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Critical Assessment of Various Techniques for the Extraction of Carotenoids and Co-enzyme Q_{10} from the Thraustochytrid Strain ONC-T18

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A variety of techniques for extracting carotenoids from the marine *Thraustochytrium* sp. ONC-T18 was compared. Specifically, the organic solvents acetone, ethyl acetate, and petroleum ether were tested, along with direct and indirect ultrasonic assisted extraction (probe vs bath) methods. Techniques that used petroleum ether/acetone/water (15:75:10, v/v/v) with 3 h of agitation, or 5 min in an ultrasonic bath, produced the highest extraction yields of total carotenoids (29−30.5 μg g^{-1}). Concentrations up to 11.5 μg g^{-1} of canthaxanthin and 17.5 μg g^{-1} of β-carotene were detected in extracts stored for 6 weeks. Astaxanthin and echinenone were also detected as minor compounds. Extracts with and without antioxidants showed similar carotenoid concentration profiles. However, total carotenoid concentrations were approximately 8% higher when antioxidants were used. Finally, an easy-to-perform and inexpensive method to detect co-enzymes in ONC-T18 was also developed using silica gel TLC plates. Five percent methanol in toluene as a mobile phase consistently eluted co-enzyme Q_{10} standards and could separate the co-enzyme fractions present in ONC-T18.

KEYWORDS: Carotenoids; *Thraustochytrium* sp. ONC-T18; antioxidants; astaxanthin; canthaxanthin; echinenone; β-carotene; ultrasonic; co-enzyme Q_{10}

INTRODUCTION

The potential toxic effects of several synthetic pigments have recently resulted in an increase in the desirability of natural alternatives (*1*). For example, natural astaxanthin is a better choice for use as a food additive when compared with synthetic pigments (*2*). Consequently, the demand for natural pigments that can be used in food, pharmaceuticals, cosmetics, nutraceuticals, textiles, and as printing dyes has been on the rise. A variety of microorganisms and microalgae found in nature is able to produce a wide range of these pigments. These include carotenoids, melanins, flavins, and quinones (co-enzymes) and, more specifically, violacein, phycocyanin, indigo, and monascins (*1*). Carotenoids are thought to provide health benefits by decreasing the risk of disorders (particularly certain cancers) and age-related macular degeneration (*3*). Thraustochytrids, marine microbial protist species, have attracted a great deal of attention due to their high production rates of omega-3 fatty acids (*4*). They can also be a potential source of carotenoids such as β-carotene, canthaxanthin, astaxanthin, and echinenone (*5*−*7*).

When recovering carotenoids from a variety of sources (including microbial), the main factor to consider is the issue of stability (*8*). Microbial carotenoids are located within the cell, attached to cellular membranes, and must be released by procedures that can break down or dissolve cell walls while at the same time minimizing oxidation. As a result, carotenoid stability may be greatly affected by the type of solvents used to extract them. In addition, factors such as high concentrations of oxygen and temperature can trigger the oxidation of carotenoids (*9*).

Several organic solvents have been used to extract carotenoids from a variety of raw materials. For example, when extracting carotenoids from crustacean waste, acetone may be used (*10*). Petroleum ether/acetone/water (15:75:10, v/v/v) can produce high yields of carotenoids when used in a proportion of 10:1 (w/v) with respect to biomass. Furthermore, carotenoid oxidation during the extraction process can be reduced by adding 0.01% of a 1:1 combination of the food grade antioxidants butylhydroxytoluene (BHT) and butylhydroxyanisole (BHA) (w/w) (*8*, *11*). Studies reporting the extractability of carotenoids rarely include an assessment of extraction methods using different organic solvents. Furthermore, the effect of extraction conditions on carotenoid stability is rarely evaluated. Using *Thraustochytrium* sp. ONC-T18, this research compares the efficiency of a variety of extraction techniques and solvent combinations in terms of total and specific carotenoid amounts.

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Similar to carotenoids, co-enzyme Q_{10} exhibits antioxidative properties that allow it to inactivate free radicals within the body. Carotenoids and co-enzymes of the Q group are isoprenoids. In plants, the biochemical pathway for the formation of these compounds (carotenoids and co-enzymes Q) is based upon the pathway of sterol biosynthesis. Furthermore, the synthesis of co-enzymes Q is affected by carotenoids such as β-carotene because this carotenoid is a precursor of vitamin A, which affects the synthesis of co-enzymes Q, cholesterol, and squalenes (*12*). Co-enzyme Q_{10} (2,3-dimethoxy-5-methyl-6-decaprenyl benzoquinone) is a fat soluble, vitamin-like quinone commonly known as ubiquinone, CoQ, and vitamin Q_{10}. Co-enzyme Q_{10} is an essential component of the mitochondria involved in the body's electron transport chain, which produces adenosine triphosphate (*13*). Co-enzyme Q_{10} has been used in the treatment of a variety of disorders related to cellular energy metabolism and appears promising for the treatment of neurodegenerative disorders such as Parkinson's disease (*14*). Animals, including humans, can only synthesize co-enzyme Q_{10}, a process that involves the synthesis of a benzoquinone structure from the amino acids tyrosine and phenylalanine, repeated synthesis of the isoprene side chain from acetyl-coA via the mevalonate path, and the condensation of these two structures (*15*, *16*). Currently, TLC methods for the analysis of co-enzymes involve the use of expensive reversed-phase TLC plates (*17*−*19*). This research aims to develop a quick and inexpensive method for the detection of co-enzymes and, in particular, co-enzyme Q_{10} using silica gel TLC plates.

MATERIALS AND METHODS

Materials. Microbial biomass of *Thraustochytrium* sp. ONC-T18 (strain surveyed in an eastern Canadian coastal site by Burja et al. (*4*)) for all extraction experiments was prepared using a single fermentation (Biostat Bplus Twin 5L Bioreactor (Sartorius BBI Systems Inc., Bethlehem, PA)) and was freeze-dried prior to sample processing. Specifically, liquid medium (pH 7.4) was prepared in artificial seawater containing 2 g L^{-1} of yeast extract (BD, Franklin Lanes, NJ), 8 g L^{-1} of monosodium glutamate that was sterilized by autoclaving, followed by the addition of 20 g L^{-1}, 0.2 μm filter sterilized glucose (*4*). A 100 mL volume inoculum culture of *Thraustochytrium* sp. ONC-T18 was prepared from an agar plate and grown for 24 h at 25 °C on a shaker at 120 RPM. This inoculum was then used to inoculate 4.9 L of medium in the bioreactor. All analytical, HPLC grade organic solvents and standards used were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

Total Carotenoid Content. Absorption spectra of carotenoids in acetone and a mixture of petroleum ether/acetone/water (15:75:10, v/v/v) were assessed using a Varian 100 Bio-spectrophotometer (Walnut Creek, CA). The absorption spectra of astaxanthin standard (1 μg mL^{-1}) were measured between 400 and 500 nm. Subsequently, the concentration of carotenoids (using astaxanthin as a standard) in the extracts was calculated using the formula (*10*, *20*)

$$\text{total carotenoid content } (\mu\text{g g}^{-1} \text{ of biomass}) = \frac{A_{477\text{nm}} V_{\text{extract}} DF}{0.2 W_{\text{sample}}}$$

where A_{477nm} is the absorbance at 477 nm (visible spectrum); $V_{extract}$ is the volume of the extract (5 mL); *DF* is the dilution factor (final volume divided by the initial volume); 0.2 is the A_{477nm} value of 1 μg mL^{-1} astaxanthin standard; and W_{sample} is the weight of the sample.

Extractions. A summary of techniques used to extract carotenoids from thraustochytrid strain ONC-T18 is indicated in **Table 1**. For each extraction technique, 0.5 g of homogeneous biomass was placed in 5 mL of solvent (1:10, w/v). Seven out of the 14 extractive methods (**Table 1**) used the protective antioxidant mixture, 0.01% BHA/BHT (1:1, w/w) in relation to the biomass. A wrist action shaker at a speed setting of 10 (Burrell model 75, Pittsburgh, PA) was used for the first two extractive techniques. Shaking using a Vortex Genie (Fisher Scientific, Bohemia, NY) at a speed setting of 5 was used for the next six extractions. Extracts were centrifuged at 2000 rpm (447*g*) for 20 min, and acetone (2 mL) was added and recentrifuged for another 20 min. To dissolve oil droplets formed in the extracts, and to avoid interference during absorbance reading, 1 mL of acetone was added to the supernatant. Absorbance was measured at 477 nm to quantify total carotenoids. The nine to 14 methods were ultrasonic assisted extractions. Ultrasonic energy was applied by using either an ultrasonic cleaning bath (Aquasonic model 75D, VWR Scientific Products, Westchester, PA) or probe (model CPX 130PB, Cole Parmer, Vernon Hills, IL). An 50 mL Erlenmeyer flask, containing the biomass−solvent mixture (0.5 g of biomass and 5 mL of solvent), was placed in the ultrasonic bath and subjected to indirect sonication at a frequency of 35 kHz for 30 min at 25 °C. For direct sonication using the ultrasonic probe, the biomass−solvent mixture was placed in glass test tubes, and the probe was immersed. The mixture was then subjected to a maximum ultrasonic energy of 20 kHz for 30 s. After sonication, samples were centrifuged and prepared for absorbance reading as previously described.

Table 1. Techniques Used To Extract Carotenoids from Thraustochytrid Strain ONC-T18

no.	technique	description
1	PAW – A (3 h)	petroleum ether/acetone/water (15:75:10), 3 h of wrist action shaking
2	PAW + A (3 h)	petroleum ether/acetone/water (15:75:10), BHA/BHT, as above
3	Ace – A (3 min)	acetone, 3 min agitation using a vortex
4	Ace + A (3 min)	acetone, BHA/BHT, 3 min agitation using a vortex
5	PAW – A (3 min)	petroleum ether/acetone/water (15:75:10), vortex 3 min
6	PAW + A (3 min)	petroleum ether/acetone/water (15:75:10), BHA/BHT, vortex 3 min
7	EA – A (3 min)	ethyl acetate, vortex 3 min
8	EA + A (3 min)	ethyl acetate, BHA/BHT, vortex 3 min
9	PAW – A (U/S: 5 min)	petroleum ether/acetone/water (15:75:10), 5 min using an ultrasonic bath
10	PAW + A (U/S: 5 min)	petroleum ether/acetone/water (15:75:10), BHA/BHT, 5 min using bath
11	PAW – A (U/S: 10 min)	petroleum ether/acetone/water (15:75:10), 10 min using an ultrasonic bath
12	PAW + A (U/S: 10 min)	petroleum ether/acetone/water (15:75:10), BHA/BHT, 10 min using bath
13	PAW – A (U/S-P: 30 s)	petroleum ether/acetone/water (15:75:10), 30 s of sonication using probe
14	PAW + A (U/S-P: 30 s)	petroleum ether/acetone/water (15:75:10), BHA/BHT, 30 s using probe

HPLC Analysis of Carotenoids. Concentrations of carotenoids were determined using a Varian 9095 HPLC system equipped with a photodiode array 9050 detector to analyze UV−vis light absorption spectra of carotenoids. Separation was performed using a 5 μm Luna C_{18} reversed-phase column 250 mm × 4.6 mm (00G-4252-EO, Phenomenex, Torrance, CA) and a safety guard column (AJO-4284, Phenomenex). A gradient mobile-phase system of water/ethyl acetate/methanol was used. A ratio of 2:10:88 (v/v/v) was used for 5 min at 0.75 mL min^{-1} for equilibration; flow was maintained for a further 10 min. Between 10 and 30 min the solvent ratio was ramped to 2:50:48 and the flow rate was adjusted to 1.5 mL min^{-1}. Concentrations of each carotenoid were analyzed and compared among extractive techniques. A six carotenoid standard, comprised of astaxanthin, zeaxanthin, canthaxanthin, β-cryptoxanthin, echinenone (CaroteNature, Lupsingen, Switzerland), and β-carotene, was used for identification purposes. These carotenoid standards were chosen as they constitute the main starting, intermediate, and final products within the microbial carotenoid pathway and have perceived commercial value. Total carotenoid content determination was performed on at least 5 replicates per extractive technique. This data, together with the concentration of individual carotenoids, was analyzed for significant differences by ANOVA, and a Duncan's multiple range test was performed for comparison of means (SPSS 13.0, Chicago, IL).

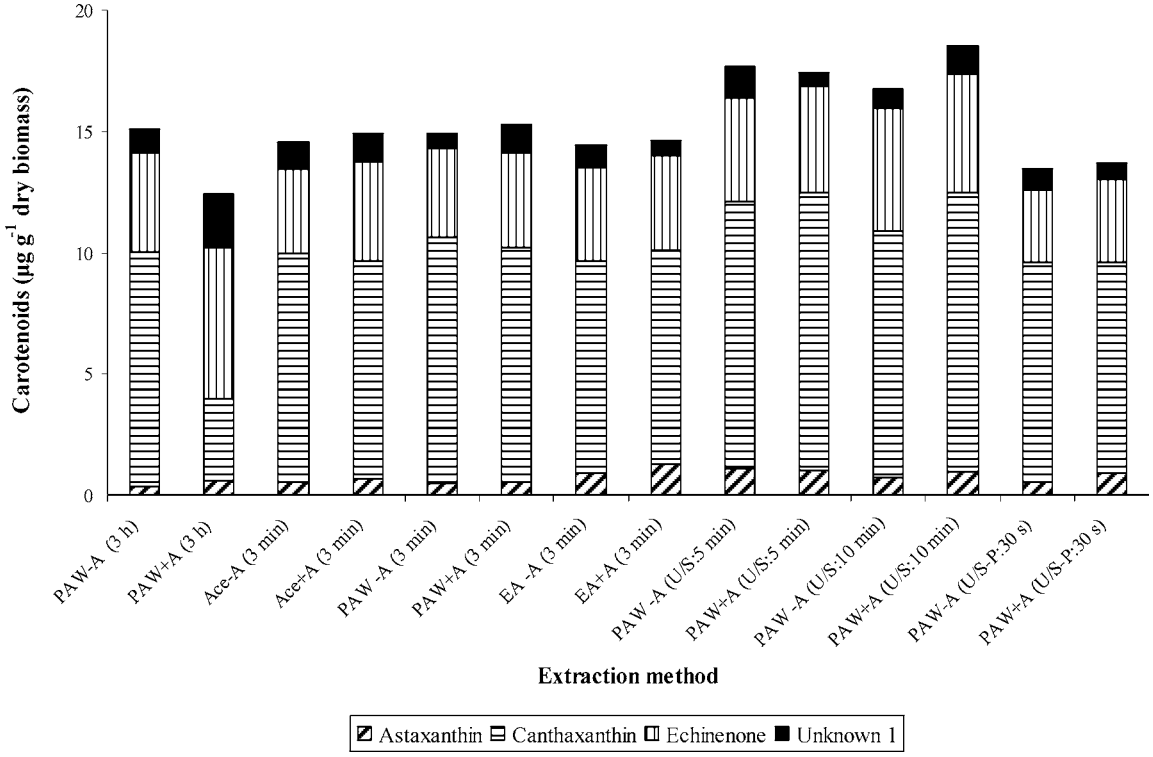

Figure 1. Carotenoid contents recovered from several extractive techniques (part 1). Standard errors (SEs) for all samples (standards and experimentals) were ≤5% ($n = 5$).

Carotenoid Retention. Retention of carotenoids (canthaxanthin, echinenone, and β-carotene) in thraustochytrid strain ONC-T18 was measured throughout the cell growth process described by Burja et al. (*4*). A 4 L fermentation was carried out for 7 days, and samples of 50 mL were collected at 24, 48, 72, 96, 120, 144, and 168 h to be analyzed by the reversed-phase LC method described previously.

Co-enzyme Analysis by TLC. At least 5 extractions to recover co-enzymes were performed from ONC-T18 and compared to a co-enzyme Q_{10} standard (Sigma-Aldrich). Freeze-dried biomass (0.4 g) was placed into a centrifuge tube, and 3 mL of methanol/hexane (3:2, v/v) was added and vortexed for 1 min. Hexane (8 mL) was added and agitated again. The sample was centrifuged for 10 min at 4000 RPM (1789*g*), and the supernatant was transferred to a scintillation vial. The procedure was repeated (from the addition of 8 mL of hexane), and the extracts were combined. The sample was evaporated to dryness using a nitrogen stream, resuspended in 3 mL of acetone, and transferred to an amber vial via a 0.45 μm syringe filter.

The mobile phases tested to separate co-enzymes on 20 cm × 10 cm glass TLC Silica Gel 60 F254 plates (Merck, Gibbstown, NJ) were 3, 5, and 10% methanol in toluene; 3 and 5% ethanol in toluene; petroleum ether/chloroform (20:80, v/v); and methanol/hexane (3:2, v/v). Five percent methanol in toluene offered the best TLC results for the qualitative assessment of the co-enzyme Q_{10} standard and was subsequently used for analysis of co-enzymes in ONC-T18. Procedures using this mobile phase are described here. A silica gel TLC plate was dried using a forced air-drying oven for 1 h at 120 °C and allowed to cool in a desiccator under vacuum for 10 min. A 100 mL solution of co-enzyme Q_{10} standard (50 μg mL^{-1}) was prepared and placed in a 1.5 mL amber vial from which 75 and 100 μL of co-enzyme Q_{10} were taken to be spotted on the TLC plate using a CAMAG Automatic TLC Sampler 4 (CAMAG Scientific Inc., Wilmington, NC). Co-enzyme extracts (1 μL) were spotted onto TLC plates with or without co-spotting of co-enzyme Q_{10} standard (100 μL). TLC plates were then placed into a developing chamber containing the mobile phase and developed until the solvent reached 1.5 cm from the top. The plate was dried and sprayed with phosphomolybdic acid (Sigma-Aldrich). To dry and develop the spots, the TLC plate was placed on a plate heater (160 °C) for 1 min (CAMAG TLC Plate Heater III, CAMAG Scientific Inc.). Finally, plates were photographed with white light from the top using the CAMAG Reprostar 3. The R_f values of spots were determined by dividing the compound distances (mid-point of spot) from the origin by the solvent front distance from the origin.

RESULTS AND DISCUSSION

Carotenoid Extractions. Total carotenoid contents from the various extraction techniques tested are shown in **Figures 1** and **2**. PAW ± A (3 h) and PAW ± A (U/S: 5 min) were found to be the most efficient treatments ($p < 0.05$) for extracting carotenoids (approximately 30.3 and 29.3 μg g^{-1}, respectively). Among all remaining treatments, there was no significant difference (24.5−26.9 μg g^{-1}), except for the ultrasonic methods using the probe (PAW ± A (U/S-P: 30 s)), which were the least efficient extractions to recover carotenoids (approximately 19.3 μg g^{-1}) ($p < 0.05$). Throughout these evaluations, the addition of BHA/BHT had no effect on extractions. Initially, a trend of lower standard errors (SEs) in the total carotenoids recovered was observed. However, on closer inspection and after performing further extractions and analyzing the results statistically, lower SEs were found to not always be related to the use of BHA/BHT (i.e., PAW + A for 3 min and EA + A for 3 min, methods 6 and 8, respectively) (**Table 1**).

In the PAW extracts, it was necessary to add 1 mL of acetone to disperse oil droplets separated from the solution (*Thraustochytrium* sp. ONC-T18 is known to produce lipids up to 81.3% of its dry biomass (*4*)). No oil droplets were observed in extracts using acetone or ethyl acetate. Petroleum ether (15%) and acetone (75%) in the PAW solvent system were not sufficient to eliminate saturation by oil in the extracts. It is probable that the water contained in the PAW solution reduced the petroleum ether and acetone oil solvating capacities, thus leading to oil separation in the samples. However, the water portion (10%) may have been responsible for the high extraction yields of the relatively polar carotenoid canthaxanthin since this xanthophyll has two oxygens that increase its level of polarity when compared with the nonoxygenated carotenoid β-carotene.

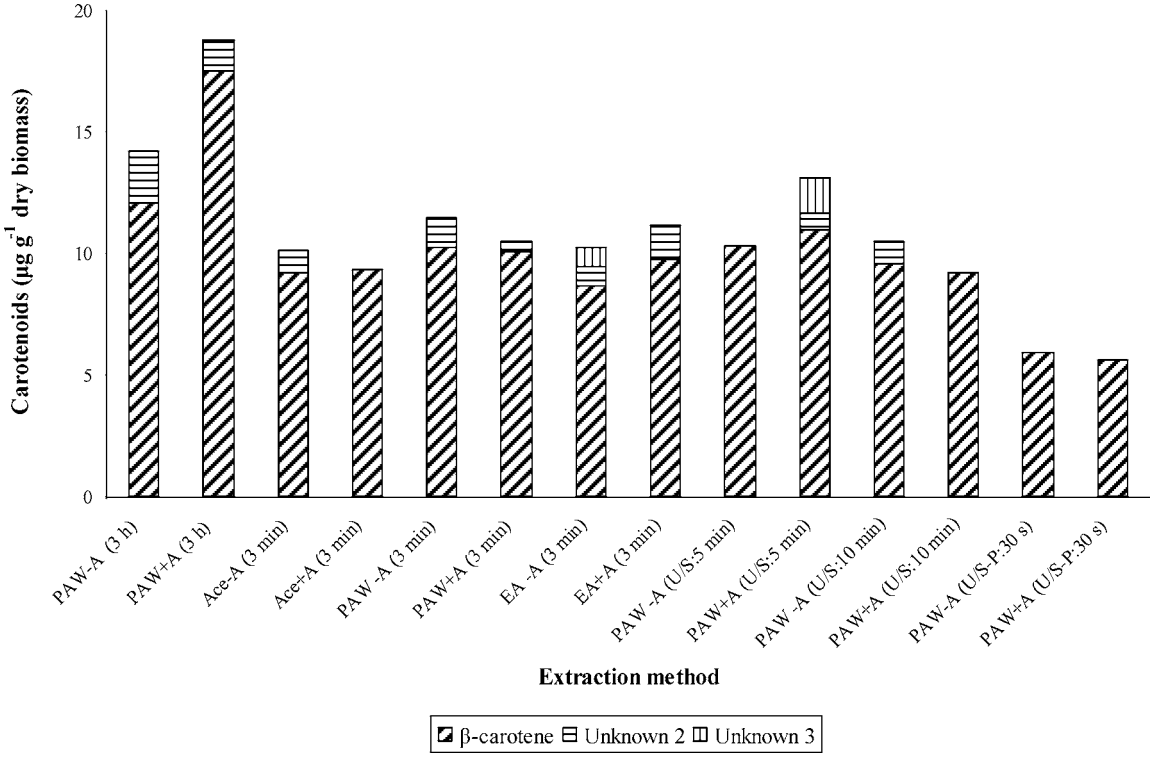

Figure 2. Carotenoid contents recovered from several extractive techniques (part 2). SEs for all samples (standards and experimentals) were ≤5% ($n = 5$).

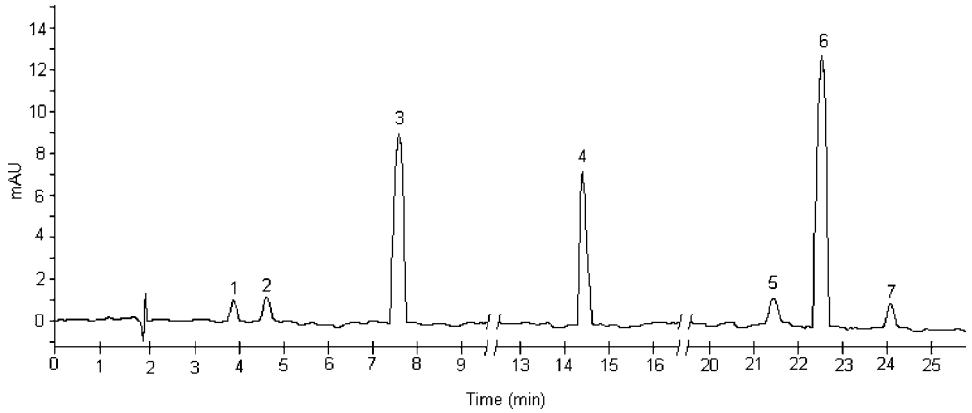

Figure 3. Reversed-phase LC chromatogram of carotenoids extracted from thraustochytrid strain ONC-T18. See **Table 2** for peak assignment.

Analysis of Carotenoids by HPLC. The mobile-phase proportion and the flow rate were changed during the HPLC analysis of carotenoids to separate and detect β-carotene. Astaxanthin, canthaxanthin, echinenone, and β-carotene were identified in all extracts tested from **Table 1**. The order in which these carotenoids were eluted using the C_{18} reversed-phase column is directly related to the number of oxygens present within each compound (**Figure 3** and **Table 2**), with oxygenated carotenoids (xanthophylls) eluting first followed by the carotenes. Thus, the elution order was found to be astaxanthin > unknown carotenoid 1 > canthaxanthin > echinenone > unknown carotenoid 2 > β-carotene > unknown carotenoid 3. One of the unknown carotenoids, eluted between astaxanthin and canthaxanthin, is hypothesized to be phoenicoxanthin (indicated as unknown 1 in **Figure 1**), a xanthophyll containing three oxygens, while the xanthophylls astaxanthin and canthaxanthin have four and two oxygens, respectively. Phoenicoxanthin has been previously reported in another thraustochytrid having a similar elution order (*5*). Aki et al. (*6*) have proposed a pathway for the synthesis of carotenoids by another thraustochytrid strain. A similar potential pathway is illustrated in **Figure 4**. This tends to suggest that when *Thraustochytrium* sp. ONC-T18 was harvested, carotenoid production that culminated in astaxanthin production was predominantly located in the first half of the pathway since the major carotenoids recovered were β-carotene and canthaxanthin.

Table 2. Carotenoids Detected by Reversed-Phase LC in Thraustochytrid Strain ONC-T18

peak no.	retention time (min)	λ_{max} (nm)	carotenoid
1	3.8	477	astaxanthin
2	4.6	nd[a]	unknown 1[b]
3	7.6	465	canthaxanthin
4	14.4	458	echinenone
5	21.4	nd	unknown 2
6	22.5	450	β-carotene
7	24.1	nd	unknown 3

[a] nd = not determined. [b] Probably phoenicoxanthin since it eluted in the same order reported for another thraustochytrid strain (*5*).

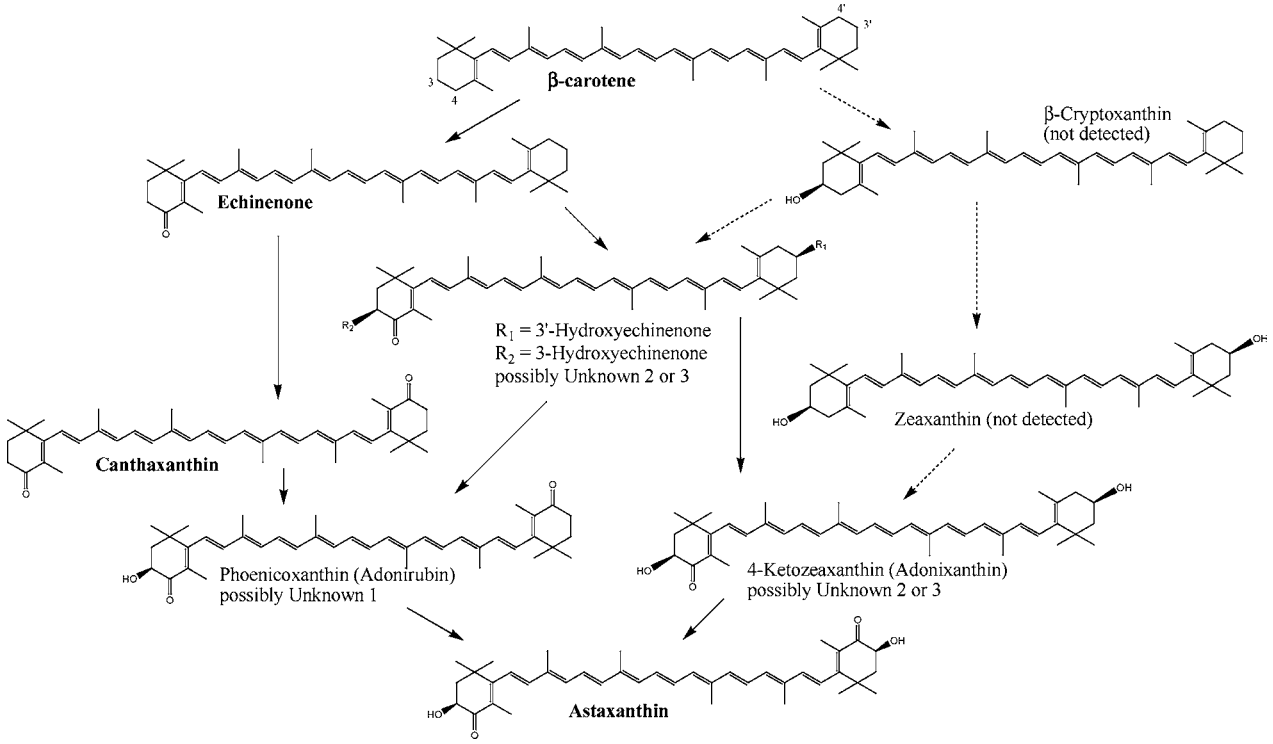

Figure 4. Postulated pathways involved in the formation of astaxanthin in *Thraustochytrium* sp. ONC-T18. Biosynthesis was modified from Fraser et al. (*23*), with compounds using RP-HPLC identified in bold. Intermediates that differ from the classical astaxanthin biosynthesis pathway are marked as not detected, and associated pathways are shown with hashed arrows. Intermediates that could most probably be unknown compounds 1–3 are also shown.

All extracts were stored at −80 °C after extraction from ONC-T18 biomass. HPLC analysis was carried out within 6 weeks of extractions. The highest amounts of β-carotene ($p < 0.05$) were recovered using PAW − A (3 h) and PAW + A (3 h) extractive techniques (12−17.5 μg g^{-1}). The addition of BHA/BHT significantly stabilized carotenoids ($p < 0.05$) in PAW + A (U/S: 5 min) and PAW + A (3 h) extracts when compared with PAW − A (U/S: 5 min) and PAW-A (3 h) extracts, respectively. PAW + A (U/S: 5 min) presented the most consistent results in terms of high extraction efficiency for astaxanthin, canthaxanthin, echinenone, and β-carotene (1, 11.4, 4.4, and 11 μg g^{-1}, respectively), whereas PAW + A (3 h) had a high extraction yield only for echinenone and β-carotene (6.3 and 17.5 μg g^{-1}, respectively) (**Figures 1** and **2**).

The use of ultrasound to extract carotenoids reduced significantly the time of extraction. Similar total carotenoid amounts were recovered using PAW + A (3 h) and PAW + A (U/S: 5 min). Ultrasonic energy produces cell fragmentation that dramatically increases surface areas and the mass transfer rate of targeted compounds into the extraction solvent (*21*). The indirect sonication (ultrasonic bath) method produced better extraction results than direct sonication using an ultrasonic probe ($p < 0.05$). Although, it may be possible that the ultrasonic probe can increase the extraction yield if the time of exposure is extended to more than 30 s. However, the power of the probe and the disruption cell rate must be balanced delicately since power ultrasound, with its associated cavitational collapse energy and bulk heating effect, can denature the cell contents once released. Moreover, extraction methods using ultrasound relate to the efficiency of cell wall breakdown to release cellular contents without destroying them at the same time (*22*).

Conversely, within the remaining extraction procedures, the addition of BHA/BHT did not affect the carotenoid content. Results also showed that the two major carotenoids extracted from ONC-T18 were β-carotene and canthaxanthin ($p < 0.05$). β-Carotene and canthaxanthin predominated in *Thraustochytrium* sp. ONC-T18, whereas in previous studies with other thraustochytrid species (*5*, *7*), astaxanthin (>200 μg g^{-1} of dry weight basis) and phoenicoxanthin (>150 μg g^{-1} of dry weight basis) were higher. A moderate amount of echinenone was detected in extracts of ONC-T18, as well as a small amount of astaxanthin. Neither β-cryptoxanthin nor zeaxanthin were detected. Three unknown carotenoids were also detected. One of them is most probably phoenicoxanthin, due to elution time matching to a previous study (*5*). The two remaining unknown compounds 2 and 3 (**Figure 2**) may either be oxidization products possibly from canthaxanthin and β-carotene since they did not present a high yield of extraction when antioxidants (BHA:BHT) were added, or they may be intermediates between echinenone and canthaxanthin (3- and 3′-hydroxyechinenone) and/or between 3- and 3′-hydroxyechineone and astaxanthin (4-ketozeaxanthin) (**Figure 4**). The detection of small amounts of unknown carotenoid oxidation products has been shown to occur even when stored at a temperature of −80 °C (*9*).

Carotenoid retention in the thraustochytrid strain ONC-T18 during fermentation is illustrated in **Figure 5**. Contrary to previous results, the carotenoid content of *Thraustochytrium* sp. ONC-T18 was not found to parallel biomass or cell growth, with a maximal stationary phase of growth being reached after 3 days of incubation (*5*). Rather, the carotenoid content was found to coincide with maximal polyunsaturated fatty acid (PUFA) production instead and may explain the 10-fold decrease in total carotenoid levels found within this strain. Specifically, ONC-T18 may synthesize these carotenoids to protect the PUFAs being produced (up to 81.3% of dry cell biomass) from oxidation (*4*). The various carotenoid components (canthaxanthin, echinenone, and β-carotene) of ONC-T18 did, however, agree with previous results, in that carotenes decreased on

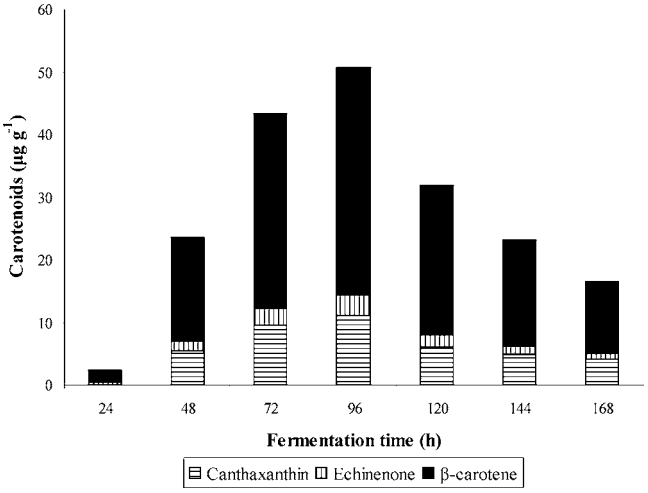

Figure 5. Carotenoid retention in thraustochytrid strain ONC-T18. SEs for all samples (standards and experimentals) were ≤5% ($n = 5$).

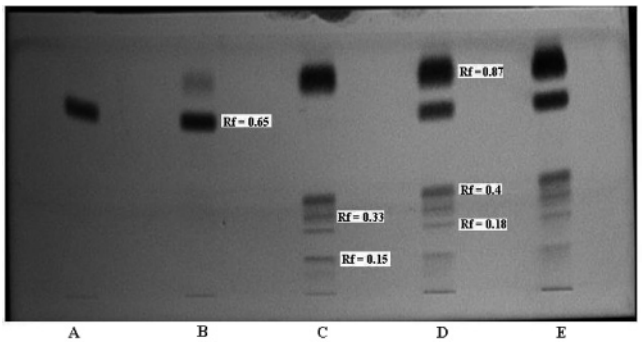

Figure 6. TLC for co-enzyme analysis (R_f co-enzyme Q10 = 0.65). A = Spotted 50 μL from a 100 μg mL^{-1} mother solution of standard co-enzyme Q10; B = spotted 100 μL from the mother solution; C = spotted 1 μL of extract from strain ONC-T18; D = spotted 1 μL of extract from strain ONC-T18 and over-spotting 50 μL from mother solution; and E = spotted 2 μL of extract and over-spotting 100 μL from mother solution.

reaching the stationary phase, while xanthophylls increased (**Figure 5**). The maximum production of carotenoids was reached at 96 h (11.16, 3.24, and 36.24 μg g^{-1} of canthaxanthin, echinenone, and β-carotene, respectively). In terms of carotenoid percentages, between 24 and 48 h on transition between exponential and stationary growth phases (growth maxima being at 36 h (*4*)), canthaxanthin, echinenone, and β-carotene went from 2.6, 12.17, and 85.22% (24 h), respectively, to 23.4, 6.3, and 70.28% (48 h) and remained constant (±3.12%) until 168 h (**Figure 5**).

Co-enzyme Analysis. The 5% methanol in toluene mobile phase was efficient for resolving a co-enzyme Q_{10} standard on silica gel TLC plates (**Figure 6**). Reported TLC methods to analyze co-enzymes only use reversed-phase high-performance TLC plates (*17*−*19*). However, these plates are costly as compared with the silica gel or normal-phase TLC plates. Altough co-enzymes can be identified and quantified by reversed-phase LC, this is a time-consuming and more expensive alternative when compared with regular TLC. The use of TLC silica gel plates is convenient to perform when the purpose is to quickly identify (not quantify) co-enzymes. Co-enzyme elution on TLC plates is related to the number of isoprene units. Therefore, co-enzymes with less isoprene units elute better on reversed-phase high-performance TLC plates; less polar compounds, like co-enzymes with a higher number of isoprene units (i.e., co-enzyme Q_{10}), interact more with the chromatographic phase and elute less, thus developing spots close to the origin.

Absorption in the normal phase presented the reverse to that seen in reversed-phase TLC silica gel; thus, co-enzymes with a higher number of isoprene units developed further from the origin. This TLC method showed there was no co-enzyme Q_{10} in extracts from thraustochytrid strain ONC-T18 (**Figure 6**). Co-enzyme Q_{10} added to extracts eluted well before other compounds, thus presenting a high R_f value (0.65−0.87). Rather, *Thraustochytrium* sp. ONC-T18 was found to possess co-enzymes having fewer isoprene units and R_f values of 0.33−0.4 and 0.15−0.18 (**Figure 6**). Positive identification via LC−MS−MS using a Varian LC 1200 determined Q_9 as being the major co-enzyme component of ONC-T18 at 182.3 μg g^{-1} of dry cell weight (data not shown).

In conclusion, thraustochytrids may represent an important source of carotenoids, antioxidants that are chemoprotective and have other health related benefits (i.e., eye health, cancer prevention, and anti-inflammation) (*1*). Reports indicate that thraustochytrid strains produce significant amounts of astaxanthin and phoenicoxanthin (*5*, *7*). In this instance, *Thraustochytrium* sp. ONC-T18 was found to produce more β-carotene and canthaxanthin. Nevertheless, the concentration of specific carotenoids in thraustochytrids depends upon a variety of culture conditions (*7*). Extractive techniques of the thraustochytrid biomass should minimize the oxidation of carotenoids, separate the lipid and carotenoid components effectively, and improve the recovery of these antioxidant compounds. Additionally, an economical and convenient method to analyze co-enzymes from *Thraustochytrium* sp. ONC-T18 using normal-phase TLC was also developed.

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