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ENHANCEMENT OF GAMMA-LINOLENIC ACID PRODUCTION BY THE FUNGUS *MUCOR* SP LB-54 BY GROWTH TEMPERATURE

Patrícia de Oliveira Carvalho[1*]; Joaquim Gilberto de Oliveira[2]; Gláucia Maria Pastore[2]

[1]Faculdade de Ciências Farmacêuticas, Universidade São Francisco, Bragança Paulista, SP, Brasil.
[2]Departamento de Ciência de Alimentos, Faculdade de Engenharia de Alimentos-FEA, Universidade Estadual de Campinas-UNICAMP, Campinas, SP, Brasil.

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ABSTRACT

As a relatively prolific producer of GLA, the strain of *Mucor* sp LB-54 was selected for a study at different growth temperatures in shaker flask culture. The strain used in our experiment was capable to accumulate a relatively high amount of intracellular lipid, 20.73 % of dry cell weight, and GLA content of 15 % of total fatty acids after 5 days of incubation at 28°C. As the growth temperature was decreased from 28 to 12°C the percentage of GLA increased from 15 to 24 % of total fatty acids. In order to optimize the culture conditions for rapid biomass production and lipid production with a high proportion of GLA, the fungus was grown at two temperature combinations associated supplies of carbon source (glucose) in the culture medium. Maximal production of GLA (74 mg/l) was obtained from the *Mucor* sp LB-54 strain after 5 days of incubation at 28°C in basal medium following glucose addition (7 % w/v) and incubation for an additional 3 days at 12°C. The identity of GLA found in the strain of *Mucor* sp LB-54 was confirmed by the coupled gas chromatography-mass spectrometry

Key words: Gamma-linolenic acid, *Mucor* sp, unsaturated fatty acids

INTRODUCTION

Gamma-linolenic acid (GLA, 6,9,12-octadecatrienoic acid) is an important intermediate in the biosynthesis of biologically active prostaglandin from linolenic acid. GLA has been reported to be effective for the prevention or curing of cardiovascular diseases (10), hyper-cholesterolemia (11), menstrual disorders (16), for applications in curing certain skin-related (19), as well as a variety of other diseases (3).

At the present time, GLA is commercially produced from the seeds of evening primrose (*Oenothera biennis*) and boragem (*Borago oficinallis*). However, the productivity of GLA from the seed oil is extremely low, since both a long period and a huge area for harvesting seed are required (8). To overcome these problems, microorganisms have been investigated as an alternative GLA source and some suitable strains have been proposed. *Mortierella ramanniana* (9), *Mucor* sp (21), *Cunninghamella japonica* (7), and *Entomophthora exitalis* (13) were reported as perspective GLA producers. Currently, only Japan is producing GLA commercially, using the fungus *Mortierella* (17).

There is sufficient information in the literature

* Corresponding author. Mailing address: Rua Santo Monte, 65, Jardim Salessi, CEP 129000-000, Itatiba, SP, Brasil. Fax: (+5511) 7806-8040

on the fatty acid composition of fungi to warrant a statement that Phycomycetes are characterized in their ability to synthesize GLA, whereas the members of Ascomycetes and Basidiomycetes, with a few exceptions, produce alfa linolenic acid (20). Most of these investigations were carried out to determine the fatty acid composition of different fungi and to compare them with other groups of organisms to obtain information on their phylogenetic relationships.

In the preceding paper (5) we reported a strain of *Mucor* sp screened in our laboratory would be a promising producer of GLA. Therefore, further works on the optimization of fermentation process are needed in order to increase the productivity of GLA. The temperature is the principal regulation factor of the degree of unsaturated in the lipids of this organism (4). In many species of fungi there is a pronounced influence of growth temperature on the biosynthesis of unsaturated fatty acids. In general, organisms grown under low temperature conditions possess a relatively high degree of unsaturation in their lipids, presumably as a part of the adaptive response to the cold environment (15).

This paper deals with the effects of growth temperature for the fungal growth, lipid and GLA contents in cellular lipid of *Mucor* sp LB-54 with an emphasis on GLA productivity.

MATERIALS AND METHODS

Strain

The fungal strain used in this study was designated *Mucor* sp LB 54 by Biochemistry Laboratory at State University of Campinas, Brazil (5). The pure cultures of fungi were stored on PDA slants and kept at 4°C until used.

Medium and culture conditions

The basal medium for fungal growth and GLA production is the same as in the previous paper containing glucose, 20g/l and yeast extract, 10g/l (5). 50ml Erlenmeyer flask containing 25ml of culture medium was inoculated with spore suspension at the final concentration of 7.10^7 spores per millilitre of cultivation medium. The initial pH of the medium was adjusted to 7.0 and liquid cultures were grown on a rotative shaker at 120 strokes per min for 1- 15 days. To study the effects of growth temperature on GLA production, mycelia were incubated at each temperature ranging from 5 to 40°C.

Measurement of dry cell weight and total lipid

The mycelia fungi were separated by centrifugation (15,000 rpm for 15 min at 10°C) and the harvested fungi were washed twice with destined water. The dry cell weight was determined by drying the cells with acetone and in a vacuum oven to constant weight at 40°C. Before extraction of the lipids, the biomass was pulverized. The lipids were extracted from the dried biomass with chloroform/ methanol/water (2).

Analysis of fatty acid composition

The lipids were saponified with 0.5M NaOH and esterified with methanol-BF_3 (1). The fatty acids methyl esters (FAME) were analyzed by gas chromatography in a Chrompack CG instrument equipped with flame ionisation detectors (FID). The separations were carried out on a 50m × 0.25mm fused silica WCOT CP-Sil 88 capillary column (Chrompack, Holland) using temperature programme of 180-220°C, 5°C/min; hydrogen was used as carrier gas. FAME were identified by comparing retention times with those of authentic standards (Sigma Chemical Co.) and determined by relative percentage. GLA was confirmed by the coupled gas chromatography-mass spectrometry (Hewlett-Packard 5890).

RESULTS AND DISCUSSION

In Fig. 1 the data obtained for the determination of growth of *Mucor* sp LB-54 at temperature ranging from 5 to 40°C are summarized. The results show that *Mucor* sp LB-54 is a slightly mesophilic fungus and that the minimum, optimum and maximum temperatures for growth are 8, 28 and 38°C respectively. It was also noted that the cultures grown at 28°C achieved maximum growth within 5 days, whereas at temperatures above or below this optimum, it took 10 days or more.

For further investigation on the biomass and lipid content, growth temperatures of 12, 28 and 38°C were selected since growth was negligible below 10°C and above 38°C. The culture was incubated for 5 days and then the production of GLA was studied. The data in Table 1 show the strain of *Mucor* sp LB-54 produced relatively high cell weight and total lipids when grown at 28°C. The yield of lipids was 20.7 % of dry cell weight of the culture, which mean the maximal concentration of 44 mg of GLA per liter of culture medium. Although the lipids of culture grown at 12°C showed the highest GLA

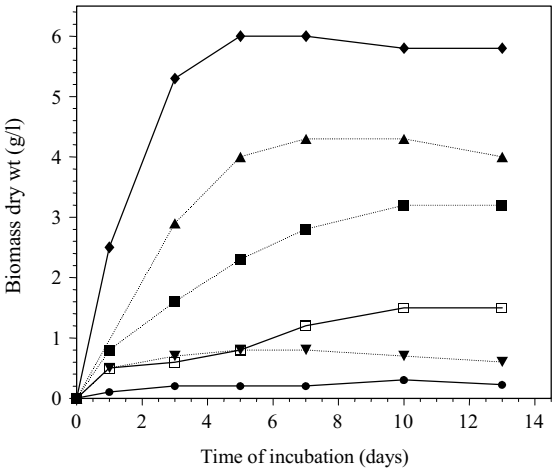

Figure 1. Growth of *Mucor* sp LB-54 on basal medium as a function of time at temperatures ranging from 5 to 45°C. The incubation temperatures (°C) are: 5 = ●—●, 8 = □—□, 12 = ■---■, 28 = ◆—◆, 38 = ▲---▲, 45 = ▼---▼. Each point is the mean of three repetitions.

content, about 24 % of total fatty acids, that seem not suitable for the production of GLA because of low lipid yield (15.8 % of dry cell weight).

The degree of unsaturation in the fatty acid composition is known to be influenced by temperature, i. e. when the growth temperature is lowered the proportion of unsaturated acids tend to increase. An earlier study with fungus has shown that there was increased production of GLA at low growth temperature with a corresponding increase in the degree of unsaturation of total lipids (6).

The temperature did not only influence growth and lipid production; it also affected the cell morphology. At 38°C big fluffy pellets were formed (10 mm in diameter) during the first 24 h, and then the pellets were transformed into mycelia that tended to clump. The mycelium formation was avoided by running the cultures at 28°C and 12°C, where stable pellets were formed; the pellets decreased in diameter with decreasing temperature (0.5 mm and 0.3 mm, respectively). This minute size of pellets would give a great advantage in the large scale and high-density cultivation of fungi when the mass transfers problems of filamentous fungal growth.

The Fig. 2 shows the time course of the main fatty acids contents in the total lipid fraction of *Mucor* sp LB-54 grown at 12, 28 and 35°C. The major fatty acids common to three growth temperatures and incubation periods were palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and GLA (18:3). A small part, about 2 – 4 % of the lipid fraction consisted of other fatty acids, myristic acid (C14:0), palmitoleic acid (C16:1) and stearic acid (18:0). The analysis of fatty acids revealed an insignificant content of fatty acids with an odd number of carbon atoms (< 1 %) and the complete absence of the alfa –isomer (alfa linolenic acid). It is also evident that at the lower temperature there was an increase in the amount of linoleic acid and GLA with a corresponding decrease palmitic acid and oleic acid. A temperature decrease to 12°C resulted in an increase in percent composition of GLA for 24 % of total fatty acids. The percent composition of other fatty acids did not vary appreciably under the influence of different temperatures.

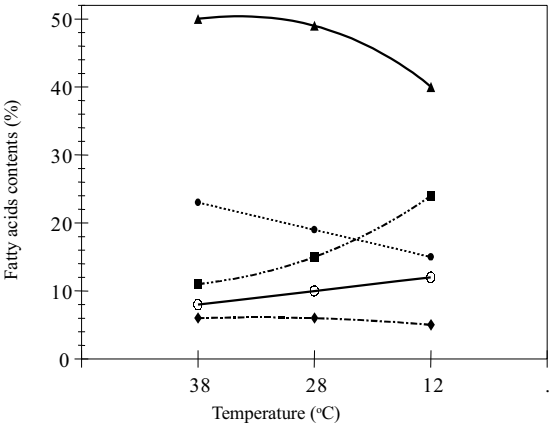

Figure 2. Changes the main fatty acids contents in lipid produced by *Mucor* sp LB-54 as a function of growth temperature. The fatty acids contents were expressed relative percentage of total fatty acids. Palmitic acid (16:0) = ●----●, Stearic acid (18:0) = ◆----◆, Oleic acid (18:1) = ▲—▲, Linoleic acid (18:2) = ○—○, γ-Linolenic acid (18:3) = ■----■. Each point is the mean of three repetitions.

Table 1. Effect of growth temperature on total lipid, biomass contents and production of GLA by *Mucor* sp LB-54.

Temperature (°C)	Biomass (g/l)	Lipid (g/l)	Total lipid/dry weight (%w/w)	GLA/total fattyacids (%w/w)	GLA (mg/l)
12	2.47	0.39	15.84	24.02	23.40
28	5.83	1.21	20.73	14.68	43.97
38	4.29	0.49	11.42	11.66	22.90

The results of this study show that the lower growth temperatures simulate the biosynthesis of highly unsaturated fatty acids, a phenomenon that has already been observed for some mesophilic and psychrophilic *Mucor* species (22). The effects of temperature on the degree of lipid unsaturation may be exerted through the influence of temperature on oxygen tension of the media. Oxygen is a necessary cofactor in enzymatic desaturation, resulting in lower levels of unsaturated fatty acids as temperature increases (15). The conversion of saturated into unsaturated fatty is known to be regulated by desaturase enzymes which require oxygen as a cofactor together with acetyl coenzyme A (acetyl CoA), acyl carrier protein (ACP), reduced nicotinamide adenine dinucleotide ($NADH_2$) and reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$) (13). Accumulation of unsaturated fatty acids at low temperature could to be a resources used for the increased biosynthesis of GLA by *Mucor* sp LB-54.

To confirm the GLA peak obtained from the cellular lipids of *Mucor* sp LB-54, a mass spectrometric analysis was applied. Fig. 3 shows a molecular ion peak at m/e 292 and intense fragment ion peak at m/e 93, 79, 67 and 41. Each peak is in good accord with the corresponding one of the authentic standard.

In order to combine the beneficial effects of rapid biomass production and a high production of GLA by *Mucor* sp LB-54, we tested the effect at two temperature combinations associated additional of glucose. Table 2 shows the results of the five culture conditions in shaker culture on total lipid, biomass contents and GLA production of *Mucor* sp LB-54.

When the culture was started at 28°C and the temperature was changed to 12°C, the proportions of GLA of total fatty acids increased (15.90 %), moreover, occurs the depletion tendency of lipid with culture time (Condition 2). This agree with the others reported (14, 23) indicating that the accumulated lipids are used as a carbon and energy supply by fungi when glucose to become exhaustion.

On the other hand, cultures with glucose added was possible to achieve both a high final lipid content and a reasonably good production of GLA by *Mucor* sp LB-54. Optimal production of GLA was obtained by incubating first for 5 days at 28°C in a basal

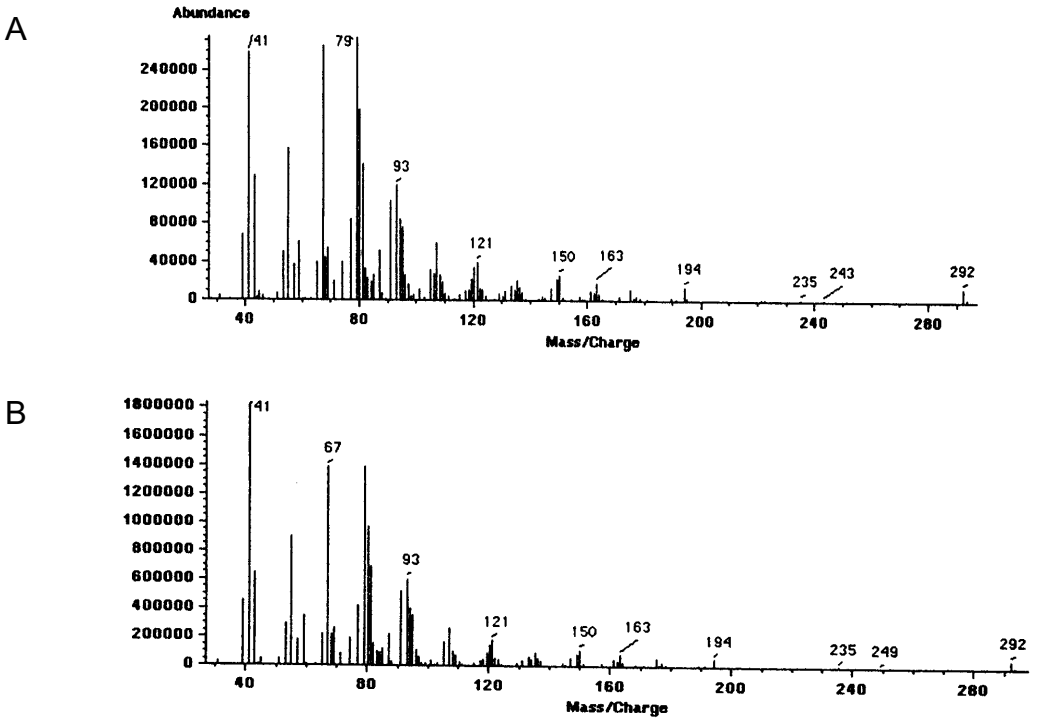

Figure 3. Mass spectra of authentic standards of GLA (A) and C18:3 acid methyl ester from cellular lipids of strain *Mucor* sp LB-54 (B).

Table 2. Effect of growth temperature and glucose addition on total lipid, biomass contents and production of GLA by *Mucor* sp LB-54

Incubation conditions	Biomass (g/l)	Lipid (g/l)	Total lipid/ dry weight (%w/w)	GLA/ total fatty acids (%w/w)	GLA (mg/l)
1	5.83	1.21	20.73	14.68	43.97
2	6.03	0.96	15.71	15.90	37.34
3	5.47	1.28	23.47	17.17	54.69
4	6.27	1.57	25.07	19.40	74.10
5	6.33	1.22	19.15	18.35	55.33

1: 5 days at 28°C in basal medium
2: 5 days at 28°C in basal medium following incubation for 3 days at 12°C (not addition glucose)
3: 5 days at 28°C in basal medium following glucose addition (3% w/v) and incubation for 3 days at 12°C.
4: 5 days at 28°C in basal medium following glucose addition (7% w/v) and incubation for 3 days at 12°C.
5: 5 days at 28°C in basal medium following glucose addition (10% w/v) and incubation for 3 days at 12°C.

medium and then supplementing with extra glucose (7 % w/v) followed by additional incubation for 3 days at 12°C (Condition 3). Using a relatively high initial growth temperature for biomass production, and glucose feeding followed by a temperature shift to 12°C, GLA production by *Mucor* sp LB-54 was enhanced to 74 mg of GLA per liter of culture medium, corresponding to 1.7 fold enrichment of GLA. This is mainly due GLA content of 19.40 % of the total fatty acids associated the increased to the content of lipid 25.07 % in dry cell which is higher than the baseline cultures (Condition 1).

Certain species of *Mucor* are capable of producing relatively large quantities of GLA. For example, *Mucor circinelloides* grown at 30°C with acetic acid as carbon source produced 90 - 120mg GLA l^{-1} (18) and strain of *Mucor* sp KCTC 8405P isolated in Korea cultured on 3% glucose and 0.1% $(NH_4)_2SO_4$ produced about 14 % (w/w) of GLA in total lipids (12).

With this strategy, we were able to stimulate GLA production by of *Mucor* sp LB-54 from the baseline level of about 44 mg l^{-1} to 74 mg l^{-1}. The data presented in this paper show the significant influence which incubation temperature and supply glucose exerts on the GLA production by *Mucor* sp LB-54. Presumably, it is not the temperature, as such, that affects the unsaturated fatty acids but the solubility of O_2, which increases at decreasing temperatures (13). These results suggest that *Mucor* sp LB-54 may have potential for commercial development for the production of GLA by fermentation techniques.

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RESUMO

Aumento da produção de ácido gama linolênico por fungo *Mucor* sp LB-54 de acordo com a temperatura de cultivo

A linhagem de *Mucor* sp LB-54, considerada uma potencial produtora de ácido gama-linolênico (GLA), foi selecionada para o estudo de diferentes temperaturas de cultivo em agitador rotativo. A linhagem usada neste experimento era capaz de acumular uma quantidade alta de lipídeos intracelulares, 20,73 % do peso seco de biomassa e conteúdo de GLA de 15 % dentre os ácidos graxos totais de sua constituição após 5 dias de incubação à 28°C. Quando a temperatura de cultivo foi diminuída de 28ºC para 12°C, o conteúdo de GLA aumentou de 15 para 24% dentre os ácidos graxos totais de sua constituição. Com o objetivo de otimizar as condições de cultivo para a produção rápida de biomassa e produção de lipídeos contendo conteúdo alto de GLA, o fungo foi cultivado em duas combinações de temperaturas associadas com a suplementação de fonte de carbono (glicose). A produção máxima de GLA (74mg/l) pela linhagem de *Mucor* sp LB-54 foi obtida após 5 dias de incubação à 28°C em meio base, seguida da adição de glicose (7% p/v) no meio de cultura e uma posterior incubação por mais 3 dias a 12°C. A identidade do GLA foi confirmada pelo sistema acoplado cromatógrafo à gás – espectrômetro de massa.

Palavras-chave: ácido gama-linolémico, *Mucor* sp, ácidos graxos insaturados

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