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Lipid and Citric Acid Production by Wild Yeasts Grown in Glycerol

Karla Silva Teixeira Souza[1], Rosane Freitas Schwan[1], and Disney Ribeiro Dias[2*]

[1]*Department of Biology, Federal University of Lavras (UFLA), Campus Universitário, 37.200-000, Lavras, MG, Brazil*
[2]*Department of Food Science, Federal University of Lavras (UFLA), Campus Universitário, 37.200-000, Lavras, MG, Brazil*

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*Corresponding author
Phone: +55-35-3829-5256;
Fax: +55-35-3829-1391;
E-mail: diasdr@dca.ufla.br

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In this study, crude glycerol was used as a carbon source in the cultivation of wild yeasts, aiming at the production of microbial lipids and citric acid. Forty yeasts of different sources were tested concerning their growth in crude and commercial glycerol. Four yeasts (*Lindnera saturnus* UFLA CES-Y677, *Yarrowia lipolytica* UFLA CM-Y9.4, *Rhodotorula glutinis* NCYC 2439, and *Cryptococcus curvatus* NCYC 476) were then selected owing to their ability to grow in pure (OD_{600} 2.133, 1.633, 2.055, and 2.049, respectively) and crude (OD_{600} 2.354, 1.753, 2.316, and 2.281, respectively) glycerol (10%, 20%, and 30%). *Y. lipolytica* UFLA CM-Y9.4 was selected for its ability to maintain cell viability in concentrations of 30% of crude glycerol, and high glycerol intake (18.907 g/l). This yeast was submitted to lipid production in 30 g/l of crude glycerol, and therefore obtained 63.4% of microbial lipids. In the fatty acid profile, there was a predominance of stearic (C18:0) and palmitic (C16:0) acids in the concentrations of 87.64% and 74.67%, respectively. We also performed optimization of the parameters for the production of citric acid, which yielded a production of 0.19 g/l of citric acid in optimum conditions (38.4 g/l of crude glycerol, agitation of 184 rpm, and temperature of 30°C). *Yarrowia lipolytica* UFLA CM-Y9.4 presented good lipid production when in the concentration of 30 g/l of glycerol. These data may be used for production in large quantities for the application of industrial biodiesel.

Keywords: Oleaginous yeasts, biodiesel, fatty acids, *Yarrowia lipolytica*

Introduction

Recent decades have seen an increase in the world's energy demand owing to population growth and global industrial development. Given that fossil fuels are not only the largest non-renewable energy source, but are also expected to be depleted in the near future, there has been growing scientific and industrial interest in biofuels. Alternative renewable fuels that can overcome these restrictions have received considerable attention [14, 21]. In particular, there has been a focus on biodiesel, which is primarily generated through the transesterification of fatty acids from various oleaginous plants. Recent studies have successfully demonstrated the production of biodiesel by microorganisms, including yeast, fungi, bacteria, and algae [12, 14, 20].

Biodiesel production releases large amounts of residual glycerol, which is the major byproduct of the transesterification process [18]. This accumulation of glycerol in the environment and the search for renewable fuels gave rise to the concept of producing biofuels using microorganisms that utilize glycerol as a carbon source. Using biotechnology, crude glycerol can be valued *via* conversion into microbial lipids and citric acid [4, 20, 22]. Other carbon sources, such as cassava starch, molasses, and other agricultural and industrial residues may also be used. These sources are low-cost raw materials and are widely available, which is fundamental for the reduction of costs of the biodiesel production [24].

Microorganisms are capable of producing lipids with similar compositions to those of vegetable oils and can thus be utilized for biodiesel production [12]. Some microorganisms, such as bacteria, yeast, and filamentous fungi, can accumulate large amounts of lipids, comprising up to 70% of their total

weight. Such microorganisms are known as oleaginous species [10, 21]. In addition to lipid production, yeasts are able to produce citric acid from various concentrations of crude glycerol. When the initial carbon/nitrogen molar ratio is high, cell growth can be followed by significant citric acid production, followed by low levels of lipid accumulation within the cell [3].

Citric acid may also be produced by fermentation using less costly substrates, as in the case of molasses. The use of natural products and of agro-industrial residues not only decreases the costs of the process but may also minimize the environmental problems [2].

The surface response methodology is a process that may make available and optimize the production of these metabolites by microorganisms. Concerning the optimization, three steps are involved: The first is to conduct the statistically planned experiments; to estimate the coefficients of a mathematical model and foresee the answer verifying the model adequacy. Several studies use this technique in the field of Applied Biotechnology to optimize different parameters [9].

The objective of this study was to measure yeast growth in media containing glycerol originating from biodiesel and commercial glycerol production and to evaluate the use of yeast for lipid and citric acid production.

Materials and Methods

Microorganisms

Thirty-seven yeast isolates from the microorganism collection of the laboratory of Microorganism Physiology and Genetics of the Federal University of Lavras (Universidade Federal de Lavras - UFLA, Brazil) and three yeast isolates from the National Collection of Yeast Cultures - NCYC (Norwich, UK) were used in this study. The evaluated yeasts were from the following genera: *Candida, Pichia, Debaromyces, Saccharomyces, Kazachistania, Torulaspora, Trichosporon, Kodamea, Lindnera, Yarrowia, Galactomyces, Cryptococcus, Hanseniaspora, Issatchenia, Rhodotorula,* and *Schizosaccharomyces* (Table 1).

Preselection of Yeasts Able to Grow in Glycerol

The yeasts were evaluated with regard to their ability to grow in culture media containing pure glycerol (Sigma Aldrich V000123), and crude glycerol (obtained from the Laboratory of Research on Oils, Fats and Biodiesel of UFLA). Yeast growth was monitored through the measurement of optical density at 595 nm every 3 h. Measurements were made in ELISA-Multiskan FC (Thermo Scientific) microplates containing 180 µl of G-1 culture medium and 20 µl of the inoculum, which represents a population of 10^6–10^7 cells/ml, and was grown for 24 h in yeast-extract peptone glucose (YEPG) medium. The incubation temperature was 28ºC for 24 h, with shaking at 150 rpm [11].

Culture Media and Cultivation Conditions

To select yeasts that were able to grow in medium containing glycerol as a carbon source, G-1 medium composed of 20 g/l glycerol (commercial or crude), 20 g/l peptone, and 20 g/l yeast extract was used. The pH was adjusted to 6.0. G-1 culture medium lacking glycerol was used as a control [11].

Yeasts that demonstrated improved growth due to growth in media lacking glycerol were submitted to additional growth tests in submerged culture in G-2 culture medium, with shaking at 150 rpm at 28 ± 2ºC for 120 h. The G-2 medium composition was adapted [16]: per L, 7.0 g $KHPO_4$, 2.5 g $NaHPO_4$, 1.5 g $MgSO_4 \cdot 7H_2O$, 0.15 g $CaCl \cdot 2H_2O$, 0.02 g $ZnSO_4 \cdot H_2O$, 0.06 g $MnSO_4 \cdot H_2O$, 0.5 g $(NH_4)SO_4$, 0.5 g yeast extract, and 5.0 g glucose, and pH 6.0. Pure and crude glycerol was used at concentrations of 10%, 20%, and 30%. The inoculum was initially grown in 2 ml of YEPG (10 g yeast extract, 20 g peptone, and 20 g glucose per liter) for 24 h.

Selection of Yeasts in Submerged Cultures

The yeasts, selected in the initial phase, were then submitted to shake-flask tests for 120 h, with shaking at 150 rpm at 28ºC in G-2 culture medium. Cells were inoculated at 10^8 cells/ml at concentrations of 10%, 20%, and 30% glycerol [18]. Samples were removed at every 24 h to count viable cells.

Production, Extraction, and Determination of Lipids

Lipid production was evaluated during growth in submerged culture in G-2 medium at the following concentrations of crude glycerol (g/l): 15, 25, 30, and 35. The yeasts were inoculated at 10^8 cells/ml, and incubated at 30 ± 2ºC, with shaking at 150 ± 10 rpm. The experiment was carried out for 48 h, as adapted from [18].

Lipid extraction was performed using a modified methodology [6]. Briefly, lipids were extracted from yeast biomass using a chloroform methanol solution (2:1). The extracted lipids were removed and the process was repeated for the remaining lipids in the sample. The solvents were then removed by evaporation.

The extracted lipids were esterified to obtain the fatty acid methyl esters [15]. Fatty acids were quantified using a gas chromatograph (GC; Shimadzu model 17A), equipped with a flame ionization detector (FID) and a silica capillary column DB Wac (30 m × 0.25 mm × 0.25 µm; J&W Scientific), with automatic injector. The analyses were performed under the following conditions: injector temperature 240ºC; detector temperature 240ºC; initial column temperature 140ºC; temperature program, 140ºC for 20 min, 2.5ºC/min up to 220ºC, and 10 min at 220ºC. The carrier gas flow rate was 1.2 ml/min, with a split ratio of 1:10. Nitrogen was used as the carrier gas [8].

Production and Evaluation of Citric Acid

To evaluate the production of citric acid by the yeast *Yarrowia lipolytica* UFLA CM-Y9.4 following growth in G-2 medium (it was selected because it showed the highest glycerol consumption in the preliminary tests), the response surface methodology was used in a central composite rotatable experimental design, with

Table 1. Yeast isolates obtained from the microbial culture collection of the Physiology of Microorganisms laboratory of UFLA used in the study of growth in crude and commercial glycerol.

Code	Species	Origin
UFLA CES-Y758	*Candida railenensis/oleophila*	Cerrado soil
UFLA FW 62	*Pichia guilliermondii*	Fermented cagaita
UFLA CES-Y660	*Debaromyces varinjiae*	Cerrado soil
UFLA CA-11	*Saccharomyces cerevisiae*	Sugar cane
UFLA CES-Y777	*Candida sojae*	Cerrado soil
UFLA CES-Y649	*Debaromyces pseudopolymorphus*	Cerrado soil
UFLA CES-Y678	*Debaromyces hansenii*	Cerrado soil
UFLA CES-Y839	*Kazachistania* sp.	Cerrado soil
UFLA CES-Y775	*Torulaspora maleae*	Cerrado soil
UFLA CES-Y631	*Trichosporon* sp.	Cerrado soil
UFLA FW 33	*Saccharomyces paradoxus*	Fermented cagaita
UFLA FW 46	*Debaromyces fabryi*	Fermented cagaita
UFLA 383	*Candida fermentati*	Coffee
UFLA FFCX-100.7	*Saccharomyces cerevisiae*	Caxiri
UFLA CES-Y64	*Torulaspora globosa*	Cerrado soil
UFLA CES-Y270	*Candida neerlandica*	Cerrado soil
UFLA CES-Y542	*Pichia kudriavzuri*	Cerrado soil
UFLA CES-Y608	*Candida ortolopsis*	Cerrado soil
UFLA CES-Y648	*Kodamea eohmeri*	Cerrado soil
UFLA CES-Y677	*Lindnera saturnus*	Cerrado soil
UFLA CES-Y818	*Candida frijolensis*	Cerrado soil
UFLA NCYC	*Pichia metanolica*	Silage
UFLA 25	*Yarrowia lipolytica*	Kefir (water)
UFLA 120	*Candida parapsilosis*	Kefir (milk)
UFLA 111	*Pichia membranifaciens*	Kefir (water)
UFLA 200	*Galactomyces geotrichum*	Kefir (milk)
UFLA CES-Y60	*Candida tropicalis*	Cerrado soil
NCYC 476	*Cryptococcus scurvatus*	NCYC
UFLA CES-Y557	*Candida glabatra*	Cerrado soil
UFLA CES-Y682	*Cryptococcus humicola*	Cerrado soil
UFLA CM-Y9.4	*Yarrowia lipolytica*	Amazon soil
UFLA 21.1	*Candida intermedia*	Cocoa
UFLA BM 14.3	*Hanseniaspora opuntiae*	Cocoa
UFLA DM 31.7	*Pichia fermentans/kluyveri*	Cocoa
UFLA BM 15.1	*Saccharomyces cerevisiae*	Cocoa
UFLA EM 5.69	*Issatchenkia orientalis*	Cocoa
NCYC 2439	*Rhodotorula glutinis*	NCYC
UFLA E.M 5.71	*Schizosaccharomyces pombe*	Cocoa
UFLA DM 25.1	*Pichia* sp.	Cocoa
UFLA CES-Y523	*Cryptococcus laurentii*	Cerrado soil

three repetitions for the central point. Experimental variables and their corresponding levels are summarized in Table 2.

Samples were collected at zero and 120 h for the evaluation of citric acid production. The analysis of citric acid was carried out by high-performance liquid chromatography (HPLC) using a Shimadzu chromatographer, model LC-10Ai (Shimadzu Corp.,

Table 2. Variables and experimental levels.

Variables	-1.68	-1	0	1	1.68
Temperature (ºC)	26.6	28	30	32	33.4
Glycerol concentration (g/l)	21.6	25	30	35	38.4
Agitation (rpm)	116	130	150	170	184

Japan), equipped with a double detection system consisting of a UV detector (SPD + 10Ai) and a refractive index detector (RID – 10A), a SCR 101H column with the respective pre-column, and a mobile phase. For each sample, a volume of 20 µl was injected at a flow rate of 0.6 ml/min, and UV-visible detection was performed at 210 nm. The drying oven temperature was 30ºC. Sample quantification was made using standard curves with citric acid and glycerol [18].

Statistical Analysis

The Statistica 4.0 software was used for carrying out the analyses. The mathematical relationship between independent variables was determined by building a second-order equation obtained from the 17 assays.

Results

Selection of Yeasts Capable of Growing in Glycerol

Following 24 h of incubation in G1 medium with commercial glycerol, crude glycerol, and or glycerol, the yeasts demonstrated different growth patterns. The crude glycerol used consisted of 2.06% phosphorus, 1.09% potassium, 0.31% calcium, 0.16% magnesium, 0.40 ppm copper, 38.08 ppm zinc, and 1,071.13 ppm iron.

The *Issatchenkia orientalis* UFLA EM5.69 strain showed reduced growth when cultivated in medium containing pure glycerol (OD_{600} 0.110). However, it showed nine times more growth in medium without glycerol. By contrast, *Pichia fermentans* UFLA DM31.7 demonstrated a reduction of 1.5 times in growth in crude glycerol (OD_{600} 0.156) and without glycerol (OD_{600} 0.101). For some yeasts, biomass production in crude glycerol was higher than when they were grown in pure glycerol. For example, *Candida railenensis/oleophila* UFLA CES-Y758 produced approximately 1.5 times higher biomass following growth in crude glycerol than in commercial glycerol (data not shown). *Kazachistania* sp. UFLA CES-Y839, *Pichia metanolica* NCYC 1381, *Hanseniaspora opuntiae* UFLA BM14.3, *Pichia fermentans* UFLA DM31.7, and *Issatechenkia orientalis* UFLA EM5.69 did not show any growth in media containing commercial glycerol. However, with the sole exception of *Pichia fermentans* UFLA DM31.7, these species did exhibit growth in medium without glycerol. The mean growth levels for all yeasts in medium containing crude, pure, and no glycerol were OD_{600} 1.538, OD_{600} 1.290, and OD_{600} 1.359, respectively. Overall, the species produced reduced biomass in culture with pure glycerol (data not shown).

At this point, we aimed to select yeasts that exhibited increased growth. To this end, we selected species that showed growth levels higher than OD 2.00 in the two types of glycerol tested. Among the evaluated yeasts, four were selected for growth in submerged culture in flasks with 10%, 20%, and 30% crude and commercial glycerol: *Lindnera saturnus* UFLA CES-Y677, *Yarrowia lipolytica* UFLA CM-Y9.4, *Rhodotorula glutinis* NCYC 2439, and *Cryptococcus curvatus* NCYC 476.

Growth in Submerged Culture

Both crude and commercial glycerol enabled yeast cell growth over the course of the incubation period, with most of the yeasts attaining stationary phase after 24 h (Table 3). However, different outcomes were observed when the yeasts were inoculated in culture medium with 30% crude glycerol. *Lindnera saturnus* UFLA CES-Y677 showed slow growth, reaching 8×10^7 CFU/ml in 24 h. After this period, its viability declined, with minimum viability occurring after 48 h of cultivation (Table 3). *Yarrowia lipolytica* UFLA CM-Y9.4 and *Cryptococcus curvatus* NCYC 476 stopped growing after 96 h. *Rhodotorula glutinis* NCYC 2439 remained viable in the stationary phase for 120 h at the highest concentrations of crude glycerol (20% and 30%).

To determine growth in medium lacking glycerol, the yeasts were also tested in medium with glucose over the same time interval (Table 3). Under these conditions, all four yeasts showed growth similar to their respective values in 10% crude and pure glycerol, demonstrating that crude glycerol did not negatively affect their growth. Chromatographic analyses showed that *Yarrowia lipolytica* UFLA CM-Y9.4 consumed the most glycerol over time in a medium initially containing 100 g/l crude glycerol. This yeast consumed 18.907 g/l at the end of the growth period, whereas *Rhodotorula glutinis* NCYC 2439 consumed 2.225 g/l, and *Cryptococcus curvatus* NCYC 476 consumed 0.309 g/l in the same medium.

Table 3. Populations of yeasts *Lindnera saturnus*, *Cryptococcus curvatus*, *Rhodotorula glutinis*, and *Yarrowia lipolytica* grown on media containing different concentrations (10% to 30%) of crude and pure glycerol for 120 h.

	Lindnera saturnus			*Cryptococcus curvatus*			*Rhodotorula glutinis*			*Yarrowia lipolytica*		
	Crude glycerol (viable cells/ml × 10^8)											
	10%	20%	30%	10%	20%	30%	10%	20%	30%	10%	20%	30%
0 h	0.8 ± 0.05	0.7 ± 0.01	1.0 ± 0.02	0.53 ± 0.02	2.2 ± 0.05	3.2 ± 0.02	1.03 ± 0.04	0.31 ± 0.02	0.52 ± 0.02	0.16 ± 0.06	0.17 ± 0.03	0.1 ± 0.03
24 h	0.7 ± 0.01	0.2 ± 0.03	1.01 ± 0.02	0.45 ± 0.02	0.92 ± 0.01	0.66 ± 0.01	0.3 ± 0.04	0.34 ± 0.03	0.22 ± 0.04	0.13 ± 0.06	0.09 ± 0.01	0.06 ± 0.01
48 h	0.3 ± 0.02	nd	nd	0.40 ± 0.02	0.55 ± 0.02	0.42 ± 0.02	0.12 ± 0.07	0.36 ± 0.02	0.09 ± 0.01	0.09 ± 0.04	0.05 ± 0.02	0.03 ± 0.02
72 h	0.3 ± 0.01	nd	nd	0.32 ± 0.02	0.30 ± 0.03	0.30 ± 0.02	0.09 ± 0.01	0.09 ± 0.01	0.07 ± 0.03	0.08 ± 0.07	0.05 ± 0.01	0.01 ± 0.05
96 h	0.2 ± 0.01	nd	nd	0.09 ± 0.01	0.49 ± 0.01	0.17 ± 0.03	0.04 ± 0.02	0.07 ± 0.05	0.05 ± 0.01	0.04 ± 0.02	0.01 ± 0.02	0.03 ± 0.02
120 h	0.1 ± 0.03	nd	nd	0.10 ± 0.04	0.1 ± 0.04	nd	0.03 ± 0.03	0.06 ± 0.01	0.02 ± 0.04	0.06 ± 0.01	0.01 ± 0.02	nd
	Pure glycerol (viable cells/ml × 10^8)											
	10%	20%	30%	10%	20%	30%	10%	20%	30%	10%	20%	30%
0 h	2.5 ± 0.03	1.4 ± 0.06	49 ± 0.03	1.73 ± 0.04	2.55 ± 0.02	1.01 ± 0.02	0.7 ± 0.01	0.59 ± 0.01	0.53 ± 0.01	1.5 ± 0.04	1.0 ± 0.03	1.5 ± 0.03
24 h	1.8 ± 0.02	1.9 ± 0.03	1.1 ± 0.04	0.6 ± 0.01	1.54 ± 0.06	0.54 ± 0.01	2.46 ± 0.03	3.82 ± 0.02	1.15 ± 0.04	1.7 ± 0.01	1.5 ± 0.05	0.9 ± 0.01
48 h	4.1 ± 0.01	1.4 ± 0.03	1.2 ± 0.03	0.95 ± 0.01	0.9 ± 0.01	0.04 ± 0.01	1.01 ± 0.04	0.75 ± 0.01	0.9 ± 0.01	2.1 ± 0.02	1.0 ± 0.06	1.4 ± 0.04
72 h	3.1 ± 0.01	2.1 ± 0.02	0.8 ± 0.01	0.96 ± 0.01	0.45 ± 0.01	0.16 ± 0.02	0.88 ± 0.01	0.75 ± 0.01	0.35 ± 0.02	1.3 ± 0.03	1.0 ± 0.04	0.9 ± 0.01
96 h	3.7 ± 0.01	2.2 ± 0.01	2.1 ± 0.04	1.47 ± 0.03	0.50 ± 0.01	0.7 ± 0.01	0.73 ± 0.01	0.56 ± 0.01	0.39 ± 0.01	1.2 ± 0.03	1.5 ± 0.01	0.7 ± 0.01
120 h	1.9 ± 0.02	1.4 ± 0.03	1.2 ± 0.05	1.55 ± 0.04	0.46 ± 0.01	0.9 ± 0.01	0.65 ± 0.01	0.53 ± 0.01	0.31 ± 0.01	1.2 ± 0.04	1.2 ± 0.03	0.6 ± 0.01

	YEPG (control without glycerol) (viable cells/ml × 10^8)			
0 h	0.02 ± 0.06	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.06
24 h	0.90 ± 0.02	0.7 ± 0.02	1.12 ± 0.03	0.89 ± 0.02
48 h	1 ± 0.04	0.77 ± 0.02	1.10 ± 0.05	0.92 ± 0.01
72 h	1.1 ± 0.07	0.85 ± 0.01	1.32 ± 0.01	0.95 ± 0.01
96 h	1.1 ± 0.07	0.92 ± 0.02	1.41 ± 0.03	1.0 ± 0.03
120 h	1.5 ± 0.08	1.3 ± 0.01	1.39 ± 0.03	1.4 ± 0.03

nd: viable cells were not detected in Neubauer chamber counting. Results are the mean ± standard deviation of triplicate assays.

Lipid Production

The greatest lipid production was observed by *Y. lipolytica* UFLA CM-Y9.4 cultivated in intermediate concentrations of crude glycerol (25 and 30 g/l), with lipid comprising 46.16% and 63.4%, respectively, of the total biomass (Fig. 1). Following 48 h growth, the lipid biomass was 37.9% in 15 g/l crude glycerol, and 38.2% in 35 g/l crude glycerol. These extreme concentrations (15 and 35 g/l) were shown to be suboptimal both for the production of biomass and the production of lipids by *Y. lipolytica* UFLA CM-Y9.4.

The highest level of growth by the yeast *Y. lipolytica* UFLA CM-Y9.4 was 8.6 × 10^7 cells/ml, observed following growth in 30 g/l crude glycerol. Thus, growth can be related to the amount of lipid produced, as the greatest production of lipids was observed in cultures with the highest cells/ml. Likewise, the lowest lipid biomass was produced by cultures with the lowest cells/ml.

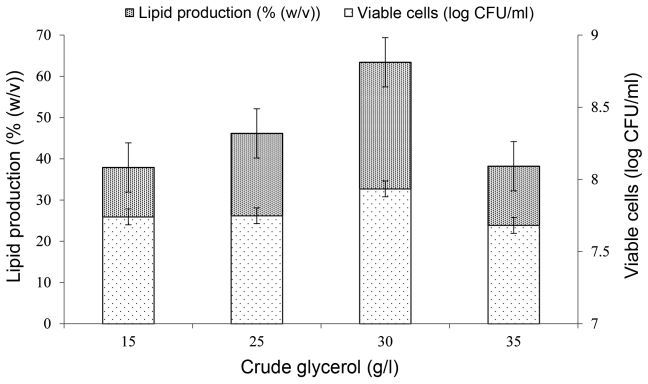

Fig. 1. Lipid production after growth in 15, 25, 30, or 35 g/l crude glycerol by the yeast *Y. lipolytica* UFLA CM-Y9.4.

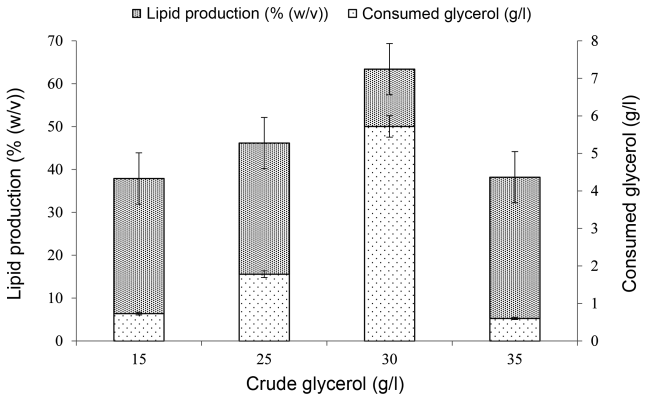

Fig. 2. Production of lipids by *Y. lipolytica* UFLA CM-Y9.4 in medium with starting crude glycerol concentrations of 15, 25, 30, and 35 g/l after 48 h, with shaking at 150 rpm at 30 ± 2ºC, as a function of the glycerol consumed by the yeast at the different concentrations.

Glycerol Consumption

The yeast *Y. lipolytica* UFLA CM-Y9.4 demonstrated higher glycerol consumption (5.72 g/l) and a greater production of lipids (63.4%). Both were observed in culture medium containing crude glycerol at a concentration of 30 g/l (Fig. 2). In culture medium that initially contained 25 g/l crude glycerol, 1.78 g/l glycerol was consumed. For crude glycerol concentrations at which lower levels of lipid production of 37.9% and 38.2% were observed, there were also lower levels of glycerol consumption: 0.73 g/l and 0.60 g/l, respectively.

Fatty Acid Profile

The profile of fatty acids produced by the yeast *Y. lipolytica* UFLA CM-Y9.4 was analyzed, and the results are shown in Table 4.

The amount of fatty acids found in the concentration at 30 g/l was higher than that reported in the remaining tested concentrations. The amount of estearic acid (C18:0), for example, was five times higher in the concentration at 30 g/l (24.73%) than the value found in the concentration at 15 g/l (4.56%) (Table 4). Palmitic acid (C16:1) had a value six times higher in the concentration at 30 g/l (15.67%) when compared with the concentration at 25 g/l (2.65%).

In total, palmitic (16:0) and estearic (18:0) acids were found in the proportions of 74.67% and 87.64%, respectively, across all crude glycerol concentrations used. Linoleic acid (18:2), a polyunsaturated fatty acid, was not found in any of the analyzed samples. For crude glycerol, saturated fatty acids comprised 71.28%, and monounsaturated fatty acids comprised 28.71% of total biomass.

Optimization of the Citric Acid Production

The three independent variables that influenced citric acid production were incubation temperature, glycerol concentration, and agitation.

The obtained results enabled us to determine regression coefficients for citric acid response (Table 5). The model parameters were significant ($p \leq 0.1$) at a 90% significance level, with the exception of the quadratic temperature. The following model with codified variables was built using the significant parameters:

$$Y = 0.0331 - 0.0084x_1 + 0.0074x_2 + 0.0150x_2^2 + 0.0272x_3 - 0.0079x_3^2 - 0.0067x_1.x_2 - 0.0065x_1.x_3 + 0.0117x_2.x_3$$

The dependent variables (population and glycerol consumption) did not show significant values and, therefore, it was not possible to find an equation defining these models. The fit of the model was confirmed by the coefficient of determination (R^2). In this case, the coefficient of determination value ($R^2 = 0.7187$) indicates that 71.87% of the variability in the response can be explained by the model. The effects of each variable on citric acid production can be observed in Fig. 3.

Table 4. Fatty acid profiles for the production (%) of lipids by *Y. lipolytica* UFLA CM-Y9.4 after growth in media with 15, 25, 30, or 35 g/l crude glycerol.

Fatty acids	Crude glycerol concentrations			
	15 g/l	25 g/l	30 g/l	35 g/l
C16:0	6.16 ± 0.16	24.15 ± 0.15	26.13 ± 1.96	18.23 ± 0.68
C17:0	-	5.57 ± 0.27	-	10.42 ± 0.22
C18:0	4.56 ± 0.23	28.87 ± 1.32	24.73 ± 0.23	29.48 ± 0.2
C16:1	8.04 ± 0.34	2.65 ± 0.45	15.67 ± 0.46	-
C18:1	8.64 ± 0.4	15.92 ± 0.5	20.92 ± 0.31	-
C18:2	-	-	-	-

C16:0 (Palmitic acid), C17:0 (Heptadecanoic acid), C18:0 (Estearic acid), C16:1 (Palmitoleic acid), C18:1 (Oleic acid), and C18:2 (Linolenic acid).

Table 5. Regression coefficient for citric acid responses.

	Regression coefficient	Pure error	t(2)	*p*-Value
Mean	0.033	0.0020	15.963	0.003
(1) T (L)	-0.008	0.0009	-8.697	0.012
T (Q)	0.002	0.0010	2.610	0.120
(2) G (L)	0.007	0.0009	7.652	0.016
G (Q)	0.015	0.0010	13.978	0.005
(3) A (L)	0.027	0.0009	27.913	0.001
A (Q)	-0.007	0.0010	-7.438	0.017
1L × 2L	-0.006	0.0012	-5.295	0.033
1L × 3L	-0.006	0.0012	-5.099	0.036
2L × 3L	0.011	0.0012	9.217	0.011

The production of citric acid found in higher value (0.14 g/l) was attained when the temperature of incubation was 32°C, with concentration of glycerol of 25 g/l, and 170 rpm of agitation. However, this statistical model does not evaluate the conditions individually but the interactions that may occur, since one of its main objectives is to reduce the number of experiments.

Citric acid production showed different responses. The effect of the interaction of the parameters is clearly evident in tridimensional plots representing an infinite number of combinations of the test variables. Plot I shows that citric acid production was higher when higher glycerol concentrations were associated with lower temperatures. Plot II illustrates that higher citric acid production occurred at higher glycerol concentrations associated with greater agitation. Plot III reveals that citric acid production was larger for greater agitation and lower temperature.

The population was not graphically represented because the values observed may not be related with the citric acid production: the population increased or decreased regardless of citric acid concentration.

As with population, glycerol consumption could not be graphically represented because the obtained values cannot be statistically explained owing to large variations in consumption between assays.

For the validation of results, the model was tested in an ideal medium, with flasks of 2,000 ml containing 1,200 ml of culture media submitted to shaking at 184 rpm, temperature of 30°C, and crude glycerol concentration of 38.4 g/l. From both the RSM experiments and validation, it was concluded that the ideal glycerol concentration is higher than 38.4 g/l. Compared with the starter medium, this optimal medium increased citric acid production by 1.35-fold and increased growth by 1.46-fold for the yeast *Y. lipolytica* UFLA CM-Y9.4.

Discussion

The results obtained in this study demonstrate that glycerol has the potential to be used as a substrate for the production of metabolic products such as lipids and organic acids by the yeast *Y. lipolytica* UFLA CM-Y9.4. The biochemical mechanisms of sugar metabolism and systems that lead to the synthesis of lipids and citric acid by the yeast *Y. lipolytica* cultivated in glycerol are not yet completely known and require further study [18].

The selection of yeasts for growth in crude glycerol and the optimization of biomass production were carried out using the response surface methodology. It was demonstrated that biomass production by some oleaginous species can be

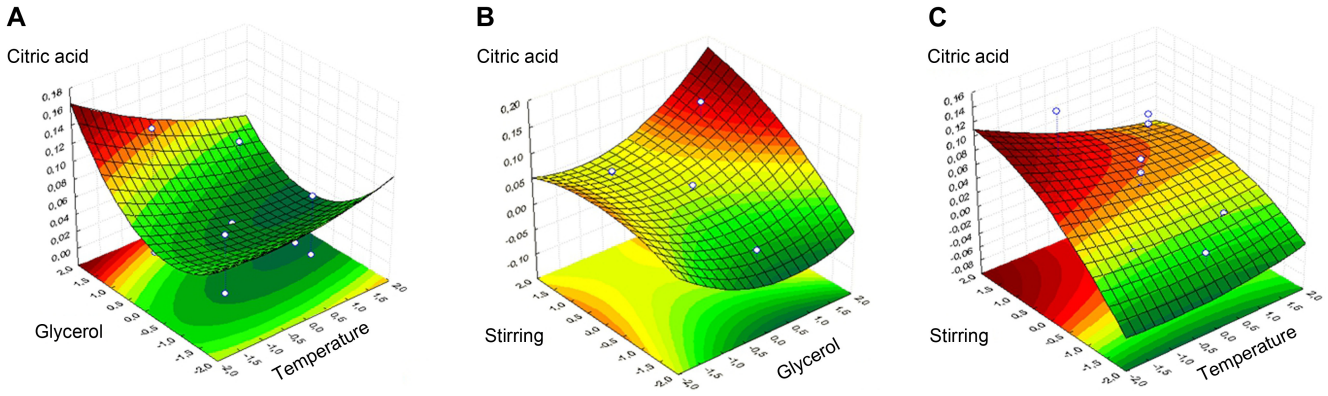

Fig. 3. Surface plots of the responses in citric acid production.
(**A**) Citric acid production as a function of glycerol concentration and temperature. (**B**) Citric acid production as a function of shaking and glycerol concentration. (**C**) Citric acid production as a function of shaking and temperature.

significantly increased by optimizing the crude glycerol concentration and the growth temperature. Yeasts are capable of converting crude glycerol into value-added products that represent a valorization of biodiesel industry byproducts [22].

In a study, the yeast *Cryptococcus curvatus* exhibited restricted growth when glycerol concentrations exceeded 64 g/l [13]. In the present study, the yeast *Cryptococcus curvatus* NCYC 476 showed cell growth for up to 96 h in medium with 300 g/l crude glycerol. The same was observed for the yeast *Y. lipolytica* UFLA CM-Y9.4. *Rhodotorula glutinis* NCYC 2439 remained viable at concentrations of 300 g/l for up to 120 h, whereas the species *Lindnera saturnus* UFLA CES-Y677 endured concentrations of 300 g/l for only 48 h. Comparisons between the literature and the obtained data reveal that the growth of yeast species in glycerol depends on cultivation conditions and the type of glycerol, and it seems that optimal conditions may be different across strains and not only across species.

Chi *et al*. [5] observed higher cell growth in crude glycerol when comparing crude and pure glycerol as carbon sources in the cultivation of the alga *Schizochytrium limacinum*. These data are in agreement with the present study, as the tested yeasts generally showed a higher growth in a crude glycerol medium. The crude glycerol used here was chemically evaluated and exhibited the following average composition: 0.271% methanol, 0.317% ethanol, 0.021% sucrose, 0.763% glucose, and 72.96% glycerol.

The present study demonstrated that the use of crude glycerol originating from biodiesel synthesis enabled a 16.12% increase in yeast growth when compared with the commercial glycerol. This result may be primarily explained by the presence of impurities in the biodiesel byproduct, including nutritional elements used by microorganisms during the fermentation process, such as phosphorus, sulfur, magnesium, calcium, nitrogen, sodium, and free fatty acids [23]. In contrast, during tests in submerged cultures where very high crude glycerol concentrations (20% and 30% (w/v)) were used, yeast growth may have been inhibited by the presence of impurities that may have negatively influenced the cell viability when found in higher concentrations.

The highest biomass yields obtained from crude glycerol are comparable to those obtained when glucose was the carbon source, suggesting that crude glycerol can be used for biomass production by yeasts. This residue also proved to be interesting for conversion into value- added products as a viable alternative for industry [22].

Rupcic *et al*. [19] evaluated lipid production by the species *Candida lipolytica* (currently known as *Yarrowia lipolytica*) using methanol as a carbon source. Upon the addition of 1% methanol to the culture medium, the authors observed that the yeast synthesized 4.9% more lipid biomass. The glycerol used in the present study was composed of only 0.27% methanol. This low concentration may have exerted little influence on growth or lipid production.

The fatty acid composition observed in this study was similar to that observed by Chatzitragkou *et al*. [4] using crude glycerol for conversion into microbial lipids. At all tested concentrations, the major cell fatty acids produced by yeasts were palmitic (C16:0), palmitoleic (C16:1), estearic (C18:0), oleic (C18:1), and linolenic (18:2) acids, whereas medium- and long-chain fatty acids were produced in smaller amounts. Linolenic acid was the sole fatty acid that was not found. The fatty acids that stood out were palmitic, estearic, and oleic acids. In another study in which cane molasses was used to stimulate lipid production by *Yarrowia lipolytica*, the fatty acids found in the largest amounts were estearic and palmitic acids [10]. These data are highly relevant because, in addition to the lipid production by yeasts being dependent on the strain used, it appears that the culture substrate also influences the amount and quality of the lipids produced.

High yields in lipid production were obtained by El Bialy *et al*. [7], after six days incubation in a medium containing 20 g/l glucose. Here, fatty acid profiles likewise varied with substrate concentration. The fatty acid concentration differed significantly at the various growth phases and for the different glycerol concentrations [1]. Consistent with this, the fatty acid concentrations observed in this study varied, with 77.16% and 87.45% of the fatty acids analyzed found at the concentrations of 25 and 30 g/l of crude glycerol, respectively.

Agro-industrial residues, including molasses as well as carrot, potato, and banana residues, are frequently used in citric acid production [2]. Studies used glycerol for the production of citric acid and observed that the greatest citric acid production (approximately 35 g/l) was obtained from crude glycerol [12, 16]. Citric acid production by *Y. lipolytica* in medium containing glycerol requires specific conditions, such as an aeration rate of 0.24–0.36, for the conversion of glycerol into citric acid. The dissolved oxygen concentration must be at 30% to 80% saturation, and cultivation time may vary between 7 and 15 days [20].

The response surface methodology was used as a more efficient method to identify the effect of individual process

variables, locate the ideal combination of variables for a multivariable process, and thus save time using less experimental data [16].

In this study, we observed that the production of citric acid occurred after the stationary growth phase, when extracellular nitrogen became a limiting factor for growth. Crude glycerol is an important factor in the production of citric acid. The use of this substrate in high concentrations, such as 80 and 120 g/l, results in a significant increase in citric acid production (33–35 g/l) [16]. The yeast *Y. lipolytica* is one of the most studied "non-conventional" yeasts. As a result of the potential of *Y. lipolytica*, many technologies, including various cultivation conditions, have been applied to the production of lipids and citric acid with high overall yields using different byproducts and agro-industrial residues as substrates [20].

Tests carried out in culture media containing pure and crude glycerol showed that the yeasts *Lindnera saturnus* UFLA CES-Y677, *Yarrowia lipolytica* UFLA CM-Y9.4, *Rhodotorula glutinis* NCYC 2439, and *Cryptococcus curvatus* NCYC 476 grew better in medium containing crude glycerol. In subsequent tests, the yeast *Yarrowia lipolytica* UFLA CM-Y9.4 stood out for its ability to consume high amounts of crude glycerol and was therefore selected for analyses of lipid and citric acid production capabilities. The highest lipid production of approximately 63.4% was obtained at a concentration of 30 g/l crude glycerol. This is an excellent result that deserves to be explored in future experiments that examine the lipid production on a larger scale for applications in the biodiesel industry.

Moreover, citric acid production was optimized, and the optimal conditions were found to be 38.4 g/l crude glycerol, shaking at 184 rpm, and a temperature of 30ºC. However, low concentrations of citric acid (0.153 g/l) were found than that which has been reported in the literature. This process still requires optimization to improve yields. The yeast *Y. lipolytica* UFLA CM-Y9.4 is able to successfully convert crude glycerol into microbial lipids and citric acid. Owing to a continuous increase in biodiesel production, crude glycerol may be a viable substrate for the production of lipids and citric acid. Additionally, the produced lipids can be used again for the production of biodiesel.

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