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Rhodotorula glutinis cultivation on cassava wastewater for carotenoids and fatty acids generation

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ABSTRACT

Yeasts from the *Rhodotorula* genus can accumulate large amounts of carotenoids and fatty acids in their cells. This study aimed to evaluate the potential of using cassava wastewater as a low-cost alternative substrate for the growth of *Rhodotorula glutinis* intended for the production of carotenoids and fatty acids. High growth of the yeast (10.28 $g{\cdot}L^{-1}$), as well as high productions of carotenoids (0.98 $mg{\cdot}L^{-1}$) and lipids (1.34 $g{\cdot}L^{-1}$), were obtained when *Rhodotorula glutinis* was cultivated in cassava wastewater as the sole source of nutrients. Moreover, the fatty acids accumulated in the yeast cells were mostly (over 50%) unsaturated, where high percentages (%) of oleic acid (59.76 ± 0.58) and linoleic acid (7.59 ± 1.18) were found when the alternative substrate was used. Results suggest that cassava wastewater presents the potential to be applied as a sole source of nutrients to support *Rhodotorula glutinis* growth and metabolites accumulation in its cells.

1. Introduction

Recently, the use of products synthesized microbiologically has been increased in various industrial sectors. The oleaginous yeast *Rhodotorula glutinis* has great potential for industrial applications as it is capable of synthesizing great amounts of lipids and carotenoid pigments (Kot, 2016). This yeast primarily synthesizes palmitic acid (16:0), oleic acid (18:1), linoleic acid (18:2), and linolenic acid (C18:3) (Kot et al., 2017). Furthermore, *R. glutinis* can synthesize three types of carotenoids: β-carotene, torulene, and torularhodin (Kot et al., 2019a).

Chemical synthesis processes have been widely used to obtain carotenoids used in food formulations, however, the widespread use of synthetic colorants has generated discussion among world health organizations, regarding the future human health impacts of these compounds (Mussagy et al., 2019). Therefore, compared with chemical synthesis, the microbial production of carotenoids presents some advantages because of the marketing appealing as a safety and natural color additive in contrast to its synthetic analogs (Cutzu et al., 2013).

Lipids are becoming an increasingly important chemical feedstock for the manufacture of biofuels, care-products and as a food source (Sargeant et al., 2017). Due to the increasing demand for these molecules, other oil sources than crops have been investigated such as microbial oils, referred to as SCO (*single cell oil*) in the literature (Kot et al., 2016; Subramaniam et al., 2010). These have many advantages such as high growth rates, year-round productivity and high lipids yield (Yen et al., 2012). However, the fatty acid profile of the lipids is extremely important in determining their eventual use. Oils high in oleic acid are the most suitable biodiesel feedstocks, whereas to replace palm oils in the cosmetic or food industries, high levels of saturated lipids are necessary (Sargeant et al., 2014). Moreover, for the food and pharmaceutical industries there is an increased demand for lipids with high concentrations of polyunsaturated fatty acids due to the health benefits of these compounds (Calder, 2015).

One of the drawbacks to the commercialization of lipids and

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carotenoids from red yeasts is the high production cost involved (Mussagy et al., 2019; Subramaniam et al., 2010). *R. glutinis* clear advantage is its capacity to grow and synthesize metabolites on substrates containing different agro-industrial byproducts (Kot et al., 2016). Therefore, in an attempt to reduce processing costs, alternative substrates like agro-industrial wastes or by-products have been used as cheap sources of nutrients (Colet et al., 2017). The main problem of using agro-industrial wastes or by-products is to find one with an adequate balance of nutrients to allow cell growth and product accumulation (Schneider et al., 2013; Yen et al., 2012). Grape must, beet molasses, soybean flour extract, cornflour extract, cheese whey, fermented radish brine, and sugar cane molasses are examples of agro-industrial wastes already evaluated for cultivation of *Rhodotorula* yeasts (Banzatto et al., 2013; Marova et al., 2012).

Cassava wastewater is a carbohydrate-rich residue generated at large amounts during the production of cassava flour, a very common food ingredient (Nitschke and Pastore, 2006). It has been estimated that the production of 1 t of cassava flour generates 300 L of wastewater (Costa et al., 2010). Cassava is among the ten most produced commodities in the world with an estimated production of 263 million t y^{-1} worldwide (FAOSTAT, 2013). The wastewater of its processing presents a wide range of both macro and micronutrients (dextrose, fructose, saccharose, magnesium (Mg^{+2}), calcium (Ca^{+2}), manganese (Mn^{+2}), iron (Fe^{+2}), zinc (Zn^{2+}) and nitrogen compounds), which can support its utilization as a culture medium to grow microorganisms (De Andrade et al., 2016).

Despite the large amount of cassava wastewater generated worldwide, it has still few applications, mainly as a fertilizer, and most of the production is disposed of in the environment without further treatments, which causes many problems due to its high chemical oxygen demand (COD). The present study aimed to assess the suitability of cassava wastewater as a growth medium for the red yeast *Rhodotorula glutinis*, to obtain carotenoids and fatty acids. Successful use of that byproduct would allow to cut the costs of production of these valuable compounds as well as to lower its impact on the environment.

2. Material and methods

2.1. Substrate preparation and characterization

Cassava wastewater (CW) was collected from local cassava flour producers in Northeastern Brazil (07° 08′ 43″ S, 35° 14′ 11″ W) and was stored at – 20 °C. Before use, CW was boiled and then centrifuged at 3248×g for 5 min to eliminate the solids (Costa et al., 2010). The supernatant was collected and analyzed for the following parameters: total nitrogen (TN), reducing sugars (RS), total carbohydrates (TC), phosphorus, calcium, sodium, iron, zinc, copper, potassium and magnesium contents, chemical oxygen demand (COD), free cyanide and pH. The analyses were performed in accordance to AOAC (2005), except for minerals and free cyanide. Minerals were evaluated through flame/-graphite furnace atomic absorption spectrometry using a spectrometer iCE 3000 series (Thermo Fisher Scientific, Cambridge, UK). Free cyanide was evaluated by using a Quantofix® cyanide test kit (Sigma-Aldrich, Brazil).

2.2. Microorganism

Rhodotorula glutinis (CTT 2182) was obtained from André Tosello Foundation (Campinas, São Paulo, Brazil). For the inoculum, the cells were transferred to 300 mL Erlenmeyer flasks containing 100 mL of culture medium: glucose (10 g·L^{-1}), peptone (5 g·L^{-1}), yeast extract (3 g·L^{-1}), and malt extract (3 g·L^{-1}). The flasks were incubated at 30 °C and shaken at 200 rpm for 24 h. Afterward, yeast cells were collected by centrifugation at 3248×g for 5 min. The cells were suspended in sterile distilled water to make a final cell concentration of approximately 10^9 cells·mL^{-1}. The cell concentration of this suspension was determined in a Neubauer chamber.

2.3. Yeast cultivation in flasks

Erlenmeyer flasks (300 mL) were prepared to contain 150 mL of medium. Ten different runs were performed (Table 1) by varying the nutrients (CW, glucose, ammonium sulfate and minerals) used in the mediums and the concentration of the carbon sources. Glucose was used in the concentrations of 10, 30 and 50 g·L^{-1} whereas CW was used based on reducing sugars (RS) concentration of 30, 20 and 10 g of RS·L^{-1}. Ammonium sulfate (5 g·L^{-1}) was used as the nitrogen source. For all runs the C/N ratio was calculated from COD/TN.

For the supplementation of the medium with minerals, macro and trace elements were used as follows: KH_2PO_4 (1 g·L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.25 g·L^{-1}), $Na_2HPO_4{\cdot}12H_2O$ (1 g·L^{-1}), 6 mL·L^{-1} $FeSO_4$ solution (4 g·L^{-1}) and 10 mL·L^{-1} trace mineral solution. Trace mineral solution contained per L: 0.36 g $CaCl_2{\cdot}2H_2O$, 0.075 g $ZnSO_4{\cdot}7H_2O$, 0.013 g $CuSO_4{\cdot}5H2O$, 0.05 g $MnSO_4{\cdot}H_2O$, 0.013 g $CoCl_2{\cdot}6H_2O$ and 0.035 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (Braunwald, 2013). Before inoculation, all media were sterilized at 121 °C for 15 min. Cells were inoculated in the medium to reach an initial concentration of 10^7 cells mL^{-1}. The flasks were incubated at 30 °C and shaken at 200 rpm for 120 h in darkness. All experiments were carried out in triplicate.

2.4. Analytical methods

Reducing sugar concentration in the culture medium was determined regularly, according to the dinitrosalicylic acid (DNS) method proposed by Miller (1959), using a spectrophotometer reading at 540 nm. Total carbohydrates were quantified spectrophotometrically at 620 nm, by using the Anthrone reagent (Yemm and Willis, 1954). For the quantification of the sugars, a glucose standard curve was adopted. Growth was monitored regularly by turbidity measurements using a spectrophotometer (model U2M, Quimis, Brazil) with absorbance reading at 600 nm and correlated to cell dry weight using a calibration curve. Medium without yeast cells was taken as blank and the cell dry weight was determined by centrifuging 2 mL of the cell suspension for 10 min at 10600×g, discarding the supernatant, and drying at 105 °C until constant weight.

Carotenoids extraction was carried out according to Cutzu et al. (2013), modified as follows: aliquots of the cultures (5 mL) were centrifuged at 2260×g for 10 min, then the supernatant was discarded and the pellets were frozen at -20 °C for 24 h. The frozen and thawed disrupted cell pellet was re-suspended in 2 mL DMSO pre-heated at

Table 1
Composition of the culture mediums in shaken flasks.

	$(NH_4)_2SO_4$ (g·L^{-1})	Glucose (g·L^{-1})	CW (g of RS·L^{-1})	Minerals supplementation	C/N
Run 1	5	–	30	–	18.2
Run 2	5	30	–	+	26.5
Run 3	5	–	30	+	18.2
Run 4	–	–	30	–	31.6
Run 5	–	10	30	–	38.0
Run 6	5	50	–	+	44.1
Run 7	–	–	20	–	31.6
Run 8	–	–	10	–	31.6
Run 9	5	–	20	+	15.0
Run 10	5	–	10	+	9.8

¶ "+" means supplemented and "-"means not supplemented.

60 °C, added with glass beads (0.5 g), vortexed for 2 min and incubated at 60 °C for 15 min. Then, 2 mL acetone, 2 mL petroleum ether and 2 mL NaCl 20% were sequentially added, the mixture was vortexed for a total time of 5 min and centrifuged at 2260×g for 10 min. The upper petroleum ether (PE) layer containing the extracted carotenoids was collected and the total carotenoids were quantified as β-carotene equivalents by using a spectrophotometer at 450 nm wavelength according to the formula provided by Rodriguez-Amaya and Kimura (2004), using the absorption coefficient of β-carotene in PE ($A^{1\%}_{1cm} = 2592$).

For intracellular lipids extraction, the cells were harvested by centrifugation (3248×g for 5 min), dried at 105 °C for 24 h and ground in a mortar to obtain a fine powder. The rupture of the dry cellular matter was performed through acid hydrolysis. For this purpose, 5 mL of 2 N HCl was added to 200 mg of dry yeast biomass and incubated in a water bath at 80 °C (model 105 Di-F, Dellta) for 1 h. The lipids were extracted with 11,6 mL of a mixture of chloroform:methanol:distilled water (1:1:0.9), according to the method described by Bligh and Dyer (1959). The transesterification into FAMEs was performed according to the procedure described by Hartman and Lago (1973). FAMEs were analyzed using a GC-FID (Trace™ 1310, Thermo Fisher Scientific, USA) equipped with an SP™-2380 capillary column (60 m × 0.25 mm ID and 0.25 μm film thickness). The different peaks were identified by comparison to a FAMEs standard (Supelco® 37 Component FAME Mix, USA) and quantification was based on their respective peak areas, and normalized.

2.5. Yields and productivities

The kinetics of substrate consumption (RS and TC), cell mass and carotenoids were followed by periodic sampling of the medium (up to 120 h). The conversion factors determined as the amount of carotenoids produced per unit weight of substrate consumed (Yp/s), the amount of biomass produced per unit weight of substrate consumed (Yx/s) and the amount of carotenoids produced per unit of dry weight of cells (Yp/x) as well as the carbon source (TC) consumption rate (Q_S) and productivity in cells (Q_X) and carotenoids (Q_P) were determined in the different runs. Total lipids production ($g \cdot L^{-1}$) was determined from the final biomass after 120 h cultivation time.

2.6. Statistical analysis

The results obtained were treated by one-way analysis of variance followed by Tukey's test, using the software Statistica 7.0 (Statsoft, Tulsa, USA). Analyses were performed considering a 95% confidence level.

3. Results and discussion

3.1. Physicochemical characteristics of cassava wastewater

Cassava wastewater presented high concentrations of TC and RS, which support its use as a carbon source in biotechnological processes (Table 2). Moreover, great amounts of macro- and micro-minerals important for microorganisms' growth were found.

The values of TC we found are quite higher than those found by Nitschke and Pastore (2006) who observed an amount of 35.3 $g{\cdot}L^{-1}$ in CW used to grow *Bacillus subtilis* for the production of surfactants. However, the contents of TC and RS found in the present study are very close to those reported by Damasceno et al. (2003), who found 58.2 and 38.0 $g{\cdot}L^{-1}$ of TC and RS, respectively, in CW used to grow *Geotrichum fragrans* for the production of volatile compounds.

The value found for free cyanide (3.0 $mg{\cdot}L^{-1}$) was low when compared with those previously reported in the literature (Leonel and Cereda, 1995). However, Souza et al. (2014) also found considerably low values of free cyanide, ranging from 5.30 to 16.60 $mg{\cdot}L^{-1}$ in CW collected in cassava flour houses located in the semiarid region of the state of Alagoas (Northeastern, Brazil). The concentration of free cyanide in CW depends on the plant genotype, cultivation and processing conditions, and storage time (Leonel and Cereda, 1995).

Table 2
Composition of the pre-treated cassava wastewater utilized in this work.

Parameter	Mean ± standard deviation
pH	5.98 ± 0.03
Free cyanide (mg L^{-1})	3.0 ± 0.0
COD (g O_2 L^{-1})	61.27 ± 5.94
Reducing sugars (g L^{-1})	40.60 ± 1.74
Non reducing sugars (g L^{-1})	17.51 ± 1.74
Total carbohydrates (g L^{-1})	58.11 ± 2.13
Total nitrogen (g L^{-1})	1.94 ± 0.08
Dry material (g 100 mL^{-1})	9.51 ± 0.29
Ca (mg L^{-1})	241.62 ± 5.56
Mg (mg L^{-1})	370.59 ± 4.35
P (mg L^{-1})	220.35 ± 3.28
Na (mg L^{-1})	147.55 ± 1.31
K (mg L^{-1})	1247.92 ± 7.36
Zn (mg L^{-1})	1.83 ± 0.16
Fe (mg L^{-1})	15.37 ± 1.75
Cu (mg L^{-1})	1.51 ± 0.02

Data are expressed as means of three replicates ± standard deviation.

A high COD (61.27 g O_2 L^{-1}) was found in the CW utilized in this work. The high COD is associated with the high concentration of sugars in CW and represents an environmental problem when this by-product is disposed of without further treatment, such as the use of anaerobic reactors to reduce pollution potential. The pH value we found was very close to 6.0 which is an optimum value for a culture medium intended to grow red yeasts (Aksu and Eren, 2007).

3.2. Biomass production

The maximum growth (12.78 $g{\cdot}L^{-1}$) was achieved in run 3 (Fig. 1), with CW (30 g of RS·L^{-1}), minerals supplementation, ammonium sulfate (5 $g{\cdot}L^{-1}$), and 120 h of culture. Run 3 also presented a significant growth from 96 h to 120 h, possibly due to the carbon availability along the entire cultivation period for all runs (Fig. 2), although that behavior was observed only for run 3. The medium containing only CW (run 4), diluted to 30 g of RS·L^{-1}, as the sole source of nutrients presented also a high growth (10.28 $g{\cdot}L^{-1}$), showing no significant differences ($p > 0.05$)

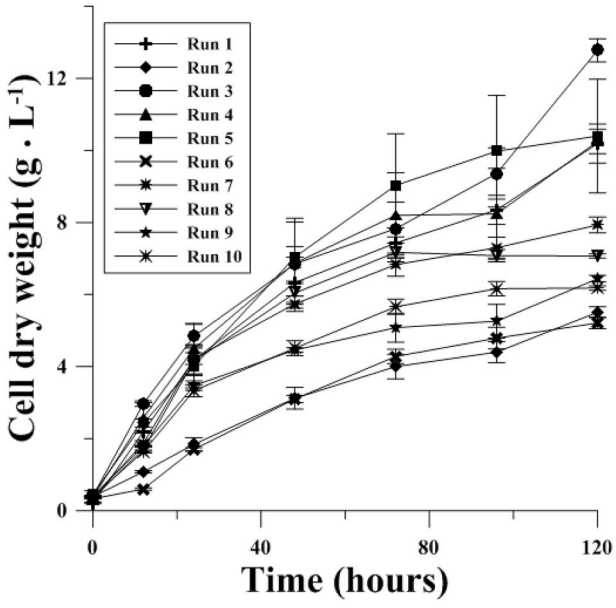

Fig. 1. Biomass concentration along the cultivation process. Data are expressed as means of three replicates ± standard deviation.

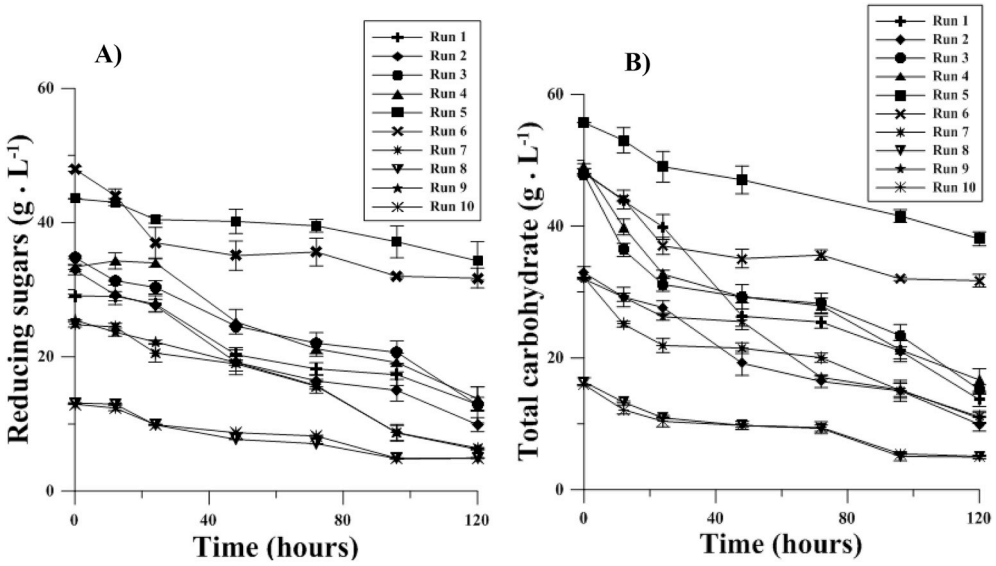

Fig. 2. Consumption of the carbon source along the cultivation Data are expressed as means of three replicates ± standard deviation.

when compared with the mediums containing CW (30 g of RS·L^{-1}) and supplemented with 5 g·L^{-1} of ammonium sulfate (run 1) or 10 g·L^{-1} of glucose (run 5), after 120 h of cultivation.

By comparison with runs 3 and 4, when CW was diluted to reach 20 g of RS·L^{-1} (runs 7 and 9) and 10 g of RS·L^{-1} (runs 8 and 10) a lower growth ($p < 0.05$) was observed as a consequence of the reduction in the carbon source available in the medium. Regarding the synthetic mediums containing glucose as the sole carbon source (runs 2 and 6) a lower growth ($p < 0.05$) was observed when compared with the treatments that used CW. After 120 h of culture, runs 2 and 6 reached a concentration of cell dry weight of 5.51 and 5.21 g·L^{-1}, respectively. It can be suggested that the higher growth obtained in the medium with CW can be due to the presence of considerable amounts of other nutrients, than sugar, which are important to support and stimulate cell growth. *R. glutinis* growth is affected by numerous parameters, such as the nature and concentration of carbon and nitrogen sources, minerals, vitamins, temperature, stress, and agitation (Hernández-Almanza et al., 2014; Yen et al., 2012).

Comparing with other studies that used agro-industrial by-products as substrates, Gientka et al. (2017) found a biomass of 14.49 g·L^{-1} for *R. glutinis* grown in a medium containing glycerol. Saenge et al. (2011) found maximum biomass of 8.05 g·L^{-1} for *R. glutinis* cultivated in palm oil mill effluent and through a response surface analysis they observed that for cell growth a relatively high COD and low C/N ratio is required. Using fermented radish brine as a substrate, Malisorn and Suntornsuk (2009) observed a biomass production of 2.6 g·L^{-1} for *R. glutinis* in batch cultivation. Marova et al. (2012) found a biomass growth of 9.16 g·L^{-1} when *R. glutinis* was cultured in deproteinized cheese whey and Park et al. (2005) found maximum biomass of 12.5 g·L^{-1} when *R. glutinis* was cultured in sugar cane molasses supplemented with urea and KH_2PO_4. Therefore, the biomass production we found can be considered satisfactory, as the values are higher or similar to those previously reported when *R. glutinis* was grown in alternative substrates. Moreover, it was observed that biomass production in the mediums composed by CW can be increased by supplementation with minerals and nitrogen and by controlling the initial concentration of reducing sugars.

3.3. Consumption of the carbon sources

The high growth obtained for all the mediums containing CW suggests that this agro-industrial by-product provides a suitable carbon source to support cell yeast growth. Cassava wastewater is reported in the literature to present a sugar composition of dextrin (2.6%), maltose (1.4%), sucrose (32.1%), glucose (38.3%), and fructose (25.6%) (Damasceno et al., 2003). The contents of RS and TC in all treatments showed a significant decrease along the cultivation time (Fig. 2A–B). Sugar consumption rates were different among the runs, which can be explained by the differences in C/N ratios of these mediums (Table 1), as shown by Braunwald et al. (2013). For the mediums containing CW a very low decrease in RS was observed after 24 h of cultivation, despite the high growth rate during this period. This result may be due to the hydrolysis of non-reducing sugars, as *R. glutinis* is known to present β-glucosidase activity (Rani et al., 2015). The consumption of TC ranged from 31.65% (run 5) to 71.62% (run 1). Runs 5 and 6 showed the lowest consumptions (%) of carbohydrates after 120 h of culture (31.65 and 33.4%, respectively), although these mediums were prepared with the highest amounts of RS. For run 5, the presence of glucose may be a reason for the lower consumption of sugars, as microbial β-glucosidases are known to be very sensitive to glucose inhibition which limits their activity toward the hydrolysis of non-reducing sugars in the medium (Saha and Bothast, 1996).

3.4. Production of carotenoids

The conversion of organic compounds in CW into carotenoids by using *R. glutinis* is of importance for its treatment and valorization. Carotenoids are widely used in the industry, i.e., as additives to food, diet supplements, and cosmetics, as well as are also an important ingredient of feedstock for poultry, fish, and mollusks (Kot et al., 2018). The accumulation of carotenoids in the yeast cells and the production of carotenoids per unit of volume are displayed in Fig. 3A and B, respectively. The highest accumulation of carotenoids in the cells (167.23 μg·g^{-1}), after 120 h, was achieved in the medium containing CW (10 g of RS·L^{-1}) supplemented with minerals and 5 g·L^{-1} of ammonium sulfate (run 10). Near values (139.18 μg·g^{-1}) were obtained for the same medium without minerals and nitrogen supplementation (run 8). Following these runs, great amounts of carotenoids were found also in the cells grown on synthetic mediums, with concentrations of 88.2 and 114.4 μg·g^{-1}, for runs 2 and 6, respectively. By comparing the mediums with CW as the sole source of nutrients, it was observed that the cellular accumulation of carotenoids was lower when the concentration of initial RS was higher: among these mediums, run 4 showed the lowest content (56.8 μg·g^{-1}) of carotenoids in the cells at the end of the cultivation process.

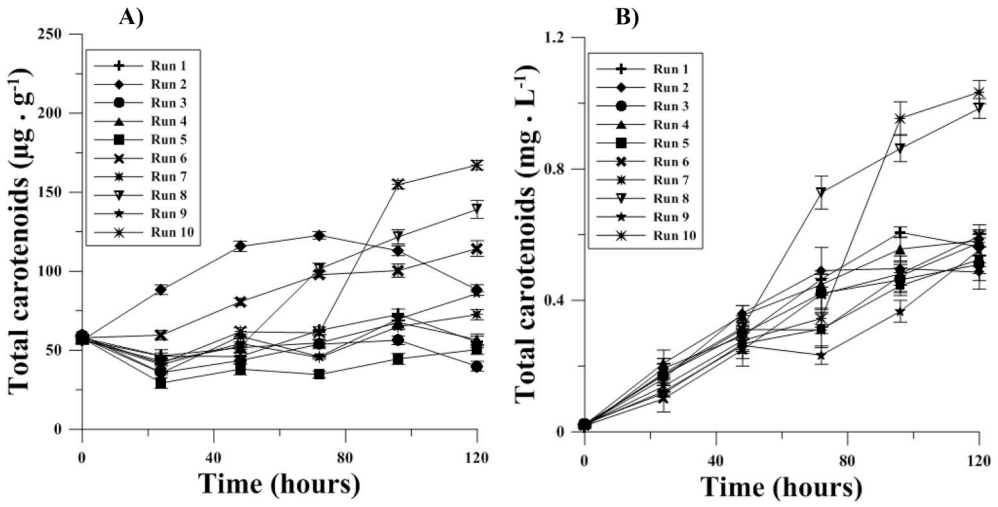

Fig. 3. Carotenoids production by *R. glutinis* (CTT 2182) along the cultivation period. Data are expressed as means of three replicates ± standard deviation.

Regarding the production of carotenoids per unit volume, no differences ($p > 0.05$) were found between runs 8 and 10, which reached means values of 0.98 and 1.03 mg·L^{-1}, respectively. These runs presented the best results for the volumetric production of carotenoids after 120 h of culture. For the other mediums, similar results for volumetric production of carotenoids were obtained, because high cellular accumulations corresponded to low growth rates and vice versa.

It can be suggested that the high production of carotenoids in the mediums containing CW, diluted to reach 10 g of RS·L^{-1}, may be a consequence of nutrients limitation, as it can lead to oxidative stress response in the yeast cells (Petti et al., 2011). Eisler et al. (2004) observed that starvation for an essential amino acid results in severe yeast cell stress, where starvation led to a constantly high burden of ROS in cells. The most important function of carotenoids in yeasts is to deactivate the free radicals produced during normal metabolism of cells, such as singlet oxygen ($^1O_2$), hydroxyl radical (OH^-), peroxides and other oxidants (Berera et al., 2010). Growth under unfavorable conditions induces stress in *R. glutinis*, which brings about a biochemical response involving an increase in the activity of enzymes and of the levels of carotenoids (Salar et al., 2013).

3.5. Yields and productivities

The mediums containing CW presented higher or similar values for the conversion of the substrate into biomass ($Y_{X/S}$) when compared with synthetic mediums with glucose as the only carbon source (Table 3). Among the mediums in which CW was used as the sole source of nutrients, those with a low initial RS concentration led to higher $Y_{X/S}$ values. By comparing with the previously reported data, Gientka et al. (2017) found a value of 0.536 for the biomass/substrate coefficient ($Y_{X/S}$) when culturing *R. glutinis* in media with glycerol as the carbon source. This value is very close to those observed in the present study for the runs 5, 8 and 10.

The conversion of the biomass into carotenoids ($Y_{P/X}$) showed its highest values (0.14–0.17) for the mediums containing 10 g of RS·L^{-1} (runs 8 and 10). The same behavior was observed for $Y_{P/S}$ (0.08–0.09). Regarding $Y_{P/S}$, the values we found are quite lower when compared to those observed by Kot et al. (2019b), who found values up to 0.220 in *R. glutinis* cells grown in media containing glycerol and deproteinized potato wastewater under exogenous stress factors. The increase in the specific yield of carotenoids is an indication of an augmented biosynthesis of carotenoids by the yeast cells and this behavior is possibly related to the changes in metabolism and cell stress (Liu et al., 2006).

By performing an evaluation of the effect of simultaneous nitrogen and minerals supplementation in the medium with CW, it can be observed that for the medium containing 30 g of RS·L^{-1} a higher biomass productivity (Q_X) was achieved, whereas for the mediums with 20 g of RS·L^{-1} and 10 g of RS·L^{-1}, a lower productivity was observed. Regarding the productivity of carotenoids (Q_P), similar results were obtained for all experiments except for the runs 8 and 10, which showed Q_P values 1.6–2.0 times superior in comparison to the other runs. For the carbon source consumption rate (Q_S) the highest values were observed for the mediums containing only CW, diluted to 30 g of RS·L^{-1}, as the carbon source (runs 1, 3 and 4), whereas the lowest values were observed for runs 8 and 10 with CW diluted to 10 g of RS·L^{-1}.

Table 3
Effect of medium composition on the conversion factors ($Y_{X/S}$; $Y_{P/X}$; $Y_{P/S}$), biomass and carotenoids productivities (Q_X; Q_P) and carbon source consumption (Q_S).

Run	$Y_{X/S}$ (g·g^{-1})	$Y_{P/X}$ (mg·g^{-1})	$Y_{P/S}$ (mg·g^{-1})	Q_X (g·L^{-1}·h^{-1})	Q_P (mg·L^{-1}·h^{-1})	Q_S (g·L^{-1}·h^{-1})
1	0.29	0.06	0.02	0.083	0.005	0.29
2	0.22	0.09	0.02	0.043	0.004	0.19
3	0.38	0.04	0.01	0.103	0.004	0.27
4	0.30	0.06	0.02	0.082	0.005	0.27
5	0.56	0.05	0.03	0.083	0.004	0.15
6	0.30	0.12	0.04	0.041	0.005	0.14
7	0.36	0.07	0.03	0.063	0.005	0.18
8	0.59	0.14	0.08	0.056	0.008	0.10
9	0.29	0.09	0.03	0.050	0.004	0.18
10	0.53	0.17	0.09	0.048	0.008	0.09

3.6. Lipids production and fatty acids composition

Lower cellular accumulation of lipids ($p < 0.05$) was observed in the mediums containing CW when compared with the synthetic mediums (Table 4). The highest ($p < 0.05$) cellular accumulation of lipids (32.39 g·100 g_{cdw}^{-1}) was observed in run 6, which presented the highest C/N ratio. The lipids accumulation in oleaginous yeasts is only triggered when a growth-required nutrient (mostly nitrogen) is limited, and carbon is still abundantly available (Papanikolaou et al., 2002). From this, it can be deduced that a high C/N ratio of the growth mediums will positively affect the lipids accumulation in these organisms.

Since the mediums containing CW diluted to 30 g of RS·L^{-1} (runs 1, 3, 4) presented a higher growth, the productions of lipids per unit volume for these mediums were similar to those obtained for the synthetic mediums and for the medium supplemented with glucose (run 5). This is a valuable result since the cost of the carbon (glucose) contributes a high share to the overall cost of the whole process, and any potential savings

Table 4
Lipids production after 120 h cultivation.

Assay	Total lipids	
	($g \cdot 100\ g_{cdw}^{-1}$)	($g \cdot L^{-1}$)
Run 1	11.80 ± 0.14^{cd}	1.19 ± 0.01^{c}
Run 2	28.28 ± 0.72^{b}	1.59 ± 0.04^{a}
Run 3	12.36 ± 0.10^{cd}	1.59 ± 0.01^{a}
Run 4	13.23 ± 0.16^{cd}	1.34 ± 0.02^{b}
Run 5	15.51 ± 0.70^{c}	1.64 ± 0.07^{a}
Run 6	32.39 ± 0.71^{a}	1.71 ± 0.04^{a}
Run 7	12.96 ± 0.38^{cd}	1.01 ± 0.03^{d}
Run 8	10.43 ± 0.16^{d}	0.73 ± 0.01^{ef}
Run 9	12.64 ± 0.48^{cd}	0.82 ± 0.01^{e}
Run 10	10.29 ± 0.74^{d}	0.67 ± 0.05^{f}

Data are expressed as means of three replicates ± standard deviation.
Values in the same column that do not share the same alphabetic superscript are significantly different at 5% levels of probability.

in pure glucose utilization will help to improve the economic features of the approach. The runs 1, 3 and 4, presented a volumetric efficiency of lipids biosynthesis similar to that observed by Gientka et al. (2017) in *R. glutinis* cells cultured in medium with glycerol as carbon source.

The lower productivities of lipids we found in run 9 (0.82 $g \cdot L^{-1}$) and 10 (0.67 $g \cdot L^{-1}$) are expected since a C/N ratio of 20 is seen as a minimum condition for lipid induction (Chi et al., 2011). In general, the production of lipids per unit volume was higher according to the carbon availability in the medium. It is agreed that only under excess carbon conditions, lipids are synthesized (Schneider et al., 2013).

The fatty acids composition was generally similar in all treatments containing CW as the carbon source (Table 5). With average values ranging from 47.28 to 59.76%, oleic acid (C 18:1 n9 *cis*) was the main fatty acid found, followed by palmitic acid (C 16:0; 13.03–15.66%) and stearic acid (C 18:0; 2.97–13.46%). According to the literature (Yen et al., 2012; Kot et al., 2019b), in this investigation palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) account for over 80% of the total fatty acids in lipids from *R. glutinis*.

When compared with the mediums containing CW, synthetic mediums (runs 2 and 6) presented lower values ($p < 0.05$) for oleic acid (C 18:1 n9 *cis*; 28.73–41.25%) and higher values ($p < 0.05$) for palmitic acid (C 16:0; 21.92–29.05%) and Gondoic acid (C 20:1 n9; 4.47–5.63%). The medium with the highest C/N ratio (Run 6) showed the lowest amount ($p < 0.05$) for oleic acid (C 18:1 n9 *cis*; 28.73%) and the highest amount ($p < 0.05$) for palmitic acid (C 16:0; 29.05%). This is also reported in the literature focusing on lipids produced by *R. glutinis*. Braunwald et al. (2013) observed that high C/N ratios led to higher contents of saturated fatty acids and lower contents of unsaturated fatty acids. Mondala et al. (2012) also found increasing levels of C 16:0 and C 18:0 with increasing initial C/N ratios.

For the mediums containing CW, oleic acid is the prevailing fatty acid with a share of over 50%, except for run 4 which showed 47.28%. Gientka et al. (2017) evaluated the use of glycerol as a carbon source for red yeasts and observed the predominance of oleic acid (C18:1) in *R. glutinis* fats, with a share of 54.6% among all fatty acids. Kot et al. (2019b) studied the effect of exogenous stress factors on the biosynthesis of lipids by *Rhodotorula* yeast strains cultured in agro-industrial waste and found a share of over 40% for oleic acid in *R. glutinis* fats, regardless of the stress applied. This result is of interest for the production of biodiesel as oleic acid is seen as optimal fatty acid for improved fuel properties (Knothe, 2005).

For all runs, the unsaturated fatty acids stand out as major contributors to the overall FAME content (over 50%). Amongst these it was found considerable amounts of the polyunsaturated ω-6 fatty acids, linoleic acid (C 18:2 n6 *cis*; 1.77–15.20%) and arachidonic acid (C 20:4 n6; 0.33–1.91%), as well as ω-3 linolenic acid (C 18:3 n3 *cis*; 0.27–1.85%). Higher values (21.4–25.1%) for linoleic acid (C 18:2) were

Table 5
Fatty acids composition (% of total) of *Rhodotorula glutinis* (CTT 2182).

	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Run 9	Run 10
C14:0	1.20 ± 0.22^{bc}	2.37 ± 0.33^{ab}	1.43 ± 0.28^{bc}	1.69 ± 0.45^{b}	1.15 ± 0.09^{bc}	2.74 ± 0.14^{a}	0.88 ± 0.05^{c}	1.73 ± 0.38^{b}	0.97 ± 0.01^{c}	1.26 ± 0.42^{bc}
C16:0	13.03 ± 1.16^{c}	21.92 ± 0.20^{b}	13.29 ± 1.23^{c}	15.66 ± 3.75^{c}	13.45 ± 0.52^{c}	29.05 ± 1.64^{a}	15.54 ± 0.12^{c}	14.07 ± 2.11^{c}	13.68 ± 0.07^{c}	13.45 ± 1.09^{c}
C16:1 n7	0.49 ± 0.17^{c}	0.87 ± 0.06^{b}	0.67 ± 0.09^{bc}	0.73 ± 0.15^{bc}	0.61 ± 0.17^{bc}	1.12 ± 0.01^{a}	0.81 ± 0.05^{b}	1.84 ± 0.36^{a}	0.60 ± 0.01^{bc}	0.82 ± 0.14^{b}
C17:0	0.84 ± 0.14^{a}	0.21 ± 0.02^{c}	0.94 ± 0.11^{a}	0.97 ± 0.21^{a}	0.80 ± 0.24^{a}	0.19 ± 0.01^{c}	0.77 ± 0.06^{a}	0.39 ± 0.13^{b}	0.99 ± 0.11^{a}	1.12 ± 0.36^{a}
C17:1 n10	0.71 ± 0.04^{a}	0.09 ± 0.03^{b}	0.88 ± 0.22^{a}	0.87 ± 0.26^{a}	0.71 ± 0.15^{a}	0.05 ± 0.00^{b}	0.64 ± 0.08^{a}	0.58 ± 0.10^{a}	0.70 ± 0.07^{a}	0.85 ± 0.35^{a}
C18:0	8.75 ± 1.66^{a}	8.34 ± 1.67^{a}	11.12 ± 1.18^{a}	13.46 ± 4.15^{a}	12.53 ± 2.08^{a}	9.63 ± 2.64^{a}	9.69 ± 0.75^{a}	2.97 ± 0.87^{b}	11.62 ± 0.21^{a}	9.75 ± 0.95^{a}
C18:1 n9 *cis*	59.76 ± 0.58^{a}	41.25 ± 2.25^{c}	55.49 ± 5.23^{ab}	47.28 ± 4.34^{bc}	54.13 ± 2.82^{ab}	28.73 ± 1.81^{d}	56.29 ± 2.67^{ab}	57.95 ± 3.50^{ab}	55.58 ± 0.10^{ab}	51.10 ± 3.41^{b}
C18:2 n6 *cis*	2.15 ± 0.12^{c}	13.01 ± 1.54^{a}	2.08 ± 0.29^{c}	2.07 ± 0.49^{c}	2.47 ± 0.69^{c}	15.20 ± 0.93^{a}	1.77 ± 0.17^{c}	7.59 ± 1.18^{b}	2.00 ± 0.13^{c}	2.73 ± 0.91^{c}
C18:3 n3	0.63 ± 0.28^{b}	0.28 ± 0.01^{c}	0.78 ± 0.19^{b}	1.85 ± 0.44^{a}	0.68 ± 0.23^{b}	0.27 ± 0.04^{c}	0.77 ± 0.05^{b}	0.89 ± 0.14^{b}	0.75 ± 0.10^{b}	0.94 ± 0.19^{b}
C20:1 n9	0.25 ± 0.06^{c}	4.47 ± 0.25^{a}	0.44 ± 0.06^{c}	0.33 ± 0.08^{c}	0.54 ± 0.16^{c}	5.63 ± 0.05^{a}	0.28 ± 0.05^{c}	1.15 ± 0.16^{b}	0.49 ± 0.03^{c}	0.52 ± 0.14^{c}
C20:4 n6	0.96 ± 0.35^{b}	0.95 ± 0.05^{b}	0.90 ± 0.29^{b}	1.91 ± 0.48^{a}	1.31 ± 0.47^{ab}	1.01 ± 0.02^{b}	1.07 ± 0.11^{b}	0.33 ± 0.05^{c}	1.00 ± 0.08^{b}	1.25 ± 0.32^{ab}
C21:0	0.12 ± 0.00^{a}	0.04 ± 0.00^{b}	0.13 ± 0.01^{a}	0.12 ± 0.04^{a}	0.09 ± 0.02^{a}	0.03 ± 0.01^{b}	0.06 ± 0.02^{ab}	0.14 ± 0.01^{a}	0.14 ± 0.06^{a}	0.16 ± 0.04^{a}
C22:0	1.75 ± 0.62^{ab}	1.42 ± 0.15^{b}	2.04 ± 0.16^{ab}	2.69 ± 0.53^{a}	2.29 ± 0.78^{ab}	1.44 ± 0.03^{b}	2.06 ± 0.11^{ab}	0.62 ± 0.07^{c}	2.07 ± 0.08^{ab}	2.30 ± 0.63^{ab}
C23:0	0.18 ± 0.04^{a}	0.09 ± 0.01^{b}	0.17 ± 0.03^{a}	0.22 ± 0.07^{a}	0.18 ± 0.03^{a}	0.09 ± 0.01^{c}	0.21 ± 0.06^{a}	0.30 ± 0.08^{a}	0.22 ± 0.02^{a}	0.22 ± 0.04^{a}
C24:0	2.44 ± 0.95^{a}	1.30 ± 0.19^{b}	3.23 ± 0.45^{a}	3.65 ± 0.76^{a}	2.55 ± 0.83^{a}	1.19 ± 0.01^{b}	2.94 ± 0.62^{a}	1.73 ± 0.16^{b}	3.21 ± 0.24^{a}	3.53 ± 0.52^{a}
Other	6.76 ± 1.63^{a}	3.37 ± 0.33^{b}	6.41 ± 0.51^{a}	6.49 ± 0.78^{a}	6.51 ± 0.65^{a}	3.63 ± 0.08^{b}	6.24 ± 0.37^{a}	7.73 ± 1.71^{a}	5.98 ± 0.34^{a}	9.98 ± 3.12^{a}

Data are expressed as means of three replicates ± standard deviation.
Values in the same line that do not share the same alphabetic superscript are significantly different at 5% levels of probability.

observed by Kot et al. (2017) when culturing *R. glutinis* in media with potato wastewater and 5% glycerol under different initial pH. The synthetic medium with the highest C/N ratio (run 6) presented the lowest content of oleic acid and the highest content of linoleic acid. A higher content of oleic acid probably is related to lower activity of enzymes converting oleic acid to linoleic acid (Kot et al., 2017).

It is known that the lipids profile of microbial oils depends upon the carbon source used in the cultivation (Yen et al., 2012) and that polyunsaturated fatty acids are associated with health benefits when included in the human diet. Therefore, due to the large presence of these fatty acids in *R. glutinis* yeast grown on CW mediums, it can be suggested that this by-product has potential to be applied as a substrate, aiming biomass generation for food and pharmaceutical industries.

4. Conclusion

Rhodotorula glutinis growth was supported by the carbohydrates present in CW. Moreover, this residue can be employed as a sole source of nutrients to grow *R. glutinis*, as the supplementation with glucose, nitrogen, and minerals was observed to be not necessary for the growth. For carotenoids production the dilution of CW to reach 10 g of RS·L^{-1} can be considered the best choice, as satisfactory growth and maximum carotenoids production were obtained. Lipids production showed to be influenced by the availability of carbon in the medium and for this purpose, the use of CW at 30 g of RS·L^{-1} would be recommended, since this concentration also provided a majority of unsaturated fatty acids in the lipids. The results support the viability of the use of CW alone as a substrate to be applied in a biotechnological process for the production of carotenoids and fatty acids by *R. glutinis*, helping to reduce the costs of the process and valorizing the main effluent of the cassava processing industry. However, other parameters of the cultivation process such as the aeration of the growth medium and stress conditions should be tested further to improve lipids and carotenoids yield in *R. glutinis* cells cultivated in CW.

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