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Eco-friendly production of nutraceutical and cosmeceutical fatty acids by oleaginous fungus *Lichtheimia hyalospora* UCP 1266 using renewable substrates

Produção ecologicamente correta de ácidos graxos nutracêuticos e cosmecêuticos pelo fungo oleaginoso *Lichtheimia hyalospora* UCP 1266 usando substratos renováveis

Producción ecológica de ácidos grasos nutracéuticos y cosmecéuticos por el hongo oleaginoso *Lichtheimia hyalospora* UCP 1266 utilizando sustratos renovables

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Abstract
Polyunsaturated fatty acids (PUFAs) are essential for human functions and can be used in nutraceuticals; however, traditional sources are environmentally unsustainable. A promising strategy to reduce the production costs of PUFAs is the use of economical agro-industrial sources as substrates in culture media for oleaginous microorganisms. In this study, we investigated the potential of the fungus *Lichtheimia hyalospora* UCP 1266 (isolated from the Caatinga soil, Brazil) in the production of PUFAs, through the metabolic conversion of alternative substrates crude glycerol (CG) and corn steep liquor (CSL). The cultivation of *L. hyalospora* UCP 1266 in medium containing glucose (Synthetic Medium for Mucorales - SMM) yielded 2.1 g/L of biomass and 0.6 g/L of total lipids. However, assay 4 of the 2^2 full-factorial design, with CG 8% and CSL 8%, provided higher yields of biomass (15.5 g/L) and total lipids (12.8 g/L). The lipids produced in this medium presented a composition of saturated, monounsaturated and polyunsaturated fatty acids (SFAs=35.13%, MUFAs=46.09% and PUFAs=18.14%). The microorganism proved to be promising in the production of omega 6 (linoleic acid, C18:2), γ-linolenic acid (C18:3), omega 3 (α-linolenic acid, C18:3) and omega 9 (oleic acid, C18:1). The results indicated *L. hyalospora* UCP 1266 as an oleaginous fungus with great potential for use in the development of nutraceutical ingredients for food or applied in cosmeceuticals and/or nutricosmetics.
Keywords: Microbial lipids; Mucorales fungi; Crude glycerol; Corn steep liquor; Agro-industrial wastes.

Resumo
Os ácidos graxos poliinsaturados (AGPIs) são essenciais para as funções humanas e podem ser utilizados em nutracêuticos; no entanto, as fontes tradicionais são ambientalmente insustentáveis. Uma estratégia promissora para reduzir os custos de produção dos AGPIs é a utilização de fontes agroindustriais econômicas como substratos nos

meios de cultura para micro-organismos oleaginosos. Neste estudo, investigamos o potencial do fungo *Lichtheimia hyalospora* UCP 1266 (isolado do solo da Caatinga, Brasil) na produção de AGPIs, através da conversão metabólica dos substratos alternativos glicerol bruto e milhocina. O cultivo de *L. hyalospora* UCP 1266 em meio com glicose (Meio Sintético para Mucorales - SMM) rendeu 2,1 g/L de biomassa e 0,6 g/L de lipídeos totais. No entanto, no ensaio 4 do planejamento fatorial completo de 2^2, com glicerol bruto 8% e milhocina 8%, proporcionou maior rendimento de biomassa (15,5 g/L) e lipídios totais (12,8 g/L). Os lipídios produzidos neste meio apresentaram composição de ácidos graxos saturados, monoinsaturados e poliinsaturados (AGS=35,13%, AGMIs=46,09% e AGPIs=18,14%). O micro-organismo mostrou-se promissor na produção de ômega 6 (ácido linoleico, C18:2), ácido γ-linolênico (C18:3), ômega 3 (ácido α-linolênico, C18:3) e ômega 9 (ácido oleico, C18:1). Os resultados indicaram *L. hyalospora* UCP 1266 como fungo oleaginoso com grande potencial de uso no desenvolvimento de ingredientes nutracêuticos para alimentos ou aplicado em cosmecêuticos e/ou nutricosméticos.
Palavras-chave: Lipídeos microbianos; Fungos Mucorales; Glicerol bruto; Milhocina; Resíduos agroindustriais.

Resumen
Los ácidos grasos poliinsaturados (AGPIs) son esenciales para las funciones humanas y pueden usarse en nutracéuticos; sin embargo, las fuentes tradicionales son ambientalmente insostenibles. Una estrategia prometedora para reducir los costos de producción de los PUFAs es el uso de fuentes agroindustriales económicas como sustratos en los medios de cultivo para microorganismos oleaginosos. En este estudio, investigamos el potencial del hongo *Lichtheimia hyalospora* UCP 1266 (aislado del suelo de la Caatinga, Brasil) en la producción de PUFAs, a través de la conversión metabólica de los sustratos alternativos glicerol crudo y licor de maceración de maíz. El cultivo de *L. hyalospora* UCP 1266 en medio con glucosa (Synthetic Medium for Mucorales - SMM) arrojó 2,1 g/L de biomasa y 0,6 g/L de lípidos totales. Sin embargo, el ensayo 4 del diseño factorial completo de 2^2, con glicerol crudo 8% y licor de maceración de maíz 8%, proporcionó mayores rendimientos de biomasa (15,5 g/L) y lípidos totales (12,8 g/L). Los lípidos producidos en este medio presentaron una composición de ácidos grasos saturados, monoinsaturados y poliinsaturados (AGS=35,13%, AGMIs=46,09% y AGPIs=18,14%). El microorganismo se mostró prometedor en la producción de omega 6 (ácido linoleico, C18:2), ácido γ-linolénico (C18:3), omega 3 (ácido α-linolénico, C18:3) y omega 9 (ácido oleico, C18:3). :1). Los resultados indicaron a *L. hyalospora* UCP 1266 como un hongo oleaginoso con gran potencial para su uso en el desarrollo de ingredientes nutracéuticos para alimentos o aplicado en cosmecéuticos y/o nutricosméticos.
Palabras clave: Lípidos microbianos; Hongos Mucorales; Glicerol crudo; Licor de maceración de maíz; Residuos agroindustriales.

1. Introduction

The term single cell oils (SCOs), also named microbial lipids or microbial oils, was created by Ratledge & Wynn since 1974, to identify those lipids of single-cell organisms – microorganisms – that would be suitable for consumption by human and animal (Ratledge, 2004). Thus, they are considered as new sources of nutraceuticals, cosmeceuticals (products applied topically) or nutricosmetics (products that are ingested orally) of great value for human use (Hyde, et al., 2010; Béligon, et al., 2016; Galán, et al., 2020; Kothri, et al., 2020; Shah, et al., 2021; Fazili, et al., 2022).

Currently, the population, especially the elderly, has been reducing fiber consumption and increasing consumption of foods rich in saturated fats, which causes harmful effects on health (Yannakoulia, et al., 2018). Thus, ingestion of polyunsaturated fatty acids (PUFAs) has been widely disseminated because of its positive effects on human health (Santos, et al., 2013; Nivetha, et al., 2021). Omega-3 fatty acids have anti-inflammatory activities that possibly reduce morbidity and mortality from infection by microorganisms, such as COVID-19, which causes severe acute respiratory syndrome and has resulted in more than 1.8 million deaths (Aburto, et al., 2022).

According to the World Health Organization (WHO) the essential fatty acids are linoleic (C18:2 ω-6), α-linolenic (ALA, C18:3 ω-3), γ-linolenic acid (GLA, C18:3 ω-6) and arachidonic acid (AA, 20:4 ω-6) (Arbex, et al., 2015; Lowry, et al., 2020). Although ALA is synthesized from linoleic acid, there is evidence of loss of this biosynthetic capability with aging and environmental factors that inhibit the activity of several desaturases and elongases involved in the synthesis of PUFAs (Lenihan-Geels, et al., 2013; Kothri, et al., 2020).

Cold-water fish such as salmon, sardines, and mackerel are among the main sources of essential fatty acids for human consumption, especially omega-3. However, the increase in consumption of these animals in some countries resulted in

environmental problems, besides the possibility of extinction of these fish (Martins, et al., 2008). Also, it should be considered that fish suffer variations in seasonality, seasons and location, are susceptible to contamination by metals and pesticides, and the difficulty of adding in diets of long periods, due to the taste of low acceptability (Domingo, et al., 2007; Sitepu, et al., 2014).

In this context, fatty acids commonly found in cosmetics can be produced by fungi (Ward & Singh, 2005; Hyde, et al., 2010). Filamentous fungi of Mucorales and Mortieralles orders, such as *Mortierella* sp., *Mortierella alpina*, *Umbelopsis isabellina*, *Cunninghamella echinulata*, *Mucor circinelloides* and *M. hiemalis*, are more intensively studied for the production of essential fatty acids (Li, et al., 2015; Zhao, et al., 2015; Kosa, et al., 2018; Kikukawa, et al., 2018; Fazili, 2022). These microorganisms produce desaturases by introducing multiple double bonds into the esterified acyl chains at the beta position in membrane lipids (Bellou, et al., 2016).

According to Zininga, et al., (2019) some industrial services use the cultivation of filamentous fungi in solid and submerged bioprocesses using agro-industrial wastes as alternative resources to produce biomass with high yields of lipids. In addition, fungi can reduce the toxicity of various agro-industrial residues, benefiting the environment (Athenaki, et al., 2018). The high cost associated with organic carbon sources is a major bottleneck for the commercialization of the fatty acids bioprocesses. Hence, the use of non-edible materials and industrial waste as glycerol source from biodiesel production could reduce overall production costs, thereby aiding the transition to large-scale microbial lipids production. To the best of our knowledge, this is the first report of the use of crude glycerol (CG) and corn steep liquor (CSL) as agro-industrial substrates for the production of nutraceutical and cosmeceutical fatty acids from *Lichtheimia hyalospora* UCP 1266.

2. Methodology

2.1 Microorganism: isolation and identification

The microorganism was isolated from Caatinga soil, Serra Talhada-PE, Brazil, collected at 7º59'31" S, 38º17'54" W, and after identification was deposited to the Cultures Collection UCP (Nucleus of Research in Environmental Sciences and Biotechnology, Catholic University of Pernambuco), registered as 927 on World Federation for Culture Collections (WFCC). The filamentous fungus was isolated by serial dilution and inoculated on Sabouraud dextrose agar (10.0 g peptone, 40.0 g glucose, 15.0 g agar, 1000 ml distilled water), amended with chloramphenicol (100 mg/L). The plates were incubated at 28°C. After growth, the colonies were transferred to test tubes with Potato Dextrose Agar (PDA; peeled potato 200 g, dextrose 20 g, agar 20 g and 1000 ml distilled water) and preserved at 5°C. Pure cultures from specimen were cultured in triplicate, in Malt Extract Agar (MEA; malt extract 20 g, peptone 5 g, agar 20 g and 1000 ml distilled water) and PDA, and incubated at 20, 25, 30, 37, 40 and 43 °C, during 7 days. The fungal material was mounted in sterile water and lactic acid with cotton blue and observed under a light microscope. The fungal were identified by analyzing the color, appearance and diameter of colony and microstructures as described by Hoffmann, et al. (2007; 2008), Hoffmann (2010) and Alastruey-Izquierdo, et al., (2010).

2.2 Agro-industrial substrates

The substrates used were corn steep liquor (CSL), kindly provided by Corn Products Ltd., and crude glycerol (CG), from the biodiesel manufacturing using cotton oil, kindly supplied by the Experimental Plant of Biodiesel Production of Northeastern Center of Strategic Technologies (CETENE, Recife-PE, Brazil). Aliquots of CSL and CG were subjected to elementary analysis to determine the amounts of carbon, hydrogen and nitrogen (%) using the model EA 1110 analyzer (Carlo Erba Instruments).

2.3 Culture conditions for production biomass e totals lipids

L. hyalospora UCP 1266 was inoculated in Synthetic Medium for Mucorales (SMM) described by Hesseltine & Anderson (1957) with the following composition: D-glucose 40 g; L-asparagine 2 g; thiamine hydrochloride 0.5 mg; potassium phosphate 0.50 g and magnesium sulphate 0.25 g per liter of distilled water, pH 5.2 and incubated at 28°C during 3 days. After this period, aliquots of 1 ml containing 10^7 spores/ml were used as pre-inoculum.

L. hyalospora UCP 1266 was cultivated in Erlenmeyer flasks of 250 ml containing concentrations different of CSL and CG according with a 2^2 full-factorial design (FFD). SMM was used as control (comparative). Culture media were adjusted to pH 5.2 and sterilized in an autoclave at 121°C for 15 min. Then, 1% (v/v) of the spore suspension (10^7 spores/ml) was inoculated in each flask and incubated during 120 h at 28 ºC, under orbital shaker at 150 rpm. The biomass was obtained from the metabolic liquid by vacuum filtration, washed with ice distilled water, filtered on filter paper, lyophilized and then kept in a desiccator until constant weight. The biomass production was determined by gravimetry.

2.4 Cytochemical analysis of lipids

Cytochemical analysis of lipids present in *L. hyalospora* UCP 1266 was performed in accordance with modifications to the method of Sheehan & Storey (1947). The cell samples were fixed in glutaraldehyde and then washed in phosphate buffered saline (PBS). They were then immersed in Sudan Black B stain for 10 min in the dark. After this step, the samples were rinsed in 70% alcohol to remove excess dye, and then washed with distilled water, counter-stained with safranin for 30 s and washed again with PBS. The slides were viewed under an optical microscope. The oil droplets present in the yeast cells were stained in black or dark blue.

2.5 Morphological analysis in Scanning Electron Microscopy (SEM)

The biomass was washed in PBS (pH 7.2) and fixed with glutaraldehyde 2.5% in cacodylate buffer, 0.1 M, pH 7.4 for 1 h at room temperature. In the post-fixation malachite green 0.05% was used in cacodylate buffer for 1 h at room temperature in dark conditions. They were then subjected to the dehydration process with ethanol in proportions of 50, 70, 90 and 100%. Samples were then placed on aluminum supports and analyzed by Scanning Electron Microscopy (LSM JEOL 5600 LV).

2.6 Extraction and quantification of total lipids

Total lipids were obtained according to the method described by Manocha, et al., (1980) using lyophilized biomass (1.0 g) by successive extractions with chloroform/methanol. The percentage of total lipids was determined by gravimetric method using Eq. 1.

$$\text{Total lipids (\%)} = \frac{\text{Lipids} \quad \text{x } 100}{\text{Dried biomass}} \qquad \text{(Eq. 1)}$$

2.7 Fatty acid profile analysis

The methylation of fatty acids was based on the methodology described by Durham & Kloos (1978) using 10.0 mg of dried biomass. The methylation was performed using boron trifluoride methane at 14% solution and benzene. The fatty acids methyl esters (FAMEs) were resuspended in n-hexane and analyzed by gas chromatography (GC) model Agilent Techologies – 7890A with automatic injector, equipped with a flame ionization detector and a capillary column HP-5 of fused silica (5% diphenyl-95% dimethyl polysiloxane) 30 m x 0.25 mm. In the column, an initial temperature of 150°C was maintained for 4 min, increased to 250°C at 4°C per min and maintained for 20 min. The temperature on the injector and the detector was 280°C and helium (1 cm^3. min^{-1}) was used as carrier gas.

2.8 Full Factorial Design (FFD)

The effects of independents variables, CSL and CG, and their interaction, were analyzed by a 2^2 FFD, using biomass and lipids yield as dependent variables. Each independent variable was examined at three levels, low (-1), high (+1) and central (0), represent four replicates, according to Table 1 The data were analyzed performed using the STATISTIC software version 10.0 (StatSoft Inc., USA) (Table 1), testing the significance of the results (p <0.05).

Table 1. Levels of the variable in 2^2 full-factorial design for production of biomass and lipids by *L. hyalospora* UCP 1266.

Variables	Factors levels		
	Low (-1)	Central (0)	High (+1)
Corn steep liquor - CSL (%, v/v)	2.0	5.0	8.0
Crude glycerol - CG (%, v/v)	2.0	5.0	8.0

Source: Authors.

3. Results and Discussion

3.1 Morphological identification of *Lichtheimia* sp. UCP 1301

Etymology: *hyalospora.* Reference to single projection in columella up to 3.5 µm, larger sporangiospores 5.5 µm smooth and rough hyaline and brownish.

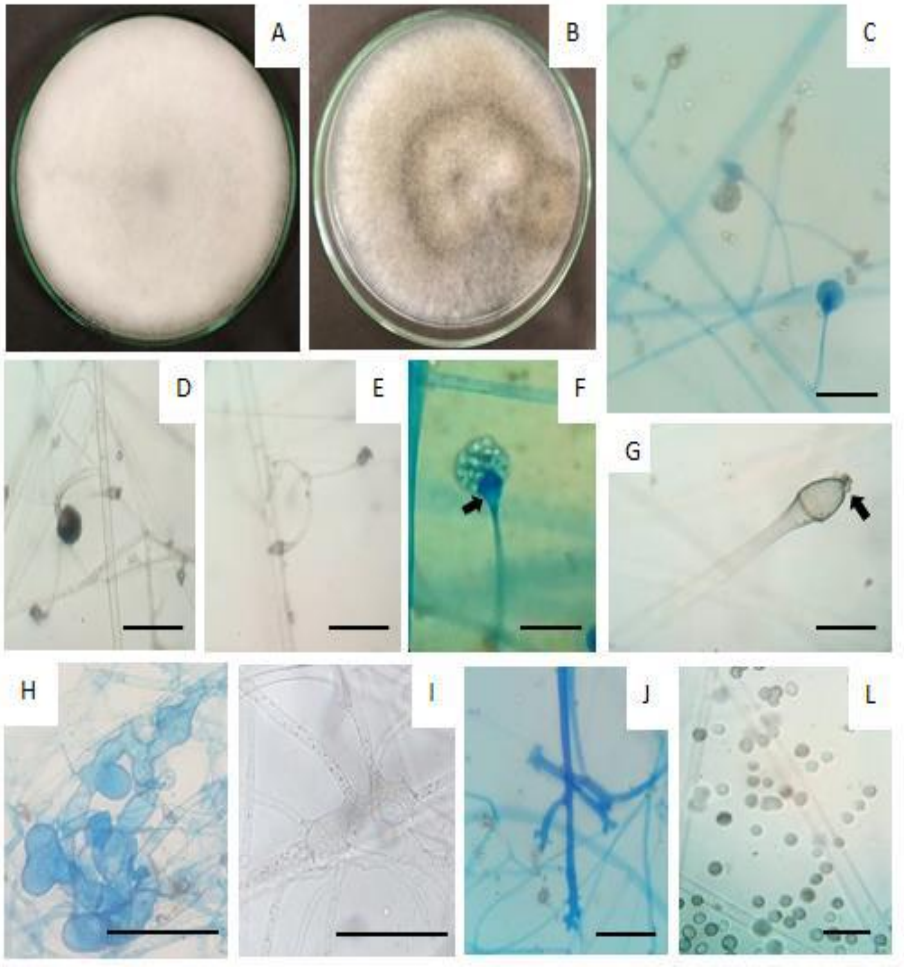
Diagnosis: *L. hyalospora* generally has a single projection, unlike species *L. ramosa* and *L. corymbifera*, which have several projections of 2.5 and 4.5 µm in length, respectively. It differs from the species *L. sphaerocystis* by the giant-cells that are apparently hypha-like swollen, irregularly and branched.

Macroscopic description: Initially white colonies then turning greyish, grey dark in the central portion (Figure 1A, 1B). At 30 °C faster growth (9 cm in 72 h) (Figure 1A) with reverse yellow. Colonies rapidly-growing mainly at optimum temperatures observed between 37ºC (9 cm/ 120 h), high level of sporulation (Figure 1B). At 43ºC slower growth (2.4 cm/ 72 h; 4.2 cm/ 120 h), poor sporulation.

Microscopic Description: Sporophores simple, erect or circinate (Figure 1C, 1D, 1E), few brached (Figure 1E). Yellowish sporangia, when young, turning dark brown or dark grey when mature (Figure 1D), multispored, pyriforme or subglobose, 20–30 µm diam, apophysate, conical apophysis (Figure 1F). Columella usually with an apical projection, 2–3.5 µm diam (Figure 1G). The presence of projections in the columellae has been cited to all known species of *Lichtheimia*. Hesseltine & Ellis (1966) reported projection up to 3.5 µm diam. Presence of giant-cells in hypha-like, simple (Figure 1H) to strongly branched (Figure 1I). Presence of rhizoids branched, occasionally with dilation in the extremities (Figure 1J). Mature sporangiospores smooth and rough, hyaline and brownish reaching 4-7.5 (8) µm diameter (Figure 1L).

The distinction of *Lichtheimia* species depends on morphology and physiology, as in the growth temperature, giant cell and the sporangiospore morphology, and with optima growth around 37ºC, all species be capable to parasitize endothermic organisms (Hoffmann, et al., 2007). The growth capacity at 37 °C, or at 55 ˚C, distinguish of group mesophilic (*Absidia*) (Hoffmann, et al., 2007; Hoffmann, et al., 2009; Further, 2009; Alastruey-Izquierdo, et al., 2010; Hoffmann, 2010). Giant cells are common in all species of *Lichtheimia*, but their size and complexity depend on the medium and the growth temperature (Alastruey-Izquierdo, et al., 2010), irregularly shaped (pleomorphic) with finger-like projections (Further, 2009).

Figure 1. *L. hyalospora* UCP 1266. (A) Colony surface (25ºC); (B) Colony surface (37ºC); (C) Sporophores, columella and sporangia; (D e E) Circinate sporangiophore (F) Simple sporangiophore and terminated in sporangia with prominent dome-shaped apophysis (arrow); (G) Columella with an apical projection (arrow); (H and I) Giant cells with finger-like; (J) Rhizoids;(L) Sporangiospores. Bars: C, D, E, G, J = 50 μm; F = 20 μm; H, I = 100 μm; L =10 μm.



Source: Authors.

3.2 Production of biomass and totals lipids

The utilization of factorial designs has as main objective to evaluate the effect of the factors (independent variables) in different conditions with a reduced number of experiments. Factorial designs are widely used as tools for evaluating factors in lipid production processes by fungi (Castanha, et al., 2014; Souza, et al., 2016, Mendonça, et al., 2021). In this study, the production of biomass and lipids by *L. hyalospora* UCP 1266 was improved using the 2^2 FFD proposed in Table 1, to point out the relationship and influence between the variables, in this case, CSL (1) and CG (2) on the efficiency of the process. The following equation (Equation 2) was used to relate the dependent and independent variables:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 \qquad \text{(Eq. 2)}$$

were Y is the response variable (biomass and lipids), b_0 is a constant; b_1 and b_2 are regression coefficients for the linear effects and b_{12} is cross-product coefficients.

Table 2 shows the experimental and predicted values of the model mathematical to obtain the greater production of biomass and lipids.

Table 2. Full-factorial design with decoded matrix for the response variables: biomass (g/L) and lipids (%) by *L. hyalospora* UCP 1566 after 120 h, using corn steep liquor (CSL) and crude glycerol (CG).

Assay	CSL (%)	CG (%)	Biomass (g/L)		Lipids (%)	
			Experimental	Predicted	Experimental	Predicted
1	2	2	12.8	12.57	44.5	43.83
2	8	2	14.2	13.97	41.0	40.33
3	2	8	10.6	10.37	42.3	41.63
4	8	8	15.5	15.27	82.4	81.73
5	5	5	13.1	13.05	51.6	51.88
6	5	5	12.8	13.05	50.3	51.88
7	5	5	13.3	13.05	52.4	51.88
8	5	5	12.1	13.05	50.6	51.88
SMM*	-	-	2.1	-	27.0	-

*SMM (Synthetic Medium for Mucorales). Source: Authors.

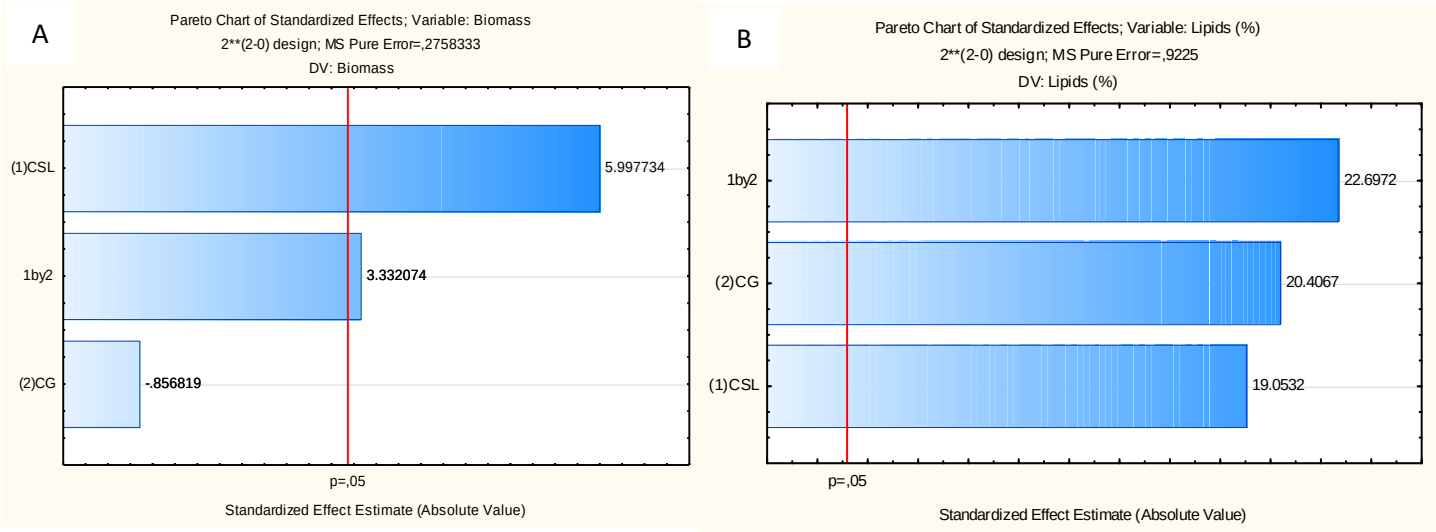
The analysis of variance (ANOVA) for the biomass response variable shows that the regression model showed a high coefficient of determination (R^2 = 0.91453). The closer the coefficient of determination value is to 1, the better the correlation between the experimental and predicted values, indicating that only 8.54% was not explained by the model. The pure error of 0.2758 and the adjusted coefficient of determination value (Adj: 0.8504) confirms the reproducibility of the experimental results.

Higher levels of CSL (1) and its interaction with CG (2) were significant for biomass production (Figure 2A). According to Berger, et al., (2014), CSL consists in an agro-industrial waste rich in amino acids and carbohydrates which favor the growth of zygomycetes. CSL used in this study is composed of 7.06% nitrogen and 34.84% carbon. The results in this study were higher than the obtained by Berger, et al., (2014) for biomass production by *Cunninghamella elegans* UCP 542 (9.93 g/L) after culture in medium containing cassava wastewater flour (5%) and CSL (8%). De Souza, et al. (2020) cultivated *Mucor subtilissimus* UCP 1262 and *L. hyalospora* UCP 1266 and obtained higher biomass yields, 4.83 g/L and 6.54 g/L, respectively, in culture medium containing cassava wastewater flour (4%) CSL (6%). Previously, similar studies were performed by Cardoso, et al., (2012) with the fungi *Rhizopus arrhizus* UCP 402, using CSL and honey, and Souza, et al., (2016) with yeast *Candida lipolytica* UCP 0988, using CSL and residual soybean oil as independent variables in a FFD. In these studies, the highest concentration of CSL favored biomass production by microorganisms. Similar to our study, Mendonça, et al., (2021) verified that high levels of CSL (5%) and CG (5%) favored the highest biomass yield (13.83 g/L) by *Absidia cylindrospora* UCP 1301.

In addition, ANOVA for the variable lipids showed a good fit expressed by the high coefficient of determination, (0.99477) and that only 0.53% was not explained by the model, confirm the reproducibility of the observed results. Figure 2,

represents the Pareto diagrams that were obtained to compare the significance of the independent variables. As seen, higher concentrations (positive effect) of CSL (1) and CG (2) are significant for the production of total lipids (Figure 2B), as well as the interaction of positively influenced factors (synergistic interaction) were critical in the responses, promoting the increase of production of lipids by *L. hyalospora* UCP 1266. These results show that CSL and CG should be set at the higher level (8%).

Figure 2. Pareto chart obtained from 2^2 full-factorial design applied for production of biomass (A) and lipids (B) by *L. hyalospora* UCP 1266. Corn steep liquor (1) and crude glycerol (2) were used as independent variables and biomass (A) and lipids (B) as the response variables. The point at which the estimates of the effect were statistically significant (p = 0.05) is indicated by the red dotted.



Source: Authors

Recently, in the study carried out by Mendonça, et al., (2021), lower levels of CSL were significant in the accumulation of lipids (41.21%) by *A. cylindrospora* UCP 1301. While the evaluated levels of CG and whey did not influence the accumulation of lipids. Glycerol is a three-carbon carbohydrate (triose) in an aerobic catabolism this substrate enters microbial cell by facilitated diffusion and/or active transport, contributing to a faster metabolism in glycolysis, requiring less enzymes than glucose for conversion to glyceraldehyde 3-P. Previously, several authors reported that impurities (salts, methanol, etc.) of CG do not affect the growth of fungi zygomycetes, presenting significant production of lipids (Fakas, et al., 2009; Chatzifragkou, et al., 2011). CG used in this study is composed of 65.18% carbon, 0% nitrogen.

In other hand, the culture medium used as control (SMM) resulted in the yield of 2.1 g/L of biomass and 27% of total lipids. This suggests a culture medium with a greater concentration of CG and CSL could favor growth by *L. hyalospora* UCP 1266.

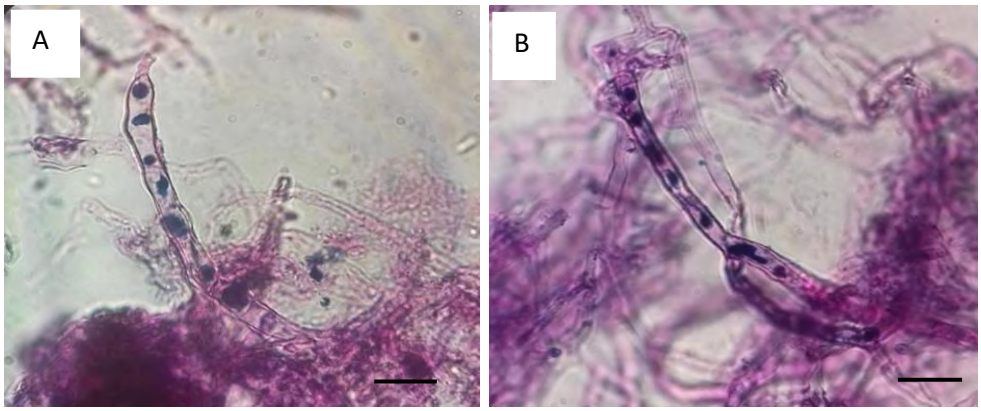
The genus *Lichtheimia* is less investigated in recent studies with the genus. Kosa, et al., (2018) evaluated the production of lipids in three strains of *L. corymbifera* (CCM-8077, VKMF-507 and VKMF-513) in medium composed by glucose and yeast extract, obtaining 27%, 28% and 29% of lipids in dry biomass, respectively. In the study of Hashem, et al. (2019), *L. corymbifera* grown in watermelon peel juice produced 39.56% of total lipids.

Species of the phylum Mucoromycota are considered promising sources of oil, because they accumulate large amounts of lipids, mainly *Mortierella isabelina* (current name *Umbelopsis isabelina*), which may produce up to 86% lipids in dry biomass in synthetic medium (Liu, et al., 2007). However, our study reports for the first time an accumulation of lipids above 80% of a Mucoromycota grown in an alternative medium, composed of agro-industrial wastes.

The study on lipid production by cultures of Mucoromycota in CG is quite limited in the literature (Liu, et al., 2007; Papanikolaou, et al., 2008; Fakas, et al., 2009; Chatzifragkou, et al., 2011; Papanikolaou, et al., 2017). In the study carried out by Fakas, et al., (2009) *Cunninghamella echinulata* achieved 25.6% lipids in 340 h of incubation and *U. isabellina* reached 53.2% lipids in 264 h of incubation, cultured on CG. Chatzifragkou, et al., (2011) demonstrated that CG is a suitable substrate for lipid production by *C. echinulata*, *Mortierella isabelina*, *M. ramanniana*, *Mucor* sp. *Thamnidium elegans* and *Zygorhynchus moelleri* (Current name *Mucor moelleri*) in submerged crops, however *T. elegans* was the most promising species, reaching a remarkable amount of 11.6 g/L corresponding to 71% of total lipids in their biomass.

A Figure 3 shows lipid bodies in hyphae in assay 4 of the FFD (CG 8% and CSL 8%) and SMM by cytochemical analysis using Sudan Black staining, thereby revealing the presence of lipids of dark color. The results obtained a significant accumulation of lipids in most *L. hyalospora* UCP 1266 hyphae occurred in SMM (Figure 3A). However, the maximum accumulation of lipids occurred in assay 4 of FFD (Figure 3B).

Figure 3. Cytochemistry staining of cells of *L. hyalospora* UCP 1266 by Sudan Black method. (**A**) Synthetic Medium for Mucorales (SMM). (**B**) Assay 4 of 2^2 full-factorial design. Bars: A = 50 μm, B = 100 μm.



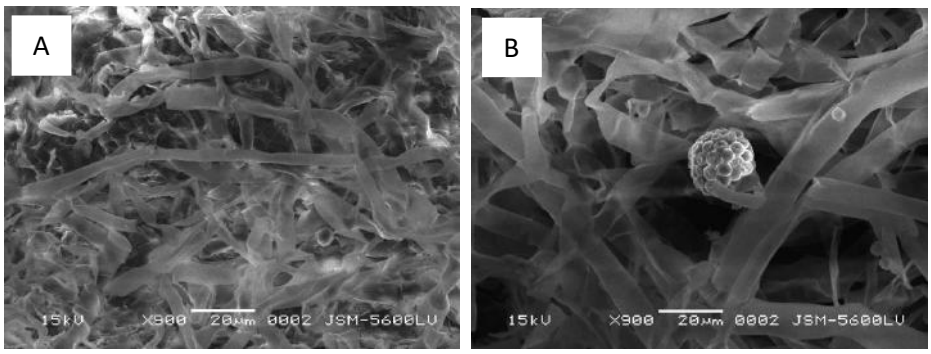
Source: Authors

Several studies have used the Sudan Black B method to detect lipid bodies in microorganisms. In the study of Souza, et al. (2016), they investigated the accumulation of lipids in daily with the aid of lipophilic dye. Kadhim and Alrubayae (2019) showed the method as fast to select filamentous oleaginous fungi. Compared to fluorescence staining methods, which require specialized instrumentation (e.g., Nile red), the Sudan Black B technique is a simple and easy to access method.

3.3 Influence of medium on the ultrastructural morphology of *L. hyalospora* UCP 1266

Scanning electron micrographs were performed by investigates the influence of CG and CSL as carbon and nitrogen sources on ultrastructural morphology of *L. hyalospora* UCP 1266 (Figure 4). The result showed that in the medium selected for production of biomass and total lipids (assay 4 of FFD) induced in the mycelium compact and electro dense hyphae with a thickness thinner, tubular, contorted and branched. But it was observed the presence of chlamydospores (Figure 4A). These CG impurities may have influenced the ultrastructural morphology. The structures of the fungus hyphae growth in control medium showed to be smoother and lax, with high transparency and with presence of spores (Figure 4B).

Figure 2. Scanning electron micrograph of mycelium of *Lichtheimia hyalospora* UCP 1266. (A) Mycelium after culture in assay 4 of the FFD (CG8% and CSL 8%) and (B) mycelium after culture in Synthetic Medium for Mucorales (SMM).



Source: Authors

3.4 Fatty acid profile of total lipids from *L. hyalospora* UCP 1266

Microorganisms also produced essential fatty acids, such as linoleic acids (18.34 - 52.36%) and α-linolenic acids (1.06 - 7.70%) (Tonato, et al., 2018). Fungi belonging Mucorales order have attracted considerable interest as industrial lipid producers as alternative source of biodiesel and good sources of high value omega-3 and omega-6 long-chain PUFAs. However, to obtain sustainable and economical oleaginous biomass, these microorganisms must be grown on low-cost substrates such as food rest materials, waste glycerol and lignocellulosic materials and curiously are able to grow and accumulate lipids on such substrates (Diwan, et al., 2018).

The lipid produced by *L. hyalospora* UCP 1266 grown in CG (8%) and CSL (8%) present composition of mostly saturated, monounsaturated and polyunsaturated fatty acids (SFAs =35.13%, MUFAs = 46.09% and PUFAs= 18.14%). According to the results obtained the fatty acid predominant were the oleic acid (C18:1, ω-9) with 45.31%, followed by α-linolenic acid (C18:3, ω-3) with 17.9% and γ-linolenic acid (C18:3, ω-6) with 0.24% (Table 3). In the study of Tonato, et al. (2018), the filamentous fungi *Nigrospora* sp. produced up to 12.85% of α-linolenic acid, but it had low total lipids in its biomass. Kosa, et al. (2018) reported that the three strains of *L. corymbifera* did not produce α-linolenic acid in medium containing glucose. However, they produced linoleic acid and γ-linolenic acid, ranging from 11-17% and 4-7%, respectively.

L. hyalospora cultured in SMM showed highest peaks to oleic acid (C18:1) followed by palmitic acid (C16:0) with yield of 41.8% and 28.4 (Table 3). However, the microorganism shown to be promising in the production of fatty acids omega 6, linoleic acid and γ-linolenic acid (GLA). When calculating the GLA content related to biomass production (2.1 g/L), *L. hyalospora* UCP 1266 obtained 36.40 mg/L. The production of GLA by *L. hyalospora* UCP 1266 (6.09%) was superior to the obtained by *L. corymbifera* AH13 cultivated in watermelon peel juice (1.42%) (Hashem, et al., 2019). In the study performed by Kosa, et al., (2018), the species *L. blakesleeana* (currently *L. hyalospora*) VKM F-993 and *L. corymbifera* VKM F-513 were grown in concentrated glucose and produced 6% GLA. Recently, *Umbelopsis isabelina*, order Umbelopsidales, a reference microorganism in the production of PUFAs, produced up to 27.3 mg of GLA/g, when grown in cereal and animal fat (Sluvý, et al., 2021). The production of GLA by *Cunninghamella echinulata* and *Mortierella rammaniana* using CG as carbon source was 5.9% and 5.6%, respectively (Papanikolaou, et al., 2007). PUFAs are significantly produced by Mucorales fungi, as summarized in Table 4.

Table 3. Fatty acid profile of total lipids from biomass of *Lichtheimia hyalospora* UCP 1266 cultured in crude glycerol (CG) added corn steep liquor (CSL) and cultivated in synthetic medium for Mucorales (SMM) by 120 h.

Fatty acid	Medium containing CG 8% and CSL 8% (%)	SMM (%)
Capric acid (C10:0)	0.16	-
Myristic acid (C14:0)	0.36	0.54
Palmitic acid (C16:0)	19.24	27.60
Palmitoleic acid (C16:1)	0.68	0.57
Stearic acid (C18:0)	2.92	11.80
Oleic acid (C18:1 –ω-9)	45.31	40.61
Linoleic acid (C18:2)	0.64	9.30
α-Linolenic acid (C18:3–ω-3)	17.9	2.04
γ-Linolenic acid (C18:3–ω-6)	0.24	6.09
Arachidic acid (C20:0)	0.35	0.77
Gadoleic acid (C20:1)	0.10	-
Behenic acid (C22:0)	12.10	0.59

Source: Authors

Papanikolaou, et al. (2017) and Bellou, et al. (2014) reported some Mucoromycota growing in the medium containing CG, which oleic acid was the main fatty acid produced, followed by palmitic and linoleic acid. Thus, the lipid produced by *L. hyalospora* UCP 1266 can also be used in cosmetics, since they have a high content of oleic and linoleic acid. Oleic acid features oxidation stability, preventing the formation of oxygenated radicals (Cicero, et al., 2007), just as its topical application intensifies the permeability of other compounds present in the oil (Mark, et al., 2014). Linoleic acid maintains the integrity of the skin's water permeability barrier (Elias, et al., 1980; Hansen, et al., 1985), in addition, in the epidermis, linoleic acid is metabolized to 13-hydroxyoctadecadienic acid, which has antiproliferative properties (Lin, et al., 2018). The dietic deficiency of this PFA results in flaking and itching of the skin (Lin, et al., 2018).

The fatty acid profile indicates that *L. hyalospora* UCP 1266 shows the ability to produce enzymes responsible for the extension of fatty acids, adding two carbon atoms (elongates) that converts palmitic acid in stearic acid and desaturation, oxidizing two carbon giving rise to double bond (desaturases), which converts stearic acid to oleic acid (Δ9 desaturase enzyme), oleic acid to linoleic acid (Δ12 desaturase enzyme) or its conversion to α-linolenic acid by the action of tears that originate double bond at Δ15 (Ochsenreither, et al., 2016**)**. In the study performed by Hashem, et al., (2020), *L. corymbifera* produced elongases and desaturases enzymes in an alternative medium containing watermelon peel, with the presence of GLA (1.43%).

Table 4. Comparation of production of total lipids and fatty acids (SFAs, MUFAs and PUFAs) by Mucorales fungi from the literature with *Lichtheimia hyalospora* UCP 1266 in this study.

Microorganisms	Substrates	Total lipids (%)	SFAs (%)	MUFAs (%)	PUFAs (%)	References
Lichtheimia hyalospora UCP 1266	CG and CSL	82.4	35.13	46.09	18.14	Present study
	Glucose and L-asparagine	27	42.63	42.39	14.71	
L. corymbifera CCM 8077	Glucose and yeast extract	27	31	50	15	Kosa, et al. (2018)
Absidia glauca CCM 451		47	28	41	27	
Mucor circinelloides VI04473	Potato hydrolysate and yeast extract	41.6	19.26	39.94	40.65	Tzimorotas, et al., 2018)
Mortierella vinacea PTCC 5262	Lactose and peptone	18.70	26.47	13.64	52.76	Nasr, et al., (2017)
M. circinelloides URM4182	Glucose and glutamic acid	44.0	35.8	41.3	20.5	Carvalho, et al. (2015)
Thamnidium elegans	Olive mill wastewater	64.1	30.5	58.1	11.3	Bellou, et al. (2014)
	Glucose	81.7	31.1	55.5	13.1	
T. elegans CCF-1465	Pure glycerol	37.2	33.4	41.1	23.4	Bellou, et al. (2012)
Zygorhynchus moelleri MUCL 143		25.7	27.4	41.7	28.5	
Cunninghamella echinulata ATHUM 4411	Glucose and $(NH_4)_2SO_4$	35.0	30.6	30.0	28.2	Papanikolaou, et al. (2007)
M. racemosus CCF – 86	Glucose and CSL	13.9	31.2	43.5	22.3	Čertík, et al. 1993)
Gongronella butleri CCF - 413		30.4	41.1	45.7	11.4	
Rhizopus arrhizus CCF – 465		18.0	28.0	49.7	21.9	

Source: Authors

In other hand, Alves, et al. (2020), report that fatty acids are able to inhibit the activation and expression of matrix metalloproteins (MMPs) responsible for damaging skin fibroblasts after exposure to UV radiation. Therefore, they are biomolecules that have potential for the development of nutricosmetic products.

Oleic acid is found in olive oil and is known for its effectiveness in reducing cholesterol levels (Puiggros, et al., 2002; Dimitrijevic, et al., 2018). Rodrigues Reis, et al. (2019) report that lipids with a high content of C18:3 fatty acids have high-value-added lipid for application in food and pharmaceutical. The α-linolenic acid (C18:3) is an essential fatty acid precursor of other x-3 fatty acids in humans, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Puiggros, et al., 2002). We should emphasize that this is the first report of significant production of α -linolenic acid in *L. hyalospora.*

4. Conclusion

The present study shows that the use of CG obtained from biodiesel manufacturing is an excellent carbon source with potential application as culture media for the growth of *Lichtheimia hyalospora* UCP 1266. Also, the chemical composition of CSL presents nutrients essential for growth and production of the fungus metabolites. The promising oleaginous fungus wild-type *L. hyalospora* UCP 1266 presents innovative lipids production, especially essential fatty acids α-linolenic acid (ω-3), precursor of eicosapentaenoic acid (EPA), oleic acid (C18:1 –ω-9) and γ-linolenic acid (C18:3–ω-6. In addition, the biomass can serve as low-cost renewable resource for saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), that can be applied as nutraceuticals and cosmeceuticals and/or nutricosmetics lipids. For this, the toxicity of the oil must be analyzed. In addition, the use of agro-industrial wastes with the combination of the application of a full-factorial design proved to be effective for obtaining low-cost nutraceutical lipids and feasibility for production on an industrial-scale.

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