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Full Length Research Paper

Fatty acid production by four strains of *Mucor hiemalis* grown in plant oil and soluble carbohydrates

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Four *Mucor hiemalis* strains (M1, M2, M3 and M4), isolated from soil at a depth of 0 - 15 cm in the Juréia-Itatins Ecology Station (JIES), in the state of São Paulo, Brazil and were evaluated for the production of γ-linolenic (GLA) and other unsaturated fatty acids. Five growth variables (temperature, pH, carbon source, nitrogen source, and vegetable oils) were studied. Liquid media containing 2% vegetable oil (palm oil, canola oil, soybean oil, sesame oil, or sunflower oil) or 2% carbohydrate (fructose, galactose, glycerol, glucose, lactose, maltose, sucrose, sorbitol or xylose) and 1% yeast extract as a nitrogen source were used. The greatest biomass production was observed with M3 and M4 strains in palm oil (91.5 g l^{-1}) and sunflower oil (68.3 g l^{-1}) media, respectively. Strain M4 produced greater quantities of polyunsaturated acids in medium containing glucose. The GLA production in the M4 biomass was 1,132.2 mg l^{-1} in glucose medium. Plant oils were inhibitors of fatty acid production by these strains.

Key words: Fatty acid production, *Mucor hiemalis,* polyunsaturated acids, γ-linolenic acid.

INTRODUCTION

The Atlantic Rainforest represents one of the most degraded ecosystems in Brazil. Remaining forested areas have been declared protected areas to guarantee the preservation of living things residing there. One of these protected areas is the Juréia-Itatins Ecology Station (JIES), in the state of São Paulo, Brazil. Previous studies have documented the occurrence of filamentous fungi in the soil in primary and secondary forests in the JIES, and the potential of the isolated species to produce enzymes of significant industrial importance (Tauk-Tornisielo et al., 2005).

Zygomycetes are able to synthesize macromolecules, such as γ-linolenic acid (GLA), and accumulate them in large amounts within the hyphae (Carvalho et al., 2003). Some of the species synthesize molecules with 12 to 24 carbons, such as saturated fatty acids (palmitic acid) and unsaturated fatty acids (oleic and linolenic acids). The fungal biomass contains 5 to 32% lipids, depending on environmental conditions and developmental stage (Pupin et al., 2000).

Humans cannot synthesize essential fatty acids and must obtain them through the diet. GLA stands out among the fatty acids because of its numerous functions, including structural component of cellular membrane, formation of prostaglandin E1, control of the permeability of skin and possibly other membranes, and regulation of metabolism and cholesterol. As a precursor of prostaglandin, this acid is used in geriatrics treatment of premenstrual syndrome, prevention of osteoporosis, reduction of inflammatory processes, and reduction of blood pressure, among others. Although plants produce this acid, the microbial process is more rapid and does not require large areas for plant cultivation.

Several studies on fungi have shown that media variables affect their growth and lipid accumulation (Hansson and Dostalek, 1988; Weinstein et al., 2000; Aki et al., 2001; Kavadia et al., 2001; Xian et al., 2001). Other studies have demonstrated the potential to produce GLA at concentrations of 15 - 25% of total fatty acids by lower fungi from the order Mucorales, especially the species *Mortierella*, *Mucor* and *Cunninghamella*. It is possible to achieve biomass densities of more than 50 g l^{-1} in sub-

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merged culture (Ward and Singh, 2005). Studies of *Mortierella ramanniana* var. *ramanniana* indicated that GLA production could be maximized by using a basal growth medium consisting of 5% dextrose and 1% yeast extract, supplemented with Mn^{2+} (Dyal et al., 2005).

In the present study, fatty acid production of four *Mucor hiemalis* isolates in culture with vegetable oils or carbohydrates as substrate. The first part of the experiments was carried through in the automated system of growth in Bioscreen C, to select the best nitrogen and carbon sources for biomass production. To follow they had been used grown submerged cultures in media containing carbohydrate or vegetable oil supplemented with yeast extract as nitrogen source, to verify the best productions of biomass, protein and fatty acids. It was evaluated and probable genetic differences between these strains were determined with the analysis of polymerase chain reaction (PCR).

MATERIAL AND METHODS

Isolation and inocula

The *M. hiemalis* strains isolated from soil at a depth of 0 - 15 cm in the Juréia-Itatins Ecology Station (JIES), in the state of São Paulo, Brazil, were stored in a malt extract medium at 4°C in test tubes and labeled M1, M2, M3, and M4. Cultures inocula were prepared with spore suspensions containing approximately 10^6 to 10^7 spores ml^{-1}, counted in a Neubauer chamber, in a (0.85% NaCl) saline solution.

Fungi cultures

The *M. hiemalis* strains were cultured in liquid medium containing 2% oils (palm oil, canola oil, soybean oil that had been used for frying, sesame oil, or sunflower oil) or 2% carbohydrates (fructose, galactose, glycerol, glucose, lactose, maltose, sucrose, sorbitol or xylose), and 1% yeast extract as a nitrogen source in 250 ml Erlenmeyer flasks with 50 ml of culture medium. Each flask was inoculated with 1 ml freshly prepared spore suspension. The cultures were agitated continuously for 72 h at 150 rpm at 25°C, and for two more days without agitation at 12°C.

The biomass produced in submerged culture was separated from the medium using vacuum filtration through No.1 Whatman filter paper. The filtrate was washed thoroughly three times in distilled water under agitation to 150 rpm during 1 h and the remaining liquid was removed by placing the filtered biomass between sheets of dry filter paper (Ruegger et al., 2002). The biomass produced in media with vegetable oil was weighed while still humid in pre-weighed, dry crucibles and left to dry in an oven at 105°C for 72 h to determine the dry weight. Total nitrogen in the samples was determined using the Kjeldhal method (Alves et al., 1994). The results were expressed in g of total nitrogen per g of dry biomass. This procedure was carried out in triplicates (n = 3) for each type of medium and the mean growth was calculated.

Lipid extraction

Approximately 100 mg of mycelia were transferred to a stoppered amber test tube and rehydrated by adding 2.0 ml of distilled water. Triplicate standards of dry mycelium were prepared to assess reproducibility. To this suspension, 2.5 ml of chloroform and 5.0 ml of methanol (0.8:1.0:2.0) were added and kept 24 h with occasional shaking. The material was centrifuged at 4,000 rpm for 11 min and the supernatant was transferred to another amber test tube and 2.5 ml of chloroform and 2.5 ml of distilled water were added. This tube was agitated for 15 s and centrifuged at 1500 rpm for 30 min. The bottom layer containing chloroform was carefully removed with a Pasteur pipette and transferred to a pre-weighed test tube. The solvent was removed in a nitrogen atmosphere, and the test tubes were placed in a vacuum dryer until they reached a constant weight, after which they were weighed using an analytical scale. The procedure was adapted from Kates (1982) and Kennedy et al. (1993).

Analysis of fatty acids

Fatty acids were analyzed as methyl esters by gas chromatography. The lipid samples were dissolved in 1 ml of hexane in 10 ml stoppered amber test tubes to which 50 µl of 2 M sodium methanol hydroxide were added. This mixture was agitated for 30 s and left to rest for 30 min. One hundred mg of anhydrous calcium chloride were added, followed by centrifugation at 4,000 rpm for 8 min. The upper phase containing the methyl esters was injected into a gas chromatographer (Finnigan-Tremetrics, model 9001) in a free fatty acid polymerase (FFAP) column, 30 m mega bore, with 1 µm film, with the following specifications: hydrogen flow, 20 ml min^{-1}; nitrogen flow, 25 ml min^{-1}; synthetic air flow, 175 ml min^{-1}; hydrogen; working pressure, 4 psi; temperature of the injector, 210ºC; temperature of the detector, 250ºC; ratio of the sample, 10:1; temperature of the column, 50ºC for 3 min, with a heating slope of 40ºC per min up to 190ºC, and an additional 5ºC per min up to a maximum temperature of 220ºC, where it remained for 10 more min. Comparing the retention time of the sample components with their known methyl ester patterns identified the fatty acids. Samples containing different concentrations of standard fatty acids were prepared to calibrate the gas chromatograph and were injected under the same conditions as the samples obtained from the *M. hiemalis* strains.

Extraction of genomic DNA

An inoculum of each strain was prepared using a stirred suspension of spores in 0.1% Tween 80 solution. One ml was inoculated in 50 ml of liquid culture medium containing 2% glucose and 2% yeast extract in a 125 ml Erlenmeyer flask. The culture was incubated at 25ºC on a rotary shaker (150 rpm) for 7 d. The mycelium was filtered through No. 1 Whatman filter paper and washed thoroughly with distilled water to eliminate the remaining culture medium. 50 mg of the mycelium was macerated in a microcentrifuge tube until completely pulverized. DNA was extracted with TNES buffer (50 mM Tris HCl, pH 7.4, 100 mM sodium chloride, 2 mM EDTA and 1% sodium dodecyl sulphate, SDS) (Kates, 1982). 50 µl of the TNES solution, previously incubated in an ice bath for 5 min, were placed in a 1.5 ml Eppendorf microcentrifuge tube. Small fragments of biomass were added and macerated on ice. An additional 550 µl of TNES solution was added, followed by 3 µl of proteinase K solution (20 mg ml^{-1}). The suspension was incubated for 3 h at 55ºC.

The sample was cooled to room temperature (25 to 28ºC); 3 µl of RNAse A was added and incubated at 37ºC for 30 min. 200 µl of 5 M NaCL was added; the tube was vortexed for 20 s at high speed and centrifuged at 15,000 g for 5 min. The supernatant containing DNA was carefully transferred to another 1.5 ml Eppendorf tube containing 600 µl of 100% isopropanol and mixed. The sample was centrifuged at 15,000 g for 3 min, the supernatant was discarded, and 600 µl of 70% ethanol were added, mixed and centrifuged at 15,000 g for 3 min. The DNA pellet was left to dry at room tempe-

Table 1. Quantity of biomass, total nitrogen, and protein of the *Mucor hiemalis* strains in vegetable oils.

Vegetable oil	Strains	Dry biomass (g l^{-1})	Protein (g l^{-1})	Protein/ g of total biomass (%)
Canola	M1	52.4	27.6	52.8
Canola	M2	54.0	28.3	52.3
Canola	M3	46.0	19.8	42.9
Canola	M4	25.6	7.8	30.6
Palm	M1	60.0	25.3	42.1
Palm	M2	64.0	32.9	51.4
Palm	M3	91.5	19.3	21.2
Palm	M4	50.4	19.2	38.0
Soybean	M1	54.4	22.1	40.6
Soybean	M2	59.5	22.9	38.4
Soybean	M3	61.6	32.5	52.8
Soybean	M4	59.6	31.1	52.2
Sesame	M1	48.0	21.4	44.6
Sesame	M2	49.9	18.2	36.6
Sesame	M3	44.3	12.3	27.9
Sesame	M4	60.4	26.0	43.1
Sunflower	M1	51.4	26.1	50.8
Sunflower	M2	61.8	19.3	31.3
Sunflower	M3	49.2	28.8	46.6
Sunflower	M4	68.3	31.4	45.9

rature. 40 µl of TE buffer was added to the DNA pellet, and the DNA solution was stored at 5ºC.

RAPD reaction and electrophoresis in agar gel

For the RAPD reactions, a "Ready-to-go" kit (Pharmacia) was used. Each Ready-To-Go PCR Bead has stabilizer, deoxynucleotides, 1 - 1.5 units of Taq polymerase and buffer which when reconstituted to 25 ml gives 200 mm dNTP in 50 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris-HCl (pH 9.0 at room temperature). The reaction mixture contained 5 ng of DNA (final volume of 25 µl with addition of distilled water). PCR (Polymerase Chain Reaction) amplification consisted of 1 cycle of 3 min at 95°C, 1 min at 55ºC and 1 min at 72ºC, followed by 39 cycles at 95ºC, for 1 min at 55ºC, and extension at 72ºC for 1 min. The extension time for the last cycle was 5 min (Voigt and Wöstemeyer, 2001). The PCR products were subjected to electrophoresis in 2% agarose gel in TBE (0.45 M tris-borate, 0.001 M EDTA) buffer at 80 V, for 90 min. The gel was exposed to 1 µg ml^{-1} of ethidium bromide and photographed under UV light. Amplified DNA fragments (bands) were reproduced and categorized as 0 (fragments absent) or 1 (fragments present) in the matrix. The results of electrophoresis in gel were analyzed using the software Gel-Pro Express for Windows, which made it possible to analyze the DNA bands and determine their molecular weights.

RESULTS AND DISCUSSION

The strains of *M. hiemalis* were selected for more detailed analysis because they were known to produce γ-linolenic acid (GLA) (Ruegger et al., 2002). Despite belonging to the same species, the strains presented differences with respect to nutritional needs as well as potential to produce GLA. The results showed that the carbon source influenced the type of lipid produced, a fact that was also observed by Somashekar et al. (2002).

Using media containing vegetable as sole carbon source, high biomass values (dry weight) were observed. In decreasing order, they were: strain M3 in palm oil (91.5 g l^{-1}); M4 in sunflower oil (68.3 g l^{-1}); M2 in palm oil (64.0 g l^{-1}) or in sunflower oil (61.8 g l^{-1}); and M3 in soybean oil (61.6 g l^{-1}). The highest protein values were observed in strain M2 grown in palm oil (32.9 g l^{-1}), M3 grown in frying (soybean) oil (32.5 g l^{-1}), and M4 in sunflower oil (31.4 g l^{-1}), Table 1. However, when the Kruskal-Wallis test (Zar, 1999; Ayres et al., 2005) was applied to compare biomass production of the strains grown in media containing different vegetable oils, no statistically significant

Table 2. Production of unsaturated fatty acids by the *Mucor hiemalis* strains in different culture media containing vegetable oils.

Vegetable oil	Strains	Fatty acids (%)					Production of GLA/ total biomass (g l^{-1})
		16:0	16:1	18:1	18:2	18:3	
Canola	M1	23.5	4.5	39.3	15.4	13.6	7.4
Canola	M2	22.4	5.4	40.6	15.4	13.1	7.2
Canola	M3	19.4	5.2	34.2	14.0	12.8	6.9
Canola	M4	24.1	4.8	36.1	12.2	13.0	6.2
Palm	M1	21.0	4.6	40.3	16.1	14.1	6.5
Palm	M2	21.2	4.1	37.3	13.6	12.2	3.1
Palm	M3	23.0	4.1	37.3	13.6	12.2	7.8
Palm	M4	18.9	5.2	26.6	13.1	10.4	6.2
Soybean	M1	23.3	4.8	36.6	11.6	12.6	8.1
Soybean	M2	19.4	4.9	21.8	8.6	8.0	4.3
Soybean	M3	21.3	4.3	39.4	12.5	12.3	7.1
Soybean	M4	20.6	4.8	26.4	14.6	12.6	6.4
Sesame	M1	22.0	5.2	32.5	11.2	14.0	8.3
Sesame	M2	18.4	5.3	36.4	11.0	13.5	8.5
Sesame	M3	23.5	4.6	40.6	9.6	13.8	7.5
Sesame	M4	20.5	4.6	40.2	10.3	12.8	7.0
Sunflower	M1	23.1	4.8	39.0	9.2	11.4	6.8
Sunflower	M2	18.5	5.3	36.2	42.1	10.7	6.6
Sunflower	M3	22.1	5.1	39.1	9.8	8.9	6.0
Sunflower	M4	21.0	4.9	36.8	12.5	12.3	5.9

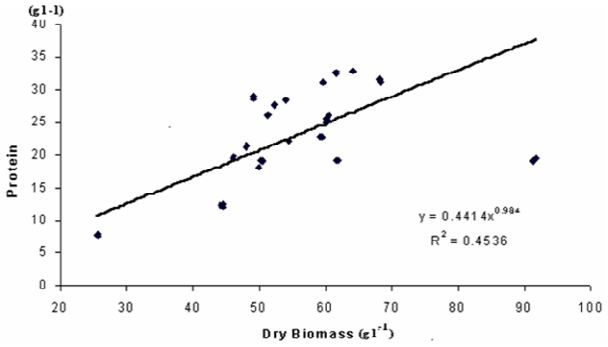

Figure 1. Adjusted regression curve for the dry biomass (independent variable) and protein (dependent variable).

Table 3. Results of the Kruskal-Wallis test (p-values) comparing fatty acid production by strains in media containing vegetable oil.

Fatty acid	(p) Kruskal-Wallis
palmitic acid	0.2670
palmitoleic acid	0.6879
oleic acid	0.4815
linoleic acid	0.5682
Γ-linoleic acid	0.4375

differences were found (p - Kruskal-Wallis = 0.794).

The greatest percentage of protein in relation to total biomass was obtained with M2 grown in palm oil and M4 grown in sunflower oil. The media containing palm oil yielded higher biomass values for M3 but did not have the same effect for the protein percentage in this biomass. Overall, the best results were observed with the culture of M3 in media containing soybean oil and the culture of M2 in media with palm oil (Table 1). The linear correlation between the dry biomass and protein was 0.47 (Students t-test, p = .05), considering all the strains with all the media containing vegetable oils. The adjusted regression curve of the dry biomass (independent variable) and protein (dependent variable) yielded the following equation: y = 0.4414 x 0.984 (Figure 1), with the coefficient explaining 45.36% of the variation in the protein.

There was greater production of unsaturated fatty acids, such as oleic acid and palmitic acid, in cultures containing vegetable oils. The best results for palmitic acid were obtained in the cultures of M1 in media with canola oil, soybean oil and sunflower oil. The biomass produced in media with sesame oil had greater amounts of palmitic acid and oleic acid. Although large amounts of GLA were not observed in the biomasses of the strains studied, the best percentages of GLA occurred with M2 (8.5 g l^{-1}) and M1 (8.3 g l^{-1}) grown in sunflower oil (Table 2). However, when the Kruskal-Wallis test was applied to verify differences in the production of fatty acids for all the strains in media with vegetable oils, no statistically significant differences were found (Table 3).

Table 4. Biomass and lipid production of the strains of *Mucor hiemalis*, in different carbon sources, after incubation at 25ºC, at 150 rpm, for 72 h.

Carbon sources	Strains	Dry biomass ($g\ l^{-1}$)	Total lipids ($g\ l^{-1}$)	Total lipids/ Dry biomass (% w/w)
Fructose	M1	5.1	<0.1	0.9
Fructose	M2	3.6	0.4	11.4
Fructose	M3	19.8	<0.1	11.2
Fructose	M4	7.1	1.0	7.9
Galactose	M1	5.6	0.1	1.9
Galactose	M2	1.5	0.1	7.9
Galactose	M3	2.3	0.1	5.0
Galactose	M4	5.7	0.9	6.8
Glycerol	M1	7.8	0.1	0.9
Glycerol	M2	5.0	0.3	5.5
Glycerol	M3	1.8	0.1	6.7
Glycerol	M4	0.0	0.0	0.0
Glucose	M1	3.2	0.3	8.5
Glucose	M2	1.5	<0.1	3.2
Glucose	M3	5.2	0.3	5.3
Glucose	M4	11.0	0.9	8.4
Lactose	M1	2.3	0.1	6.1
Lactose	M2	5.0	<0.1	1.1
Lactose	M3	0.4	<0.1	0.2
Lactose	M4	0.0	0.0	0.0
Maltose	M1	10.7	0.2	11.6
Maltose	M2	1.3	0.2	12.0
Maltose	M3	6.0	<0.1	0.9
Maltose	M4	24.0	1.6	15.8
Sucrose	M1	1.1	<0.1	1.6
Sucrose	M2	9.8	0.2	1.7
Sucrose	M3	6.1	0.3	4.3
Sucrose	M4	20.0	1.6	14.6
Sorbitol	M1	7.9	0.1	1.1
Sorbitol	M2	9.9	0.3	2.7
Sorbitol	M3	6.7	0.2	2.4
Sorbitol	M4	17.6	1.1	6.4
Xylose	M1	7.6	0.1	1.3
Xylose	M2	9.9	0.3	2.7
Xylose	M3	9.1	0.3	3.0
Xylose	M4	11.1	1.2	11.0

In an *Aspergillus niger* or a mixed culture of yeast and fungus with vinasse sugar cane as a nutrient source, the nitrogen level in the fungal biomass was 0.69 g l^{-1}, biomass 3.10 - 6.71 g l^{-1}, and protein 19.9 – 22.9% (Ceccato-Antonini and Tauk-Tornisielo, 1994). These values are lower than those obtained for the *M. hiemalis* strains grown in media containing vegetable oil (Table 1).

The largest amount of biomass was observed in the cultures of M4 in media with maltose (24.0 g l^{-1}) or with sucrose (20.0 g l^{-1}). The relation of total lipids and dry biomass (%, w/w) in these cultures were of 15.8 and 14.6, respectively. Applying the Kruskal-Wallis test to compare the biomass production of the strain groups in relation to carbohydrate media (Table 4), no statistically significant differences were found (p) Kruskal-Wallis = 0.3301). As with the data presented in Table 3, one concludes that the differences observed between the groups are not statistically significant.

Biomass production was approximately 8 to 10% greater in media containing vegetable oils than in those containing carbohydrate as the single carbon source. In the different cultures, reduction of lipid biosynthesis was ob-

Table 5. Quantity of fatty acids (mg l^{-1}) in the cultures of the strains of *Mucor hiemalis* in media with different carbon sources, after incubation at 25 ºC, at 150 rpm, for 72 h.

Carbon sources	Strains	Fatty acids (mg.l^{-1})				
		Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	γ-Linolenic acid
Fructose	M1	5.6	2.0	19.9	7.6	8.7
Fructose	M2	6.5	1.1	21.6	7.6	10.1
Fructose	M3	9.9	2.0	11.0	9.9	11.0
Fructose	M4	127.1	20.6	139.2	123.5	144.6
Galactose	M1	17.4	5.0	49.8	156.8	20.2
Galactose	M2	69.1	5.8	203.0	55.4	7.6
Galactose	M3	25.3	4.1	31.7	26.2	29.7
Galactose	M4	86.6	12.5	108.6	78.1	93.5
Glycerol	M1	14.0	3.1	28.1	12.5	13.3
Glycerol	M2	22.0	0.0	50.1	15.0	31.4
Glycerol	M3	18.2	4.0	32.6	28.1	37.3
Glycerol	M4	0.0	0.0	0.0	0.0	0.0
Glucose	M1	52.5	11.5	107.2	44.5	54.1
Glucose	M2	42.0	5.0	136.5	53.0	37.5
Glucose	M3	58.8	11.4	72.3	55.1	47.3
Glucose	M4	650.4	129.6	1,330.4	763.2	1,123.2
Lactose	M1	15.6	2.4	49.2	22.3	47.4
Lactose	M2	7.5	0.0	22.5	8.1	10.6
Lactose	M3	9.5	2.0	14.4	8.2	10.6
Lactose	M4	0.0	0.0	0.0	0.0	0.0
Maltose	M1	43.9	2.4	46.0	37.4	245.0
Maltose	M2	10.5	0.0	25.5	8.0	9.5
Maltose	M3	9.0	1.2	16.8	14.4	10.2
Maltose	M4	181.3	0.0	422.4	225.3	301.0
Sucrose	M1	24.5	5.3	49.7	20.8	4.0
Sucrose	M2	20.3	0.0	74.5	21.6	20.6
Sucrose	M3	55.5	12.2	73.8	58.6	65.3
Sucrose	M4	238.6	47.7	330.8	319.7	264.2
Sorbitol	M1	15.0	0.0	30.8	11.1	11.8
Sorbitol	M2	31.4	0.0	74.5	21.6	20.6
Sorbitol	M3	34.8	7.4	65.0	26.1	22.8
Sorbitol	M4	89.8	0.0	209.1	110.7	165.1
Xylose	M1	9.1	3.0	21.3	8.4	9.1
Xylose	M2	54.4	0.0	133.8	30.0	31.8
Xylose	M3	41.0	6.4	78.3	71.0	72.8
Xylose	M4	109.3	89.8	64.7	67.2	81.5

served in the M2 and M3 strains grown on lactose or maltose as the single carbon sources (Table 4). No growth was observed of any of the strains of *M. hiemalis* in media containing sodium acetate, nor of the M4 in media with glycerol or lactose as the sole carbon sources.

The biomass production of M4 grown on maltose was 24.0 g l^{-1} (Table 4), which was higher than that obtained with *Mucor inaquisporus* grown on glucose, 17.2 g l^{-1} (Emelyanova, 1997); however, both were below results obtained for *Thamnidium elegans* cultured in a glucose medium at 24ºC for 8 d with shaking, which yielded 17.9% total lipids (Stredansky et al., 2000).

Although large amounts of biomass were produced in the cultures containing vegetable oils, the results presented in Table 2 indicate that this type of substrate reduced the production of unsaturated fatty acids, when these results are compared with those obtained in media with carbohydrate (Table 5). The highest values of fatty acids were observed in media with glucose as sole carbon source. The M2, M3 and M4 produced more biomass in media with vegetable oil, but the production of

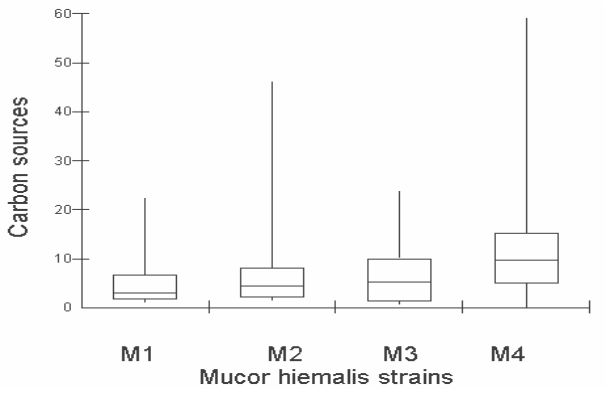

Figure 2. Production of palmitic acid by strains on media with different carbon sources.

Table 6. Results of the Kruskal-Wallis test (p-values) comparing fatty acid production by strains in media containing carbohydrate.

Fatty acid	(p) Kruskal-Wallis
palmitic acid	0.7168
stearic acid	0.3795
oleic acid	0.4969
linoleic acid	0.2009
γ-linoleic acid	0.1455

fatty acids by theses strains was highest in media containing sugar as the single carbon source. When the Kruskal-Wallis test was applied to compare fatty acid production, for example of palmitic acid, in carbohydrate media, no statistically significant differences were found ($p = 0.7168$). Great variability was observed among the different media within each strain regarding production of this fatty acid (Figure 2), which may mask possible differences between the strains. When the same statistical test was applied for an only type of acid, no statistically significant differences were found between the strains. Analysis of other fatty acids led to similar conclusions (Table 6).

Results of lipid formation and GLA production by 48 species of Mucorales fungi grown in sunflower oil showed 42.7 – 65.8% lipids in the biomass (7.7 - 13.4 g l^{-1}) (Certik et al., 1997). This same species (*M. hiemalis* IPD 51), when cultivated in media containing maltose or glucose as the sole carbon source, and yeast extract used as the nitrogen source (in addition to other nutrients), produced 41.1% GLA after being cultured for 6 d at 25ºC, shaken at 140 rpm (Kennedy et al., 1993). When the results regarding the yield of each culture were calculated, it was found that the best values depend on the type of carbon source and the strain under consideration. For all strains, with respect to the unsaturated fatty acids studied, there was little production of stearic acid, and the contrary for oleic acid. The results show no correspondence between the highest yield of biomass and fatty acids and the carbon source.

Environmental factors of the culture have a large influence on these microorganisms; despite being from the same species, they demonstrate individual behavior. Under the same conditions, the M2 and M4 presented differentiated growth. Thus, if the main objective is production of biomass from the *M. hiemalis* strains, the ideal is the use of media containing vegetable oils. However, if the objective is the production of fatty acid, the results demonstrated better productivity in media containing carbohydrates as the sole carbon source.

Regarding the other strains, differences were found in the extractions, with a larger quantity of extracted DNA occurring with the M4. The presence or absence of amplified bands was observed, showing that the bands r11, r12, r16, and r19 occurred in all four strains, and that this is therefore a region of homology, and perhaps a sequence that is typical of the species. No other homology was observed between the M1 and M3, M1 and M4, M2 and M3, and M3 and M4. Among the M1, M2, and M4, homology was observed for bands r4, r6, r7, r9, r14, and r15. The bands r2, r3, r4, and r18 occurred in the M1 and M2, showing that there is great phylogenetic proximity between these two strains. Of the 20 regions amplified, 16 occurred in M1 as well as M2, and the M4 also presented proximity in relation to these two strains, having 10 regions in common in their genomes. The r20 band occurred only in M3, and M2 was the only strain where band r8 was not observed.

Ecological factors become extremely important for the metabolic reactions, which also depend on the medium in which the microorganism is found. Advances in studies of molecular techniques have facilitated the discovery of regions that express the proteins responsible for the different metabolisms of unique individuals, even from the same species. Upcoming studies will look at the use of primer to amplify the specific genetic sequence of an enzyme related to the metabolism of fatty acids, so that these strains can be compared not only with respect to phylogenetic proximity, but also modifications in the sequence of interest.

The results showed that the vegetable oils resulted in greater quantities of *M. hiemalis* biomass, although the same was not true of the levels of production of GLA and other unsaturated fatty acids studied here. The culture conditions of the strains studied were defined to be 72 h at 25ºC, as the log phase of some strains can occur after approximately 50 h of culture. The best biomass production did not always correspond to the greatest quantity of protein and GLA; in addition, the levels of these compounds were dependent on the composition of the culture media for the same strain of *M. hiemalis*.

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