Biocatalysis and Agricultural Biotechnology 20 (2019) 101208

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Use of agroindustrial byproducts as substrate for production of carotenoids with antioxidant potential by wild yeasts

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ARTICLE INFO

Keywords:
Sugar cane molasses
Raw glycerol
Corn steep liquor
Carotenogenic extracts
Antioxidant potential

ABSTRACT

In order to produce carotenoids with antioxidant potential from the wild yeast *Rhodotorula mucilaginosa, Sporidiobolus pararoseus* and *Pichia fermentans,* isolated from the ecosystem of Escudo Sul-Rio Grandense (Brazil), cultivations were performed with: raw glycerol and corn steep liquor (M1), sugar cane molasses and corn steep liquor (M2) and Yeast Malt (YM) broth. Production of *β*-carotene more than 63% was obtained in all the experiments. The antioxidant activity of the extracts was analyzed by the methods DPPH, ABTS and FRAP. *S. pararoseus* is highlighted, showing 635 and 830 $\mu g.L^{-1}$ of carotenoids in M1 and M2, respectively. The extracts of *P. fermentans* in M2 were promising in relation to the antioxidant activity, presented results not reported in the literature until the present moment. Based on these results and the importance of obtaining carotenoid compounds from alternative sources, the proposed means, as well as the yeasts used, were shown to be promising choices. The use of agroindustrials media and microorganisms isolated from the ecosystems intensify the importance of this study providing new sources of biocompounds from the biodiversity and biotechnological potential of Brazil.

1. Introduction

The carotenoids are the most widespread class of pigments in nature (Maldonade et al., 2008; Ngamwonglumlert and Devahastin, 2019; Rodriguez-Concepcion et al., 2018). Most carotenoids are C40 tetraterpenoids, composed of 8 isoprenoid units, linked in such way that the molecules is linear and symmetric, with the order inverted in the center. The basic structure can be modified by hydrogenation, dehydrogenation, cyclization and oxidation. The light absorption characteristic of these pigments occurs due to conjugated double bonds chain which acts as chromophore (Stahl and Sies, 2003; Valduga et al., 2009b). The characteristic structure of the carotenoid determines their actions and the potential of their biological functions (Britton, 1995; Cheng and Yang, 2016; Hempel et al., 2016; Poliak et al., 2018).

Carotenoids are known for their anticancer activity and pro-vitamin A function. These compounds are largely used in dietary supplements, food coloring agents and cosmetic and pharmaceutical additives (Ngamwonglumlert and Devahastin, 2019; Ví and Forjá, 2011). Furthermore, these important group of compounds are widely studied due to their antioxidant activity (Edge et al., 1997; Esteban et al., 2015; Papaioannou et al., 2011; Ribeiro et al., 2018; Thaipong et al., 2006; Wang et al., 2010). They act as membrane-protective antioxidants that efficiently scavenge O_2 and peroxyl radicals. Possibly its antioxidant activity is related to their structure (Marova et al., 2012). Carotenoids can be obtained by many bacteria, fungi, and plants (Amorim-Carrilho et al., 2014; Rodriguez-Amaya, 2010). Carotenoids obtaining by microbial way has advantages such as availability of cultivation throughout the year, and the possible industrial production level using less area (Valduga et al., 2009a), especially with the use of micro-organisms such as yeasts. These microorganims are highlighted by using simple nitrogen and carbon sources for its growth, being easy to adapt on inexpensive substrates. In addition, many of the potential producers are considered GRAS (Generaly recognized as safe) (Oliveira et al., 2017; Rodriguez-Amaya, 2001; Rodriguez-Amaya, 2010; Valduga et al.,

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https://doi.org/10.1016/j.bcab.2019.101208
Received 28 November 2018; Received in revised form 23 May 2019; Accepted 18 June 2019
Available online 23 June 2019
1878-8181/

2009a).

The production of carotenoids by fermentation is limited by the processing costs (Maldonade et al., 2008), but the use of industrial byproducts as a source of nutrients, can reduce costs and to make possible the industrial production of these compounds (Aksu and Tugba Eren, 2005; Rios et al., 2015). According to BCC Research (2018) the carotenoid market value was estimated at about $ 1.5 billion in 2017, and is expected to reach $ 2.0 billion by 2022 with an annual growth rate of 5.7%.

The use of alternative substrates, the agroindustrial byproducts, and the cheapening of costs can generate the reduction of environmental problems such as discard and the cost of waste treatment (Makkar and Cameotra, 2002; Strati and Oreopoulou, 2014). In this context, raw glycerol, sugar cane molasses and corn steep liquor can become an alternative for producing carotenoids (Machado and Burkert, 2015). These products are found in abundance due to the production of biodiesel, sugar and corn processing, which may contribute both as source of carbon and/or nitrogen and other nutrients for microbial metabolism (Imandi et al., 2007; Valduga et al., 2014; Saenge et al., 2011).

Due to the importance of these compounds, the search for new carotenoid-producing microorganisms and new ways to improve production are constant goals (Cipolatti et al., 2015; Marova et al., 2012; Yoo et al., 2016). Thus, the study of the use of alternative substrates for the production of microbial carotenoids is of great importance to minimize environmental impacts, in addition to enabling the reduction of costs of obtaining these pigments with antioxidant activity. The objective of this study was to produce carotenoids from culture media containing corn steep water and raw glycerol, and corn steep liquor and sugar cane molasses in liquid media with the yeasts: *Rhodotorula mucilaginosa, Sporidiobolus pararoseus* and *Pichia fermentans*, initially isolated from the *Litoral Médio* and *Escudo Sul-Rio-Grandense* (Rio Grande do Sul – Brazil), and evaluation of the antioxidant activity of extracts. The obtaining of high value-added compounds such as antioxidants from agro-industrial byproducts adds value to the raw material and can cheapen the costs of antioxidants. In addition, no antioxidant activity studies of carotenoids obtained by *Pichia fermentans* have been found to date.

2. Material and methods

2.1. Agroindustrial substrates

The byproducts used were obtained in agro industries: raw glycerol, derived from the biodiesel synthesis (BS Bios Indústria e Comércio de Biodiesel Sul Brasil S/A - Passo Fundo - RS); sugar cane molasses (Guimarães Indústria e Comércio Ltda - RS) and corn steep liquor (Corn Products - Balsa Nova - PR).

2.1.1. Partial characterization of agroindustrial byproducts

The determinations of carbon, nitrogen and hydrogen were performed for corn steep liquor in an Elemental Analyzer CHNS/O 2400 Series II da PerkinElmer. The same determinations were made for raw glycerol (Silva et al., 2012), for sugar cane molasses and the components of YM medium (Otero et al., 2019) Except the determination the carbon percentage in the sugar cane molasses was performed in a total organic carbon analyzer (modelo TOC-V CPH/CPN, Shimadzu, Japan).

2.2. Microorganisms

Yeasts isolated from environmental samples were used the experiments. They were isolated from the *Litoral Médio* and *Escudo Sul-Rio-Grandense* (Rio Grande do Sul – Brazil) identified as *Sporidiobolus pararoseus, Pichia fermentans* and *Rhodotorula mucilaginosa* (Otero et al., 2019). The microorganisms are deposited in the André Tosello Tropical Culture Collection: *Sporidiobolus pararoseus* (CCT 7689), *Rhodotorula mucilaginosa* (CCT 7688) and *Pichia fermentans* (CCT 7677).

2.3. Maintenance and reactivation of microbial cultures

The yeasts were maintained in GYMP agar slants tubes ($w.v^{-1}$) (20 $g.L^{-1}$ of glucose, 10 $g.L^{-1}$ of malt extract, 5 $g.L^{-1}$ of yeast extract, 2 $g.L^{-1}$ of $NaHPO_4$ and 2 $g.L^{-1}$ of agar) at 4 °C (Fonseca et al., 2011). The maintenance of the microorganism in GYMP is made every 3 months. For reactivation, the microorganisms were transferred to Yeast Malt agar slants (YM): 3 $g.L^{-1}$ of yeast extract, 3 $g.L^{-1}$ of malt extract, 5 $g.L^{-1}$ of peptone, 10 $g.L^{-1}$ of glucose, added 0,2 $g.L^{-1}$ of KNO_3 (Parajó et al., 1998) at 25 °C for 72 h.

2.4. Inoculum

From the tubes containing the micro-organisms in YM agar slants, a cell suspension was carried out (one mL) in sterile peptone water (0.1%) and added to

9 mL of YM broth and incubated at 25 °C for 48 h. The inoculum was cultivated in Erlenmeyer flasks of 250 mL containing 90 mL YM broth previously sterilized at 121 °C for 15 min, plus the cell suspension, and incubated at 25 °C, 150 rpm for 48 h or enable time for reach $1x10^8$ cells.mL^{-1} by counting in a Neubauer chamber (Michelon et al., 2012).

2.5. Cultivation

First, it was determined the C/N ratio of the YM broth, in order to adjust a similar ratio in the preparation of culture media for yeasts with the byproducts. To achieve this ratio, it was used the SOLVER tool, available in the EXCEL software. The C/N ratio was 6.20 (similar to YM medium), carbon concentration was 8.50% and nitrogen concentration was 1.37%. The formulated media contained:

4.8 $g.L^{-1}$ of raw glycerol and 35.6 $g.L^{-1}$ of corn steep liquor (M1); and

6 $g.L^{-1}$ of sugar cane molasses and 36.5 $g.L^{-1}$ of corn steep liquor (M2).

Yeast cultivations were performed in 500 mL Erlenmeyer flasks with 153 mL of the medium (M1, M2 or YM) at initial pH 6.0, with 10% inoculum, and the operating conditions of the process were 180 rpm for 168 h at 25 °C (Michelon et al., 2012; Fonseca et al., 2011).

2.6. Determination of biomass concentration

Aliquots were taken from cultures at the starting time (0 h) and at 12, 24, 36, 48, 72, 96, 120, 144 and 168 h and centrifuged (1745 × *g*) for 10 min. The supernatants were separated to pH determination (AOAC, 2000) and the precipitates were washed with distilled water and resuspended in volumetric flasks. The biomass concentration ($g.L^{-1}$) was estimated by absorbance reading at 620 nm, and a standard curve previously determined for each micro-organism (Kusdiyantini et al., 1998). Where y is the biomass concentration ($g.L^{-1}$) and x is the absorbance.

$$R.\ mucilaginosa: y = 0.0428 + 1.0427 * x(\mathrm{r} = 0.9982) \quad (1)$$

$$P.\ fermen\ \mathrm{tan}\ s:\ y = 0.1002 + 1.6712 * x(r = 0.9991) \quad (2)$$

$$S.\ pararoseus:\ y = 0.0354 + 0.9729 * x(\mathrm{r} = 0.9938) \quad (3)$$

2.7. Determination of total carotenoids

The biomass obtained at the end of cultivation (168 h) was dried at 35 °C for

24 h and frozen for 48 h at −18 °C. It was macerated in a mortar and pestle and sieved into 115 mesh corresponding to particulate sizes of > 0.125 mm (Cipolatti et al., 2015).

To disrupt the cell wall, 2 mL of dimethylsulfoxide (DMSO) was

used in direct contact with biomass (0.05 g), then vortexing was performed for 1 min at 15 min intervals, totaling 1 h (Cipolatti et al., 2015; Fonseca et al., 2011; Pires-Cabral et al., 2005). After the rupture, it was added 6 mL of acetone to extract the carotenoids. The sample was centrifuged at 1745 × *g* for 10 min, the acetonic phase was separated and the disruption procedure was repeated until the disappearance of the yellowish color of biomass. In the phase containing the carotenoids 10 mL of sodium chloride solution 20% (w/v) and 10 mL of petroleum ether were added. After stirring and separation of phases, the excess water was removed with sodium sulfate (Na_2SO_4), giving rise to carotenogenic extracts. The determination of the concentration of total carotenoids in the extracts was performed in a spectrophotometer at 448 nm (Silva et al., 2012). The results of concentration of total carotenoids were obtained by Equation (4) (Moriel et al., 2005), expressed in terms of its major carotenoid β-carotene (specific absorptivity in petroleum ether of 2592, experimentally obtained) (Davies, 1976).

$$TC = \frac{A * V * 10^6}{A_{1cm}^{1\%} * 100 * m_{amostra}} \quad (4)$$

where: TC = total carotenoids ($\mu g.g^{-1}$); A = absorbance; V = volume (mL); m_{sample} = dry cell mass (g); $A_{1cm}^{1\%}$ = specific absorptivity.

To calculate total carotenoids ($\mu g.L^{-1}$) with the results of the specific concentration and biomass concentration, unit conversion was performed.

2.8. Identification and quantification of carotenoids

Carotenoids obtained at the end of the cultivations (168 h) were identified and quantified using high-performance liquid chromatography (HPLC) (Shimadzu, Quioto, Japan), consisting of a pump system LC-20AT, degasifier DGU $20A_5$, controller CBM-20A, manual injector with loop of 20 µL and spectrophotometric detection system

SPD-20A at 450 nm (Supplementary material).

The equipment control and data acquisition was done by LC Solution software. The determinations were performed using a chromatographic column Reverse Phase Discovery Bio Wide Pore C18, 10 µm (25 cm × 4.6 mm), maintained at room temperature. It was used, as mobile phase for the elution of carotenoids, a mixture of acetonitrile: methanol: ethyl acetate (70:30:0 $v.v^{-1}$ in the gradient mode, with a flow of

1 mL min^{-1}, and the total run time of 17 min (Cipolatti et al., 2015). The quantitation was performed by the standard curves showed in equations (5)–(7).

$$y_{astaxanthin} = 127978{,}1x - 32563{,}73 (r = 0.9946) \quad (5)$$

$$y_{lutein} = 287419{,}9x - 17246{,}59 (r = 0.9996) \quad (6)$$

$$y_{B-carotene} = 143743{,}8x - 107361{,}3 (r = 0.9977) \quad (7)$$

2.9. Determination of total reducing sugars (TRS)

Samples of the medium with the products were hydrolyzed with 2 $mol.L^{-1}$ HCl in a bath at 55 °C for 30 min, followed by addition of 2 $mol.L^{-1}$ NaOH (Liu et al., 2012). The determination of total reducing sugars (TRS) was performed using the 3–5 dinitrosalicylic acid (DNS) spectrophotometric method (Miller, 1959), using a glucose standard curve (Equation (8)), whose concentration ranged between 0.1 and 1 $g.L^{-1}$. Where y is the glucose concentration ($g.L^{-1}$) and x is the absorbance measured at 540 nm.

$$y = 0.028x - 1.6696 (r = 0.9957) \quad (8)$$

2.10. Determination of kinetic parameters of cultivation

To obtain the conversion factors, maximum specific growth rate and productivity, the data from analytical determinations were used. These parameters were calculated according to the following equations:

$$\mu_{máx} = \frac{1}{X}\frac{dX}{dt} \quad (9)$$

$$Y_{P/S} = \frac{r_P}{r_S} \quad (10)$$

$$Y_{X/S} = \frac{r_X}{r_S} \quad (11)$$

$$Y_{P/X} = \frac{r_P}{r_X} \quad (12)$$

where: Y = conversion factor; X = biomass ($g.L^{-1}$); S = substrate (glucose) ($g.L^{-1}$); P = product (carotenoid) ($\mu g.g^{-1}$).

The productivities in biomass (P_X in $g.L^{-1}.h^{-1}$) and in carotenoids (P_C in $\mu g.L^{-1}.h^{-1}$) were calculated by equations (13) and (14).

$$P_X = \frac{X_m - X_o}{t_f} \quad (13)$$

$$P_C = \frac{P_m - P_o}{t_f} \quad (14)$$

where: X_m = maximum biomass concentration ($g.L^{-1}$) achieved in t_f; X_o = initial biomass concentration ($g.L^{-1}$); t_f = cultivation time related to X_m (h); P_m = maximum concentration of carotenoids ($\mu g.L^{-1}$) achieved in t_f; P_o = initial concentration of carotenoids ($\mu g.L^{-1}$); t_f = cultivation time related to P_m (h).

2.11. Determination of antioxidant activity

The carotenogenic extracts obtained from yeast biomass were concentrated on a rotary evaporator and resuspended in petroleum ether to determine the antioxidant activity under different oxidizing conditions (Cipolatti et al., 2015). The scavenging of DPPH (2,2-diphenyl-1-picrylhydrazyl) was determined according to the method by (Sousa et al., 2007), with modified reaction time. A solution of 5 $mmol.L^{-1}$ DPPH in methanol was prepared and, protected from light, was mixed with a known quantity of carotenogenic extract. After 60, 120 and 180 min, the absorbance was determined at 515 nm. The results were expressed as percentage inhibition of DPPH compared to samples without carotenoids.

The 2,2 ′-azinobis (3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) method was applied in accordance with Nenadis and coauthors. (Nenadis et al., 2004), with modified reaction time. A stock solution of 7 $mmol.L^{-1}$ ABTS was prepared, from which the radical $ABTS^{\cdot+}$ was prepared, and this consisted of reaction between 5 mL stock solution with 88 µL of 140 mM potassium persulphate solution. The mixture was protected from light at room temperature for 16 h. Afterwards, it was diluted with ethyl alcohol to obtain an absorbance of 0.70 ± 0.05 at 734 nm. In the dark, the radical $ABTS^{\cdot+}$ was added to test tubes along with carotenogenic extracts to complete 4 mL in each tube. The reaction was monitored every 15 min at 734 nm, the total time of analysis was 75 min.

The reduction power of iron (ferric reducing antioxidant power –FRAP Assay) was determined based on the protocol developed by Benzie and Strain (Benzie and Strain, 1996). The FRAP reagent was prepared from a solution of 0.1 $mol.L^{-1}$ acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-striazine (TPTZ) and 20 $mmol.L^{-1}$ ferric chloride (10:1:1 v/v/v) (Chang et al., 2007). The reagent was heated to 37 °C and the carotenoid extract was added at the end of this time. The reduction of Fe(III)- TPTZ was monitored every 15 min by absorbance reading at 593 nm. The values were expressed as percentage of reduction of ferric ion.

Astaxanthin (Sigma-Aldrich) was used as a standard for antioxidant activity, in the above described methods. It was dissolved in acetone at a concentration of 5 µg mL^{-1}.

Table 1
Partial characterization of substrates in the culture medium.

Sample	% C	% H	% N
Corn steep liquor	17.83	2.41	3.80
Sugar cane molasses[a]	36.50	–	0.30
Glycerol[b]	49.30	–	1.05
Yeast extract	38.40	5.65	10.67
Malt extract	39.24	6.54	10.93
Peptone	43.47	6.71	14.47

[a] (Otero, 2011).
[b] (Silva et al., 2012).

2.12. Statistical analysis

Analyses were performed in triplicate and the results were submitted to analysis of variance (ANOVA). The mean values were compared by Tukey's test at a 5% significance level using the Statistica software (version 5.0, StatSoft, Inc., USA).

3. Results and discussion

3.1. Partial characterization of substrates

Partial characterization of the substrates of the culture medium are described in Table 1. In order to produce a nutrient-rich and low-cost culture medium, two culture media were formulated, named as M1 and M2. The composition of the medium was: 35.6 $g.L^{-1}$ of corn steep liquor and 4.8 $g.L^{-1}$ of raw glycerol for the M1 and 36.5 $g.L^{-1}$ of corn steep liquor and 6.0 $g.L^{-1}$ of sugar cane molasses for the M2. The media were formulated to present a C/N ratio of 6.20, similar to YM medium, with carbon concentration of 8.50% and nitrogen concentration of 1.37% $w.v^{-1}$, aiming to compare the performance of YM medium with the alternative media M1 and M2. YM medium was used as a standard because it is widely used in yeasts cultivation. The substrates used directly affect the microbial community, and therefore the metabolic pathways. Carbon is an essential element necessary for microbial growth. Nitrogen (N) is required for the formation of nucleic acids, cell wall components and proteins. Therefore, the study of C/N ratio becomes of great importance, as an attempt to improve cell growth and carotenoid production. In general, the synthesis of carotenoids is stimulated with higher C/N ratios (Braunwald et al., 2013).

The use of raw glycerol in biotechnological processes is reported by several authors, since it is showing increased production due to high production of biodiesel, therefore, it is interesting to use this coproduct to obtain compounds with high added value (Machado and Burkert, 2015; Robert et al., 2017; Saenge et al., 2011; Taccari et al., 2012).

3.2. Kinetics of the cultivations for the production of carotenoids

The yeasts showed different behaviors in each medium, which is directly associated with their metabolism (Fig. 1). *R. mucilaginosa*, known as a favorable food additive as it is rich in lipids, proteins and vitamins (Bhosale and Gadre, 2001), showed higher biomass production at 96 h of cultivation in YM and M2 (13.5 and 6.4 $g.L^{-1}$, respectively) and 7.9 $g.L^{-1}$ at 72 h for M1. *R. mucilaginosa* showed a very interesting production of carotenoids in the M1, with 650 $\mu g.L^{-1}$ in just 36 h. *R. mucilaginosa* presented consume of the sugars (TRS) in all media (Fig. 1). For comparation, Maldonade et al. (2008) isolated yeasts of the Brazilian ecosystem and cultivated in YM medium for 5 days, thereby obtained a production of carotenoids of 881 $\mu g.L^{-1}$ for *R. glutinis*, 594 $\mu g.L^{-1}$ for *R. graminis,* 590 $\mu g.L^{-1}$ for *R. mucilaginosa-137,* 562 $\mu g.L^{-1}$ for *R. mucilaginosa-108*, 545 $\mu g.L^{-1}$ for *R. mucilaginosa-135*, 168 $\mu g.L^{-1}$ for *R. minuta* and 237 $\mu g.L^{-1}$ for *S. roseus*.

The yeast that presented a higher production of carotenoids was the *Sporidiobolus pararoseus*, with 1310 $\mu g.L^{-1}$ in the M2 at 144 h, 950 $\mu g.L^{-1}$ at 24 h in the M1 and 63 $\mu g.L^{-1}$ in the YM at 72 h. *S. pararoseus* presented a biomass production of 7.3, 9.8 and 12.2 $g.L^{-1}$ for M1, YM and M2, respectively. This yeast is indicated as a producer of carotenoids with high levels of torulene, torularrodine and γ-carotene (Davoli et al., 2004).

P. fermentans stands out from the other yeasts in terms of biomass production in alternative media. The maximum values were achieved at 48 h for all media: 12.5, 12.6 and 14.2 $g.L^{-1}$ for M2, YM and M1, respectively. Values higher than to that found by (Huang et al., 2001) when cultivating the yeast *P. fermentans* for production of 2-phenylethanol in medium containing 0.25% of yeast extract, 18% of sucrose and 10% phenylalanine reaching 2.5 $g\,L^{-1}$ of biomass at 75 h. *P. fermentans* showed lower production of carotenoids among the yeasts studied in 168 h in YM medium, which justifies the lack of studies in the literature with carotenoid production by this yeast. The production of carotenoids by *Pichia* is still poorly reported in the literature. However, there are research works with some species of *Pichia*, such as *Pichia pastoris* for the production of lycopene (Bhataya et al 2009). No studies have been found to date on the production of carotenoids by *Pichia fermentans*.

In general, yeasts are applied in numerous fermentation processes, which utilize the sugars in the medium, and the ability to assimilate various substrates is an important factor in relation to other microorganisms. The medium containing corn steep liquor and molasses (M2) showed higher concentration of reducing sugar relative to the medium containing corn steep liquor and glycerol (M1), most of them from sugar cane molasses. The yeast *S.pararoseus* consumed 96% of the sugars in the M2, while *R. mucilaginosa* and *P. fermentans* consumed 88% in the total cultivation time.

The pH at the start of cultivation was adjusted to 6.0, but in the process suffered influence of compounds released and formed, as can be seen in Fig. 1 which shows the development of pH along the 168 h for the yeasts *P. fermentans, R. mucilaginosa* and *S. pararoseus*. In the cutivations in YM and M2, the pH decreased during the first hours, and increased in the following hours, that due to organic acids released during substrate consumption (sugar), demonstrated by a decrease in the concentration of sugars in the first 36 h. After this time, the released intermediates were assimilated and carotenogenesis was stimulated, due to stress conditions which the micro-organism is submitted by substrate depletion (Silva et al., 2012). For M1, it was not observed a sharp decline during the first hours of cultivation, since this medium has a low content of total reducing sugars, releasing few acid intermediates. In the final hours, the pH remained constant for all yeasts, indicating the end of cultivation. *S. pararoseus* showed the highest carotenoids concentration in M1 (634.5 $\mu g.L^{-1}$), followed by *R. mucilaginosa* (224.8 $\mu g.L^{-1}$) and *P. fermentans* (201.6 $\mu g.L^{-1}$).

Table 2 presents the content of specific and volumetric carotenoids obtained by the yeasts studied in different media at the end of 168 h of culture, where it can be seen that alternative media were favorable for the development and production of carotenoids. The yeast that was able to produce more carotenoids in the alternative media was *S.pararoseus*, followed by *R. mucilaginosa* and *P. fermentans* (634.5, 224.8 and 201.6 $\mu g.L^{-1}$ in M1 and 830.3, 150.6 and 66.2 $\mu g.L^{-1}$ in M2, respectively).

3.3. Determination of kinetic parameters

Table 3 presents the main kinetic parameters related to the cultivation of yeasts in YM, M1 and M2. The maximum specific growth rate (μ_{max}) ranged from 0.28 to 1.39 h^{-1} depending on the medium and the yeast used. The highest μ_{max} of *S. pararoseus* was in M2 (1.17 h^{-1}), following by M1 (1.05 h^{-1}) and YM (0.60 h^{-1}). For *P. fermentans* it was observed μ_{max} 1.39, 1.26 and 1.15 h^{-1} for the M1, YM and M2, respectively. Lower specific rates have been found for yeast *R. mucilaginosa.*

Taccari and coauthors (Taccari et al., 2012) isolated 113 yeast

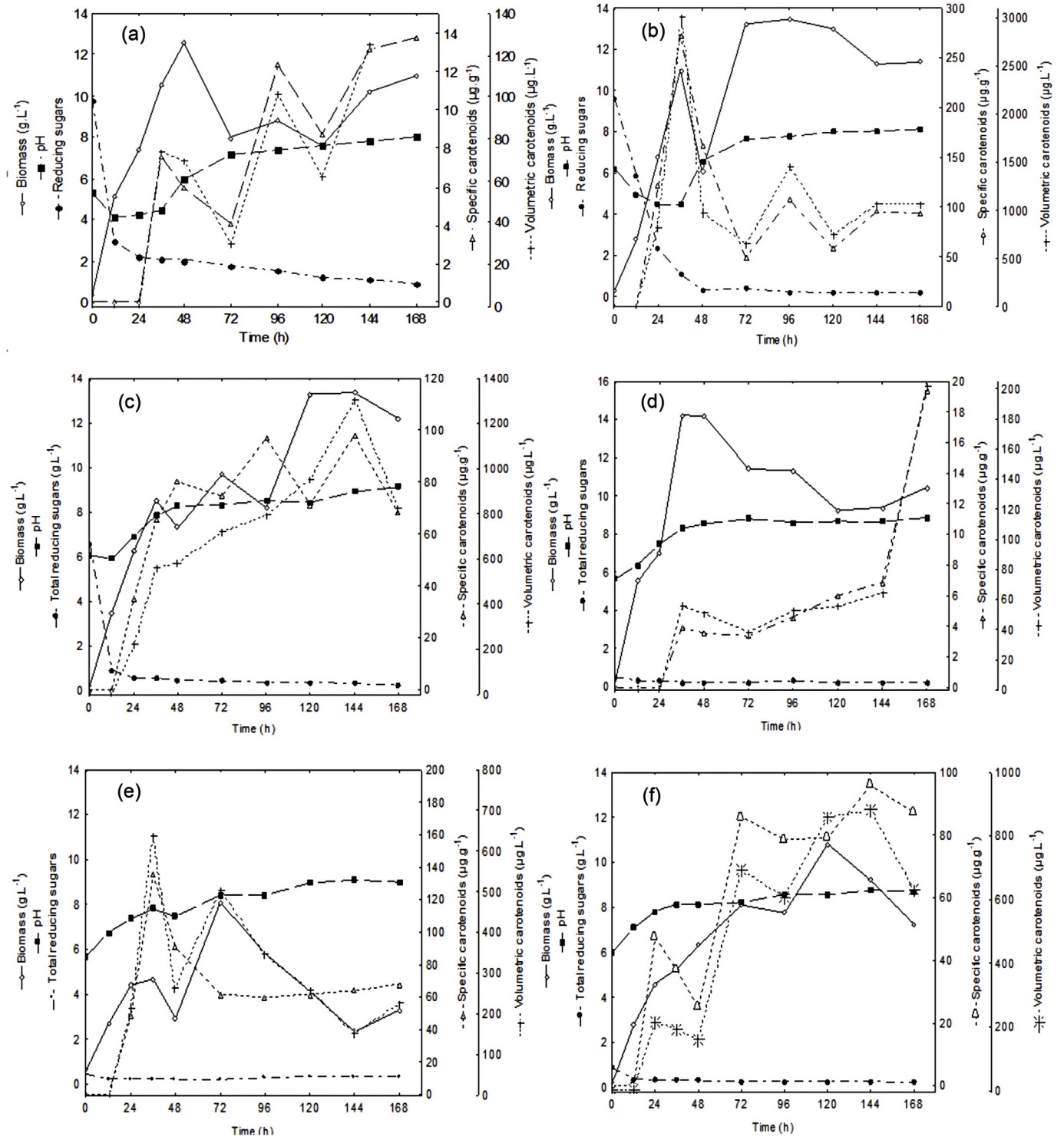

Fig. 1. Kinetics of growth of yeast. *P. fermentans* in YM (a), M1 (b) and M2 (c); *R. mucilaginosa* in YM (d), M1 (e) and M2 (f); and *S. pararoseus* in YM (g), M1 (h) and M2 (i). Operational conditions: 168 h of cultivation at 25 °C and 180 rpm.

strains, which 45 were able to grow in a medium containing crude glycerol (at 25 °C and 150 rpm) with growth rates ranging from 0.11 to 0.37 h^{-1}, which confirmed that raw glycerol can be used as a carbon source for the yeast biomass production, results similar to this work.

The highest productivity in carotenoids were found for yeast *R. mucilaginosa* in the YM medium (83.5 $\mu g.L^{-1}.h^{-1}$) and in the alternative media (17.6 and 38.1 $\mu g.L^{-1}.h^{-1}$ for M1 and M2, respectively). This yeast also highlighted in relation to the substrate to product (carotenoid) conversion factor, 211.8 and 403.8 $\mu g\ g^{-1}$ for M2 and YM, respectively. The productivity in biomass presented maximum values for *P. fermentans*, 0.39; 0.35 and 0.25 $\mu g.L^{-1}.h^{-1}$ for M1, M2 and YM, respectively. So, for *S. pararoseus* the productivity in biomass (P_x), the productivity in carotenoids (Pc) and the $Y_{P/X}$ proved superior in YM medium, while the specific growth rate ($\mu_{máx}$) and conversion factors $Y_{P/S}$ e $Y_{X/S}$ proved superior in M2.

The yeast *P. fermentans* obtained its best performance in alternative media with maximum values for maximum specific growth rate, $Y_{P/X}$, productivity in biomass (P_x) and productivity in carotenoids (Pc) for M1

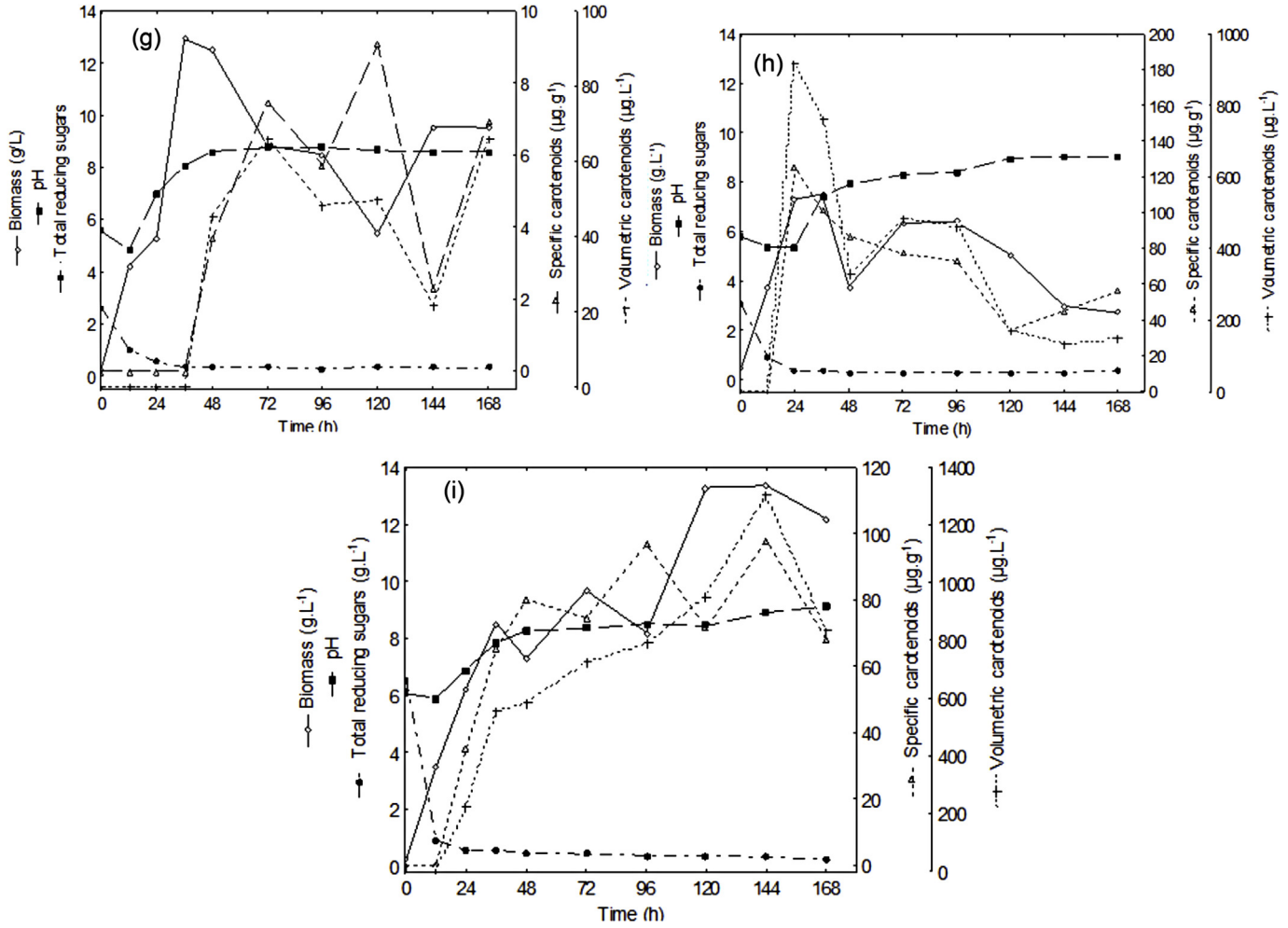

Fig. 1. (*continued*)

medium. However, the conversion factors $Y_{P/S}$ e $Y_{X/S}$ proved superior in M2. Except for maximum productivity in biomass P_x and the conversion factor $Y_{X/S}$ in M2 for *R. mucilaginosa*, the other kinetic parameters showed the best performance in YM medium.

3.4. Identification of carotenoids produced in different substrates

For identification of produced carotenoids, the following standards were used: β-carotene, astaxanthin and lutein. The percentages of identified carotenoids obtained in each medium are showed in Table 4. Different behaviors were observed for the yeast in the different media used, where, in general, there was a greater production of β-carotene. For *P. fermentans,* the change of YM medium to alternative media showed a change in the production of astaxanthin from 27 and 31% in the substrates M1 and M2, respectively, maintaining practically constant the concentration of lutein and β-carotene.

The carotenoids chosen as standards are among the most important of this class, they are widely studied and have high antioxidant activity (Rodriguez-Amaya, 2010; Stringheta et al., 2006). Astaxanthin is an important carotenoid, mainly due to its high antioxidant activity. Its instability can be bypassed through the encapsulation (Machado et al., 2016). Lutein is appointed as a versatile phyto-nutraceutical (Madaan et al., 2017). While *β*-carotene is one of the most studied carotenoids and has known antioxidant activity (Duarte-Almeida et al., 2006).

In a medium containing molasses (M2) for *R. mucilaginosa* it was not detected the presence of astaxanthin. Lutein was produced independent of production medium, similar to *P. fermentans*. In general, the carotenogenic extracts showed a higher concentration of β-carotene. The yeast *S.pararoseus* also showed higher production of *β*-carotene. This yeast presents lutein with concentrations higher than the other microorganisms, however the presence of astaxanthin in cultivated biomass was not detected.

Table 2
Specific (SC) and volumetric carotenoid (VC) content of the biomass obtained from the yeast.

Microorganism	YM		M1		M2	
	SC (µg.g^{-1})	VC (µg.L^{-1})	SC (µg.g^{-1})	VC (µg.L^{-1})	SC (µg.g^{-1})	VC (µg.L^{-1})
R. mucilaginosa	93.9 ± 2.0^{a}	1068.5 ± 33.1^{a}	68.1 ± 12.7^{b}	224.8 ± 11.2^{b}	55.7 ± 8.0^{b}	150.6 ± 16.0^{c}
P. fermentans	12.8 ± 3.0^{ab}	140.4 ± 20.0^{a}	19.3 ± 3.7 ^{a}	201.6 ± 39.3^{a}	7.0 ± 1.1 ^{b}	66.2 ± 10.7^{b}
S. pararoseus	71.1 ± 10.5^{a}	697.8 ± 57.8^{b}	87.3 ± 1.8^{a}	634.5 ± 10.0^{b}	68.1 ± 13.8^{a}	830.3 ± 27.0^{a}

YM: Malt and yeast extract; M1: raw glycerol and corn steep liquor; M2: sugar cane molasses and corn steep liquor. Different letters indicate significant difference between the means in same line ($p < 0.05$) for same determination.

Table 3
Kinetic parameters of the cultivations.

	kinetic parameters	YM[a]	M1[a]	M2[a]
S. pararoseus	μ_{max} (h^{-1})	0.60	1.05	1.17
	$Y_{P/S}$ ($\mu g.g^{-1}$)	42.2	–	49.5
	$Y_{X/S}$ ($g.g^{-1}$)	0.4	–	0.6
	$Y_{P/X}$ ($\mu g.g^{-1}$)	105.5	45,1	85.8
	P_X ($g.L^{-1}.h^{-1}$)	0.11	0.09	0.09
	P_C ($\mu g.L^{-1}.h^{-1}$)	11.9	6.1	9.1
P. fermentans	μ_{max} (h^{-1})	1.26	1.39	1.15
	$Y_{P/S}$ ($\mu g.g^{-1}$)	5.4	–	17.9
	$Y_{X/S}$ ($g.g^{-1}$)	0.7	–	2.8
	$Y_{P/X}$ ($\mu g.g^{-1}$)	7.7	13.3	6.5
	P_X ($g.L^{-1}.h^{-1}$)	0.25	0.39	0.35
	P_C ($\mu g.L^{-1}.h^{-1}$)	0.8	1.2	0.4
R. mucilaginosa	μ_{max} (h^{-1})	0.61	0.28	0.57
	$Y_{P/S}$ ($\mu g.g^{-1}$)	403.8	–	211.8
	$Y_{X/S}$ ($g.g^{-1}$)	1.1	–	1.6
	$Y_{P/X}$ ($\mu g.g^{-1}$)	368.2	155.4	136.2
	P_X ($g.L^{-1}.h^{-1}$)	0.14	0.10	0.20
	P_C ($\mu g.L^{-1}.h^{-1}$)	83.5	17.6	38.1

[a] YM: malt and yeast extract medium, M1: glycerol + corn steep liquor; M2: sugar cane molasses + corn steep liquor.

Table 4
Astaxanthin (1), Lutein (2) and *β*-carotene (3) present in the microbial extracts (%).

	P. fermentans			*R. mucilaginosa*			*S.pararoseus*		
	YM	M1	M2	YM	M1	M2	YM	M1	M2
1	0.0	27.2	30.7	6.9	23.3	0.0	0.0	0.0	0.0
2	7.2	5.7	5.6	1.3	5.5	7.6	4.0	10.8	5.6
3	92.8	67.2	63.7	91.8	71.2	92.4	96.0	89.2	94.4

*YM: Malt and yeast extract, M1: glycerol and corn steep liquor; M2: molasse and corn steep liquor.

3.5. Antioxidant activity of the carotenogenic extracts

Several techniques have been used to determine antioxidant activity in vitro to allow the rapid selection of potentially interesting substances and/or mixtures as DPPH (Cheng et al., 2019; Derf et al., 2019; Zhang et al., 2019), FRAP (Sharayei et al., 2019; Young and Nitin, 2019), ABTS (Baria et al., 2019; Cruz et al., 2019), ORAC (Rocchetti et al., 2019; YIN et al., 2019), linoleic acid-carotenoid system (Duarte-Almeida et al., 2006; Silva et al., 2018). However, the methods used in this work to measure the antioxidant potential of the samples were selected because of their sensitivity, speed and reliability (Ozkan et al., 2019).

The antioxidant activity was measured over time, and at 60, 120 and 180 min for DPPH, 15, 30, 45, 60 and 75 min for ABTS and intervals of 15 min until 210 min for FRAP (Table 5). The contact time between radical and carotene has fundamental importance to the interaction/scavenging. DPPH radical absorbance at 515 nm, but upon reduction by an antioxidant or a radical species, its absorption decreases (Gülçin, 2010). The same happens with other methods that require monitoring reaction time to the stabilization of the measured absorbance. That time will depend on the method and the antioxidant used. For example, Thaipong et al. (2006) affirmed that que guava fruit extracts react rapidly with ABTS (2 h) and with ferric ion, using the FRAP method (30 min), while the reaction with DPPH time was longer (24 h). That is, the reaction time will depend on the method used and of the antioxidant. Table 4 refers to the last reaction time for each of the tested oxidizing systems.

In general, according to Table 5, the extracts were capable of interacting with the ABTS radical, and reduce the iron through the FRAP method, however, scavenging free radical DPPH results were not satisfactory, only the extract from M2 with *P. fermentans* showed a high activity (130% inhibition.μg^{-1}). Some carotenoids are not capable of inhibiting DPPH free radical, as shown in the study by Müller et al. (2011) (Müller et al., 2011), where carotenoids obtained from carrot and tomato juices, and sunflower, olive and walnut oils, were not able to scavenging this free radical, ie the resulting antioxidant potential will depend on the extracted carotenoid. Fu et al (2011) evaluated the antioxidant activity of three carotenoids extracted and purified from water spinach (*Ipomoea aquatica* cv. *Slim leaf*), violaxanthin, lutein and β-carotene. Among them, β-carotene was the most efficient in scavenger the free radical DPPH, with similar activity between β-carotene and lutein, confirming that the structure is very important in the choice of antioxidant.

P. fermentans showed the lower quantification of total carotenoids among the studied yeast. However, it had higher antioxidant activity, that can be assigned to this greater content of astaxanthin with this yeast (30.7% of the carotenoids identified). It is not mentioned in the literature, to date, carotenoid antioxidant activity data from *P. fermentans*. *S. pararoseus* also deserves attention because of its high iron reduction power with maximum value in M1 (1205.5 %reduction μg^{-1}).

All the carotenogenic extracts had antioxidant activity, although they present different profiles. The extracts may contain components such as carbohydrates with carboxyl, amine, or sulfonyl may efficiently target different radicals. Or, a small structural difference could give the difference of antioxidant activities (Yoo et al., 2016). From the promising results of the antioxidant activity of carotenogenic microbial extracts with the selected methods, it is possible to note of the importance of the extracts obtained in this work. We propose that in a future work a system such as linoleic acid-carotenoid can be used in order to evaluate the antioxidant activity in a more real system.

This work showed that the yeasts studied were able to growth in alternative media containing corn steep water and glycerol (M1), and corn steep liquor and molasses (M2), showing different behaviors in relation to production of carotenoids and biomass. The carotenoids obtained had a satisfactory antioxidant activity. The yeasts studied

Table 5
Antioxidant activity by different methodologies of microbial extracts from *R.mucilaginosa*, *P. fermentans* and *S. pararoseus*.

Yeast	Medium	DPPH (%inhibition.μg^{-1})	ABTS (%inhibition.μg^{-1})	FRAP (%reduction μg^{-1})
R. mucilaginosa	YM	2.7 ± 0.1	29.4 ± 1.6	73.7 ± 2.0
	M1	14.7 ± 0.2	102.3 ± 4.0	1084.4 ± 3.5
	M2	5.1 ± 0.1	61.3 ± 7.4	1142.7 ± 1.3
P. fermentans	YM	2.5 ± 0.0	293.5 ± 16.7	14.7 ± 0.3
	M1	10.4 ± 0.3	31.4 ± 0.5	32.6 ± 1.1
	M2	129.6 ± 0.3	293.1 ± 3.8	1778.7 ± 1.5
S. pararoseus	YM	5.8 ± 0.5	59.6 ± 1.6	2997.0 ± 3.3
	M1	7.5 ± 0.3	32.0 ± 6.0	1205.5 ± 5.0
	M2	4.1 ± 0.1	22.2 ± 4.1	536.5 ± 0.8

*YM: Malt and yeast extract, M1: glycerol and corn steep liquor; M2: molasse and corn steep liquor.

produced mainly β-carotene. *S. pararoseus* highlighted with 96, 89 and 94% of astaxanthin, lutein and β-carotene present in the microbial extracts (%) produced in YM, M1 and M2, respectively. The use of byproducts in cultivation can be an advantageous alternative to well explored and implemented, thus helping to minimize environmental impact and lowering costs with culture mediums.

The carotenoids chosen as standards are among the most important of this class, they are widely studied and have high antioxidant activity (Rodriguez-Amaya, 2010; Stringheta et al., 2006). Astaxanthin is an important carotenoid, mainly due to its high antioxidant activity. Its instability can be bypassed through the encapsulation (Machado et al., 2016). Lutein is appointed as a versatile phyto-nutraceutical (Madaan et al., 2017). While *β*-carotene is one of the most studied carotenoids and has known antioxidant activity (Duarte-Almeida et al., 2006).

Acknowledgements

The authors are grateful to the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq). This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) [grant numbers 001]. The authors also thank the companies BS Bios Indústria e Comércio de Biodiesel Sul Brasil S/A - Passo Fundo – RS (Brazil), Guimarães Indústria e Comércio Ltda – RS (Brazil) and Corn Products - Balsa Nova – PR (Brazil).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101208.

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