UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE BIOCIÊNCIAS

LIPASE: A NOVEL *Metarhizium anisopliae* ENZYME. PRODUCTION AND SOLUBILIZATION

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Monografia apresentada para a obtenção do título de Bacharel em Ciências Biológicas – ênfase em Biologia Molecular, Celular e Funcional da Universidade Federal do Rio Grande do Sul.

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Porto Alegre, agosto de 2003.

O artigo a seguir foi redigido segundo as normas da revista ENZYME AND MICROBIAL TECHNOLOGY.

Lipase: a novel *Metarhizium anisopliae* enzyme. Production and solubilization

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Abstract

Lipase from a Brazilian strain of *Metarhizium anisopliae* was investigated. The effect of different lipid sources added to basal medium was verified to improve enzyme production. Biomass was highest for sunflower oil (21.88 $mg.mL^{-1}$), however lipase specific activity was highest olive oil (108.38 $U.mg^{-1}$). In the presence of surfactants, the highest lipase activity always occurred when SDS, Tween 20 and Tween 80 were added after 50h *M. anisopliae* growth, being SDS (4.54$\pm$ 0.46 $U.mL^{-1}$) and Tween 80 (4.15 $\pm$ 1.13 $U.mL^{-1}$) the best surfactants. This is the first report investigating lipase production by an entomopathogenic fungus.

Keywords: *Metarhizium anisopliae*; lipase; entomopathogenic fungus; commercial enzymes; lipase solubilization.

1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are serine hydrolases of considerable physiological significance and industrial potential, which catalyze the hydrolysis, and the synthesis of esters bonds formed from glycerol and long-chain fatty acids. In contrast to esterases, lipases are activated only when adsorbed to an oil-water interface [1]. The number of available lipases has increased considerably since the 1980s in response to an increasing demand for these biocatalysts [2].

Since lipases are extremely versatile because can catalyze numerous different reactions, they have been widely used in industrial applications, such as in dairy and food manufacture, leather and detergent industry, production of cosmetics and pharmaceuticals and inorganic synthesis reactions especially reactions in non-aqueous media [3, 4]. Microbial lipases represent the major commercial source of this enzyme. In recent years, research in lipases, mainly of microbial origin, has increased because of their great commercial potential.

Fungi are one of the most important lipase sources for industrial application because fungal enzymes are usually excreted extracellularly, facilitating extraction from fermentation media. A large number of filamentous fungi have been studied for lipase production [5-7].

The filamentous fungus *Metarhizium anisopliae* is one of the most important and best-studied entomopathogen for control of many plagues [8]. It has been proven the participation of proteases and chitinases at its site of action being suggested that lipases can also be involved in the process [9]. Therefore, to understand the penetration process in the host is important characterize the proteins involved.

Increasing of lipase production during the cultivation process is also an important step in industrial application of this enzyme. Different environmental factors have been extensively studied to increase lipase productivity, as carbon sources, pH, and temperature, among others [5, 6, 10-14].

The aim of this study is to analyze different growth conditions and solubilization to obtain an enhanced lipase production from *M. anisopliae*.

2. Material and methods

2.1. Microorganism and culture conditions

M. anisopliae strain E6 isolated from *Deois flavopicta* in Espirito Santo State, Brazil was maintained on agar slants in Cove's complete medium (MCc) at 4^0C [15]. For lipase production, the composition of the basal medium with an initial pH value of 7.2 consisted of glucose 0.2% (w/v), peptone 0.5% (w/v), $MgSO_4$ 0.01% (w/v) and K_2HPO_4 0.1% (w/v), supplied with olive oil 2% (v/v). Different animal fat (pig fat and bovine fat) and vegetable oils (babassu palm oil, soybean, sunflower, sesame, palm, rice, canola, corn, lineseed, olive and hydrogenated vegetable fat) were tested at a concentration of 2% (v/v) as a lipid source. All media were heat sterilized (121^0C for 15 min). After cooling, the fats and oils, previously sterilized by dry heat (180^0C for 60 min), were added to the culture medium.

2.2. Growth conditions:

Growth experiments were performed in triplicate in 125 mL Erlenmeyer flasks containing 16 mL of sterile growth medium. The medium was inoculated with 10^6 spores mL^{-1} and the flasks were incubated for 50 h in an orbital shaker operating at 150

rpm at 28°C. For evaluation of the best pH for lipase production, the basal media containing soybean oil 1% (v/v) as lipid source, was buffered with 0.1 M sodium phosphate in the following pH: 5.7, 6.3, 7.0 and 8.0. Growth temperatures of 28°C, 32°C and 36°C were also tested.

For biomass determination the culture supernatants were separated from the mycelium by filtration through Whatman no. 1 filter paper and dried at 80°C to a constant weight.

2.3. Enzyme solubilization:

For enzyme solubilization 0.25% (v/v) of Triton X-100 (Sigma), Tween 80 (Sigma), Tween 20 (Sigma), SDS (Sigma) or Polyethylene Glycol (PEG-Dow) were added to the medium before or after *M. anisopliae* growth [16].

2.4. Analytical determinations:

For determination of lipase activity the culture supernatants (CS) were used as enzyme source. Lipase activity was assayed in the enzyme-containing CS with p-nitrophenyl palmitate (pNPP - Sigma) as substrate [5,17]. CS (0.1 ml) were mixed with 0.9 mL of substrate solution containing 3 mg of pNPP dissolved in 1 mL propanol-2-ol emulsified, diluted in 9 mL of 50 mM Tris-HCl pH 8.0, with 40 mg of Triton X-100 and 10 mg of gum arabic. After 30 min of incubation at 37°C the absorbance was measured spectrophotometrically at 410 nm against an enzyme-free control. One lipase unit (U) was defined as the amount of enzyme that liberates 1 μmol p-nitrophenol per min. Specific activity was expressed as U mg^{-1} protein. Enzyme assays were carried out in triplicate and the average values were calculated.

For determination of protease activity the culture supernatants (CS) were used as enzyme source. Protease activity was assayed in the enzyme-containing CS with azocasein 2% (Sigma) as substrate [18]. CS (0.1 ml) were mixed with 0.1 mL of substrate solution containing 0.5 mg of azocasein and 0.2 mL of sodium phosphate buffer 50 mM pH 8.0. After 15 min of incubation at 50^0C 0.8 mL of trichloroacetic acid 20% (TCA-Sigma) was added. The blank was made with addition of 0.8 mL TCA 20% before incubation. After centrifugation (5 min 13.000 rpm) the absorbance was measured spectrophotometrically at 400 nm. One protease unit (U) was defined as the amount of enzyme that increased the absorbance in one unit.$mL^{-1}.h^{-1}$. Enzyme assays were carried out in triplicate and the average values were calculated.

Protein measurements were carried out by the method of Bradford [19], using bovine serum albumin as standard.

3. Results and discussion

Twelve major producers and 400 minor suppliers satisfy the world enzyme demand. Around 60% of the total world supply of industrial enzymes is produced in Europe but relatively little work has been done on development of robust lipase bioreactor systems for commercial use [4]. In Brazil, there are large amounts of fat waste from vegetable oil refineries that could be used as carbon sources with commercial potential.

Lipase production is influenced by the type and concentration of carbon and nitrogen sources, and the culture pH and temperature. Previous work on the physiology of lipase production showed that the mechanisms regulating biosynthesis vary widely in different microorganisms [4]. 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 1 higher biomasses were obtained with olive, sunflower and soybean oils or with bovine and pig fats. All lipid-related substrates supported lipase activity levels ranging from 1.64 to 4.97 $U.mL^{-1}$ for media supplemented with 2% sesame and 4% sunflower oils, respectively. However, the total protein of the culture supernatants did not correlate with extracellular lipase activities, with 3% sunflower exhibiting maximum protein production with lower lipase activity, when compared with 4% sunflower and 2% olive oil that gives maximum specific activity of the supernatant (108.38 U.mg of protein). Protease activity was negligible in all conditions used.

It is well know that various compounds, such as surfactants, can increase cell permeability, facilitating the export of several molecules across the cell through its membrane. We investigated the addition of SDS, PEG, Tween 20, Tween 80 or Triton X-100 on lipase production. The surfactants were added at the beginning of cultivation, together with fungal inoculum, or after 50h *M. anisopliae* growth. These compounds may affect the permeability of cell increasing protein secretion, or by facilitating the contact between enzyme and substrate. Apart from PEG, the highest lipase activity always occurred when the surfactants (SDS, Tween 20 and Tween 80) were added after 50h *M. anisopliae* growth (Fig. 1), being SDS (4.54 ± 0.46 $U.mL^{-1}$) and Tween 80 (4.15 ± 1.13 $U.mL^{-1}$) the best surfactants. The difference encountered related to the time of application may be caused by a possible toxic effect interfering with *M. anisopliae* conidia germination and consequently, with mycelia growth. The effects of surfactants on improving secretion of lipase have been studied in several microorganisms with different results [20]. Dalmau, *et al* (2000) reported a low biomass of *Candida rugosa* when Tween 80 was used as the unique carbon source, because of

its possible toxicity [13]. For *M. anisopliae* when the surfactant was applied after growth, there was no difference between Tween 80 and Triton X-100 However, Mahadik *et al* (2002), found a twofold-increased recovery of *Aspergillus niger* lipase when Triton X-100 was used as surfactant [6]. 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 [21]. Li *et al* (2001) obtained good recoveries for lipase from *Acinetobacter radioresistens* using Tween 80 as carbon source [22].

In general, lipase production in microorganisms is enhanced varying not only the lipid source but also its concentration [5, 10, 11, 12, 20, 23, 24]. The different lipid sources that best induced lipase production in *M. anisopliae* were rice oil (4.46 $\pm$ 0.63 $U.mL^{-1}$), soybean oil (4.26$\pm$ 1.18 $U.mL^{-1}$), olive oil (4.25 $\pm$ 0.69 $U.mL^{-1}$), sunflower oil (4.23 $\pm$ 0.34 $U.mL^{-1}$), sesame oil (3.51 $\pm$ 0.78 $U.mL^{-1}$) and hydrogenated vegetable fat (3.50 $\pm$ 1.00 $U.mL^{-1}$) (Fig. 2a). Based on local cost and availability, sunflower and soybean oils were chosen as the most suitable inducers for test the effect of different lipid concentrations. From the results obtained (Fig. 2b) there was no statistical difference through the Tukey's test (α 0.05) on lipase production. Soybean oil supported a lipase activity of 4.90 $\pm$ 0.63 $U.mL^{-1}$, 4.85 $\pm$ 1.18 $U.mL^{-1}$ and 3.94 $\pm$ 1.21 $U.mL^{-1}$ at concentrations of 1%, 2 and 3%, respectively. Lipase production using sunflower oil were 3.93 $\pm$ 0.53 $U.mL^{-1}$, 4.9 7$\pm$ 0.54 $U.mL^{-1}$ and 4.27 $\pm$ 1.04 $U.mL^{-1}$, using 1, 2, or 3% concentrations. Soybean oil 1% was adopted for using in the experiments because represents smaller costs. The best lipase production was when the medium was obtained when basal medium was buffered with pH 5.7 or without buffering (Table 2). The best temperature in basal medium was 32°C.

The study of lipases in *M. anisopliae* is important for two main reasons: (i) being a filamentous fungi the process of protein extraction is facilitated, since these enzymes are secreted, lowering the costs for a future industrial production, and (ii) being an entomopathogen, the lipases produced may be involved in pathogenicity.

These data will be used for further improvements in lipase production in a pilot plant bioreactor by using different operational strategies.

Acknowledgments

This work was supported by grants and fellowships from: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS).

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Table 1: Effect of different lipid sources on *M. anisopliae* growth and lipase production.

Lipids source	Lipase activity* ($U.mL^{-1}$)	Protein ($mg.mL^{-1}$)	Dry weight ($mg.mL^{-1}$)	Protease activity ($U.mL^{-1}$)	Specific lipase activity
Babassu palm oil 2%	3.30±0.89	0.125	7.02	0.24	26.30
Olive 2%	4.25±0.69	0.039	12.19	1.56	108.38
Sunflower 2%	4.23±0.34	0.062	12.15	0.20	68.27
Lineseed 2%	2.72±0.06	0.116	4.09	0.00	23.35
Soybean 2%	4.26±1.18	0.102	11.35	0.08	41.94
Corn 2%	3.45±0.18	0.119	5.31	0.00	28.89
Cotton seed 2%	2.51±0.61	0.092	7.38	0.84	27.18
Canola 2%	2.17±0.16	0.055	6.71	0.98	39.81
Rice 2%	4.48±0.63	0.124	6.38	0.62	36.09
Palm 2%	2.20±0.37	0.169	9.27	0.00	13.02
Pig fat 2%	1.64±0.35	0.055	11.63	0.48	29.66
Sesame 2%	3.51±0.78	0.126	6.34	0.00	27.88
Bovine fat 2%	2.57±0.72	0.044	10.78	0.00	58.25
HVF 2%**	3.51±1.00	0.098	7.56	0.00	35.70
Sunflower 1%	1.98±0.75	0.166	8.75	0.52	11.91
Sunflower 2%	3.93±0.53	0.082	11.88	0.10	48.06
Sunflower 3%	4.97±0.54	0.656	15.29	0.02	7.57
Sunflower 4%	4.27±0.65	0.326	21.88	0.00	13.09
Soybean 1%	4.90±0.63	0.102	10.97	3.08	47.87
Soybean 2%	4.85±1.18	0.086	12.06	0.14	56.47
Soybean 3%	3.94±1.21	0.334	11.52	0.00	11.79
Soybean 4%	3.20±1.04	0.304	13.85	0.66	10.52

*Lipase was extracted with SDS 0.25%

± Standard deviation (based on three replicates)

**HVF: hydrogenated vegetable fat

Table 2: Effect of different pH in basal medium buffered with sodium phosphate 0.1 M on *M. anisopliae* lipase production.

pH	Lipase activity* ($U.mL^{-1}$)
Control**	5.41 ± 0.56^{ab}
pH 5.7	5.60 ± 0.63^{a}
PH 6.3	4.95 ± 0.13^{b}
pH 7.0	3.62 ± 0.38^{c}
pH 8.0	3.52 ± 0.67^{c}

*Lipase was extracted with SDS 0.25%. Culture conditions: basal medium with 1% soybean oil, incubated at 28°C, in shaker at 150 RPM for 50h.

**Growth without buffered

± Standard deviation (based on three replicates)

[a,b,c,] Means followed by the same letter are not significantly different according to Tukey's test (α=0.05).

Table 3: Effect of different temperature on *M. anisopliae* lipase production.

Temperature	Lipase activity* ($U.mL^{-1}$)
28 °C	5.03±0.70
32 °C	6.27±0.40
36 °C	1.12±0.30

*Lipase was extracted with SDS 0.25%. Culture conditions: basal medium with 1% soybean oil, in shaker at 150 RPM for 50h.

± Standard deviation (based on three replicates)

Figure 1. Lipase solubilization in the presence of different surfactants added in the initial *M. anisopliae* cultivation (a) or after 50 h growth (b). Culture conditions: basal medium with 2% olive oil, incubated at 28°C, in shaker at 150 RPM for 50h. The growth was done in triplicate. ^{A,B,C,D,E,F} Means followed by the same letter are not significantly different according to Tukey's test (α=0.05).

Figure 2. Production of lipase by *M. anisopliae* in basal medium: (a) using different lipid source at 2% concentration, (b) sunflower and soybean oils at 1%, 2%, 3% and 4% concentrations. Culture conditions: basal medium supplemented with the respective lipid source, incubated at 28°C, in shaker at 150 RPM for 50h. SDS 0.25% was added after *M. anisopliae* growth as a surfactant. This test was performed in triplicate.^{A,B,C,D,E,F,G,H,I,J,K}Means followed by the same letter are not significantly different according to Tukey's test (α=0.05).

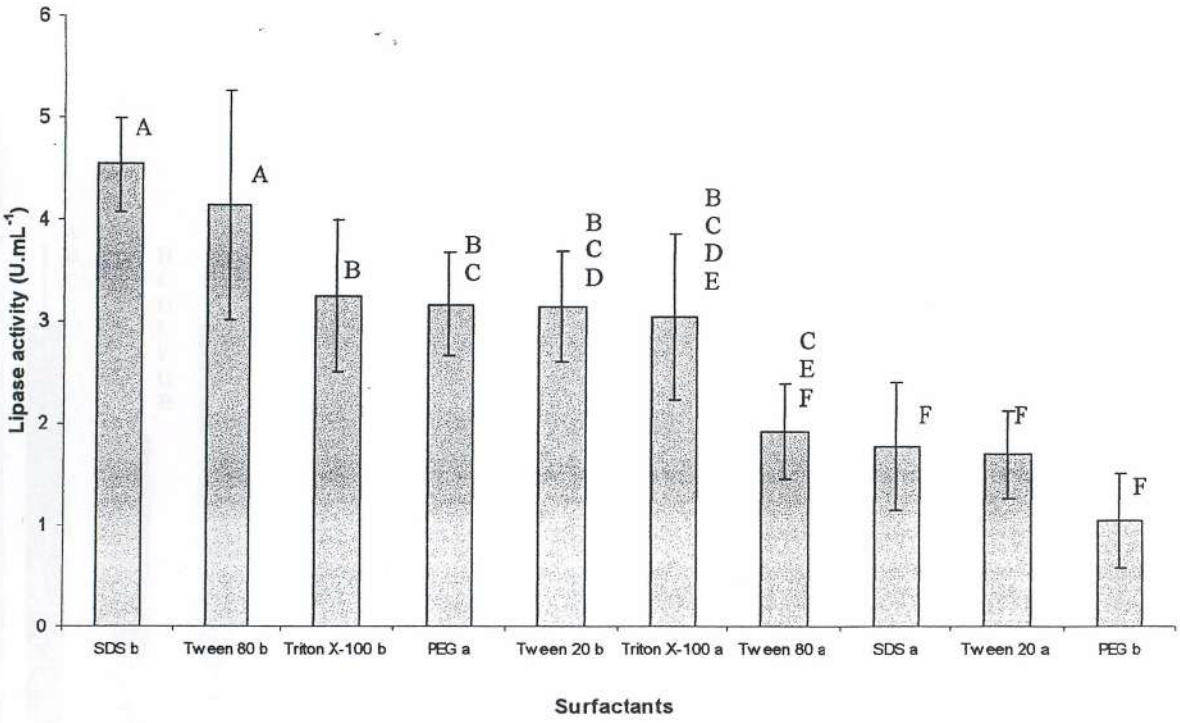
Lipase activity (U.mL-1)
0
1
2
3
4
5
6
A
A
B
B C
B C D
B C D E
C E F
F
F
F
SDS b
Tween 80 b
Triton X-100 b
PEG a
Tween 20 b
Triton X-100 a
Tween 80 a
SDS a
Tween 20 a
PEG b
Surfactants

Figure 1

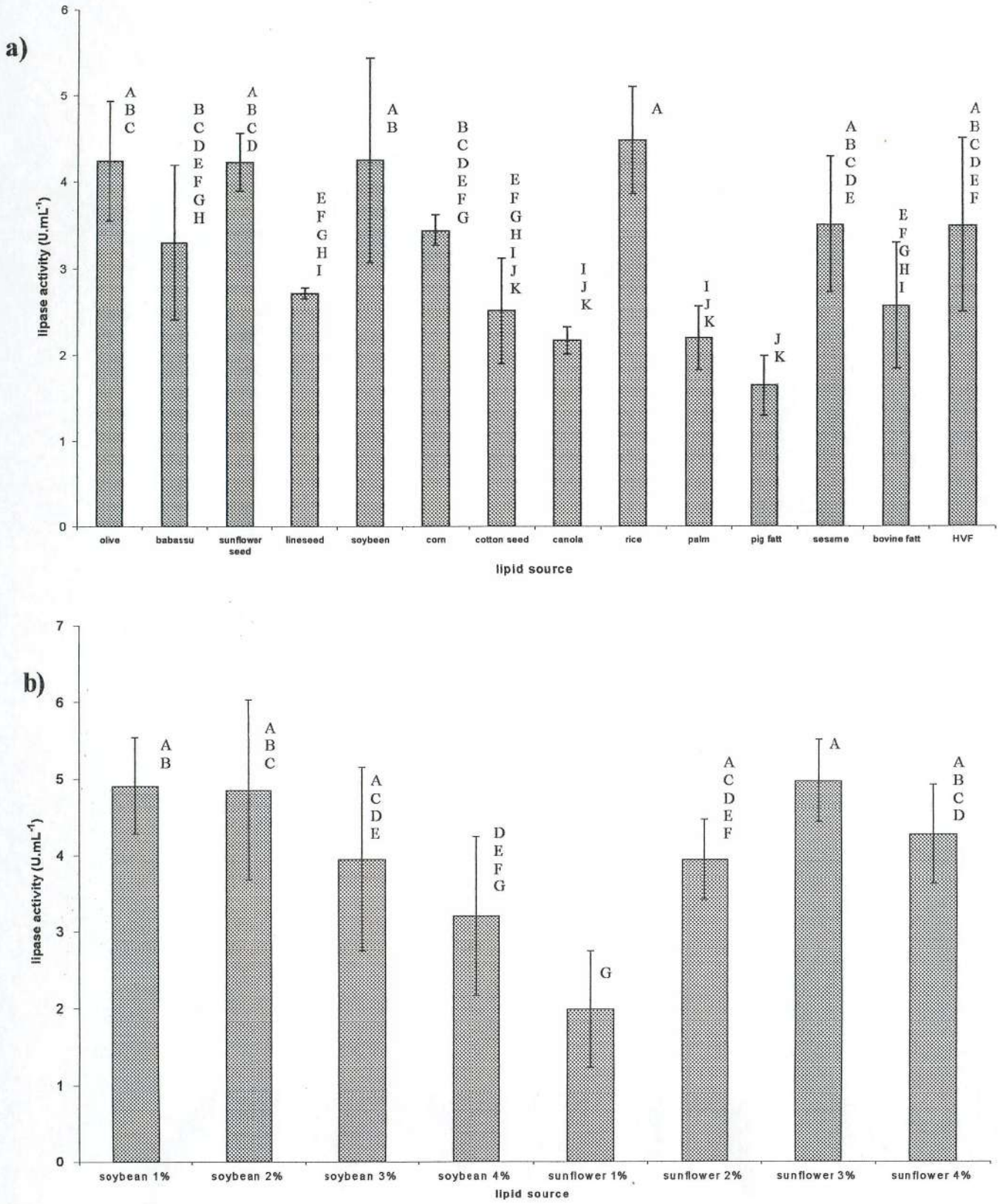
a)
lipase activity (U.mL-1)
0
1
2
3
4
5
6
A B C
B C D E F G H
A B C D
E F G H I
A B
B C D E F G
E F G H I J K
I J K
A
I J K
J K
A B C D E
E F G H I
A B C D E F
olive
babassu
sunflower seed
lineseed
soybeen
corn
cotton seed
canola
rice
palm
pig fatt
sesame
bovine fatt
HVF
lipid source
b)
lipase activity (U.mL-1)
0
1
2
3
4
5
6
7
A B
A B C
A C D E
D E F G
G
A C D E F
A
A B C D
soybean 1%
soybean 2%
soybean 3%
soybean 4%
sunflower 1%
sunflower 2%
sunflower 3%
sunflower 4%
lipid source

Figure 2