Deesterification of astaxanthin and intermediate esters from Haematococcus pluvialis subjected to stress

Janeth I. Galarza, Bertha O. Arredondo Vega, Jimmy Villón, Vitalia Henríquez

**A R T I C L E   I N F O**

**A B S T R A C T**

Haematococcus pluvialis is the richest biological source of astaxanthin under unfavorable growing conditions. Many reports have discussed the optimal astaxanthin extraction methods. Free-astaxanthin could be still hindered by microalgae extracts composition or by prolonged extraction times. In this study we evaluated the effect of enzymolysis and saponification deesterification processes of astaxanthin and its carotenoid precursors under high irradiance and nitrogen deprivation stress time conditions. Results showed that cholesterol esterase facilitated astaxanthin deesterification (975.65 μg mg⁻¹ DW) while saponification positively affected zeaxanthin (1038.68 μg mg⁻¹ DW).

**1. Introduction**

Astaxanthin is a secondary ketocarotenoid, 3,3′-dihydroxy-β, β-carotene-4,4′-dione, which belongs to the family of xanthophylls. It is a lipophilic pigment with unique chemical properties stemming from its molecular structure [1–3]: 2 carboxyl groups, 2 hydroxyl groups, and 13 ethylene conjugated double bonds [4]. The presence of keto and hydroxyl groups in each ion ring reveal some important characteristics, such as the ability to be esterified, high antioxidant activity, and a greater polar nature than other carotenoids [5–8]. The beneficial effects of astaxanthin lie in its antioxidant, anticarcinogenic, anti-inflammatory, skin-protective and natural coloration properties, which can be used for various bioactive, cosmetic, and nutraceutical purposes [8–11], or for improved feeds in commercial production of fish and crustaceans. In short, there is extensive interest in industrial scale production [9,12–19].

One of the most important biological sources of astaxanthin is the green algae Haematococcus pluvialis (H. pluvialis) [20,21], which accumulates large amounts of astaxanthin under unfavorable growing conditions, e.g., nitrogen and phosphorus deficiencies, saline stress, light intensity, and/or different temperatures [5,6,22–29]. Biosynthesis in Haematococcus follows a general carotene pathway towards formation of β-carotene, which is the main precursor for astaxanthin [30]. In H. pluvialis, astaxanthin content represents 90% of total carotenoids [5,31] and is synthesized through two pathways: a β-carotene ketolase (BKT) enzyme converting β-carotene to canthaxanthin via echino- nene; and a carotene β-hydroxylase (CrrK-b) enzyme converting β-carotene to zeaxanthin via β-cryptoxanthin [30,32,33]. Astaxanthin produced from H. pluvialis exists in ratios of approximately 70% monoesters, 25% diesters, and 5% free form. H. pluvialis contains a variety of fatty acids [5,34] stored as triacylglycerol (TAG) in the form of cytoplasmic lipid droplets [29,35–37]. Furthermore, intermediaries of astaxanthin, carotenoids like β-carotene, zeaxanthin, canthaxanthin, echinolones, etc. [38,39], can be found in free forms or in esterified forms with fatty acids [17,35,40,41].

Several reports have discussed astaxanthin extraction methods from H. pluvialis using organic solvents: acetone, ethanol, ethyl acetate, n-hexane, dichloromethane, and methanol [17,42–46]. However, quantification of free astaxanthin has been hindered by pigments, esters, waxes, and/or fatty acids; or, in the case of carotenoid extracts, esterified xanthophylls, chlorophylls, and/or lipids [17,47,48]; or by prolonged extraction times [5,49,17,50]. Deesterification of carotenoids like astaxanthin is usually processed through saponification with KOH or NaOH to release the lipids [45,46,51]; or by enzymolysis with alkaline lipase [52] or cholesterol esterase [45,53,54]. The latter technique has

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been shown to improve chromatographic analysis, concentrations, and pigment yields [47,17,45], and previous studies mention the use of deesterification in carotenoid extracts to improve performance [17,55].

In spite of the above, optimal stress variables to achieve total carotenogenesis in *H. pluvialis* strains are still under investigation, given differences among strains and genetic variability. On the other hand, concentrations of other deesterified carotenoids are not known when astaxanthin processes of saponification or enzymolysis from *H. pluvialis* are extracted at different stress times. In this research, we have evaluated both enzymolysis and saponification deesterification processes of astaxanthin and its carotenoid precursors under high irradiance and nitrogen deprivation stress time conditions.

2. Materials and methods

2.1. *Haematococcus pluvialis* strain, cultivation and stress conditions

Microalgae *H. pluvialis* (CCAP 34/7) was obtained from the Culture Collection of Algae and Protozoa (CCAP), Windermere, England, and grown in 400 mL of Bold’s basal medium (BBM) as recommended by The Culture Collection of Algae at the University of Texas, Austin, USA, under the following conditions: a photon flux density of 60 μmol m⁻² s⁻¹, provided by white light fluorescent lamps; photoperiod, 12:12 h (light:dark); temperature 25 ± 1 °C; constant aeration; and pH 7.5. Growth was evaluated based on culture cell concentrations (cell mL⁻¹). Cell counts were performed daily in a Zeiss brand optical microscope using a 0.1 mm deep Neubauer camera. Cultures were grown until late exponential phase [56]. For stress tests, cultures were centrifuged at 2500 rpm for 5 min at 10 °C, then suspended in 400 mL of nitrogen-free BBM (BBM-N) and exposed to photon flux density of 1200 μmol m⁻² s⁻¹ over 6 days. Samples for pigment analysis were taken initially (T0), at 4 days (T4) and 6 days (T6) of stress conditions. Vegetative growth samples and stressed samples were evaluated in triplicate.

2.2. Extraction and deesterification of astaxanthin

*H. pluvialis* cysts (20 mg) were lysed in vortex with metal beads (1.00 and 2.38 mm). Total carotenoids were extracted with 100% acetone and maintained at -20°C for 24 h. Samples were repeatedly sonicated and centrifuged at 4 °C until colorless, giving a final extraction volume of 50 mL Galarza et al. [53]. Deesterification of astaxanthin through enzymolysis was performed using 1 mL of total carotenoid extract mixed with 1 mL of cholesterol esterase (4 units mg⁻¹) (Sigma, cat # C9281) following Gómez et al. [54] with some modifications. Deesterification of astaxanthin through saponification was performed using 3 mL of total carotenoid extract dried under gaseous nitrogen and re-dissolved in 3 mL 100% methanol, and a 1 mL solution of 0.05 M NaOH in methanol. The sample was placed under a nitrogen atmosphere to evaporate to a concentration of 3 mL. Once saponification of astaxanthin esters was complete, the sample was kept at 5 °C under nitrogen atmosphere and in darkness for 12 h. Aliquots of extracts from both methods were stored in amber vials at -80 °C prior to high-performance liquid chromatography (HPLC) analysis [46,51].

2.3. HPLC quantification of astaxanthin and other pigments

Extracts were analyzed by HPLC with an automatic injection pump (Agilent Technology HPLC 1260) and diode array detector (model G4212B) in 5 μm columns (ZORBAX SB-C8, 4.6 cm x150 mm). The mobile phase was activated by a quaternary pump (model VL 1260). Solvents used were: solvent A, methanol, 1 N ammonium acetate (70:30 v/v); and solvent B, methanol at a flow rate of 1 mL min⁻¹ for 20 min at room temperature. The gradient system was according the following procedure (minutes; %solvent A, % solvent B): (0; 75, 25), (1; 50, 50), (15; 0, 100), (18; 0, 100), and (18.5; 75, 25) [57]. Astaxanthin and other carotenoids were identified at 440 nm and compared against known standards for retention time and absorption spectrum. Known standards were astaxanthin, zeaxanthin, β-carotene, canthaxanthin, and chlorophyll-a (Sigma-Aldrich).

2.4. Statistical analysis

Results were expressed as mean value ± standard deviation for the three extractions and independent treatments. Differences were considered significant at *p* < 0.05. All statistical analyses were performed using the MINITAB statistical package.

3. Results

3.1. *Haematococcus pluvialis* growth

*H. pluvialis* was grown in BBM at pH 7.5–8.0, containing NaNO₃ (8.82 mM) as a nitrogen source by recommendation of the University of Texas. Microalgae vegetative growth reached high cell concentrations (3 × 10⁵ cell mL⁻¹) by the fourth day of cultivation; the stationary phase started on the fifth day of cultivation (Fig. 1). Cell activity was evident during the exponential phase (Fig. 2A). Stress conditions induced palmella (Fig. 2B) and cyst (aplanospore) stages (Fig. 2C and D).

3.2. Analysis of astaxanthin deesterification via enzymolysis and saponification

Astaxanthin deesterification was performed via enzymolysis and saponification. HPLC analyses showed significant differences between the two treatments. The cholesterol esterase enzyme was highly effective in astaxanthin hydrolysis from *H. pluvialis* samples subjected to six days of stress with respect to the control sample (T0), reaching concentrations of 975.65 μg mg⁻¹ (T6). Saponified astaxanthin, through hydrolysis with NaOH, reached lower concentrations (356.39 μg mg⁻¹ dry weight) compared to enzymatic hydrolysis. Concentrations of free astaxanthin at T0 and T4 were relatively low for both treatments (Fig. 3).

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**Fig. 1.** Growth curve of *H. pluvialis* CCAP34/7 during six days of culture in Bold’s Basal Medium (BBM).
3.3. Analysis of deesterified astaxanthin pigment contents

HPLC analyses revealed the presence of intermediary astaxanthin carotenoids: zeaxanthin, β-carotene, and canthaxanthin. Cholesterol esterase enzymolysis for T0 samples resulted in zeaxanthin concentrations of 791.18 μg mg⁻¹ dry weight (DW); and β-carotene at 466.16 μg mg⁻¹ DW. Saponification treatment with NaOH of samples at T0 also resulted in high concentrations of zeaxanthin (697.66 μg mg⁻¹ DW) and β-carotene (489.7 μg mg⁻¹ DW). β-carotene concentration was considerably higher at T0 (925.74 μg mg⁻¹ DW), followed by zeaxanthin (537.01 μg mg⁻¹ DW). The Kruskal-Wallis test determined that there are significant differences in the concentration of pigments in the two treatments and the control, except for β-carotene, (Table 1).

Enzymolysis pigment deesterification in samples subjected to 4 days of stress resulted in higher concentrations of zeaxanthin (601.61 μg mg⁻¹ DW), followed by astaxanthin esters (305.24 μg mg⁻¹ DW) and β-carotene (244.88 μg mg⁻¹ DW); while saponification deesterification had surprising amounts of zeaxanthin (829.5 μg mg⁻¹ DW), followed by β-carotene (266.8 μg mg⁻¹ DW). In control samples at T4, concentrations were higher for zeaxanthin (943.83 μg mg⁻¹ DW), followed by canthaxanthin (540.08 μg mg⁻¹, dry weight) and astaxanthin esters (223.20 μg mg⁻¹ DW). The Kruskal-Wallis test determined that there are significant differences in the concentration of pigments in the two treatments and the control, except for zeaxanthin (Table 2).

Samples from T6 undergoing enzymolysis deesterification had lower astaxanthin (126.61 μg mg⁻¹ DW) and canthaxanthin (108.62 μg mg⁻¹ DW), while zeaxanthin and β-carotene were negligible. From saponification deesterification, zeaxanthin had considerably higher concentrations (1038.7 μg mg⁻¹ DW), while other pigment concentrations were low. Under the same stress conditions, control cells showed high concentrations of canthaxanthin (614.54 μg mg⁻¹ DW) and astaxanthin esters (590.31 μg mg⁻¹ DW). Finally, the control from T6 had canthaxanthin (614.53 μg mg⁻¹ DW) and astaxanthin esters (590.31 μg mg⁻¹ DW). The Kruskal-Wallis test determined that there are significant differences in the concentration of pigments in the two treatments and the control, (Table 3).

4. Discussions

4.1. Cell growth and stress conditions for astaxanthin production

*H. pluvialis* cultures achieve high concentrations of vegetative cells under optimal growth conditions [58]; in this sense, our strain reached cell concentrations of 3 × 10⁴ cells ml⁻¹ (Fig. 1) by the fourth day. The relative number of mobile vegetative cells during growth was high (90%), and were deep green with translucent cell walls (Fig. 2A). *H. pluvialis* under stress went through palmella (Fig. 2B) and cyst (aplanospore) stages (Fig. 2C and D). Optimal growing conditions were switched to stress conditions on the fifth day of culture, when cells were in their palmella stage, following Cifuentes et al. [22] and Wang et al. [59]. Stress conditions for *H. pluvialis* CCAP 34/7 were nitrogen deficiency stress (BBM-N) and high photon flux (1200 μmol m⁻² s⁻¹), which proved to be effective in stimulating astaxanthin production within days of being subjected to stress, building on previous studies [4, 40, 53, 60–62].

4.2. Analysis of astaxanthin deesterification by enzymolysis and saponification

Fig. 3 shows data for samples stressed at T0, T4, and T6, saponified with NaOH 0.05 M at 5 °C, for which concentrations of free astaxanthin were highest at T6 (356.39 μg mg⁻¹ DW). For astaxanthin hydrolyzed with cholesterol esterase, astaxanthin content was three times greater than saponification at T6 (975.65 μg mg⁻¹ DW). This result is in accordance with Su et al. [45], who point out that free astaxanthin obtained by enzymolysis was higher than by saponification in samples of *H. pluvialis*. The
Table 2

<table>
<thead>
<tr>
<th>Pigments</th>
<th>Control</th>
<th>NaOH Saponification</th>
<th>Cholesterol esterase Enzymolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cantaxanthine</td>
<td>548.09 ± 12.93</td>
<td>46.06 ± 1.84</td>
<td>180.08 ± 3.71</td>
</tr>
<tr>
<td>Astaxanthin esters</td>
<td>423.21 ± 0.28</td>
<td>106.05 ± 0.34</td>
<td>305.24 ± 0.30</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>638.20 ± 33.30</td>
<td>829.46 ± 11.89</td>
<td>601.61 ± 13.15</td>
</tr>
<tr>
<td>β-carotene</td>
<td>222.81 ± 3.01</td>
<td>266.84 ± 1.14</td>
<td>244.88 ± 2.08</td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>568.42 ± 12.43</td>
<td>218.52 ± 5.82</td>
<td>376.64 ± 4.65</td>
</tr>
</tbody>
</table>

Significant differences by Kruskal-Wallis test.

Table 3

<table>
<thead>
<tr>
<th>Pigments</th>
<th>Control</th>
<th>NaOH Saponification</th>
<th>Cholesterol esterase Enzymolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cantaxanthine</td>
<td>614.54 ± 1.51</td>
<td>50.61 ± 1.09</td>
<td>108.62 ± 0.40</td>
</tr>
<tr>
<td>Astaxanthin esters</td>
<td>590.31 ± 0.72</td>
<td>56.40 ± 0.52</td>
<td>126.61 ± 1.17</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>108.50 ± 9.10</td>
<td>1038.70 ± 1.91</td>
<td>97.72 ± 0.40</td>
</tr>
<tr>
<td>β-carotene</td>
<td>59.99 ± 1.28</td>
<td>36.54 ± 0.77</td>
<td>46.53 ± 0.93</td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>60.04 ± 4.87</td>
<td>8.71 ± 0.57</td>
<td>40.73 ± 0.98</td>
</tr>
</tbody>
</table>

Significant differences by Kruskal-Wallis test.

results of the present study confirm this, with a significant difference between deesterification treatments (p ≤ 0.05).

Generally, saponification with NaOH favors ester hydrolysis at higher temperatures, but the longer process times can result in a significant degradation of astaxanthin [45, 46, 51], losses of astaxanthin isomers, and more degradation products, such as semi-astacene and astacene [45]. That said, experiments by [46, 51] obtained complete astaxanthin ester hydrolysis without perceived degradation. Those investigations suggest that NaOH saponification should be performed at low temperatures to minimize astaxanthin degradation during hydrolysis of astaxanthin esters. This study attempted to replicate this, applying lower temperatures; however, rather than astaxanthin, results yielded higher concentrations of zeaxanthin. Regardless, the presence of astaxanthin esters in samples suggests more studies are needed regarding NaOH concentrations and reaction times [51, 63].

4.3. Analysis of enzymolysis carotenoid deesterification

In order to demonstrate enzymatic deesterification treatments of chlorophyll and carotenoid precursors to astaxanthin, pigments extracted from stress induced *H. pluvialis* cultures at different times (T0, T4, and T6) were quantified by HPLC.

Enzymolysis treatment with cholesterol esterase enzymes at times T0 and T4 resulted in high concentrations of zeaxanthin (791.18 μg mg⁻¹ and 601.61 μg mg⁻¹ DW, respectively); at time T6, zeaxanthin was low (Tables 1 and 2). Xanthophyll carotenoids from this treatment measured β-carotene at T0 and T4 (466.16 μg mg⁻¹ and 244.88 μg mg⁻¹ DW, respectively), and at time T6, canthaxanthin (108.62 μg mg⁻¹ DW). At time T6, zeaxanthin and β-carotene were relatively low.

β-carotene content in deesterified extracts decreased as *H. pluvialis* underwent stress, supporting the assumption that astaxanthin is synthesized from β-carotene through zeaxanthin and canthaxanthin [32, 33, 38, 64, 65]. At T6, tests showed high concentrations of carotenoids and low chlorophyll, providing evidence for stress-induced microalgae carotenoid production (Tables 1–3).

Interestingly, chlorophyll derived from T0 samples presented high concentrations of carotenoids (902.82 μg mg⁻¹ DW). Samples taken from vegetative cells were during fully developed photosynthetic activity [14, 66]. As stress time progressed, chlorophyll was reduced to aplanospores, and carotenoid concentrations rose [61, 67, 68] (see Tables 1–3). Chlorophyll is still detectable in totally stressed cells, since red aplanospores are photosynthetically active, if not with moderately decreased photosynthetic rates [10, 69–71].

4.4. Analysis of carotenoid deesterification via saponification

As compared to the non-deesterified sample, this work found that carotenoids deesterified by saponification with 0.05 M NaOH for 12 h at 5 °C gave increased zeaxanthin for T6 samples (1038.68 μg mg⁻¹ DW), as well as low concentrations of astaxanthin, canthaxanthin, and β-carotene at all stress times (Table 3).

The present work confirms the presence of astaxanthin precursors like zeaxanthin, canthaxanthin, and β-carotene at different times (T0, T4, and T6) of having been subjected to stress. The presence of other xanthophyll carotenoids in NaOH deesterified astaxanthin has been previously demonstrated. Doria et al. [72] found xanthophylls lutein, canthaxanthin, neoxanthin, and violaxanthin in *H. pluvialis* cells subjected to lighting stress; [46, 51] found lutein, canthaxanthin, β-carotene, and echinone; and, in contrast, Su et al. [45] only recorded astacene and semi-astacene as degradation products of astaxanthin.

Here, carotenoid losses during saponification depend on deesterification conditions like temperature, hydrolysis time, and the use of potassium hydroxide (KOH) or sodium hydroxide (NaOH) at different concentrations [73, 74]. Prolonged saponification times can also cause carotenoids to degrade [73]. Although Yuan and Chen [51] suggested that a higher astaxanthin content could be obtained via cold saponification for 12 h, and other studies have suggested that the best deesterification results are achieved over longer periods of time [17, 55], the present study found only trace detectable zeaxanthin and considerably low carotenoid content in samples.

In addition to treatment time, NaOH concentration can affect carotenoid saponification: several researchers have analyzed concentrations of less than 0.1 M NaOH in astaxanthin and other carotenoid saponification from *H. pluvialis* [45, 46, 51, 72]. The present study found that the addition of 0.05 M NaOH positively affected zeaxanthin concentrations at all stress times (T0, T4, and T6). In contrast, control samples only had measurable zeaxanthin at time T4, and at lower concentrations; β-carotene and canthaxanthin were the main carotenoid contents at T0 and T6, respectively (Tables 1–3).

The saponification process used in this study, though less effective in deesterifying astaxanthin, yielded positive results for zeaxanthin concentrations, although zeaxanthin did not show significant differences (0.051 > 0.05), with no treatment. This technique could feasibly be used to obtain this carotenoid of great biotechnological importance [75–78].

In conclusion, among both carotenoid deesterification processes applied in this study, cholesterol esterase was shown to facilitate astaxanthin deesterification (975.65 μg mg⁻¹ DW) while saponification positively affected zeaxanthin (1038.68 μg mg⁻¹ DW). Deesterified astaxanthin was shown to be always accompanied by precursors zeaxanthin, canthaxanthin, and β-carotene. Progressively decreasing β-carotene concentrations from T0 to T6 samples sustains even more that this pigment is indeed the main precursor of the astaxanthin metabolic pathway.

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