Production and analysis of secondary carotenoids in green algae

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Journal of Applied Phycology, October 2000, Volume 12, Issue 3, pp 553–556

Received 1 November 1999; in revised form 17 May 2000; accepted 18 May 2000

Cite this article as:

Orosa, M., Torres, E., Fidalgo, P. et al. Journal of Applied Phycology (2000) 12: 553. doi:10.1023/A:1008173807143

The final publication is available at Springer via http://dx.doi.org/10.1023/a:1008173807143

Abstract

The microalgae *Neochloris wimmeri*, *Scenedesmus vacuolatus*, *Scotiellopsis oocystiformis*, *Chlorella zofingiensis* and *Protosiphon botryoides* were grown under secondary carotenoid inductive conditions. The results indicate that nitrogen deficiency and high light intensity are potential inducers of astaxanthin formation in the five microalgae studied. All these microalgae accumulate significant quantities of secondary carotenoids, mainly as astaxanthin esters and canthaxanthin. They also showed high resistance to environmental conditions. All these qualities make these microalgae good candidates for successful culture in open ponds.

Key words: Astaxanthin; Carotenoids; Green microalgae

Chlorophycean algae generally contain the same set of major carotenoids as higher plants, namely ß-carotene, α-carotene, lutein, violaxanthin and neoxanthin (Goodwin & Britton, 1988). Under unfavorable culture conditions, it has been reported that some microalgae have the ability to synthesize very high amounts of a complex mixture of secondary carotenoids, especially astaxanthin, canthaxanthin and echinenone (Droop, 1954; Brown et al., 1967; Rise et al., 1994). These ketocarotenoids are commonly used as a feed supplement in aquaculture for the production of salmon, trout and shrimp.

In recent years the green microalga *Haematococcus pluvialis* has been considered as a possible natural source for the production of astaxanthin and it has been widely studied. However, one of the main problems, according to Bubrick (1991), in the production of astaxanthin from Haematococcus is contamination with fast-growing unicellular green and/or blue-green algae due to the relative slow growth of *Haematococcus*.

A search for other fast-growing astaxanthinproducing microalgae with the capability of overcoming the *Haematococcus* slow growth problem is necessary. Secondary carotenoid inductive conditions of *Haematococcus* in other green algae should be studied to determine if these conditions cause the same effect in these algae, and if the carotenogenic process and the profile of accumulated pigments are similar. The aim of the study reported here was to assess the capability of some Chlorophycean microalgae to accumulate secondary carotenoids under stress conditions, together with the pigment profile of the cultures.

The microalga *Neochloris wimmeri* CCAP-213/4 was obtained from the Culture Collection of Algae and Protozoa of the Windermere Laboratory, Cumbria, UK. *Scenedesmus vacuolatus* SAG-211/15, *Scotiellopsis oocystiformis* SAG-277/1, *Chlorella zofingiensis* SAG-211/14 and *Protosiphon botryoides* SAG- 731/1a were obtained from the Culture Collection of the University of Göttingen, Germany.

The strains were all cultured in modified Bristol's medium (Brown et al., 1967) supplemented with ALGAL trace elements solution (Fábregas et al., 1984). Cultures were carried out in aerated minireactors containing 400 mL medium and maintained at 18 ºC and 68 µmol photon m^{-2} s^{-1}, with a dark: light cycle of 12:12 h. The comparison of the biomass production of the microalgae was performed under these conditions. The

microalgae were grown under conditions that induce the accumulation of secondary carotenoids in culture medium without $NaNO_3$ and 350 μmol photon m^{-2} s^{-1} to induce the accumulation of carotenoids.

Dry weight was determined according to Vonshak (1986). For pigment analyses, 10-mL samples were centrifuged at 6000 × g for 10 min, and the pellet extracted with 5 mL acetone. The extracts were centrifuged again and chlorophyll a, chlorophyll b and total carotenoids were determined spectrophotometrically, recording the absorption at 661.6, 644.8 and 470 nm and using the equations of Lichtenthaler (1987).

At the end of the culture (day 24), the pigment extracts in acetone were separated on a Hewlett Packard HPLC equipped with a photodiode array detector. A reversed-phase 250 × 4 mm Hypersil C18 (5 μm) column (Hewlett Packard) was used. The elution gradient was run with water, methanol and acetone. The flow rate was 1 mL min^{-1}. The detection wavelengths for integration were 445 and 476 nm. β-carotene (Sigma) and astaxanthin, canthaxanthin and echinenone (F. Hoffman La Roche Ltd) were used as standards.

The culture were grown under conditions which promote production and accumulation of large amounts of secondary carotenoids: high photon flux density and nitrogen starvation. During this time no effect on carotenoid accumulation was observed under inductive conditions in *Scenedesmus vacuolatus* and *Protosiphon botryoides* and their carotenoid levels remained constant thoughout the experiment; however, their pigment pattern showed a significant change. They synthesized a variety of ketocarotenoids, mainly canthaxanthin, astaxanthin and its esters (Figure 1). These inductive conditions also resulted in primary carotenoids and chlorophylls content decline.

This secondary carotenoid accumulation inducing treatment had a similar effect on *Neochloris wimmeri, Chlorella zofingiensis* and *Scotiellopsis oocystiformis* (Figure 1), although with some qualitative and quantitative differences. The main secondary carotenoid peaks were identified as astaxanthin esters, but their proportions in relation to the rest of pigments were very variable, between 50% for total pigments in *S. oocystiformis* and *Chlorella zofingiensis* (Figure 1), and more than 80% in *Neochloris wimmeri* and *Protosiphon botryoides* (Figure 1).

Scenedesmus vacuolatus also accumulated high amounts of ketocarotenoids. However, in this case the main secondary carotenoid accumulated was canthaxanthin; astaxanthin was also detected in its free and esterified form, although in a lower concentration than in the other microalgae studied. The highest total carotenoid levels occurred in *Chlorella zofingiensis*, where the total carotenoid concentration ranged during the process of secondary carotenoid accumulation from 0.4 mg L^{-1} at the beginning of the experiment to approximately 3 mg L^{-1} on the final day of culture (Figure 1). *Neochloris wimmeri* and *Scotiellopsis oocystiformis* also accumulated high amounts of secondary carotenoids until day 15, after which the carotenoid levels remained almost constant. *Scenedesmus vacuolatus* showed a faster growth rate than the other species, but its carotenoid production was very low (Table 1).

Table 1. Maximum values of biomass and carotenoid content (mg $L^{-}1$) in red and green cultures and maximum carotenoid production (mg $L^{-}1$ d^{-1}) in different microalgae

	Biomass mg L-1	Carotenoids in red algae mg L-1	Carotenoids in green algae mg L-1	Carotenoids production mg L-1 d-1
N. wimmeri	280 ± 01	2,22 ± 0,13	0,76 ± 0,05	0,220 ± 0,007
P. botryoides	900 ± 20	0,50 ± 0,01	0,46 ± 0,04	0,080 ± 0,003
S. oocystiformis	720 ± 40	0,83 ± 0,02	0,26 ± 0,03	0,068 ± 0,005
S. vacuolatus	1680 ± 40	0,25 ± 0,01	0,19 ± 0,03	0,041 ± 0,002
C. zofingiensis	720 ± 40	2,81 ± 0,02	0,34 ± 0,04	0,213 ± 0,005

One of the aims of this study was to find out the effect on other algae of the conditions used for secondary carotenoid induction in *Haematococcus*. The results clearly indicate that nitrogen deficiency and high light intensity, as in the case of *H. pluvialis*, are potential inducers of astaxanthin formation in the five microalgae studied. All these microalgae accumulate significant quantities of secondary carotenoids; however, the intracellular changes during the secondary carotenoid accumulation appear to be different (Hanagata & Dubinsky, 1999).

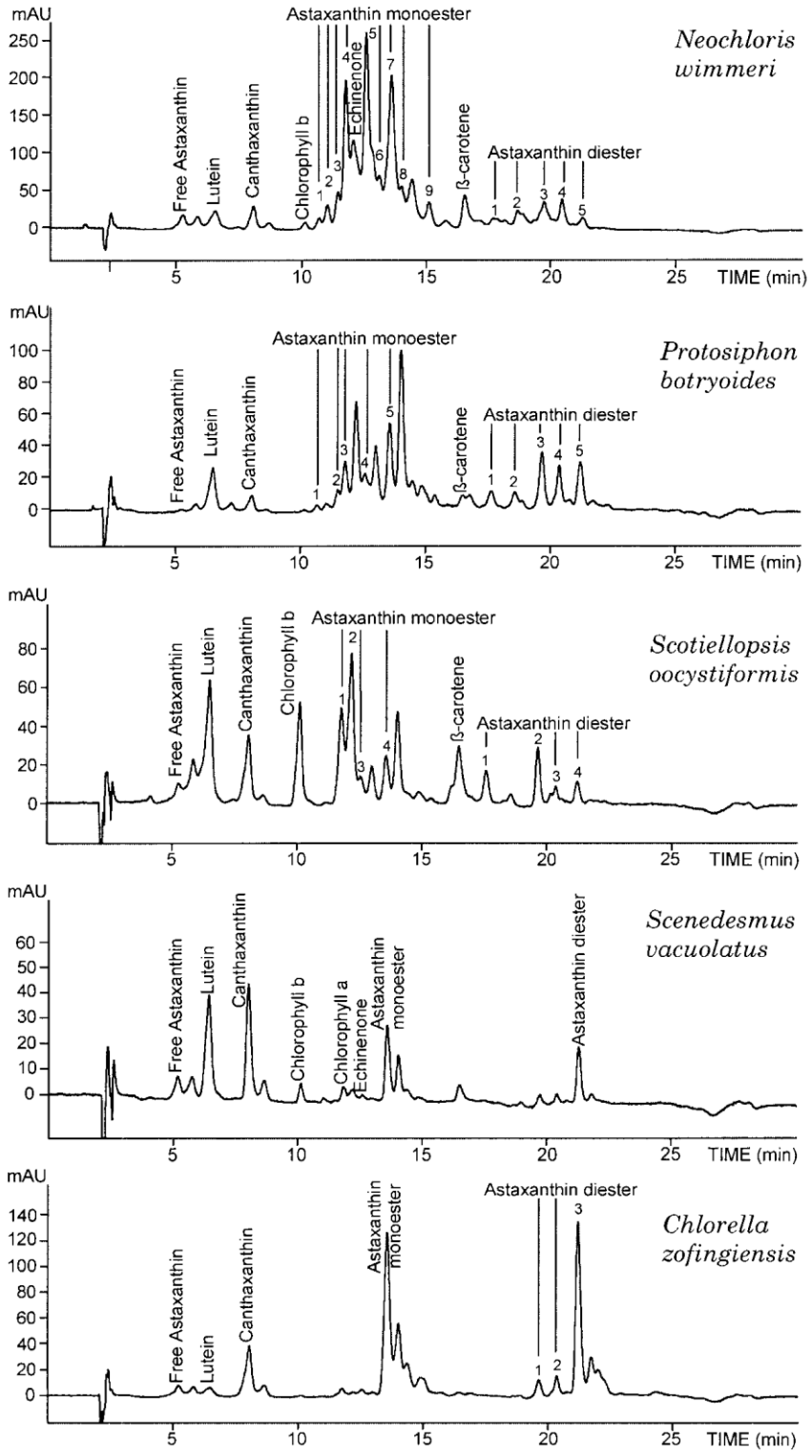

Figure 1. Pigment analysis by reversed-phase HPLC of *Neochloris wimmeri*, *Protosiphon botryoides*, *Scotiellopsis oocystiformis*, *Scenedesmus vacuolatus* and *Chlorella zofingiensis*.

Under these inductive carotenoid conditions astaxanthin and canthaxanthin were the main secondary carotenoids for all of them, and astaxanthin appeared in an esterified

form in all the microalgae studied. Free astaxanthin was also found, but in a lower concentration. The esterified form of astaxanthin seems to be the optimal and the most commonly found form for accumulation of this compound, as observed in astaxanthin-producing microalgae (Renstrom et al., 1981; Rise et al., 1994; Gouveia et al., 1996; Zhang et al., 1997). These results show that the secondary carotenogenesis process is similar in green microalgae. However, the relative concentration of each pigment and each ester, as well as, the number of different mono- and di-esters vary in each microalgae studied.

One of the basic parameters for monitoring the perfomance of algal production systems is the estimation of algal biomass in culture. Apart from *Neochloris wimmeri*, these algae all showed very fast growth and a high capability for biomass accumulation in a short time. These characteristics could make these organisms suitable candidates for overcoming the slowgrowth problem of *Haematococcus* and that would permit successful culture in open ponds. The best growths were obtained with *Scenedesmus vacuolatus* and *Protosiphon botryoides*, but these species did not show higher astaxanthin accumulation. Although *Chlorella zofingiensis* and *Scotiellopsis oocystiformis* accumulated less biomass, they did show a higher secondary carotenoid content. These species have the additional advantage of resistance to high irradiance (as solar light) and fluctuating temperature and salinity conditions. Their relative salt tolerance could also be used to control the growth of unwanted contaminants (Richmond, 1986). All these qualities make these microalgae good candidates for successful culture in open ponds. However, more work is needed on their possible uses as natural coloring source in animal feed, and also on enhancement of ketocarotenoid synthesis in response to changes in culture conditions or selection for mutants showing higher astaxanthin production.

Acknowledgements

This work was supported by a grant from the Xunta de Galicia (XUGA 10301B96). M. Orosa holds a fellowship award from Xunta de Galicia.

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