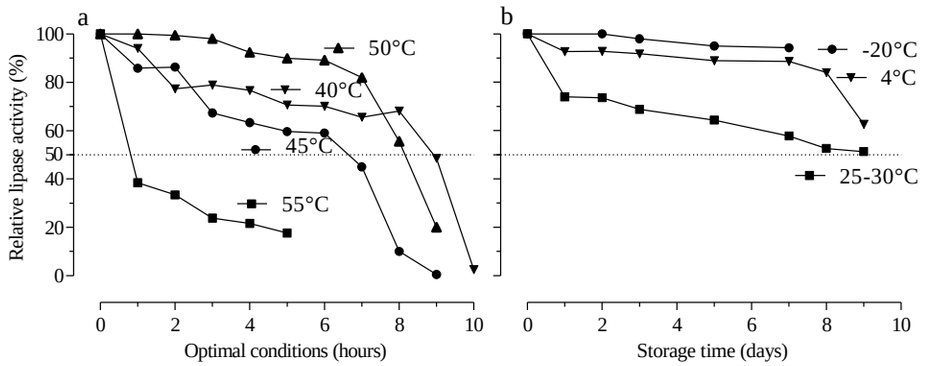



NaCl concentrations from 0.1 to 2M had no influence on activity of lipases produced by *B. bassiana* on Alves broth supplemented with OR from canola (Table 2). These results, together others presented above to lipase stability, are very good information to helps the choice of purification methodologies of these lipases.

**Table 2** Effect of NaCl concentrations on activity of lipase produced by *B. bassiana* on Alves broth supplemented with OR from canola

| NaCl | Lipase activity | |
|---|---|---|
| (M) | U $ml^{-1}$ | % |
| 0.0 | 203.49 | 81.76 |
| 0.1 | 220.29 | 88.51 |
| 0.2 | 239.28 | 92.14 |
| 0.4 | 247.90 | 96.30 |
| 1.0 | 248.88 | 100.00 |
| 2.0 | 185.83 | 74.66 |

**Figure 3** Stability of ultra filtrate lipase extract produced by *Beauveria bassiana* CG432: (a) Incubated at optimal activity conditions at pH 8.5 and 40, 45, 50 and 55°C over 10 hours. (b) Storaged at frozen -20°C; room temperature, 25-30°C; and under refrigeration, 4°C over 10 days. All assays were carried out for *p*-NPP emulsion hydrolysis.

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