Review

The Enhancement of Astaxanthin Production Using Medium Cultures Components, Stressors, Nanoparticles, Elicitors, and Gene Engineering in Haematococcus pluvialis

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Posted Date: 3 February 2023

doi: 10.20944/preprints202302.0063.v1

Keywords: Microalgae; Bio acids; Phytohormones ; Medium Culture; Stress

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The Enhancement of Astaxanthin Production Using Medium Cultures Components, Stressors, Nanoparticles, Elicitors, and Gene Engineering in *Haematococcus pluvialis*

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Abstract: Natural astaxanthin is a precious substance obtained from some organisms such as microalgae. This plant has many benefits for humans, so research into its cost-effective and economical production has recently increased. For this purpose, some methods such as the use of different culture media, gene engineering, different stresses, nanoparticles, bio acids, and phytohormones are important. Accordingly, this review study was conducted to demonstrate the effect of the factors mentioned above for the high production of astaxanthin in microalgae, especially *Haematococcus pluvialis* (*H.p*).

Keywords: microalgae; bio acids; phytohormones; medium culture; stress

Introduction

Green microalgae have more than 7,000 species that live in a variety of habitats. They produced beneficial components for humans, such as astaxanthin, carotenoids, canthaxins, and so on. Astaxanthin (3,3-dihydroxy-β, β-carotene-4, 4′-dione) is a precious keto carotenoid and a coloring agent in aquaculture (Borowitzka, 1992b, Borowitzka, 1992a, Kobayashi *et al.*, 1993). Naturally, astaxanthin (AX) pigments are synthesized by several types of microalgae, plants, bacteria, and fungi. (Gao and Meng, 2012). The most important natural producer of astaxanthin is *H. pluvialis* (*Chlorophyceae*, *Volvocales*) with 80% dry weight of astaxanthin. (Garshasbi *et al.*, 2020), *H.p* is a unicellular freshwater microalgae. The microalga has two life cycles, moving green and fixed red, the second cycle is called cyst, at which stage astaxanthin is produced (Xi *et al.*, 2016). Various stresses and inducers on *H.p* stimulate astaxanthin production. Researchers are looking for economical approaches to maximizing this product. The use of different stresses, inducer materials, culture media, and gene engineering are the main methods. Stresses include excess light (Xi *et al.*, 2016), salt (Johnson and An, 1991)and nitrogen deficiency (Boussiba, 2000). Inducers include biological acids, including salicylic(Raman and Ravi, 2011), linoleic, Jasmonic acids (Xi *et al.*, 2016), and nanoparticles, such as TiO2, SiO2, Cd, etc. (Xia *et al.*, 2015). Such research has been done on other organisms such as *Chlorella sorokiniana* (hassan jalili, 2016), *Bifidobacterium animalis* subsp. lactis Bb 12 (Jalili *et al.*, 2010), and *Chlorella vulgaris* (Jafari *et al.*, 2016). In this review, the outcomes of researchs firstly on the structure of astaxanthin, secondly the best medium cultures, effective genes and the effect of different stressors and inducers on microalgae, especially on *H.pluvialis* for AX production, were collected.

Astaxanthin structure

The main structure of AX consists of a lycopene structure with typically 40 carbon atoms, two polar ring ends, and dual hydrogen bonds. (Higuera-Ciapara *et al.*, 2006a). Astaxanthin has different structures depending on the type of fatty acid used in it, these fatty acids include palmitic, oleic, stearic, linoleic, or none of them. Natural AX is esterified while artificial one is not. (Johnson and An,

 doi:10.20944/preprints202302.0063.v1

1991). Astaxanthin (3,3′-dihydroxy-ß-carotene-4,4′-dione) is a member of the lutein, lycopene, and β-carotene family, synthesized by some microalgae, yeast, plants, bacteria, and could be found in some living creatures including salmon, trout, lobster, shrimp, and fish eggs (Higuera-Ciapara *et al.*, 2006b), (Ambati *et al.*, 2014). There are three forms of AX in crustaceans (Arango, 1996).AX is found in nature in two forms and three variable configurations (Yang *et al.*, 2013),(Al-Bulishi *et al.*, 2015). Astaxanthin is produced in *microalgae* for defense and cellular storage purposes and is synthesized from pyruvate and glyceraldehyde-3-phosphate via the carotenoid pathway. The antioxidant power of astaxanthin is 65, 54, and 10 times higher than that of vitamin C, beta-carotene, and zeaxanthin, respectively. (Miki, 1991), (Koller *et al.*, 2014, Pérez-López *et al.*, 2014), (Pérez-López *et al.*, 2014).Today, more than 95% of the consumable Astaxanthin used in the world is produced industrially; while the natural one of *H. pluvialis* is about <1% (Koller *et al.*, 2014). The synthetic Astaxanthin contains C10 -aldehyde and asta-C15 - triarylphosphonium salt that connect in a Wittig reaction (Krause *et al.*, 1997), The natural antioxidant AX has 20 times the capacity compared to the industrial sample and is very useful for humans (Lorenz and Cysewski, 2000). The appropriate natural one is a preferred choice for human consumption, because of its benefits and antioxidant power (Li *et al.*, 2011). This microalgae (*H. pluvialis*) has a complex life cycle that includes four different morphological stages (Micro, Macrospore, Palmela, and Apalanspur). The life cycle change of *Haematococcus pluvialis* depends on environmental conditions can affect the antioxidant power of astaxanthin, which means that if the microalgae cells in the aplanospore (red blood cell) stage are placed in a suitable culture medium without any stress, they re-enter the microspore (green cell) stage. (Boussiba, 2000), (Li *et al.*, 2011).

The best medium cultures for Astaxanthin production

Depending on the purpose, there are many medium cultures for growing microalgae. For example, in the *Phaffia rhodozyma* mutant (NCHU-FS501), the addition of glucose to agar in malt yeast culture had a high positive effect on AX production. (Fang and Chiou, 1996). A new two-stage mix trophic culture system using NIES-C media (Park *et al.*, 2014) and an attached cultivation system using the immobilized biofilm in BG-11 medium(Zhang *et al.*, 2014) were invented for Astaxanthin production for *H.pluvialis*. In the autotrophic media for cell growth and Astaxanthin production, BBM, in the mixotrophic media also KM1, MM2 and KM2 were the best respectively, for carotenoid synthesis KM1in Agar was the most appropriate medium. KM2 with trace elements and B vitamins was excellent for the production of astaxanthin (Fang and Chiou, 1996). Optimal Astaxanthin production (0.8 g.l mg.l^{-1} or 3.8% of Dry wigth (DW))was obtained in initial biomass densities (IBDs) with no nitrogen, and maximum Astaxanthin (16.0 mg. l^{-1}) earned in 0.8 (g.l^{-1}) (DW), with 4.4 mM nitrogen in medium culture (Wang *et al.*, 2013a). No P and low N with Photosynthetically active radiation 240 µmol photons m^{-2} sec^{-1} (PAR) in BBM medium culture produced the best biomass containing 33% lipids that had more saturated fatty acids, and less unsaturated fatty acids (linoleic, oleic, and α/γ-linolenic) (Saha *et al.*, 2013). Researchers displayed that the best compounds for optimal *H .lacustris* growth rate were 0.25 (g.l^{-1}) potassium nitrate, 0.51 (g.l^{-1}) sodium acetate, 0.63 (ml.l^{-1}) the major element stock solution and 0.2 (ml.l^{-1}) trace elements. In another study it was displayed that the special compounds for the most biomass production were1.64 (g.l^{-1}) sodium acetate, 0.37 (g.l^{-1}) potassium nitrate, 2.52 (ml.l^{-1}) the major element stock solution and 0.03 (ml.l^{-1}) the trace element stock.(Gong and Chen, 1997) .

H.pluvialis (strain vischer 1923/2) grows the best at high nitrate concentration (about 0.5– 1.0 g.l^{-1}), phosphate concentration of (0.1.g.l^{-1} K2HPo4) and much amount of Fe^{2+}. Nitrate Decreasing or phosphate increasing induces the exchange of green cells to reddish palmella cells. Acetate improves green and red cells that contain Astaxanthin. The maximum temperature and salinity for *H.pluvialis* are 28C° and approximately above1% NaCl (Li *et al.*, 2011). *H.pluvialis* grows well in high nitrate concentration (about 0.5 to 1.0 g.l^{-1} KNO3), the average rate of phosphate (about 0.1 g.l^{-1} K_2HPO_4) and most of Fe. Low concentration of nitrate and high rate of phosphate initiate Astaxanthin production (Li *et al.*, 2011). Addition of acetate and ferrous ion to the basal medium culture (BBM) of *H.pluvialis* exchange green cell to red cells containing Astaxanthin. For carotenoid and AX precursor formations,

 doi:10.20944/preprints202302.0063.v1

mevalonate and pyruvate are useful and pyruvate is very special (Kobayashi *et al.*, 1991). *H. pluvialis* in rich medium culture with FeZ^{+} and acetate in high light produced the most amount of Astaxanthin. Continuous illumination was better than cyclic illumination. Also, the type of light is important for increasing astaxanthin. Red and blue lights are the best spectra for AX formation (Kakizono *et al.*, 1992). Environmental factors on cell growth and astaxanthin production were studied in *H.pluvialis.* A group of researchers discovered that maximum growth rate of *H.pluvialis* earned in BBM medium culture in 28°C, continues white fluorescent illumination and, direct aeration, while maximum Astaxanthin production determined in BAR medium culture, continuous illumination and sodium acetate without aeration (Domínguez-Bocanegra and Torres-Muñoz, 2004). *H.pluvialis* can grow both heterotrophically and mixotrophically by acetate in the dark and light, respectively. There are two independent metabolic pathways for mixotrophic growth of *H.pluvialis* (Kobayashi *et al.*, 1992). Acetate and especially acetate plus Fe^{2+} can change *H.pluvialis* green cells to red cyst cell. Acetate and Fe^{2+}induce green and red cyst cells, respectively (Kobayashi *et al.*, 1993). In a study, two microalgae (*Tetraselmis* sp *and C. sorokiniana*) were investigated for Astaxanthin production. It was produced in aerated and unaerated conditions.(Rasid *et al.*, 2014) . Scientists have developed a new method for extracting astaxanthin using hydrochloric acid at specific time and temperature (Sarada *et al.*, 2006). Distilled water with CO2 without N with illumination of 546 µmol photons (m^{-2} s^{-1}) was the best medium culture for the production of astaxanthin from Haematococcus.(Imamoglu *et al.*, 2009). Growth factors for *H.pluvialis* growth were measured in an experimental design. The best temperature for astaxanthin production was 25 ° C to 28 ° C and saturation radiation of 90 µmol quantum ($m^{-2}s^{-1}$) (Fan *et al.*, 1994). An *H.pluvialis* medium culture optimization test was performed between 18 compounds in semi-continuous culture to determine the highest productivity. The best result was obtained in %20 OHM medium culture that contained 3.77×10^{5} cells. Ml^{-1}, it was three times as much activity compared with BBM medium culture, and higher cell density was obtained using three times nutrition (Fábregas *et al.*, 2000). Scientists invented a two-stage culture system to produce astaxanthin from *H. pluvialis*. In this method, green cells were grown in semi-continuous cultures and then exposed to high light in batch cultures to produce astaxanthin. (Fábregas *et al.*, 2000) In an experiment performed by a group of scientists, many factors were tested for the formation of astaxanthin. Nitrogen and phosphate deficiency, high levels of iron and radiation alone increase AX formation(Harker *et al.*, 1996). The effect of some factors such as high light intensity and starvation of iron, sulfur and phosphate to produce more astaxanthin in medium culture was tested in an experiment, so especially sulfur starvation had the best results. (He *et al.*, 2007). In a comparison experiment between new photobioreactors for *H.pluvialis* culture, NIES-144 and 3-lC-ALPBR photobioreactors had the highest cell growth rate and 90-l FP -ALPBR had the highest economic efficiency (Issarapayup *et al.*, 2009). The effects of surface response (RSM) NaNo3, Co2, light intensity and inoculation volume on the growth of *Haematococcus pluvialis* cells were studied. Good results were obtained in NaNo3 1.06 g $.l^{-1}$, Co2 1.54, light intensity 2.42 Klux and inoculation volume 24.97 (Issarapayup *et al.*, 2009). In one study, the effects of temperature and light / dark cycle on astaxanthin production were investigated. The results showed that increasing the night temperature reduces astaxanthin, while increasing the light temperature increases its content. (Wang *et al.*, 2014). A new open pound system was tested to determine growth cells and produce astaxanthin (two-stage growth one-step process) in four strains of *Haematococcus pluvialis,* two strains were selected for mass culture, *H. pluvialis* 26 and *H. pluvialis* WZ which can produce 51.06 and 40.25 mg biomass per liter. (Zhang *et al.*, 2009). The important factors for Astaxanthin production in a variety of microalgae, *Haematococcus pluvialis* UTEX 16, were identified.

Among high irradiation, nitrogen deficiency, addition of acetate and addition of iron ions with acetate, addition of iron ions to acetate had a negative effect on astaxanthin production. (Choi *et al.*, 2002)In one study, important factors for biomass and astaxanthin production by RSM method in *Haematococcus pluvialis* (CCAP 7.34) were investigated. They were photon flux density (50–60 µmol.m^{-2} s^{-1} for biomass production, and ~1600 µmol $.m^{-2}$ s^{-1} for Astaxanthin accumulation), low amount of NaCl, and temperature between 14–15 °C, the most effective factor was irradiation (Harker *et al.*, 1995). When green *Haematococcus* was starved for phosphate or nitrogen separately or together, the

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rate of Astaxanthin accumulation raised up to 4% cell dry weight. Also Nitrogen starvation accumulated Astaxanthin faster and more than phosphate starvation (Boussiba *et al.*, 1999). The biomass of *Haematococcus pluvialis* in Chemostat culture with dilution rate of 0.9 per day, brightness level of 1.220 (µE/m2 · s) and different nitrate levels from 1.7 to 20.7 (mM), is related to nitrate level. NaNo3 0.15 ($g.l^{-1}$) was the best factor for astaxanthin production and cell growth. The use of malonate in culture increased carotenoid synthesis by an average of 13 times (Orosa *et al.*, 2005). Batch culture with three medium cultures containing medium cultures of BBM, BG11 and 3NBBM was studied. The best BBM culture medium was the best for growth. BBM medium with 3 times phosphate produced 86% growth and maximum cell density, also growth rate of *H.pluvialis* cells with inoculation size of 105 2 2 cells per liter in BBM medium with 0.046 (mM) iron or 0.185 (mM) boron Was greater than cell growth with two or half iron levels (Nahidian *et al.*, 2018). A new system for cultivation of *H.pluvialis* microalgae designed. In this system, a photo bioreactors (PBR) fence for green cells in addition to a raceway pond for red cells was designed. The result was excellent(Panis and Carreon, 2016). It is determined that photorespiration did not affect dry Wight, chlorophyll and OJIP transients. Photosystem II (PSII) system performance and total photosynthetic O2 evolution capacity were clearly reduced by inhibition of light respiration. Therefore, astaxanthin content is positively correlated with light respiration, so it plays an important role in the AX production cycle (Zhang *et al.*, 2016).

AX production was optimized by heterotrophic-photoautotrophic culture in *H.pluvialis* . The optimum situation for AX production was PH 8 and 25°C, different acetate concentrations were between 10 ($mM.l^{-1}$) and 30 ($mM.l^{-1}$) for cell growth. When growth environment was changed from heterotrophic to photoautotrophic, a decrease in a cell number was seen but AX accumulation was more significant. AX concentrations and productivity were 114 ($mg.L^{-1}$) and 4.4($mg.L^{-1}.day^{-1}$), respectively, which are high in current methods (Hata *et al.*, 2001). Maximum AX production was determined in 12-14 hours of light in *H.pluvialis* (Praveenkumar *et al.*, 2015). A new method for producing AX on an industrial scale was discovered. This method is based on Aquasearch Growth Module(AGM), the results of this method are: 1- Increasing biomass density 2- Increasing AX production (Olaizola, 2000). In a new study, a fed-batch medium culture was used to further grow *H.pluvialis* cells and produce AX, which contained 10 times more nutrients in addition to blue LEDs. In batch feeding culture, cell growth increased also AX production increased under the influence of blue LED. (Lababpour *et al.*, 2005).

The effects of light, temperature and C/N ratio on cell growth and AX production were measured in *H.pluvialis* . Light increased cell growth and AX production 6-fold. The cell growth in multidirectional light (three-directional) was increased 4 times more than control. Biomass in the low temperature (25°C) was increased while in higher temperature (35°C) AX production increased. (Tripathi *et al.*, 2002).Irradiation (170 µmol $.m^{-2}s^{-1}$), low phosphate and salt increasing (NaCl 0.8%) increased AX synthesis and decreased cell biomass in *Haematococcus pluvialis*. One result of this study was that AX synthesis increased when *Haematococcus pluvialis* cell division was inhibited. (Boussiba *et al.*, 1999). A three-dimensional reconstruction experiment was performed to understand how AX accumulates in *H.pluvialis* cells. This study showed that the formation of AX in motile cells and cysts is directly related to the resizing of intracellular organs, some of which are chloroplasts, nuclei and mitochondria. The chloroplast has inverse proportion with AX in cyst cells, also starch grain, cell walls, Golgi apparatus, nucleus, and mitochondria in a cyst cell become smaller than those in movable stage cells. In the AX synthesis step, chloroplasts break down but are not completely removed in red cyst cells (Wayama *et al.*, 2013) . A new method was designed by combining two methods to increase commercial biomass and AX. This method uses two strategies: 1- maximum production in minimum time and 2- continuous cultivation under limited stress. The volume of biomass and AX was about 4% of dry biomass and AX 1.5 ($mg .L^{-1}$), which can be increased at any stage and other stresses such as radiation can be used. (Aflalo *et al.*, 2007). The effect of primary hematococcal biomass on astaxanthin accumulation in outdoor culture was measured that the optimal biomass was 0.8 ($g .l^{-1}$) and the highest AX accumulation was 1.17 ($mg . day^{-1}$). (Wang *et al.*, 2013b). Inoculation density of 10 M and radiation intensity of 100 (µmol . m^{-2} s^{-1}) with 1.8 (g . m^{-2}) NaNO3 in BG-11 medium culture

 doi:10.20944/preprints202302.0063.v1

were the best conditions for *H.pluvialis* growth and AX production (Katsuda *et al.*, 2006). BBM was the best medium culture for autotrophic growth, KM1 was the best medium culture for AX production. Its production in *H.pluvialis* was induced by adding trace element and vitamin B to MM1 and MM2 medium cultures (Fang and Chiou, 1996). In continuous cultures of Chemostat with N deficiency in *Haematococcus lacustris* (Gir.) Rostaf (UTEX 16), the highest AX accumulation was obtained with the lowest dilution rate. One study found that pigment production was positively correlated with surface radiation density. AX content was independent of culture elements (Lee and Soh, 1991). The percentage of *Haematococcus pluvialis* MUR 145 Aplanospore components was determined in an assay. It contained (3*S*, 3′*S*)-astaxanthin diester 34%, canthaxanthin (4%), echinenone (4%), β-carotene (5%), (3*S*, 3′*S*)-astaxanthin monoester (46%), lutein (6%) and (3*S*,3′*S*)-astaxanthin (1%) (Chen *et al.*, 2015).

Astaxanthin producing Genes and engineering

Some scholars displayed that Astaxanthin synthesis is influenced by 8 genes in *H.pluvialis*, Astaxanthin synthesis is more controlled by carotenoid hydroxylase and less by isopentenyl isomerase، phytoene desaturase، phytoene synthase and carotenoid oxygenase, respectively (Shah *et al.*, 2016). In a study of the metabolic pathway of astaxanthin in *Haematococcus pluvialis* and *Chlorella zofingiensis*, two different pathways and their genes were discovered. (Danxiang Han &Yantao Li 2013)The transcripts of 8 genes that Participated in Astaxanthin biosynthesis were investigated in *H.pluvialis* , it was exposed on 2, 4-epibrassinolide (EBR) then transcripts were tested by the qRT-PCR method. The results showed that 25 and 50 mg $.l^{-1}$ EBR concentrations were the best for AX accumulation. Also 25($mg.l^{-1}$) EBR had more effect on the expression of ipi-1, ipi-2, crtR- B, *lyc*, and crtO relative to psy, pds, and *bkt*, also 50($mg.l^{-1}$) EBR more affected on some genes than other genes (Gao *et al.*, 2013b). Using the β-end ketolase gene from *Agrobacterium aurantiacum*, the metabolic pathway of Zeaxanthin β-d-diglucoside, as well as the new substances astaxanthin β-d-diglucoside and adonixanthin β-d-diglucoside were synthesized (Danxiang Han &Yantao Li 2013). It determined that Biosynthetic processes of carotenoids were performed with several enzymes produced by carotenoid genes in *H.pluvialis*. (Huang *et al.*, 2006). The biosynthetic pathway of Astaxanthin in *H. pluvialis* was demonstrated using the inhibitors and some of the related genes that were cloned. (Grünewald *et al.*, 2000). For example, two genes in *H. pluvialis* that encode isopentyl pyrophosphate isomerase, ipi1 and ipi2, are upregulated at the transcriptional level to facilitate the synthesis of pyrophosphate from isopentyl (EONSEON *et al.*, 2006). Phytoene synthase (psy) is the first step in the biosynthesis of carotenoids to form phytoene, which make up the entire carotenoid precursor (EONSEON *et al.*, 2006). Two enzymes that are similar in structure and activity, phytoene desaturase (pds) and carotene desaturase (zds), combine to convert colorless python to red lycopene, but their isomer structure has not yet been determined. (EONSEON *et al.*, 2006). Lycopene to β-carotene cycle contains lycopene beta catalysis enzymes (lyc) that are necessary for Carotene to Astaxanthin conversation under environmental stressors (EONSEON *et al.*, 2006). In the conversion of beta-carotene to astaxanthin, the enzymes beta-carotene ketolase (bkt2) (Gao *et al.*, 2012), beta-carotene oxygenase (crtO) and beta-hydroxylase (crtRB) perform the necessary oxygenation interaction. (Vidhyavathi *et al.*, 2008). Gene Regulation can be seen in RNA, especially in response to environmental stressors. (EONSEON *et al.*, 2006). For example, psy and crtR-B gene expression will be increased comprehensively when *H. pluvialis* exposed to light and salt, so Astaxanthin accumulation will be increased in the cell (Steinbrenner and Linden, 2001). The results show that Astaxanthin biosynthesis is associated with the regulation of gene expression (Vidhyavathi *et al.*, 2008). A new biosynthetic metabolic pathway involving five genes for astaxanthin biosynthesis has been developed by scientists (Misawa *et al.*, 1995). The new cDNA was obtained from *H.pluvialis* which can produce AX, and also contains the beta-carotene genes of the bacterium *Erwinia uredovora* (Kajiwara *et al.*, 1995). A gene isolated from *Xanthophyllomyces dendrorhous* was cloned into the beta-carotene mutant gene to produce Astaxanthin. The results showed that this gene has two functions, the first is ketolase and the second is hydroxylase. (Ojima *et al.*, 2006). Researchers found that the CrtS gene plays an important role in the conversion of β-carotene to xanthophyll 12. (Álvarez *et al.*,

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2006).Two genes, crtW38 and crtW148, were cloned from a cyanobacterium, Nostoc punctiforme PCC 73102, into Escherichia coli and expressed, both of which convert β-carotene to canthaxanthin, but only crtW148 was able to produce astaxanthin. (Steiger and Sandmann, 2004). In a gene engineering experiment, the gene that produces the enzyme carotenoid biosynthesis (phytoene desaturase) was extracted from *Haematococcus pluvialis* and mutated by site-induced mutagenesis. In a gene engineering experiment, a gene that produces the carotenoid biosynthesis enzyme and phytoene desaturase, was extracted from *Haematococcus pluvialis* and mutated by locally induced mutagenesis. In this mutation, the leucine codon in code 504 was changed to the arginine codon. The results showed that phytoene desaturase enzyme was active in the conversion of phytoene to ζ-carotene, and was 43 times more resistant about to herbicide norflurazon, also some transformed *H.Pluvialis* had higher Astaxanthin production than wild type in the high irradiation (Verwaal *et al.*, 2007). In one study, the light regulation of the beta-carotenoid gene and the synthesis of astaxanthin in *H.pluvialis* including four genes: lycopene cyclase, phytoene synthase, phytoene desaturase and carotenoid hydroxylase were studied. All four transcription genes showed higher synthesis of astaxanthin and carotenoids in high light than in normal light. Also, under blue and red light, transcription of carotenoid synthesis genes is increased and photosynthetic plastoquinone pool is used as a redox sensor to control carotenoid synthesis genes in microalgae. (Steinbrenner and Linden, 2003). AX gene expression was measured in three *H.pluvialis* strains under salinity stress for 10 days. Strains were New South Wales (NSW), South Australia (SA) and Queensland (QLD)). Transcription of seven carotenogenic genes (ipi-1, ipi-2, psy, lyc, crtR-B, bkt2 and crtO) increased differently between the three strains. In QLD and NSW strains, the expression of psy, lyc, bkt2, crtR-B and crtO was much higher, the level of gene expression was not exactly related to the AX content in the three strains. (Gao *et al.*, 2015)AX metabolite pathway contains the main enzyme β-carotene ketolase (BKT) that exchange β-carotene to echinenone and canthaxanthin. In the gene engineering experiment, the BKT gene was isolated from *H.pluvialis* and cloned into pRT100 as a vector and then transferred to the pCAMBIA 1304 binary vector. The TDNA of this vector using of Agrobacterium was put in *H.pluvialis* . Transformed *H.pluvialis* , tested with Southern blotting and also by PCR analysis. 2-3 folds AX and β-carotene, 8–10 folds echinenone and canthaxanthin were seen in results, also other genes that intermediated in AX production like phytoene synthase (*psy*), phytoene desaturase (*pds*), lycopene cyclase (*lcy*), *bkt*, and β-carotene hydroxylase (*bkh*) showed higher expression than untransformed cells (Gao *et al.*, 2015). One study showed that the synthesis of AX and fatty acids in *H.pluvialis* is not fully coordinated and that the relationship between them is coordinated through feedback in the metabolite pathway. AX esterification leads to the synthesis and accumulation of AX. Production and esterification of AX occurs in the endoplasmic reticulum, and the enzyme diacylglycerol acyltransferases is suggested to guide the diacylglycerol acyltransferases pathway. (Chen *et al.*, 2015). In one experiment, a new blue phototropin receptor (PHOT) cDNA was fabricated, cloned, and bioinformatics analyzed. The algal *phot* gene was first discovered in *H.pluvialis* , which cooperates in the control of AX biosynthesis (Cui *et al.*, 2017). The association between AX and fatty acid biosynthesis was demonstrated using a molecular model. Results showed that AX synthesis and esterification occurred in the endoplasmic reticulum, also a special diacylglycerol acyltransferases enzyme esterifies AX (Chen *et al.*, 2015). Some researchers have shown that the expression of PDS and CHY genes in *H.pluvialis* increased by 86.89 with the addition of folic acid. (Zhao *et al.*, 2015). Three beta-carotene ketolase (bkt) genes were identified in the metabolic pathway of AX biosynthesis. Bkt1, Bkt2, formerly called crtO, and newly discovered Bkt3, Bkt3 with Bkt2 share 95% of the DNA in the *H.pluvialis* nucleus. These genes are activated rapidly against stress (Huang *et al.*, 2006). In order to rapidly determine the pathway of AX biosynthesis, *H.pluvialis* mutants that were sensitive to inhibition were generated by random mutation. The result of this assay was the production of a new strain with cell biomass and AX production up to 79% and 153% more than control under normal light irradiation, respectively. (Zhao *et al.*, 2015). In another experiment, growing microalgae cells were exposed to mutagenic agents such as UV, EMS and NTG and then exposed to the herbicide Glufosinate. The mutants showed a 23 to 59 percent increase in carotenoid and AX production. The enzyme expression levels of phytoene desaturase, phytoene synthase, beta-

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carotene ketolase, lycopene cyclase and beta-carotene hydroxylase increased up to 22 times to the control (Kamath *et al.*, 2008). By adding MJ and GA3 to *H.pluvialis* culture, the expression level of three beta-carotene ketolase (bkts) genes increased. Activated Cis elements MJ and GA3 were identified upstream of the 5′ BKT regions(Lu *et al.*, 2010).

Effect of different stresses, Nanoparticles, Bio acids, and Phytohormones on microalgae growth and Astaxanthin enhancement.

1-Different stresses

A mutant in *Phaffia rhodozyma* (NCHU-FS501) showed that temperature and pH have very little effect on carotenoid production volume but play an important role in astaxanthin production and growth rate. (Wang *et al.*, 2013a). Salt stress can effectively cause astaxanthin to accumulate in *H. pluvialis* ،(Steinbrenner and Linden, 2001, Sarada *et al.*, 2002a)Salt stress (0.1% NaCl, MgCl2, KCl or CaCl2) increased astaxanthin production and cysts in *Haematococcus pluvialis* during 8 days of darkness. (Kobayashi *et al.*, 1997b). Increasing Salt and light, nitrogen and phosphate deficiency increased Astaxanthin production in the microalgae (Harker *et al.*, 1996). Microalgae cells exposed to high light (170 μmol . $m^{-2}s^{-1}$) and deficient in iron, sulfur and phosphate showed increased production of astaxanthin. (He *et al.*, 2007). High light and nitrogen starvation increased oleic acid and AX in *Haematococcus pluvialis*(Zhekisheva *et al.*, 2002).A study showed that high light and low nitrogen with a type cascade process such as increasing of D1 protein induce AX production and protects cellular PSII system with AX accumulation in the cell, but when the microalgae was covered from light, AX accumulated around Nucleolus(Wang *et al.*, 2003). Results of a test displayed that flashing blue Light Emitting Diode (LED) with light intensity (2–12 μmol m^{-2} s^{-1}), work cycle (17–67%) and regularity (25–200 Hz) was applied for maximum grow cell and AX accumulation in *H. Pluvialis*(*H.pluvialis*). (Katsuda *et al.*, 2006). The optimum situation for AX accumulation (>1.4%w/w) and AX syntheses in *H.pluvialis* (13 $mg.l^{-1}$) was in addition of sodium acetate 31–50 mM and sodium chloride 0.55–0.63% w/v to medium culture, also culture age was more important than two above objects. (Sarada *et al.*, 2002a). Also, the optimal growth of *H. pluvialis* was obtained in the concentration of (about 0.5 to 1.0 g $.l^{-1}$ KNO_3) nitrate, (about 0.1 $g.l^{-1}$ K_2HPO_4) phosphate concentration in addition to the wide range of iron. If the nitrate concentration decreases or the phosphate exceeds the amount mentioned, it accelerates the conversion of green microalgae cells into palmella and aplanospore and begins to produce AX. (Li *et al.*, 2011). The results of an experiment displayed that the best nitrate concentration for the microalgae growth and AX production in *H.pluvialis* was $NaNO_3$ 0.15 ($g.l^{-1}$) (Li *et al.*, 2011). Some scholars announced that the most effective factor in cell growth and AX production is light irradiation. Light intensity 75μE/m^2s induced the best result for biomass production (2.7$g.l^{-1}$) and light intensity of 160μE/m^2/s had the most effect on AX production (6.5$mg.l^{-1}$). The best result was obtained for the production of AX (30.07 mg . g^{-1}) in *Haematococcus pluvialis* in distilled water with CO2 without any nitrogen at 546 (μmol photon . m^{-2} s^{-1}) (Park and Lee, 2001). AX production was related to a high carbon/nitrogen ratio (Kakizono *et al.*, 1992). In batch culture, low temperature at night and suitable food increased dry biomass, also using blue and white light with a ratio of 3: 1, green cells were changed to red cells (Sun *et al.*, 2015). In a test *Haematococcus pluvialis* mutant(with ^{60}Co-γ irradiation) was exposed to 6% CO_2 and high light so the amount of AX and biomass increased (Sun *et al.*, 2015). The researchers showed that AX production in *H.pluvialis* increases with phototrophic conditions, nutritional deficiencies along with dilution and increased light radiation. (Ranjbar *et al.*, 2008). Each picogram of astaxanthin in *Haematococcus pluvialis* was associated with 3-5 picograms of oleic, palmitic and linoleic acids with triacylglycerol under stress (Ranjbar *et al.*, 2008). The highest accumulation of AX was obtained with high light irradiation and ٪15 Co2 in *H.pluvialis* . (Christian *et al.*, 2018). Four forms of oxygen include singlet oxygen, superoxide anion radical, hydrogen peroxide, and peroxy radical were able to exchange with Fe^{2+} in carotenoid formation of *H.pluvialis* (Kobayashi *et al.*, 1993). The results of an experiment showed that cell growth and AX production were good using three forms of iron containing $FeC_6H_5O_7$, Fe(OH) x ^{32x} and EDTA-Fe, and $FeC_6H_5O_7$ was the best. Cell density and AX accumulation were 2 and 7 times higher than those

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restricted to iron (Cai *et al.*, 2009). Excessive light exposure, nitrogen deficiency and acetate addition increase the AX content in the cell, Iron ions, along with acetate, also increase cellular AX in *H.pluvialis* . (Choi *et al.*, 2002). The results of one experiment showed that the highest AX production was obtained at pH 7, salinity ٪25 and 10 ppm Ag (Tan, 2016). Researchers published that NaCl (>1.0% w/v) was toxic for *H.pluvialis* culture, also the age of culture was effective for AX production. Calcium nitrate and PH=7 increased AX production in *H.pluvialis* (Sarada *et al.*, 2002b). Potassium acetate increased AX syntheses and productivity (Pan-utai *et al.*, 2017). AX production in *H.pluvialis* medium culture was considered by two ionic shortage, N^- and Mg^{2+}, accompanied by low and high light irradiation, 40 and 230 μmol m^2 s^{-1}, respectively. The most AX production (49.5 $\mu g.ml^{-1}$) was earned under N^- shortage with high light irradiation. Mg^{2+} shortage produced lower AX than N^- deficiency, 7 and 26 ($\mu g.ml^{-1}$) of AX was earned for the least and highest light irradiation respectively. Without the two elements, AX was lower than N deficiency alone but greater than Mg2 + deficiency. N deficiency alone had a greater effect than high light intensity on AX production (Fábregas *et al.*, 1998). The scientists claimed that a comparison between *H.pluvialis* and other green microalgae that were deficient in N in the medium culture showed that *H.pluvialis* produced the highest AX. The amount of AX in *H.pluvialis* was (22.7 mg/g biomass) while in *Scenedesmus vacuolatus*, *Protosiphon botryoides*, *Scotiellopsis oocystiformis*, *Chlorella zofingiensis,* and *Neochloris wimmeri* was 6.8, 2.7, 14.3, 10.9 and 19.2 mg AX /g biomass, respectively (Orosa *et al.*, 2001). AX synthesis has been shown to be depressed by some inhibitors such as norflurazon and diphenylamine (DPA), but neutral lipid biosynthesis is not inhibited. Sethoxydim, a fat biosynthesis inhibitor, rapidly reduces fat concentration but gradually reduces AX production in *H.pluvialis*. The synthesis of TAG (oleic acid) with high irradiation is not exactly related to the production of AX. Rather, TAG is the most required substance for AX esterification in *H.pluvialis* (Zhekisheva *et al.*, 2005). The researchers tested three *H.pluvialis* strains including New South Wales (NSW), South Australia (SA) and Queensland (QLD) to produce AX under 0.17 M NaCl for 10 days. AX content increased in all strains. SA, NSW, and QLD were up to 5.6, 16.2, and 17.7 $mg.g^{-1}$ dry weight (DW), respectively. (Gao *et al.*, 2015). The scientists measured the effect of CO2, temperature, pressure and ethanol on the extraction of AX from *H.pluvialis* . The results showed that at 2−4 ml/min CO2 motion, 313-353 K temperature, 20-55 MPa pressure and 67−7.5% (v/v) ethanol, the AX content in the extracted solvent was increased. When the rate of CO2 became higher than before, the amount of extracted solvent could be increased while AX content in the extract was stable, 12.3%, but the role of Ethanol as the receiver was enhancing of extracted AX (80.6%) along with average temperature and pressure (Machmudah *et al.*, 2006). A new method was discovered to convert E-AX to Z and 13-AX using ethyl acetate (70 ° C) plus I-TiO2 as catalyst. A new method was discovered for changing of E-AX to Z and 13- AX using of ethyl acetate (70 °C) with I-TiO_2 as a catalyst, Because 13-AX has the highest antioxidant activity (Yang *et al.*, 2017).

2- Nanoparticles

Nanoparticles usually have a toxic effect on growth but have a positive stimulatory effect on AX biosynthesis in microalgae, here are some studies: Cd Nanoparticle in 10$mg.l^{-1}$ enhanced AX accumulation in *H. pluvialis* (Hong, 2016). The results of several observations showed that nanoparticles of ZnO, TiO2, Ag and platinum in contact with the microalga *p.subcapitata* slow down its growth(Déniel *et al.*, 2019). 50 $\mu M.l^{-1}$ iron(II)NPs along with high temperature (23.4–33.5 ◦C) enhances AX production up to 147% compared with that of low temperature (17.5–27.3 ◦C) (Hong *et al.*, 2016). Cerium oxide NP in contact with microalga causes the accumulation and sedimentation of it. Also, silicon dioxide nanoparticles cause the microalga *Chlorella kessleri* to fade(Déniel *et al.*, 2019). A group of scientists demonstrated that carbon, copper oxide and cobalt NPs by blocking light and shading to *Platymonas subcordiforus, Chaetoceros curvisetus* and *Skeletonema costatum* microalgae reduce their growth, respectively.(Déniel *et al.*, 2019). Amino clay-conjugated TiO_2 NPs increased AX, KR-1 and fatty acid in *Chlorella sp* and *H.pluvialis* (Lee *et al.*, 2014). Cerium oxide NP covered with polystyrene (80 nm; 0,01$g.l^{-1}$) ruins the microalgae photosystem II (PS II) and destroy chlorophyll in C. *reinhardtii* (Déniel *et al.*, 2019). Ag (2.83 ± 0.70 $mg.l^{-1}$) and Cd (4.98 ± 0.09 $mg.l^{-1}$) nanoparticles increased AX and cell density in *H.pluvialis* (Cheng *et al.*, 2018). Fe and Zn

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nanoparticles enhanced the production of AX, chlorophyll and biomass in *H.pluvialis* (Rastar *et al.*).The highest accumulation of astaxanthin (0.008 ± 0.458 mg .l^{-1}) was obtained under 10mg.l^{-1} Ag nanoparticles after 14 days in *H.pluvialis* . Lead nanoparticles have a negative effect on AX production and cell density in *H.pluvialis* (Rasheed, 2018). Copper nanoparticles at high concentrations have no effect on AX production in *H.pluvialis* . (Thinakren, 2018). The researchers used gold nanoparticles to slightly damage the *H.pluvialis* cell wall, which increased AX production in the recovery process. (Praveenkumar *et al.*, 2015). The effect of silver nanoparticles on green microalgae *Pithophora oedogonia* and *Chara Vulgaris* was investigated. The results showed that Ag nanoparticles reduced chlorophyll, chromosomes, mitotic chromosome distribution and cell growth(Dash *et al.*, 2012). Some scientists have shown that polystyrene nanoparticles can increase the response of microalga (*Chlamydomonas reinhardtii*) to certain environmental stresses, such as nitrogen deprivation (Déniel *et al.*, 2020). AuNPs (5 nm) were able to increase carotenoid synthesis by 42.7% compared to untreated microalgae (*Chlorella zofingiensis*). (Li *et al.*, 2020). Liquid fluorescent carbon nanotubes (C-paints) can increase the efficiency and production of astaxanthin in *H.pluvialis* (Choi *et al.*, 2020). The toxic effect of ZnO NPs from 10-200 µg . ml^{-1} in 24 h for 96 h on the production of astaxanthin in *H.pluvialis* was demonstrated. (Djearamane *et al.*, 2019). 10 ppm for both Ag (0.83 mg .l^{-1}) and Cd (4.98 mg .l^{-1}) nanoparticles increased the concentration of astaxanthin in *H.pluvialis* (Cheng *et al.*, 2018). Silver nanoparticles when applied simultaneously with silver salt on *C. reinhardtii* microalga produced oxygen free radicals which reduced the growth of microalga. (Déniel *et al.*, 2019) CU NPs diminished growth and astaxanthin content in *H.pluvialis* (Wong *et al.*). Scientists have shown that *Dunaliella salina*, when exposed to salicylic acid, treatment with AgNPs cannot affect its astaxanthin (Bahador *et al.*, 2019). Some researchers have shown that magnesium aminoclay (MgAC) NPs at a concentration of 1.0 g.l^{-1} increase the amount of astaxanthin 13.7 times compared to the control treatment in *H.pluvialis* (Kim *et al.*, 2020a). It was shown that 2.49 mg.l^{-1} Fe NPs plus 4.41 mg.l^{-1} Zn NPs enhances astaxanthin, fatty acid, and chlorophyll to the highest concentration which is 7.29 mg.l^{-1} for astaxanthin (Kim *et al.*, 2020a). The scientists showed that Ag NPs with 10 mg.l^{-1}could increase the concentration of astaxanthin to 0.458 ± 0.002 mg.l^{-1}, but at higher concentrations of 100 and 200 mg.L^{-1}, the AX content decreased. (Hu *et al.*, 2020). Magnesium aminoclay (MgAC) nanoparticles in 10 mg.l^{-1} treatment could enhance astaxanthin, fatty acid content and the cell size in *Haematococcus pluvialis* compared to the control treatment (Kim *et al.*, 2020b). Addition of ZnO NPs at the rate of 200-72 µg.ml^{-1} to *H.pluvialis* culture was associated with reduced astaxanthin, biomass, bioavailability, intracellular and extracellular damage. (Djearamane *et al.*, 2019). Al203 nanoparticles increased the growth of *Chlorella sp* at a concentration of 1000 mgl^{-1} by 18.9% after 4 days.(Ji *et al.*, 2011). A group of scientists showed that when copper oxide nanoparticles are placed in the culture medium of *Chlamydomonas reinhardtii*, it increases growth at low concentrations (0.1 mg L^{-1}) and decreases its growth at high concentrations (1000 mg L^{-1}). A similar result was obtained in the study on *C. reinhardtii* when exposed to Cr_2O_3 nanoparticles at concentrations of 0.1 and 1 g .l^{-1}. (da Costa *et al.*, 2016). A group of scientists showed that 2.49 mg L^{-1} Fe NPs and 4.41 mg L^{-1} Zn NPs had the greatest effect on the growth and production of chlorophyll and astaxanthin in *Hematococcus polyvialis* microalgae compared to other forms and concentrations of these two elements(Rastar *et al.*, 2018). One scientific study determined that CU NPs in different concentrations from 10 to 200 mg L^{-1} has negative effects on biomass and astaxanthin production in *H.pluvialis.*

3- Bioacids, Phytohormones, etc

Some materials such as biocides, hormones, in different concentrations enhance either biomass and AX synthesis or one of them in different microalgae. There are many studies in this case, such as the following: In low irradiation (30 µmol. M^{-2} s^{-1}), methyl jasmonate (10µM) had little effect on AX enhancement while Salicylic acid (100 µM) enhanced AX production to 6.8 times compared to the control (Sarada *et al.*, 2002a). Some scientists have reported that ethanol and linoleic acid increased astaxanthin production by 142% and 282% in *H.lacustris*, respectively. (Liu *et al.*, 2020). A scientific study showed that linoleic acid and methyl jasmonate have the ability to increase astaxanthin alone and with the help of salt several times more than the control in *C. sorokiniana* and *S. acutus*

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microalgae(Khalili *et al.*, 2019). The group also displayed that the addition of 32 μM LA to the culture medium of *Chlorella sorokiniana* with and without light stress increases the content of astaxanthin and biomass in microalgae(Khalili *et al.*, 2020). A scientific study showed that Jasmonic acid increases biomass in *Chlorella vulgaris.(Jusoh et al., 2015)*. Some researchers have reported that fulvic acid (FA) increases both lipid and astaxanthin levels in high light and nitrogen deficiency in *H.pluvialis*. (Zhao *et al.*, 2019).Production and accumulation of AX in *H.pluvialis* were increased using jasmonic acid (JA) treatment (50 $mg.L^{-1}$) (Gao *et al.*, 2012). Malonate has a positive effect on the production of carotenoids up to 13 times in *H.pluvialis* . Malonate has a positive effect on carotenoid production until 13 times in *H.pluvialis* (Li *et al.*, 2011). Butyl hydroxyanisd enhanced AX accumulation and biomass culture in *H.pluvialis* (Shang *et al.*, 2016). The highest amount of AX (31.32$mg.g^{-1}$) or 2.36 times the control treatment was obtained after treatment with 10$\mu M.L^{-1}$ melatonin. (Ding *et al.*, 2018). Abscisic acid (ABA) can convert green cells into cyst cells but does not stimulate AX production in *H.pluvialis* (Kobayashi *et al.*, 1997a). The results showed that Arachidonic acid (AA) at moderate concentrations (62.5 $mg.L^{-1}$) increased *H.pluvialis* cell growth but at low concentrations (0.1-12.5 $mg.L^{-1}$) and high concentrations (312.5-1562.5 $mg.l^{-1}$) reduced it, but AA in high concentrations(312.5 -1562.5 $mg.L^{-1}$) increased AX production by 48.8% compared to control(Wang *et al.*, 2010). Fulvic acid increased AX accumulation in *H.pluvialis* LUGU by 86.89% and 9.78% at 5 and 10 $mg.L^{-1}$, respectively (Zhao *et al.*, 2015). The effect of methyl jasmonate (MJ) and gibberellin A3 (GA3) on the AX biosynthesis pathway were measured. *H.pluvialis* cells exposed to MJ and GA3 showed some increase in AX production (Lu *et al.*, 2010). A group of academics found that increasing both light and salicylic acid together increased the production of astaxanthin and fat, and that there was a link between the gene network of them. (Hu *et al.*, 2021). Some scholars showed that addition of 100 μM gibrelin acid to the medium culture of the marine Chrysophyte *Monodopsis subterranean* increased biomass and total fatty acid up to 3.3 and 3.9 folds respectively, also the addition of 1 μM methyl jasmonate to it increased it's biomass up to 2.7 folds. (Arora and Mishra, 2021).

Auxins

In a study it was displayed that addition of an auxin-producing symbiotic bacterium (*Achromobacter* sp) to *Haematococcus pluvialis* increased growth (biomass) and extended exponential growth phase(Lee *et al.*, 2019). A group of scientists in a scientific study stated that a concentration of 10 μM IAA produced the highest production of biomass (431 $mg\,l^{-1}$), protein and carotenoids among the other two auxins, including 1-naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acetic acid (2,4-D) in the green microalga *Ankistrodesmus falcatus* CMSACR1001. (Vijay *et al.*, 2020). The results of one scientific study showed that auxins such as Indole Propionic acid and Indole Acetic acid promote astaxanthin and lipid content in the microalga *Chromochloris zofingiensis(Chen et al., 2020).* In general, the effect of auxins in microalgae and plants is similar.(Garshasbi *et al.*, 2009, Garshasbi *et al.*, 2012)

Cytokinins

Cytokines have a stimulating effect on the growth and carotenoids of microalgae, much like those of plants. Preliminary research has shown that the use of two types of cytokines increases cell division and dry weight of *C. vulgaris* microalgae(Burkiewicz, 1987). The results of a scientific study showed that increasing kinetin (a type of cytokinin) in the medium culture of *C. pyrenoidosa* increases cell division by up to 16% compared to the control treatment(Vance, 1987). The results of one study showed that cytokines (CKs) increase cellular growth and defense in microalgae. (Romanenko *et al.*, 2016). Scientists showed that cytokine(zeatin) has not any effect on Astaxanthin accumulation in *H.pluvialis*.(Yu *et al.*, 2015). Some scientists showed that cytokines in forty-five (45) microalgal species increased cell division, growth processes, and photosynthetic activity, also they had a cellular protective role. (Romanenko *et al.*, 2016). In two separate studies on two microalgae varieties of Chlorella including *C.pyrenoidosa* and *C.vulgaris*, the effect of 7 different types of cytokines was measured on them and it was found that adding N-6-benzylaminopurine (BAP), N-6-furfurylaminopurine (kinetin, Kin), and allantoin/5-ureidohydantoin (AT) to *C. pyrenoidosa* increases

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chlorophyll a, b, water-soluble proteins, and aldohexoses (Czerpak *et al*., 1999), also showed the greatest stimulant effect of N,N0 diphenylurea (DPU), kinetin (Kin), and trans-zeatin (Z) hormones in concentrations of 10^{-6} to 10^{-8} M on the microalga, *C.vulgaris,* While the order of the effect of cytokines on its growth and stimulation was as follows; DPU>Z> Kin>BA.(Piotrowska and Czerpak, 2009).

Gibberellins

The effect of gibberellin on the growth and development of algae is same as on plants, which means that up to a certain concentration increases growth in them(Brian, 1959).Some scholars stated that Gibberellins in microalgae lag phase decrease and increase cellular growth and division, respectively. Also increase biomass, carotenoids and proteins, but reduce the effects of heavy metals.(Romanenko *et al*., 2016). The results of a group of researchers showed that the addition of two compounds, methyl jasmonate and gibberellin A3 with an effect on the beta-carotene ketolase (bkt) gene caused more astaxanthin production in the microalga, *H. pluvialis*. (Lu *et al*., 2010). Some researchers have reported that gibberellin GA_3 at concentrations of 20 and 40 mg $.l^{-1}$increases the expression of some genes of the astaxanthin production pathway, such as bk2, ipi-2, psy, compared to other genes in this pathway in *H.pluvialis*. (Gao *et al*., 2013a). The results of a scientific study showed that the adding of 100 M gibberellin(GA_3) to *Scenedesmus obliquus* medium culture increased fresh weight by up to 30% and fatty acids by up to 42.6% of the control treatment.(Mansouri and Ebrahim Nezhad, 2020) . The results of a scientific study showed that *H. pluvialis* under stress with GA_3 5.0 mg L^{-1} produced earlier and more astaxanthin (6-46%) than GA_3 20 mg L^{-1} and control. (MENG and GAO, 2007).

Absic acids(ABA)

Abscisic acid often decreases growth, astaxanthin, and other growth parameters in microalgae such as plants. Results of one study revealed that ABA changes vegetative green cells to red cyst cells but not has any effect on astaxanthin formation in *H.pluvialis*.(Kobayashi *et al*., 1997a).

ABA decreased growth, astaxanthin, and lipids in the green microalga, *Chromochloris zofingiensis* (Chen *et al*., 2020). It showed that between some plant growth regulators (PGRs) such as 1-naphthaleneacetic acid (NAA); salicylic acid (SA); 2,4-epibrassinolide (EBR); 6-benzylaminopurine (6-BA); gibberellin (GA_3); ethephon (ETH); abscisic acid (ABA); and spermidine (SPD), ABA increased Lipid but decreased carotenoid content in *Chlorella vulgaris* (Lin *et al*., 2018). Indole-3-acetic acid, salicylic acid, abscisic acid, and methyl jasmonate were tested on BR2 gene expression of the carotenoid metabolic pathway in *Chlorella* sp. Results showed that only Indole-3-acetic acid induced carotenoid synthesis and ABA and other phytohormones decreased it (Alsenani *et al*., 2019).

Conclusion

In this review, many types of research and results about AX structure, different medium cultures, genes, and engineering also effects of different material, environmental stresses, nanoparticles, bio acids, and hormones on different microalgae especially *H. pluvialis* were studied. It was shown that there are many medium cultures for different microalgae to produce AX. There are generally 8 genes in the pathway of astaxanthin synthesis in *Haematococcus*. Most substances, bioacids and stresses increase the production of Astaxanthin to a special level in different microalgae. The effect of nanoparticles is not general. Some increase astaxanthin production, but most of them are toxic and reduce it and a few are ineffective. Often Auxins, Cytokinins and Gibberellins increase but Absic acids(ABA) decrease AX production in different microalgae. It is hoped that studies on AX and *H. pluvialis* will become easier every day, and find more efficient ways to produce natural AX because it has many benefits for humans.

Acknowledgments: We thank everyone who helped us complete this article, especially the professors of the University of Tehran.

 doi:10.20944/preprints202302.0063.v1

References

1. Aflalo, C., Meshulam, Y., Zarka, A.andBoussiba, S. (2007). On the relative efficiency of two-vs. one-stage production of astaxanthin by the green alga Haematococcus pluvialis. *Biotechnology and bioengineering,* 98**,** 300-305.
2. Al-Bulishi, M. S. M., Changhu, X.andTang, Q.-J. (2015). Health aspects of astaxanthin: a review. *Canad. J. Clin. Nutr,* 3**,** 71-78.
3. Alsenani, F., Wass, T. J., Ma, R., Eltanahy, E., Netzel, M. E., et al. (2019). Transcriptome-wide analysis of Chlorella reveals auxin-induced carotenogenesis pathway in green microalgae. *Algal research,* 37**,** 320-335.
4. Álvarez, V., Rodríguez-Sáiz, M., De La Fuente, J. L., Gudiña, E. J., Godio, R. P., et al. (2006). The crtS gene of Xanthophyllomyces dendrorhousencodes a novel cytochrome-P450 hydroxylase involved in the conversion of beta-carotene into astaxanthin and other xanthophylls. *Fungal Genet Biol,* 43.
5. Ambati, R. R., Phang, S.-M., Ravi, S.andAswathanarayana, R. G. (2014). Astaxanthin: sources, extraction, stability, biological activities and its commercial applications—a review. *Marine drugs,* 12**,** 128-152.
6. Arango, I. (1996). Magnitude scaling factors for soil liquefaction evaluations. *Journal of Geotechnical Engineering,* 122**,** 929-936.
7. Arora, S.andMishra, G. (2021). Effect of gibberellin, methyl jasmonate and myoinositol on biomass and eicosapentaenoic acid productivities in the eustigmatophyte Monodopsis subterranea CCALA 830. *Journal of Applied Phycology,* 33**,** 287-299.
8. Bahador, E., Einali, A., Azizian-Shermeh, O.andSangtarash, M. H. (2019). Metabolic responses of the green microalga Dunaliella salina to silver nanoparticles-induced oxidative stress in the presence of salicylic acid treatment. *Aquatic Toxicology,* 217**,** 105356.
9. Borowitzka, M. (1992a). Comparing carotenogenesis in Dunaliella and Haematococcus: implications for commercial production strategies. *Profiles on biotechnology. Universidade de Santiago de Compostela, Santiago de Compostela***,** 301-310.
10. Borowitzka, M. A. (1992b). Algal biotechnology products and processes—matching science and economics. *Journal of Applied Phycology,* 4**,** 267-279.
11. Boussiba, S. (2000). Carotenogenesis in the green alga Haematococcus pluvialis: Cellular physiology and stress response. *Physiologia Plantarum,* 108**,** 111-117.
12. Boussiba, S., Bing, W., Yuan, J.-P., Zarka, A.andChen, F. (1999). Changes in pigments profile in the green alga Haeamtococcus pluvialis exposed to environmental stresses. *Biotechnology letters,* 21**,** 601-604.
13. Brian, P. (1959). Effects of gibberellins on plant growth and development. *Biological reviews,* 34**,** 37-77.
14. Burkiewicz, K. (1987). The influence of gibberellins and cytokinins on the growth of some unicellular Baltic algae.
15. Cai, M., Li, Z.andQi, A. (2009). Effects of iron electrovalence and species on growth and astaxanthin production of Haematococcus pluvialis. *Chinese Journal of Oceanology and Limnology,* 27**,** 370.
16. Chen, G., Wang, B., Han, D., Sommerfeld, M., Lu, Y., et al. (2015). Molecular mechanisms of the coordination between astaxanthin and fatty acid biosynthesis in Haematococcus pluvialis (Chlorophyceae). *The Plant Journal,* 81**,** 95-107.
17. Chen, J.-H., Wei, D.andLim, P.-E. (2020). Enhanced coproduction of astaxanthin and lipids by the green microalga Chromochloris zofingiensis: Selected phytohormones as positive stimulators. *Bioresource Technology,* 295**,** 122242.
18. Cheng, W. H., Wong, L. S., Hong, Y. Z., Tan, Y. M.andAhmad, Z. A. (2018). The effect of Argentum and Cadmium towards astaxanthin content in green algae, Haematococcus pluvialis. *Asian Journal of Microbiology, Biotechnology and Environmental Sciences,* 20**,** 43-47.
19. Choi, S.-A., Jeong, Y., Lee, J., Huh, Y. H., Choi, S. H., et al. (2020). Biocompatible liquid-type carbon nanodots (C-paints) as light delivery materials for cell growth and astaxanthin induction of Haematococcus pluvialis. *Materials Science and Engineering: C,* 109**,** 110500.
20. Choi, Y.-E., Yun, Y. S.andPark, J. (2002). Evaluation of factors promoting astaxanthin production by a unicellular green alga, Haematococcus pluvialis, with fractional factorial design. *Biotechnology progress,* 18**,** 1170-1175.
21. Christian, D., Zhang, J., Sawdon, A. J.andPeng, C.-A. (2018). Enhanced astaxanthin accumulation in Haematococcus pluvialis using high carbon dioxide concentration and light illumination. *Bioresource Technology,* 256**,** 548-551.
22. Cui, H., Chen, J., Wang, L., Hu, Z.andQin, S. (2017). Gene cloning and bioinformatics analysis of novel blue photoreceptor phototropin from green alga Haematococcus pluvialis. *Southwest China Journal of Agricultural Sciences,* 30**,** 2639-2647.
23. Czerpak, R., Krotke, A.andMical, A. (1999). Comparison of stimulatory effect of auxins and cytokinins on protein, saccharides and chlorophylls content in Chlorella pyrenoidosa Chick. *Polskie Archiwum Hydrobiologii,* 46.

24. Da Costa, C. H., Perreault, F., Oukarroum, A., Melegari, S. P., Popovic, R., et al. (2016). Effect of chromium oxide (III) nanoparticles on the production of reactive oxygen species and photosystem II activity in the green alga Chlamydomonas reinhardtii. *Science of The Total Environment,* 565**,** 951-960.
25. Danxiang Han &Yantao Li , Q. H. (2013). Astaxanthin in microalgae: pathways, functions and biotechnological implications. *ALGAE,* 28**,** 131-147.
26. Dash, A., Singh, A. P., Chaudhary, B. R., Singh, S. K.andDash, D. (2012). Effect of silver nanoparticles on growth of eukaryotic green algae. *Nano-micro letters,* 4**,** 158-165.
27. Déniel, M., Errien, N., Daniel, P., Caruso, A.andLagarde, F. (2019). Current methods to monitor microalgae-nanoparticle interaction and associated effects. *Aquatic Toxicology,* 217**,** 105311.
28. Déniel, M., Errien, N., Lagarde, F., Zanella, M.andCaruso, A. (2020). Interactions between polystyrene nanoparticles and Chlamydomonas reinhardtii monitored by infrared spectroscopy combined with molecular biology. *Environmental Pollution,* 266**,** 115227.
29. Ding, W., Zhao, P., Peng, J., Zhao, Y., Xu, J.-W., et al. (2018). Melatonin enhances astaxanthin accumulation in the green microalga Haematococcus pluvialis by mechanisms possibly related to abiotic stress tolerance. *Algal research,* 33**,** 256-265.
30. Djearamane, S., Lim, Y. M., Wong, L. S.andLee, P. F. (2019). Cellular accumulation and cytotoxic effects of zinc oxide nanoparticles in microalga Haematococcus pluvialis. *PeerJ,* 7**,** e7582.
31. Domínguez-Bocanegra, A.andTorres-Muñoz, J. (2004). Astaxanthin hyperproduction by Phaffia rhodozyma (now Xanthophyllomyces dendrorhous) with raw coconut milk as sole source of energy. *Applied Microbiology and Biotechnology,* 66**,** 249-252.
32. Eonseon, J., Lee, C.-G.andPolle, J. E. (2006). Secondary carotenoid accumulation in Haematococcus (Chlorophyceae): biosynthesis, regulation, and biotechnology. *Journal of microbiology and biotechnology,* 16**,** 821-831.
33. Fábregas, J., Domínguez, A., Álvarez, D. G., Lamela, T.andOtero, A. (1998). Induction of astaxanthin accumulation by nitrogen and magnesium deficiencies in Haematococcus pluvialis. *Biotechnology letters,* 20**,** 623-626.
34. Fábregas, J., Domínguez, A., Regueiro, M., Maseda, A.andOtero, A. (2000). Optimization of culture medium for the continuous cultivation of the microalga Haematococcus pluvialis. *Applied Microbiology and Biotechnology,* 53**,** 530-535.
35. Fan, L., Vonshak, A.andBoussiba, S. (1994). Effect of temperature and irradiance on growth of Haematococcus pluvialis (Chlorophyceae). *Journal of phycology,* 30**,** 829-833.
36. Fang, T.andChiou, T. (1996). Batch cultivation and astaxanthin production by a mutant of the red yeast, Phaffia rhodozyma NCHU-FS501. *Journal of industrial microbiology,* 16**,** 175-181.
37. Gao, Z.andMeng, C. (2012). Induction of salicylic acid (SA) on transcriptional expression of eight carotenoid genes and astaxanthin accumulation in Haematococcus pluvialis. *Enzyme and Microbial Technology,* 51**,** 225-230.
38. Gao, Z., Meng, C., Chen, Y. C., Ahmed, F., Mangott, A., et al. (2015). Comparison of astaxanthin accumulation and biosynthesis gene expression of three Haematococcus pluvialis strains upon salinity stress. *Journal of Applied Phycology,* 27**,** 1853-1860.
39. Gao, Z., Meng, C., Gao, H., Li, Y., Zhang, X., et al. (2013a). Carotenoid genes transcriptional regulation for astaxanthin accumulation in fresh water unicellular alga Haematococcus pluvialis by gibberellin a3 (ga 3).
40. Gao, Z., Meng, C., Gao, H., Zhang, X., Xu, D., et al. (2013b). Analysis of mRNA expression profiles of carotenogenesis and astaxanthin production of Haematococcus pluvialis under exogenous 2, 4-epibrassinolide (EBR). *Biological Research,* 46**,** 201-206.
41. Gao, Z., Meng, C., Zhang, X., Xu, D., Zhao, Y., et al. (2012). Differential Expression of Carotenogenic Genes, Associated Changes on Astaxanthin Production and Photosynthesis Features Induced by JA in H. pluvialis. *PLOS ONE,* 7**,** e42243.
42. Garshasbi, H., Fakheri, B., Jalili, H., Rahaie Jahromi, M.andMahdinezhad, N. (2020). Astaxanthin Production and Expression of its Metabolic Pathway Genes under the influence of Linoleic Acid and Nanoparticles in Microalga (Hematococcus Lacustris). *Fisheries Science and Technology,* 9**,** 144-155.
43. Garshasbi, H., Omidi, M., Torabi, S.andDavodi, D. (2012). The study of phytohormones and explants on callus induction and regeneration of sainfoin (Pnobrychis sativa). *Pak. J. Agri. Sci,* 49**,** 289-292.
44. Garshasbi, H., Omidi, M., Torabi, S.andDavoudi, D. (2009). اثر هورمون گیاهی و ریز نمونه برکالزایی و باززایی اسپرس زراعی (Onobrychis sativa L.). *Iranian Journal of Crop Sciences,* 11**,** 101-108.
45. Gong, X.andChen, F. (1997). Optimization of culture medium for growth of Haematococcus pluvialis. *Journal of Applied Phycology,* 9**,** 437-444.
46. Grünewald, K., Eckert, M., Hirschberg, J.andHagen, C. (2000). Phytoene Desaturase Is Localized Exclusively in the Chloroplast and Up-Regulated at the mRNA Level during Accumulation of Secondary Carotenoids in Haematococcus pluvialis (Volvocales, Chlorophyceae). *Plant Physiology,* 122**,** 1261-1268.

47. Harker, M., Tsavalos, A. J.andYoung, A. J. (1995). Use of response surface methodology to optimise carotenogenesis in the microalga, Haematococcus pluvialis. *Journal of Applied Phycology,* 7**,** 399-406.
48. Harker, M., Tsavalos, A. J.andYoung, A. J. (1996). Factors responsible for astaxanthin formation in the chlorophyte Haematococcus pluvialis. *Bioresource Technology,* 55**,** 207-214.
49. Hassan Jalili, A. K. (2016). Effect of linoleic acid on pigments content of Chlorella sorokiniana under autotrophic and light shock condition. *Tehran university*.
50. Hata, N., Ogbonna, J. C., Hasegawa, Y., Taroda, H.andTanaka, H. (2001). Production of astaxanthin by Haematococcus pluvialis in a sequential heterotrophic-photoautotrophic culture. *Journal of Applied Phycology,* 13**,** 395-402.
51. He, P., Duncan, J.andBarber, J. (2007). Astaxanthin accumulation in the green alga Haematococcus pluvialis: effects of cultivation parameters. *Journal of Integrative Plant Biology,* 49**,** 447-451.
52. Higuera-Ciapara, I., Felix-Valenzuela, L.andGoycoolea, F. (2006a). Astaxanthin: a review of its chemistry and applications. *Critical Reviews in Food Science and Nutrition,* 46**,** 185-196.
53. Higuera-Ciapara, I., Félix-Valenzuela, L.andGoycoolea, F. M. (2006b). Astaxanthin: A Review of its Chemistry and Applications. *Critical Reviews in Food Science and Nutrition,* 46**,** 185-196.
54. Hong, M.-E., Choi, Y. Y.andSim, S. J. (2016). Effect of red cyst cell inoculation and iron(II) supplementation on autotrophic astaxanthin production by Haematococcus pluvialis under outdoor summer conditions. *Journal of Biotechnology,* 218**,** 25-33.
55. Hong, Y. Z. 2016. Effect of different Concentrations of Cadmium Nanoparticle, PH and Salinity on Production of Astaxanthin in Haematococcus Pluvialis. INTI International University.
56. Hu, Q., Hu, S., Fleming, E., Lee, J.-Y.andLuo, Y. (2020). Chitosan-caseinate-dextran ternary complex nanoparticles for potential oral delivery of astaxanthin with significantly improved bioactivity. *International Journal of Biological Macromolecules,* 151**,** 747-756.
57. Hu, Q., Huang, D., Li, A., Hu, Z., Gao, Z., et al. (2021). Transcriptome-based analysis of the effects of salicylic acid and high light on lipid and astaxanthin accumulation in Haematococcus pluvialis. *Biotechnology for Biofuels,* 14**,** 1-20.
58. Huang, J.-C., Chen, F.andSandmann, G. (2006). Stress-related differential expression of multiple β-carotene ketolase genes in the unicellular green alga Haematococcus pluvialis. *Journal of Biotechnology,* 122**,** 176-185.
59. Imamoglu, E., Dalay, M. C.andSukan, F. V. (2009). Influences of different stress media and high light intensities on accumulation of astaxanthin in the green alga Haematococcus pluvialis. *New biotechnology,* 26**,** 199-204.
60. Issarapayup, K., Powtongsook, S.andPavasant, P. (2009). Flat panel airlift photobioreactors for cultivation of vegetative cells of microalga Haematococcus pluvialis. *Journal of Biotechnology,* 142**,** 227-232.
61. Jafari, Y., Sabahi, H.andRahaie, M. (2016). Stability and loading properties of curcumin encapsulated in Chlorella vulgaris. *Food Chemistry,* 211**,** 700-706.
62. Jalili, H., Razavi, H., Safari, M.andAmrane, A. (2010). Kinetic analysis and effect of culture medium and coating materials during free and immobilized cell cultures of Bifidobacterium animalis subsp. lactis Bb 12. *Electronic Journal of Biotechnology,* 13**,** 2-3.
63. Ji, J., Long, Z.andLin, D. (2011). Toxicity of oxide nanoparticles to the green algae Chlorella sp. *Chemical Engineering Journal,* 170**,** 525-530.
64. Johnson, E. A.andAn, G.-H. (1991). Astaxanthin from Microbial Sources. *Critical Reviews in Biotechnology,* 11**,** 297-326.
65. Jusoh, M., Loh, S. H., Chuah, T. S., Aziz, A.andSan Cha, T. (2015). Elucidating the role of jasmonic acid in oil accumulation, fatty acid composition and gene expression in Chlorella vulgaris (Trebouxiophyceae) during early stationary growth phase. *Algal research,* 9**,** 14-20.
66. Kajiwara, S., Kakizono, T., Saito, T., Kondo, K., Ohtani, T., et al. (1995). Isolation and functional identification of a novel cDNA for astaxanthin biosynthesis from Haematococcus pluvialis, and astaxanthin synthesis in Escherichia coli. *Plant molecular biology,* 29**,** 343-352.
67. Kakizono, T., Kobayashi, M.andNagai, S. (1992). Effect of carbon/nitrogen ratio on encystment accompanied with astaxanthin formation in a green alga, Haematococcus pluvialis. *Journal of Fermentation and Bioengineering,* 74**,** 403-405.
68. Kamath, B. S., Vidhyavathi, R., Sarada, R.andRavishankar, G. (2008). Enhancement of carotenoids by mutation and stress induced carotenogenic genes in Haematococcus pluvialis mutants. *Bioresource Technology,* 99**,** 8667-8673.
69. Katsuda, T., Shimahara, K., Shiraishi, H., Yamagami, K., Ranjbar, R., et al. (2006). Effect of flashing light from blue light emitting diodes on cell growth and astaxanthin production of haematococcus pluvialis. *Journal of Bioscience and Bioengineering,* 102**,** 442-446.
70. Khalili, Z., Jalili, H., Noroozi, M.andAmrane, A. (2019). Effect of linoleic acid and methyl jasmonate on astaxanthin content of Scenedesmus acutus and Chlorella sorokiniana under heterotrophic cultivation and salt shock conditions. *Journal of Applied Phycology,* 31**,** 2811-2822.

71. Khalili, Z., Jalili, H., Noroozi, M., Amrane, A.andAshtiani, F. R. (2020). Linoleic-acid-enhanced astaxanthin content of Chlorella sorokiniana (Chlorophyta) under normal and light shock conditions. *Phycologia,* 59**,** 54-62.
72. Kim, Y.-E., Matter, I. A., Lee, N., Jung, M., Lee, Y.-C., et al. (2020a). Enhancement of astaxanthin production by Haematococcus pluvialis using magnesium aminoclay nanoparticles. *Bioresource Technology***,** 123270.
73. Kim, Y.-E., Matter, I. A., Lee, N., Jung, M., Lee, Y.-C., et al. (2020b). Enhancement of astaxanthin production by Haematococcus pluvialis using magnesium aminoclay nanoparticles. *Bioresource Technology,* 307**,** 123270.
74. Kobayashi, M., Hirai, N., Kurimura, Y., Ohigashi, H.andTsuji, Y. (1997a). Abscisic acid-dependent algal morphogenesis in the unicellular green alga Haematococcus pluvialis. *Plant Growth Regulation,* 22**,** 79-85.
75. Kobayashi, M., Kakizono, T.andNagai, S. (1991). Astaxanthin production by a green alga, Haematococcus pluvialis accompanied with morphological changes in acetate media. *Journal of Fermentation and Bioengineering,* 71**,** 335-339.
76. Kobayashi, M., Kakizono, T.andNagai, S. (1993). Enhanced carotenoid biosynthesis by oxidative stress in acetate-induced cyst cells of a green unicellular alga, Haematococcus pluvialis. *Applied and environmental microbiology,* 59**,** 867-873.
77. Kobayashi, M., Kakizono, T., Nishio, N.andNagai, S. (1992). Effects of light intensity, light quality, and illumination cycle on astaxanthin formation in a green alga, Haematococcus pluvialis. *Journal of Fermentation and Bioengineering,* 74**,** 61-63.
78. Kobayashi, M., Kurimura, Y.andTsuji, Y. (1997b). Light-independent, astaxanthin production by the green microalga Haematococcus pluvialis under salt stress. *Biotechnology letters,* 19**,** 507-509.
79. Koller, M., Muhr, A.andBraunegg, G. (2014). Microalgae as versatile cellular factories for valued products. *Algal research,* 6**,** 52-63.
80. Krause, W., Henrich, K., Paust, J.andErnst, H. (1997). Preparation of astaxanthin. *Google Patents*.
81. Lababpour, A., Shimahara, K., Hada, K., Kyoui, Y., Katsuda, T., et al. (2005). Fed-batch culture under illumination with blue light emitting diodes (LEDs) for astaxanthin production by Haematococcus pluvialis. *Journal of Bioscience and Bioengineering,* 100**,** 339-342.
82. Lee, C., Jeon, M. S., Kim, J. Y., Lee, S. H., Kim, D. G., et al. (2019). Effects of an auxin-producing symbiotic bacterium on cell growth of the microalga Haematococcus pluvialis: Elevation of cell density and prolongation of exponential stage. *Algal research,* 41**,** 101547.
83. Lee, Y.-C., Lee, H. U., Lee, K., Kim, B., Lee, S. Y., et al. (2014). Aminoclay-conjugated TiO2 synthesis for simultaneous harvesting and wet-disruption of oleaginous Chlorella sp. *Chemical Engineering Journal,* 245**,** 143-149.
84. Lee, Y. K.andSoh, C. W. (1991). Accumulation of astaxanthin in haematococcus lacustris (chlorophyta) 1. *Journal of phycology,* 27**,** 575-577.
85. Li, J., Zhu, D., Niu, J., Shen, S.andWang, G. (2011). An economic assessment of astaxanthin production by large scale cultivation of Haematococcus pluvialis. *Biotechnology Advances,* 29**,** 568-574.
86. Li, X., Sun, H., Mao, X., Lao, Y.andChen, F. (2020). Enhanced Photosynthesis of Carotenoids in Microalgae Driven by Light-Harvesting Gold Nanoparticles. *ACS Sustainable Chemistry & Engineering,* 8**,** 7600-7608.
87. Lin, B., Ahmed, F., Du, H., Li, Z., Yan, Y., et al. (2018). Plant growth regulators promote lipid and carotenoid accumulation in Chlorella vulgaris. *Journal of Applied Phycology,* 30**,** 1549-1561.
88. Liu, Z., Hou, Y., He, C., Wang, X., Chen, S., et al. (2020). Enhancement of linoleic acid content stimulates astaxanthin esterification in Coelastrum sp. *Bioresource Technology,* 300**,** 122649.
89. Lorenz, R. T.andCysewski, G. R. (2000). Commercial potential for Haematococcus microalgae as a natural source of astaxanthin. *Trends in Biotechnology,* 18**,** 160-167.
90. Lu, Y., Jiang, P., Liu, S., Gan, Q., Cui, H., et al. (2010). Methyl jasmonate-or gibberellins A3-induced astaxanthin accumulation is associated with up-regulation of transcription of β-carotene ketolase genes (bkts) in microalga Haematococcus pluvialis. *Bioresource Technology,* 101**,** 6468-6474.
91. Machmudah, S., Shotipruk, A., Goto, M., Sasaki, M.andHirose, T. (2006). Extraction of Astaxanthin from Haematococcus pluvialis Using Supercritical CO2 and Ethanol as Entrainer. *Industrial & Engineering Chemistry Research,* 45**,** 3652-3657.
92. Mansouri, H.andEbrahim Nezhad, S. (2020). Improvement in biochemical parameters and changes in lipid profile of Scenedesmus obliquus by plant growth regulators under mixotrophic condition. *Biomass and Bioenergy,* 140**,** 105708.
93. Meng, C.-X.andGao, Z.-Q. (2007). Effect of Extraneous Gibberellin (GA_3) on Astaxanthin Accumulation of Haematoccus pluvialis [J]. *Fisheries Science,* 5.
94. Miki, W. (1991). Biological functions and activities of animal carotenoids. *Pure and Applied Chemistry,* 63**,** 141-146.
95. Misawa, N., Satomi, Y., Kondo, K., Yokoyama, A., Kajiwara, S., et al. (1995). Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at the gene level. *J Bacteriol,* 177.

96. Nahidian, B., Ghanati, F., Shahbazi, M.andSoltani, N. (2018). Effect of nutrients on the growth and physiological features of newly isolated Haematococcus pluvialis TMU1. *Bioresource Technology,* 255**,** 229-237.
97. Ojima, K., Breitenbach, J., Visser, H., Setoguchi, Y., Tabata, K., et al. (2006). Cloning of the astaxanthin synthase gene from Xanthophyllomyces dendrorhous (Phaffia rhodozyma) and its assignment as a β-carotene 3-hydroxylase/4-ketolase. *Molecular Genetics and Genomics,* 275**,** 148-158.
98. Olaizola, M. (2000). Commercial production of astaxanthin from Haematococcus pluvialis using 25,000-liter outdoor photobioreactors. *Journal of Applied Phycology,* 12**,** 499-506.
99. Orosa, M., Franqueira, D., Cid, A.andAbalde, J. (2005). Analysis and enhancement of astaxanthin accumulation in Haematococcus pluvialis. *Bioresource Technology,* 96**,** 373-378.
100. Orosa, M., Valero, J., Herrero, C.andAbalde, J. (2001). Comparison of the accumulation of astaxanthin in Haematococcus pluvialis and other green microalgae under N-starvation and high light conditions. *Biotechnology letters,* 23**,** 1079-1085.
101. Pan-Utai, W., Parakulsuksatid, P.andPhomkaivon, N. (2017). Effect of inducing agents on growth and astaxanthin production in Haematococcus pluvialis: Organic and inorganic. *Biocatalysis and Agricultural Biotechnology,* 12**,** 152-158.
102. Panis, G.andCarreon, J. R. (2016). Commercial astaxanthin production derived by green alga Haematococcus pluvialis: A microalgae process model and a techno-economic assessment all through production line. *Algal research,* 18**,** 175-190.
103. Park, E.-K.andLee, C.-G. (2001). Astaxanthin production by Haematococcus pluvialis under various light intensities and wavelengths. *Journal of microbiology and biotechnology,* 11**,** 1024-1030.
104. Park, J. C., Choi, S. P., Hong, M.-E.andSim, S. J. (2014). Enhanced astaxanthin production from microalga, Haematococcus pluvialis by two-stage perfusion culture with stepwise light irradiation. *Bioprocess and biosystems engineering,* 37**,** 2039-2047.
105. Pérez-López, G., Plata-Díaz, A. M., Zafra-Gómez, J. L.andLópez-Hernández, A. M. (2014). Operaciones fuera de presupuesto (off budget), factores políticos y deuda municipal: Un estudio empírico aplicando una metodología de datos de panel. *Gestión y política pública,* 23**,** 185-218.
106. Piotrowska, A.andCzerpak, R. (2009). Cellular response of light/dark-grown green alga Chlorella vulgaris Beijerinck (Chlorophyceae) to exogenous adenine-and phenylurea-type cytokinins. *Acta Physiologiae Plantarum,* 31**,** 573-585.
107. Praveenkumar, R., Gwak, R., Kang, M., Shim, T. S., Cho, S., et al. (2015). Regenerative Astaxanthin Extraction from a Single Microalgal (Haematococcus pluvialis) Cell Using a Gold Nano-Scalpel. *ACS Applied Materials & Interfaces,* 7**,** 22702-22708.
108. Raman, V.andRavi, S. (2011). Effect of salicylic acid and methyl jasmonate on antioxidant systems of Haematococcus pluvialis. *Acta Physiologiae Plantarum,* 33**,** 1043-1049.
109. Ranjbar, R., Inoue, R., Shiraishi, H., Katsuda, T.andKatoh, S. (2008). High efficiency production of astaxanthin by autotrophic cultivation of Haematococcus pluvialis in a bubble column photobioreactor. *Biochemical Engineering Journal,* 39**,** 575-580.
110. Rasheed, N. 2018. The Production Of Astaxanthin In Freshwater Algae, Haematococcus Pluvialis with Lead Nanopparticle Exposure. INTI INTERNATIONAL UNIVERSITY.
111. Rasid, E. N. B. I., Mohamad, S. E., Jamaluddin, H.andSalleh, M. M. (2014). Screening factors influencing the production of astaxanthin from freshwater and marine microalgae. *Applied biochemistry and biotechnology,* 172**,** 2160-2174.
112. Rastar, M., Hosseini Shekarabi, S., Shamsaie Mehrgan, M.andSabz, S. (2018). Effects of iron and zinc concentrations on growth performance and biochemical composition of Haematococcus pluvialis: A comparison between nanoparticles and their corresponding metals bulks. *Journal of Algal Biomass Utilization,* 9**,** 59-67.
113. Rastar, M., Shekarabi, S. P. H., Mehrgan, M. S.andSabzi, S. Effects of iron and zinc concentrations on growth performance and biochemical composition of Haematococcus pluvialis: a comparison between nanoparticles and their corresponding metals bulks.
114. Romanenko, K., Kosakovskaya, I.andRomanenko, P. (2016). Phytohormones of microalgae: Biological role and involvement in the regulation of physiological processes. *International Journal on Algae,* 18.
115. Saha, S. K., Mchugh, E., Hayes, J., Moane, S., Walsh, D., et al. (2013). Effect of various stress-regulatory factors on biomass and lipid production in microalga Haematococcus pluvialis. *Bioresource Technology,* 128**,** 118-124.
116. Sarada, R., Bhattacharya, S., Bhattacharya, S.andRavishankar, G. (2002a). A response surface approach for the production of natural pigment astaxanthin from green alga, Haematococcus pluvialis: effect of sodium acetate, culture age, and sodium chloride. *Food Biotechnology,* 16**,** 107-120.
117. Sarada, R., Tripathi, U.andRavishankar, G. A. (2002b). Influence of stress on astaxanthin production in Haematococcus pluvialis grown under different culture conditions. *Process Biochemistry,* 37**,** 623-627.

118. Sarada, R., Vidhyavathi, R., Usha, D.andRavishankar, G. A. (2006). An Efficient Method for Extraction of Astaxanthin from Green Alga Haematococcus pluvialis. Journal of Agricultural and Food Chemistry 54, 7585-7588. *Journal of agricultural and food chemistry,* 54**,** 7585-7588.
119. Shah, M. M. R., Liang, Y., Cheng, J. J.andDaroch, M. (2016). Astaxanthin-Producing Green Microalga Haematococcus pluvialis: From Single Cell to High Value Commercial Products. *Frontiers in Plant Science,* 7.
120. Shang, M., Ding, W., Zhao, Y., Xu, J.-W., Zhao, P., et al. (2016). Enhanced astaxanthin production from Haematococcus pluvialis using butylated hydroxyanisole. *Journal of Biotechnology,* 236**,** 199-207.
121. Steiger, S.andSandmann, G. (2004). Cloning of two carotenoid ketolase genes from Nostoc punctiforme for the heterologous production of canthaxanthin and astaxanthin. *Biotechnology letters,* 26**,** 813-817.
122. Steinbrenner, J.andLinden, H. (2001). Regulation of two carotenoid biosynthesis genes coding for phytoene synthase and carotenoid hydroxylase during stress-induced astaxanthin formation in the green alga Haematococcus pluvialis. *Plant Physiology,* 125**,** 810-817.
123. Steinbrenner, J.andLinden, H. (2003). Light induction of carotenoid biosynthesis genes in the green alga Haematococcus pluvialis: regulation by photosynthetic redox control. *Plant molecular biology,* 52**,** 343-356.
124. Sun, H., Kong, Q., Geng, Z., Duan, L., Yang, M., et al. (2015). Enhancement of cell biomass and cell activity of astaxanthin-rich Haematococcus pluvialis. *Bioresource Technology,* 186**,** 67-73.
125. Tan, Y. M. 2016. The Effects of Ag Nanopaticles to the Production of Astaxanthin Algae with Different pH and Salinity Growth Conditions. INTI International University.
126. Thinakren, B. 2018. QUANTIFICATION OF ASTAXANTHIN PRODUCTION BY Haematococcus pluvialis INDUCED BY COPPPER NANOPARTICLES. INTI INTERNATIONAL UNIVERSITY.
127. Tripathi, U., Sarada, R.andRavishankar, G. A. (2002). Effect of culture conditions on growth of green alga — Haematococcus pluvialis and astaxanthin production. *Acta Physiologiae Plantarum,* 24**,** 323-329.
128. Vance, B. D. (1987). Phytohormone effects on cell division in Chlorella pyrenoidosa Chick (TX-7-11-05)(Chlorellaceae). *Journal of Plant Growth Regulation,* 5**,** 169-173.
129. Verwaal, R., Wang, J., Meijnen, J. P., Visser, H., Sandmann, G., et al. (2007). High-level production of beta-carotene in Saccharomyces cerevisiae by successive transformation with carotenogenic genes from Xanthophyllomyces dendrorhous. *Appl Environ Microbiol,* 73.
130. Vidhyavathi, R., Venkatachalam, L., Sarada, R.andRavishankar, G. A. (2008). Regulation of carotenoid biosynthetic genes expression and carotenoid accumulation in the green alga Haematococcus pluvialis under nutrient stress conditions. *Journal of experimental botany,* 59**,** 1409-1418.
131. Vijay, A. K., Prabha, S., Thomas, J., Kurian, J. S.andGeorge, B. (2020). Effect of auxin and its synthetic analogues on the biomass production and biochemical composition of freshwater microalga Ankistrodesmus falcatus CMSACR1001. *Journal of Applied Phycology,* 32**,** 3787-3797.
132. Wang, B., Zarka, A., Trebst, A.andBoussiba, S. (2003). Astaxanthin accumulation in Haematococcus pluvialis (Chlorophyceae) as an active photoprotective process under high irradiance. *Journal of phycology,* 39**,** 1116-1124.
133. Wang, C., Kim, J.-H.andKim, S.-W. (2014). Synthetic biology and metabolic engineering for marine carotenoids: new opportunities and future prospects. *Marine drugs,* 12**,** 4810-4832.
134. Wang, J., Han, D., Sommerfeld, M. R., Lu, C.andHu, Q. (2013a). Effect of initial biomass density on growth and astaxanthin production of Haematococcus pluvialis in an outdoor photobioreactor. *Journal of Applied Phycology,* 25**,** 253-260.
135. Wang, J., Sommerfeld, M. R., Lu, C.andHu, Q. (2013b). Combined effect of initial biomass density and nitrogen concentration on growth and astaxanthin production of Haematococcus pluvialis (Chlorophyta) in outdoor cultivation. *ALGAE,* 28**,** 193-202.
136. Wang, L., Li, H.andGong, Y. (2010). The effects of arachidonic acid (AA) on the cell growth and astaxanthin content in alge Haematococcus pluvialis. *Fisheries Science (Dalian),* 29**,** 142-146.
137. Wayama, M., Ota, S., Matsuura, H., Nango, N., Hirata, A., et al. (2013). Three-dimensional ultrastructural study of oil and astaxanthin accumulation during encystment in the green alga Haematococcus pluvialis. *PLOS ONE,* 8**,** e53618.
138. Wong, L. S., Batumalai, T., Cheng, W. H.andOng, G. H. The Effect of Copper Nanoparticle to Astaxanthin Content in Microalgae.
139. Xi, T., Kim, D. G., Roh, S. W., Choi, J.-S.andChoi, Y.-E. (2016). Enhancement of astaxanthin production using Haematococcus pluvialis. *Applied Microbiology and Biotechnology,* 100**,** 6231-6238.
140. Xia, B., Chen, B., Sun, X., Qu, K., Ma, F., et al. (2015). Interaction of TiO 2 nanoparticles with the marine microalga Nitzschia closterium: Growth inhibition, oxidative stress and internalization. *Science of The Total Environment,* 508**,** 525-533.
141. Yang, C., Zhang, L., Zhang, H., Sun, Q., Liu, R., et al. (2017). Rapid and Efficient Conversion of All-E-astaxanthin to 9Z- and 13Z-Isomers and Assessment of Their Stability and Antioxidant Activities. *Journal of agricultural and food chemistry,* 65**,** 818-826.

142. Yang, Y., Kim, B.andLee, J. (2013). Astaxanthin structure, metabolism, and health benefits. *J. Hum. Nutr. Food Sci*, 1**,** 1-1003.
143. Yu, X., Niu, X., Zhang, X., Pei, G., Liu, J., et al. (2015). Identification and mechanism analysis of chemical modulators enhancing astaxanthin accumulation in Haematococcus pluvialis. *Algal research,* 11**,** 284-293.
144. Zhang, B. Y., Geng, Y. H., Li, Z. K., Hu, H. J.andLi, Y. G. (2009). Production of astaxanthin from Haematococcus in open pond by two-stage growth one-step process. *Aquaculture,* 295**,** 275-281.
145. Zhang, C., Zhang, L.andLiu, J. (2016). The role of photorespiration during astaxanthin accumulation in Haematococcus pluvialis (Chlorophyceae). *Plant Physiology and Biochemistry,* 107**,** 75-81.
146. Zhang, W., Wang, J., Wang, J.andLiu, T. (2014). Attached cultivation of Haematococcus pluvialis for astaxanthin production. *Bioresource Technology,* 158**,** 329-335.
147. Zhao, Y., Shang, M., Xu, J.-W., Zhao, P., Li, T., et al. (2015). Enhanced astaxanthin production from a novel strain of Haematococcus pluvialis using fulvic acid. *Process Biochemistry,* 50**,** 2072-2077.
148. Zhao, Y., Xing, H., Li, X., Geng, S., Ning, D., et al. (2019). Physiological and metabolomics analyses reveal the roles of fulvic acid in enhancing the production of astaxanthin and lipids in Haematococcus pluvialis under abiotic stress conditions. *Journal of agricultural and food chemistry,* 67**,** 12599-12609.
149. Zhekisheva, M., Boussiba, S., Khozin-Goldberg, I., Zarka, A.andCohen, Z. (2002). ACCUMULATION OF OLEIC ACID IN HAEMATOCOCCUS PLUVIALIS (CHLOROPHYCEAE) UNDER NITROGEN STARVATION OR HIGH LIGHT IS CORRELATED WITH THAT OF ASTAXANTHIN ESTERS 1. *Journal of phycology,* 38**,** 325-331.
150. Zhekisheva, M., Zarka, A., Khozin-Goldberg, I., Cohen, Z.andBoussiba, S. (2005). INHIBITION OF ASTAXANTHIN SYNTHESIS UNDER HIGH IRRADIANCE DOES NOT ABOLISH TRIACYLGLYCEROL ACCUMULATION IN THE GREEN ALGA HAEMATOCOCCUS PLUVIALIS (CHLOROPHYCEAE)1. *Journal of phycology,* 41**,** 819-826.