

Original Research Article

Production of Very Long Chain Polyunsaturated Omega 3 and Omega 6 Fatty Acids by *Candida glabrata* Strains

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ABSTRACT

Lipids are a group of biological substances, mainly composed of non-polar substances (triglycerides, diglycerides, monoglycerides and sterols) and polar compounds (free fatty acids, phospholipids and sphingolipids). This work was developed for the biotechnological studies of polyunsaturated lipids ($\omega 3$ e $\omega 6$) production from agro-industrial wastes (corn steep liquor and milk whey) by two strains of *Candida glabrata* (UCP 1002 and 1556). The production was carried by submerged cultivation method in control medium containing glucose, yeast extract, peptone and inorganic salts and a medium of production the base of agro-industrial wastes, with 40% milk whey in replacing glucose and 20% corn steep liquor replacing peptone. The biomass yield was calculated by gravimetrically and the lipids extracted biomass. The fatty acids were methylated and identified by gas chromatography. The results demonstrated that the strains of *C. glabrata* (UCP 1002 and 1556) showed biomass yield of 6.8 and 8.8 g/L⁻¹, respectively. The total lipids production by strains of *C. glabrata* (UCP 1002 and 1556), showed values of 16.20 and 21.50%, respectively. The two strains of *C. glabrata* (UCP 1002 and 1556) showed ability to produce polyunsaturated fatty acids (PUFAs), with emphasis for production of α -linolenic acid ($\omega 3$), with 75.61 and 90.05%, respectively. Our results indicate that the two strains of *Candida glabrata* have the ability to metabolize agro-industrial wastes and produce biotechnologically polyunsaturated fatty acids (PUFAs).

Keywords

Lipids,
PUFAs,
Candida glabrata,
Agro-industrial wastes

Introduction

Biotechnology offers new perspectives with microorganisms and the use of industrial waste as a substrate in fermentation processes for the production of secondary metabolites, stands out as an innovative

alternative to contain large amounts of organic matter, reducing the cost of production as well as the disposal in the environment. In addition, this market is promising for attracting interest in the study

of lipid metabolism in various microorganisms (Meng *et al.*, 2009; Taskin *et al.*, 2015).

Using yeast as a source of lipids has been extensively studied for biotechnological applications. In them the accumulation, composition, quality and quantity of lipids vary from species to species according to their genetic makeup, factors such as culture conditions (temperature, pH, time, etc.) and medium composition (Beopoulos *et al.*, 2011; Javier, Antonio, 2011).

The lipids produced by microorganisms feature composition and energy value similar to vegetable oils and animals, but as lipid-producing microorganisms do not compete by food resources, especially if the carbon source is low cost, as raw materials, by-products and industrial surpluses, has great generation of speed, and its production is not subject to weather and cyclical seasonal variations, it requires less area of production and better control of production and product (Rossi *et al.*, 2011; Harder *et al.*, 2014).

The yeast *Candida glabrata*, single-celled microorganism devoid of endotoxin and capable of improvement genetic suitable for use in large-scale. Among the various kinds of lipids are fatty acids having primarily structural function. Within the diversity of fatty acids, there are those that the body is capable of synthesis, but others do not.

These fatty acids whose biosynthesis is inadequate are called essential: α -linolenic acid (ω -3) and γ -linolenic acid (ω -6) are considered precursors of polyunsaturated fatty acids (PUFAs) of long chain: arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Papanikolaou *et al.*, 2008; Sitepu *et al.*, 2013).

Fatty acids synthesized by yeast has potential for use in the pharmaceutical industry for technical or nutritional supplements purposes. Recently, some studies have shown in laboratory tests that the topical use of essential fatty acids have great benefits for the skin (Daudt *et al.*, 2013; Zhau *et al.*, 2014).

This study aimed to conduct a biotechnological process to produce polyunsaturated fatty acids - PUFAs (ω 3 and ω 6) by *Candida glabrata* (UCP 1002 and 1556) using a culture medium with alternative substrates the basis of agro-industrial wastes (milk whey and corn steep liquor).

Materials and methods

Microorganisms

Candida glabrata UCP 1556 isolated from the semi-arid land (Serra Talhada, Pernambuco, Brazil) and *C. glabrata* UCP 1002 isolated from mangrove sediments (Rio Formoso, Pernambuco, Brazil), was kindly provided by Collection Research Center of cultures in Environmental Sciences and Biotechnology (NPCIAMB) of the Catholic University of Pernambuco (UNICAP), registered in the World Federation Culture Collection-WFCC.

Agro-industrial substrates

The production medium was composed by agro-industrial wastes: corn steep liquor (CSL), a byproduct of corn processing industry and milk whey (MW) from the milk industry of São Bento do Una, Pernambuco, Brazil.

Maintenance medium

The medium for maintaining the yeast was

Yeast Mould Agar (YMA), with the following composition: yeast extract 3g; peptone, 5g; malt extract 3g; glucose 10g; agar 15g; Distilled water 1000 ml, pH 5.8 and incubated for 48h at 28°C.

Dry biomass production medium

The control medium used was that described by Natori *et al.*, (1978), comprising: Yeast extract (1g/ml), peptone (1g/ml), glucose (2g/ml), CaCO₃ (0.12g/ml) and MgSO₄ (0.03g/ml). The medium of production organic wastes base comprises milk whey (40%) as a carbon source in place of glucose and corn steep liquor (20%) as a nitrogen source in place peptone. The yeast extract and the concentration of minerals in the production medium remained constant.

Microbiological methods

Preparation of the inoculum

The inoculum was standardized transferring cells strains of *Candida glabrata* (UCP 1002 and 1556) to the Cald Yeast Mold (CYM), maintained at temperature of 28°C and 150rpm for 24 hours to obtain 10⁷ cells/ml.

Biomass production by *Candida glabrata* (UCP 1002 and 1556) and determination of pH

The cultures for production of biomass were performed in Erlenmeyer flasks of 500ml capacity, containing 250ml of the medium control and the medium with agro-industrial wastes (40% milk whey and 20% corn steep liquor), inoculated with suspension 10⁷cells/ml. The bottles were kept under orbital agitation 150rpm, incubated for 168 hours at 28°C temperature. Aliquots were collected at 4, 12 hours and thereafter every 24 hours totaling 168 hours. The samples were centrifuged at 4.000g, followed by filtration (120F silkscreen nylon membrane)

to separate the cells of metabolic liquid. The biomass obtained was washed with deionized water and lyophilized, kept in a desiccator until a constant weight, estimated by gravimetrically. To determine the pH was used potentiometer Orion (Model 310).

Determine the maximum growth rate (μ_{max}) and generation time (GT)

The maximum growth rate (μ_{max}) and the generation time (GT) of the strains of *C. glabrata* grown in control medium and in the medium of the base of agro-industrial wastes (40% whey and 20% corn steep liquor), were described according to the Pirt formulas (1982). To calculate the growth rate equation was used:

$$\mu_{\max} = (\ln X - \ln X_0)/(T - T_0) \quad \text{Eq.1}$$

Wherein: X = Final biomass; X₀ = Initial biomass; T = End time; T₀ = initial time. The generation time (GT) was calculated as follows:

$$GT = \ln 2/\mu_{\max} \quad \text{Eq. 2}$$

Analytical Methods

Extraction of total lipids

Lipids were extracted according to the method described by Manocha *et al.*, (1980), where the biomass lyophilized, lipids were extracted by nonpolar solvents and polar system. The lyophilized biomass (1.0g) was subjected to successive extractions of lipids three times with chloroform: methanol (2: 1, 1: 1, 1: 2 v/v). The material was stirred for 15 min. at 5°C temperature. The supernatant was separated from the biomass by centrifugation at 5.000g for 10 minutes and the biomass subjected to a new extraction. The supernatants containing the extracts were pooled and evaporated to dryness in a

rota-evaporator. The answers for the production of total lipids were calculated in terms of concentration by the formula: (%) = Dry total lipids of the lipid weight (g) / weight of sample (g) x 100.

Methylation of fatty acids

The extraction of fatty acids was based on Dunlap methodology and Perry (1967) cited in Durham and Kloos (1978). For extraction of dry biomass 10.0mg were placed in test tubes with screw cap, suspended in 3 ml of boron trifluoride-methano-solution 14% and 3 ml of hexane, incubated at 60°C, overnight. After incubation, 4 ml of distilled water were added and stirred under vortex for 5 minutes. The mixture was centrifuged at 5.000g for 10 minutes at 5°C. The hexane was separated by centrifugation removed and evaporated with nitrogen. The methyl esters of fatty acids were suspended in n-hexane.

Identification of fatty acids

The analysis of fatty acids was performed by gas chromatography (GC) and identifying the components by comparing the retention times corresponding to the peaks of the samples.

We used a Varian model gas chromatograph with injector automatic (NPCIAMB) equipped with a flame ionization detector capillary column HP-5 fused silica (5% diphenyl and 95% dimethylpolysiloxane), 30m x 0.25mm. The temperature of the column oven has the following heating ramp: initial temperature 150°C for 4 min⁻¹; increasing a ratio of 4°C min⁻¹ to 250°C is reached, remaining for 20 minutes. The temperatures of the injector and detector were 280°C, and helium (1cm³min⁻¹) as carrier gas.

Results and Discussion

Growth and dry biomass production by *C. glabrata* (UCP 1002 and 1556)

Due to the rapid growth and adaptation of the yeast in different culture medium and with different substrates, they are presented as an biotechnological innovation alternative. Research shows that the use of agro-industrial wastes stands out in the cultivation of microorganisms as an innovative alternative by contain large amounts of organic matter, reducing the cost of production as well as in discharge to the environment (Pelizer, 2007; Taskin *et al.*, 2015; Dengyue *et al.*, 2015).

The pH of the control medium and the medium of production to agro-industrial wastes base, showed similar behavior during 168 hours of cultivation. At the beginning of the cultivation the pH was maintained with acid values of about 4.0, and the end of the cultivation the pH remained around 5.5 to 6 (Figure 1A, 1B, 1C and 1D), the results corroborate with studies of Dias *et al.*, (2015) in the production of lipids by oleoginosa yeast *Rhodosporidium toruloides* in optimal medium. Biomass production by strains of *C. glabrata* (UCP 1002 and 1556) showed similar behavior production, getting evident that after 72 hours of cultivation, the maximum production was reached by yeasts at the end of exponential phase, and then entered the stationary phase (Figure 1A, 1B, 1C and 1D).

C. glabrata (UCP 1002 and 1556) in production medium in basis to agro-industrial wastes showed biomass higher yield compared to control medium, with values of 6.8 and 8.8g/L⁻¹, respectively (Figure 1B and 1D).

Harder *et al.*, (2014), using *Yarrowia lipolytica* to produce lipids in a medium containing industrial residues (vinasse and molasses) and municipal (sewage), obtained a yield of biomass with and without stirring, with values 3.55 and 3.49g/L⁻¹, respectively. In studies conducted by Faria (2010), using molasses supplemented with zinc, magnesium and manganese was evidenced that the medium supplemented with magnesium sulphate resulted in a higher biomass (8.69g/L⁻¹), while a lower value found for the medium with zinc sulfate (7.39g/L⁻¹) in experiments with the yeast *Rhodotorula rubra*.

Cazetta and Celligoi (2006) used *Candida lipolytica*, *Rhodotorula mucilaginosa* and *Saccharomyces cerevisiae* to produce biomass using a medium the based molasses (50%) and vinasse (50%), obtaining values of 6.9; 2.63 and 7.49g/L⁻¹, respectively.

The kinetic parameters demonstrated a production behavior, where the maximum growth rate and generation time of *C. glabrata* (1002 and 1556) in control medium and in waste-based production medium are reported in table 1.

Quantification of total lipids

The formation of lipid depends on the physiology of the microorganisms, starting production of lipids during the late exponential phase and continues during the stationary phase (Raschke, Knorr, 2009). Under the limiting conditions and in the presence of a source carbon of excess, microorganisms begin lipid storage. Thus the composition of carbon/nitrogen in the culture medium is a basic requirement for the accumulation of lipids (Beopoulos *et al.*, 2009). Microorganisms may accumulate lipid to more than 20% of its biomass are defined as oleaginous (Ratledge, Wynn, 2002; Banzatto *et al.*, 2013).

During 168 hours of cultivation it was evident that the greater production of biomass was obtained with 72 hours, and this time interval determined in total lipids. *C. glabrata* (UCP 1002 and 1556) was able to produce a total lipid value of 16.20 and 21.50%, respectively (Table 2) using milk whey and corn steep liquor as substrates in the production medium. The use of a low commercial value substrate is directly related to the low cost of production to obtain the microbial oil.

Delabio *et al.* (2013), held a biotechnological process with some yeasts, including the *Yarrowia lipolytica*, in medium composed of sewage, vinasse, bamboo broth and yeast extract, with and without agitation. Through the obtained results, it is evident that the two medium showed relatively equal values for the production of biomass with agitation 3.5g/L⁻¹ and without agitation 3.4g/L⁻¹. The total lipids produced had very different values being generated 5.2% of lipids in the medium without agitation and more of in the medium with agitation, 16.22%. The results demonstrate the importance of unrest in lipid production process, influenced directly in its quantification.

Cazetta and Celligoi (2006) used the yeast *Candida lipolytica*, *Rhodotorula mucilaginosa* and *Saccharomyces cerevisiae* to produce total lipids using based medium molasses (50%) and vinasse (50%) to give values of 16.80; 19.50 and 26.90%, respectively. *Y. lipolytica* S6 cultured in a bioreactor using crude glycerol with carbon source produced 12.3g/L⁻¹ of biomass and 11% total lipids (Juszczuk, *et al.*, 2013). Although the biomass production by *Y. lipolytica* S6 is higher than the one obtained in this work, on the other side with respect to production of lipids *Y. lipolytica* S6 showed lower yields than those obtained with *C. glabrata*.

Table.1 Kinetics growth of *C. glabrata* (UCP 1002 and 1556) in the control medium and in medium the basis of agro-industrial waste

Kinetics of growth	Control medium	UCP 1002	Control medium	UCP 1556
Maximum growth rate (μ_{max}) (h^{-1})	0.078	0.056	0.086	0.045
Generation time (G_T) (h)	7.7	6.2	7.70	5.2

Table.2 Results of the biomass yield and total lipid production with *C. glabrata* (UCP 1002 and 1556) in control medium and in medium the basis of agro-industrial waste by 72h

Micro-organisms	Biomass (g/L^{-1})	Total Lipids (%)
<i>C. glabrata</i> UCP 1002 *	4.5	8.84
<i>C. glabrata</i> UCP 1002**	6.8	16.20
<i>C. glabrata</i> UCP 1556*	4.8	15.80
<i>C. glabrata</i> UCP 1556**	8.8	21.50

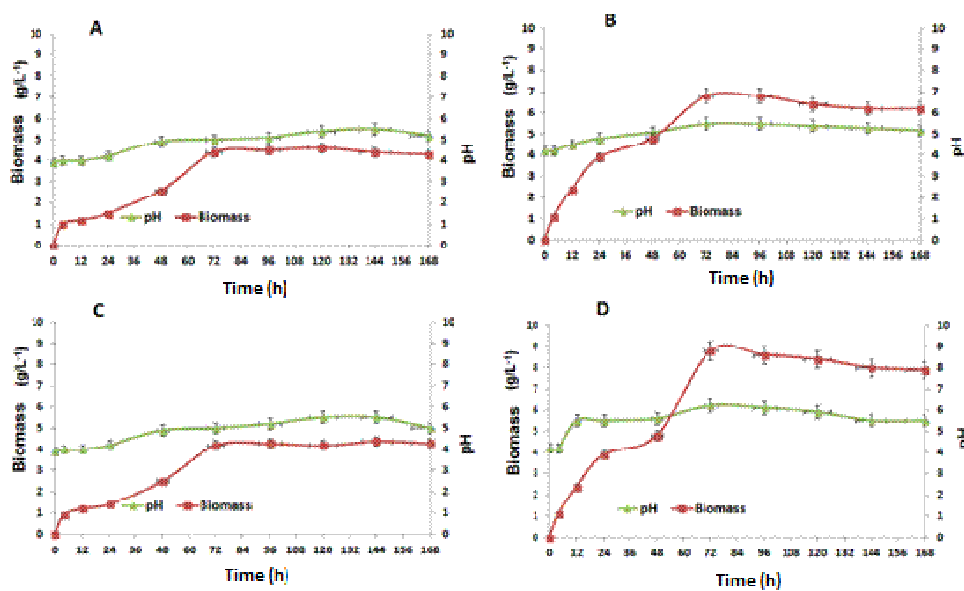
*Control medium

**Production medium

Table.3 Composition of fatty acids of *C. glabrata* (UCP 1002 and 1556)

Composition of fatty acids	UCP 1002 (%)	UCP 1556 (%)
Palmitic acid (C16:0)	-----	0.42
Palmitoleic acid (16:1)	0.81	1.02
Stearic acid (C18:0)	1.16	3.94
Oleic acid (C18:1)	2.52	5.01
Linoleic acid (18:2)	2.24	6.05
γ-linolenic acid (C18:3 - ω6)	3.22	7.95
α-linolenic acid (C18:3 - ω3)	90.05	75.61

Figure.1 Determination of pH and biomass production of the strains of *C. glabrata* UCP 1002 (1A) and UCP 1556 (1C) in the control medium and *C. glabrata* UCP 1002 (1B) and UCP 1556 (1D) in production medium the basis of agro-industrial wastes



Identification of fatty acids

The fatty acids were identified and analyzed in the condition selected for best production of biomass and total lipids (72h), only with mediums of production the basis of agro-industrial wastes.

C. glabrata (UCP 1002 and 1556), showed ability to produce polyunsaturated fatty acids (PUFAs), γ -linolenic acid ($\omega 6$) with an production of 3.22 and 7.95%, respectively, and especially with production α -linolenic acid ($\omega 3$) of 75.61 and 90.05%, respectively (Table 3).

Pirozzi *et al.*, (2014), a biotechnological process performed with the *Lipomyces starkeyi* yeast in a agro-industrial wastes medium with milk whey basis for the production of fatty acids and biomass, obtained 9.5g/L^{-1} biomass for 120 hours and identification of fatty acids, obtained of palmitic acid 24.2%, stearic acid 14.6%, oleic acid 48.5% and linoleic acid 5.5%.

Duarte *et al.* (2014) using *Candida* sp. (LEBM3) isolated from the Pantanal, obtained a predominance of oleic acid in cultivation in pure glycerol and linoleic acid for growing raw glycerol. Testing with low agitation and aeration favored the formation of fatty acids.

Souza *et al.* (2014) conducted a study for the production of microbial fatty acids with forty yeasts from different sources were tested for growth amid a gross and commercial glycerol. The *Y. lipolytica* using crude glycerol as a carbon source was selected for its ability to maintain cell viability at concentrations of 30% raw glycerol. This yeast obtained a profile of fatty acids with predominance of stearic acid 22.3 and palmitic acid 30.3%.

Sitepu *et al.* (2015) noted an increase in fatty acids such with nitrogen deprivation and longer incubation, indicating that each strain of yeast could display a particular behavior on the composition of fatty acids.

Candida glabrata (UCP 1002 and 1556) showed ability to assimilate and metabolize nutrients from agro-industrial wastes, corn steep liquor and milk whey as carbon and nitrogen source, and produced biotechnologically biomass, lipids and especially of polyunsaturated fatty acids (PUFAs). The interaction between the substrates (milk whey and corn steep liquor), promising conditions also provides for the industrial-scale production of these byproducts. Noting that the lineage of *C. glabrata* obtained values for lipids above 20% and is considered a oleoginosa yeast. The production of polyunsaturated fatty acids (PUFAs) by *C. glabrata* (UCP 1002 and 1556) is promising for obtaining the: α -linolenic acid (ω 3) and γ -linolenic acid (ω 6), highlighting the production of α -acid linolenic (ω 3) in two strains reached values above 75%. The polyunsaturated fatty acids (PUFAs) synthesized by yeast has potential for use in pharmaceutical industry.

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