Astaxanthin Production in Cysts and Vegetative Cells of the Microalga *Haematococcus Pluvialis* Flotow

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Citation:

Herrero, C., Orosa, M., Abalde, J., Rioboo, C. and Cid, A. 2012. Astaxanthin Production in Cysts and Vegetative Cells of the Microalga *Haematococcus Pluvialis* Flotow. Pages 359-371 *in* M. N. Johnsen, ed. *Microalgae: Biotechnology, Microbiology and Energy*. Nova Science Publishers, Hauppauge, NY.

INTRODUCTION

Carotenoids are isoprenoid polyene pigments widely distributed in nature. They are the main source of the red, orange or yellow colour of many edible fruits (lemons, peaches, apricots, oranges, strawberries, cherries, and others), vegetables (carrots and tomatoes), mushrooms (milk-caps), and flowers. They are also found in animal products: eggs, crustaceans (lobsters, crabs and shrimps) and fish (salmonids) (De Saint Blanquat, 1988).

Carotenoids are synthesized *de novo* by all photosynthetic organisms, including microalgae. Some fungi and non-photosynthetic bacteria can also produce certain carotenoids. Animals lack the ability to synthesize carotenoids *de novo*, but some vertebrates and invertebrates are capable of metabolizing some carotenoids ingested in their diets producing chemical modifications of their structure.

Carotenoids are also found in different organs of higher plant, such as leaves (mainly 13-carotene, lutein, violaxanthin, neoxanthin), fruits (liycopene in the tomato), rhizomes and roots (β-carotene in carrots), seeds (β-carotene in wheat), etc. (Goodwin & Britton, 1988).

All algae contain carotenoids; each algal species usually has between five and ten main compounds. Algal carotenoids exhibit high structural diversity, more than those found in higher plants. Although some carotenoids are widely distributed in algae (β -carotene, violaxanthin, neoxanthin), others are restricted to a few species.

Carotenoids concentration in microalgae ranges from 0.1 % to 2% of dry weight; however, some species accumulate much higher quantities under certain conditions. An example of this is *Dunaliella salina,* which accumulates as much as 14% of β-carotene under conditions of nutritional stress, high salinity, and high luminosity (Borowitzka *et al.*, 1984). Another example is the accumulation of astaxanthin by the green freshwater microalga *Haematococcus pluvialis.* The reported astaxanthin conten of this microalgae varied from 1 % up to 5% of the dry weight (Zhang *et al., 2009).*

Primary carotenoids are associated with chlorophylls in the thylakoid; they act as light harvesting molecules with subsequent energy transfer to chlorophylls in photosystems. The light harvesting complex (LHC) consists of carotenoid-chlorophyll-protein complexes.

Carotenoids also playa photoprotective role, stabilizing the molecules of chlorophyll against oxygen radicals and extreme radiation levels. Due to this function, as lipid anti-oxidants, carotenoids have been proposed as anti-cancer agents (Peto *et al.,* 1981). Secondary carotenoids may occur in the stigma of algae, where they act as photoreceptors for phototaxis. In chloroplast and thylakoid membranes some carotenoids undergo epoxidation and deepoxidation induced by light

Other secondary carotenoids are located in the cytoplasm, as is the case of the freshwater microalga *Haematococcus pluvialis,* in which astaxanthin is accumulated in the perinuclear region of the cytoplasm of the aplanospores (Santos & Mesquita, 1984). Astaxanthin accumulataion is carried out under stress conditions, such as nutrient limitation, like chloroplastidic β-carotene of *Dunaliella.* However, carotenoids synthesis pathways in *Haematococcus* and *Dunaliella* should be quite different, since an excess of β-carotene in *Dunaliella salina* and *D. parva* leads to the formation of vesicles in the chloroplast (BenAmotz *et al.,* 1982 ; Borowitzka *et al.,* 1984), while the astaxanthin of *Haematococcus* forms vesicles surrounding the nucleus (Lang, 1968). It is possible that Golgi bodies are involved in the formation of astaxanthin (Borowitzka & Borowitzka, 1988).

It is remarkable the production of astaxanthin in the yeast *Phaffia rhodozyma,* which this carotenoid appears to protect against oxidative stress; its synthesis is stimulated by oxygen radicals. In *Phaffia,* carotenoids appear to be associated with lipid globules and concentrated near the nuclear envelope (Johnson, 1992).

In photosynthetic organisms carotenoids have two well-defined functions, one in the photosynthesis itself, and another in the protection of the photosynthetic structures from photo-oxidation. In non-photosynthetic tissues of higher plants, in fungi, and in nonphotosynthetic bacteria, carotenoids also carry out a photo protective function, but the mechanism appears to be different from that in photosynthetic tissues (Goodwin, 1980).

In animals, in addition to their anti-oxidant function, carotenoids also affect growth and reproduction (Nagasawa *et al., 1989*). Thus, juvenile salmonids accumulate pigment in their muscle, and once sexual maturity is reached the pigment is mobilized to the reproductive organs, in which pigment appears to play a role in stimulating fertility and reproduction (Schiedt *et al.,* 1985). On the other hand, β-carotene is the precursor of retinal, the chromophore of all known visual pigments.

INDUSTRIAL APPLICATIONS OF CAROTENOIDS

Carotenoids have a wide range of industrial applications. They are mainly used as coloring agents on an industrial scale, being used in the feed industry, aquaculture and poultry. Beyond their role in pigmentation, carotenoids benefit in various aspects of animal and human health. They basically protect the cells of the body acting as quencher of reactive oxygen species (Cardozo *et al., 2007*). Therefore, carotenoids are used in the nutraceutical market as potent antioxidants.

As food coloring agents, the most used are β -carotene and lycopene, but β,ε-carotene (α carotene), γ-carotene (β, ψ-carotene), β-apo 8' carotenal, β-apo-8' -carotenic ethylic acid ester are also used (De Saint Blanquat, 1988).

Since animals cannot synthesize carotenoids *de novo,* intensive aquaculture requires a diet including astaxanthin to produce coloration similar to wild fish for market acceptance (Choubert & Heinrich, 1993). The ketocarotenoids astaxanthin (3,3' -dihydroxy- β - β -carotene-4,4' -dione) and canthaxanthin (B-B-carotene-4,4' -dione) are widely used to obtain the desired flesh coloration of wild salmonids. Although the pigment canthaxanthin can also be beneficial, astaxanthin is preferable because it produces identical pigmentation to that of the flesh of wild salmonids; in addition, astaxanthin is more efficiently deposited (Torrissen, 1986) and remains more stable during the process of pigmentation in the fish (Skrede & Store bakken, 1987).

Therefore, astaxanthin is the most important and expensive colorant in the feed industry for the production of salmonids (salmon, rainbow trout), crustaceans (shrimp, lobster) and poultry (Cardozo *et al., 2007*). Astaxanthin is one of the most expensive components of salmon farming, accounting for about 15% of total production costs. (Cardozo *et al., 2007).* In nature the main source of this pigment are crustaceans, but in intensive aquaculture the carotenoids must be added in the diets (Choubert & Heinrich; 1993). Furthermore, precursors of astaxanthin and astaxanthin itself contribute to the characteristic flavor of salmon (An *et al.,* 1989). It has also been demonstrated that these substances increase the survival time of eggs and the percentage of fertilized eggs, by protecting them against extreme conditions (Craik, 1985) and stimulating their growth (Torrissen, 1984).

In addition to its role in the coloration of aquatic animals, astaxanthin possesses several important bioactivities, including antioxidation, enhacement of immune response and anticancer activities (Zhang *et al., 2009*). The ketocarotenoid astaxanthin is believed to play a key role in the amelioration/prevention of several human pathological processes, such as skin UV - mediated photooxidation, inflammation, prostate and mammary carcinogenesis, ulcers due to *Helicobacter pilori* infection and age-related diseases (Cardozo *et al., 2007).*

Numerous studies have shown that astaxanthin has health-promoting effects in the prevention and treatment of various diseases, such as cancers, chronic inflammatory diseases, metabolic syndrome, diabetes, diabetic nephropathy, cardiovascular diseases, gastrointestinal diseases, liver diseases, neurodegenerative diseases, eye diseases, skin diseases, exercise induced fatigue, male infertility, and HgCl2-induced acute renal failure (Y uan *et al.,* 20 11).

HAEMATOCOCCUS AS A SOURCE OF ASTAXANTHIN

While astaxanthin is ubiquitous in nature, it exists in low abundance. Natural sources of astaxanthin were mainly dependent on extraction from by-products of crustacean and certain yeast species such as *Phaaffia rodozyma.* These sources were often limited by the availability of natural resources and the low astaxanthin content (0 .005%-0.4%) in *Phaffia* yeast. The microalga *Haematococcus pluvialis* (Flotow) has been reported to be the richest source of natural astaxanthin. The organism has since drawn great attention from many researchers, technology development to maximize the astaxanthin content in this alga has also become an attractive research area (Zhang *et al., 2009).*

H pluvialis is an ubiquitous single cell biflagellate microalga, often found in puddles, eaves and birth bathes. It frequently shows an eye-catching red to deep purple color due a massive accumulation of the secondary carotenoid astaxanthin as well as its fatty acid monoor diesteres. Cells are in akinete forms within this resting stage and are able to survive unfavorable environmental conditions such as high light, nutrient depletion and eve complete desiccation (Grewe & Griehl, 2008).

H pluvialis has two types of cell morphology depending on its environmental conditions; green motile and non-motile forms. Under optimal growth conditions the cells are green vegetative cells capable of actively swimming with two flagella and of increasing in number. The pigmentation profile of *Haematococcus* in its green vegetative stage is essentially the same as that of higher plants: chlorophylls *a* and *b,* and the carotenoids β-carotene, lutein, violaxanthin, neoxanthin and zeaxanthin (Ricketts, 1970).

Under unfavorable conditions, or under various forms of environmental stress, the green vegetative cells cease to be motile, increase their volume drastically, lose their flagella, form a hard, thick cyst-like wall, which may contain sporopollenin (Burczyk, 1987) and enter a resting stage. In this stage astaxanthin is synthesized in the cyst cells, which are then marked by a red color due to astaxanthin accumulation (Kang et al., 2006). Astaxanthin is first deposited around the nucleus and then radially extended until all the protoplast acquires a red coloring. The two processes, encystment and astaxanthin accumulation, are generally coupled, but they are in fact distinct processes and can be experimentally separated in time (Bubrick, 1991).

Resistant forns of this microalga can accumulate as much as 1 %-2% astaxanthin in the total dry biomass of microalgae (Borowitzka, 1992), mainly in the form of the monoester fatty acids $C_{16:0}$, $C_{18:0}$, $C_{20:0}$ and $C_{18:1}$, in the early stationary phase, when cells lack a thick cell wall (Renstrom *et al.,* 1981). In later stages, when cells present a thick cell wall, di-esters are predominant, indicating a esterification in the final stages of development (Grung *et al.,*1992). Mature cysts may contain as much as 3% dry weight in esterified astaxanthin, and values as high as 5% have been reported (Czygan, 1968; Renstrom *et al.,* 1981). Astaxanthin esterification with fatty acids would be a mechanism to concentrate this chromophore in cytoplasmic globules in order to maximize its photo protective efficiency (Renstrom *et al.,* 1981). These resistant red cells are photosynthetically competent, but their photosynthetic activity is very reduced. This reduction in the photosynthetic activity is mainly due to the lack of cytochrome *f,* and, consequently, the absence of an electron flow from photosystem II (PS II) to photosystem I CPS I), and, to a lesser extent, a decrease in some components of PS II and PS I. The loss and decrease of photosynthetically essential proteins in the aplanaspores may be due to a decrease in the processes of new synthesis and repair, because of the stress imposed by unfavorable environmental conditions (Tan *et al., 1995).*

Some species of the genus *Haematococcus* can accumulate up to 6-8% (w/w) astaxanthin (Tsavalos *et al.,* 1992); however, there are important problems for the large-scale production of astaxanthin from these microalgae due to their complex life cycles, production process design, and scale-up. Relatively low growth rates and intolerance to high temperatures and high light have limited the use of these microalgae to obtain astaxanthin in open-systems (Lee & Zhang, 1999).

Haematococcus is not the only microalga able to accumulate significant amounts of astaxanthin and other related ketocarotenoids. The capability to synthesize secondary carotenoids under

environmental stresses, such as nutrient deficiency (nitrogen, phosphate, etc.), intense light, high temperature, acidic pH, etc, is widely spread over the green microalgae, probably as a defense mechanism against environmental injuries (Borowitzka; Goodwin; Orosa *et al.* , 2001 a; Orosa *et al.,* 2000). Astaxanthin accumulated in these microalgae is mainly as isomers 3S and 3'S (Renstrom *et al.,* 1981), with differences in the composition and the quantity of ketocarotenoids among the different species (Tsavalos *et al.,*1992).

PATHWAY FOR THE SYNTHESIS OF THE CAROTENOID ASTAXANTHIN IN THE GENUS *HAEMATOCOCCUS*

Higher plants and green algae share the same carotenoid biosynthetic pathway to β - carotene. β-carotene ketolase and β-carotene hydroxylase catalyze further steps leading to astaxanthin in *H. piuvialis.* β -carotene ketolase is the only enzyme that exclusively participates in the secondary carotenoid pathway leading to astaxanthin (Lu *et al.,* 2010).

Astaxanthin is synthesized from β- carotene by two different ways (Chumpolkulwong *et al.;* Fan *et al.,* 1995; Fraser *et al.;* Grewe & Griehl, 2008). With the use of diphenylamine, an astaxanthin synthesis inhibitor, a synthesis pathway for *Haematococcus* has been proposed (Fan *et al.,* 1995), via echinenone, cantaxanthin and adonirubin:

β-carotene → echinenone → cantaxanthin →adonirubin → astaxanthin

Astaxanthin biosyntesis also takes place via another route: from β-carotene via β -cryptoxanthin, zeaxanthin and adonixanthin:

β -carotene → cryptoxanthin → zeaxanthin → adonixanthin → astaxanthin

In the case of the yeast *Phaffia rhodozyma* the pathway proposed by Andrewes *et at.* (Andrewes *et al.)* for the synthesis of astaxanthin differs significantly:

β-carotene → echinenone → hydroxy-echinenone → adonirubin → astaxanthin

accumulating in this case the isomer *(3R,3 'R)* (Andrewes *et al.,* 1976), whereas in the case of *Haematococcus* is accumulated the stereoisomer *(3S,3 'S)* (Andrewes *et al., 1974).*

FACTORS AFFECTING GROWTH AND ACCUMULATION OF ASTAXANTHIN IN THE GENUS *HAEMATOCOCCUS*

There are two proposed strategies for commercial production. One separates in time the production of biomass (optimal growth, green stage) and pigment (permanent stress, red stage), while the other uses an approach based on continuous culture under limiting stress at steady state.

In general astaxanthin production from *H pluvialis* is achieved though a two-stage culture: vegetative (green) and aplanospore (red) stages. In the vegetative stage, the slow growth rate, low cell concentration, and susceptibility to contamination are the major problems. In this respect, various studies were performed to improve the growth mainly on the optimization of the culture medium, light intensity and organic carbon nutrition. Although the use of sodium acetate as an organic carbon source under mixotrophic condition seems to be a favourable way of boosting cell concentration and growth rate in *H pluvialis,* it also increases the contamination

risk, particularly with bacteria and *Chiarella* sp., a green microalga able to grow very fast on organic substances. In traditional mixotrophic algal cultures an organic carbon source is present in the medium together with the other inorganic nutrients before inoculation. However, it has been developed a new approach where the cultures were initially grown phototrophically by inorganic nutrients, and sodium acetate addition was done subsequently at the end of the log phase under different light intensities. This alternative mixotrophy has several advantages against traditional mixotrophy such as a much higher cell density in a batch culture period and minimized risks of contamination owing to the shorter exposure of cells to organic carbon sources (Goksan *et al.).*

A two-stage growth process could solve the contradiction between vegetative cell growth and astaxanthin accumulation and culturing *H pluvialis* in a closed system could reduce the risk of contamination. Different closed bioreactors were used to culture *H pluvialis* through a two-stage growth process. Efforts were made to simplify the two-stage production process and to develop efficient one-step production process in continuous cultures. But in terms on relative efficiency the two-stage systems performs better than the one-stage systems. The productivities, efficiencies and yields for the pigment accumulation in each case have been compared and analyzed in terms of the algal basic physiology. The two-stage system performs better (by a factor of 2.5-5) than the one-stage system, and the former is best fit in an efficient mass production setup (Aflalo *et al.,* 2007). One-stage production uses an approach based on continuous culture under limiting stress at steady state, the biomass production and astaxanhin production occur simultaneously. Two-stage-production separates in time the production of biomass (green stage) and pigments (red stage). So far, both one-stage and two-stage production of astaxanthin requires closed bioreactors (at least in green stage). Culture in photobioreactors are expensive compared with open culture systems. However Zhang et al. (2009) developed a two-stage growth one-step process for cultivation of *H pluvialis* in open pond, obtaining an average astaxanthin conten in cyst of 2.10 g per 100g-1 wt.

Many induction methods have been developed for the transformation of green cells to red cysts containing high astaxanthin contents. Induction methods can be divided into two classes according to the role of astaxanthin as an antioxidative storage molecule or as a protoprotective substance. The first class uses various environmental stresses, except for strongh light, to cause retardation of cell multiplication; for example, nitrogen starvation, excess acetate addition, salt stress, or addition of specific inhibitors of cell division (Borowitzka *et al.,* 1991; Cordero *et al.,* 1996; Harker *et al.,* 1996a; Kakizono *et al., 1992;* Tjahjono *et al.,* 1994a). A nitrogen deficiency would appear to be the most important factor in the triggering of cellular encystmen. If only carbon sources are available upon cessation of cell division, the cells accumulate astaxanthin as a storage material with an antioxidant function. The other class is the light induction method, which makes use of the photoprotective role of astaxanthin under high-intensity light (Boussiba & Vonshak, 1991).

When *Haematococcus* cells are exposed to high-intensity light, astaxanthin accumulation is accelerated to protect the cells against photodamage and oxidative damage. Moreover, a highintensity light induction method, using acetate addition, has also been applied to enhance astaxanthin accumulation. Excess acetate addition generates a relative shortage of nitrogen, resulting in a high carbon/nitrogen (C/N) ratio, which triggers cyst formation and astaxanthin accumulation N (Kakizono *et al.,* 1992; Orosa *et al.,* 2001a; Orosa *et al.,* 2005). However, a productive photoautrophic Induction system was established for the production of antioxidative astaxanthin by the green microalga. A favorable CO_2 concentration and controlled specific

radiation rate leads to the productive encystement of *H. pluvialis* and enhanced astaxanthin synthesis (Kang *et al., 2006).*

Recently, it has been reported that methyl jasmonate- and gibberellins A(3) constitute molecular signals in the network of astaxanthin accumulation. Methyl jasmonate- and gibberellins A(3) treatment increased the transcription of three β-carotene ketolase genes (bkts) in *H. pluvialis* enhancing astaxanthin synthesis and accumulation. Methyl jasmonateand gibberellins A(3) are involved in the stress responses of plants. Induction of astaxanthin accumulation by methyl jasmonate- or gibberellins A(3) without any other stimuli presents anattractive application potential. (Lu *et al.,* 20 10).

OBTAINING VEGETATIVE CELLS RICH IN AST AXANTHIN IN *HAEMATOCOCCUS*

There are several problems for the industrial production of astaxanthin from *Haematococcus* related to the microalgal growth, the synthesis and accumulation of the pigment and the extraction of the astaxanthin from the biomass or the use of entire biomass. As it has been cited above astaxanthin production from *H. pluvialis* generally is carried out in a two-stage culture. The first stage is carried out in optimum conditions to achieve high growth rates and high cellular densities; at this stage there are not nutrient depletion, high light intensity or stress conditions. Once the desired cell density is reached, or when the microalgae have reached the stationary growth phase, different induction methods are used for the transformation of green cells to red cysts containing high astaxanthin contents. *Haematococcus* cysts rich in astaxanthin present a thick cell wall, probably composed of sporopollenin (Burczyk, 1987). The walls of the encysted *Haematococcus* cells are extremely resistant and difficult to break (Johnson & An, 1991), so the extraction of the pigment is difficult and expensive.

However, it has been reported that the synthesis of astaxanthin and the formation of resistant cells in *Haematococcus* are two completely different processes, normally coupled but that can be experimentally separated (Bubrick). Therefore, it seems possible to obtain vegetative flagellated *Haematococcus* cells rich in astaxanthin.

It has also been reported the accumulation of secondary carotenoids in flagellated vegetative *Haematococcus* cells under conditions of nitrogen deficiency and high intensity light (Grünewald *et al.,* 1997; Hagen *et al.,* 2000). However, it has been described that under these conditions flagellated green cells quickly lose their flagela and produce a thick cell wall, becoming first 'webbed' cells and then resistant cells or hematocysts (Borowitzka *et al.,* 1991; Boussiba *et al.,* 1992; Orosa *et al.,* 2001a).

Orosa obtained vegetative cells rich in astaxanthin from *H pluvialis.* The microalga was cultured in a modified BBM medium (Orosa *et al.,* 2005), at a temperature of 18 ± 1°C under intense light (350 µmol. photon. m^{-2}. s^{-1}), and without nitrate deficiency (12 mM); in these conditions carotenogenesis was induced whereas vegetative cells were not transformed into resistant cells or hematocysts. Astaxanthin was the main accumulated carotenoid, mainly as monoesters. However, the concentration of chlorophylls and primary carotenoids remained relatively high (Abalde *et al.,* 2005). Under the culturing conditions used for the synthesis of astaxanthin in flagellated cells of *Haematococcus pluvialis,* the cells were not subjected to any type of nutrient

limitation, an organic carbon source was not needed to induce synthesis, and the temperature was not excessively high.

Regarding the discussion about the most important factor in inducing the synthesis of astaxanthin (nitrogen deficiency or high intensity light), in the case of vegetative cells it is quite clear that this pigment is synthesized and accumulated without any nitrogen limitation in the culturing medium.

High light intensity has been considered the most important factor inducing carotenogenesis in *H plicatilis* (Harker *et al.);* light intensity of 50-60 µmol. photon. m^{-2}. s^{-1} was the optimal intensity for growth, while the optimal intensity for synthesis of astaxanthin was much higher, about 1600 µmol. photon. m^{-2}. s^{-1}, referring always to the production of astaxanthin in two stages: one for obtaining maximum biomass and then provoking conditions to induce the synthesis of astaxanthin. A light intensity of 350 µmol. photon. m^{-2}. s^{-1} yields a good production of astaxanthin, accompanied by good growth and a high level of biomass production, in a one-stage culture system by Orosa *et at.* (2001b). Culturing times did not extend beyond the sixth day, in order to ensure that more than 90% of the cells were in a vegetative form; longer time periods lead to the appearance of resistant cells, probably due to a nutritional deficiency.

Obtaining vegetative cells of *Haematococcus pluvialis* rich in astaxanthin supposes important advantages for the commercialization of this product; because these cells do not have thick cell walls indigerible for animals, it is possible used them directly as a food supplement in the diets of salmonids and other organisms, to obtain the desirable flesh color. There are another advantages in the production process itself, since, biomass production and astaxanthin production occurs in a one-stage system of short duration, in contrast to two stage systems currently used. However, the maximum astaxanthin concentration reached is lower to that obtained in two-stage production systems. Astaxanthin accumulation can be improved supplementing the culturie medium with an organic carbon source.

AST AXANTHIN OVERPRODUCING MUTANTS

Algal strain with improved growth rate and enhanced carotenoid accumulation makes the commercial process of astaxanthin production more feasible. Genetic manipulation techniques have contributed to this purpose, but random screening is the procedure widely used for the improvement of strains (Rowlands, 1984). The main disadvantage of nonspontaneous mutations is the appearance of hidden mutations (Boura-Halfon *et al., 1997),* that can affect cell growth.

Ultraviolet radiation has been used for the production of mutations but chemical mutagens such as ethyl methane sulphonate (EMS) or N-methyl-N-nitro N nitrosoguanidine (NTG) have also been used. Herbicides affecting carotenoid synthesis pathway are used to select resistant mutants.

Induction and selection of mutants has been a widely employed technique for strain improvement as well as for studying mechanisms of metabolic processes (Tjahjono *et al.*, 1994b). It has been reported astaxanthin formation using norflurazon and floridone in *H. pluvialis* cultures. Mutants of *Phaffia rodozyma* have been obtained by UV exposure and by ethyl methane sulphonate (EMS) or l-methyl-3-nitro 1 nitrosoguanidine (NTG) treatment for hyperproduction of astaxanthin. It has also been reported mutation *of Haematococcus* by UV

and EMS followed by the selection of mutants resistant to compactin, nicotine, diphenylamide, fluridone or norflurazon. However, the information on astaxanthin overproducing mutants is limited.

Orosa (2001) used UV radiation to obtain *H pluvialis* mutants using diflufenican, norfluazon, fluometuron and amitrol to select astaxanthin hyperproducing mutants. Two astaxanthin overproducing mutants were obtained: RNC-l (resistant to the herbicide Norflurazon) and RDC-1 (resistant to Diflufenican). RDC 1 mutants showed an increase in astaxanthin production of 1.7 (w/w) in flagellated vegetative cells, without differences in the production of biomass. The concentration of total astaxanthin in RNC-I, although lower than that found in RDC-1, was also improved respect to the wild strain (1.5 times higher). These mutations were not different from the wild strain in their carotenoid profile; monoesters and diesters of astaxanthin were the main secondary carotenoids accumulated under conditions of inducing synthesis. The mutants RNC-1 and RDC-l only showed decreased biomass production in autotrophic cultures; however, in mixotrophic growth there were no significant differences.

Growing culture of green alga *H.pluvialis* was exposed to mutagens such as UV, ethyl methane sulphonate (EMS) and l-methyl-3-nitro 1 nitrosoguanidine (NTG) and further screened over herbicide glufosinate. The survival rate of cells decreased with increasing concentrations of mutagens and herbicides. The mutants exhibited 23-59% increase in total carotenoids and astaxanthin contents. The NTG treated glufosinate resistant mutants showed increased (2.2 to 3.8 % w/w) astaxanthin content. The NTG treated glufosinate resistant mutants showed increased 82.2 to 3.8 % w/w) astaxanthin content (Kamath *et al., 2008).*

Another stable astaxanthin overproduction mutant (MT 2877) was obtained by chemical mutagenesis with MNNG (methyl nitro nitrosoguanidina) of a wild type (WT) of the green alga *H. pluvialis.* MT2877 was identical to the WT with respect to morphology, pigment composition and growth kinetics during the early vegetative stage of the life cycle. However, it has the ability to synthesize and accumulate about twice the astaxanthin content of the WT under high light, or under high light in the presence of excess amounts of ferrous sulphate and sodium acetate. MT2877, or other astaxanthin overproduction *Haematococcus* mutants, may offer dual benefits as compared with the wild type, by increasing cellular astaxanthin content while reducing cell mortality during stress-induces carotenogenesis (Hu *et aI., 2008).*

All of these data support the use of mutants for the production of astaxanthin, mainly inthe form of vegetative cells rich in this carotenoid.

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