

Production and Extraction of Extracellular Lipase from Entomopathogenic Fungus *Metarhizium anisopliae* (Clavicipitaceae: Hypocreales)

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Abstract.- Lipases are important cuticle degrading enzymes involved in the infection process of entomopathogens by hydrolyzing the ester bonds of lipoproteins, fats and waxes present in the insect integument. Production of extracellular lipase by using *Metarhizium anisopliae* Sorokin (Clavicipitaceae; Hypocreales) isolate M408 was investigated by different combinations of basal medium components. The effect of different vegetable oils added to basal medium at different concentrations was studied to improve enzyme production. Maximum lipase activity (97.44 ± 1.96 U/ml) as well as maximum biomass production (12.92 ± 0.11 mg/ml) was observed for olive oil when used at a concentration of 2% (v/v) of the basal medium. In the presence of surfactants, the highest lipase activity occurred when SDS and Tween 80 were added at the time *M. anisopliae* inoculation. SDS proved to be the best surfactant having 79.68 ± 1.26 U/ml lipase activity. Iron inhibited the lipase activity, whereas magnesium slightly increased the lipase activity. The optimum pH for lipase production was 5.7, while 32°C was the best temperature for lipase production.

Key Words: *Metarhizium anisopliae*, lipase, vegetable oils, entomopathogenic fungus.

INTRODUCTION

Entomopathogenic fungi are widely distributed throughout the fungal kingdom. Some insect-pathogenic fungi have restricted host ranges, while others have a wide host range with individual isolates being more specific (Maia *et al.*, 2001). Several species of fungi are potent biocontrol agents of plant pathogenic fungi and arthropods. Isolates of *Zoophthora radicans* (Pell *et al.*, 1993), *Metarhizium anisopliae*, *Fusarium* sp., and *Beauveria bassiana* can infect different insect species in screen house or field conditions (Ibrahim and Low, 1993; Vandenberg and Ramos, 1997; Vandenberg *et al.*, 1998; Shelton *et al.*, 1998). *Metarhizium anisopliae* Sorokin (Clavicipitaceae; Hypocreales) is one of the most promising fungal species currently being investigated as a biological control agent against diamondback moth, whiteflies

and other insect pests (Vandenberg *et al.*, 1998; Altre *et al.*, 1999).

The insect integument is composed of proteins and chitin with associated lipids and phenolic compounds which serve as a barrier against invading microorganisms (St.Leger, 1991). Through the combined action of hydrolytic enzymes such as chitinase, protease and lipase, the fungal mycelia are able to penetrate through these barriers (Bidochka *et al.*, 1987; St. Leger *et al.*, 1986). The importance of any one of these enzymes is dependant upon the cuticular characteristics of insects and invasion mechanism of the fungus. The participation of proteases and chitinases in the infection process of *M. anisopliae* has been demonstrated, and it has been suggested that lipases can also be involved in the process by hydrolyzing the ester bonds of lipoproteins, fats and wax layers of the insect integument (Clarkson and Charnely, 1996; Kachatourians and Qazi, 2008). Without the action of lipases, some of these materials would be a barrier to *M. anisopliae* entry. James *et al.* (2003) showed that cuticular lipids and silverleaf whitefly (*B. argentifolii*) affect germination of *B. bassiana*

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and *P. fumosoroseus* conidia. Cuticular lipids are toxic or inhibitory to conidia of *B. bassiana* and *P. fumosoroseus*. The thick coating of long-chain wax esters produced by whitefly nymphs affects spore germination. Lord and Howard (2004) also proposed that cuticular fatty amides of *Liposcelis bostrychophila* have a role in preventing adhesion of dry-conidial preparations of *B. bassiana*, *P. fumosoroseus*, *A. parasiticus* and *M. anisopliae*. Also, some of the fats present in the cuticle have antifungal activity and they would be of no use as a substrate for *M. anisopliae* without the action of lipases and lipoxygenases (Nesbit and Gunasekran, 1993).

In order to increase the virulence through optimizing production of lipases by *M. anisopliae* during penetration of the host, it is imperative to determine the optimal conditions for their production. Different environmental factors such as carbon sources, pH, and temperature have been extensively studied as factors that increase lipase productivity among other fungal species (Maia *et al.*, 2001; Mahadik *et al.*, 2002). In this report, we describe the effect of different growth factors, such as lipid sources, surfactants, divalent metal salts, pH and temperature on enzyme activity to obtain an enhanced lipase production from *M. anisopliae*

MATERIALS AND METHODS

Fungal strains

M. anisopliae isolate M408, originally isolated from soil (Liu, 2006), maintained in tubes containing sabouraud dextrose agar (SDA) and deposited in the Engineering Research Center of Biological Control, South China Agricultural University, was cultured on potato dextrose agar (PDA) and incubated at $26\pm 2^\circ\text{C}$ for 10 days. Conidia were harvested with deionized water containing 0.03% Tween 80 and sieved through filter paper into sterile vials. Conidia were counted in a compound microscope using a hemocytometer (0.0625m^2 ; Fuchs-Rosenthal Merck Euro lab, Darmstadt, Germany) to calibrate a suspension of 1×10^6 conidia/ml.

Basal medium

Basal medium (pH 7.2) consisted of glucose

0.2% (w/v), peptone 0.5% (w/v), MgSO_4 0.01% (w/v), K_2HPO_4 0.1% (w/v) and SDS 0.25 % (w/v).

Effect of different oils on lipase activity and biomass production

To study the effect of different oils on lipase production, different vegetable oils (soybean, sunflower, canola, corn, olive, and peanut) were added to the basal medium as a lipid source at concentrations of 1 and 2% (v/v). All media were heat sterilized (121°C for 15 min). After cooling, the oils (15 ml each), previously sterilized by dry heat (180°C for 60 min), were added to the culture medium (Walter *et al.*, 2005). Basal medium without oil served as a control.

Growth experiments were performed in 250 ml Erlenmeyer flasks containing 50 ml of sterile growth medium. The medium was inoculated with one ml of 10^6 spores/ml and the flasks were incubated for 96 h in an orbital shaker operating at 150 rpm and 28°C .

Effect of different surfactants on lipase activity

To observe the effect of chemical surfactants on enzyme production, 0.25%(v/v) of Triton X-100, Tween 80, Tween 20 or SDS were added to the basal medium described above (applied at the time of inoculation or 24 hrs after *M. anisopliae* growth). Fifty milliliters of the sterile growth medium was added to 250 ml Erlenmeyer flasks and inoculated with one ml of spore suspension (10^6 spores/ml). The flasks were incubated for 96 h in an orbital shaker at 150 rpm and 28°C . All the treatments were replicated three times.

Effect of different salts on lipase activity

The effect of the salts, MgSO_4 , MgCl_2 and FeSO_4 on lipase activity was studied by adding 1mM (v/v) of these salts to the basal medium described above while basal medium without any salt served as a control. Fifty milliliters of sterile growth medium inoculated with 1 ml of 10^6 spores/ml was incubated for 96 h in an orbital shaker at 150 rpm and 28°C .

Evaluation of optimum pH and temperature for lipase production

To evaluate the best pH for lipase production,

the basal media described above was buffered with 0.1M sodium phosphate to obtain pHs of 5.7, 6.3, 7.0 and 8.0, whereas non buffered basal medium was used as control. Fifty milliliters of the sterile growth medium inoculated with 1 ml of 10^6 spores/ml was incubated for 96 h in an orbital shaker at 150 rpm and 28°C.

To determine the optimum temperature for lipase production, 50 ml of basal medium inoculated with one ml of 10^6 spores/ml was incubated for 96 h at three different temperatures (28, 32 and 36°C) in an orbital shaker at 150 rpm.

Analytical determinations

Lipase activity was determined as described by Pignede *et al.* (2000). The substrate emulsion was prepared as a 1:1 mixture of olive oil and gum arabic (10% w/v). The reaction mixture contained 1 ml cultured fungal filtrate, 5 ml substrate emulsion and 2 ml of 50 mM phosphate buffer (pH 6.8) and incubated for 1 h at 37°C with shaking. The reaction was stopped with 4 ml of acetone-ethanol (1:1) containing 0.09% phenolphthalein as an indicator. Enzyme activity was determined by titration of the fatty acid released with 50 mM sodium hydroxide. One international unit was defined as enzyme activity that produced 1µmole of fatty acid per hour.

For biomass determinations, culture supernatants were separated from the mycelium by filtration through Whatman filter no. 1 and dried at 80°C until constant weight.

Chemicals and reagents

Triton X-100, Tween 80, Tween 20 and SDS were purchased from Sigma (St Louis, MO, USA). All the other chemicals were obtained from Guangzhou Jinhua chemical reagent company, Guangzhou, China. All oils used were of commercial grade (95% purity), purchased from Nan Hai Oil Industry, Shenzhen, Guangdong, China.

Statistical analysis

Each study was conducted three times with freshly prepared fungal suspension. Lipase activities as well as biomass production under different conditions were analyzed by Analysis of Variance (ANOVA) and treatment means were compared using Tukey's studentized range test for mean

comparisons at 5% level of significance. All statistical analysis was performed using SAS 8.01(SAS, 2000).

RESULTS

Effect of different oils on lipase activity and biomass production

Lipase activity of *M. anisopliae* was significantly affected by different kinds of the vegetable oils (Table I). Olive oil proved to be the most active inducer of lipase activity showing average lipase activities of 90.24 ± 2.50 and 97.44 ± 1.96 U/ml respectively for 1% and 2% concentration. The lowest lipase activity (71.28 ± 2.59 U/ml) was shown by peanut oil when used at a concentration of 1% whereas lipase activity of 55.23 ± 1.29 U/ml was observed for control. In short, olive, soybean and sunflower oils showed comparatively higher lipase activity as compared with other oils used (Table I).

Table I.- Effect of different vegetable oils on lipase activity and biomass production.

Lipids Sources	Lipase activity (U/ml)	Biomass (mg/ml)
Soybean 1%	88.56 ± 1.90 BC	5.59 ± 0.32 FG
Soybean 2%	81.60 ± 2.16 CD	8.95 ± 0.51 C
Corn 1%	72.00 ± 1.90 E	6.94 ± 0.29 EF
Corn 2%	79.68 ± 2.13 D	7.34 ± 0.17 DE
Canola 1%	83.04 ± 1.45 C	6.25 ± 0.73 FG
Canola 2%	78.48 ± 2.07 DE	7.81 ± 0.96 D
Peanut 1%	71.28 ± 2.59 E	6.32 ± 0.14 FG
Peanut 2%	78.72 ± 2.28 D	7.56 ± 0.67 D
Sunflower 1%	80.88 ± 1.87 D	5.47 ± 0.38 G
Sunflower 2%	81.84 ± 2.91 CD	6.94 ± 0.17 EF
Olive 1%	90.24 ± 2.50 AB	12.92 ± 0.11 B
Olive 2%	97.44 ± 1.96 A	16.33 ± 0.27 A
Control	55.23 ± 1.29 F	4.32 ± 0.53 H
F, d.f, P	17.34; 12; <0.0001	22.59; 12; <0.0001

Mean in the same column with different letters are significantly different from each other (Tukey's, $P < 0.05$)

±: Standard error (Based on three replicates)

Highly significant differences were observed among different oils for the biomass production of *M. anisopliae* ($F=22.59$; $df = 12$; $P < 0.0001$). All lipids related substrates supported the biomass production levels ranging from 16.33 ± 0.27 mg/ml

(2% olive oil) to 5.47 ± 0.38 mg/ml (1% canola). As indicated in Table I higher biomasses were obtained with olive and soybean oils.

Effect of different surfactants on lipase activity

Different surfactants (applied at the time of inoculation or 24 hrs after *M. anisopliae* inoculation) significantly affected the lipase activity of *M. anisopliae* ($F=47.49$; $df=7$; $P<0.0001$). Apart from Triton X-100 the highest lipase activity always occurred when the surfactants (Tween 20, Tween 80 and SDS) were added at the time of fungal inoculation, and SDS was the best inducer of lipase activity when used at the time of inoculation with a mean value of 79.68 ± 1.26 U/ml (Fig.1). The lowest lipase activity (43.20 ± 1.78 U/ml) was observed for Tween-20 when applied after 24 hrs of the fungal inoculation (Fig. 1).

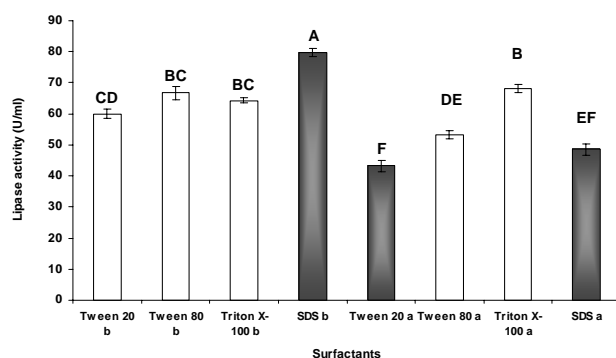


Fig 1. Lipase activity of *M. anisopliae* in the presence of different surfactants added at the time of fungal cultivation (a) or after 24 hrs of growth. Legends in the figure followed by different letters are significantly different from each other (Tukey's, $P<0.05$). Bars represent Standard error of means (Based on three replicates).

Effect of different salts on lipase activity

Cationic salts of metal ions such as, iron and magnesium showed significant effects on the lipase activity of *M. anisopliae* ($F=32.71$; $df=3$; $P=0.0001$). The divalent metal ions of ferric inhibited the lipase activity having a mean value of 70.56 ± 2.68 U/ml when compared to the control while divalent ions of magnesium showed higher lipase activities compared with control (Fig. 2).

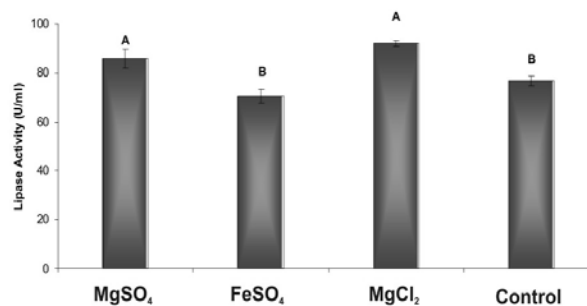


Fig 2. Effect of different salts on lipase activity of *M. anisopliae*. Means followed by different letters are significantly different from each other (Tukey's, $P<0.05$). Bars represent Standard error of means (Based on three replicates)

Evaluation of best pH and temperature for lipase production

Lipase activity of *M. anisopliae* differed significantly at different pH levels ($F=38.79$; $df=4$; $P<0.0001$). The best lipase production was obtained when media was buffered with pH 5.7 having mean lipase activity of 91.68 ± 1.45 U/ml. Lipase activity decreased as the pH value of media were increased (Table II).

Table II.- Effect of pH of basal medium on lipase production by *Metarhizium anisopliae*.

pH	Lipase activity (U/ml)
Control	82.80 ± 1.24 B
5.7	91.68 ± 1.45 A
6.3	81.84 ± 1.87 B
7.0	77.04 ± 0.72 B
8.0	67.44 ± 1.46 C
F, d.f, P	38.79 ; 4 ; <0.0001

Mean in the same column with different letters are significantly different from each other (Tukey's, $P<0.05$)
 \pm : Standard error (Based on three replicates)

Examination of the relationship between growth temperature and lipase production indicated that growth temperature had a significant effect on lipase production ($F=28.63$; $df=2$; $P=0.002$). A growth temperature of 36°C produced substantially lesser lipase activity (55.92 ± 3.12 U/ml). The best temperature for lipase production in the basal medium was 32°C (Table III).

Table III. Effect of temperature on lipase production by *Metarhizium anisopliae*.

Temperature (°C)	Lipase activity (U/ml)
28	73.68 ± 2.94 B
32	86.40 ± 1.90 A
36	55.92 ± 3.12 C
F, d.f, P	28.63;2;0.002

Mean in the same column with different letters are significantly different from each other (Tukey's, $P < 0.05$)

±: Standard error (Based on three replicates)

DISCUSSION

Lipase production can be influenced by the type and concentration of carbon and nitrogen sources. Previous work on the physiology of lipase production showed that the mechanisms regulating biosynthesis vary widely in different microorganisms (Sharma *et al.*, 2001). In this work, a range of different lipids was screened for their capacity to support *M. anisopliae* growth and lipase production. As indicated in Table I higher biomasses were obtained with olive and soybean oils while olive oil (2%) gave maximum lipase activity as well as biomass production. It is noteworthy that saturated fat (HSF) did not interfere with lipase activity in *M. anisopliae* when compared to soybean oil (Table I) which is similar to the findings of Walter *et al.* (2005). The effect of 0.02–5.0% olive oil, having approximately 60–85% trioleic acid, on lipase production in *B. bassiana* was also investigated by Hegedus and Khachatourians (1988). In stationary phase cultures of *B. bassiana*, in the absence of the inducer, lipase activity observed was 100 units/ml while the addition of increasing concentrations of olive oil resulted in an increase in lipase as well as biomass production.

It is well documented that various compounds, such as surfactants, can increase cell permeability, facilitating the export of several molecules across the cell membrane. These compounds may alter cell permeability increasing protein secretion, or by facilitating the contact between enzyme and substrate (Walter *et al.*, 2005). We investigated the addition of SDS, Tween 20, Tween 80 and Triton X-100 on lipase production. The surfactants were

added prior to cultivation, together with fungal inoculum, or after 24 h *M. anisopliae* inoculation. Apart from Triton X-100 the highest lipase activity always occurred when the surfactants (Tween 20, Tween 80 and SDS) were added at the time of fungal inoculation (Fig. 1). The effects of surfactants improving lipase secretion have been studied in several microorganisms with different results. Dalmau *et al.* (2000) reported a low biomass of *Candida rugosa* when Tween 80 was the sole carbon source, and suggested possible surfactant toxicity. In *M. anisopliae* when the surfactant was added after a period of growth, there were significant difference between Tween 80 and SDS (Fig. 1) which is similar to the findings of Mahadik *et al.* (2002) found a two-fold increased recovery of *Aspergillus niger* lipase when Triton X-100 was used as surfactant. The addition of Tween 20 and Tween 80 in the media could stimulate lipase production since some authors place these compounds in the category of artificial lipids (Thomson *et al.*, 1999). Li *et al.* (2001) obtained good recoveries for lipase from *Acinetobacter radioresistens* using Tween 80 while Hegedus and Khachatourians (1988) showed that Tween-80 inhibited lipase activity when introduced into the reaction vessel at concentrations as low as 0.4% (w/v).

The divalent metal ions, ferric significantly inhibit lipase activity whereas magnesium ions slightly increase the activity at low concentrations. This agrees with data presented by Iwai *et al.* (1970) on the inhibition of *Aspergillus niger* lipase by the salts of ferric and ferrous ions. They showed that iron ions bind to the enzyme and inactivate it. With regards to the stimulatory effect of magnesium ions, this reaction could take place through their binding to a fatty acid, thus driving the reaction to completion as suggested by Walter *et al.* (2005). Hegedus and Khachatourians (1988) also showed similar inhibition by the ferric ions. Our data is consistent with the above, and would indicate a similar mechanism of inhibition caused by the copper ions.

Lipase production by *M. anisopliae* was also affected by the pH and temperature changes (Tables II, III). Maximum lipase activity was observed at a pH of 5.7 and a temperature of 32°C which is

similar to the findings of Walter *et al.* (2005), who reported maximum lipase production at a pH of 5.7 by the entomopathogenic fungus *M.anisopilae* they also concluded that the best temperature for lipase production was 32°C. Hegedus and Khachatourians (1988) also studied the effect of temperature on the lipase production by *B.bassiana*. A growth temperature of 20°C produced substantially lesser amounts of lipase even though biomass production reached the same level as at 27-30°C. Growing the culture at 15°C also produced lesser amounts of lipase but biomass production was also inhibited. At 35°C which is restrictive for growth of the fungus both growth and lipase production were minimal.

The study of lipases in *M. anisopliae* is important because being an entomopathogen the lipases produced may be involved in pathogenicity by helping the pathogen to spread and infect lipid containing cells and utilize fatty acids as a means of nutrition (Nesbit and Gunasekran, 1993) or by reacting with lipoproteins, fats, and waxy layers of insect integument, some of which can act as a barrier to *M. anisopliae* for its entry as some have anti-fungal activity, and they would be of no use as a substrate for *M. anisopliae* (Kacahtourians and Qazi, 2008). These data presented in this work can be used for enhancement of lipase production.

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