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Conditions promoting astaxanthin production in *Haematococcus*, *Haematococcaceae*, *Chlorophyceae,* with special reference to *H. pluvialis*

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Abstract

In this third part of the review on *Haematococcus* special emphasis is given to the understanding of the production of the red pigment astaxanthin, the stage in developmental cycle in which pigment synthesis occurs and the influence of physical and biological stress conditions on its accumulation. Cellular biosynthesis of astaxanthin, its location in the cell and biological role in photoprotection of chlorophyll have also been briefly discussed. Finally applications of *H. pluvialis* as feed in aquaculture as well as certain nutraceutical and therapeutic applications have been discussed.

Key words: *Haematococcus pluvialis*, astaxanthin production, accumulation, culture conditions, high light, high temperature, stress factors, biological role, applications.

Introduction

The first part of this review (Jeeji Bai et al., 2016a) dealt with nomenclature, taxonomy, life cycle and ecology. The second part (Jeeji Bai et al., 2016b) reviewed the growth dynamics of the green motile cells in laboratory and open-air cultures and the physicochemical conditions influencing it for maximum biomass production. In this third and last part of the series, special emphasis is given to the understanding of the production of the economically important red pigment astaxanthin from the point of view of influence of physical and biological stress conditions such as nutrient depletion, high light intensity, high temperature and salinity stress. The red astaxanthin pigment, a ketocarotenoid, which was earlier called euglenorhodone or haematochrome was later identified as the ketonic carotenoid, astaxanthin (Lang, 1968) with manifold applications in aquaculture, food and nutraceutical industries. This predominantly red-coloured pigment was first recognized in marine crustaceans and fish as the pigment responsible for the pink colour of their flesh, which increases their table value. The early reports of the production of this pigment by microorganisms were those from certain yeasts and marine bacteria. Among the various algae, which are capable of synthesizing this pigment, the unicellular green alga, *Haematococcus* has shown great promise as a preferred source because of its high pigment content, ease of isolation and culture with manipulation of culture conditions (Pringsheim, 1966).

Earlier studies on this alga mainly concern cell morphology, location of pigment deposition and some studies on vegetative propagation (Borowitzka et al. 1991). Later studies, however, concern specifically the growth conditions which stimulate pigment production and accumulation with special reference to certain stress conditions (Kobayashi et al., 1991; Fan et al., 1994), the biosynthesis and cellular location of the pigment (Lee and Zhang, 1999; Margalith, 1999; Zlotnik et al., 1993), induction of pigment synthesis (Boussiba, 2000; Fábregas et al., 2000; Gong and Chen, 1997) and the role of astaxanthin in the cell (Hagen et al., 1994; Kobayashi and Sakamoto, 1999; Yong and Lee, 1991).

Factors influencing astaxanthin production

Haematococcus has drawn the attention of scientists from the time of its discovery by its striking red colour due to the massive accumulation of astaxanthin. It was first recognized that the synthesis of this pigment occurred in aged cells when they entered the "resting" non-motile aplanospore stage (cysts). Most of the studies pertaining to the conditions promoting the biosynthesis of this pigment were carried out almost exclusively on strains of *H. pluvialis*, also named *H. lacustris* by some investigators (see Jeeji Bai et al., 2016a, for nomenclature). Pringsheim (1914) and Chodat and Mayer (1927) were the first to point out that the important factors for haematochrome formation were N-deficiency and strong illumination. Lwoff and Lwoff (1930) found that acetic acid promoted pigment formation. Droop (1954) carried out detailed studies on the effect of CO_2, acetate and nitrate in light and darkness as well as certain stress conditions like Mg-deficiency and salt stress on pigment formation. He observed that all these factors promoted pigment synthesis and this effect was more pronounced in light although dark synthesis also occurred with acetate (Table 1). He concluded that the accumulation of haematochrome was favoured by those substances, which inhibited cell division without impairing carbon assimilation and hampered by those which reduced carbon availability.

Many studies, which followed also showed a positive correlation between the arrest of cell division (formation of aplanospores) and astaxanthin accumulation. Boussiba and Vonshak (1991) demonstrated this using a cell division inhibitor vinblastine and NaCl with 1.5 g L^{-1} of $NaNO_3$ at light intensity of 8 µmol photon $m^{-2}s^{-1}$. Astaxanthin production was 10.7 pg $cell^{-1}$ (4 days) and 14.9 pg $cells^{-1}$ (7 days) at 2 µg mL^{-1} of vinblastine and 14.9 pg $cell^{-1}$ (4 days) and 23.5 pg $cell^{-1}$ (7 days) at 5 µg mL^{-1} of vinblastine. With 0.8% NaCl, astaxanthin production was 33.3 and 84.9 µg $cell^{-1}$ after 4 and 7 days respectively.

Zlotnik et al. (1993) observed that the cell division rate decreased from 2.34 per week in growing green cells to almost '0' in aplanospore stage. Hagen et al. (2001) also reported that the accumulation of the pigment accompanied normally, but not exclusively, the formation of resting state in the developmental cycle. More recent studies have shown high light intensity as the main factor although other factors such as acetate supply, N- and P-deficiencies and different types of stress have also been shown to influence pigment production. These are discussed in the relevant sections in the following. Some of them are summarized in table 2.

High light intensity:

Fan et al. (1994) showed that high light intensity of 200 µmol photon m^{-2} s^{-1} increased the ratio of astaxanthin to chlorophyll till a steady state was reached whereas in low light conditions (50–100 µmol photon $m^{-2}s^{-1}$) the ratio was constant with no astaxanthin accumulation. However, Harker et al. (1996) reported cellular astaxanthin accumulation of over 300 pg $cell^{-1}$ after 30 days even in low light intensity (<37 µmol photon $m^{-2}s^{-1}$) and 500 pg $cell^{-1}$ after 20 days at a light intensity of 90 µmol photon $m^{-2}s^{-1}$. According to Lee and Soh (1991) in a continuous system with daily dilution, progressively high light intensities in combination with N- limitation brought about a progressive increase in both chlorophyll and astaxanthin formation when dilution rates were increased from 0.113 d^{-1} to 0.230 d^{-1} and a decrease at a higher dilution rate of 0.356 d^{-1}. They concluded that in growing cultures the rate of pigment accumulation was determined by light intensity but the cellular pigment content was a function of the nitrogen status. The highest cellular astaxanthin content depended on the intrinsic capacity of the cell to form the compound rather than the culture parameters. However, Ding et al. (1994) attributed the higher rates of pigment synthesis at higher dilution rates to greater exposure to incident light. Therefore, the steady state rate of astaxanthin accumulation was a function of irradiance and not the absorbed light energy. Studying the effect of different light dark cycles and light quality, Kobayashi et al. (1992b) reported a higher stimulation of pigment synthesis in 24 hour high light than in light–dark cycles and blue light also caused enhanced pigment biosynthesis (table 2). They concluded that the period of exposure to light was more important than the intensity of light. Nagaraj et al. (2012) reported in *H. pluvialis* a maximum value of 73.57 µg mL^{-1} total carotenoids at 40 µmol photon $m^{-2}s^{-1}$ light intensity.

In a study to optimize the conditions for astaxanthin production in a strain of *H. pluvialis* received from University of Concepción, Chile, Cifuentes et al. (2003) cultured the alga in batch mode under autotrophic and mixotrophic conditions and carotenogenesis was induced by the addition of NaCl (0.2 and 0.8 %), N-deprivation and high light (150 µmol photon $m^{-2}s^{-1}$). They found that light was the best inductive carotenogenic factor and the highest carotenoid production of 4.9 mg L^{-1} and 25 pg $cell^{-1}$ was obtained in cultures pre-grown in nitrate at low light. The highest astaxanthin content of 22 pg $cell^{-1}$ and dry weight content of 10.3 mg g^{-1} (1% w/w) were obtained at 85 µmol photon $m^{-2}s^{-1}$ with 0.8% NaCl. Brinda et al. (2004) reported that high light favours astaxanthin accumulation even in flagellated cells of *H. pluvialis*. Torzillo et al. (2005) found that carotenoid content increased with increasing irradiance and chlorophyll content decreased. A sharp increase in carotenoid- content occurred above 200 µmol photon $m^{-2}s^{-1}$.

High temperature:

The effect of above optimum temperatures (30°C) on astaxanthin formation in *H. pluvialis* was investigated by Tjahjono et al. (1994a) as stress factor. They observed a 3-fold increase in astaxanthin at 30°C as compared to that at 20°C. A further 2-fold increase was brought about by acetate and $FeSO_4$ addition. They observed that at 30°C, when growth was arrested, endogenous production of oxygen from photosynthesis resulted in hyperaccumulation of carotenoids. They suggested the use of a high temperature photobioreactor with low light intensities for cost reduction and higher pigment production. On the other hand, Hong et al. (2015) reported that high temperatures beyond 30°C inhibit astaxanthin accumulation in *H. pluvialis* under photoautotrophic conditions. The inhibition of carotenogenesis is primarily attributed to the excess intracellular less reactive oxygen species (LROS) i.e. superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) levels generated under high temperature conditions. However, they demonstrated an improvement in astaxanthin production with heat stress through acceleration of iron-catalyzed Haber–Weiss reaction which converts LROS to more reactive oxygen species (MROS) i.e. oxygen (O_2) and hydroxyl radical (OH), thus facilitating lipid- peroxidation. During 18 days of photoautotrophic induction astaxanthin

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concentration of cells cultured in high temperatures in the presence of 450 µM iron showed remarkable increase of 75 % at 30°C and 133 % at 36°C when compared to that of cells exposed to heat stress alone. They suggested that the heat stress-mediated Haber–Weiss reaction will be useful for economically producing enhanced photoautotrophic astaxanthin by reducing energy cost, particularly in outdoors utilizing natural solar radiation including heat and light for photo-induction.

Nutrient status:

Chodat (1938) first suggested the importance of the carbon- nitrogen balance for pigment formation. Droop (1954) however found that C: N ratio alone is not sufficient to account for pigment formation as the pigment was formed even with excess nitrogen. He drew certain important conclusions concerning haematochrome formation: 1. light and carbon-dioxide were interdependent in their action; 2. organic carbon acted independently of light; 3. pigment synthesis was independent of nitrogen but not C-supply, and 4. cell multiplication precluded accumulation and involved even loss of haematochrome (Table 1). These are corroborated by many later studies.

Table 1: Effect of nitrate, CO_2 and Acetate on haematochrome formation in light and dark in *H. pluvialis*

Numbers refer to pairs of cultures differing in treatment

		Light				**Dark**				**Totals**
Condition	Acetate	**+**	**+**	**–**	**–**	**+**	**+**	**–**	**–**	
	CO_2	**+**	**–**	**+**	**–**	**+**	**–**	**+**	**–**	
Result	No difference	0	0	0	0	2	2	5	4	13
	Less haematochrome with more nitrate	7	7	7	7	5	5	2	3	43
Condition	Acetate	**+**	**+**	**–**	**–**	**+**	**+**	**–**	**–**	
	Nitrate	**+**	**–**	**+**	**–**	**+**	**–**	**+**	**–**	
Result	More haematochrome with more CO_2	6	7	1	7	0	3	1	1	26
	No difference	1	0	2	0	6	4	6	5	24
Condition	CO_2	**+**	**+**	**–**	**–**	**+**	**+**	**–**	**–**	
	Nitrate	**+**	**–**	**+**	**–**	**+**	**–**	**+**	**–**	
Result	More haematochrome with more acetate	5	6	1	6	5	5	5	5	38
	No difference	2	1	2	1	2	2	2	2	14

Data condensed from Tables I, II & III of Droop, 1954

Czygan and Kessler (1967) characterized the different secondary carotenoids developed under N-deficiency conditions in a variety of green algae including *H. pluvialis*. Boussiba and Vonshak (1991) also found that nitrogen deficiency caused rapid pigment accumulation and quick leveling off whereas continued and excess supply of nitrogen resulted in a slow rate of accumulation but reached higher levels finally. However, Kakizono et al. (1992) obtained cellular astaxanthin accumulations of 22, 18, 19 and 12 pg $cell^{-1}$ respectively with C/N ratios of α, 6.2, 2.8 and 1.1, after 48 hour- exposure of light of 120 $\mu E\ m^{-2}s^{-1}$. These ratios were obtained by adjusting different levels of $NaNO_3$ with a constant level of 47.3mM acetate (Table 2). In all subsequent studies, astaxanthin synthesis was induced by high light and N-deficiency with or without certain additional stress conditions.

The maximum values of total carotenoids reported by Nagaraj et al. (2012) after 21 days were 63.3 $\mu g\ mL^{-1}$ (with 2mM urea), 67.5 $\mu g\ mL^{-1}$ (with 0.2M $NaNO_3$), 66.2 $\mu g\ mL^{-1}$ (with 2mM NH_4Cl), 77.6 $\mu g\ mL^{-1}$ (with 0.06M

K_2HPO_4) and 68.3 μg mL^{-1} (with 0.06M NaCl). They also observed that as nutrients got depleted in older cultures astaxanthin accumulation became enhanced.

According to Borowitzka et al. (1991) palmella formation and pigment synthesis were stimulated in the presence of KNO_3 and this effect was much lower with urea and quite inhibitory with NH_4Cl. Red palmella cells constituted over 90% of the total cell number at the highest concentration of PO_4 (0.2 g L^{-1}). At lower concentrations of 0.05 and 0.1 g L^{-1} they constituted only around 80% in 30 days and at still lower levels of 0.001 and 0.005 g L^{-1}, they hardly formed 20% of the total cell number even after 40 days. Stimulation of astaxanthin synthesis by P-starvation was also observed by Boussiba and Vonshak (1991) and Harker et al. (1996). Acetate induced the formation of red palmella stage earlier at pH 7.5 than at 6.5. Kobayashi et al. (1992a) found mixotrophic condition with acetate in low light to be more effective than dark heterotrophic condition for pigment synthesis (Table 2). Gong and Chen (1998) studied the effect of five variables of medium components on astaxanthin content using a statistical design of experiments. They showed increased response with increasing levels of acetate, decreased response with increasing levels of KNO_3 with a striking lowering effect exerted by acetate at 0.55g L^{-1} of KNO_3. They evolved optimal levels of nutrients for growth and highest pigment production and suggested a 2-step process for green cell growth and red palmella formation. Fábregas et al. (1998) tested the effect of N- and Mg- deficiency singly and in combination on astaxanthin formation in high light intensity and found maximum cellular astaxanthin content of 381 pg $cell^{-1}$ in N-deficient medium, 74.7 pg $cell^{-1}$ in media without Mg and 163.3 pg $cell^{-1}$ without N and Mg. However, according to Boussiba et al. (1999) low amounts of nitrogen appear to be very essential for sustained pigment production. Addition of $FeSO_4$ and NaCl did not show any significant increase in astaxanthin content. Acetate addition stimulated astaxanthin biosynthesis consistently irrespective of other conditions.

In an earlier study on the German strain SAG 34-1b of *H. pluvialis*, it was reported (Jeeji Bai, 2002) that the percentage of astaxanthin in different clone cultures showed differences – with clones 1, 3 and 4 showing more pigment in BBM and BG11 media with acetate and NaCl addition and lower values in SAG medium in laboratory cultures (Fig. 1). Under both laboratory and open air conditions both N- and P- deficiency led to increased astaxanthin accumulation. Cifuentes et al. (2003) reported that though increasing concentrations of NaCl caused an increase in carotenoid content per cell, it resulted in high cell mortality and did not produce any increment in carotenoid content per volume compared to cultures grown at high light intensities.

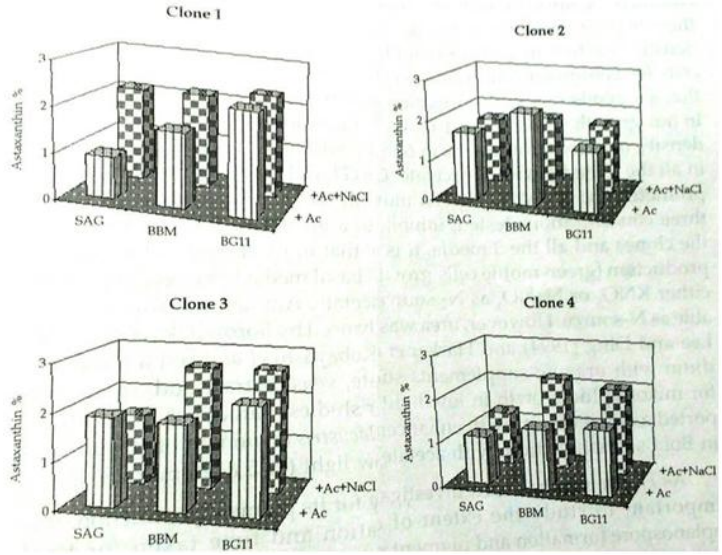

Fig 1: Astaxanthin percentage in the four clones of *Haematococcus pluvialis* in different media with different treatments
(Adopted from Jeeji Bai, 2002)

Ferrous ions, in combination with acetate also promoted astaxanthin synthesis. Kobayashi et al. (1991) studied the effect of increasing levels of $FeSO_4$ with a constant level of acetate on astaxanthin synthesis during palmella stage and found 450 μM level of $FeSO_4$ to exert maximum effect (Table 2). Harker et al. (1996) found that high Fe^{2+} concentration inhibited cell growth and yielded 50 pg $cell^{-1}$ of astaxanthin in 30 days. Besides, manganese and cadmium also increased astaxanthin production. Kobayashi et al. (1993) found that after encystment, inhibitors of carotenoid synthesis did not affect synthesis in the presence of acetate and iron. By using a combination of inhibitors they showed that oxidative stress also caused enhanced carotenoid biosynthesis after encystment. According to

Margalith (1999), enhancement of carotenogenesis by ferrous ions is not blocked by inhibitor in aphlanospores, whereas in vegetative cells, it is inhibited. Tjahjono et al. (1994a) confirmed this by studying the effect of acetate alone and in combination with iron and with iron and H_2O_2 and showed progressively increased synthesis of the pigment. As tiron and potassium iodide, which are oxygen scavengers, severely blocked carotenogenesis, they concluded that the levels of endogenous oxygen were responsible for stimulated carotenogenesis. Lee and Ding (1995) also reported increased astaxanthin accumulation in chemostat cultures at increasing dissolved oxygen partial pressures.

Table 2: Summary of conditions used for maximum astaxanthin accumulation in *H. pluvialis*

Days of treatment	Cell no. x10^4 mL^{-1}	Conditions for cyst formation	Light intensity for 24 hrs (µEm^{-2}s^{-1})	Chlorophyll (*pg cell^{-1})	Astaxanthin (*pg cell^{-1})	Reference
6	70	14.6 mM Acetate	21#	21.4	11.6	Kobayashi et al. 1991
4	50	216 µM $FeSO_4$	63	17.5	26.2	
4	50	45mM Acetate 450µM $FeSO_4$	90	8.0	40.2	
8	75	15mM Acetate	20	22.7	7.0	Kobayashi et al. 1992a
8	45	22.5 mM Acetate	Dark	16.7	6.2	
8	40	45 mM Acetate 450µM $FeSO_4$	68	12.0	28.0	Kobayashi et al. 1992b
8	40	-do-	281	12.0	50.0	
8	40	-do-	Blue Light	12.0	48.0	
8	55	45 mM Acetate	120	7.0	20.0	Kobayashi et al. 1993
8	50	450 µM $FeSO_4$	120	22.0	12.0	
8	50	45 mM Acetate 450µM $FeSO_4$	120	8.0	48.0	Tjahjono et al. 1994a
6	18	-do-	103	75.7	449.3	
30°C temperature stress						
5 wild type	50	45 mM Acetate 450µM $FeSO_4$	120	-	28.0	Tjahjono et al. 1994b
5 mutant	30	-do-	120	-	84.0	
8	30	45 mM Acetate	120	11.0	100.0	Kobayashi et al. 1997a
8	30	-do-	Dark	12.5	30.0	
0.1% NaCl stress						

#12:12 hrs light : dark cycle; *Values directly reproduced or calculated from figures or tables

In a two-step batch cultivation scheme developed by Hagen et al. (2001), the first step involving germination of aplanospores under optimal conditions and the second-step involving exposure of the resulting flagellated cells to a decreased level of 0.4 mM KNO_3 and high light intensity of 150 µmol photon m^{-2}s^{-1} for optimal induction and enhancement of carotenoid biosynthesis. Addition of acetate at a concentration not higher than 10 mM increased carotenoid synthesis only slightly whereas partial or complete phosphate deficiency or salt stress (40 mM NaCl) did

not bring about any increase. By evaluating different nutrient-deficiency conditions to achieve high astaxanthin accumulation in flagellated cells of *H. pluvialis*, Brinda et al. (2004) found that a combination of N- and P-limitation was effective in enhancement of astaxanthin content to 2.0±0.2% (dry weight basis) with production level of 35–38 mg L^{-1}. Different inhibitors added to understand regulation did not yield any result as they caused growth inhibition. Results showed that a combination of nutrient stress and high light favour astaxanthin formation in flagellated cells. Domínguez-Bocanegra et al. (2004) obtained a maximal astaxanthin production of 98 mg g^{-1} biomass in modified BBM medium with continuous illumination at 345 µmol photon $m^{-2}s^{-1}$ with 1 g L^{-1} of sodium acetate without aeration.

Under simulated out-door conditions resembling batch cultures, Garcia-Malea et al. (2005) observed carotenogenesis under nutrient-limiting conditions only with 0.2- or 0.3-fold standard medium strength whereas severe nutrient deprivation (0.1-fold strength) arrested carotenogenesis. Del et al. (2008) found that a specific nitrate input and average irradiance were decisive in determining biomass astaxanthin content. Increasing irradiance resulted in enhancement of astaxanthin accumulation when nitrogen input was limiting, but never under N-sufficiency. González et al. (2009) observed that total carotenoid content differed among the strains of *H. pluvialis* isolated from different microhabitats and also when grown under autotrophic and mixotrophic conditions under two light intensities of 20 and 85 µmol photon $m^{-2}s^{-1}$. Though the mixotrophic condition assayed did not substantially improve the growth rate of any strain, it did improve the total carotenoid yield in one of the strains. Tripathi et al. (1999) reported that addition of trace elements and B vitamins to mixotrophic growth media such as MM1, MM2 and KM2 (see Jeeji Bai et al., 2016b) gave 1.5-, 1.35- and 2.02- fold increases in astaxanthin content respectively over the standard medium. KM2 medium was the best with the yield of 2.2% (w/w).

Stress conditions:

Salt stress with 0.8% NaCl together with PO_4- starvation under high light and low nitrogen conditions induced high astaxanthin synthesis (Boussiba and Vonshak, 1991). In a statistical experimental design called Response Surface Methodology, the optimal conditions for astaxanthin synthesis were determined by Harker et al. (1995) as: close to '0' nitrogen, 1600 to 1700 µmol photons m^{-2} s^{-1} light intensity and 25 to 30 mM NaCl concentration. Cordero et al. (1996) used different concentrations of acetate and NaCl in N-deficient medium at light intensity of 140 µmol photon $m^{-2}s^{-1}$ and obtained 3% (18.6 mg L^{-1}) as the highest content on dry weight basis with 0.2% NaCl. Using NaCl concentrations between 0.05 to 1% Kobayashi et al. (1997a) showed that in cells pre- grown in heterotrophic conditions, a 5-fold enhancement in carotenoid / chlorophyll ratio was observed in cultures grown in light under 0.1% salt stress over that without salt stress. In dark heterotrophic conditions salt-stressed cultures showed 50% higher astaxanthin content over those grown in light with a high C/N ratio. Comparing different salts they observed highest carotenoids / chlorophyll ratio with $MgCl_2$, closely followed by NaCl and low values with $CaCl_2$, KCl and LiCl. In all cases where hyperaccumulation of pigment was observed with temperature of 30^{o}C and excessive salt stress of 0.1%, a significant reduction in cell number was observed so that the overall pigment production was indeed much lower. In a recent study Hagen et al. (2001) induced carotenoid biosynthesis in flagellated cells by increased irradiance (150 µmol photons $m^{-2}s^{-1}$) and low nitrogen level (0.4 mM KNO_3) and obtained 2% carotenoid production on dry mass basis on 4th day. A further increase occurred with acetate at 10mM level whereas partial or complete PO_4- deficiency and NaCl (40 mM) stress did not cause any increase. Ping et al. (2007) found phosphate deficiency to enhance total carotenoids content to 11.0 pg $cell^{-1}$.

Choi et al. (2002) evaluated the factors affecting the astaxanthin production by *H. pluvialis* UTEX 16 with sequential fractional factorial design. To simulate an actual production mode, a two-stage process was adapted for astaxanthin production: the alga was first cultivated under vegetative growth conditions, and then astaxanthin production was induced by applying various induction methods. A high dose of irradiation was most effective for the production of astaxanthin both in weight (mg g^{-1}) and in cellular (pg $cell^{-1}$) contents. A combination of nitrogen deficiency and acetate addition also significantly increased the astaxanthin content of cells on a dry weight basis whereas acetate addition alone increased only the cellular content of astaxanthin. Although the addition of ferrous ion alone had a negative effect on the weight content of astaxanthin, simultaneous addition of ferrous ion and acetate was effective for increasing the cellular content of astaxanthin. Irrespective of type of stress, better results could be obtained if stress conditions were given after aplanospore formation as done by a majority of workers. In a majority of studies, therefore, high light and N-deficiency have been used for astaxanthin production. Carbon requirement in inorganic and organic (acetate) form has also been shown unequivocally in a large number of studies as emphasized by Droop even as early as 1954. In some cases, ferrous form of iron was used to increase pigment synthesis. Additional stress factors, such as Mg-deficiency, NaCl addition, oxidative conditions etc. also increased astaxanthin biosynthesis.

By evaluating different nutrient-deficient conditions to achieve high astaxanthin accumulation in flagellated cells of *H. pluvialis*, Brinda et al. (2004) found that a combination of nitrogen and phosphate limitation was effective in enhancement of astaxanthin content to 2.0 ± 0.2% (on dry weight basis), resulting in overall production of 35–38 mg L^{-1}. They administered different inhibitors to understand their involvement in the regulatory aspects of secondary carotenoid formation, but as expected, all of them inhibited growth when added at the time of inoculation. The results indicated that a combination of nutrient stress and high light will favour astaxanthin formation in flagellated cells.

Orosa et al. (2001) obtained maximum total carotenoid yield in cultures with low acetate concentrations (0.25, 0.5 and 1% w/v). Increased acetate levels caused growth inhibition and decreased carotenoid yield. Similarly, in malonate stressed alga total carotenoid content increased and higher malonate concentration (2% w/v) resulted in 5-fold increase in carotenoid production. In spite of growth inhibition at higher concentrations, acetate and malonate exhibited strong effects on stimulation of astaxanthin synthesis, the accumulation being more than 4 times higher than in cultures without these compounds.

Physiological changes occurring during carotenogenesis

Zlotnik et al. (1993) studied other physiological changes accompanying astaxanthin synthesis during aplanospore formation. They observed a slight decrease in chlorophyll 'a' (from 16.0 to 14.8 pg $cell^{-1}$), a marked increase in chlorophyll 'b' (from 8.3 to 15.7 pg $cell^{-1}$) and a significant increase in total carotenoids (from 5.0 to 94.1 pg $cell^{-1}$) and astaxanthin (from 0 to 76.7 pg $cell^{-1}$) after 4 weeks under N-deficiency. Similarly cellular photosynthetic rate decreased to a third of that of green vegetative cells, dark respiration increased and excretion decreased.

Hagen et al. (1993a) demonstrated by fluorescence spectroscopy a decrease in light absorption by chlorophyll and primary carotenoids in the photosynthetic apparatus of aplanospores with secondary carotenoids. In another study (1993b) they obtained motile cells and aplanospores with different secondary carotenoid levels – 1) by cultivating in low or high light with nutrient limitation and 2) by using inhibitors of pigment accumulation and cell division and found that the former was more suitable for the purpose. Still later Hagen et al. (2000) reported an increase in the photosynthetic productivity per unit chlorophyll in motile cells which had accumulated secondary carotenoids. Shi et al. (1995) found decreased levels of cytochrome 'f' in red cells as compared to green cells and much higher respiration rates and conversely much lower O_2 evolution. According to Torzillo et al (2002), the maximum quantum yield of PSII (F_v/F_m) gradually declined from 0.76 at the lowest irradiance of 50 µmol photon m^{-2} s^{-1} to 0.66 at 600 µmol photon $m^{-2}s^{-1}$.

Boussiba et al. (1999) tested the hypothesis of chlorophyll reduction during aplanospore formation and the question of final yield of astaxanthin in different stress conditions and found that a decrease in chlorophyll occurred with N- deficiency but not with P- deficiency. However, a minimum of 40% chlorophyll was necessary for astaxanthin synthesis. Astaxanthin occurred almost exclusively in the form of esters constituting 99% of the total carotenoids. They concluded that the nature of stress was very important as many stresses led to excessive chlorophyll reduction resulting in decreased astaxanthin production.

Orosa et al. (2001) reported maximum values of total carotenoids under N-deficiency while chlorophylls reached a minimum value. According to them, the source of nitrogen for continued protein synthesis for the massive pigment accumulation could not be from the culture media but from intracellular N-store as RuBisCo as it supports cell survival and even growth for several hours under N-starvation.

Astaxanthin biosynthesis

The first detailed study of the biosynthetic pathway of astaxanthin synthesis was that of Donkin (1976) using ^{14}C- labelled acetate. Acetate was added: 1) throughout the experiment, 2) in the initial growing phase and 3) in the reddening phase after palmella formation. The results showed that: the demand for carbon was greater after encystment; significant labelling was in precursors and greater proportion of ketocarotenoids was from unlabelled acetate and rate of biosynthesis of the entire pool of ketocarotenoids could not be accounted for by the intermediates (primary carotenoids). He concluded that acetate was directly incorporated into ketocarotenoids and *de novo* synthesis from exogenous carbon sources was a major pathway. This paper remained largely unknown and was quoted subsequently only by Santos and Mesquita (1984). Harker and Young (1995) observed the accumulation of intermediates such as xanthophylls, phytoene- a, β-carotene and lycopene by using certain specific herbicides.

At present it is generally accepted that there are two biosynthetic pathways of astaxanthin synthesis: 1) via echinenone, canthaxanthin and adonirubin and 2) via zeaxanthin and adonixanthin. Grünewald et al. (1997) obtained

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motile cells with secondary carotenoids in order to facilitate extraction and characterized the pigment accumulation with and without inhibitors of carotenoid synthesis. They observed that after 2 days when steady state was reached carotenoid accumulation started and reached maximum values during resting state. Unlike other studies, zeaxanthin was an intermediate in the biosynthetic pathway from β- carotene to astaxanthin, which was attributed to strain difference (fig. 2). In a more recent paper Hagen et al. (2001) studied the pigment accumulation in motile cells. They observed that under N- deficiency and enhanced irradiance of 150 μmol photons $m^{-2}s^{-1}$, not only astaxanthin esters but also some amounts of canthaxanthin and echinenone accumulated in the lipid vesicles outside the chloroplast. Following this, Grünewald and Hagen (2001) reported their findings on localization of an early and late enzyme in astaxanthin synthesis. They suggested that the early site of synthesis was the chloroplast and after cyclization, the intermediate carotenoid was transported into the cytoplasmic lipid vesicles via the carotenoid binding proteins.

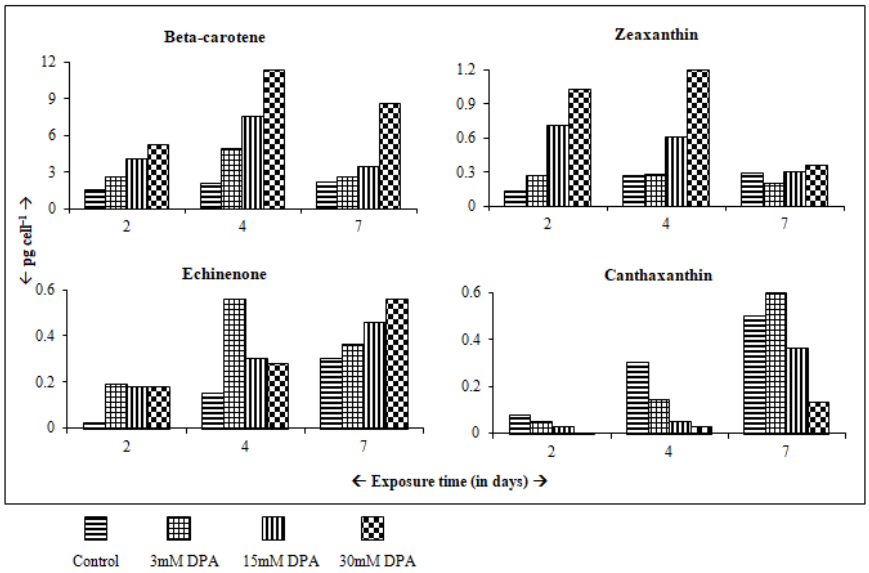

Figure 2: Changes in beta-carotene, zeaxanthin, echinenone and canthaxanthin content of *H. lacustris* at different concentrations of DPA (Diphenylamine)
(From graphs of figures 2, 4 & 7 of Grünewald et al. 1997)

The pathway of astaxanthin biosynthesis in *Haematococcus* is discussed in detail by Lee and Zhang (1999, figure 8.1). They have listed the individual enzymes and genes encoding them for the different steps involved starting from mevalonic acid, which is known to be the starting point of biosynthesis of carotenoids.

Renstrøm et al. (1981) obtained 1% of free astaxanthin out of the total carotenoids from a strain of *H. pluvialis* with 76% mono- and 7% di-ester of astaxanthin. They also characterized the different optical isomers of astaxanthin. Boussiba et al. (1999) obtained 99% of astaxanthin esters out of the total carotenoids constituting 4% of cellular dry weight, the maximum reported thus far from their strain. In an earlier study, Tsavalos et al. (1992) reported a still higher astaxanthin content of 6–8% of biomass in a species of *Haematococcus*. For pigment extraction, Yuan and Chen (1999) studied the reaction kinetics of hydrolysis of astaxanthin esters and showed that reaction rates and degradation were directly proportional to the concentration of sodium hydroxide in the saponified solution and a higher temperature should be avoided to minimize degradation. Free '*trans*'-astaxanthin from a high-yielding strain of *H. pluvialis* containing 3.67% *trans*- and 1.35% *cis*-astaxanthin in dry cells was obtained by Yuan and Chen (2000). According to them saponification at a low temperature of 5°C reduced degradation, giving a final yield of 32.2 mg *trans*-astaxanthin from 1g of dry cells.

Sarada et al. (2006) evaluated the extractability of astaxanthin from cyst cells by treating cells with various solvents and pre-treating the cells with organic and mineral acids at 70°C followed by acetone extraction. Hydrochloric acid treatment facilitated 86–94% extractability of astaxanthin. Treatment time, temperature, and concentration of the acid were found to be critical factors for maximum extractability. It was also found that the treatment did not affect the astaxanthin ester profile and the treated cells could be preserved until further use. According to Lemoine and Schoefs (2010) astaxanthin synthesis corresponds to a multifunctional response to stress. The accumulation of astaxanthin constitutes a convenient way to store energy and carbon, which will be used for further synthesis under less stressful conditions. They reviewed and compared the various biochemical, genetic, and

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molecular data related to the biosynthesis of ketocarotenoids by *Haematococcus pluvialis* and other taxa. They also proposed a tentative regulatory model of the biochemical network driving astaxanthin production.

Role of astaxanthin

The red astaxanthin pigment of *Haematococcus* has been considered to protect the assimilatory pigment chlorophyll from damage due to high light intensities. This pigment develops usually in aged cells, which undergo encystment after completion of vegetative growth. Droop (1955) suggested that it might be a storage product. Yong and Lee (1991) carried out *in situ* observations of cells exposed to different light intensities and showed that the pigment was confined to the central region in low light and dispersed towards the periphery of cell in high light. A quantitative increase in pigment content was observed according to the intensity and duration of light. They also showed that the pigment conferred resistance to photo-inhibition. They failed to record increased pigment synthesis in high light intensities (4000 μmol photons $m^{-2}s^{-1}$). Hagen et al. (1994) used the *in vivo* chlorophyll fluorescence method to demonstrate protection against photo-inhibition. They showed strong quenching of chlorophyll fluorescence corresponding to the degree of secondary carotenoid accumulation. They supported the view of Yong and Lee (1991) that in strong light there is only an intracellular redistribution and no *de novo* synthesis of the pigment. According to Lee and Ding (1992) the recovery process from photo-inhibition involving *de novo* protein synthesis in *H. pluvialis*, had only a low capacity to prevent deterioration of energy conversion efficiency and hence the alga had adopted the protective mechanism of accumulation of secondary carotenoids. Kobayashi et al. (1997b) showed the presence of anti-oxidative enzymes in vegetative cells of *H. pluvialis* and secondary carotenoids having anti-oxidant activity in cysts, so that they functioned as anti-oxidants against excessive oxidative stress. Studying the singlet oxygen quenching activities of β-carotene, free astaxanthin, and its mono- and di- esters *in vitro*, Kobayashi and Sakamoto (1999) showed that the astaxanthin esters functioned as powerful antioxidant agents under both hydrophobic and hydrophilic conditions. However, Fan et al. (1996) questioned the photoprotective role of astaxanthin. They demonstrated that the red cells are more sensitive to damage than green cells when exposed to high light. As singlet oxygen generator triggered astaxanthin accumulation, they proposed that astaxanthin biosynthesis is an enzymatic antioxidant process and an outcome of the protection process.

According to Lemoine and Schoefs (2010) the presence of high amount of astaxanthin enhances the cell resistance to oxidative stress generated by unfavorable environmental conditions including excess light, UV-B irradiation and nutrition stress and therefore confers a higher survival capacity to the cells. This better resistance results from the quenching of oxygen atoms for the synthesis itself as well as from the antioxidant properties of the astaxanthin molecules.

Applications

Astaxanthin is a natural pigment, which is a preferred supplement in artificial feeds for fish and poultry for the attractive colour of eggs and flesh and skin of salmon, rainbow trout etc. Adding the pigment also gives attractive colour to crustaceans such as prawn and shrimp.

In order to make the pigment bioavailable in fish feeds and other applications, the thick sporopollenin wall of aplanospores must be broken. Conventional methods consist of physical grinding, sonication or homogenization. Mendes-Pinto et al. (2001) compared the efficacy of different physical and chemical processes in terms of astaxanthin recovery, which was assessed by the extent of leaching of the pigment into an organic solvent. Out of the many processes tested, they found homogenization and autoclaving (30 min., 121°C, 1 atm.) to be the most effective.

Choubert and Heinrich (1993) compared the effect of supplementation of feed with freeze-dried spores of *Haematococcus* up to 6% level and synthetic pigment on muscle colour of rainbow trout. Carotenoid deposition of 6.2 mg kg^{-1} of fish muscle was observed with natural pigment, which was low as compared to synthetic carotenoid. However, a 50: 50 mixture of synthetic and natural carotenoid had a synergistic effect of increasing the pigment deposition to a much higher level. For salmonids, astaxanthin level of 4 mg Kg^{-1} and above is necessary for satisfactory colour of flesh. Miki (1991) showed the superior antioxidant activity of natural astaxanthin, by virtue of its being an oxygenated ketocarotenoids, when compared to β-carotene and α-tocopherol. Jyonouchi et al. (1995) showed that astaxanthin, which had no vitamin A activity, enhanced the T–antigen specific humoral immune response. In a review on natural sources of astaxanthin, Johnson and An (1991) indicated that the low pigment concentration (2%) and slow growth rate of *Haematococcus* are the deterrent factors in its commercial exploitation.

By employing a newly developed fluorometric assay to measure the antioxidant activities of astaxanthin and related carotenoids, Naguib (2000) showed that astaxanthin had high antioxidant activity toward peroxyl radicals.

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Fassett and Coombes (2012) demonstrated the potential of astaxanthin as an antioxidant and anti-inflammatory therapeutic agent in models of cardiovascular disease. Clinical studies conducted in over 180 humans using astaxanthin as a dietary supplement to assess its safety, bioavailability and clinical aspects relevant to oxidative stress, inflammation or the cardiovascular system reported no adverse outcomes. Studies in a variety of animals using a model of myocardial ischemia and reperfusion have demonstrated protective effects from prior administration of astaxanthin both intravenously and orally.

Palozza et al. (2009) studied the growth-inhibitory effects of the astaxanthin-rich *Haematococcus pluvialis* on HCT-116 colon cancer cells and suggested that *H. pluvialis* may protect from colon cancer. *H. pluvialis* extract (5–25 μg mL^{-1}) inhibited cell growth in a dose- and time-dependent manner, by arresting cell cycle progression and by promoting apoptosis.

According to Rao (2011), *H. pluvialis* extracts exhibited 80% antioxidant activity in clams. Studying the anti-bacterial properties they showed that chloroform extract exhibited highest antibacterial effect followed by ethyl acetate extract. Astaxanthin and its esters also showed significant antioxidant activity and hepatoprotective ability. Astaxanthin from *Haematococcus* was effective in retinol formation and accumulation in serum and liver in *in-vivo* experiments. Pre-feeding of rats with astaxanthin extracts at 250 μg Kg^{-1} body weight prior to treatment with carcinogen showed 3–4 fold reduction in tumor index.

Conclusions

Haematococcus is a preferred source of astaxanthin because of the ease of isolation and culture especially under controlled conditions in the laboratory. However, its life cycle involving palmella and aplanospore formation triggered by deficiency conditions in aged cultures and adverse conditions of light intensity and temperature limits its possibility of sustained growth in open-air conditions. Again, the highest cellular dry weight content of 2% astaxanthin in *H. pluvialis* reported so far in open-air conditions is only 50% of that achieved under laboratory conditions. As the cells in green motile stage are capable of vegetative propagation yielding 16 or even 32 daughter cells it may be possible to achieve a replicable process to aid proliferation of green cells before subjecting them to a process whereby large red aplanospores with high astaxanthin content may be obtained. A few investigators, however, have also attempted to achieve continued and high pigment synthesis even in growing motile stage. There is thus an urgent need not only to increase biomass production of green cells but also to achieve the highest concentration of cellular astaxanthin. Even in 1954, Droop came to the conclusion that whatever factor inhibits cell division leads to increased astaxanthin production. There seems to be a synergistic effect of different factors in astaxanthin synthesis and accumulation and it is essential to study combinations of factors and their time sequence of influence. Although acetate has been reported to promote pigment synthesis in most of the studies, the isolated report of malonate stimulation by Orosa et al. (2001) needs further study. In many of the studies it is not clear whether the astaxanthin content reported refers to the motile red cells or thick walled aplanospores. As the cysts are more resistant to changes in ambient conditions, it may be worthwhile to look into the economic feasibility of taking advantage of the life cycle to achieve quick (albeit low biomass) production of red spores with highest possible astaxanthin content. This may also obviate the necessity of growing the green cells in artificially cooled bioreactors.

Yet another aspect which needs further study is the comparative estimate of pigment content in motile and aplanospore stages under appropriate stress conditions in order to select the most suitable stage and conditions for maximum pigment production under the shortest time interval.

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