Evaluation of different cell disruption processes on encysted cells of *Haematococcus pluvialis:* effects on astaxanthin recovery and implications for bio-availability

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Abstract

Although *Haematococcus pluvialis* is one of the most important natural sources of the carotenoid astaxanthin as a pigmentor for the aquaculture industry, the thick sporopollenin cell wall in the cysts hinders astaxanthin extraction and its subsequent bio-availability to fish. A range of physical and chemical processes were tested to promote the disruption of the encysted cells. The efficacy of these processes was evaluated in terms of astaxanthin recovery, which was assessed by determining the extent of leaching of astaxanthin into an organic solvent. The processes tested were: autoclave 30 min, 121 °C, 1 atm; HCl 0.1 M, 15 min and 30 min; NaOH 0.1 M, 15 min and 30 min; enzymatic treatment with a mixture of 0.1% protease K and 0.5% driselase in a phosphate buffer, pH 5.8, 30 °C, for one hour; spray drying, inlet 180 °C, outlet 115 °C; and mechanical disruption, with a cell homogeniser developed for this purpose. The mechanical (homogenisation) and autoclave treatments were the most effective in terms of extraction and availability.

Introduction

Haematococcus pluvialis Flotow (Chlorophyceae) has a complex life-cycle involving several stages from motile flagellated zooids through to palmella and encysted stages (Elliot, 1934). This alga is one of the most important natural sources of the carotenoid pigment astaxanthin (3,3′-dihydroxy-β,β'-carotene-4,4′-dione) and is cultivated commercially by several companies. Under optimum conditions, astaxanthin is completely absent from the cells. Upon exposure of the cells to growth-limiting conditions (typically as a result of nutrient – especially nitrogen – limitation or exposure to very high irradiance or high temperature: Droop, 1954; Fan et al., 1994; Harker et al., 1996a), astaxanthin is synthesised and accumulated within oil droplets.

Haematococcus can accumulate up to 8% dry weight of astaxanthin, in the form of 3*S*,3′*S* enantiometer, as a mixture of mono and di-esters (Harker et al., 1996b). The composition of the aplanospores is somewhat dependent upon the age of the culture so that the ratio of mono-esters: di-esters decreases with time (Harker et al., 1996b).

Astaxanthin biosynthesis in this alga is generally, but not exclusively (A. Hartley & A.J. Young, unpublished data), linked to the generation of aplanospores and the associated formation of a thick sporopollenin wall around the cell. The subsequent bio-availability of the astaxanthin is limited due to this wall in the astaxanthin-rich cysts (Good & Chapman, 1979). Intact astaxanthin-rich cysts of *Heamatococcus* do not cause pigmentation in salmonids (Sommer

et al., 1991). Thus there is a need to extract or rupture these cysts prior to use in order to achieve a desired level of pigmentation in the target organism. Whilst it is possible to obtain a 'cell-free' extract of carotenoids, this is not practical on a large scale. Nor is it feasible to extract and purify 'free' astaxanthin from *Haematococcus*, in part due to the sensitivity of astaxanthin to oxidation. A range of alternative processes have been suggested for processing algal cells. For example, Bubrick (1991) described a process incorporating cryogenic (–170 °C) grinding of dried *Haematococcus* biomass in the presence of butylated hydroxytoluene.

The main aim of this study was to evaluate a range of different physical and chemical treatments on the disruption of encysted cells of *H. pluvialis*, in terms of astaxanthin recovery and the effect on subsequent 'potential bioavailability' of pigment from the processed biomass.

Materials and methods

Haematococcus pluvialis (34/7 strain of CCAP – Culture Collection of Algae and Protozoa, Windermere,UK) was cultivated in modified Bold's Basal Medium (Nichols & Bold, 1964). The culture was grown at 21 ± 1 °C for 14 days in plastic sleeves of 10-L volume into which ambient air was used to agitate the culture. Illumination was provided by cool white fluorescent lamps (L36 W/21 Hellweiss Luminux Coolwhite, Osram, Lisbon, Portugal) at an irradiance of 46 μmol photon m^{-2} s^{-1}. After 14 days, the irradiance was increased to 80 μmol photon m^{-2} s^{-1} in order to stimulate astaxanthin biosynthesis in the alga.

Processing of algal biomass

After sedimentation and centrifugation (10 min, 1000 × g) of the encysted cells, the de-watered astaxanthin-rich biomass was divided into aliquots and submitted to the following processes: (i) autoclaved 30 min, 121 °C, 1 atm; (ii) HCl 0.1 M, 15 min and, (iii) 30 min; (iv) NaOH 0.1 M, 15 min and, (v) 30 min; (vi) enzymatic treatment for 1 hour with a mixture of 0.1% protease K and 0.5% driselase in a phosphate buffer, pH 5.8, 30 °C; (vii) spray drying, inlet 180 °C, outlet 115 °C; and, (viii) mechanical disruption using a cell homogeniser developed for this purpose (patent pending). A steady stream of nitrogen was applied during the biomass mechanical processing in order to prevent or minimise oxidation reactions that might occur. All the treatments were carried out in triplicate. Following treatment, the astaxanthin content and composition of the processed biomass was determined by HPLC (see below).

Astaxanthin determination

After the addition of 2 mL ethanol and glass beads to the test tubes, triplicates (30 mg) were submitted to a combination of vortex mixing (1 min) and ultrasound treatment (15 min). Samples were centrifuged (5 min at 1000 × g), the supernatant removed and the pellet re-suspended in 2 ml diethyl ether / ethanol (1:1, v/v). After repeating all the steps the pellet was extracted with 2 ml diethyl ether and finally with 3 ml *n*-hexane. All supernatants were pooled and the sample was evaporated to dryness under a steady stream of oxygen-free nitrogen.

Extraction into acetone

Pigment availability in processed algal biomass was assessed by extraction of astaxanthin into acetone (2 mL), from 30 mg cells (centrifuged as above). The material was gently mixed using a magnetic stirrer and left for 16 hours at room temperature. The sample was filtered prior to analysis to remove any cell debris. These conditions were previously found to permit maximum extraction of cellular astaxanthin from astaxanthin-rich materials, without any degradation of carotenoid. This assay was used to compare the effects of different processing methods on subsequent astaxanthin availability from cells of *Haematococcus*.

Spectrophotometry

Astaxanthin content was determined using a double beam UV-VIS spectrophotometer (Shimadzu 1601) within a wavelength range 400–700 nm. For astaxanthin quantification, the extracts originated from the solvent mixture extraction were re-suspended in *n*-hexane; those obtained from the contact with acetone were analysed directly in acetone. The extinction coefficient of astaxanthin in both solvents is 2100 and the λ_{max} is 470 nm in *n*-hexane and 476 nm in acetone (Britton, 1995).

Thin-Layer Chromatography (TLC) – one dimensional separation of carotenoids

Pre-coated silica gel H60 thin layer chromatography plates 20 × 20 cm (Merck, Darmstadt, FRG) were used for the separation of carotenoids. Acetone, *n*-hexane, methanol (all from Merck, Darmstadt, FRG) and citric acid (Sigma) were laboratory grade. The carotenoid standards, β-carotene and astaxanthin (purchased from Sigma) were dissolved in a small volume of *n*-hexane at 1mg/ml; 2.5 μg was applied to the TLC plate. Samples of 30 mg from all treatments were evaporated to dryness and reconstituted individually in 500μl *n*-hexane, from which 1.2 mg was applied to the TLC plate. All samples and standards were kept dried, under oxygen-free nitrogen, in the dark at –4 °C until used.

All separations were carried out in developing chambers presaturated with the appropriate solvent system, at room temperature. Carotenoids were separated using a solvent system of acetone: *n*-hexane, 3:7 (v/v) (Kobayashi et al., 1991). Before immersing the plate into the solvent system, the plate was prerun in a 2.5% (w/v) solution of citric acid in methanol. This prevented the tailing of carotenoids that posses a 3-hydroxy, 4-keto end-group such as astaxanthin and astacene. The TLC plates were then dried at 40 °C for 10 min.

Analysis of chromatograms was made using an imaging densitometer (Model GS-700, Bio-Rad) operating with a reflectance resolution of 56 μm (450 dpi), with a blue filter at a pixel depth of 8.

High Performance Liquid Chromatography (HPLC)

The HPLC analyses were carried out using a modified method based on that described by Vecchi et al. (1987). Samples were analysed by normal phase HPLC using a diode array detector (HP1100, Hewlett Packard). A Lichrosorb column (12.5 × 4.6 cm, 5μm particles) was pre-coated with 1% H_3 PO_4 in methanol (v/v) before used, to prevent tailing of astaxanthin. The mobile phase was *n*-hexane: acetone, 86:14 (v/v; Merck, Darmstadt FRG) operating at a flow rate of 1.2 ml/min. In order to improve separation efficiency, the polarity of the mobile phase was changed during the run (run time 25 min); the gradient elution was 1:1 (v/v) *n*-hexane: acetone. The sample volume applied was 20 μl following solubilisation of the carotenoid extract in 100 μl *n*-hexane.

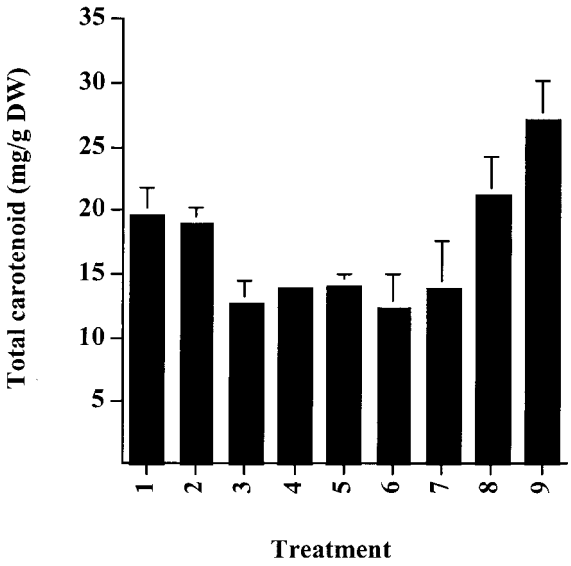

Figure 1. Total carotenoid content of *Haematococcus* cysts following processing: 1. Control (intact); 2. Autoclave; 3. HCl 15 min; 4. HCl 30 min; 5. NaOH 15 min; 6. NaOH 30 min; 7. Enzyme; 8. Mechanical disruption; 9. Spray drying. See Materials and Methods for details.

Scanning Electron Microscopy (SEM)

The *Haematococcus* processed biomass was examined by scanning electron microscopy using a JEOL JSM-840 microscope at an accelerating voltage of 1–15 KV. The samples were lyophilised (CHRIS ALPHA 1–4,B-Braun Biotech International) at –52 °C, and coated with a thin gold-palladium film (10–15nm) using a Polaron E5000 sputter unit.

Results

The carotenoid composition of the *Haematococcus* biomass used in this study was 70.5% astaxanthin mono-esters, 24.7% astaxanthin di-esters and 4.8% lutein. Traces of β-carotene were observed but were not quantified. The astaxanthin content of the algal biomass was approximately 2% (w/dw) which is comparable to the commercial source of this material currently available (NatuRose, Cyanotech Corp.). This pigment composition is typical of encysted cells of this alga, with high levels of astaxanthin mono-esters predominating (Grung et al., 1992; Harker et al., 1996b).

The effects of processing of the algal biomass on the carotenoid content of the cells is shown in

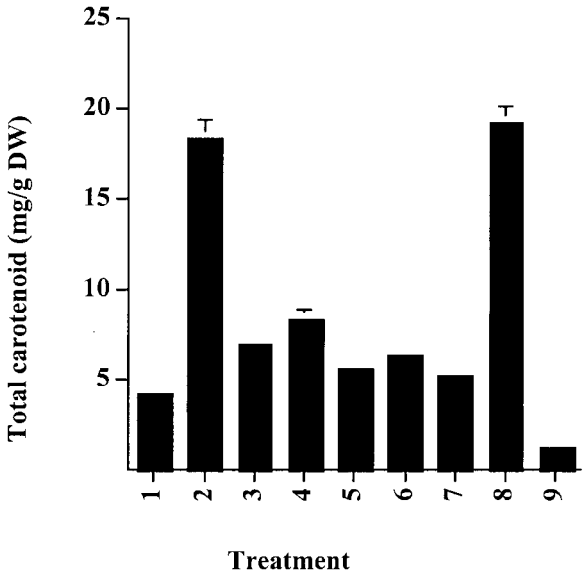

Figure 2. Extraction of total carotenoids into acetone from processed *Haematococcus* biomass. 1. Control (intact); 2. Autoclave; 3. HCl 15 min; 4. HCl 30 min; 5. NaOH 15 min; 6. NaOH 30 min; 7. Enzyme; 8. Mechanical disruption; 9. Spray drying. See Materials and Methods for details.

Figure 1. This shows that, compared to the control, the highest recovery of astaxanthin was obtained in the autoclaved, spray-dried and mechanical disrupted cells. None of these processes has a detrimental effect on the carotenoid content and composition of the algal biomass. However, enzymatic treatment of *Haematococcus* cells or exposure to alkali or acid, resulted in a significant loss of carotenoid (20–35% of total carotenoid) as a direct result of processing. Carotenoids such as astaxanthin which possess the 3-hydroxy, 4-keto end-group are unstable, especially in the presence of alkali. However, the presence of oxidative products of astaxanthin, namely astacene and semi-astacene, could not be confirmed by HPLC analysis. Chromatographic analysis of these processed materials revealed that selective degradation of carotenoids did not occur and that losses were seen for all carotenoids. The high yield of carotenoid from the biomass obtained by spray-drying may be explained by the reduced water content facilitating better extraction by the organic solvents.

The use of algal astaxanthin as a pigmentor in the aquaculture industry is dependent upon the bio-availability of the carotenoid from the algal biomass. The physical barrier of the sporopollenin cell wall in the cysts of *Haematococcus* is thought to be a key factor in limiting carotenoid bio-availability in the gastro-intestinal tract of salmonids. The leaching of carotenoids, under gentle conditions, from biological materials into an organic solvent provides a simple, rapid estimation of the extent to which the cell wall is disrupted. Intact cysts of *Haematococcus* only release ~20% of astaxanthin into acetone over a 16 hour period (Figure 2). Similar low yields were observed for cells that were subject to enzymatic treatment, spray-drying or exposure to acid or alkali. High recoveries of carotenoid (>85%) were only observed for cells that had been autoclaved or mechanically processed. This suggests that these two methods of processing algal biomass may be most effective in terms of pigmentation as the cell contents are readily accessible.

In general, the leaching of carotenoids into acetone did not result in a high recovery of lutein and β-carotene (data not shown). This may be due to their binding to the various pigment-protein complexes within the chloroplast, whilst the astaxanthin esters are accumulated in extra-chloroplastic globules.

In addition to using solvent extractability as a means of assessing the effectiveness of processing on the algal biomass the direct effect that processing had on the cell wall of the alga can be observed by scanning electron microscopy (Figure 3 a-d). The unprocessed cysts of *Haematococcus* (Figure 3a) are fully intact with no signs of pitting or damage to the cell wall. In contrast, the cells subjected to mechanical processing (Figure 3b) and autoclaving (Figure 3d) are clearly disrupted. A noticeable effect of spray-drying the algal biomass (Figure 3c) was the clumping of cells, although the cells are not damaged by this process. Exposure to NaOH did result in some distortion and collapse of some cells, especially those exposed to alkali for a longer period. Some pitting of the cell wall was observed with treatment by HCl, whilst enzymatic treatment had no visible effect on cell morphology (data not shown).

Discussion

The bio-absorption or bio-availability of carotenoids in animals and humans is a critical aspect concerning the use of these natural pigments / anti-oxidants in the food and feedstuff industries (Castenmiller & West, 1997). In aquaculture, the relatively poor retention (typically only a few percent; Torrissen et al., 1989) of astaxanthin and canthaxanthin in salmonids has significant implications in terms of cost. Natural sources of astaxanthin include krill waste, the

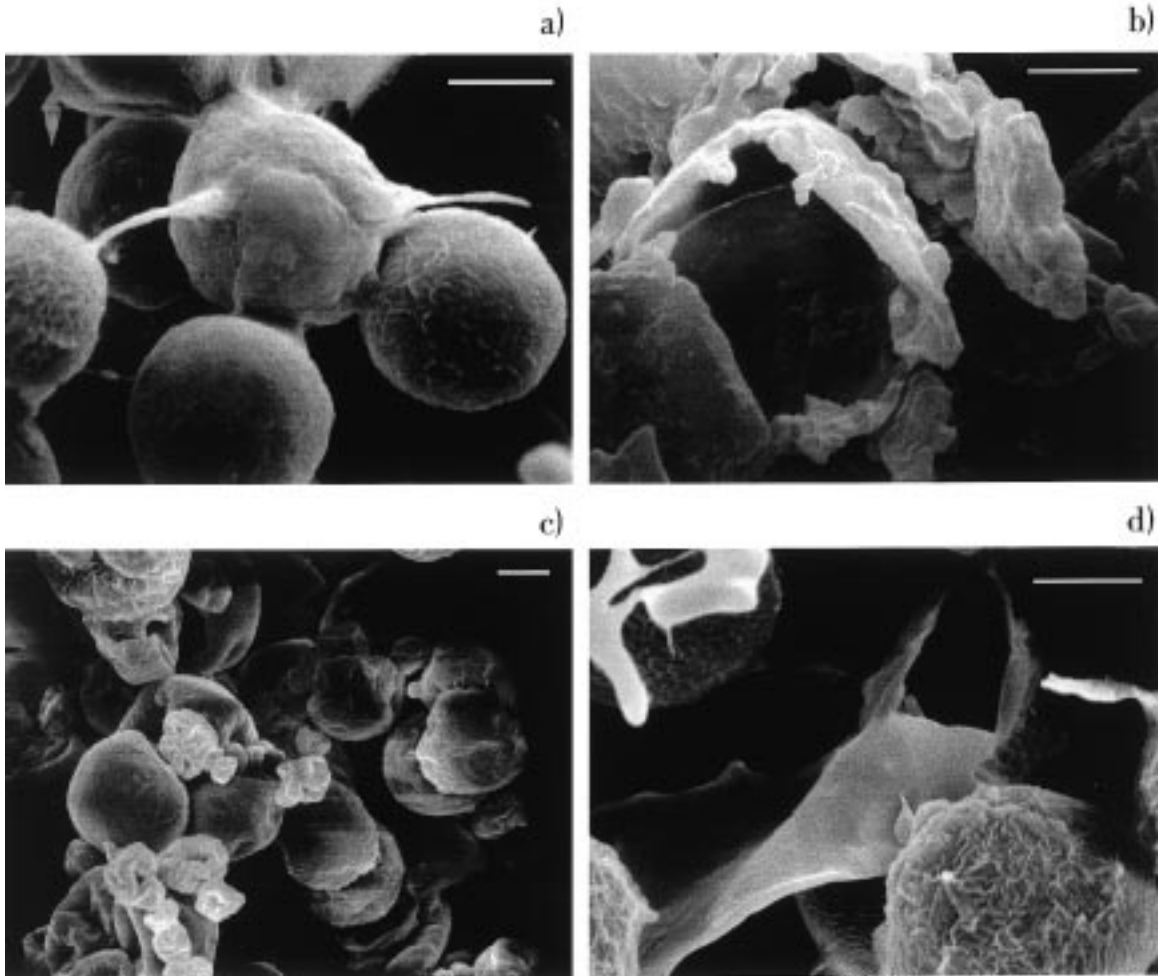

Figure 3. Scanning electron micrographs of *Haematococcus pluvialis* ($\times$ 2000), bar size 10 μm: (a) control (intact) cells, (b) mechanical disrupted cells, (c) spray-dried cells, and (d) cells autoclaved.

yeast *Phaffia rhodozyma*, flowers of *Adonis annua* and a number of microalgae, notably *Haematococcus pluvialis* (Bernhard, 1990).

Salmonids appear to lack the digestive enzymes necessary to break down the sporopollenin wall found in cysts of *Haematococcus*. A number of factors may be responsible for limiting the bio-availability of astaxanthin from *Haematococcus* cells. The use of astaxanthin-rich cells of this alga in pigmentation trials on rainbow trout has shown that intact cysts do not permit pigmentation and that cellular astaxanthin only becomes bio-available once the cyst is broken or disrupted (Johnson & An, 1991; Sommer et al., 1991; J. Bowen, S. Davies, S. Lagocki, A.J. Young, unpublished). In addition, astaxanthin accumulated in algae such as *Haematococcus* is esterified, principally as mono-esters, whilst the synthetic products are free carotenoid. A study by Foss et al. (1987) demonstrated that racemic astaxanthin di-palmitate did not pigment salmonids as effectively as free astaxanthin. A more recent study in rainbow trout has however demonstrated that both the natural mono- and di-esters of astaxanthin from *Haematococcus*, when administered to fish in an isolated form, pigment the muscle tissues as effectively as synthetic astaxanthin (J. Bowen J, S. Davies, R. Serwata, & A.J. Young, unpublished data). Another factor that distinguishes algal astaxanthin from the synthetic form is the configuration of the carotenoid. In algae, astaxanthin occurs as the (3*S*, 3′*S*) form (Renstrøm et al., 1981) whilst the synthetic product is a racemic mixture of the (3*R*, 3′*R*), (3*S*, 3′*S*) and the meso form (3*R*, 3′*S*) (Bernhard, 1990). Foss

et al., (1987) demonstrated that all three epimers of astaxanthin are equally utilised by salmonids, which suggests that the algal form of the carotenoid would not be discriminated against by the fish.

The cracking or disruption of the algal cell therefore appears to be the single most important factor in utilisation of algal astaxanthin. It is, however, important to note that astaxanthin accumulation in *Haematococcus* is not restricted to the formation of cysts and that this carotenoid can be biosynthesised at a number of stages of the alga's complex life cycle (see Introduction). Similarly the formation of the sporopollenin cell wall, and hence the apparent 'toughness' of the cell, alters with the age of the cyst (Burczyk, 1987).

Several methods have been proposed to disrupt algal cells (Farrow & Tabenkin, 1966; Ruane, 1977; Nonomura, 1987) although most methods are not very efficient for disrupting the sporopollenin wall of *Haematococcus* cysts. These cysts are similar to those found in many microalgae and in pollen from higher plants (VanWinkle, Swift & Rickoll, 1997). They are particularly resistant to chemical attack, including KOH and acetolysis but can be attacked by chromic acid. Indeed, resistance to acetolysis is used in part to characterise such cell walls. According to the results obtained from this study it can be observed that both mechanical disruption and autoclaving of the astaxanthin-rich algal biomass are effective treatments. Both processes result in a very high yield of astaxanthin (Figure 1; i.e. no detrimental effects were observed due to processing *per se*). In addition, the extractability of the astaxanthin (Figure 2) from these processed cells was very efficient suggesting effective cracking of the cyst wall. This was confirmed by SEM for both products (Figure 3).

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