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SIMULTANEOUS LIPID AND CAROTENOID PRODUCTION BY STEPWISE FED-BATCH CULTIVATION OF *Rhodotorula mucilaginosa* WITH CRUDE GLYCEROL

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Abstract - In the present study, an innovative cultivation strategy of *Rhodotorula mucilaginosa* CCT 7688 to accumulate both lipids and high added value carotenoids was proposed, with the potential to contribute to the economic feasibility of microbial oils in comparison with vegetable oils. The stepwise fed-batch cultivation with crude glycerol feeding at 24 h, 96 h, and 144 h, with the addition of magnesium to the initial culture medium, resulted in a lipid content of 51.0 ± 0.3 % CDW, biomass concentration of 21.00 ± 0.48 g L^{-1}, total lipid production of 10.72 ± 0.18 g L^{-1}, lipid productivity of 0.037 ± 0.001 g L^{-1} h^{-1}, volumetric carotenoid production of 2843.2 ± 282.0 μg L^{-1}, and carotenoid productivity of 9.87 ± 0.98 μg L^{-1} h^{-1}. These values represent a 6-fold increase in lipid content, a 19-fold increase in lipid production, and a 2-fold increase in carotenoid production compared to batch cultivation.

Keywords: Feeding strategies; Oleaginous yeasts; Microbial lipids; β-carotene.

INTRODUCTION

Lipids have broad potential applications in various industrial sectors and can be used in food, food supplementation, oleochemicals, and biofuel production, including biodiesel. The growing biodiesel production is essentially dependent on vegetable oils, which has led to many discussions regarding the competition with food and feed production (Koizumi, 2015; Koutinas et al., 2014).

In this context, alternative raw materials have been investigated, with several studies on the production of microbial lipids (Angerbauer et al., 2008; Béligon et al., 2015; Chang et al., 2013; Chen et al., 2013; Zhao et al., 2008). Oleaginous yeasts stand out among the lipid-producing microorganisms due to their high growth rate and ability to assimilate a wide variety of carbon sources (Moliné et al., 2012), such as crude glycerol from biodiesel production.

Glycerol is the main byproduct of biodiesel production and approximately 10% of the weight of biodiesel is generated in glycerol (Quispe et al., 2013). With the growing demand for biodiesel, this byproduct is increasingly easy to obtain, reducing its commercial value. According to the UFOP report for 2017/2018, world biodiesel production amounted to 34.08 million tonnes in 2016. The most important biodiesel producer was the European Union (12.61 million tonnes), followed by the United States (6.21 million tonnes) and Brazil (3.30 million tonnes). Therefore, solutions are required regarding the use of the crude glycerol generated in this production.

Different oleaginous yeasts have demonstrated an ability to synthesize lipids using the crude glycerol

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derived from biodiesel production as a carbon source (Liang et al., 2010; Saenge et al., 2011; Thiru et al., 2011; Duarte et al., 2013). The use of this byproduct to obtain microbial oil may be a promising alternative, since it can add value to the biodiesel production chain, thus making it more competitive (Silva et al., 2009). However, microbial oils (mainly from yeasts) are not a viable alternative for both biodiesel production and other processes in the substitution of vegetable oils, mainly due to the cultivation costs (e.g. aeration, agitation, and sterilization) (Koutinas et al., 2014; Tchakouteu et al., 2017).

The yeast *Rhodotorula mucilaginosa* has shown a great potential for lipid production and, as a red yeast, has the capacity to synthesize carotenoids, a high value-added bioproduct that, if produced simultaneously, can make the process economically viable. Carotenoids are natural pigments widely used in the food, pharmaceutical, cosmetic, and animal feed industries due to their biological properties, such as antioxidant activity (Valduga et al., 2009). Greater consumer awareness with regard to health and functional foods has triggered an increase in the demand for carotenoids, highlighting the biotechnological production due to the proven biological activity of these biomolecules (Cipolatti et al., 2015).

Thus, the conditions that favor cell growth and lipid and carotenoid accumulation should be investigated, including the cultivation mode. In the stepwise fed-batch process, feeding is intermittent and allows the regulation of the feeding of the substrate, preventing an inhibitory activity in biomass growth and lipid accumulation (Shuler and Kargi, 2012). Furthermore, some authors found an increase in lipid production in the fed-batch process compared with the batch process (Fei et al., 2016; Saenge et al., 2011). This cultivation strategy may be a promising alternative, attracting the attention of several researchers regarding the production of microbial oils by this process (Anschau et al., 2014; Béligon et al., 2015; Cescut et al., 2014; Chang et al., 2013; Fei et al., 2016; Pirozzi et al., 2014; Raimondi et al., 2014; Rakicka et al., 2015). However, studies that involve the simultaneous lipid and carotenoid production by fed-batch processes remain scarce.

Therefore, the main goal of this study was to evaluate different stepwise fed-batch cultivation strategies for the yeast *R. mucilaginosa* CCT 7688 in crude glycerol-based medium and the effects on biomass concentration, lipid production, and carotenoid production.

MATERIAL AND METHODS

Yeast strain

The yeast *Rhodotorula mucilaginosa* CCT 7688 (André Tosello Foundation, Campinas, Brazil) was previously isolated and identified by our research group, and characterized by PCR fingerprinting, i.e., the mini/microsatellite-primed PCR technique (MSP-PCR), in accordance with Libkind et al. (2003).

The strain was reactivated by transferring it to Yeast Malt (YM) agar slants, composed of (g L^{-1}): 10 glucose, 5 peptone, 3 malt extract, 3 yeast extract, and 20 agar. The tubes were incubated at 25 °C for 48 h (Spier et al., 2015).

Crude glycerol

The crude glycerol was purchased from BS Bios Indústria e Comércio de Biodiesel Sul Brasil S/A, located in Passo Fundo (Brazil), obtained from the biodiesel production of degummed soybean oil by the methanolic route. The crude glycerol contained (%): 81.92 glycerol (Official monographs USP XXI, 1985), 11.29 moisture (AOCS, 2004), 5.38 ashes (Official monographs USP XXI, 1985), and 1.41 nonglyceridic organic matter (by difference), pH 5.39.

Pre-inoculum and inoculum preparation

To prepare the pre-inoculum, two tubes of the reactivated microbial culture were scraped with 10 mL of 0.1 % peptone diluent to each tube for the removal of the microorganism cells and transferred to an Erlenmeyer flask containing 180 mL of YM broth. The culture medium was prepared in concentrated form to attain the desired composition after inoculation (10 g L^{-1} of glucose, 5 g L^{-1} of peptone, 3 g L^{-1} of malt extract and 3 g L^{-1} of yeast extract) and incubated in a rotary shaker (Tecnal TE-424, Brazil) at 25 °C and 180 rpm (Spier et al., 2015). After 48 h, 20 mL of the pre-inoculum were transferred to the inoculum medium (180 mL) with the same composition and incubated under the same temperature and stirring conditions. After 72 h, the number of cells was counted in a Neubauer chamber (10^8 cells mL^{-1}).

Batch cultivation

The batch cultivation was carried out in 500 mL Erlenmeyer flasks with an initial volume of 200 mL, using a rotary shaker (Tecnal TE-424, Brazil) at 180 rpm and 25 °C. The inoculum corresponded to 10 % of the initial volume and the total cultivation time was 168 h. The composition of the medium (YMG) was adapted from the YM broth, replacing the glucose with crude glycerol, maintaining the same C/N ratio (7.63). The concentrations of the components were established using Excel software (Solver tool) (Microsoft Inc., USA), considering this C/N ratio and the composition of the components in terms of carbon and nitrogen contents, determined in a CHNS/O Elemental Analyzer (Model 2400 Series II, Perkin Elmer, USA), using acetanilide as a reference (Table 1). The medium composition was (g L^{-1}): 10.4 crude glycerol, 4.3 peptone, 2.6 malt extract, and 3.4 yeast extract, without pH adjustment.

Table 1. Carbon, nitrogen and hydrogen content of the culture medium components.

Component	% C	% H	% N
Peptone	43.47	6.32	15.32
Yeast extract	39.12	6.23	10.66
Crude glycerol	31.50	2.27	< 0.07
Malt extract	40.47	5.25	1.22

Stepwise fed-batch cultivation

First, the cultivation conditions and the medium composition were the same in the batch mode, and the microbial growth curve was used as the basis for establishing the fed-batch cultivation strategies. The first two strategies (YMG24 and YMG96) consisted of feeding 20 mL of aqueous solution (10% initial volume), containing only crude glycerol (104 g L^{-1}), at different feeding times: a pulse in the exponential phase (24 h) or the stationary phase (96 h). New strategies were then proposed (Table 2), increasing the number of feedings (YMG1, YMG2 and YMG3). Cultivations with the addition of 3 g L^{-1} magnesium sulfate and/or pH adjustment of the culture medium to 4.5 were also performed (YMG4, YMG5 and YMG6).

Analytical methods

During the cultivation, samples were taken at regular intervals to determine the biomass concentration, glycerol concentration, and pH. The lipids and carotenoids in the recovered biomass were determined at the end of the process.

Biomass

Samples were centrifuged (Eppendorf 5804 R, Germany) at 10414 × g for 15 min, and the cells (pellet) were washed with distilled water, centrifuged again and resuspended. The optical density was then measured in a spectrophotometer (Bioespectro SP 220, China) at 600 nm. Using a previously established standard curve for the microorganism, the biomass concentration in cell dry weight (g L^{-1}) was determined for each sample (Choi and Park, 2003).

pH

The pH of the supernatant was measured with a pH meter, in accordance with AOAC (2000).

Glycerol

The glycerol in the supernatant was determined by the method described by Bondioli and Bella (2005), based on the reaction with sodium periodate, with an absorbance reading at 410 nm. The absorbance values were converted to glycerol concentration (g L^{-1}) using a predetermined standard curve (Equation 1).

$$\text{Glycerol concentration}\left(\text{g L}^{-1}\right) = 0.0996 \times \text{Absorbance} \quad (1)$$

Lipid content

The lipid content, expressed as a percentage of cell dry weight (% CDW), was determined in the dry biomass (300-500 mg) using the method of Bligh and Dyer (1959), adapted by Manirakiza et al. (2001). The dry biomass was obtained through the centrifugation (10414 × g for 15 min) of the remaining medium at the end of the cultivation, washing with distilled water, centrifugation again, drying at 35 °C (Michelon et al., 2012) until constant weight and crushing to powder in a mortar and pestle. Total lipid production (g L^{-1}) was calculated by multiplying the biomass concentration by the lipid content (Spier et al., 2015). Lipid productivity (g L^{-1} h^{-1}) was calculated by dividing total lipid production by time.

Fatty acid profile

To determine the fatty acid profile, the lipid fraction was esterified to obtain fatty acid methyl esters (Metcalfe et al., 1966). The samples were then analyzed using a gas chromatograph (Shimadzu 2010 Plus, Japan), equipped with a split/splitless injector, a Rtx®-1 capillary column (30 m × 0.25 mm internal diameter × 0.25 μm particle diameter) and a flame ionization detector. Hydrogen was used as the carrier gas at a flow rate of 1.25 mL min^{-1}, and the injector and detector temperatures were 260 °C. The initial column temperature was 50 °C, rising to 200 °C at a rate of 6 °C min^{-1}, remaining 4 min at this temperature. This was followed by a further increase of 2 °C min^{-1}, until the temperature reached 240 °C, at which it remained for 10 min. Sigma-Aldrich standards were used to identify the fatty acids, which were quantified by area normalization (Massarolo et al., 2016).

Table 2. Strategies for stepwise fed-batch cultivations.

Assay	Feeding times (h)	Pulse feeding (mL)	Other conditions
YMG24	24	20	-
YMG96	96	20	-
YMG1	24/96	20/20	-
YMG2	24/96/144	20/20/20	-
YMG3	24/72/120	20/20/20	-
YMG4	24/96/144	20/20/20	Initial pH at 4.5
YMG5	24/96/144	20/20/20	Addition of $MgSO_4.7H_2O$
YMG6	24/96/144	20/20/20	Addition of $MgSO_4.7H_2O$ and initial pH at 4.5

Carotenoid concentration

The total carotenoids (TC) were determined according to the methodology of Fonseca et al. (2011), modified by Cipolatti et al. (2015). For this purpose, 2 mL of dimethylsulfoxide (55 °C) was mixed with 0.05 g dry biomass (35 °C for 48 h), ground, sieved (Tyler 115) and frozen (-18 °C for 48 h). The suspension was stirred for 1 min at 15 min intervals, totaling 1 h. Afterwards, 6 mL acetone was added, and the sample was centrifuged (1745 x g for 10 min) to remove the solvent. The procedure was repeated until the cells were colorless. Following this, 10 mL of 20 % w/v NaCl and 10 mL petroleum ether were added to the solvent extract. After stirring and phase separation, the excess water was removed with sodium sulfate (Michelon et al., 2012). The absorbance was then measured in a spectrophotometer at 448 nm (Machado and Burkert, 2015) and the total carotenoids were calculated using Equation 2, as reported by Davies (1976).

$$TC = \frac{A \times V \times 10^6}{A_{1cm}^{1\%} \times 100 \times W} \quad (2)$$

where TC is the total carotenoid concentration (μg g^{-1}), A is the absorbance, V is the volume of carotenogenic extract (mL), $A_{1cm}^{1\%}$ is the specific absorptivity, and W is the cell dry weight (g).

To calculate the volumetric concentration of carotenoids (μg L^{-1}), the TC was multiplied by the biomass concentration (cell dry weight) (Machado and Burkert, 2015).

Statistical analysis

The experiments were conducted in triplicate, using ANOVA followed by Tukey's test to verify significant differences between the cultivation conditions at a 95 % confidence level ($p \leq 0.05$), using Statistica 5.0 software (Stat Soft Inc., USA).

RESULTS AND DISCUSSION

Batch and stepwise fed-batch cultivation with YMG medium

As shown in Table 3, no significant lipid accumulation was observed in *R. mucilaginosa* CCT 7688 for the batch cultivation (lipid content of 8.5 % CDW and total lipid production of 0.57 g L^{-1}), probably due to the C/N ratio used (7.63), with no nitrogen limitation. Nitrogen limitation is generally the most efficient type of regulation for inducing lipid accumulation, since the carbon is distributed among the different macromolecular groups (carbohydrates, lipids, nucleic acids, and proteins) during the microbial growth phase, and nitrogen is essential for the synthesis of nucleic acids and proteins, which are required for cell growth. With nitrogen limitation, the growth rate decreases rapidly, while the carbon assimilation rate decreases more gradually. Thus, the carbon flux is channeled into the lipid synthesis, allowing the accumulation of triacylglycerols (Beopoulos et al., 2009).

Angerbauer et al. (2008) studied the yeast *Lipomyces starkeyi* using glucose as carbon source and observed an increase in lipid content from 34 % CDW to 68 % CDW, and total lipid production from 4.1 to 6.4 g L^{-1} when the C/N ratio changed from 15 to 150.

Based on the growth curve of *R. mucilaginosa* CCT 7688 in batch cultivation (Figure 1), strategies YMG24 and YMG96 were established (Table 2), i.e., crude glycerol feeding within 24 h or 96 h, respectively. It is important to note that all stepwise fed-batch cultivations began with the same medium used in batch cultivation in order to provide initially a C/N ratio that promotes microbial growth, followed by crude glycerol feedings at specific time intervals that provide an excess of carbon and an increase in

Table 3. Comparison of lipid production between the batch and stepwise fed-batch process.

Assay	Lipid content (% CDW)	Biomass (g L^{-1})	Total lipid (g L^{-1})	Lipid productivity (g L^{-1} h^{-1})	Total time of cultivation (h)
Batch	8.5 ^{e} ± 0.5	6.77 ^{c} ± 0.76	0.57 ^{e} ± 0.09	0.003 ^{e} ± 0.001	168
YMG24	9.5 ^{e} ± 1.9	14.47 ^{b} ± 1.52	1.38 ^{d,e} ± 0.27	0.008 ^{d,e} ± 0.002	168
YMG96	9.1 ^{e} ± 0.5	13.22 ^{b} ± 1.87	1.21 ^{d,e} ± 0.24	0.007 ^{c,d,e} ± 0.001	168
YMG1	16.0 ^{d} ± 1.3	14.54 ^{b} ± 2.28	2.35 ^{c,d} ± 0.55	0.014 ^{c} ± 0.003	168
YMG2	21.4 ^{c} ± 1.5	14.23 ^{b} ± 2.35	3.04 ^{c} ± 0.39	0.013 ^{c,d} ± 0.002	240
YMG3	15.9 ^{d} ± 4.1	17.57 ^{a,b} ± 0.99	2.78 ^{c} ± 0.70	0.012 ^{c,d} ± 0.003	240
YMG4	44.3 ^{b} ± 1.3	17.61 ^{a,b} ± 0.54	7.81 ^{b} ± 0.47	0.027 ^{b} ± 0.002	288
YMG5	51.0 ^{a} ± 0.3	21.00 ^{a} ± 0.48	10.72 ^{a} ± 0.18	0.037 ^{a} ± 0.001	288
YMG6	50.9 ^{a} ± 1.2	17.08 ^{a,b} ± 1.02	8.68 ^{b} ± 0.47	0.030 ^{b} ± 0.002	288

Mean values ± standard deviation, n = 3.
Different letters in the same column indicate a significant difference at 95% confidence level ($p<0.05$).
YMG24: 20 mL crude glycerol within 24 h; YMG96: 20 mL crude glycerol within 96 h; YMG1: 20 mL crude glycerol within 24 h, and 20 mL within 96 h; YMG2: 20 mL crude glycerol within 24 h, 20 mL within 96 h, and 20 mL within 144 h. YMG3: 20 mL crude glycerol within 24 h, 20 mL within 72 h, and 20 mL within 120 h; YMG4: 20 mL crude glycerol within 24 h, 20 mL within 96 h, and 20 mL within 144 h, with initial pH adjustment at 4.5; YMG5: 20 mL crude glycerol within 24 h, 20 mL within 96 h, and 20 mL within 144 h, with the addition of 3 g L^{-1} $MgSO_4.7H_2O$ to the initial medium; YMG6: 20 mL crude glycerol within 24 h, 20 mL within 96 h, and 20 mL within 144 h, with pH adjustment at 4.5, and the addition of 3 g L^{-1} $MgSO_4.7H_2O$ to the initial medium.

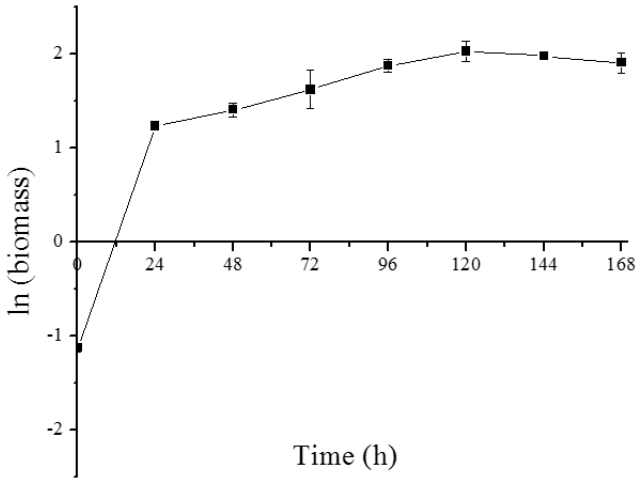


Figure 1. Growth curve of the microorganism in batch cultivation. Mean values ± standard deviation (n = 3).

C/N ratio that promotes lipid accumulation, because a high total lipid production depends on both biomass and lipid content. Therefore, this cultivation strategy seeks to reconcile both high biomass and lipid content to improve lipid production.

According to Table 3, an increase in biomass concentration was observed (from 6.77 to 14.47 and 13.22 g L^{-1}, respectively), probably due to the greater availability of the carbon source and the presence of nitrogen. However, there was no change in lipid content, with no significant differences between these two strategies.

In contrast, when more than one feeding with crude glycerol was used (YMG1, YMG2, and YMG3), the lipid synthesis capacity also increased, probably due to the significant amount of carbon introduced into the medium, resulting in an increase in the C/N ratio, favoring the lipid accumulation in the cells. One of the differences among the strategies YMG1, YMG2, and YMG3 was the feeding time (Table 2). Moreover, YMG2 and YMG3 exhibited one more feeding compared to YMG1 (Table 2). This higher carbon availability, together with the feeding time, allowed a greater lipid accumulation in YMG2. Compared with the batch process, the total lipid production increased by more than 400 % in this condition. Saenge et al. (2011) compared the results from batch and fed-batch processes for the yeast *R. glutinis* TISTR 5159, and reported that the fed-batch led to an increase of approximately 41 % in total lipid production (from 4.33 g L^{-1} to 6.10 g L^{-1}). Thus, the lipid productivity also increased from 0.058 g L^{-1} h^{-1} to 0.085 g L^{-1} h^{-1}. Fei et al. (2016) compared the batch and the fed-batch processes and observed that the latter provided an increase in productivity from 0.28 g L^{-1} h^{-1} to 0.40 g L^{-1} h^{-1}.

No significant differences were observed in the biomass concentration when YMG24 and YMG96 are compared with both YMG1 and YMG2 (Table 3), despite the addition of increasing quantities of glycerol. During the growth phase, yeast metabolism results in the balanced distribution of carbon between the four main macromolecules (carbohydrate, lipid, nucleic acid and protein). In conditions of nitrogen limitation (increase in the C/N ratio), caused by an excess of glycerol feeding, nucleic acid and protein synthesis are repressed and the growth rate slows down (Beopoulos et al., 2009).

The different times of cultivation for the different strategies are also shown in Table 3. The respective times were established according to stationary phase achievement, where lipid accumulation increases. This fact is associated with the different strategies used because the glycerol feedings lead to a higher substrate availability that impacts cell growth and extends the exponential phase. The oleaginous yeasts tend to accumulate lipids in the stationary phase, when the nitrogen present in the medium is already exhausted, channeling the carbon flow to the lipid synthesis instead of the growth, allowing the accumulation of triacylglycerols. Comparing the YMG1 and YMG2 strategies, an increase in time (from 168 h to 240 h, respectively) was observed, since the YMG2 strategy presents three feedings instead of two, increasing the quantity of glycerol in the medium, which provided an extension in the consumption of this carbon source. The same occurred for the assays YMG4, YMG5 and YMG6, which presented longer cultivation times when compared with the strategies with one or two feeds.

Effect of initial pH adjustment

During the first cultivation with no pH adjustment, an expressive increase in pH was observed, reaching pH 8 (data not shown). These values were above those recommended in the literature for microbial growth, since the most frequently reported pH values ranged from 3 and 6 (Ageitos et al., 2011). This behavior indicated the need to evaluate the influence of the initial pH on the performance of the microorganism. Therefore, an assay with adjustment of the initial pH to 4.5 was proposed (YMG4).

The cultivation YMG4 led to an increase in lipid content from 21.4 % CDW to 44.3 % CDW and total lipids from 3.04 g L^{-1} to 7.81 g L^{-1} compared with the YMG2 subjected to the same feeding, without adjustment of the initial pH. The final pH of the cultivation YMG4 was 4.32. Similar behavior was observed by other authors. Karatay and Dönmez (2010) found an increase in lipid content from 31.5 % CDW to 59.9 % CDW for *Candida lipolytica* with an increase in pH of the medium from 4 to 5. Angerbauer et al. (2008) evaluated the effect of pH on the cultivation of *Lipomyces starkeyi* and found a lipid content of approximately 7 % CDW at pH 7.0 and approximately 56 % CDW at pH 5.

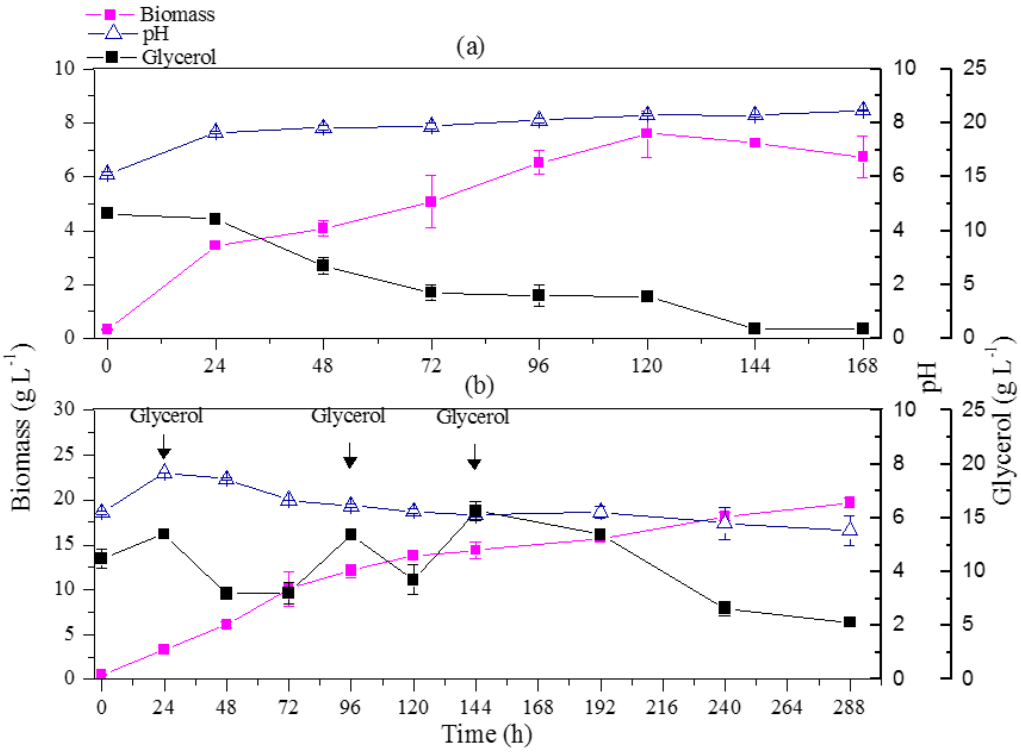
Effect of the addition of magnesium

As reported by Taha et al. (2013), the addition of magnesium to the medium favors the lipid accumulation in the microorganism. Based on previous studies (data not shown), assays have been proposed with the addition of magnesium sulfate to the initial culture medium (YMG5, YMG6, with and without pH adjustment, respectively). YMG5 resulted in lipid content of 51.0 % CDW, biomass concentration of 21.00 g L^{-1}, total lipid production of 10.72 g L^{-1}, and lipid productivity of 0.037 g L^{-1} h^{-1}, with significant differences from the other conditions tested in relation to total lipid production and lipid productivity (Table 3). Compared with the batch process (Table 3), this strategy represented an increase in lipid accumulation and biomass production of approximately 500 % and 200 %, respectively, and an increase in lipid productivity and lipid production of approximately 12 and 19 times, respectively.

The kinetic profile of both cultivations is shown in Figure 2. In the batch cultivation, the pH remained close to 8.0, a value considered above that recommended in the literature for the growth of microorganisms. For YMG5 cultivation, after an increase to 8, the pH values decreased to 6 due to stepwise feedings that favored lipid accumulation. A decrease in pH is expected because lipid accumulation is followed by citric acid production, leading to a pH reduction (Beopoulos et al., 2009). In relation to the glycerol consumption in the YMG strategy, after each feeding time, glycerol concentration almost doubled in relation to the amount present in the culture medium, and was then quickly consumed by the yeast, whereas in the batch strategy, almost all of the glycerol was consumed.

When compared, YMG2 and YMG5, which differ only with regard to the addition of magnesium, an increase in lipid content and lipid production was observed, with values ranging from 21.4 % CDW to 51.0 % CDW and 3.04 g L^{-1} to 10.72 g L^{-1}, respectively. This higher lipid accumulation in the presence of magnesium sulfate may be due to the action of Mg^{2+} on the cellular metabolism during the lipid biosynthesis.

According to Beopoulos et al. (2009), the triacylglycerol synthesis occurs from the Kennedy pathway and monoacylglycerol, in which glycerol-3-phosphate is acylated to lysophosphatidic acid (LPA) by the enzyme glycerol-3-phosphate acyltransferase (SCT1), which generates phosphatidic acid (PA) after acylation by the enzyme lysophosphatidic acid acyltransferase (SLC1). It undergoes dephosphorylation by the enzyme phosphatidic acid phosphohydrolase (PAP), becoming diacylglycerol (DAG), which leads to the formation of triacylglycerol (TAG) after the acyl-CoA reaction, catalyzed by the enzymes DGAT1 and DGAT2. According to Taha et al. (2013), magnesium can influence this process in two steps, as follows: on PAP1 (Mg^{2+}-dependent), because this enzyme requires Mg^{2+} as a cofactor for



YMG5: 20 mL crude glycerol within 24 h, 20 mL within 96 h, and 20 mL within 144 h, with the addition of 3 g L^{-1} $MgSO_4.7H_2O$ to the initial medium.

Figure 2. Kinetic profile of batch and stepwise fed-batch cultivations. (a) batch; (b) YMG5 stepwise fed-batch strategy.

the catalytic activity, resulting in greater activity and, consequently, greater conversion to DAG; and on the enzymes DAGT1 and DAGT2, which may have their catalytic activities favored by the concentration of available magnesium, resulting in a greater conversion to TAG (Yen et al., 2008).

Similar behavior was observed by other authors with other microorganisms. Singh et al. (2016) studied the behavior of the microalga *Aurantiochytrium* sp. in the presence of magnesium in the culture medium (5 g L^{-1}), and observed an increase in lipid content of approximately 17 % CDW compared with the magnesium-free cultivation. With regard to the yeast *Trichosporon cutaneum*, a higher lipid accumulation and biomass concentration were observed with the addition of 0.3 g L^{-1} of magnesium sulfate to the culture medium containing corncob hydrolysate (Chen et al., 2013). For *Lipomyces starkeyi*, an increase in lipid content from 41.6 % CDW to 56.1 % CDW was observed compared with the addition of 0.5 and 1.0 g L^{-1} of $MgSO_4.7H_2O$ to the culture medium (Zhao et al. 2008). Thus, it can be stated that the addition of magnesium to the culture medium stimulates lipid production by the microorganism.

Fatty acid profile

As shown in Table 4, linoleic acid was the main fatty acid found in the oil produced by *R. mucilaginosa* CCT 7688. It is common in corn, sunflower, and soybean oil, which are important vegetable oils used in biodiesel production (Ma and Hanna, 1999).

Kot et al. (2017) studied the yeast *Rhodotorula glutinis* CCT 7688 cultivated in a culture medium containing glycerol with pH between 4 and 7, and found an increase in the synthesis of linoleic acid in detriment of the oleic acid, which is also found in microbial oils, probably due to an increase in the activity of the enzymes that convert oleic acid to linoleic acid. Liang et al. (2010) studied *Cryptococcus curvatus* ATCC 20509 using crude glycerol as a substrate, and found oleic (39.6 %), palmitic (23 %), stearic (16.7 %) and linoleic (15.2 %) acids as the major fatty acids, while Duarte et al. (2013) found linoleic (54.9 %), oleic (25.5 %), palmitic (9.8 %), stearic (13.5 %) and palmitoleic (0.4 %) acids in the biomass from *Candida* sp. Even though the main fatty acids are the same, in the present study *R. mucilaginosa* CCT 7688 presented different proportions in comparison with the findings of these authors, with a higher linoleic acid content (76.8%) and an expressive proportion of palmitoleic acid (13.5%) that is generally found in small quantities. Furthermore, a small content of saturated fatty acids was observed, e.g., palmitic acid, in relation to the other microorganisms.

Moreover, it is important to emphasize the presence of essential fatty acids such as arachidonic, γ-linolenic, and α-linolenic acids in the oil from *R. mucilaginosa* CCT 7688, albeit in small amounts. Humans cannot synthesize α-linolenic acid, which is a precursor of two other important polyunsaturated fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Therefore, it should be ingested at dietary levels (Nelson and Cox, 2013).

Table 4. Fatty acids profile of the biomass obtained with the YMG5 stepwise fed-batch strategy.

Fatty acid	Distribution of fatty acids (% w/w)
Myristoleic (C14:1)	0.4
Palmitic (C16:0)	1.4
Palmitoleic (C16:1)	13.5
Oleic (C18:1n9c)	3.4
Linoleic (C18:2n6c)	76.8
γ-Linolenic (C18:3n6)	0.4
α-Linolenic (C18:3n3)	1.7
Eicosatrienoic (C20:3n3)	0.5
Arachidonic (C20:4n6)	0.4
Tricosanoic (C23:0)	0.5
Nervonic (C24:1n9)	0.6
Elaidic (C18:1n9t)	0.2

YMG5: 20 mL crude glycerol within 24 h, 20 mL within 96 h, and 20 mL within 144 h, with the addition of 3 g L^{-1} $MgSO_4.7H_2O$ to the initial medium.

Carotenoids

R. mucilaginosa CCT 7688 was able to synthesize carotenoids associated with lipid accumulation. In this study, the cultivation strategy YMG5 corresponded to the best condition for lipid production, resulting in a volumetric carotenoid production of 2843.2 μg L^{-1}, which represents around twice as many carotenoids as the batch process (Table 5). No significant differences

Table 5. Comparison of carotenoid production between the batch and stepwise fed-batch process at the end of the cultivation.

Assay	Volumetric carotenoids (μg L^{-1})	Carotenoids productivity (μg L^{-1} h^{-1})
Batch	$1345.1^{c,d} \pm 118.8$	$8.01^{c,d} \pm 0.71$
YMG24	$3216.9^{a} \pm 525.9$	$19.15^{a} \pm 3.13$
YMG96	$2427.4^{a,b} \pm 249.6$	$14.45^{a,b} \pm 1.49$
YMG1	$3141.6^{a} \pm 586.3$	$18.70^{a} \pm 3.49$
YMG2	$1928.6^{b,c} \pm 268.2$	$8.04^{c,d} \pm 1.12$
YMG3	$2368.2^{a,b} \pm 272.5$	$9.87^{b,c} \pm 1.14$
YMG4	$3087.0^{a} \pm 155.4$	$10.72^{b,c} \pm 0.54$
YMG5	$2843.2^{a,b} \pm 282.0$	$9.87^{b,c} \pm 0.98$
YMG6	$2714.6^{a,b} \pm 103.8$	$9.43^{b,c,d} \pm 0.36$

Mean values ± standard deviation, n = 3.
Different letters in the same column indicate a significant difference at 95% confidence level ($p<0.05$).
YMG24: 20 mL crude glycerol within 24 h; YMG96: 20 mL crude glycerol within 96 h; YMG1: 20 mL crude glycerol within 24 h, and 20 mL within 96 h; YMG2: 20 mL crude glycerol within 24 h, 20 mL within 96 h, and 20 mL within 144 h. YMG3: 20 mL crude glycerol within 24 h, 20 mL within 72 h, and 20 mL within 120 h; YMG4: 20 mL crude glycerol within 24 h, 20 mL within 96 h, and 20 mL within 144 h, with initial pH adjustment at 4.5; YMG5: 20 mL crude glycerol within 24 h, 20 mL within 96 h, and 20 mL within 144 h, with the addition of 3 g L^{-1} $MgSO_4.7H_2O$ to the initial medium; YMG6: 20 mL crude glycerol within 24 h, 20 mL within 96 h, and 20 mL within 144 h, with pH adjustment at 4.5, and the addition of 3 g L^{-1} $MgSO_4.7H_2O$ to the initial medium.

were observed among the other strategies, except for YMG2. However, carotenoid productivity was 9.87 μg L^{-1} h^{-1}, which is lower than that observed for YMG24 and YMG1.

Saenge et al. (2011) produced both lipid and carotenoids from the yeast *Rhodotorula glutinis*, using crude glycerol as a substrate. This resulted in an increase of only 5 % of total carotenoids when passing from batch cultivation to the stepwise fed-batch process, while the lipid content increased approximately 40 %. This result may be due to the concurrent production of carotenoids and lipids, since they can compete with each other, as both production pathways require Acetyl Co-A as a common precursor (Schneider et al., 2013). However, in the present study, YMG5 resulted in an approximately 111 % increase in carotenoid production, concomitant with the increase in lipid accumulation, compared with the batch mode.

The present results are consistent with those found by other authors. Kot et al. (2017) studied the concomitant production of carotenoids and lipids in a batch process, with values of 3500 μg L^{-1} and 2.2 g L^{-1} of total carotenoids and lipids, respectively, for *Rhodotorula glutinis* cultivated in a culture medium containing glycerol and potato wastewater.

On the other hand, the addition of magnesium and adjustment of the pH of the medium did not lead to an increase in the synthesized carotenoids, contrary to the results obtained by other authors. Cheng and Yang (2016) evaluated carotenoid productivity by *R. mucilaginosa* in a batch process and reported a 21.4 % increase in the amount of volumetric carotenoids (3082.4 μg L^{-1}) with the addition of magnesium.

Therefore, the simultaneous lipid and carotenoid production may be an attractive alternative, with the possibility of synthesizing two bioproducts of commercial interest in the same cultivation, thus adding value to the process.

CONCLUSIONS

This study demonstrated that the stepwise fed-batch cultivation of *R. mucilaginosa* CCT 7688, as well as the addition of magnesium and the adjustment of initial pH, led to expressive increments in total lipid production (19-fold increase), lipid content (6-fold increase) and carotenoid content (2-fold increase) compared with the batch cultivation. This innovative cultivation strategy constitutes a potential technique to contribute to the economic viability of microbial oils in comparison with vegetable oils, with the simultaneous production of high added value carotenoids.

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