Brazilian Journal of Microbiology (2009) 40: 747-756
ISSN 1517-8382

SCREENING OF MICROBIAL LIPASES AND EVALUATION OF THEIR POTENTIAL TO PRODUCE GLYCERIDES WITH HIGH GAMMA LINOLENIC ACID CONCENTRATION

Patricia B.L. Fregolente[1], Leonardo V. Fregolente[1], Maria R.W. Maciel[1], Patricia O. Carvalho[2]*

[1]Universidade Estadual de Campinas, Faculdade de Engenharia Química, Departamento de Processos Químicos, Campinas, SP, Brasil; [2]Universidade São Francisco, Laboratório de Biotecnologia, Bragança Paulista, SP, Brasil.

Submitted: June 03, 2008; Returned to authors for corrections: August 27, 2008; Approved: May 19, 2009.

ABSTRACT

Gamma-linolenic acid (GLA, 18:3, cis- 6,9,12-octadecatrienoic acid), an important compound in n-6 eicosanoid family biosynthesis, occurs in the lipids of a few plant and microbial sources. This study focused on the screening of microbial strains with suitable lipase activity for enrichment of GLA by selective hydrolysis of the borage oil (21.6 % of GLA/total fatty acids). Firstly, 352 microrganisms were tested for their lipolytic capacity using screening techniques on agar plates containing borage oil, strains were then selected and screened for their activity (U/mg) using both submerged fermentation (SmF) and solid state fermentation (SSF). The rate of hydrolysis and the selective preference of these hydrolytic enzymes towards fatty acids, with a special focus on enrichment of GLA were studied and compared with those obtained by two commercially-available lipases. Only one of the lipases tested during this study displayed selectivity, discriminating the GLA during the hydrolysis reaction. Using the enzymatic extract from *Geotrichum candidum* as a biocatalyst of the reaction, it was possible to obtain a percentage of 41.7% of GLA in acylglycerols fraction when the borage oil was treated in a fixed-bed reactor for 24 hours at 30ºC.

Key words: gamma linolenic acid, borage oil, lipase, *Geotrichum candidum*

INTRODUCTION

Polyunsaturated fatty acids (PUFA) present important actions in physiological functions, therefore intensive efforts have been made to identify the possible effects of eicosapentaenoic acid (EPA), docosahexaenoic (DHA) and gamma-linolenic acid (GLA) in treating some diseases. GLA has been reported to be an effective nutritional supplement to treat premenstrual syndrome (2), cancer (3), certain skin diseases (11) and hypertriglycemidemia (18). Several studies have demonstrated a significant role of the dietary ômega-6 PUFA in hypertension treatment (10). Some processes have been developed for the concentration of these fatty acids to incorporate them in food supplies, since higher animals are unable to synthesize all the fatty acids they need and the dosages necessary to reach the desired biological effect would demand the ingestion of a great amount of oils in their natural forms. Urea complexation (19), low temperature

***Corresponding Author. Mailing address:** Universidade São Francisco, Av. São Francisco de Assis, 218 CEP 12916-900, Bragança Paulista, SP, Brasil.; Tel: 55 11 2454-8298.; E-mail: patcarvalho@saofrancisco.edu.br

crystallization (7) and supercritical extraction (32) are some optional methods for the enrichment of GLA. However, lipase-catalyzed reactions are an advantageous alternative for this objective since, owing to the high selectivity of some lipases toward unsaturated fatty acids, PUFA can be concentrated in the remaining acylglycerols, with consequent nutritional advantages (6).

In particular, three plants have become important sources of GLA: borage (*Borago officinalis*), evening primrose (*Oenothera biennis*) and blackcurrant (*Ribes nigrum*). These natural sources generally contain variable quantities of GLA (31), rarely exceeding 25 % of GLA in the oil (e.g. borage oil).

Among the high numbers of lipases described in the literature, only the enzymes belonging to a few species have been demonstrated to have adequate stability and selectivity to allow routine use for the concentration of PUFA. Some researchers have reported that lipases from *Candida* species are effective for the enrichment of PUFA via selective hydrolysis (27, 13, 28, 29, 22). Few studies have been carried out to exploit the capacities of novel microbial lipases to concentrate the content of PUFA in oils (12, 21, 25). In a preliminary study, we reported that some native lipases provide good results for the concentration of GLA from a fungal oil derived from *Mucor* sp (4).

In this present study, we describe the selection, by screening techniques, of lipase-producing microrganisms and the preliminary characterization of the catalytic performance of native lipase preparations to concentrate the content of GLA in the acylglycerols fraction in borage oil by selective hydrolysis in a fixed-bed reactor. We used lipase-producing microrganisms, isolated from natural sources, and compared their lipases with commercially-available lipases from *Candida cylindracea* and *Aspergillus niger* as biocatalysts. In addition, a preliminary study with respect to the influence of growing media, enzyme concentration and time reaction in the concentration of GLA in the borage oil of selected lipase was carried out.

MATERIALS AND METHODS

Chemicals

Yeast extract and Bacto peptone were purchased from Difco Laboratories, (Detroit, MI, USA). The components from culture media, chemical reagents and the other solvents were obtained from Merck (Darmstadt, Germany) and from Sigma-Aldrich Chemical Co. in the highest purity available. The Borage oil was purchased from SP Farma Ltda (São Paulo, Brazil). The commercial lipases employed were *Candida cylindracea* (Sigma - -Aldrich Co. St. Louis, MO) and *Aspergilus niger* (Aldrich Chemical Company, Inc., Milwaukee, WI). Low acidity olive oil (Carbonel) and wheat flour were purchased at a local market.

Isolation and preliminary screening of lipase-producing microrganisms

All the microrganisms used in this study were isolated from soil and fruit samples and collected from Southwest Brazil. The samples were suspended in a buffer (0.85 % saline solution) and the isolation process was performed by serial dilution of the samples, according to standard techniques (16). Preliminary screening of lipolytic filamentous fungi and yeast was carried out on BYPO agar plates supplemented with a 10 % (v/v) borage oil emulsion prepared in 10 % (w/v) Arabic gum solution. Culture plates were incubated at 30ºC and periodically examined for 4 days. Colonies showing clear zones around them were picked out and used for lipase production.

Lipase production

The production of lipase was studied using both submerged fermentation (SmF) and solid state fermentation (SSF). The SmF was carried out in a basal medium, with an initial pH value of 6.0, consisting of 1 % glucose, 2 % peptone, 0.5% yeast extract, 0.1% $NaNO_3$, 0.1%, KH_2PO_4, 0.05% $MgSO_4$. $7H_2O$ and 1 % olive oil. The cultures were grown in Erlenmeyer flasks (500 mL) containing 120 mL of the growth medium and the flasks were agitated by a rotary

shaker (130 -150 rpm) at 30ºC for 72 h. The lipase production in SSF was carried out in a medium containing 10 g of wheat flour (40 % water w/w) moistened with 10 mL of the liquid medium described above. The Erlenmeyer flasks (500 mL) were incubated at 30ºC for 96 h. After SSF, 100mL of aqueous solution of NaCl (1%) was added to each flask and the mixture shaken on a rotary shaker (180 rpm) for 2 h at room temperature for extraction of enzyme. Both culture broths were collected, centrifuged (3000 rev/min for 15 min.) and the supernatants treated with ammonium sulphate (80% saturation). The precipitates were dialyzed overnight against the phosphate buffer pH 7.0, lyophilized and the resultant powder was used as crude enzyme preparation.

Identification of the isolates

Morphological identification of isolates to the species level was accomplished using established procedures (8) and the taxonomic identification was performed by the Centro de Micologia da Universidade de Lisboa, Portugal.

Lipase-catalyzed hydrolysis of the borage oil

The conditions used for the enzymatic hydrolysis reaction were: 12g of Borage oil (22.1% of GLA), 28 g of distilled water, 300 U of enzyme /g of oil, stirring at 500 rpm and a temperature of 30º C. The reaction was conducted using a fixed-bed reactor of 70mL and, to interrupt the reaction, the enzyme was deactivated by heating the reaction to approximately 90ºC for 15 min. All of the lipases selected (native and commercial) were used in the enzymatic hydrolysis reactions fixing a total time of reaction of 24 hours to verify which present a higher enrichment of GLA.

Assay of lipase activity

Lipolytic activity was determined at 35ºC, pH 7.0, by the titration of free fatty acids with 0.1 N NaOH released from olive oil hydrolysis and the quantitation was carried out with a standard oleic acid curve. The enzymatic activity (U) was expressed as micromoles of acid released per minute and milligrams of crude lipase preparation (μmol min^{-1} mg^{-1}). All experiments were performed with duplicate flasks, with the results reported as the mean of the duplicates.

Separation of the free fatty acids and acylglycerols

At the end of each enzymatic reaction, acylglycerols (triacylglycerol (TG), diacylglycerol (DG) and monoacylglycerol (MG)) and free fatty acids (FFA) fractions were separated according to a modified procedure outlined in previous study (23). Ten mg of the reaction mixture were then mixed with 70 mL of KOH solution 0.5N (30% ethanol) and the acylglycerols were extracted with 10 mL of n-hexane. pH was then returned to acid pH (pH above 2) by adding 4 N HCl. The fatty acids present in the aqueous phase were then extracted by adding 100 mL of n-hexane and separated by evaporation.

Fatty acid composition analysis

Both acylglycerols and FFA were converted to methyl esters by treatment with methanol-BF_3 as described by AOCS (30) and analyzed by gas chromatography in a Chrompack CG instrument equipped with flame ionization detector (FID). The separations were carried out on a fused silica WCOT CP-Sil 88 capillary column (Chrompack, Holland) using a programmed temperature of 180-220°C, 5°C/min. Hydrogen was used as a carrier gas. The composition of fatty acids was identified by comparing retention times with authentic standards (Sigma Chemical Co.) and determined by relative percentage.

Acylglycerols and FFA analysis

Gel-permeation chromatography, also called high-performance size-exclusion chromatography (HPSEC) was used for the acylglycerols and FFA analyses. The chromatographic system consists of an isocratic pump, model 515 HPLC Pump (Waters, Milford, MA), a differential refractometer detector model 2410 (Waters, Milford, MA), and an oven for columns thermostatted at 40ºC by a temperature control module (Waters, Milford, MA). The samples were injected using a manual injector, rheodyne

7725i model, with a 20 µL-sample loop. The mobile phase used was HPLC-grade tetrahydrofuran from Tedia Inc. (Fairfield, OH) and the flow rate was 1 mL/min.

RESULTS AND DISCUSSION

The screening process and the hydrolytic activity of the lipase preparations selected are shown in Table 1. Microrganisms (352) isolated from soil and fruits samples, including yeasts (48) and filamentous fungi (304) were employed in this work. A total of 23 microrganisms produced a clear halo around them in plates containing borage oil, thus indicating a predominant presence of lipases acting on long chain triglycerides. These strains, selected in the first step, were then cultured in SmF and SSF and were used to quantify their lipase activity using the standard method of titrating fatty acids liberated from olive oil. From this analysis, 5 strains were selected demonstrating the best lipase yields (lipase activity higher than 1U/mg of enzyme for one or for both medium cultures). The strains produced were taxonomically identified as follows: *Aspergillus niger*, *Fusarium oxysporum*, *Geotrichum candidum*, *Penicillium solitum* and *Rhizopus javanicus*. *A. niger*, *G. candidum* and *R. javanicus* are well known as lipase producers (20). *F. oxysporum* have also been previously found to have lipolytic activity in solid and in liquid media (15, 9). To our knowledge, this is the first report of *Penicillium solitum* as a lipase producer. Low growth of these strains was observed when SSF was used, explaining the lower values of enzyme activity in the presence of wheat flour than those obtained with liquid culture (SmF).

Table 1. Number of microorganisms selected in this study through the screening process and lipolytic activity of the crude enzymatic extracts using olive oil.

Screening step	Number of selected microrganisms	
Soil and fruit sample isolation	352	
Solid medium screening [(a)]	23	
Submerged and solid state fermentation (SmF and SSF)	5	
Sources of lipases	**Lipase activity (U/mg) [(b)]**	
	SmF [(c)]	**SSF [(d)]**
Aspergillus niger	5.96 ± 0.23	2.84 ± 0.29
Fusarium oxysporum	4.89 ± 0.12	4.67 ± 0.09
Geotrichum candidum	4.32 ± 0.24	2.21 ± 0.22
Penicillium solitum	1.34 ± 0.07	0.63 ± 0.21
Rhizopus javanicus	1.89 ± 0.05	0.74 ± 0.12

[(a)] BYPO agar plates supplemented with a 10 % (v/v) borage oil emulsion prepared in 10 % (w/v) Arabic gum solution
[(b)] Activity expressed in U/mg of enzyme. One unit of lipase activity (U) is defined as the amount of enzyme that releases 1 µmol of fatty acid from olive oil per minute of reaction at 37°C.
[(c)] Submerged fermentation : 1 % glucose, 2 % peptone, 0.5% yeast extract, 0.1% $NaNO_3$, 0.1%, KH_2PO_4 0.05% $MgSO_4$. $7H_2O$ and 1 % of olive oil.
[(d)] Solid state fermentation: 10 g of wheat flour (40 % water w/w) moistened with 10 mL of the liquid medium described above

Twenty-five percent of the tested microrganisms produced detectable levels of lipase; this percentage of lipase-producing species may be regarded as high. Nevertheless, few studies have been carried out to exploit the capacities of these native microbial lipases for enrichment of PUFA by selective hydrolysis of unsaturated oil. To concentrate a specific fatty acid by selective hydrolysis, it is necessary to select lipases that act slightly on the ester of that

fatty acid, i.e., if there is a lipase that does not hydrolyse GLA esters, GLA will be concentrated in glycerides by hydrolyzing GLA containing oil with this lipase.

Lipases from different sources demonstrate different substrate specificities such as fatty acid (typoselectivity), position (regioselectivity) and stereospecificity. Most of the lipases are classified into two groups: sn-1,3 regiospecific, which mediate selective reactions at the positions sn-1 and sn-3 of the glycerol and glyceride system (e.g. lipases from *A. niger* and *R. javanicus*) and non-regiospecific lipases, which act on all three positions of triacylglycerol (e.g. lipases from *C. cylindracea* and *G. candidum* (20). *G. candidum* produces a lipase that demonstrates pronounced specificity for the hydrolysis of esters of a particular type of long-chain fatty acid with a *cis* double bond at C9 (14).

In the next experiment, we compared the properties of these native enzyme preparations with those of two commercially available lipases, *Candida cylindracea* (Sigma) and *Aspergilus niger* (Aldrich), since the lipase from *Candida cylindracea* has been found to be suitable for PUFA concentration in glycerides by the hydrolysis reaction (27, 17). During the enzymatic hydrolysis reaction of the borage oil by the selective lipases, the concentrations of the TG, DG, MG and FFA were monitored. Figure 1 shows the acylglycerols and FFA contents after 24 h incubation for each lipase employed in the hydrolysis reaction.

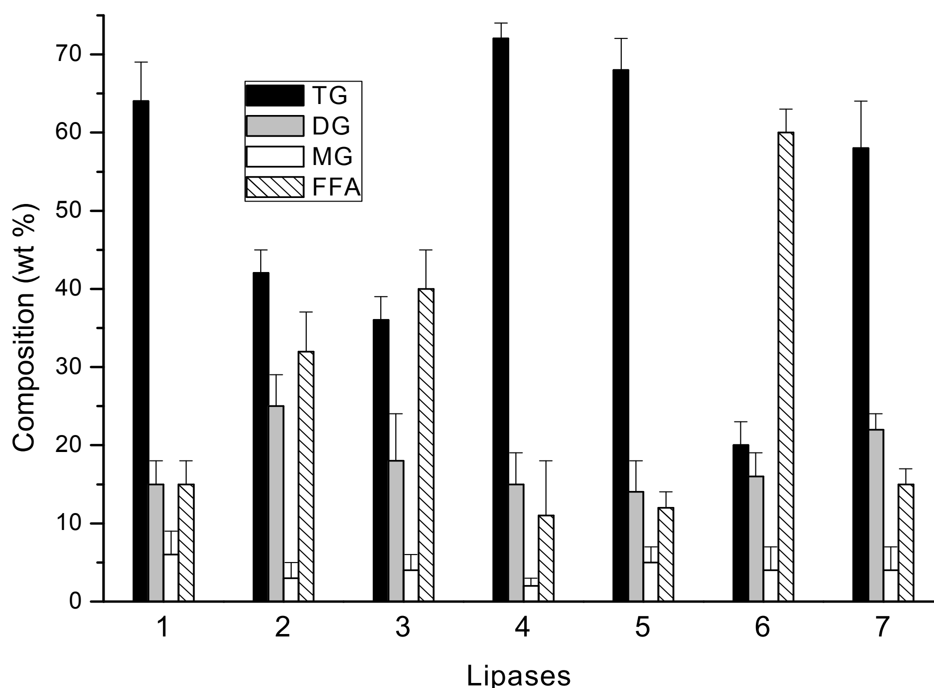

Figure 1. Content of the acylglycerols (TG, DG and MG) and FFA, derived by hydrolysis of borage oil with selected lipases. 1. *A. niger*, 2. *F oxysporum*, 3. *G. candidum*, 4. *P. solitum*, 5. *R. javanicus*, 6. *C. cylindracea* (Sigma), 7. *A. niger* (Aldrich). Reaction conditions are described in Materials and Methods

As expected, the commercial *C. cylindracea* lipase presented a high potential to hydrolyze the acylglycerols, obtaining a maximum of 60 % of FFA. Efficient results were also obtained with native *G. candidum* and *F. oxysporum*

lipases used as biocatalysts (40 and 32 % of FFA, respectively). Poor results (less than 15% of FFA) were obtained with the lipases from *A. niger* (commercial and native), *P. solitum* and *R. javanicus*. The ability of the lipases to hydrolysis borage oil was higher for non-specific lipases (commercial *C. cylindracea* and native *G. candidum*), compared to 1,3-specific lipases (*A. niger* and *R. javanicus*), which is in agreement with other studies (5, 17).

The compositions of the individual fatty acids in the acylglycerols (TG, DG and MG were analyzed as the only collective fraction) and FFA fractions are summarized in Table 2.

Table 2. Fatty acid composition (%) in the acylglycerols and FFA fractions derived by hydrolysis of borage oil with native and commercial lipases.

	Fatty acid composition (%) in acylglycerols fraction [(a)]								
	16:0	18:0	18:1	18:2	18:3	20:0	20:1	24:0	Enrichment of GLA
Original oil	12.8	4.7	17.5	36.6	21.5	0.4	4.6	1.8	---
Native lipases [(b)]									
Aspergillus niger	10.5	4.1	13.3	37.1	29.7	0.3	3.3	1.7	1.4
Fusarium oxysporum	7.9	3.8	15.6	35.4	30.8	0.4	4.5	1.6	1.4
Geotrichum candidum	5.1	2.7	12.7	37.1	39.5	0.2	2.3	1.4	1.8
Penicillium solitum	15.2	7.5	15.9	33.1	20.4	0.5	4.9	2.5	---
Rhizopus javanicus	11.8	4.8	17.4	36.8	22.3	0.6	4.4	1.9	---
Commercial lipases									
Aspergillus niger	6.7	3.3	14.7	36.3	32.4	0.2	4.6	1.5	1.5
Candida cylindracea	6.4	3.2	15.5	36.4	42.3	0.1	3.3	1.4	1.9
	Fatty acid composition (%) in FFA fraction[(a)]								
	16:0	18:0	18:1	18:2	18:3	20:0	20:1	24:0	Enrichment of GLA
Native lipases [(b)]									
Aspergillus niger	14.4	4.9	19.9	35.8	16.5	0.9	5.7	1.9	---
Fusarium oxysporum	15.9	6.5	19.1	35.3	14.7	0.9	5.5	2.1	---
Geotrichum candidum	19.2	8.3	17.8	32.8	13.7	0.8	5.9	1.5	---
Penicillium. solitum	8.0	3.4	18.3	37.9	25.8	0.5	4.4	1.7	1.2
Rhizopus. japonicus	14.2	5.8	17.9	35.4	20.6	0.6	4.2	1.3	---
Commercial lipases									
Aspergillus niger	14.7	6.3	19.0	33.2	19.3	0.5	4.5	2.5	---
Candida cylindracea	18.4	7.6	20.3	33.6	11.4	0.8	5.7	2.2	---

--- not detected. [(a)]16:0 Palmitic acid; 18:0 Stearic acid; 18:1 Oleic acid; 18:2 Linoleic acid; 18:3 Gamma-linolenic acid; 20:0 Arachidic acid; 20:1 Eicosenoic acid; 24:0 Lignoceric acid.

[(b)] The native lipases were produced in SmF and used as crude enzyme preparation, according to the description in materials and methods. The enzymes were tested using the same lipase activity (U/mg).

The selective hydrolysis of borage oil with *C. cylindracea* (commercial) and *G. candidum* (native) lipases led to an significant increase in the GLA content from 21.5 % in the original oil to approximately 40 % in the unhydrolysed acylglycerols (1.8 - 1.9-fold enrichment). It is also evident that the GLA enrichment was accompanied by a decrease in the levels of palmitic (16:0), stearic (18:0) and a moderate decrease of oleic acid (18:1) in the unhydrolysed acylglycerols. For both lipases, linolenic acid (18:2) and GLA (18:3) were detected in the acylglycerols fraction in higher quantities, in comparison to the fraction of FFA, showing its preferentially release of saturated fatty acids from borage oil.

Hydrolysis with *A. niger* (commercial and native) and *F. oxysporum* lipases also enriched GLA in the unhydrolysed acylglycerols (1.4 - 1.5-fold enrichment); however, these lipases were not as effective as *C. cylindracea* and *G. candidum* lipases. On the other hand, *P. solitum* and *R. javanicus* lipases did not increase in the GLA contents in the unhydrolysed acylglycerols. As shown in Figure 1, the hydrolysis by the last two lipases was almost the same (approximately 12 % of FFA released). However, the fraction containing FFA obtained by *P. solitum* lipase-mediated hydrolysis showed a high content of unsaturated fatty acids (86%), obtaining a 1.2-fold enrichment of GLA. All other lipases showed selectivity to saturated fatty acids and preferentially released saturated fatty acids.

The ability of the lipase from commercial *C. cylindracea* to enrich GLA by selective hydrolysis of evening primrose and borage seed oil was previously reported (27). Many other reports confirm that *C. cylindracea* lipase is the most effective for concentrating other PUFAs in glyceride, such as arachidonic acid in oil extracted from *Mortierella* (22) and DHA in fish oil (28, 29). In this study, good results were also obtained with native *G. candidum* lipase (1.8-fold enrichment) used as biocatalyst for borage oil hydrolysis in the unhydrolysed acylglycerols fraction.

Figure 2 shows that the maximum enrichment of the GLA in the acylglycerols fraction occurs at between 20 and 24 hours of reaction, when catalyzed by native *G. candidum* lipase. Under these conditions, an enrichment of 39.5% of GLA was achieved when the extract produced in SmF was used as a biocatalyst of the reaction. In this time, only 13.7% of GLA was found in the FFA fraction. A further increase in reaction time leads to a decrease in the extent of enrichment of GLA. In general, lipase yields obtained for *G. candidum* cultured in 500mL flasks in SmF were similar to those obtained in 2 l flasks (data not shown). When the extract produced in SSF underwent the same procedure, a maximum of 28% of GLA was found after 24 h of reaction.

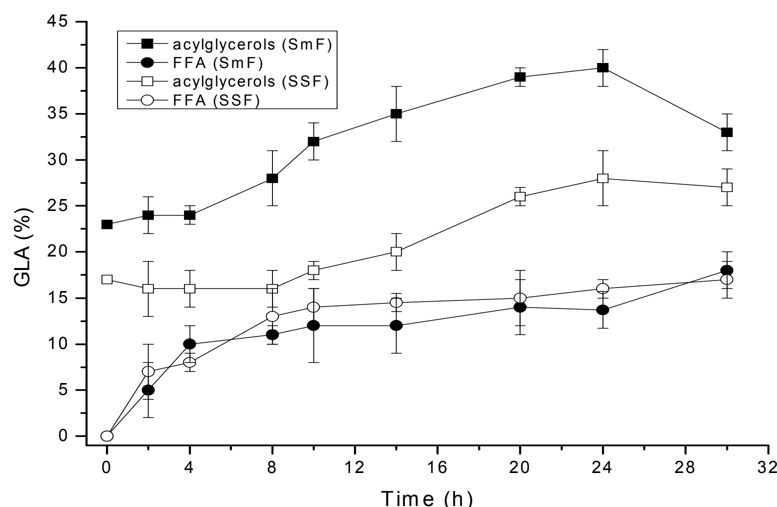

Figure 2. Change in GLA content in the acylglycerols (TG, DG and MG) and FFA fractions during the course of hydrolysis of borage oil, catalyzed by native *G. candidum* lipase produced in two different growing media. Reaction conditions are described in Materials and Methods

This study shows also the effect of culture medium composition on the ability of the *G. candidum* lipase to enrich GLA. The different activity and optimal fermentation conditions of the substrates could be explained by the different digestibility of these carbon sources. However, activity and specificity studies of crude extracts can be complicated by the presence of more than one lipase. Some differences in the substrate specificity of various strains were observed, suggesting that the differences between the crude lipases could originate from either different isoforms or, more likely, from different ratios of these isoforms in various preparations (24). Although many studies show than *G. candidum* lipase features specificity towards fatty acids with a *cis* double bond at C9 (14), subsequent attempts to purify and identify lipases from *Geotrichum* strains resulted in contradictory reports regarding their substrate specificity (24, 1, 26). Different forms of *Geotrichum* sp lipases were identified and described as differing in substrate specificities. Two forms (I and II) of this lipase show non-positional specificity, while the other two remaining forms (III and IV) showed unusual positional specificity; they cleaved the inside (2-position) ester bond of triolein at nearly twice the rate of the cleavage at the 1 (3)-position (26).

The use of crude lipase preparations from *G. candidum* for the enrichment of GLA in glycerides by hydrolysis of blackcurrant oil was recently reported (25). This recent study shows the effect of culture medium composition on the isoenzyme pattern of the lipases produced by selected strains of *Geotrichum* sp. The enzymes from *G. candidum* produced in Medium A (urea, olive oil and minerals) preferentially released unsaturated fatty acids from blackcurrant oil triacylglycerols, as well as extracellular lipases from *G. ludwiggi* and *G. candidum* produced in Medium B (peptone, glucose, olive oil and minerals). On the other hand, a higher content of unsaturated fatty acids was found in the acylclycerols fraction (TG, DG and MG) obtained from the hydrolysis of blackcurrant oil by extracellular lipase from *G. ludwiggi* induced in Medium A.

To decide on the optimal proportion between the amount of *G. candidum* lipase and borage oil content, the reaction was carried out for 24 h with 104.7 – 388.8 U/g of oil, as shown in Table 3. By increasing the content of the borage oil and, principally, increasing the concentration of the *G. candidum* enzyme, a higher enrichment of GLA was obtained in the acylglycerols fraction (~ 42% of total fatty acid).

Table 3. Effect of the ratio of oil and enzyme on the enrichment of GLA in the acylglycerols and FFA fractions after 24h of hydrolysis at 30º C with *Geotrichum candidum* lipase

Lipase (U/g oil)	**GLA (% total fatty acids)**	
	Acylglycerols	**FFA**
104.7[d]	35.2	15.4
300.0[a]	39.5	13.7
317.8[b]	41.7	14.6
388.8[c]	40.2	14.9

Quantity of borage oil and lipase: [a]12g and 0,83g; [b]14g and 1.03; [c]20g and 1.80; [d]26 g and 0.63.

Figure 2 and Table 3 demonstrate that the concentration of GLA in the FFA fractions does not present great changes, with its concentration remaining at around 15 %. Values of above 40% for the enrichment of GLA were obtained following catalysis by native *G. candidum* lipase, in accordance to the data reported in the literature for commercial lipases. The use of commercial *C rugosa* lipase resulted a 46% GLA-containing oil, by carrying out the hydrolysis of borage oil (GLA content, 22%) at 35ºC for 15h in a solvent-free system (23). A maximum GLA content of 51.7% in the unhydrolyzed acylglycerols was reported in the hydrolysis of borage oil (GLA content, 23.6 %) with immobilized *Candida rugosa* lipase in isooctane at 30ºC (13).

ACKNOWLEDGEMENTS

This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo of Brasil. The authors thank the Centro de Micologia da Universidade de Lisboa (Portugal) and Novozymes Latin America Ltda (Brasil).

REFERENCES

1. Asahara, T.; Matori, M.; Ikemoto, M.; Ota, Y. (1993). Production of two types of lipases with opposite positional specificity by *Geotrichum* sp FO 401B. Biosci Biotechnol Biochem 57:390-394
2. Budeiri D, Li Wan PA, Dornan CJ (1996) Is evening primrose oil of value in the treatment of premenstrual syndrome? *Control Clin Trials* 17: 60-68.
3. Cai, J.; Jiang, W.G.; Mansel, R.E. (1999). Inhibition of angiogenic factor- and tumour-induced angiogenesis by gamma linolenic acid. *Prostaglandins Leukot Essent Fatty Acids* 60:21-29.
4. Carvalho, P.O.; Pastore, G.M. (1998). Enrichment of Gamma-Linolenic acid from fungal oil by lipases. *Food Biotechnol* 12:57-71.
5. Carvalho, P.O.; Campos, P.R.B.; Bastos, D.H.M.; Oliveira, J.G. *et al* (2002). Enzymic Enhancement of ω3 Polyunsaturated Fatty Acids Content in Brazilian Sardine Oil. *Acta Farm Bonaerense* 21: 85-88.
6. Carvalho, P.O.; Campos, P.R.B.; Noffs, M.D.; Oliveira, J.G. *et al* (2003). Aplicações de lipases microbianas na obtenção de concentrados de ácidos graxos poliinsaturados. *Quim Nova* 26:75-80.
7. Chawla, P.; DeMan, J.M. (1990). Measurement of the Size Distribution of Fat Crystals Using a Laser Particle Counter. *J Am Oil Chem Soc* 67:329-332.
8. Domsch, K.H.; Gams, W.; Anderson, T.H. (1993). Compendium of Soil Fungi v1 Eching, IHV-Verlag, Germany.
9. Dos Prazeres, J.N.; Cruz, J.A.B.; Pastore, G.M. (2006). Characterization of alkaline lipase from *Fusarium oxysporum* and the effect of different surfactants and detergents on the enzyme activity. *Braz J Microbiol* 37:505-509.
10. Dokholyan, S.R.; Albert, C.M.; Appel, J.L.; Cook, R.N. *et al.* (2003). A Trial of Omega-3 Fatty Acids for Prevention of Hypertension. *Am J Cardiol* 93:1041-1043.
11. Eriksen, B.B.; Kare, L.D. (2006). Open trial of supplements of omega 3 and 6 fatty acids, vitamins and minerals in atopic dermatitis. *J Derm Treat* 17:82-85.
12. Hoshino, T.; Sasaki, T.; Watanabe, Y.; Nagasawa, T. *et al.* Purification and some characteristics extracellular lipase from *Fusarium oxysporum* f sp Lini. *Biosci Biotechnol Biochem* 56:660-664.
13. Huang, F.C.; Ju, Y.H.; Huang, C.W. (1997). Enrichment of γ-Linolenic Acid from Borage Oil via Lipase-Catalyzed Reactions. *J Am Oil Chem Soc* 74:569-573.
14. Jensen, R.G. (1983). Detection and determination of lipase (acylglycerol hydrolase) activity from various sources. *Lipids* 18:650-657.
15. Mase, T.; Matsumiya, Y.; Akiba, T. (1995). Purification and characterization of a new lipase from *Fusarium* SP YM-30. *Biosci Biotechnol Biochem* 59:1771-72.
16. Nakayama, K. (1981). Sources of industrial microorganisms. In: Rehm HJ, Reed G (ed) Biotechnology VCH Verlag, Veinheim, vol 1, pp 355-410.
17. Okada, T.; Morrissey, M.T. (2007). Production of n-3 polyunsaturated fatty acid concentrate from sardine oil by lipase-catalyzed hydrolysis. *Food Chem* 103:1411-1419.
18. Pejic, N.R.; Lee, T.D. (2006). Hypertriglyceridemia. *J Am Board Fam Med* 19:310-316.
19. Ratnayake, W.M.N.; Olsson, B.; Matthews, D.; Ackman, R.G. (1988). Preparation of Omega-3 PUFA Concentrates from Fish Oils via Urea Complexation. *Wood Sci Technol* 90:381-386.
20. Saxena, R.K.; Ghosh, P.K.; Gupta, R.; Sheba Davidson, W.S.; Bradoo, S.; Gulati, R. (1999). Microbial lipases potential biocatalysts for the future industry. *Curr Sci* 77:101-115.
21. Shimada, Y.; Maruyama, K.; Okazaki, S.; Nakamura, M. *et al.* (1994). Enrichment of polyunsaturated fatty acids with *Geotrichum candidum* lipase. *J Am Oil Chem Soc* 71:951-954.
22. Shimada, Y.; Sugihara, A.; Maruyama, K.; Nagao, T. *et al*. (1995). Enrichment of arachidonic acids: selective hydrolysis of a single-cell oil from *Mortierella* with *Candida cylindracea* lipase. *J Am Oil Chem Soc* 72:1323-1327.
23. Shimada, Y.; Fukushima, N.; Fugita, H.; Honda, Y. *et al*. (1998). Selective Hydrolysis of Borage Oil with *Candida rugosa* Lipase: Two Factors Affecting the Reaction. *J Am Oil Chem Soc* 75:1581-1586.
24. Sidebottom, C.M.; Charton, E.; Dunn, P.P.; Mycock, G. *et al*. (1991). *Geotrichum candidum* produces several lipases with markedly different substrate specificities. *Eur J Biochem* 202:485-491.
25. Stránský, K.; Zarevúcka, M.; Kejík, Z.; Wimmer, Z. *et al*. (2007). Substrate specificity, regioselectivity and hydrolytic activity of lipases activated from *Geotrichum* sp. *Biochem Eng J* 34:209-216.
26. Sugihara, A.; Shimada, Y.; Nakamura, M.; Nagao, T. *et al*. (1994). Positional and fatty acid specificities of *Geotichum candidum*. *Protein Eng* 7:585-588.
27. Syed Rahmatullah, M.S.K.; Schukla, V.K.S.; Mukhergee, K.D. (1994). Enrichment of γ-Linolenic Acid from Evening Primrose Oil and Borage Oil via Lipase-Catalyzed Hydrolysis. *J Am Oil Chem Soc* 71:569-573.

28. Tanaka, Y.; Hirano, J.; Funada, T. (1992). Concentratiom of docosahexaenoic acid in glyceride by hydrolysis of fish oil with *Candida cylindracea* lipase. *J Am Oil Chem Soc* 69:1210-1214.
29. Tanaka, Y.; Funada, T.; Hirano, J.; Hashizume, R. (1993). Triglyceride specificity of *Candida cylindracea*: Effect of docosahexaenoic acid on resistance of triglyceride to lipase. *J Am Oil Chem Soc* 70:1031-1034.
30. Walker, R.O. (1978). Official and Tentative of the American Oil Chemists' Society - Method Ce 2-66, American Oil Chemists' Society, 3rd ed, Champaign.
31. Wille, H.J.; Wang, J. (1996). Enzymatic enrichment of γ-Linolenic acid from blackcurrant seed oil. In: Huang YS, Mills DE (ed) γ-Linolenic, metabolism and its roles in nutrition and medicine, AOCS Press, Champaign, pp 33-41.
32. Yin, J.Z.; Wang, A.Q.; Wei, W.; Liu, Y. *et al*. (2005). Analysis of the Operation Conditions for Supercritical Fluid Extraction of Seed Oil. *Sep Purif Technol* 43:163-167.