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# Biology of growth conditions, nutrition and biomass development in *Haematococcus pluvialis*, Haematococcaceae, Chlorophyceae

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**Abstract**

This is the second part of the review on the genus *Haematococcus*. Here studies on biology and growth conditions of different strains of *H. pluvialis* are dealt with under appropriate sections, such as nutritional requirements – especially N- and P-sources, N / P ratio, organic carbon sources and vitamins; light intensity and temperature range. Optimization of culture conditions have been carried out in batch cultures as well as semi-continuous cultivation systems. Some studies on growth in photobioreactors have also been included.

**Keywords:** Growth conditions, Nutrients, Autotrophy, Organic C-sources, Light intensity, Temperature, Photobioreactors

## Introduction

Vegetative cultivation of *Haematococcus* is the most challenging task owing to its slow growth rate, low cell density and susceptibility to contamination (Lee and Zhang, 1999). Therefore, optimization of the culture conditions becomes essential to support higher cell productivities. In an earlier review on *Haematococcus* (Jeeji Bai et al., 2016) aspects of nomenclature, taxonomy, life cycle and ecology were discussed. In this paper, the physico-chemical conditions, which promote persistence of green motile stage for continued cell division and biomass production, are dealt with. The interest in laboratory cultures started with the realization of the high value of the red pigment astaxanthin in aquaculture of Salmon fish for the pink colour of its flesh. This resulted in a few reviews on astaxanthin synthesis and accumulation (Johnson and An, 1991; Lee and Zhang, 1999). The present work includes some information on conditions of culture, nutrition and growth besides different modes of culture employed by different investigators.

## Laboratory culture, growth mode and nutritional requirements

Jacobsen (1912) obtained the first unialgal culture of *H. pluvialis* using dilute mineral solutions and enrichment of cultures wherein the natural source water was enriched with some essential nutrients. Chodat (1913) also found dilute media to be most suitable in his experiments. However, these investigators did not succeed in getting sub-cultures. This was probably due to the requirement of certain unknown nutrients. Addition of soil extract helped to overcome this problem to a certain extent (Pringsheim, 1914; Droop, 1954). Similarly, agar cultures were better than liquid cultures (McLachlan, 1963). Many organic substrates such as acetate, peptone, tryptone, beef extract etc. have also been used commonly to culture this alga. Pringsheim (1966) found that the levels of acetate requirement varied in the different strains studied, although almost all strains did perform better with acetate addition at levels between 0.001 and 0.01%. Peptone and beef extract also promoted luxuriant growth. Studying the organic nutrition of *H. pluvialis*, Droop (1961) observed that although the alga was capable of total autotrophy, a variety of organic acids and sugars were utilized and acetate was utilized even in the dark. Besides acetate, many other simple organic compounds such as urea, uric acid, arginine, guanine and tryptophan could be utilized as N- source under facultative heterotrophy. Glycollate, however, was not utilized as C- source (Droop and Mc Gill, 1966). Stross (1960) found that of the variety of organic acids tried, acetate was most suitable. McLachlan (1963) found glycylglycine to be better than tris (hydrxymethyl-aminomethane) as a buffer.

With the revival of interest in the study of this alga from 1990 onwards for production of the red pigment astaxanthin, many studies on nutritional physiology were taken up for producing maximum biomass of the green motile cells, which later formed the red aplanospores. *H. pluvialis* is reported to use three growth modes: (a) photoautotrophic – inorganic carbon + light, (b) mixotrophic – organic and/or inorganic carbon + light, and (c) heterotrophic – organic and/or inorganic carbon + dark (Lau, 1998). For the photoautotrophic mode, three inorganic nutrient media MCM (Borowitzka et al., 1991), BG11 (Boussiba and Vonshak, 1991) and A9 media (Lee and Soh,

1991) were shown to be effective in supporting the algal growth and accumulation of astaxanthin. However, Kobayashi et al., (1991) showed that the basal mineral medium with simple and inexpensive ingredients [sodium acetate – 1.2, yeast extract – 2, magnesium chloride – 0.2, ferrous sulphate – 0.01, calcium chloride – 0.02, all values in g $L^{-1}$] promoted the algal growth better than the other 3 media and therefore, it is recommended as the desirable medium for large-scale cultivation of *Haematococcus*. They also used other organic supplements such as yeast extract and asparagine to demonstrate the mixotrophic growth of the alga in low light conditions and heterotrophic growth with acetate under dark. In a later study (Kobayashi et al., 1992) they demonstrated that both photosynthesis and oxidative metabolism of acetate could occur concomitantly during mixotrophic growth on acetate by inhibiting photosynthesis by DCMU (dichlorophenyl-dimethyl-urea).

Studying the organic nutrition of *H. pluvialis*, Droop (1961) observed that although the alga was capable of total autotrophy, a variety of organic acids, sugars and acetate were utilized even in the dark. Glycollate, however, did not prove to be a significant C-source (Droop and McGill, 1966). Stross (1960) found that of the variety of organic acids tried, acetate was the most suitable. Among the various carbon sources such as potassium hydrogen carbonate, sodium carbonate, dipotassium carbonate, sodium bicarbonate used in the study, Rao (2011) obtained a maximum yield of 2.9 g $L^{-1}$ in 3 mM ammonium carbonate.

Under mixotrophic culture conditions, Jeon et al. (2006) found that acetate concentration of 30 mM enhanced the growth rate up to 0.243 g $L^{-1}$ $day^{-1}$. However, in a fed-batch culture, Hata et al. (2001) reported no significant difference in specific growth rate of *H. pluvialis* with increasing concentrations of acetate from 10 mM to 30 mM. The inhibitory effect observed at higher concentrations was due to the formation of non-motile cyst stage. Nonetheless, with repeated fed-batch processes, the cells could be maintained in the vegetative form resulting in more than twofold increase in cell number.

The growth of *H. pluvialis* was monitored by Tripathi et al. (1999) in autotrophic (BBM, Z8 and A9) and mixotrophic (KM1 with acetate and yeast extract; BBM with sodium acetate, MM1; BBM with sodium acetate and L-asparagine, MM2; KM1 without yeast extract, KM2) culture conditions. Among autotrophic media, BBM was best for growth ($15\times10^4$ cells $mL^{-1}$). Cell counts of $30\times10^4$, $32.5\times10^4$ and $42\times10^4$ cells $mL^{-1}$ were obtained in KM1, MM2 and KM2 media, respectively showing that acetate alone was sufficient to bring about growth stimulation. Domínguez-Bocanegra et al. (2004) too obtained maximum growth of $35\times10^4$ cells $mL^{-1}$ in BBM medium.

Different basal media were used with nitrate or urea as N-source for autotrophic cultures and acetate with or without organic N-source for mixotrophic cultures. Besides acetate, many other simple organic compounds such as urea, uric acid, beef extract, peptone, tryptone, arginine, guanine, tryptophan etc. have also been used as N-source to culture this alga, which resulted in significant growth stimulation. Ammonium-N is usually utilized preferentially by many green algae. However, *H. pluvialis* is exceptional in preferentially utilizing nitrates when supplied with both nitrate-N and ammonium-N (Morris, 1974). There are several reports on the optimization of culture medium for *Haematococcus*, but many of which mainly focus on the optimum concentrations of $KNO_3$ and $NaNO_3$ (Borowitzka et al., 1991; Gong and Chen, 1997; Fábregas et al., 2000; Orosa et al., 2005).

Borowitzka et al. (1991) determined the optimal concentrations of nitrate and phosphate, singly and in combinations for 2 strains of *H. pluvialis* concentration ranges of 0.5 to 5.0 g $L^{-1}$ $KNO_3$ and 0.001 – 0.02 g $L^{-1}$ $K_2HPO_4$ were found to be suitable and highest levels yielding maximum growth. Urea also supported equally good growth whereas ammonium salts were not suitable. With acetate, a marked growth enhancement was obtained. They observed a slightly higher initial cell yield at pH 6.5 as compared to 7.5; however, in both cases the final yields were similar. They concluded that acetate helped in pH regulation by the release of $CO_2$ during its oxidation. No significant difference in growth was observed in the wide range of iron levels (as $FeCl_3.6H_2O$ – 18, 36 and 72 mM) tested by them. However, Harker et al. (1996) have reported that high $Fe^{2+}$ concentration inhibited cell growth in *H. pluvialis.*

Rao (2011) obtained a maximum biomass yield of 3.3 g $L^{-1}$ in 25 mM $NaNO_3$ whereas with same concentration of $NH_4NO_3$, the yield was only 1.4 g $L^{-1}$. In a study on optimization of culture medium for continuous cultivation of *H. pluvialis*, Fábregas et al. (2000) found an N:P ratio of 20:1 and a $KNO_3$ concentration of 8 mM to be optimum at 20% renewal rate. They also found that Zn, B, I and V were non-essential and Se, Cr and vitamins $B_1$, $B_{12}$ and biotin were essential for optimal growth. Fábregas et al. (2000) and Kaewpintong et al. (2007) suggested that

addition of vitamin B to the growth medium could appreciably enhance the final cell density of vegetative cells to achieve sustainable high cell density. While these reports highlight the importance of vitamins as essential for increasing the productivity of vegetative cells, the optimum levels of the vitamins in batch cultures have not been specified.

Göksan et al. (2011) found nitrate (Na, K, $NH_4$ salts) as N-source yields higher biomass than urea as N-source. The best growth of *H. pluvialis* was achieved with 1% $NaNO_3$, 0.05% $KNO_3$ and 0.025% $NH_4NO_3$ as nitrogen sources with cell densities of 25.3, $26.3\times10^4$ and $18\times10^4$ cells $mL^{-1}$ respectively. However, the use of urea and ammonium salts has not been studied sufficiently. They also determined the optimal doses of vitamins – thiamine (0.1 mg $L^{-1}$), biotin (1 mg $L^{-1}$) and $B_{12}$ (0.1 mg $L^{-1}$) for maximum cell yields of $29\times10^4$, $28\times10^4$ and $29\times10^4$ cells $mL^{-1}$, respectively. Their study also demonstrated that thiamin alone was sufficient instead of the complex vitamin mix. Pringsheim even as early as 1966 showed that among vitamins, thiamine is the best as growth factor for *H. pluvialis*, while $B_{12}$ stimulated growth but was not essential. Gong and Chen (1997) and Fábregas et al. (2000) also reported that biotin, thiamine and $B_{12}$ had no significant effect on growth rate and final dry weight. This was substantiated by Imamoglu et al. (2007) by cultivating the microalga in an economical Rudic's culture medium (Rudic and Dudnicenco, 2000) without vitamin supplement.

Lee and Ding (1994) studied the developmental sequence of motile and stationary phases in relation to urea levels in the medium. They observed that with low levels of urea (0.85 g $L^{-1}$), the number of motile cells increased up to 110 hours and then up to 275 hours the number of non-motile palmella cells increased with a slight drop at 208 hours. In higher urea concentration (1.7 g $L^{-1}$), the increase in the number of motile cells was more rapid reaching a maximum in 50 hours and then the number of non-motile cells continued to gradually increase up to 304 hours by vegetative division to produce 4 to 32 aplanospores. They found that the size of non-motile cells and number of daughter cells formed were inversely proportional to the growth rate of the culture and the increase in biomass was largely contributed by the growing non-motile cells, despite their slower rate of division as compared to motile cells. In a later paper (1995) they found that in chemostat cultures at high oxygen partial pressures, steady state cell densities reduced to almost half of that at low $O_2$ partial pressures. At high dissolved oxygen levels the fraction of non-motile cells also increased.

In general, many investigators found Bold basal medium with nitrate as N-source yields higher biomass both under autotrophic and mixotrophic (with sodium acetate) growth conditions. As an exception, Cifuentes et al. (2003) reported that ammonium chloride was the best N-source for the algal growth and a further slight increment in growth occurred with increasing acetate concentration.

Harker et al. (1995) used a statistical approach of Response Surface Methodology (RSM) with visual representation and found $NaNO_3$, $KNO_3$ and urea to be more or less equally good but urea at 2.5 – 3.0 mM gave better results than $KNO_3$. A central, composite rotatable design was employed by Gong and Chen (1997) to minimize the number of experiments for culture medium optimization and for determining the optimum levels of acetate (0.1 to 0.5 g $L^{-1}$), $KNO_3$ (0.25 g $L^{-1}$), major element stock (0.63 mL $L^{-1}$) and trace elements stock (0.2 mL $L^{-1}$). They found an insignificant interaction with other nutrients for acetate and considerable interaction for nitrate. In a later study, Gong (1997) employed a novel conductance change method to ascertain that acetate is better than glucose for heterotrophic growth of *H. pluvialis*. Using the same methodology (RSM), Sarada et al. (2002) found the optimum values of $NaNO_3$ and $CO_2$ to be 1.06 g $L^{-1}$ and 1.54% respectively to bring about a significant effect on cell growth.

Brinda et al. (2004) achieved high biomass yield of 3.5 g $L^{-1}$ under autotrophic conditions on phosphate free medium. Of the various nutrient deficient combinations, 1/4 magnesium and 1/10 phosphate resulted in high biomass yields whereas 1/10 of nitrogen and phosphate in combination gave lower biomass yields.

The feasibility of autotrophic production of vegetative cells was analyzed by García-Malea (2005) under conditions resembling outdoors. Growth was kept nutrient-limited by using nutrients concentration below the standard inorganic medium (10 mM nitrate). The results showed that when no nutrient-limitation occurred, growth rate and biomass productivity were 0.57 $day^{-1}$ and 0.28 g $L^{-1}$ $day^{-1}$ respectively. These were similar to the maximum values reported regardless of the nutritional regime: photoautotrophic, mixotrophic or heterotrophic.

Dalay et al. (2007) used agricultural fertilizers as an economical alternative for the cultivation of *H. pluvialis*. The results showed that maximum cell concentration of 0.9 g $L^{-1}$, corresponding to a growth rate of 0.15 $d^{-1}$ was found with an N-P-K 20:20:20 fertilizer under a light intensity of 75 µmol photons $m^{-2}$ $s^{-1}$ on the 12$^{th}$ day of cultivation. Tocquin et al. (2012) used low-cost hydroponic fertilizers successfully for achieving high density cultures of vegetative cells and obtained a maximal cell density of $2\times10^6$ cells $mL^{-1}$ in a medium having a high level of phosphate relative to nitrate resulting in a much lower N:P ratio than in commonly used media. In this medium, cells remained in vegetative motile stage for a prolonged period of time. Both high cell density culture and motile stage persistence was found to be related to the N: P status of the medium, and the macrozoid stage was favored under high-P and low-N supply.In an investigation by Sipaúba-Tavares et al. (2015) to verify the use of NPK fertilizer (20-5-20) as an alternative medium for cultivation of *H. pluvialis* in the laboratory, it was observed that highest number of cells was achieved ($5.4\times10^5$ cells $mL^{-1}$) on the 28$^{th}$ day.

**Light and temperature requirements**

Light intensities above 75 µmol photons $m^{-2}$ $s^{-1}$ are detrimental to green cells leading to arrest of cell divisions accompanied by mortality. Light intensities as low as 10 – 30 µmol photons $m^{-2}$ $s^{-1}$ were tested for mixotrophic growth (Kobayashi et al., 1991; Barberà et al., 1993; Gong, 1997). Intensities up to 90 µmol photons $m^{-2}$ $s^{-1}$ were tried by Fan et al. (1994) for autotrophic growth of green motile cells with varying degrees of success. Borowitzka et al. (1991) used a light intensity of 120 µmol photons $m^{-2}$ $s^{-1}$ in a 10:14 light / dark cycle for autotrophic growth of *H. pluvialis*. Harker et al. (1995) found 50–60 µmol photons $m^{-2}$ $s^{-1}$ to be the optimum for growth as determined by RSM. Fábregas et al. (1998) found less growth (a maximum of $6.2\times10^6$ cells $mL^{-1}$) in complete medium in low light (40 µmol photons $m^{-2}$ $s^{-1}$) as compared to high intensities (230 µmol photons $m^{-2}$ $s^{-1}$) when cultured in a mini-reactor with 12:12 light / dark cycle. In a later study (2000), they tested four different light intensities and found 40 µmol photons $m^{-2}$ $s^{-1}$ level to be suitable for continuous cultivation at 20% renewal rate of medium. Göksan et al. (2011) reported that the cell growth was significantly higher in 75 and 150 µmol photon $m^{-2}$ $s^{-1}$ illuminations compared to 20 and 40 µmol photon $m^{-2}$ $s^{-1}$. Higher levels of chlorophylls 'a' and 'b' were observed in low light intensities which promoted the growth of green motile cells (Lee and Soh, 1991; Kobayashi et al., 1991; Fan et al., 1994). Kobayashi et al. (1992) reported higher chlorophyll content with acetate than with $CO_2$ in low light intensities (39 to 68 µE $m^{-2}$ $s^{-1}$).

*H. pluvialis* BM1 isolated from the coastal rocks on Kost'yan island, White sea when cultivated in BG-11 medium at 27 ºC, with the light intensity of 40 µE $m^{-2}$ $s^{-1}$, the "green" cell cultures reached a maximum cell density of $160\times10^4$ $mL^{-1}$ yielding about 1 g $L^{-1}$ of biomass (DW) in 5–7 days corresponding to the specific growth rate (µ) of 0.095 $day^{-1}$ at the exponential phase (Chekanov et al., 2014). Fan et al. (1994) found that the saturated irradiance for the growth of *H. pluvialis* in batch culture was 90 µmol photon $m^{-2}s^{-1}$; under higher irradiances (e.g. 400 µmol photon $m^{-2}$ $s^{-1}$) astaxanthin accumulation was induced. Imamoglu et al. (2007) obtained maximum cell density of $9.5\times10^5$ cells $mL^{-1}$ with a corresponding growth rate of 0.195 $d^{-1}$ in *H. pluvialis* when grown in Rudic's medium with light intensity of 40 µmol photons $m^{-2}$ $s^{-1}$. Only 7% decrease in cell population was observed when grown in basal culture medium at the same light intensity.

Studies conducted by Sarada et al. (2002) using response surface methodology (RSM) revealed the significant effect of light intensity on the growth of *H. pluvialis*. According to them, the optimum light intensity to obtain the maximum predicted value of biomass was 2.42 Klux. Under continuous illumination (177 µmol photon $m^{-2}$ $s^{-1}$) of white fluorescent light Domínguez-Bocanegra et al. (2004) obtained maximum growth of *H. pluvialis* ($3.5\times10^5$ cells $mL^{-1}$) in BBM medium.

In a study to find the optimal photon irradiance for the growth of green cells of *H. pluvialis*, Torzillo et al. (2005) reported that productivity of cultures increased with irradiance. However, the rate of increase was higher in the range 50–200 µmol $m^{-2}$ $s^{-1}$. Photosynthetic activity showed a drop at the end of the light period, but recovered fully during the following dark phase. A steep increase in non-photochemical quenching was observed when cultures were grown at irradiances above 200 µmol $m^{-2}$ $s^{-1}$. According to them, with green cells grown in a 5-cm light path device, 200 µmol $m^{-2}$ $s^{-1}$ was optimal for growth and represented a threshold above which important changes in photochemical parameters occurred. Jeon et al. (2006) partially optimized the mixotrophic culture conditions by using a sequential factorial design and found that light is one of the major factors affecting the algal growth rate. A light intensity of 170 µE $m^{-2}$ $s^{-1}$ enhanced the growth rate up to 0.243 g $L^{-1}$ $day^{-1}$. However, when vegetative cells were

grown autotrophically under conditions resembling outdoors, García-Malea et al. (2005) found that the cultures were not photoinhibited even under the maximum irradiance-level tested (2500 μE $m^{-2}$ $s^{-1}$).

Li et al. (2010) determined the growth kinetics in cultures of the wild type (WT) and mutant (MT 2877) of *H. pluvialis* grown under various photon flux densities (PFD). Cultures were first maintained under a low PFD of 20 μmol photons $m^{-2}$ $s^{-1}$ and 22 °C for 4 days and then subjected to continuous illumination of various PFDs (i.e. 50, 125, 300, and 600 μmol photons $m^{-2}$ $s^{-1}$). At a low PFD of 20 μmol photons $m^{-2}$ $s^{-1}$ cell numbers increased before they got stabilized in culture. When exposed to the higher PFDs, significant cell mortality occurred in cultures of both strains, with the net result in the cultures; the higher PFD, the greater the cell mortality.

Comparing the growth among four strains of *H. pluvialis* isolated from different microhabitats in two geographical locations in Chile under autotrophic and mixotrophic conditions and under two photon flux densities (PFD) of 20 and 85 μmol $nrV^{-1}$, González (2009) found that most of the strains grew better under the autotrophic conditions; the highest exponential growth rates were exhibited at the high PFD in all the strains with the exception of a strain, which showed the same exponential growth rate with either PFD. He concluded that a high intraspecific variability in the physiological attributes analyzed existed among the strains under study.

Initial biomass density (IBD), which influences the net light availability, is an important factor that affects the viability and productivity of microalgae particularly when sunlight is used for photosynthesis. Wang et al. (2013) established the severe photoinhibition of photosynthesis at low IBD (0.1 g $L^{-1}$) cultures, especially in the winter season. On the other hand, severe light limitation to individual cells in high IBD cultures (>2.7 g $L^{-1}$) were found to be responsible for reduced astaxanthin production.

Among the various stress conditions tested by Saha et al. (2013) to maximize the biomass and lipid production in *H. pluvialis* SCCAP K-0084, photosynthetically active radiation (PAR), warm white light emitting diode (WWLED), and white light emitting diode (WLED) at illumination of 240 μmol photons $m^{-2}$ $sec^{-1}$ were found to be the best stress-regulatory factors.

Green motile cells have usually been grown for biomass production at temperatures between 20 and 25 °C with 20 °C being used most commonly. In a few studies slightly lower temperatures such as 18 °C (Shi et al., 1995) and 14–15 °C (Harker et al., 1995) have been employed. Fan et al. (1994) found a steady increase in photosynthetic oxygen evolution from 25 to 28 °C and a gradual drop from 28 to 33 °C and a sudden drop at higher temperatures. They observed 28 °C to be the optimum for photosynthesis. However, growth rate in terms of cell numbers showed maximum values from 25 to 28 °C with doubling times of 12–13 hours and a steep drop at higher temperatures. At higher temperatures cell division stopped but increase in cell volume occurred. Tjahjono et al. (1994) reported that in mixotrophic cultures, growth in terms of cell numbers reduced to nearly 50% at 25 °C and to 15% at higher temperatures as compared to that at 20 °C. Borowitzka et al. (1991) used day and night temperatures of 20 and 15 °C in a 10:14 hrs light / dark cycle. They observed equally good growth at 15 and 25 °C. However 28 °C was inhibitory and 35 °C proved lethal. While determining the growth characteristics of *H. pluvialis* in batch culture, Fan et al. (1994) found that optimal temperature for growth of the alga was between 25 and 28 °C at which the specific growth rate was 0.054 $h^{-1}$. At higher temperatures, no cell division was observed and cell diameter increased from 5 to 25 μm.

Harker et al. (1995) found 14 – 15 °C as optimum temperature in their study using RSM. Hata et al. (2001) cultivated *H. pluvialis* heterotrophically to high cell concentration under an optimum pH and temperature of 8 and 25 °C respectively, whereas Domínguez-Bocanegra et al. (2004) obtained maximum growth in BBM medium at 28 °C with continuous aeration (1.5 vvm). Tripathi et al. (2002) obtained higher cell counts at a low temperature of 25 °C in both autotrophic and heterotrophic media.

**Mass and medium-scale cultures, photobioreactors, immobilized cultures**

The importance of astaxanthin pigment in aquaculture, human health and medicine has not only led to certain basic studies on the conditions of culture which promote pigment production, but also to a few others on mass culture, immobilization, photobioreactors etc. Ding and Lee (1994) grew the cells of *H. lacustris* entrapped in calcium alginate gel to overcome problems of slow growth, contamination, sensitivity to mechanical and aeration stress and

adverse effect of temperatures of 30 $^{o}$C and above. They used 2 L air-lift fermenter aerated with 5% $CO_2$ at 2 L min$^{-1}$ and a light intensity of 100 μmol photons m$^{-2}$ s$^{-1}$ with medium replacement every 5 days. They found a slightly higher rate of growth in beads as compared to free cells; a greater thermo-tolerance (up to 32 $^{o}$C) and a greater adaptability to airlift recycle system. The low stability of alginate beads posed a problem of disintegration after 2 weeks which could be prevented by periodic washing with $CaCl_2$.

Lee and Zhang (1999) believe that under outdoor cultivation high productivity is difficult to achieve because of the low light requirement for the growth of the green motile cells. The limited number of doublings of the motile cells also poses a problem in reaching high cell densities. However, a process for commercial production of natural astaxanthin from *H. pluvialis* was reported by Bubrick (1991) using the conventional raceway ponds. With a continuous scale-up process from 2.6 to 4500 m$^2$ ponds with retention times of 5 to 7 days at each stage, he could obtain a cell density of 3 to 6×10$^5$ cells mL$^{-1}$. However, protozoan contamination caused nearly 90% loss in 72 hours. Besides, special drying and grinding process was required to break the sporopollenin walls of the cysts causing cost escalation.

Olaizola (2000) described the first and only large-scale photobioreactor, the Aquasearch Growth Module, presently operating in Hawaii. The module consists of three reactors each of 25,000 L capacity, with tubes of 0.18–0.41 m. diameter, laid parallel on impermeable surface and occupying an area of roughly 100 m$^2$. Mixing was by an air-lift system with a flow velocity of 2×10$^3$ to 2×10$^5$ Re (Reynold's number) and cooling by deep sea water to a temperature range of 15 to 25 $^{o}$C. This was suitably exposed to the sun to produce a quick growing green culture and then transferred to conventional raceway ponds with nutrient depletion and exposure to strong light for carotenogenesis. The average biomass yields were 50 to 90 g m$^{-2}$ with a productivity of 9 to 13 g m$^{-2}$ d$^{-1}$. The astaxanthin content ranged from 0.7 to 3.4% on cell dry weight basis.

Kaewpintong et al. (2007) investigated the effects of the bioreactor configurations and their design variables on the cultivation of vegetative cells of *H. pluvialis* to achieve sustainable high cell density. The addition of vitamin B to F1 growth medium could appreciably enhance the final cell density. In this medium, cultivation in the airlift bioreactor growth to outperformed the bubble column at the same operating conditions. Maintaining low level of aeration at 0.4 cm s$^{-1}$ minimized shear stress was crucial for proper growth in the airlift bioreactor. A smaller riser also showed a positive influence on cell growth. Air supply with 1%$CO_2$ considerably enhanced the growth rate and the most suitable light intensity was 20 μmol photons m$^{-2}$ s$^{-1}$. This semi-continuous culture system was successfully implemented under optimal conditions every four days with the specific growth rate of 0.31 d$^{-1}$.

A novel flat panel airlift photobioreactor (FP-ALPBR) was proposed by Issarapayup et al. (2009) as an alternative system for the cultivation of *H. pluvialis* strain NIES-144. Changes in the efficiency of the system were tested in response to variations in two engineering parameters: the ratio between the downcomer and riser cross-sectional areas ($A^d/A^r$) and the size of the system (as determined by the length of the panel) and to one operating parameter: the superficial gas velocity ($u^{sg}$). The best growth performance was obtained by operating the system at a superficial velocity of 0.4 cm s$^{-1}$ and with a downcomer-to-riser cross-sectional area ratio of 0.4. The 17 L FP-ALPBR system was capable of giving reasonable growth characteristics with a maximum cell density of 4.1×10$^5$ cells mL$^{-1}$ and specific growth rate of 0.52 day$^{-1}$. A similar level of performance was obtained from the 90 L FP-ALPBR system, i.e., cell density = 4×10$^5$ cells mL$^{-1}$ but with a slight decrease in specific growth rate to 0.39 day$^{-1}$. The performances of these two differently sized FP-ALPBRs were compared with two conventional cylindrical airlift photobioreactors (C-ALPBRs) of different dimensions. The 90 L FP-ALPBR exhibited reasonably good performance when compared with the two 17 L systems (both C- and FP-ALPBRs); however, the best growth rate was observed using the 3 L C-ALPBR. Semi-continuous cultures, which could be periodically harvested at a reasonably high growth rate, were successfully created. Of all the systems investigated in this study, the 90 L FP-ALPBR was found to be the most cost-effective, as it could cultivate 18 g of alga for approximately US$ 21.

Sipaúba-Tavares et al. (2013) compared the growth rate and medium parameters between two bench scale volumes (13 L and 250 L). Vegetative cell growth was higher when cultured in 13 L with 1.33×10$^5$ cells mL$^{-1}$ on the 12$^{th}$ day than when cultured in 250 L. Significant difference ($P < 0.05$) in the biology and water culture of *H. pluvialis* was reported between the two volumes with the exception of dry weight, ash, nitrite and ammonia. Light was of paramount importance on the direct performance of *H. pluvialis*.

**Conclusions**

For autotrophic growth of green cells of *H. pluvialis*, most studies referred in this review have used Bold basal medium. F1 medium (Fábregas et al., 1998) and BG11 medium (Boussiba and Vonshak, 1991) have also been used in a few studies for autotrophic culture of green cells irrespective of the strain investigated. Nitrate as N-source is preferred irrespective of the anions used although in most studies $NaNO_3$ is used. In only one study (Cifuentes et al., 2003), $NH_4Cl$ has been recommended as the best source. Similarly urea is generally not a preferred N-source. Media with high N : P ratios have been used in a majority of the studies with consistently good growth yields. Equal proportions of NPK in the form of fertilizers are recommended in 1 study (Dalay et al., 2007). In another study in which hydroponic fertilizers were used (Tocquin et al. 2012) high phosphate levels and the consequent very low N/P ratios yielded maximum cell density besides prolonging the green motile stage. Sodium acetate is a preferred C-source at concentrations between 10 and 30 mM enhances mixotrophic growth yields to a large extent. B vitamins especially thiamin have also been found to be essential for growth of green vegetative cells (Kaewpintong et al., 2007; Göksan et al., 2011).

Green cells, which are capable of multiplication into 4, 8, 16 and even 32-cells prefer somewhat low intensities of light around 50 µmol $m^{-2}$ $s^{-1}$ and not exceeding 80 µmol $m^{-2}$ $s^{-1}$ depending on the volume of culture and size of vessel used for the study. In general, mixotrophic cultures prefer to grow at lower light intensity levels. In those cases where bioreactors of large capacities between 13 L and 250 L were used, higher light intensities of 90 and 180 µmol $m^{-2}$ $s^{-1}$ were employed for optimal growth yields (Sipaúba-Tavares et al., 2013).

Temperature range preference of *H. pluvialis* is rather narrow – between 20–25 $^{o}$C as shown by the majority of the studies referred. This is probably because the adaptability of the alga is low and hence, the failure of most large scale open-air systems. The only case referred here is from a warm region where the open-air tubular photobioreactors are cooled by deep seawater (Olaizola 2000).

The closed photobioreactor systems may have some promise but the cost factor makes it unviable for scaling up. There is an urgent need to study the possibility of induction of cell division of parent cells to form 16 and 32 daughter cells simultaneously to achieve high densities with “growth-spurts”. This method should be made reliable as well as repeatable for sustainability.

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