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Carotenoids from *Phaffia rhodozyma*: Antioxidant activity and stability of extracts

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The main goal of this work was to establish the stability and antioxidant activity of the extracts obtained through different techniques for recovering carotenoids from *Phaffia rhodozyma* NRRL-Y 17268. The best conditions for extracting carotenoids through cell rupture with dimethylsulfoxide (DMSO) were found to be a particle size of 0.125 mm submitted to freezing temperature (-18°C) for 48 h (272 µg/g). For DMSO extracts, freezing negatively affected the antioxidant activity by 2,2 '-azinobis (3-ethyl benzothiazoline-6-sulfonic acid)) and DPPH (2,2-diphenyl-1-picrylhydrazyl (DPPH) methods. The carotenogenic extracts obtained by enzymatic disruption proved to be more promising in relation to its antioxidant activity.

Key words: Microbial carotenoids, antioxidant properties, cell wall disruption.

INTRODUCTION

Carotenoids are widespread in nature and found in several plants, animals and microorganisms (Maldonade et al., 2008). According to BCC Research (2008), the global market for carotenoids was estimated at about $1.2 billion in 2010, with the potential to grow to $1.4 billion in 2018. They are mostly produced by chemical synthesis. However, the biotechnological production of these pigments by yeasts has been highlighted for possibly using low cost substrates in cultivation, control of metabolites with biological activity, designation of natural substances, the small space required for production, independence of environmental conditions such as weather, season or soil composition, and control of culture conditions (Zeni et al., 2011).

The yeast *Phaffia rhodozyma*, also known as *Xanthophyllomyces dendrorhous*, stands out as a natural source of carotenoids. It has a pattern of relatively rapid growth and nutritional quality as well as being approved as a Generally Recognized as Safe (GRAS) microorganism by the Department of Health and Human Services of the Food and Drug Administration (FDA, 2000). *P. rhodozyma* produces different carotenoids depending on the growth conditions. This yeast produces astaxanthin in its configuration (3R, 3'R), and so far it is the only known

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natural source of this stereoisomer. It is also capable of producing β-carotene, which is a dicyclic carotene produced from neurosporene through lycopene (An et al., 1999; Rodriguez-Amaya, 2001; Grewe et al., 2007; Schmidt et al., 2010; Chang et al., 2015; Xiao et al., 2015).

Several potential vegetable sources of carotenoids with antioxidant activity have been studied, such as mango wine (Varakumar et al., 2011), tomatoes (Li et al., 2012), carrots, green beans and broccoli (McInerney et al., 2007), as well as the macrofungi *Phenillinus merrilli* (Chang et al., 2007). However, few studies have been conducted on the antioxidant activity of carotenoids obtained from microbial sources.

On the other hand, different cell disruption methods are available for carotenoid recovery from yeast biomass, including chemical, physical and enzymatic techniques (Michelon et al., 2012). Furthermore, some works have demonstrated that biomass freezing can improve extraction yield (Fonseca et al., 2011). However, there is no information regarding the effects of these treatments on the antioxidant activity of carotenogenic extracts.

In this context, the main goal of this study was to establish the antioxidant activity and stability of the carotenogenic extracts from *P. rhodozyma* NRRL-Y 17268. For this, the effect of particle diameter of biomass on carotenoid recovery was evaluated, and its stability during freezing was taken into consideration. Moreover, antioxidant activities of extracts obtained by chemical and enzymatic techniques of cell disruption were evaluated.

MATERIALS AND METHODS

Microorganism, maintenance and reactivation

This study used the yeast, *P. rhodozyma* NRRL-Y 17268 supplied by Northern Regional Research Laboratory (Peoria, USA). Prior to the experiments, the yeast was maintained at 4°C on yeast malt (YM) agar supplemented with 0.2 g/L of KNO_3 with the following composition (g/L): 3 yeast extract; 3 malt extract; 5 peptone; and 10 glucose (Parajó et al., 1998). For reactivation of stock cultures, transfers to tubes containing the same medium were made and the tubes were incubated for 48 h at 25°C. They were scraped with 10 mL of 0.1% (w/v) peptone diluent for each tube and transferred to 500 mL Erlenmeyer flask (Santos et al., 2012) containing YM medium (3 g/L yeast extract; 3 g/L malt extract; 5 g/L peptone; and 10 g/L glucose) and incubated under the same conditions.

Inoculum

The suspension obtained (10 mL) was transferred to a 500 mL Erlenmeyer flask containing 90 mL YM broth supplemented with 0.2 g/L of KNO_3 (Grewe et al., 2007). The suspension was maintained at 25°C in a rotary shaker at 150 rev/min for 48 h or the time required to achieve $1x10^8$ cells/mL by counting in a Neubauer chamber (Zhang et al., 2005).

Shaken flask cultivation

The cultivation medium was inoculated with 10% (v/v) of inoculum (reaching 10^7 cells/mL). The cultivation was carried out in 500 mL Erlenmeyer flasks containing 153 mL YM medium on a rotary shaker (180 rev/min) at 25°C for 168 h (Fonseca et al., 2011). The initial pH was adjusted to 6.0 and the medium was sterilized at 121 °C for 15 min.

At the end of cultivation (168 h), aliquots were taken and centrifuged (1745 xg) for 10 min. pH was determined in the supernatant (Horwitz, 2000) and the precipitate was washed and resuspended with distilled water. Biomass concentration was estimated by measure of absorbance at 620 nm and conversion to dry weight (g/L) using a biomass standard curve previously determined (Kusdiyantini et al., 1998).

Cell disruption techniques

Two different methods for cell disruption were used: chemical disruption with dimethylsulfoxide (DMSO) and enzymatic disruption. The method with DMSO used 0.05 g dry biomass (48 h at 35°C) submitted to freezing (48 h at -18°C) and 2 mL DMSO. The mixture was homogenized by vortexing every 15 min for 1 h (Fonseca et al., 2011).

The enzymatic method used commercial enzyme preparation Glucanex® (Novozymes) from *Trichoderma harzianum*, containing the enzymes β-1,3-glucanase, protease, chitinase and cellulase. A sample (0.011 g dry biomass) was mixed with sodium acetate buffer 0.2 M, pH 4.5 and enzyme extract with initial activity of 0.6 U/mL. The final mixture was incubated at 55°C in an agitated bath for 30 min, centrifuged at 1745 x*g*, and the supernatant was separated for carotenoid extraction (Michelon et al., 2012).

Biomass particle size effect

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 different fractions (<32, 32, 42, 80 and 115 mesh) corresponding to particulate sizes of >0.500, 0.500, 0.355, 0.180 and 0.125 mm, respectively. Disruption with DMSO and subsequent extraction with petroleum ether were used to determine the total carotenoids.

Stability of carotenoids during biomass freezing

Dry biomass (0.05 g) was submitted to freezing (-18°C) in flasks with a lid, and compared with the carotenoid content of the unfrozen sample. Disruption with DMSO and subsequent extraction with petroleum ether were used to determine the carotenoids, followed by determination of antioxidant activity.

Extraction and determination of total carotenoids

After the disruption, 6 mL acetone was added to facilitate carotenoid extraction. The sample was centrifuged at 1745 xg for 10 min, then solvent was removed and the disruption procedure was repeated until cells were colorless. The solvent extracts were mixed with 10 mL NaCl 20% (w/v) and 10 mL petroleum ether was added. After stirring and phase separation, the excess water was removed with sodium sulfate (Fonseca et al., 2011). The total carotenoids in the extracts were determined as astaxanthin using a spectrophotometer (Biospectro SP-220, China) at 474 nm (Rodriguez-Amaya, 2001) and the values were defined as in Equation 1, using the specific absorptivity coefficient in petroleum ether (2100 mol/L.cm) (Chumpolkulwong et al., 1997; Domínguez-Bocanegra and Torres-Muñoz, 2004).

Table 1. Gradient elution of the mobile phase.

Time (min)	Acetonitrile (%)	Methanol (%)	Ethyl acetate (%)	Flow (mL/min)
0	70	30	0	1.0
5	80	10	10	0.3
6	30	0	70	1.0
7	30	20	30	1.0
10	70	30	0	1.0
17	70	30	0	1.0

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

Where, C = total concentration of carotenoids (µg/g dw); A = absorbance; V = volume (mL); m_{sample} = dry cell mass (g); $A_{1cm}^{1\%}$ = specific absorptivity.

HPLC-VWD analysis of carotenoids

The carotenoids obtained at the end of yeast growth (168 h) without freezing were identified using a high-performance liquid chromatographer (Shimadzu, Kyoto, Japan), consisting of a system of LC-20AT pumps, a DGU 20A5 degasser, a CBM -20A controller, manual gun with 20 µL handle and a SPD-20A spectrophotometric detection system at 450 nm. Instrument control and data acquisition were conducted using LC Solution software. Determinations were made using Discovery Bio Wide Pore C18 Reverse Phase column chromatography, 10 µm (25 cm x 4.6 mm) maintained at room temperature (20°C) using a mobile phase acetonitrile : methanol : ethyl acetate (70:30:0 v/v) in gradient mode (Table 1) with 1 mL/min flow rate and 17 min total run time, and linearity between 0.1 and 7 µg/mL for astaxanthin and β-carotene and between 0.05 and 6 µg/mL for lutein. As chromatographic standards, astaxanthin, lutein and β-carotene were used.

Antioxidant activity of carotenogenic extracts

To determine the antioxidant activity of extracts, these were concentrated in a rotary evaporator at 30°C, and petroleum ether was used as solvent for all the assays. 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 mM 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 2,2 '-azinobis (3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) method was applied in accordance with Nenadis et al. (2004), with modified reaction time. A stock solution of 7 mM ABTS was prepared, from which the radical $ABTS^{\bullet+}$ was prepared, and this consisted of reaction between 5 mL stock solution with 88 µL 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 $ABTS^{\bullet+}$ 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 reduction power of iron (ferric reducing antioxidant power – FRAP Assay) was determined based on the protocol developed by Benzie and Strain (1996). The FRAP reagent was prepared from a solution of 0.1 M acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) and 20 mM 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.

6-Hydroxy-2,5,7,8-tetramethylchloromane-2-carboxylic acid (Trolox; Sigma-Aldrich Chemical) was used to construct calibration curves and all the results are expressed as Trolox equivalents (in mM of Trolox per µg of sample).

Statistical analysis

Results were submitted through 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).

RESULTS AND DISCUSSION

Influence of particle size and freezing time of biomass on carotenoid extraction using cell disruption with DMSO

Biomass of *P. rhodozyma* NRRL-Y 17268 obtained at 168 h cultivation was 4.4 ± 0.4 g-dw/L, final pH was 8.4 ± 0.1 and, after disruption with DMSO and extraction, the content of total carotenoids in dried biomass was 215.0 ± 4.4 µg/g-dw, prior to standardization of the particle size of the biomass of the yeast.

The extraction of carotenoids was facilitated by decreasing the particle size, which can be shown through statistical analysis (Table 2). The total carotenoids content extracted from the biomass and collected in sieves opening 115 mesh was highest (264 µg/g-dw) and significantly different from the others, probably due to the cell/solvent contact area. Biomass with a larger particle size (> 0.500 mm) resulted in lower recovery of carotenoids, while in the case of intermediate sizes (between 0.500 and 0.180 mm), there was no significant difference between them. Therefore, biomass particle was established as 0.125 mm in order to obtain a higher yield of carotenoids. The particle sizes considered in this work are the average of the particles that pass through the sieve.

The method using DMSO is the cell disruption process

Table 2. Total carotenoids extracted from biomass of *P. rhodozyma* NRRL-Y 17268 with different particle sizes using cell disruption with DMSO.

Mesh	Aperture diameter of sieves (mm)	Total carotenoids (µg/g-dw)*
>32	> 0.500	26.0 ± 4.5[c]
32	0.500	192.0 ± 9.9[b]
42	0.355	204.0 ± 0.2[b]
80	0.180	215.0 ± 10.4[b]
115	0.125	264.0 ± 3.9[a]

*Mean values ± standard deviation for n determinations (n=3). Different letters in the same column indicate a significant difference ($p < 0.05$).

Table 3. Total carotenoids from *P. rhodozyma* NRRL-Y 17268 biomass (0.125 mm) subjected to freezing (-18ºC) for increasing periods of time, using cell disruption with DMSO.

Freezing time (h)	Total carotenoids (µg/g-dw)*
0	92.0± 3.6[c]
48	272.0 ± 8.1[a]
120	265.0 ± 5.1[a]
192	251.0 ± 2.9[a]
264	176.0 ± 2.4[b]
336	170.0 ± 3.2[b]
408	144.0 ± 9.5[b,c]
480	125.0± 5.7[c,d]
552	102.0± 5.9[c,d,e]
624	92.0 ± 3.9[d,e]
720	80.0 ±3.56[e]

*Mean ± standard deviation for n determinations (n=3). Different letters in the same column indicate a significant difference ($p < 0.05$).

most widely used for the extraction of total carotenoids from *P. rhodozyma* biomass as it promotes a change in composition and permeability of membranes (Michelon et al., 2012). This method proves to be more efficient for total carotenoids recovery. However, due to its high toxicity, DMSO is usually used for quantification on an analytical scale, as in the case of this study.

The results of freezing at different times on the recovery of carotenoids are shown in Table 3. The content of total carotenoids extracted from the frozen biomass for 48 h is three times higher and significantly different when compared with biomass without freezing. This confirms that the freezing procedure was indeed favorable and facilitates the extraction of carotenoids. However, with the increase in freezing time, the recovery of carotenoids decreased (at 264 h, a significant difference was observed in comparison with the observations made after 192 h), revealing a negative effect on the extraction process, with a gradual decrease until 720 h. Between 48 and 192 h of freezing, no significant differences were observed. Therefore, it was not necessary to freeze the biomass for more than 48 h.

Several authors have studied the influence of storage temperatures on carotenoids from fruits and vegetables. Leong and Oey (2012) studied the effects of processing (98°C for 10 min) and freezing (-20°C in liquid nitrogen), as well as drying and freezing, on the contents of anthocyanins, carotenoids and vitamin C from cherries, nectarines, apricots, peaches, plums, carrots and red peppers. They found that freezing induced the formation of ice crystals favoring extraction and reallocation of molecules and water within cell structure. They also showed that when cultivars were submitted to freezing, most had an increased content of carotenoid, as found in this study, although a different freezing method was used. However, these authors did not evaluate the antioxidant activity of carotenoids.

Influence of freezing time of biomass on antioxidant activity of carotenogenic extracts obtained by cell disruption with DMSO

Carotenogenic extracts obtained from different biomass freezing intervals (0, 24, 48 and 72 h) were evaluated in relation to the ability to inhibit DPPH radical, ABTS radical and reduction power of iron (FRAP), in order to measure antioxidant activity. The methods for the determination of antioxidant activity were expressed in mM trolox/µg of carotenoids (Table 4), which relates the concentration of carotenoids in each extract and the maximum time of reaction for each method.

The carotenoids obtained from biomass without freezing had significantly higher antioxidant activity than carotenoids extracted from frozen biomass in all the methods under study, with 0.77, 2.05 and 4.30 mM trolox/µg for ABTS, FRAP and DPPH (Table 4), respectively.

In general, the antioxidant activity presented in Table 3 decreased with increased freezing time for all methods in question. The method that suffered the greatest decline, 4.30 to 0.96 mM trolox/µg, was the DPPH. The FRAP method also present a decline with the freezing time in

Table 4. Antioxidant activity (mM trolox/µg) for the carotonogenic extracts of *P. rhodozyma* NRRL-Y 17268.

Freezing time (h)	DPPH	ABTS	FRAP
0	4.30±0.05^{aA}	0.77±0.03^{aC}	2.05±0.24^{aB}
24	0.96±0.09^{bcB}	0.75±0.11^{aB}	1.62±0.05^{bA}
48	0.99±0.03^{bA}	0.37±0.01^{bB}	0.99±0.09^{cA}
72	0.84±0.00^{cB}	0.72±0.04^{aB}	1.45±0.05^{bA}

Mean values ± standard deviation (n=3). Different lowercase letters in the same column indicate significant difference. Different uppercase letters in the same row indicate significant difference ($p<0.05$).

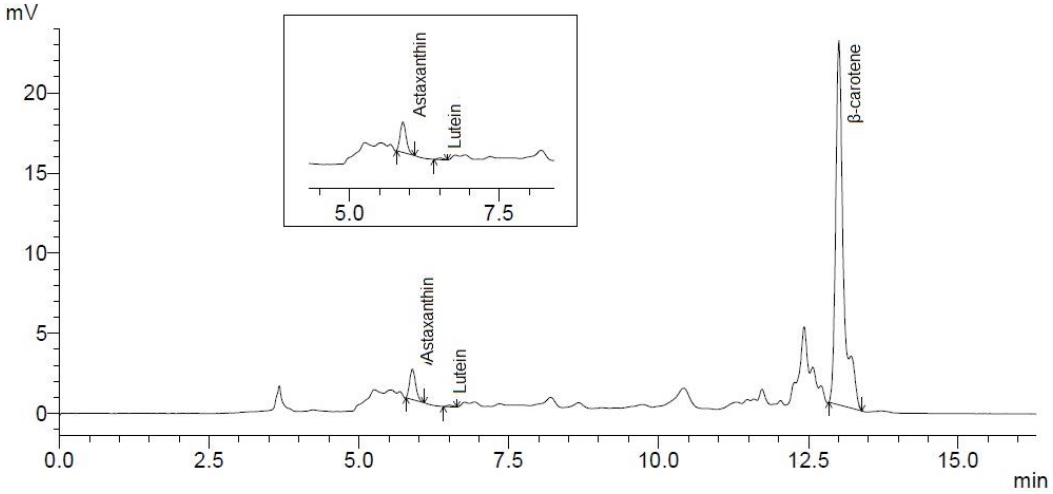

Figure 1. Chromatogram of carotenoids obtained from carotenogenic extract from *P. rhodozyma* NRRL-Y 17268 using cell disruption with DMSO without freezing. SPD-20A Spectrophotometric detection system 450 nm. Reversed Phase Chromatography Discovery Bio Wide Pore C18, 10 µm (25 cm x 4.6 mm).

the first 24 h, from 2.05 to 1.62 mM trolox/µg, while for the ABTS method, the values at 0 and 24 h, there was no significant difference.

Considering that the ice expands, disrupting the cell wall and potentially making carotenoids more accessible to solvents, increasing recovery, the antioxidant activity would also be expected to increase with the growing concentration of carotenoids. However, such behavior was not observed in this study.

Figure 1 shows the chromatogram of carotenoids obtained from carotenogenic extracts of *P. rhodozyma* NRRL-Y 17268 using cell disruption with DMSO without freezing. It was possible to verify the presence of β-carotene, lutein and astaxanthin, while 81% of the carotenoids identified correspond to the first. These are indicated as singlet oxygen binders, and this is related to a conjugated double bond system, which is likely to have resulted in the highest antioxidant activity without freezing.

The three carotenoids cited have nine double bonds in the main chain, which confer greater activity (Rodriguez-Amaya, 2001). Thus, with the freezing, there may have been a release of some compounds during this process, which may have inhibited the activity or have affected the determination of antioxidant potential. The degradation of these carotenoids may also have occurred. Another hypothesis for the decreased antioxidant activity throughout the freezing is the formation of cis isomers, which have lower biological activity (Rodriguez-Amaya et al., 2008). Furthermore, carotenoids may have been oxidized by exposure to air (Britton, 1995) in such a way as not to have a high antioxidant activity, although freezing may have promoted a more efficient cell wall disruption, reflected in higher levels of pigment recovered (Table 3).

The values of antioxidant activity of the carotenogenic extracts depend on the method employed and the conditions of the reaction. Bolanho et al. (2014) studied formulations of cookies enriched with *Spirulina platensis* (which contains high level of total phenolic compounds).

They obtained antioxidant activities ranging from 31.1 to 36.5 mmol trolox/kg with the DPPH method, 0.7 to 1.1 mmol trolox/kg with the ABTS method, and 12.1 to 17.0 with the FRAP method. Also, in terms of comparison values, Hayes et al. (2011) analyzed the antioxidant capacity of four commercial phytochemical products (olive leaf extract, lutein, sesamol and ellagic acid). The best results were for ellagic acid with 1.97 and 1.70 mmol trolox/100 g DW for the ABTS and FRAP, respectively.

Due to its structure, the carotenoids can interact differently in each methodology of antioxidant activity, so it is necessary to use several methods, which help to elucidate its mechanism of action (Parrila et al., 2005; Fu et al., 2011). The proposed methods had differences and similarities, resulting in different behaviors in relation to interaction with microbial carotenoids. The DPPH radical decreased over biomass freezing time, while ABTS and FRAP decreased until 48 h.

Besides the yeasts, other microorganisms are capable of producing carotenoids with antioxidant potential. Goiris et al. (2012) studied the antioxidant potential of micro-algae in relation to its content of phenolic compounds and carotenoids, using ABTS, FRAP and AIOLA (a method that measures the ability of the antioxidant to prevent oxidation of linolenic acid), obtaining maximum values of 48.9, 89.7 and 46.3 µmol trolox eq/g for *Phaeodactylum tricornutum*, stating that the carotenoids present in the extracts are mainly responsible for the antioxidant activity of this microalga.

Antioxidant activity of carotenogenic extracts from *P. rhodozyma* NRRL-Y 17268 obtained by cell wall disruption by enzymatic method

Since the aim is to obtain an extract with potential application in foods, enzymatic lysis of the cell wall would be ideal because it does not present the possible risks of toxicity shown by chemicals. Thus the DDPH, ABTS and FRAP antioxidant activity methods were conducted with the extract obtained from the enzymatic rupture of *P. rhodozyma* cells.

Although the freezing led to a greater recovery of carotenoids from *P. rhodozyma* NRRL-Y 17268 (Table 3), a negative influence was observed concerning anti-oxidant activity with increasing freezing time (Table 4). Therefore, biomass was used without freezing in the enzymatic lysis procedure.

The cell disruption method for biomass without freezing was applied using commercial enzyme preparation Glucanex®, obtaining total carotenoids of 267.3 ± 0.6 µg/g, statistically equal to values obtained with DMSO; 264.0 ± 3.9 µg/g (Table 2), according to test t ($p<0.05$).

The interaction of carotenoids obtained from enzymatic cell disruption with the DPPH radical was not observed. This non-detection may be associated with the release of secondary cell wall compounds or the release of the enzymatic preparation itself, which also has the enzymes protease, cellulase and chitinase. Moreover, interaction between microbial carotenoids and the ABTS radical was observed (Table 5), achieving values of 4 mM trolox/µg in 75 min, which are higher than those found previously using chemical disruption with DMSO. This increase was not observed in the extracts obtained by chemical disruption with DMSO (data not show).

Table 5. Antioxidant activity (mM trolox/µg) for the carotonogenic extracts of *P. rhodozyma* NRRL-Y 17268 using an enzymatic method of cell disruption.

DPPH (min)	ABTS (min)	FRAP (min)
ND (0 min)	2.42 ± 0.03 (15)	2.16 ± 0.04 (15)
ND (120 min)	2.71 ± 0.07 (30)	2.24 ± 0.03 (30)
ND (180 min)	2.89 ± 0.05 (45)	2.24 ± 0.04 (45)
-	3.49 ± 0.05 (60)	3.00 ± 0.05 (60)
-	4.00 ± 0.01 (75)	3.66 ± 0.11 (75)

Mean ± standard deviation. (n=3). ND, not detected. Different lowercase letters in the same column indicate significant difference. Different uppercase letters in the same row indicate significant difference ($p<0.05$).

The method using the enzymatic disruption was milder and more ideal for cell disruption of *P. rhodozyma* NRRL-Y 17268. Although the antioxidant activity of carotenogenic extract was not detected by the method using the free radical DPPH, the other methods showed a significant improvement, suggesting that this method of cell disruption affects favorably antioxidant activity.

Therefore, through the use of enzymatic cell wall disruption, the highest values of antioxidant activity of this study were obtained for both the ABTS and FRAP methods: 4.00 and 3.66 mM trolox/µg, respectively.

Conclusions

This study aimed to explore two different disruption methods in relation to the antioxidant activity. Although the use of freezing favored the extraction of carotenoids, the carotenoids extracted not necessarily had higher antioxidant activity. Using the enzymatic method of disruption of the cell wall, it was possible to obtain the highest values of antioxidant activity, using the ABTS and FRAP methods, with results of 4.00 and 3.66 mM trolox/µg, respectively. The extent of this activity was shown to be influenced by three different methods: the DPPH, ABTS and FRAP. There is still much to be explored in this field, mainly because of the importance of this study, and the future application of microbial carotenoids obtained by submerged cultivation is promising. Therefore, this study has contributed to the use of microbial carotenoids as a source of biologically active compounds, also contributing to future studies in this field.

Conflict of interests

The author(s) did not declare any conflict of interest.

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REFERENCES

An GH, Cho MH, Johnson EA (1999). Monocyclic carotenoid biosynthetic pathway in the yeast *Phaffia rhodozyma (Xanthophyllomyces dendrorhous).* J. Biosci. Bioeng. 88:189-193.

BCC Research (2008). The global market for carotenoids (FOD025C). Wellesley: BCC Research, 2008. http://www.bccresearch.com

Benzie IFF, Strain JJ (1996). The ferric reducing ability of plasma (FRAP) as a measure of ''Antioxidant Power'': the FRAP assay. Anal. Biochem. 239:70-76.

Bolanho BC, Egea MB, Jácome ALM, Campos I, Carvalho JCM, Danesi EDG (2014). Antioxidant and nutritional potential of cookies enriched with *Spirulina platensis* and sources of fibre. J. Food Nutr. Res. 53:171-179.

Britton G (1995). Structure and properties of carotenoids in relation to function. FASEB J. 9:1551-1558.

Chang HY, Ho YL, Sheu MJ, Lin YH, Tseng MC, Wu SH, Huang GJ, Chang YS (2007). Antioxidant and free radical scavenging activities of *Phellinus merrillii* extracts. Bot. Stud. 48:407-417. ISSN: 1999-3110.

Chang JJ, Thia C, Lin HY, Liu HL, Ho FJ, Wu JT, Shih MC, Li WH, Huang CC (2015). Integrating an algal β-carotene hydroxylase gene into a designed carotenoid-biosynthesis pathway increases carotenoid production in yeast. Bioresour. Technol.184:2-8.

Chumpolkulwong N, Kakizono T, Nagai S, Nishio N (1997). Increased astaxanthin production by *Phaffia rhodozyma* mutants isolated as resistant to diphenylamine. J. Ferment. Bioeng. 83:429-434.

Domínguez-Bocanegra AR, Torres-Muñoz JA (2004). Astaxanthin hyperproduction by *Phaffia rhodozyma* (now *Xanthophyllomyces dendrorhous*) with raw coconut milk as sole source of energy. Appl. Microbiol. Biotechnol. 66:249-252.

Fonseca RAS, Rafael RS, Kalil SJ, Burkert CAV, Burkert JFM (2011). Different cell disruption methods for astaxanthin recovery by *Phaffia rhodozyma*. Afr. J. Biotechnol. 10:1165-1171.

Food and Drug Administration (FDA) (2000). Federal register. Rules and regulations. Vol. 65, No. 130. Washington: FDA, 2000. <www.fda.gov/OHRMS/DOCKETS/98fr/070600e.txt>

Fu H, Xie B, Ma S, Zhu X, Fan G, Pan S (2011). Evaluation of antioxidant activities of principal carotenoids available in water spinach (*Ipomoea aquatica*). J. Food Compost. Anal. 24:288-297.

Goiris K, Muylaert K, Fraeye I, Foubert I, Brabanter J, Cooman L (2012). Antioxidant potential of microalgae in relation to their phenolic and carotenoid content. J. Appl. Phycol. 24:1477-1486.

Grewe C, Menge S, Griehl C (2007). Enantioselective separation of all-E-astaxanthin and its determination in microbial sources. J. Chromatogr. A. 1166:97-100.

Hayes JE, Allen P, Brunton N, O'Grady MN, Kerry JP (2011). Phenolic composition and *in vitro* antioxidant capacity of four commercial phytochemical products: Olive leaf extract (*Olea europaea L.*), lutein, sesamol and ellagic acid. Food Chem. 126:948-955.

Horwitz W (2000).(Ed): Official methods of analysis of AOAC international. 17th edition. Gaithersburg: Association of Official Analytical Chemists, 2000. ISBN 0935584676.

Kusdiyantini E, Gaudin P, Goma G, Blanc PJ (1998). Growth kinetics and astaxanthin production of *Phaffia rhodozyma* on glycerol as a carbon source during batch fermentation. Biotechnol. Lett. 20:929-934.

Leong SY, Oey I (2012). Effects of processing on anthocyanins, carotenoids and vitamin C in summer fruits and vegetables. Food Chem. 133:1577-1587.

Li H, Deng ZY, Liu R, Loewen S, Tsao R (2012). Ultra-performance liquid chromatographic separation of geometric isomers of carotenoids and antioxidant activities of 20 tomato cultivars and breeding lines. Food Chem. 132:508-517.

Maldonade IR, Rodriguez-Amaya DB, Scamparini ARP (2008). Carotenoids of yeast isolated from the Brazilian ecosystem. Food Chem. 107:145-150.

McInerney JK, Seccafien CA, Stewart CM, Bird AR (2007). Effects of high pressure processing on antioxidant activity, and total carotenoid content and availability, in vegetables. Innovative. Food Sci. Emerg. Technol. 8:543-548.

Michelon M, Borba TM, Rafael RS, Burkert CAV, Burkert JFM (2012). Extraction of carotenoids from *Phaffia rhodozyma*: A comparison between different techniques of cell disruption. Food Sci. Biotechnol. 21:1-8.

Nenadis N, Wang JF, Tsimidou M, Zhang HY (2004). Estimation of scavenging activity of phenolic compounds using the ABTS assay. J. Agric. Food Chem. 52:4669-4674.

Parajó JC, Santos V, Vázquez M (1998). Optimization of carotenoid production by *Phaffia rhodozyma* cells grown on xylose. Process Biochem. 33:181-187.

Parrila EA, Rosa LA, Torres-Rivas F, Rodrigo-Garcia J, González-Aguilar (2005). Complexation of apple antioxidants: Chlorogenic acid, quercetin and rutin by β-cyclodextrin (β -CD). J. Inclusion Phenom. Macrocyclic Chem. 53:121-129.

Rodriguez-Amaya DB (2001). A guide to carotenoid analysis in foods [online]. Washington: ILSI Press, 2001. ISBN 1-57881-072-8. < http://pdf.usaid.gov/pdf_docs/PNACQ929.pdf>

Rodriguez-Amaya DB, Kimura M, Godoy H, Amaya-Farfan J (2008). Updated Brazilian database on food carotenoids: Factors affecting carotenoid composition. J. Food Compos. Anal. 21:445–463.

Santos EO, Michelon M, Furlong EB, Burkert JFM, Kalil SJ, Burkert CAV (2012). Evaluation of the composition of culture medium for yeast biomass production using raw glycerol from biodiesel synthesis. Braz. J. Microbiol. 43:432-440.

Schmidt I, Schewe H, Gassel S, Jin C, Buckingham J, Humbelin M, Sandman G, Schrader J (2010). Biotechnological production of astaxanthin with *Phaffia rhodozyma/Xanthophyllomyces dendrorhous*. Appl. Microbiol. Biotechnol. 89:555-571.

Sousa CMM, Silva HR, Vieira-Jr GM, Ayres MCC, Costa CLS, Araújo DS, Cavalcanti LCD, Barros EDS, Araújo PBM, Brandão MS, Chaves MH (2007). Fenóis totais e atividade antioxidante de cinco plantas medicinais. Quim. Nova 30:351-355.

Varakumar S, Kumar YS, Reddy OVS (2011). Carotenoid composition of mango (*Mangifera indica L.*) wine and its antioxidant activity. J. Food Biochem. 35:1538-1547.

Xiao A, Jiang X, Ni H, Yang Q, Cai H (2015). Study on the relationship between intracellular metabolites and astaxanthin accumulation during *Phaffia rhodozyma* fermentation. Electronic J. Biotechnol. 18:148-153.

Zeni J, Cence K, Grando CE, Tiggermann L, Colet R, Lerin LA, Cansian RL, Toniazzo G, Oliveira D, Valduga E (2011). Screening of pectinase-producing microorganisms with polygalacturonase activity. Appl. Biochem. Biotechnol. 163:383-392.

Zhang Y, Rittmann BE, Wang J, Sheng Y, Yu J, Shi H, Qian Y (2005). High-carbohydrate wastewater treatment by IAL-CHS with immobilized *Candida tropicalis*. Process Biochem. 40:857-863.