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# Extraction of valuable biomolecules from microalga *Haematococcus pluvialis* assisted by electrotechnologies

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**Abstract:** The freshwater microalgae *Haematococcus pluvialis*, is well known as the cell factory of natural astaxanthin which composes up to 4-7 % of its total dry weight. The bioaccumulation of astaxanthin in *H. pluvialis* cysts seems to be a very complex process that depends on different unfavorable conditions during its cultivation. The red cysts of *H. pluvialis* develop a thick and rigid cell walls under unfavorable growing conditions. The biomolecules extraction typically requires application of rather complex cell disruption, extraction, and purification techniques. This short review provides the analysis of different steps in *H. pluvialis*'s up and downstream processing including cultivation and harvesting of biomass, cell disruption, extraction, and purification techniques. The useful information on structure of *H. pluvialis* cell, biomolecular composition, properties, and bioactivity of astaxanthin is collected. The special emphasis is given to the recent progress in application of different electrotechnologies during the growth stages and for assistance of the recovery of different biomolecules from *H. pluvialis*.

**Keywords:** microalga; *Haematococcus pluvialis*; extraction; astaxanthin; biomolecular composition; electrotreatment

## 1. Introduction

In recent years, the up and downstream processing problem of *H. pluvialis* microalgae attracts great attention [1–5]. The bio-production at low cost of natural astaxanthin using this microalga is still one of the market's most concerned issues. It remains a challenge to biosynthesis of natural astaxanthin using microalgae like *H. pluvialis* at a large scale. The main reason for the high cost of natural astaxanthin is that the recovery of the final product necessitates additional manufacturing steps. It includes the cultivation and downstream process of algal biomass; both require extensive energy. It involves cultivation, biomass harvesting, drying, cell wall perturbation, and extraction of targeted compounds.

The cultivation of *H. pluvialis* for natural astaxanthin production (known as a king of antioxidants), involves two phases green and red. During the second one, the red cysts or aplanospores accumulate a high amount of astaxanthin which accounts for ≈80% of the total carotenoids content. Accumulation of astaxanthin is rather complex process that typically includes specific abiotic stress application like nutrient starvation [6], pH [7], illumination stresses [8–10], temperature [11], irradiation or pressure stresses [12] and mixture of different stresses [13]. Particularly, the synergetic combination of different factors allowed improving of astaxanthin accumulation [13,14].

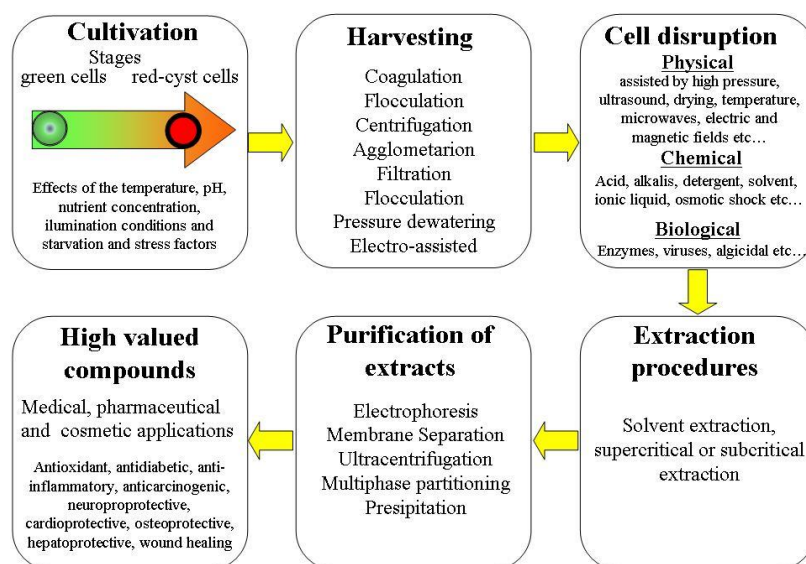
Application of electrotreatments during the growth stages allows genetic transformation, inactivation of culture contaminants, improvement of growth kinetics, accumulation of bio-actives and increasing of permeability of cell walls [15–17]. The cells of *H. pluvialis* are covered by extraordinarily thick, rigid and indigestible cell walls and the availability of internal biomolecules is very limited [18,19]. Therefore,

the selective recovery of these biomolecules and particularly astaxanthin from *H. pluvialis* requires application of rather complex cell disruption, extraction, and purification techniques. The topic is a very hot issue in contemporary studies of extraction from various microalgae [19]. The processing of *H. pluvialis* using the pulsed electrotechnologies (pulsed electric fields and (or) high-voltage electric discharges) is very attractive because at proper adaptation these techniques allows effective extraction and obtaining high purity extracts [20]. However, the thick cell walls of *H. pluvialis* cysts can restrict the extraction efficiency and applications of pulsed electrotechnologies. Therefore, careful adaptation and optimization of such treatments is required [21]. Nowadays, the existing literature on application of pulsed electrotechnologies for *H. pluvialis* is very limited.

In this review, the analysis of different steps of up and downstream processing in *H. pluvialis* biorefinery including cultivation and harvesting of biomass, cell disruption, extraction and purification techniques is presented. The useful information on structure of *H. pluvialis* cell, biomolecular composition, properties, and bioactivity of astaxanthin is collected. The special emphasis on recent progress in application of different pulsed electrotechnologies for recovery of biomolecules from *H. pluvialis* is given.

## 2. Main steps *Haematococcus pluvialis* biorefinery

In both laboratory and industrial scale, many important steps are included in the processing biorefinery of *H. pluvialis* microalgae. The general overviews of these steps are presented in Figure 1.



**Figure 1.** Overview of different steps in *H. pluvialis* microalgae processing biorefinery.

Each of these steps may be crucial in determination of quantity and quality of extracted bioactive molecules. In recent years, the different ways of *H. pluvialis* processing optimization are intensively discussed [1–3]. These ways include optimization of favorable and unfavorable conditions on cultivation stages, and screening of optimized techniques for harvesting of biomass, cell disruption, extraction, and purification processes.

## 3. Cultivation and growth stages of *Haematococcus pluvialis*. Effects of electrotreatment

The freshwater microalgae *H. pluvialis* was described for the first time in 1841 [22]. It has been reported that this microscopic object can occur in green and red forms. The green forms were observed in juvenile states. In cold seasons (winter, spring, and autumn), the green forms were mainly observed [23,24]. A detailed discussion of early studies of *H. pluvialis* was presented in 1899 [25]. *H. pluvialis* occurs in different regions of Europe, Africa, and North America [26].

### 3.1. Cultivation and growth stages

It is still acknowledged that the green microalga *H. pluvialis* recognized for its capacity to produce significant quantities of natural astaxanthin via the application of different stress conditions and its high specific growth rate  $1.3 \text{ d}^{-1}$ .

*H. pluvialis* astaxanthin production for the market is relatively new. The induction of astaxanthin bioaccumulation in *H. pluvialis* cells is carried out by subjecting cells to stress growth conditions. In last two decades, the two-stage strategy cultivation has been widely adopted both in laboratory and large-scale, where the first stage involves growing *H. pluvialis* under favorable conditions for green biomass production (green stage), followed by a second stage (red stage) in which the astaxanthin biosynthesis is induced by changing favorable growth conditions to unfavorable ones. The natural response to harsh environmental conditions involves the inhibition of normal growth, and the accumulation of astaxanthin is for the purpose to protect the photosynthetic apparatus from photooxidative damage by absorbing the excess light. The role of astaxanthin is important to protect cells scavenge free radicals and react with reactive oxygen species (ROS). This role is to ensure the maintain of the photosynthesis during red stage stress.

Obviously, the life cycle of *H. pluvialis* microalgae is complicated. The two stages culturing implies different cells forms. This life cycle can be roughly divided into a motile (bi-flagellated cells) and non-motile stage (aplanospores, astaxanthin-accumulating aplanospores, red cysts cells, sporangium and zoospores released from the sporangium) [19,27–29]. Each transition, from the motile to the non-motile cell form, involves remarkably changes in the cell wall structure.

Photosynthetic activity is highest in the initial vegetative green growth phase (days 1 to 12) [7]. In this green vegetative phase, the cells do not accumulate astaxanthin, and their carotenoids pattern is mainly composed of lutein (75–80%) and  $\beta$ -carotene (10–20%) [30]. However, the astaxanthin accumulation is in the second phase triggered by specific stress application such as nitrogen starvation, oxidative stress, salinity stress, high temperature, high irradiation. These cited factors affect significantly the astaxanthin accumulation.

The recent literature on cultivation and growth conditions of *H. pluvialis* is rather vast. It includes discussions of the effects of temperature [11], pH [7], nutrient concentration (nitrogen, phosphorus and carbon starvation) [6,31], and illumination conditions [8–10,32–34]. The effects of light illumination on the growth of biomass and accumulation of bioastaxanthin were analyzed and it was demonstrated that red light is more effective for increasing the biomass, while blue light supports the accumulation of bioastaxanthin [10]. A comprehensive examination of the effects of different photo-, mixo-, and heterotrophic cultivation conditions on *H. pluvialis* cell germination has been recently presented [35].

The effects of temperature (20–30.5 °C) on the cell growth and accumulation of astaxanthin has been discussed [11]. It was demonstrated that increased temperature allows enhanced astaxanthin accumulation combined with nitrogen starvation stress. It was demonstrated that the photosynthetic properties are differentially modulated in response to nitrogen starvation/high light stresses and a correct balance between these stresses is required for efficient astaxanthin production [36]. Effects of nuclear irradiation and high concentration of carbon dioxide  $\text{CO}_2$  stress on enhancing the growth rate and astaxanthin yield were demonstrated [12]. Advanced methods for genetic engineering of *H. pluvialis* were discussed in [37].

The economical two stage strategy with low light illumination in the initial phase (growth for biomass production) and combination of high light illumination and elevated carbon dioxide levels (5 or 15%) in the second phase (astaxanthin accumulation) has been tested [8]. The applied procedures allowed significantly increase (2–3 times) the accumulation of astaxanthin. A two-step process was proposed to maximize algal growth and astaxanthin yield [13]. During the first step (biomass production), the different nitrogen sources were tested. During the second step (carotenogenesis induction) a mix of moderate stressors (mild light, nitrogen limitation, the addition of sodium acetate) was used. The synergetic combination of different factors allowed promoting of astaxanthin accumulation. The new sequential heterotrophy-dilution-photoinduction cultivation strategy was shown to be rather effective for

production of astaxanthin from *H.pluvialis* [14]. The effects of continuous and interrupted (light/dark cycles) illumination conditions on the production of vegetative green cells and astaxanthin accumulation were investigated [38]. It was demonstrated that light/dark cycles are optimal to produce green cells whereas continuous illumination is better for astaxanthin accumulation. The effects of fulvic acid on biomass growth and astaxanthin accumulation were investigated [39]. It was demonstrated that the astaxanthin content increased by 86.89% in 5 mg/L treated cells.

Recently the details of laboratory and commercial scale cultivation procedures in the production of natural astaxanthin derived by *H.pluvialis* have been discussed [40–42]. The effects of different cultivation conditions and strategies, technological innovations, and types of cultivation systems and an economic assessment for astaxanthin production were also reviewed [43–50]

### 3.2. Electrotreatments during the growth stages

The effects of electrotreatments applied during the growth of *H.pluvialis* on cell viability, cell number density, and accumulation of astaxanthin attract great attention. General review on the application of different electrotechnologies for genetic transformation, inactivation of culture contaminants, and improvement of growth kinetics of different species of microalgae is given in [15]. Application of electrotreatments of *H.pluvialis* during the different growing stages may reduce the strength and impermeability of cell walls and positively affect the accumulation of bio-actives.

The effects of pulsed electric field (PEF) treatments with square wave and exponential decay wave pulses on cell viability during the growth stages were discussed [51]. Nanosecond PEF treatment (with very short pulses of 25–50 ns and high electric field strength 40 kV/cm) has been applied to *H.pluvialis* cells during the growth stage [16]. It was demonstrated that such treatment can modulate the expression of the astaxanthin biosynthesis genes. PEF treatment increased both the cell mortality (by up to 20%) and astaxanthin accumulation (by 20%–30%) from the initial values. Such results were explained by the expression of carotenoids pathway-related genes.

Electrical treatment of *H.pluvialis* cells was studied in the bio-electrochemical chambers [52,53]. Such treatment enhanced the nitrogen consumption and chlorophyll biosynthesis, and it resulted in considerable increase of electro-treated cell number density (by 20%) after the cultivation for seven days [52]. However, such treatment did not significantly affect the intracellular astaxanthin content (10% increase). The special adaptation of electrotreatment parameters (electric strength and duration) allowed significant increasing of the astaxanthin content in electrostimulated *H.pluvialis* (by 36.9%) [53].

Application of electrical treatment by low temperature plasma in *H.pluvialis* biotechnology have been also discussed. It was demonstrated that low temperature plasma promotes growth of *H.pluvialis* [54]. The plasma treatment is widely used as a safe and environmentally friendly sterilization technology [55] and it may be applied during the growth stages as strong oxidative stress and mutagenesis tool without damaging the microalgae cells [17]. Atmospheric pressure argon dielectric barrier discharge plasma was used for mutation induction in *H.pluvialis* [56]. The presence of the enhanced astaxanthin accumulation in the mutants was observed and it was attributed to genetic modification by the mechanism of carotenogenesis. It was indicated that plasma mutation is suitable for effective *H.pluvialis* breeding with promising industrial application [57]. The optimal plasma treatment effectively improved the growth of *H.pluvialis* and its astaxanthin accumulation [58,59]. The possible mechanisms of astaxanthin accumulation induced by plasma treatment were discussed.

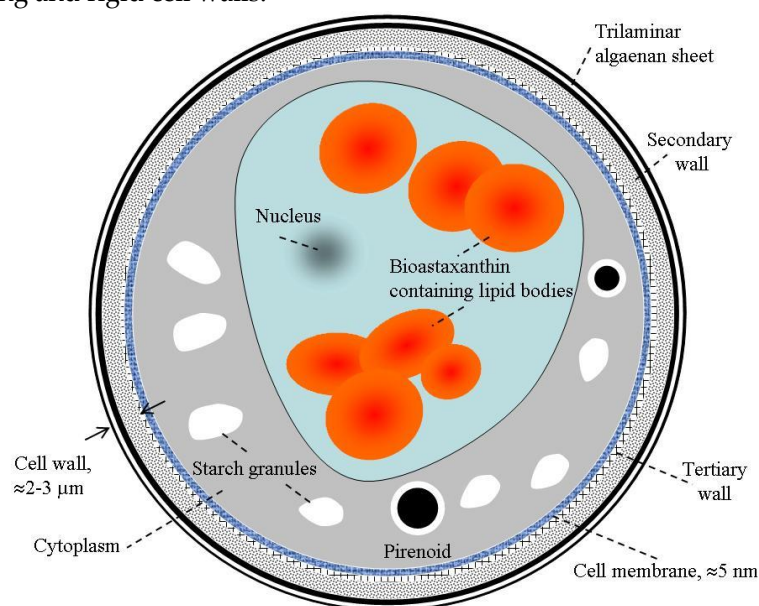
## 4. Structure of cell and biomolecular composition of *Haematococcus pluvialis*

### 4.1. Cell structure

Figure 2 gives a schematic presentation of *H.pluvialis* cell structure at the resistant cyst stage. On the final aplanospore stage of cyst formation, the rigid and resistant multilayered cell walls are formed [60–65]. The total size of the cells at this stage is approximately 20–30  $\mu\text{m}$  [27]. The cell internal compartment contains cytoplasm starch granules, sub-cellular micro-compartments of pyrenoids, and astaxanthin deposited in extra-plastidial oil globules (lipid droplets). This internal space is covered by lipid membrane with thickness of  $\approx 5 \mu\text{m}$ .

The outside part of the cell is covered with an aplanospore cell wall (with total thinness of 2–3  $\mu\text{m}$ ). This wall is composed of an outer primary algaenan-based wall (very stable sporopollenin-like wall),

secondary wall (composed of mannose and cellulose with homogeneous arrangement), and tertiary wall (composed of mannose and cellulose with heterogeneous arrangement). Therefore, the cell is protected by complex, strong and rigid cell walls.

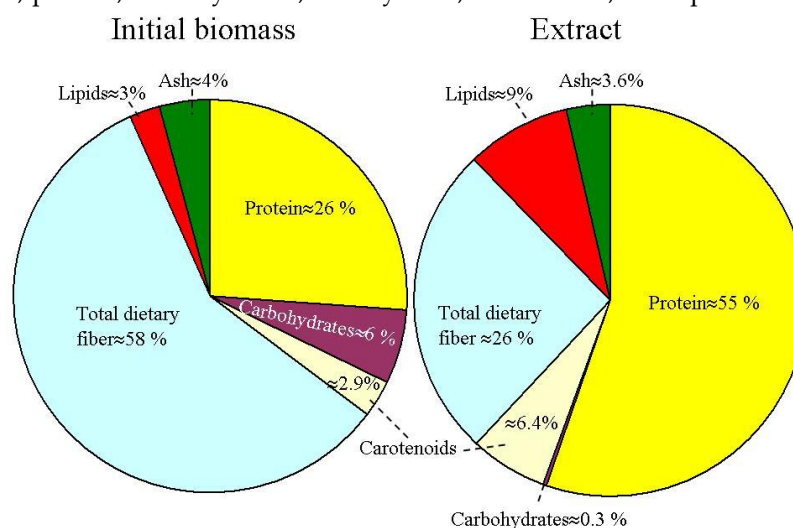


**Figure 2.** Description of *H. pluvialis* cell structure at the resistant cyst stage. Based on the data collected in [60–65].

The three layered cell structure acts as a strong barrier to different external physical and chemical stresses, and it provides a high resistance against extreme environmental stress conditions [18,19]. Consequently, this cell wall developed is indigestible and reduces the bio-accessibility of internal biomolecules such as astaxanthin. Moreover, the thickness of *H. pluvialis* cell wall reached after stress culturing stage is considered as an important bottleneck limiting large production of natural astaxanthin.

#### 4.2. Biomolecular composition

Chemical constituents of extracellular extract of *H. pluvialis* were recently analyzed [66]. *H. pluvialis* cell composition includes ash, protein, carbohydrates, dietary fiber, carotenoids, and lipids.



**Figure 3.** Initial biomass and extract compositions for *H. pluvialis* red phase. Based on the data obtained in [67].

Figure 3 presents initial biomass and extract compositions for *H. pluvialis* red phase [67]. The total dietary fiber and protein were observed as the main intracellular constituents in the initial biomass. Carotenoids include astaxanthin (66-70 %),  $\beta$ -carotene (3.5-6.5 %) and lutein (27-28%). Content of proteins, lipids and carotenoids in extract obtained by supercritical CO<sub>2</sub> was higher in comparison to that of the biomass. Inverse situation was observed for the content of total dietary fibers (58 % in biomass as compared with 26 % in extract).

#### 4.2.1. Lipids, proteins, and carbohydrates

The content of lipids, proteins, and carbohydrates in the *H. pluvialis* cells strongly depends on the stage of cell evolution [27,41,68]. Lipids content in the *H. pluvialis* cells may be rather high during the green vegetative stage ( $\approx$ 20-25% of the total biomass). During the red stage evolution, lipids content increases ( $\approx$ 32-37%), and it can be manipulated by operation conditions and stress imposed. The protein and carbohydrate contents also change with transition from the green phase to the red phase. The protein content is  $\approx$ 29-45% in the green stage and it reduces up to 17-25% in the red stage. The carbohydrate content is  $\approx$ 15-17% in the green stage and it increases up to 36-40% in the red stage.

#### 4.2.2. Carotenoids

During green stage, other carotenoids of *H. pluvialis* are mainly presented by lutein ( $\approx$ 50- 70% of total carotenoids), neoxanthin,  $\beta$ -carotene, and violaxanthin ( $\approx$ 8-13% for each of them) with no content of astaxanthin [41,69]. During the red stage, the total carotenoids content is significantly increased (mainly due to the astaxanthin accumulation), and it can reach more than 4% of the biomass dry weight. At this stage astaxanthin is the main cell compound, and other carotenoids represent  $\approx$ 5-20% of the total content.

Astaxanthin (the chemical IUPAC's name is 3,3'-dihydroxy- $\beta,\beta$ -carotene-4,4' -dione) is a liposoluble red dietary carotenoid. It has molecular formula C<sub>40</sub>H<sub>52</sub>O<sub>4</sub>, molecular weight 596.86 g/mol, boiling point 774.04 °C, density 1.071 g/cm<sup>3</sup> and melting point 182-183 °C. Astaxanthin contains 13 conjugated double bonds with two benzene rings, and hydroxyl groups at the ends [70]. Due to the presence of hydroxyl groups the molecule of astaxanthin is polar. At room temperature (T=25 °C) astaxanthin is practically insoluble in water, but it is highly soluble in different organic solvents (Table 1).

**Table 1.** Solubility of astaxanthin at room temperature T=25°C in different organic solvents.

Solvent	Solubility (25 °C), references
Water	$\approx$ 10 <sup>-12</sup> g/L, [71]
Ethanol	0.038 g/L, [72]
Methanol	0.04 g/l, [72]
Acetone	0.55 g/L, 0.2 g/L, [73]
DMSO	1.64 g/L[72], 0.5 g/L [73]
DMSO:Acetone mixture (1:1)	2.03 g/L, [72]
Chloroform	10 g/L, [73]
Dichloromethane	30 g/L, [73]

It was also demonstrated that solubility of astaxanthin in acidic condition was 10-20 times higher than in neutral and basic conditions [74].

Astaxanthin is rather unstable and can be degraded during production process at heating even for a very short time, long-term storage, in acidic condition under UV irradiation and in the presence of oxygen [75]. Effect of extraction and drying methods on antioxidant activity of astaxanthin was discussed in [76]. The general overview of different chemical and biochemical properties of astaxanthin is presented in [41].

The microalga *H.pluvialis* can accumulate the optical pure isomer of astaxanthin (3S, 3'S) which is approximately 20 times stronger the synthetic astaxanthin. Several studies have shown that astaxanthin accumulated within cysts of *H.pluvialis* exists in esterified form, mainly as monoesters (70%), di-ester (25%), free form (5%) with other occurring carotenoids. The astaxanthin can be esterified on its one or both hydroxyl groups with various fatty acids such as palmitic, stearic, and oleic acids.

For the commercialization on natural astaxanthin, the free form is only approved in the market, that means the purification of final astaxanthin of *H. pluvialis* needs to pass by additional step of deesterification, namely known as the saponification. Two methods exist for the deesterification of extracted astaxanthin: 1) deesterification through enzymolysis using a cholesterol esterase. 2) deesterification of astaxanthin through saponification in darkness at low or room temperature using an alkaline solution of NaOH or KOH in methanol. But this step of deesterification could induce a loss of carotenoids due to their degradation. Three factors could negatively affect the final pigments, treatment time and NaOH or KOH concentrations, and temperature.

## 5. Harvesting of biomass

The harvesting procedure of *H.pluvialis* is a very important step before application of cell disruption technique. It consists in separation of biomass from the bulk culture medium, which can be done using gravity sedimentation, centrifugation, filtration, flotation or flocculation technique [77,78]. Gravity sedimentation or centrifugation are rather effective for the large cells of *H.pluvialis* and can be successfully employed. The filtration technique is based on the liquid biomass flowing through the membrane. Flotation is a gravity separation technique, and it involves attaching micro-sized air bubbles to microalgae cells. Flocculation technique is based on the aggregation of *H. pluvialis* cells caused by addition of flocculants. A combination of these techniques can be useful to achieve good harvest efficiency of *H. pluvialis* [79].

## 6. Cell disruption, extraction, purification techniques

Industrial perspective of downstream processing of *H.pluvialis* and different steps for optimization of bioactives recovery are widely discussed in current literature [80,81].

### 6.1. Cell disruption

Cell disruption can use different physical, chemical and biological techniques (Fig. 1). Physical treatments including mechanical, thermal and different innovative methods are widely applied for cell disruption and separation of the value-added biocompounds [77,82,83], phycobiliproteins, carotenoids [84] and astaxanthin [19,41,85–89]. Traditional mechanical treatments use bead milling, grinding, high pressure homogenization, hydraulic and screw pressing [90,91]. Generally, mechanical treatments are non-selective, non-toxic, they are suitable for large scale production. The mechanical cell disruption requires high energy input. The thermal treatments use conductive, ohmic or radio frequency heating, autoclaving, or application of freezing-thawing. These techniques are also non-selective and non-toxic. However, they are highly energy consuming and some of them may cause the thermal degradation of biomolecules. The new innovative methods assisted by electric and magnetic [92] fields, ultrasound [93–95] and microwaves [96,97] may be more selective, they are non-toxic, but may require more sophisticated equipment and special adaptation for large scale production.

Chemical treatments use acid, alkalis, detergent, salt, surfactant, nanoparticle, polymer, solvent, ionic liquid, oxidative and osmotic shock treatments [98–101]. These treatments do not require advanced equipment, but they are non-selective, toxic, less suitable for large scale production and they may add chemical contaminants to the extracted biomolecules.

Biological treatments use enzymes, viruses, and algicidal bacteria [86,102]. They are relatively selective and less toxic as compare with chemical techniques, but they are rather expensive, time consuming and less suitable for large scale production.

All these treatments can be applied individually, but for enhanced disruption and increased selectivity the combinations of different techniques may be useful.



## 6.2. Extraction

Once the cell walls are disrupted, the biomolecules with antioxidant and antimicrobial activity, and particularly astaxanthin must be rapidly recovered from *H. pluvialis* microalgae to prevent their degradation. Popular methods of extraction are the subcritical water extraction (SWE), supercritical fluid extraction and extraction with solvents and oils. The supercritical carbon dioxide (S-CO<sub>2</sub>) extraction is considered as the most efficient and it has been widely used for industrial applications [69,103]. SWE at 200 °C was demonstrated to be a green and fast extraction method to obtain antioxidant and antimicrobial extracts in red growing phase and it allowed obtaining the high extraction yields of vitamin E and simple phenolics (up to 30% of dry weight) [104]. However, astaxanthin destruction is rather significant at high temperatures. In many works the different modes of S-CO<sub>2</sub> extraction at moderate critical temperatures and pressures were tested [105–113] (see [114] for a recent review). Supercritical CO<sub>2</sub> is non-polar, non-toxic, not flammable, and inexpensive. The solubility of astaxanthin in supercritical CO<sub>2</sub> is rather high ( $\approx 10^{-2}$  g/L at 25° C and 100 bar) [115]. In addition, the CO<sub>2</sub> at a supercritical state has high diffusion coefficient and low viscosity that provides its rapid penetration into the porous matrix of *H. pluvialis*. In some works modified CO<sub>2</sub> using addition of ethanol [105,108] and ionic liquids [111] were used to enhance extraction efficiency. Economic assessments of S-CO<sub>2</sub> extraction of astaxanthin from *H. pluvialis* were analyzed in [113].

In a simple liquid-solvent extraction the biomolecules recovery is commonly assisted by safe solvents (ethanol, ethyl lactate, and ethyl acetate) [116,117] with some limitations of use the more toxic solvents (acetone, toluene, hexane, benzene, petroleum ether, methanol, dimethylaminocyclohexane and methyl-tert-butyl ether) [115].

## 6.3. Purification

The necessity of subsequent purification step is mainly defined by selected cell disruption and extraction methods. Typically, the purification methods are based on the application of electrophoresis, membrane separation (ultra- or electro-membrane filtration), ultracentrifugation, liquid multi-phase flotation (for a review, see, for example [103]).

## 7. Processing of *Haematococcus pluvialis* by pulsed electrotechnologies

The modern innovative cell disruption techniques based on pulsed electrotechnologies (pulsed electric field (PEF), moderate electric field (MEF), and high-voltage electric discharges (HVED)) have been previously applied to assist extraction of biomolecules from different types of microalgae [118–121]. These techniques typically require rather small energy input, lower amounts of solvent, and shorter extraction times as compared with traditional methods [20].

PEF treatment is based on the application of short-duration and high-voltage electric pulses. This treatment can cause perforation of membrane and the formation of nanopores inside them (phenomenon of so-called electroporation). In turn electroporation facilitate the solvent mediated extraction of different intracellular biomolecules from the microalgae species. Effects of electroporation significantly depends on the parameters of PEF treatment such as pulse duration  $t_i$ , number of pulses  $n$ , total time of PEF treatment  $t_{PEF}=nt_i$ , electric field strength  $E$ , specific energy input  $W$ , and other details of PEF protocol. The pulses may be unipolar or bipolar and have exponential or square-wave shape. The pulse duration ranges from several nanoseconds to several milliseconds, and electric strength ranges in the interval  $E=100$  V/cm -80 kV/m with application of moderate (100-200 V/cm), intermediate (typically under 1000 V/cm), high (1-20 kV/cm), and extra-high (above 20 kV/cm) electric fields in dependency of the type of treated microalgae. At the moderate specific energy inputs ( $W=0.5$ – $5$  kJ/kg) electroporation may be reversible, and it becomes definitely irreversible at more high specific energy inputs ( $W=50$ – $200$  kJ/kg). PEF-assisted extraction typically involves moderate specific energies below 20 kJ/kg. At the extra-high electric field strength, the high voltage electric discharges typically occur, and they accompany with different phenomena like pressure shock waves, bubble cavitation, liquid turbulence, etc. HVED treatment can affect the integrity of cell wall and enhance the extraction of bioactive compounds.

Pulsed electric field (PEF) was frequently applied in previous studies and there exist many recent reviews on applications of PEF for treatment of different biomolecules [82,103,122,123], antioxidants [19,124], pigments and carotenoids [125–128], phycobiliproteins (fluorescent proteins) [84], proteins and

carbohydrates [129], polyphenols [130,131], and lipids [132]. Moreover, the efficiency of different electric field-based techniques for enhanced microalgae biomolecules extraction (PEF, electrolysis, high voltage electrical discharges and moderate electric field) were recently compared [133]. In general PEF-treatment can be considered as suitable technique for extraction of the intracellular proteins and pigments from microalgae [134].

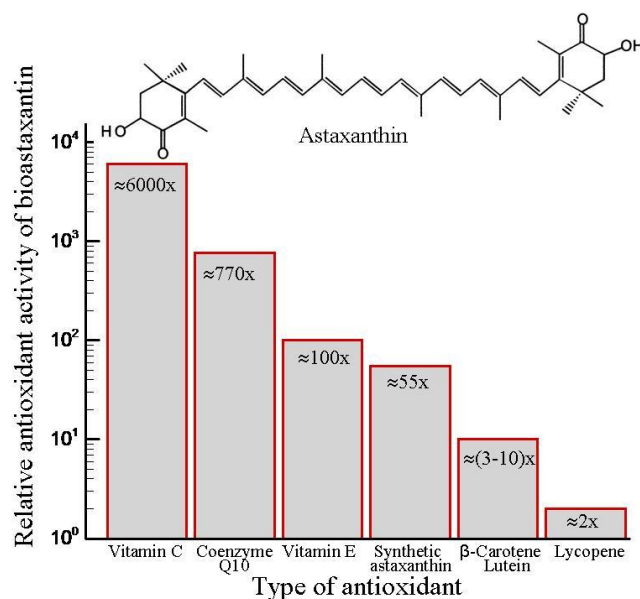
However, as far as we know, the reported in literature data on application of pulsed electrotechnologies for the recovery of biomolecules from *H.pluvialis* concern just the proteins [135,136] and astaxanthin [87,137] extraction. It was stated that larger cells of *H.pluvialis* are more susceptible to electroporation [77]. However, the thick rigid cell walls of *H.pluvialis* can lead to inefficient extraction of biomolecules even at very high PEF intensities and number of pulses [21].

In study of proteins extraction, the *H.pluvialis* suspension was PEF treated in a distilled water at  $E=3\text{--}6$  kV/cm (9 bipolar pulses with duration of 2 ms), and this treatment was followed by a 24 h incubation period in salty buffer. Protein recovery was compared for the PEF treated and untreated samples [135]. PEF treatment significantly increases the protein recovery (by 10-20 times) and the content of extracted proteins significantly depends on the temperature (4 and 20 °C), and concentration of cells ( $10^5$  cells/mL or  $10^6$  cells/mL). A post-pulse incubation step was found to be necessary to allow oozing. It was assumed that PEF treatment induces some alteration of cell walls, creases their porosity that allows the leakage of cytoplasmic proteins. In other work, the PEF treatment in water at  $E=1$  kV/cm allowed extraction of 46% of total quantity of proteins (10.2 µg protein per mL of culture) [136]. The non-destructive electro-extraction from *H.pluvialis* with cultures recovered after 72 h was observed. A biocompatibility index (fraction of surviving cells after PEF treatment) was estimated.

Rather contradictory results on the PEF-assisted astaxanthin extraction from *H. pluvialis* were obtained [87,137]. For example, a very low astaxanthin extraction efficiency (3.1-6.5%) was observed using PEF pretreatment in methanol (10–30 kV/cm, 30 min) and additional 2 h incubation [87]. PEF treatment led to the lowest percentage of astaxanthin extractability compared to ultrasounds, high-pressure microfluidization, hydrochloric acid, and ionic liquids techniques. For instance, high-pressure microfluidization led to the maximum extractability value of 91.4%. PEF treatment was considered as inefficient for the cell walls disintegration of *H.pluvialis*. In other work, the PEF treatment was applied to extract astaxanthin from the Nordic microalga *H.pluvialis* [137]. A high extraction efficiency (96% of the total carotenoid content) was observed using PEF treatment in water (10 pulses of 5 ms at 1 kV/cm and 20° C). The value around of  $E=1$  kV/cm was considered as a threshold for the effective extraction from this Nordic *H.pluvialis* strain. It was demonstrated that PEF treatment resulted in a 1.2-fold more efficient extraction compared to the classical disruption methods (bead-beating and thermal treatment). It was stated that efficient PEF-assisted extraction of carotenoids requires proper optimization of the incubation time after PEF application. These differences between results obtained in [87] and [137] may reflect the significant variations in the PEF-assisted extraction protocols and differences in the studied strains [19].

## 8. Bioactivity of *Haematococcus pluvialis* extracts

The bioactivity of biocompounds extracted from *H.pluvialis* was intensively studied in the current literature [67,138–142]. Many of these studies are related with applications of natural bioastaxanthin. Figure 4 demonstrates that the natural astaxanthin has exceptionally high antioxidant activity as compared to other known antioxidants. The biosynthetic (natural) astaxanthin produced by the *H.pluvialis* has superior therapeutic properties and it is highly preferable in compare to astaxanthin produced by chemical synthesis from petrochemical sources in the form of a mixture of isomers [47]. Moreover, the synthetic astaxanthin is not recommended for use as a human nutraceutical supplement [143].



**Figure 4.** Relative antioxidant activity of the natural astaxanthin compared to other antioxidant in neutralisation of destructive reactive oxygen species (ROS) found in the human body. Based on the data collected in [26,75,144,145].

The estimated cost of bioastaxanthin production from *H. pluvialis* is around \$3000–\$3600 kg<sup>-1</sup> compared to the cost of production of synthetic astaxanthin, which is around \$1000 kg<sup>-1</sup>[88]. The bioastaxanthin demonstrated significantly stronger (by order of magnitudes) bioactivity as compared to astaxanthin synthesized from petrochemicals (see Fig. 4). Recent advancements in bioastaxanthin production from *H. pluvialis*, multifunctional applications and world market were analyzed in [88,146–148]. The natural astaxanthin is more powerful and stronger antioxidant for the neutralization of destructive reactive oxygen species (ROS) found in the human body in compare to all other antioxidants [26,27,47,75,144]. Particularly, it is in ≈6000 times more active than vitamin C and in ≈55 times more active than coenzyme Q10 (CoQ10), in 100 times more potent than vitamin E, and in five times more powerful than β-carotene for the neutralization of destructive reactive oxygen species (ROS) found in the human body.

Nowadays the bioastaxanthin is approved by FDA (United States Food and Drug Administration) as a health food supplement and food colorant for the animal and fish feed [149]. Bioastaxanthin has been also approved as a dietary supplement for human consumption in many European countries, USA and Japan. Recommended or approved doses of astaxanthin are ranged between 2 and 24 mg/day [149].

Bioastaxanthin demonstrates many attractive anti-aging, anti-inflammatory, sun proofing, and immune system boosting effects on living organisms. The possibilities and limitations of the use of astaxanthin in food technology were analysed in [150]. The possible applications of natural astaxanthin also evolve pharmaceutical [138,139,151], and cosmetic [67,152] (Oliveira et al., 2022) industries. The medical and human health applications [139,153], different anti-cancer, anti-diabetic, anti-atherosclerotic effects, and immune, neuroprotective, and genetic engineering potential exerted by astaxanthin have been discussed in [26,139,153–155]. The different effects related with impact of natural astaxanthin on oxidative stresses, aging (skin and brain aging), skin physiology, central nervous system functioning [75], and inflammation-associated diseases [156] were also discussed.

## 7. Conclusions

Pulsed electrotechnologies and particularly pulsed electric field (PEF) are very perspective for the extraction of different biomolecules from *H. pluvialis*. However, the thick rigid cell walls of *H. pluvialis* can effectively protect the cell membrane against electroporation and lead to inefficient extraction of biomolecules even under the heavy PEF loads (high intensities and big number of pulses). PEF treatment is usually referred as a mild treatment method that could not significantly affect the structure of cell

walls. The PEF treatment of *H. pluvialis* requires careful optimization of all PEF treatment parameters, selection of the type of organic solvent and combined application of different cell destruction techniques. Therefore, the more careful future studies on application of PEF in combination with other cell disruption techniques for *H. pluvialis* are needed.

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