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Review

Novel Insights into the Biotechnological Production of *Haematococcus pluvialis*-Derived Astaxanthin: Advances and Key Challenges to Allow Its Industrial Use as Novel Food Ingredient

Samuel Jannel [1,2], Yanis Caro [1,3], Marc Bermudes [2] and Thomas Petit [1,3,*]

[1] Laboratoire de Chimie et Biotechnologies des Produits Naturels, CHEMBIOPRO (EA 2212), Université de la Réunion, 15 Avenue René Cassin, 97490 Sainte Clotilde, Réunion Island, France; samuel.jannel@univ-reunion.fr (S.J.); yanis.caro@univ-reunion.fr (Y.C.)

[2] Green Mascareignes Technologies, 2 Rue Maxime Rivière, 97490 Sainte-Clotilde, Réunion Island, France; mbermudes@green-mascareignes.com

[3] IUT de la Réunion, Département HSE, 40 Avenue de Soweto Terre-Sainte, BP 373, 97455 Saint-Pierre CEDEX, Réunion Island, France

* Correspondence: thomas.petit@univ-reunion.fr

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Abstract: Astaxanthin shows many biological activities. It has acquired a high economic potential and its current market is dominated by its synthetic form. However, due to the increase of the health and environmental concerns from consumers, natural forms are now preferred for human consumption. *Haematococcus pluvialis* is artificially cultured at an industrial scale to produce astaxanthin used as a dietary supplement. However, due to the high cost of its cultivation and its relatively low biomass and pigment productivities, the astaxanthin extracted from this microalga remains expensive and this has probably the consequence of slowing down its economic development in the lower added-value market such as food ingredient. In this review, we first aim to provide an overview of the chemical and biochemical properties of astaxanthin, as well as of its natural sources. We discuss its bioavailability, metabolism, and biological activities. We present a state-of-the-art of the biology and physiology of *H. pluvialis*, and highlight novel insights into the biotechnological processes which allow optimizing the biomass and astaxanthin productivities. We are trying to identify some lines of research that would improve the industrial sustainability and economic viability of this bio-production and to broaden the commercial potential of astaxanthin produced from *H. pluvialis*.

Keywords: astaxanthin; *Haematococcus pluvialis*; carotenoids; microalgae; microalgae cultivation; downstream process; biological activities

1. Introduction

Astaxanthin is a high added value xanthophyll carotenoid naturally synthetized by some yeasts, bacteria, microalgae, and to a lesser extent by plants. It is also present in some animals, including fishes, crustaceans, and birds that consume these primary producers, unable to synthetize this molecule. Thereby, astaxanthin is extensively used in aquaculture as a feed additive to colorize farmed salmonids, shrimps, and other crustaceans, and to a lesser extent as an antioxidant in the dietary supplement and cosmetic sectors. While numerous in vitro, in vivo, and clinical studies have demonstrated its biological activities, particularly due to its strong antioxidant and anti-inflammatory properties, astaxanthin has acquired a great economical potential for human applications, such as a potent food ingredient for human nutrition and health, as well as in pharmaceutic and cosmetic.

In 2014, the global market value for astaxanthin was estimated at 280 metric tons valued at USD 447 million [1]. In 2018, its market size exceeded USD 600 million and some economical analysts have estimated that it will probably reach USD 880 million in 2026 [2]. More than 95% of this market refers to synthetically derived astaxanthin produced from petrochemical sources [1] and only approved by the United States Food and Drug Administration (US FDA) and the European Food Safety Authority (EFSA) as a feed additive in aquaculture [3,4]. The production cost of synthetic astaxanthin is about USD 1000 per kg for a market price above USD 2000 per kg [1,5] and the companies Dutch State Mines (DSM) and Badische Anilin- & Soda-Fabrik (BASF) are leading the production [3].

However, due to the increase of the health and environmental concerns from consumers, natural forms of astaxanthin, even if more expensive, are now preferred for direct human consumption. Among natural sources, the freshwater green microalga *Haematococcus pluvialis* can accumulate the largest amounts of astaxanthin [6] and it is already cultivated at an industrial scale to produce this carotenoid for human applications. Besides, *H. pluvialis* has received approval from the US FDA and EFSA for use as a human nutritional supplement [3,7]. However, astaxanthin extracted from *H. pluvialis* accounts for less than 1% of the total world production, and its market price is estimated at USD 7000 per kg due to its higher production cost, estimated at best at about USD 1800 per kg in an adequate location [1]. The major commercial producers of *H. pluvialis* astaxanthin worldwide are Algatechnologies Ltd. in Israel, AstaReal Inc. in Hawaii, USA, a subsidiary of Fuji Chemical Industry in Toyama, Japan, BGG (Beijing Ginko Group) in Yunnan Province, China, Cyanotech Corporation in Hawaii, USA and Parry's Pharmaceuticals in India [8].

Considering the dynamism of the dietary supplement market, the increasing consumer demand for natural products and the remarkable technical properties of this molecule, the market share of astaxanthin produced through the cultivation of *H. pluvialis* is expected to grow very significantly in the coming years and it represents an attractive economic opportunity, particularly for countries where the climate is adapted to its cultivation. The objective of the present review is to provide an overview of the scientific and technical knowledge dealing with astaxanthin, its biological activities, and the main technical issues related to the cultivation of *H. pluvialis* and astaxanthin bioproduction. We first aim to provide an overview of the chemical and biochemical properties of astaxanthin, as well as an overview of its natural sources. We discuss its bioavailability, metabolism, and biological activities in human health. We also present a state-of-the-art account of the biology and physiology of *H. pluvialis*, and highlight novel insights into the biotechnological processes which allow optimizing the growth and the biomass and astaxanthin productivities. Subsequently, we are trying to identify some lines of research that would improve the industrial sustainability and economic viability of this bioproduction and to broaden the commercial potential of astaxanthin produced from *H. pluvialis*.

2. Astaxanthin

2.1. Biochemistry

Astaxanthin, or 3,3′-dihydroxy-β,β-carotene-4,4′-dione, is a carotenoid with a reddish-orange color that belongs to the family of xanthophylls, the oxygenated derivatives of carotenoid. Its molecular formula is $C_{40}H_{52}O_4$ and its molecular mass is 596.85 g.mol^{-1} (C 80.5%, H 8.78%, O 10.72%). Solid at room temperature and fat-soluble, its melting point is 182.5 °C and log P (octanol/water partition) is equal to 13.27. The Chemical Abstract Services Registry Number (CAS) of astaxanthin is 472-61-7 and the corresponding number in the EC database is 207-451-4. Its synthetic form is approved at the United States and European Union levels as a feed additive only for coloring of salmon, trout, and ornamental fish. It is listed by the European Commission under the number E161j [9]. So far, astaxanthin sourced from *H. pluvialis* has been approved as a dietary supplement for human consumption in Europe, Japan, and the USA [10].

Astaxanthin is a tetraterpene (40 carbon atoms) which contains two polar terminal β-ionone rings joined by a nonpolar central polyene chain [11] (Figure 1). This molecule contains 13 conjugated

double bonds that confer to astaxanthin a strong antioxidant activity due to the ability to neutralize free radicals and to scavenge Reactive Oxygen Species (ROS) [5,12,13].

Each ring contains one hydroxyl (OH) and one keto (C=O) moieties that participate in the antioxidant activity of astaxanthin and confer to the molecule its ability to be esterified and a more polar nature than other carotenoids [14]. Due to the presence of hydroxyl groups, each molecule has two chiral centers in C-3 and C-3′. Therefore, astaxanthin may present three configurational isomers: two enantiomers (3S, 3′S and 3R, 3′R) and a meso form (3R, 3′S) [11] (Figure 1a–c).

As for other carotenoids, the polyene system gives to astaxanthin its distinctive molecular structure, its chemical properties, and its light-absorption characteristics [11], and thus it constitutes the chromophore of the molecule [5]. Each carbon–carbon double bond from the polyene chain may exist in two configurations: as geometric isomers *trans* and *cis* (Figure 1a,d,e). *Cis*-isomers are thermodynamically less stable than the *trans*-isomers, thus most carotenoids found in nature, such as astaxanthin, are predominantly all-*trans*-isomers [11]. However, *trans*-astaxanthin (Figure 1a) may be readily isomerized to *cis*-*trans* mixtures, especially 9-*cis* and 13-*cis* (Figure 1d,e). For instance, the astaxanthin produced by *H. pluvialis* is naturally present at 73% as *trans*-astaxanthin and 27% as *cis*-astaxanthin [15]. Isomerization can be promoted using organic solvents or at high temperatures. However, *trans*-astaxanthin cannot be isomerized completely to its *cis*-isomers, therefore isomerization of *trans*-astaxanthin is a reversible reaction that follows a first-order reversible reaction kinetic model [16].

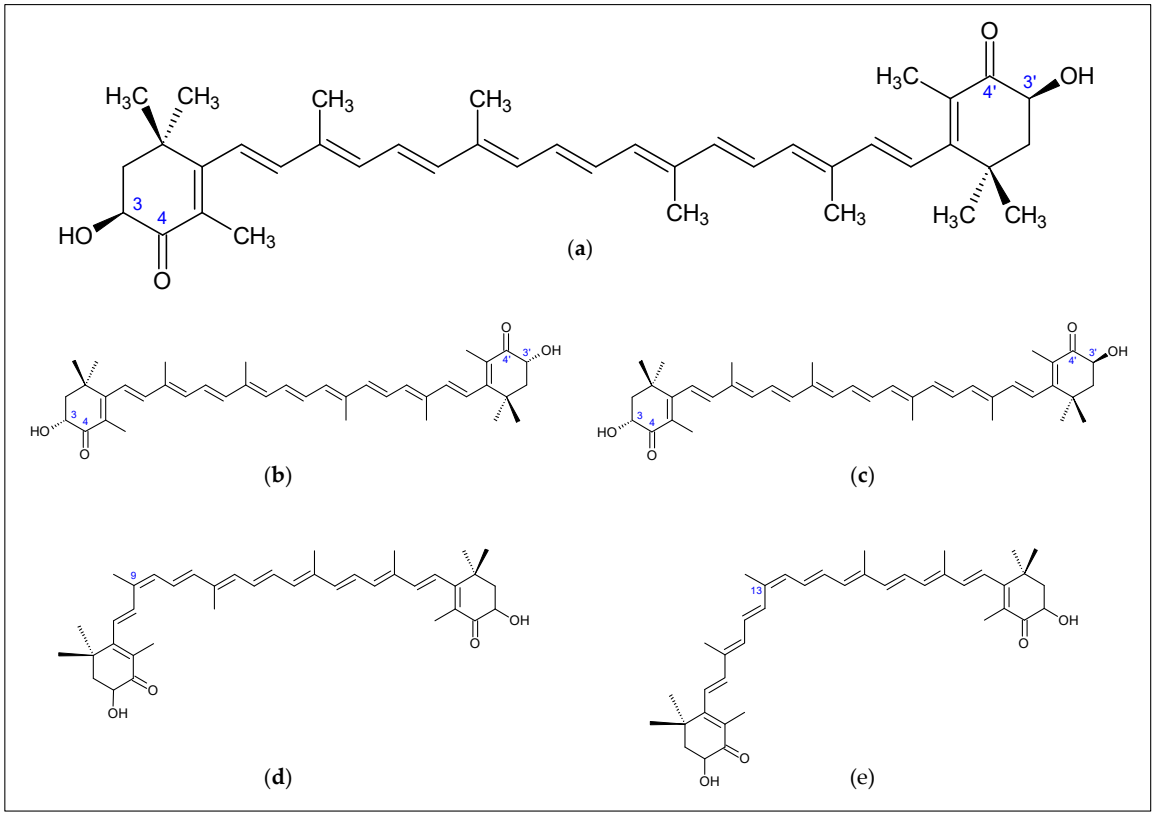

Figure 1. Molecular structures of some configurational and geometrical isomers of astaxanthin [17,18]. (**a**) (3S,3′S)-all-*trans*-astaxanthin; (**b**) (3R,3′R)-all-*trans*-astaxanthin; (**c**) (3R,3′S)-all-*trans*-astaxanthin; (**d**) 9-*cis*-astaxanthin; (**e**) 13-*cis*-astaxanthin.

In nature, astaxanthin can be found in different forms: either free, conjugated with proteins or lipoproteins, or esterified on the hydroxyl groups with one or two fatty acids to form mono- or diesters, respectively [11]. Free astaxanthin is particularly sensitive to oxidation [14]. The esterification of astaxanthin increases its hydrophobicity and therefore its solubility in globules made of triacylglycerols.

In *H. pluvialis*, the fatty acid composition of the astaxanthin esters consists mostly of oleic acid ($C_{18:1,\,n-9}$) [19], then palmitic ($C_{16:0}$) and linoleic ($C_{18:2,\,n-6}$) acids [20]. In this microalga, astaxanthin $C_{18:1}$ and astaxanthin $C_{16:1}/C_{18:1}$ are the main astaxanthin monoester and diester [21].

2.2. Sources of Astaxanthin

Synthetic astaxanthin is widely used in aquaculture. It is produced since 1990 at large scale, particularly by Hoffmann-La Roche (DSM Nutritional Products) and it consists of a mixture of isomers (3S, 3S′), (3R, 3S′) and (3R, 3R′) in a 1:2:1 ratio [11].

However, natural sources of astaxanthin are nowadays the only acceptable ones for human applications. Among them, the main primary producers of natural astaxanthin, i.e., able to synthesize it, are the microalga *Haematococcus pluvialis* and the yeast *Phaffia rhodozyma* (or *Xanthophyllomyces dendrorhous*) [22]. Microalgae *Chlorella zofingiensis* and *Chlorococcum* sp. as well as the marine bacteria *Paracoccus* sp. (formerly called *Agrobacterium aurantiacum*) are also able to synthesize the molecule. Astaxanthin can also be found in some flowering plants, such as *Adonis aestivalis* [23], and other bacteria and fungi but to a lesser extent as compared to microalgae [24]. In *H. pluvialis*, astaxanthin is mainly found in the (3S, 3′S) configuration and in esterified forms where monoesters are predominant as compared to diesters [19,25] (Figure 2a), while *C. zofingiensis* has a higher percentage of diesters [6]. Astaxanthin in *P. rhodozyma* is in the free form and the 3R, 3′R configuration [18] (Figure 2b). With advances in metabolic engineering, astaxanthin can also be produced by non-carotenogenic microbes such as *Escherichia coli* and *Saccharomyces cerevisiae* [26].

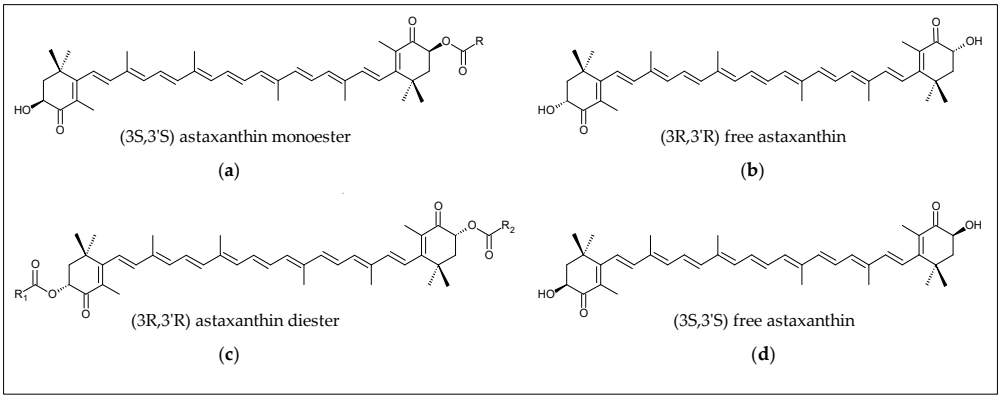

Figure 2. Predominant forms of astaxanthin in different living organisms [9,18,19,27]. (**a**) *H. pluvialis*; (**b**) *P. rhodozyma*; (**c**) *E. superba*; (**d**) wild salmonids. R, R_1, R_2 = carbon chains.

Astaxanthin cannot be synthesized by animals but it can be present in those that feed these primary producers, particularly in the shell of some crustaceans such as crabs, shrimps, lobsters, and krill [9,11]. For example, in Antarctic krill (*Euphausia superba*), the molecule is mainly in the 3R, 3′R configuration, and in esterified forms where diesters are predominant as compared to monoesters [27] (Figure 2c). Other animals consumed by humans contain astaxanthin in their flesh, such as salmonids in which astaxanthin is in the free form. Unlike farm salmonids, wild salmonids have a high content of astaxanthin, which is mainly in the (3S, 3′S) configuration (Figure 2d). Among them, some *Oncorhynchus* (e.g., *O. nerka*) can have around 26 to 38 mg/kg of astaxanthin in their flesh. Farmed Atlantic salmon, fed with synthetic astaxanthin, can contain in its flesh up to 6 to 8 mg/kg of astaxanthin mainly in the (3R, 3′S) configuration. Farmed rainbow trout is available on the European market at 6 mg/kg of flesh and 25 mg/kg on the Japanese market [9].

2.3. Bioavailability and Metabolism of Carotenoids and Astaxanthin in Human

Due to their high hydrophobicity and thus their poor solubility in digestive fluid, carotenoids are less bioavailable than other fatty compounds such as α-tocopherol and triacylglycerols [28].

Their bioavailability depends on many factors including the nature of the food matrix which contains them, their structure, and the presence of other dietary compounds [29].

After ingestion, carotenoids must first be released from the food matrix into the gastrointestinal tract, and this mechanism is more or less effectively depending on the nature of the matrix [28]. For example, because of the rigid structure of the cell walls, the release of carotenoids from raw vegetables is made more difficult than those extracted from fat or processed and heated foods [28,29]. Then, carotenoids are dispersed as an emulsion in the digestive fluid [28]. In the small intestine, carotenoid esters are hydrolyzed to free carotenoids by digestive enzymes, most probably cholesterol esterase [30] before it is solubilized into the micelles [28]. While the presence of dietary lipids improves carotenoid dispersion and micelle incorporation, other dietary compounds, such as fibers or phytosterols, on the contrary, inhibit their absorption [29].

Following absorption, carotenoids are incorporated into chylomicrons and transported to the liver via lymph and blood [17]. In the liver, astaxanthin is not converted into vitamin A unlike provitamin A carotenoids such as β-carotene [31]. It is partly secreted in blood in lipoproteins for distribution to cells of various tissues. The main part of plasma astaxanthin is present in very-low-density lipoproteins containing chylomicron (VLDL/CM), then low-density lipoproteins (LDL) and high-density lipoproteins (HDL). Only a small part of total plasma astaxanthin is present in precipitated particles [17].

In general, polar carotenoids such as xanthophylls are preferably incorporated into micelles and tend to be more bioavailable than non-polar carotenoids like β-carotene and lycopene. Likewise, free xanthophylls and *cis*-isomers seem to be more absorbed than the corresponding esterified molecules and the *trans*-isomers [29]. Geometrical *cis*-isomers of astaxanthin are preferentially absorbed or selectively accumulated in human blood, in the same way as the optical (3R, 3′R) isomer [17]. Pharmacokinetic studies showed that the maximum plasma concentration (C_{max}) was higher and was reached more quickly after oral administration of unesterified astaxanthin [32] than with esterified astaxanthin [17]. Indeed, it has been reported that for a single dose of 100 mg unesterified astaxanthin, the C_{max} (1.3 mg/L or 2.18 μmol/L) was reached 6.7 h after administration (t_{max}) and the elimination half-life ($t_{1/2}$) was 21 h [32]. Comparatively, for a single dose of 100 mg astaxanthin equivalents of astaxanthin fatty acyl diesters, the C_{max} (0.28 mg/L or 0.47 μmol/L) was reached at a t_{max} of 11.5 h, and $t_{1/2}$ was about 52 h [17]. This difference may be explained, on the one hand, by the molecule polarity, and thus the gastrointestinal solubility, and on the other hand by the requirement for ester hydrolysis before uptake by enterocytes which slowed down the absorption. It was also shown that for the administration of a single dose of 10 mg astaxanthin fatty acyl diesters, the C_{max} was equal to 0.08 mg/L (0.134 μmol/L) suggesting that the dose–response was non-linear [17].

Because carotenoid absorption is facilitated by the presence of dietary compounds such as lipids, the bioavailability of astaxanthin is affected by the timing of its ingestion. A pharmacokinetic study has shown that its bioavailability is significantly higher for administrations after a meal than before a meal. This study also indicated that smoking negatively affected the bioavailability of astaxanthin [33]. Some formulation techniques are adapted to astaxanthin and astaxanthin-rich lipid extracts and can also influence their bioavailability. For example, micro- and nano-encapsulation in particles of different sizes and structure can improve astaxanthin stability, bioaccessibility, bioavailability, and bioactivity. These techniques were recently well-reviewed in the literature [34,35].

There was no clear data regarding the distribution of astaxanthin in human tissues but it has been studied in animals. For example, in broiler fed with *Phaffia rhodozyma*, the highest concentration of astaxanthin was observed in the small intestine, followed by adipose tissues, spleen, liver, heart, kidneys, and skin, and finally in the muscles to a lower extent [36]. In mice, astaxanthin was accumulated in the liver and it was also detected in the heart and brain after oral administration of synthetic astaxanthin [37]. In rat, astaxanthin was accumulated primarily in the spleen, kidneys, adrenals, liver, skin, and eyes [38].

The mechanisms and functions of astaxanthin metabolism are not yet well understood. However, four metabolites generated through the catabolism of astaxanthin have been identified both

in vitro, in human hepatocyte cultures, and in vivo, in human plasma 24 h after a single oral intake of 100 mg of astaxanthin. These compounds were identified as 3-hydroxy-4-oxo-β-ionone, 3-hydroxy-4-oxo-β-ionol, and their reduced forms 3-hydroxy-4-oxo-7,8-dihydro-β-ionone and 3-hydroxy-4-oxo-7,8-dihydro-β-ionol, respectively [31] (Figure 3).

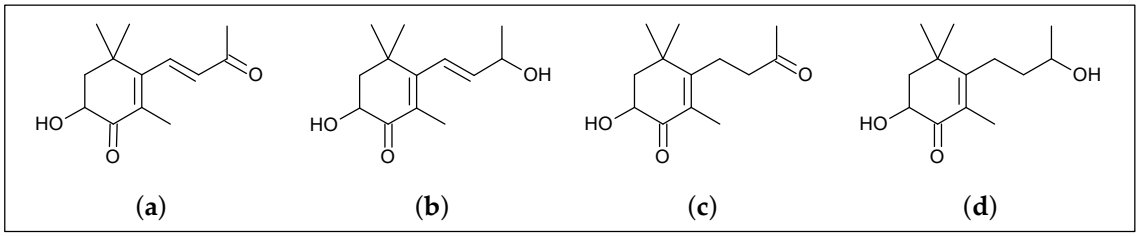

Figure 3. Molecules generated through the catabolism of astaxanthin in humans [31]. (**a**) 3-hydroxy-4-oxo-β-ionone; (**b**) 3-hydroxy-4-oxo-β-ionol; (**c**) 3-hydroxy-4-oxo-7,8-dihydro-β-ionone; (**d**) 3-hydroxy-4-oxo-7,8-dihydro-β-ionol.

2.4. Toxicological Issues of Astaxanthin

Safety testing of an astaxanthin enriched extract from *H. pluvialis* did not produce any clinically important changes in blood pressure, hematology, and blood chemistry parameters in healthy adults at a dose of 6 mg per day during eight weeks [39] or 20 mg per day during four weeks [40]. When administrated at the dose of 8 mg/day for three months, astaxanthin was shown to be a safe dietary supplement that does not cause any gastrointestinal tract distress or any other side effects [41]. No adverse effects following a single oral administration of 100 mg astaxanthin equivalents from its fatty acyl diesters [17] or free astaxanthin [32] were reported in healthy men. Likewise, no adverse effects or toxicity were observed during daily oral administration of a food supplement containing 4 mg of astaxanthin during 12 months in patients with age-related macular degeneration [42], nor in patients with functional dyspepsia treated daily with 40 mg of astaxanthin from *H. pluvialis* during four weeks [43], neither in overweight and obese adults at a dose of 20 mg/day during three weeks [44].

In 2019, the scientific panel of the European Food Safety Authority (EFSA) on additives and products or substances used in animal feed (FEEDAP), in its scientific advice on the safety and efficacy of synthetic astaxanthin-dimethyldisuccinate (DMDS) as a coloring additive for feeding salmonids, crustaceans, and other fish, has confirmed that astaxanthin is neither genotoxic nor carcinogenic. The scientific panel has established an acceptable daily intake (ADI) of 0.2 mg astaxanthin/kg body weight per day by applying an uncertainty factor of 200 at the lowest observed adverse effect level (LOAEL) of 40 mg/kg body weight per day for the increased incidence of multinucleated hepatocytes observed in a two-year carcinogenicity study in rats [4]. In its recent advice on the safety of astaxanthin for its use as a novel food in food supplements, the scientific group of the EFSA on nutrition, novel foods, and food allergens (NDA) has considered that this ADI also applies to astaxanthin from *H. pluvialis*. Taking into account the combination with a high exposure of consumers to astaxanthin from salmonids and crustaceans consumption, the experts have concluded that an intake of 8 mg/day of food supplements was safe for adults and that the ADI was reached in 14–18 years old adolescents and was exceeded below 14 years [7].

2.5. Biological Activities of Astaxanthin

Numerous in vitro, in vivo, and clinical studies have demonstrated various health benefits and physiological responses of astaxanthin, which appear to result primarily from its antioxidant and anti-inflammatory properties. This chapter does not claim to be exhaustive, but its objective is to present the best-studied activities and their probable mechanisms of action. Biological activities described in this chapter and demonstrated by in vitro and in vivo studies are summarized in Table 1 while some clinical studies are presented in Table 2.

Table 1. Biological activities of astaxanthin revealed in in vitro and in vivo experiments.

Biological Activities	Experimental Models	References
antioxidant activity	liposomes rat, human cells mouse, rat	[45–48] [49–56] [49,50,57,58]
anti-inflammatory activity	mouse, human cells mouse, rat	[59–64] [61,62,65–67]
preventive effects on cardiovascular disease	mouse, human cells mouse, rat, rabbit	[60,68] [50,69–74]
prevention effects on diabetes	rat, pig cells mouse, rat	[75,76] [77–83]
protective effects on liver	rat, human cells mouse, rat	[53,84–86] [71,87–89]
immuno-modulating effects	mouse cells mouse, dog, cat	[90] [90–93]
Anti-cancer activity	mouse, rat, human cells mouse, rat, hamster	[94–97] [57,66,67,96,98–104]
effects on nervous system, cerebral and visual functions	mouse, rat, human cells mouse, rat	[49,51,105–112] [49,69,105,113]

Table 2. Summary of clinical studies investigating the biological activities of astaxanthin.

Study Design	Population	Duration	Dosage mg/day	Outcomes	Ref.
open labelled	24 healthy volunteers	14 days	1.8, 3.6, 14.4, 21.6	↑ LDL oxidation lag time	[68]
randomized double-blinded, placebo-controlled	40 healthy non-smoking male volunteers	3 months	8	↓ 12- and 15-hydroxy fatty acids	[41]
randomized double-blinded, placebo-controlled	42 healthy young female subjects	8 weeks	2, 8	↓ 8-OHdG and CRP, ↑ NK cell cytotoxic activity, ↑ total T and B cells, ↑ IFN-γ and IL-6	[114]
randomized double-blinded	23 overweight and obese healthy adults	3 weeks	5, 20	↓ MDA and ISP, ↑ SOD and TAC plasma levels	[44]
randomized trial	39 heavy smoker subjects	3 weeks	5, 20, 40	↓ MDA and ISP, ↑ SOD and TAC plasma levels	[115]
randomized double-blinded, placebo-controlled	61 healthy subjects with mild hyperlipidemia	12 weeks	6, 12, 18	↓ triglyceride, ↑ HDL-cholesterol and adiponectin	[116]
randomized, double-blinded, placebo-controlled	30 middle-aged and senior healthy subjects	12 weeks	6, 12	↓ erythrocyte PLOOH levels	[117]
single-blinded, placebo-controlled	20 healthy adult male subjects	10 days	6	↓ whole blood transit time	[118]
randomized, placebo- controlled	12 biopsy-confirmed NASH patients	24 weeks	12	improvement of steatohepatitis, ↓ total NAS score	[89]
open-labelled	10 subjects with age-related forgetfulness	12 weeks	12	↑ performances in CogHealth and P300 cognitive tests	[40]
randomized, double-blinded, placebo-controlled	96 subjects with age-related forgetfulness	12 weeks	6, 12	↑ scores in the CogHealth and GMLT cognitive tests	[119]
randomized, controlled	27 patients with non-advanced AMD	12 months	4	↑ multifocal electroretinogram RAD for retinal eccentricity of 0° to 5°	[42]
randomized, double-blinded, placebo-controlled	26 VDT workers	4 weeks	5	improvement of accommodation amplitude	[120]

Abbreviations: LDL, low-density lipoprotein; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; CRP, C-Reactive protein; NK, natural killer; IFN, interferon; IL, interleukin; MDA, malondialdehyde; ISP, isoprostane; SOD, superoxide dismutase; TAC, total antioxidant capacity; HDL, high-density lipoprotein; PLOOH, phospholipid hydroperoxide; NASH, non-alcoholic steatohepatitis; NAS, NAFLD activity score; GMLT, Groton maze learning test; AMD, age-related macular degeneration; RAD, response amplitude densities; VDT, visual display terminal. The arrows symbolize the increase (upward) or decrease (downward) of the physiological outcomes.

2.5.1. Antioxidant Activity

It is well known that carotenoids display antioxidant activities due, firstly, to the electron-rich conjugated system of their polyene chains that can quench singlet oxygen and to scavenge free radicals to terminate oxidative chain reactions [45,121]. Secondly, because carotenoids are hydrophobic molecules, they fit inside the hydrophobic core of the phospholipid bilayer of cell membranes. Most xanthophylls, like zeaxanthin, in particular in the all-*trans* conformation, span the lipid bilayer and have their polar groups anchored in the opposite polar zones of the membrane. Due to the van der Waals interactions between their polyene chains and the acyl chains of membrane lipids, zeaxanthin stiffens the fluid phase of the membranes and limits the penetration of oxygen to the core of the hydrophobic membrane sensitive to oxidative degradation, in particular on polyunsaturated fatty acids [122]. Given the structural similarities with zeaxanthin, it can be assumed that astaxanthin acts in the same way.

Some in vitro studies performed with liposomes as a membrane-mimicking system have shown that astaxanthin has a strong antioxidant activity on a broad spectrum of free radicals, reactive oxygen species (ROS), and reactive nitrogen species (RNS). For example, it exhibited a greater activity to scavenge peroxyl radicals ($RO_2\bullet$) as compared to α-tocopherol and other carotenoids, such as β-carotene, lycopene, and lutein [46]. In addition, astaxanthin was also able to scavenge hydroxyl radicals (HO•), $RO_2\bullet$, and hypochlorous acid (HOCl) to a greater extent compared to these other carotenoids but also than zeaxanthin, fucoxanthin, and canthaxanthin. Even if β-carotene was the most efficient against peroxynitrite anion ($ONOO^-$), astaxanthin was found to be the more potent scavenger of this RNS compared to α-tocopherol or lycopene [47]. In polyunsaturated fatty acids-enriched liposomes, astaxanthin exhibited significant antioxidant activity on lipid peroxidation, reducing lipid hydroperoxide (LOOH) generation and preserved membrane structure, unlike β-carotene and lycopene that harbored a potent pro-oxidant effect and disturbed membrane bilayer [45]. This stronger activity against lipid peroxidation is explained by the fact that astaxanthin traps radicals not only at its conjugated polyene chain but also in its polar terminal ring moieties, and thus has antioxidant activity both inside of the membrane and at the membrane surface via inter- and intramolecular hydrogen bonding [48]. It has also been suggested that these polar moieties allow interactions with extra-membrane hydrophilic cofactors such as vitamin C, which, by accepting the generated radical cations, restore the electron transfer capacity of degraded astaxanthin [123].

The antioxidant activity of astaxanthin has also been described in various cellular models exposed to different stresses. For example, in cultured rat retinal ganglion cells exposed to H_2O_2 or serum deprivation, astaxanthin has been shown to reduce ROS-induced intracellular oxidation via hydrogen peroxide (H_2O_2), superoxide anion ($O_2\bullet^-$), and hydroxyl radicals (HO•) scavenging. Furthermore, it decreased the generation of free radicals in mitochondria [49]. It was also reported that it suppressed 1-methyl-4-phenylpyridinium ion (MPP^+)-induced cytotoxicity and oxidative stress in cultured PC12 rat pheochromocytoma cells via the suppression of ROS generation, as well as the enhancement of nuclear factor erythroid 2-related factor 2 (Nrf2), which regulates the expression of a variety of genes for endogenous antioxidant defense and xenobiotic metabolism [50], and heme oxygenase-1 (HO-1) mRNA and protein expression, and finally the suppression of NAD(P)H oxidase 2 (NOX2) mRNA and protein regulation [51]. In cultured rat alveolar macrophages exposed to phorbol 12-myristate 13-acetate (PMA) or lipopolysaccharides (LPS), astaxanthin increased cell viability and showed a suppressive effect on the generation of both free radical superoxide ($O_2\bullet^-$) and nitric oxide (NO) [52]. In cultured primary rat hepatocytes treated with 2,3,7,8,-tetrachlorodibenzo-p-dioxin (TCDD) which is a highly toxic and persistent organic pollutant, astaxanthin increased the reduced total antioxidant capacity (TAC) ratios in a dose-dependent manner and decreased the level of 8-hydroxy-deoxyguanosine (8-OHdG), an indicator of the oxidative DNA damage [53]. Furthermore, in cultured HeLa human cervical cancer cells, undifferentiated PC12 rat pheochromocytoma cells, and Jurkat immortalized human T lymphocyte cells, astaxanthin has been shown to decrease physiological endogenous oxidative stress and to protect cultured cells against strong oxidative stress induced by a mitochondrial respiratory inhibitor, such as

Antimycin A. In addition, under basal conditions, astaxanthin exhibited significant positive effects on mitochondrial function by improving the maintenance of high mitochondrial membrane potential and stimulation of mitochondrial respiration. This study concluded that astaxanthin improved the mitochondrial redox state by maintaining mitochondria in a reduced state under oxidative challenge [54]. In cultured human lymphocytes, treatment with astaxanthin could partially prevent fatty acid induced oxidative stress probably by quenching free radical production. It promoted a reduction in superoxide $O_2\bullet^-$, H_2O_2, and more generally in ROS production. In addition, it partially restored thiobarbituric acid reactive substances (TBARS) levels, which is a good indicator of lipid peroxidation [55]. In cultured human dermal fibroblast exposed to UVA irradiation, astaxanthin inhibited apoptosis, decreased membrane perturbation as well as levels of ROS and TBARS, and increased antioxidant enzymes activities. Its preventive effects against photo-oxidative induced alterations were superior compared to β-carotene and canthaxanthin [56].

In vivo experiments performed with mice retina showed that astaxanthin reduced the expression of 4-hydroxy-2-nonenal (4-HNE)-modified protein, an indicator of lipid peroxidation, and 8-OHdG induced by intravitreal N-methyl-D-aspartate (NMDA) injection [49]. In the liver of apolipoprotein E knockout (apoE$^{-/-}$) mice fed with a high-fat and high-cholesterol diet, astaxanthin extracted from *H. pluvialis* induced expression of the *Nrf2* gene, as well as its target genes, such as glutamate-cysteine ligase modifier subunit (*GCLm*), superoxide dismutase 1 and 2 (*SOD-1, SOD-2*), and reduced glutathione (GSH) peroxidase 1 (*GPX-1*). Concomitantly, the level of glutathione disulfide (GSSG, an oxidized form of GSH) was reduced and the expression of uncoupling protein 2 (UCP-2), which inhibited ROS production, was increased [50]. In rat model, both pre- and post-treatment with astaxanthin attenuated cyclophosphamide-induced oxidative stress and DNA damage in the liver as evidenced by a decrease of malondialdehyde (MDA), a lipid peroxidation marker, and an increase of GSH levels. It was further observed that the level of nuclear factor Nrf2 and the associated protective phase-II enzymes, e.g., quinine oxidoreductase-1 (NQO-1) and HO-1, were increased with astaxanthin-treatment [57]. In Wistar rats, supplementation with astaxanthin extracted from *H. pluvialis* offered extended protection to auto-induced lipid oxidation in membranes of neutrophils activated by PMA. In non-activated neutrophils, it increased the GSH-recycling enzymes glutathione peroxidase (GPX) and the glutathione reductase (GR) [58].

In an ex vivo study on plasma sampled from healthy subjects which had ingested during 14 days up to 21.6 mg/day of astaxanthin extracted from krill, the LDL oxidation lag time induced by the oxidant agent V-70 was significantly increased in a dose-dependent manner. In this study, its capacity to reduce LDL oxidability was stronger than that of α-tocopherol and lutein in vitro [68]. A randomized, double-blind, placebo-controlled trial on healthy non-smoking men showed that diet supplementation with *H. pluvialis*-extracted astaxanthin at 8 mg/day for three months could decrease lipid peroxidation, as evidenced by the reduction of 12- and 15-hydroxy fatty acids plasma levels, biomarkers of lipid peroxidation [41]. Another similar study performed on young healthy women supplemented with capsules of oleoresin extracted from *H. pluvialis* showed that astaxanthin decreased the plasmatic 8-OHdG level at 2 mg/day during four weeks [114]. Overweight and obesity induce an increase in oxidative stress. In a prospective randomized and double-blind study performed on overweight and obese healthy adults (BMI > 25 kg/m^2), oral administration of astaxanthin extracted from *H. pluvialis* at doses of 5 and 20 mg/day for three weeks significantly suppressed lipid peroxidation and stimulated the activity of the endogen antioxidant defense system, as evidenced by the reduced plasmatic levels of MDA and isoprostanes (ISP), which is another lipid peroxidation marker, and the increased SOD and TAC levels [44]. Smoking also increases oxidative stress. Similar results were observed in a study on heavy smokers to whom astaxanthin supplementation at doses from 5 to 40 mg/day for three weeks also prevented oxidative damage by suppressing peroxidation and stimulating the activity of the antioxidant system [115] (Table 2).

2.5.2. Anti-Inflammatory Activity

It is well recognized that persistent inflammation contributes to the pathogenesis of many diseases, including cancer, heart disease, and atherosclerosis [59]. During the inflammatory process, the production of nitric oxide (NO) increases up to levels that induce cytotoxicity of the compound to the cells by reacting with superoxide anions to generate peroxynitrite (ONOO-). This NO production by macrophages depends upon inducible NO synthase (iNOS), which can be activated by various agents, including lipopolysaccharides (LPS), interferon-gamma (IFN-γ), and tumor necrosis factor-α (TNF-α). ROS can upregulate pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 [52]. Nuclear factor-κB (Nf-κB) is a transcription factor which controls the expressions of numerous genes, such as pro-inflammatory genes encoding TNF-α, IL-1β, IL-6, COX-2, and iNOS [60].

A study performed in vitro has shown that astaxanthin inhibited the production of pro-inflammatory mediators, including NO and prostaglandin E_2 (PGE_2), that are synthesized by iNOS and cyclooxygenase-2 (COX-2) respectively, and cytokines, such as TNF-α and interleukin-1β (IL-1β), in LPS-stimulated RAW264.7 murine macrophage cells. It also inhibited activation of NF-κB, which activates pro-inflammatory genes encoding iNOS, COX-2, TNF-α, IL-1β, and IL-6, as well as iNOS promoter activity. It directly inhibited the intracellular accumulation of ROS in these cells, as well as H_2O_2-induced NF-κB activation and iNOS and COX-2 expression. Moreover, astaxanthin blocked nuclear translocation of NF-κB p65 subunit and IκB-α degradation, which was correlated to its inhibitory effect on IκB kinase (IKK) activity. This study suggests that astaxanthin inhibits the production of inflammatory mediators by blocking NF-κB activation and consequent suppression of IKK activity and IκB-α degradation [61]. In the same model, another study showed that astaxanthin decreased production of NO, the enzymatic activity of iNOS, and the production of PGE_2 and TNF-α in a dose-dependent manner [62]. In LPS-stimulated BV2 mouse microglial cells, astaxanthin inhibited the production of NO as well as the mRNA and protein expression of iNOS and COX-2. The results suggested that astaxanthin, probably due to its antioxidant activity, inhibited the production of inflammatory mediators by blocking iNOS and COX-2 activation [63]. In THP-1 human macrophages, astaxanthin also significantly suppressed the mRNA expression of iNOS and COX-2, as well as pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6). Furthermore, astaxanthin inhibited the phosphorylation of NF-κB [60]. Similar results have been reported in cultured U-937 human monocytes exposed to H_2O_2. In addition, it was shown that this property is elicited by the restoration of the basal expression level of SHP-1, a protein tyrosine phosphatase which regulates negatively the immune cytokine signaling, and reduced NF-κB (p65) nuclear expression [59]. Furthermore, in cultured human primary keratinocytes, pre- or post-treatments of astaxanthin have significantly suppressed UVB-induced secretion of PGE_2 and IL-8 through the down-regulated expression of COX-2 and IL-8 by an inhibited phosphorylation of NF-kB [64].

In vivo, it has been observed that oral administration of astaxanthin in BALB/cA mice infected by *Helicobacter pylori* reduced the inflammation score of the gastric mucosae [65]. Astaxanthin suppressed the serum levels of NO, PGE2, TNF-α, and IL-1β in LPS-administrated septic mice. The results of this study suggested that astaxanthin prevents inflammatory processes by blocking the expression of pro-inflammatory genes as a consequence of suppressing NF-κB activation [61]. In mice fed with astaxanthin, the expression of NF-κB and inflammatory cytokines, including TNF-α and IL-1β in azoxymethane/dextran sulfate sodium (AOM/DSS)-induced colonic adenocarcinomas was suppressed. It also lowered the protein expression of NF-κB and the mRNA expression of inflammatory cytokines, including IL-1β, IL-6, and COX-2, TNF-α, and iNOS in DSS-induced colonic inflammation [66]. Administration of astaxanthin also suppressed the development of LPS-induced rat uveitis (EIU), an animal model of acute anterior segment intraocular inflammation, in a dose-dependent manner. The study suggests that astaxanthin displays a dose-dependent ocular anti-inflammatory effect, by suppression of NO, PGE_2, and TNF-α production, through directly blocking NOS enzyme activity [62]. Finally, in Wistar male rats, astaxanthin attenuated the inflammation induced by the 1, 2 dimethyl

hydrazine (DMH) by decreasing expression of NF-κB, possibly by blocking its nuclear translocation, and COX-2 [67].

In the clinical study of Park, supplementation with astaxanthin extracted from *H. pluvialis* at a dose of 2 mg/day for eight weeks reduced plasma concentration of C-reactive protein, an acute-phase protein marker of inflammatory status [114] (Table 2).

2.5.3. Roles of Astaxanthin in the Prevention of Cardiovascular Disease

Cardiovascular disease (CVD) is the leading cause of death worldwide. The coexistence of dyslipidemia, impaired glucose tolerance, and hypertension with visceral fat accumulation (or obesity) is known as metabolic syndrome, which synergistically increases the risk of CVD. Metabolic syndrome is typically characterized by oxidative stress, which can lead to the oxidation of biological molecules, including unsaturated lipids in low-density lipoprotein (LDL), and can cause dysfunctional endothelial cells. This can play a central role in the pathogenesis of several CVD, including atherosclerosis, which leads to myocardial infarction and ischemic stroke [69,70,124]. Also, growing evidence suggested that astaxanthin may exert protective actions on CVD by improving oxidative stress, inflammation, lipid metabolism, and glucose metabolism [124].

It is well known that dyslipidemia and oxidative stress contribute to atherogenesis [50]. For instance, it was shown that astaxanthin reduced LDL oxidability in vitro [68] and lowered plasma total cholesterol and triglyceride (TG) levels in obese mice fed high-fat diet [71] and in apolipoprotein E Knockout (apoE$^{-/-}$) mice fed a high-fat and high-cholesterol diet, the most commonly used mouse model of atherosclerosis [50] in vivo. The hypocholesterolemic effect is probably linked to the enhanced expression in the liver of the transcription factor sterol regulatory element binding-protein-2 (SREBP-2). Consequently, the expression of LDL receptor (LDLR) and 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMG-CoA reductase), which are involved in the hepatic cholesterol metabolism and regulated by SREBP-2, was also enhanced. The TG-lowering effect of astaxanthin may be due to the increased expression of the enzymes involved in the fatty acid β-oxidation in the liver, namely carnitine palmitoyl transferase 1 (CPT-1), acyl CoA oxidase (ACOX), and acetyl-CoA carboxylase-β (ACC-β). Because CPT-1 and ACOX expression is transcriptionally regulated by the peroxisome proliferator-activated receptor α (PPARα), the authors speculated that astaxanthin may activate the regulator PPARα. This study also reported that the endogenous antioxidant defense mechanisms are stimulated by astaxanthin supplementation via the activation of Nrf2 pathway (see above). Thus, improvement of cholesterol and lipid metabolism as well as antioxidant defense mechanisms could help mitigate the progression of atherosclerosis [50].

The susceptibility of atherosclerotic plaques to rupture, which increases the risk of thrombus formation, is partly dependent on their composition. A reduction in lipid content and inflammatory activity minimized the risk of plaque disruption, thereby avoiding adverse clinical events. Other factors related to plaque stability included the number of macrophages and smooth muscle cells, apoptosis of arterial cells, and the collagen content in atherosclerotic lesions. In the thoracic aorta of Watanabe heritable hyperlipidemic (WHHL) rabbits, astaxanthin significantly improved atherosclerotic plaque stability by decreasing macrophage, collagen, and smooth muscle cells infiltration, matrix metalloproteinase 3 (MMP-3) expression, and apoptosis [72]. The class A scavenger receptor (SR-A) and CD36 are integral membrane proteins responsible for the major part of oxidized LDL uptake by macrophage, suggesting their pro-atherogenic roles. In THP-1 human macrophages, astaxanthin remarkably decreased the expression of SR-A and CD36 and reduced the activity of metalloproteinases, which are increased in atherosclerotic lesions and linked to a weakening of the vascular wall due to degradation of the extracellular matrix [60].

The normal vascular endothelial function appears to be strongly dependent on the balance between nitric oxide (NO) and ROS, such as superoxide anion and hydrogen peroxide. ROS increase vasoconstriction and platelet activation and aggregation, while NO inhibits platelet activities and is involved in vasorelaxation [70,73,74]. Accordingly, long-term administration of astaxanthin induced a

significant reduction in blood arterial pressure (BP) in spontaneously hypertensive rats (SHR) and SHR prone to stroke (SHR-SP) in vivo. Moreover, it delayed the incidence of stroke in SHR-SP [69]. In SHR, it was demonstrated that astaxanthin can modulate the oxidative condition and may improve some vascular histopathological changes associated with hypertension such as increased vascular elastin fiber number and coronary arterial wall thickness [73]. Furthermore, in SHR-SP, a study has shown that long-term astaxanthin-feeding suppressed the increase of pressure of systolic blood and thrombogenesis in cerebral vessels. The results of this study suggested that the antithrombotic and antihypertensive effects of astaxanthin are linked to an increase in bioavailable NO, possibly mediated by a reduction of the inactivation of NO by ROS [70]. Another study performed on SHR reported that the antihypertensive effect of chronic administration of astaxanthin is a consequence of lowering of blood pressure associated with improved endothelium-dependent vasodilatation and cardiovascular remodeling, and decreased NAD(P)H oxidase-stimulated $\bullet O_2^-$ production associated with NO bioavailability [74]. As a consequence, it could be concluded that the antihypertensive and antithrombotic effects of astaxanthin are most likely related to its strong antioxidant activities [70].

An ex vivo study performed on human plasma showed that astaxanthin extracted from krill increased LDL oxidation lag time induced by an oxidant agent in a dose-dependent manner and thus reduced LDL oxidability [68]. In a randomized double-blind, placebo-controlled study on non-obese healthy subjects with mild hyperlipidemia, the administration of astaxanthin extracted from *H. pluvialis* at doses from 6 to 18 mg/day for 12 weeks significantly reduced the serum triglyceride content at doses comprised between 12 and 18 mg/day, while serum HDL-cholesterol, a robust negative risk factor for atherosclerotic cardiovascular disease, was increased significantly at lower doses comprised between 6 and 12 mg/day. Serum adiponectin, which is one of the adipocytokines involved in insulin sensitivity and thus in glucose and lipid metabolism, was increased at doses of 12 and 18 mg/day with a positive correlation with HDL-cholesterol and inversely a negative correlation with TG and VLDL-cholesterol. Thus astaxanthin was found to be able to reverse cholesterol transport from peripheral tissues to the liver [116]. In another randomized, double-blind, placebo-controlled trial, it was also shown that this type of supplementation attenuated phospholipid peroxidation of erythrocyte membranes rich in polyunsaturated fatty acids (PUFAs) at doses of 6 or 12 mg/day for 12 weeks, as shown by the low erythrocyte and plasma phospholipid hydroperoxide (PLOOH) concentrations [117]. In a single-blind, placebo-controlled study, administration of astaxanthin extracted from *H. pluvialis* at a dose of 6 mg/day for 10 days significantly decreased the whole blood transit time, which reflects an improvement in blood rheology [118] (Table 2).

2.5.4. Roles of Astaxanthin in the Prevention of Diabetes

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia and insufficient secretion or action of endogenous insulin, predisposing to an increase of micro- and macro-vascular complications. Chronic hyperglycemia can lead to an increase of ROS and RNS production by mitochondria, glucose auto-oxidation, activation of the polyol pathway, formation of advanced glycation end-products (AGEs), antioxidant enzyme inactivation, and imbalance of the glutathione redox status [77]. Thus, it can promote significant oxidative stress which results in various tissue damage and contributes to the development and progression of diabetes and its consequences such as neuropathy, nephropathy, retinopathy, and vascular disorders [78].

In diabetes mellitus, the β-cells of the islets of Langerhans in the pancreas do not produce enough insulin or the insulin receptors are not working properly and insulin deficiency may lead to hyperglycemia. In db/db mice (an obese model of type 2 diabetes), oral astaxanthin treatment has been shown to lower non-fasting blood glucose levels and preserves the ability of pancreatic β-cells to secrete insulin. The results of this study suggested that astaxanthin may reduce the oxidative stress caused by hyperglycemia in the pancreatic β-cells [79]. In alloxan-induced diabetic mice, it was reported that oral administration of astaxanthin extracted from shrimp waste is also effective in suppressing hyperglycemia in this rodent model of type 1 diabetes mellitus [78].

Oxidative stress promotes insulin resistance in obesity and type 2 diabetes. Insulin resistance in insulin target tissues such as adipose tissue and skeletal muscle leads to the development of type 2 diabetes. A concomitant decrease in endogenous antioxidants was observed in diabetic patients [75]. The metabolic mechanism of insulin is mediated by a pathway involving the insulin receptor substrates (IRS), phosphatidylinositol-3-kinase (PI3K), and protein kinase B (Akt) (IRS-PI3K-AKT signaling pathway). Insulin resistance occurs when the pathway is downregulated, including through the phosphorylation of the insulin receptor and its substrates (IRS)-1 and 2 by serine kinases, such as c-Jun NH2-terminal kinase (JNK) and inhibitor kappa kinase beta (IKKβ) [80]. Using an in vitro experiment based on a model skeletal muscle cell line (L6 rat muscle cells), it was shown that astaxanthin enhanced insulin-stimulated GLUT4 translocation to the plasma membrane and consequently glucose uptake, which is associated with an increase in insulin receptor substrate (IRS)-1 tyrosine and Akt phosphorylation and a decrease in c-Jun NH2-terminal kinase (JNK), a regulator of the negative feedback in the insulin signaling pathway, and IRS-1 serine phosphorylation. Furthermore, astaxanthin restored the lack of insulin-stimulated GLUT4 translocation or glucose uptake induced by inflammatory cytokines, such as TNF-α, or fatty acids, such as palmitate, which are key mediators in insulin resistance in high-fat diet obese animals. Concomitantly, astaxanthin decreased the ROS generation through an Akt-involving signaling pathway [75]. The improvement of insulin sensitivity via the IRS-PI3K-Akt pathway of insulin signaling in skeletal muscle was also demonstrated in high fructose-fat diet (HFFD)-fed mice [81]. In this model, another study showed that astaxanthin reduced ROS production, lipid droplet deposition, expression of JNK and IKKβ, as well as activation and nuclear translocation of Nf-κB, which were increased in the liver after HFFD-diet. This study concluded that astaxanthin can reverse the induction of hepatic inflammation and endoplasmic reticulum stress in a diet-induced obese mouse model of insulin resistance [80].

The immune system is particularly vulnerable to oxidative damage because many immune cells (such as neutrophils) produce ROS and RNS as part of the body's defense mechanisms to destroy invading pathogens. Neutrophils are extremely sensitive to oxidative damage due to their high PUFA content and diabetes promotes significant oxidative stress in neutrophils. An in vivo study showed that astaxanthin-treatment can partially reverse lipid and protein damage, as shown by decreased levels of TBARS and of protein carbonyl groups (an indicator of damaged proteins) in neutrophils from alloxan-induced diabetic rats stimulated with phorbol myristate acetate (PMA), a potent inducer of respiratory burst [77].

Diabetic nephropathy is characterized by persistent proteinuria, hypertension, deterioration of the renal function [82], and enlargement of glomerular mesangium due to the accumulation of extracellular matrix protein. In type 1 and 2 diabetes, hyperglycemia is responsible for the development and progression of vascular complications, including diabetic nephropathy, through metabolic derangements. Indeed, hyperglycemia increases oxidative stress, renal polyol formation, activation of protein kinase C-mitogen-activated protein kinases, and accumulation of AGE products, as well as such hemodynamic factors as systemic hypertension and increased intraglomerular pressure [83]. In db/db mice, it was shown that chronic astaxanthin-treatment reduced the oxidative stress on the kidneys and prevented renal cell damage. Indeed, it lowered the level of blood glucose, improved glomerular histologic changes, decreased urinary albumin and 8-OHdG, and reduced 8-OHdG expression in mesangial cells [83]. Similar results were obtained in streptozotocin (STZ)-induced diabetic Osteogenic Disorder Shionogi (ODS) rats. In this study, dietary astaxanthin in combination with α-tocopherol was able to improve the oxidative injury in the kidney through the suppression of oxidative stress induced by diabetes. This effect was shown by the decrease of urinary 8-OHdG excretion, serum creatinine (Cr) level, creatinine clearance (Ccr), urinary albumin excretion, and urinary protein concentration, as the renal function parameters and of lipid peroxide levels in the serum, liver, and kidneys [82]. Also, hyperglycemia causes cytotoxicity in proximal tubular epithelial cells (PTECs). In cultured porcine PTECs exposed to high-glucose which induced oxidative stress, inflammation, and apoptosis, astaxanthin improved the overall oxidative stress of the cells by its ability to suppress

lipid peroxidation and generation of total reactive species, $ONOO^-$, •O2, and NO•. Additionally, it suppressed the inflammatory process by modulating COX-2, iNOS, and NF-κB nuclear translocation. Furthermore, astaxanthin upheld anti-apoptotic Bcl2 protein levels while suppressed pro-apoptotic Bax protein levels. This study then suggested that the modulation of oxidative stress, inflammation, and apoptosis was the likely mechanism underlying the beneficial role of astaxanthin on diabetic nephropathy in high-glucose-treated PTECs [76].

2.5.5. Protective Effect of Astaxanthin on Liver

Several studies have shown that astaxanthin could minimize the cytotoxic effect induced by toxic compounds on hepatocytes. An in vitro study performed on cultured primary rat hepatocytes treated with TCDD, shown that astaxanthin improved cell viability, and decreased the extracellular level of lactate dehydrogenase (LDH), a marker of hepatocellular damage. It also increased the total antioxidant capacity (TAC) reduced by TCDD and minimized micronucleated hepatocytes (MNHEP) rates, a marker of DNA damage, in a dose-dependent manner, and leveled of 8-OH-dG [53]. Another in vivo study demonstrated that astaxanthin impaired the increase of glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GTP) activity and TBARS in response to carbon tetrachloride (CCl4) in rat liver, while it generated an increase in glutathione (GSH) levels and SOD activities. Thus, astaxanthin was assumed to protect liver damage induced by CCl4 by inhibiting lipid peroxidation and stimulating the cellular antioxidant system [87].

Non-alcoholic fatty liver disease (NAFLD) is a common cause of chronic liver disease and a major indicator of metabolic syndrome, especially obesity and diabetes, and is characterized by excessive hepatic lipid accumulation. Non-alcoholic steatohepatitis (NASH) is a more severe form of NAFLD that is broadly defined by the presence of steatosis with inflammation and progressive fibrosis, ultimately leading to cirrhosis and hepatocellular carcinoma (HCC). Insulin resistance (that leads to hepatic steatosis) and oxidative stress (which leads to steatohepatitis and fibrosis) are among the major risk factors associated with NAFLD. Also, fatty liver is susceptible to oxidative stress and inflammation. Consequently, it was found that the liver activated the resident immune cells like Kupffer cells (which lead to the development of insulin resistance) and hepatic stellate cells (HSC), which are primarily responsible for extracellular matrix (ECM) production and fibrosis development in the liver [84,88,125]. It was then concluded that astaxanthin has the potential to improve NASH in humans. [89,125].

It was shown that astaxanthin prevented in vivo diet-induced obesity as well as hepatic triglyceride accumulation and steatosis in mice [71]. In lipid loaded HepG2 human hepatocytes, it was demonstrated that astaxanthin significantly reduced cellular cholesterol and TG concentration by regulating the peroxisome proliferator-activated receptor (PPAR) activity. Astaxanthin was found to bound directly to PPAR-α and PPAR-γ with moderate affinity and to activate PPAR-α, which stimulates fatty acid catabolism via increased β-oxidation and uptake of fatty acids, and to inhibit PPAR-γ, a pre-steatotic factor which increases TG accumulation in hepatocytes. The results of this study also indicated that astaxanthin improved insulin resistance [85].

TGF-β1 is a cytokine secreted by hepatic stellate cells (HSC) and this protein plays a central role in the early stage of fibrogenesis by promoting the accumulation of several extracellular matrix proteins, especially collagen by inhibiting its degradation and activating its biosynthesis. A study based on obese mice fed with HFFD showed that astaxanthin treatment prevented liver injury by decreasing oxidative stress, fat accumulation, and inflammatory cell infiltration. Indeed, it decreased the hepatic activities of cytochrome P4502E1 (CYP2E1), a pro-oxidative enzyme, and myeloperoxidase (MPO), an indicator for inflammatory cell infiltration and tissue injury. It reduced nitro-oxidase stress and improved the antioxidant status by the enhancement of SOD, CAT, GPX, and glutathione-S-transferase (GST), in the plasma and the liver. Astaxanthin treatment also abolished lipid deposition and increased TGF-β1 expression in the liver induced by HFFD and restored plasma and liver lipid levels. It also controlled the levels of lipid peroxidation products (such as lipid hydroperoxide and TBARs) and protein oxidation products (such as protein carbonyl). In this study, the sensitivity to insulin of the

whole body was substantially increased by astaxanthin [88]. In the normal liver, quiescent HSCs are present in the space of Disse and contain cytoplasmic lipid droplets mainly consisting of retinyl esters. In response to injury, quiescent HSCs transdifferentiate to myofibroblast-like cells, which highly express α-smooth muscle actin (α-SMA), a myofibroblast marker, and ECM proteins, e.g., procollagen type I and alpha 1 (Col1A1). The activated HSCs are highly proliferative and migrate to the sites of injury. HSC activation was found to be a reversible process when stimulants, such as oxidant or fibrogenic cytokines are removed and activated HSCs are either eliminated through apoptosis or reverted to an inactive phenotype [84]. It was reported in immortalized human HSC cell line LX-2 that ROS accumulation induced by tert-butyl hydrogen peroxide and TGF-β1, the most potent pro-fibrogenic cytokine, was abolished in vitro by astaxanthin. It exerted anti-fibrogenic effects by blocking TGFβ1-signaling, consequently inhibiting the phosphorylation, nuclear translocation, and activation of Smad3, which regulates the expression of α-SMA and procollagen type 1 and alpha 1 (Col1A1) in HSCs [86]. In primary HSCs from C57BL/6J mice, astaxanthin prevented the activation of quiescent HSCs, as evidenced by the presence of intracellular lipid droplets and the reduction of α-SMA. It also reversed activated HSCs to a quiescent phenotype with the reappearance of lipid droplets with a concomitant increase in lecithin retinol acyltransferase (LRAT), an enzyme that esterifies retinol to retinol esters, and a decrease in α-SMA and Col1A1 expressions. The cellular accumulation of ROS was significantly reduced by astaxanthin, which is attributable to a decrease in NADPH oxidase 2 expression [84].

In an in vivo lipotoxic model of the NASH experiment, where mice were fed with a high-cholesterol and high-fat diet, it was demonstrated that astaxanthin attenuated insulin resistance, excessive hepatic lipid accumulation and peroxidation, the proportion of M1-type macrophages/Kupffer cells, stellate cell activation and fibrosis. These effects were associated with attenuated MAPK (JNK/p38 MAPK) signaling and NF-κB activation, decreased T cell accumulation, as well as enhanced alternative M2 macrophage activation in the liver. In addition, astaxanthin improved simple steatosis, the early stage of NAFLD, in both genetically (ob/ob) and diet-induced obese mice, more potently than do vitamin E, which has become a standard treatment for NASH [89].

In a randomized, placebo-controlled trial on biopsy-confirmed NASH patients, oral administration of astaxanthin at a dose of 12 mg/day for 24 weeks had markedly improved hepatic steatosis, as evidenced by the reduced grade of steatosis and the alleviated lobular inflammation. This study also suggested that astaxanthin can reduce the total NAFLD activity score and alleviate human NASH [89] (Table 2).

2.5.6. Immuno-Modulating Effects

The immune system is a vital defense against tumors, cancerous growth, and infectious diseases. Immune cells are particularly sensitive to oxidative stress due to a high percentage of PUFA in their plasma membranes and their ability to produce oxidative products [91]. Astaxanthin harbored a potential value as a therapeutic or preventing agent for the management of immune diseases because astaxanthin significantly influenced the immune function in several in vitro and in vivo assays. For instance, it was shown that astaxanthin modulated the humoral immune response in vitro, and that this effect was more significant than β-carotene. Several studies have indicated that astaxanthin enhanced the antibody (Ab) production in cultured mouse splenocytes in response to T cell-dependent Ag and induced production of polyclonal antibodies G and M in murine spleen cells. It also stimulated cell proliferation of murine splenocytes and thymocytes in vitro. Astaxanthin was also able to enhance Ig production by peripheral blood mononuclear cells (PBMNC) from human blood samples. It increased IgM production in response to T-dependent Ag and a T-dependent polyclonal stimulant, IgG production in response to a recall Ag and IgA production in response to a T-dependent polyclonal stimulant [10,90,114]. It was also reported in in vivo experiments that Ab production, in response to T-dependent antigens (TD-Ag), was significantly enhanced by astaxanthin in mice and that the number of IgM- and IgG-secreting cells increased. Furthermore, it was shown that astaxanthin

supplementation partially restored the in vivo Ab production in response to TD-Ags which is decreased in old mice. Similarly, it was demonstrated that lutein and β-carotene could also produce a similar effect, but to a lesser extent [10,90,114].

Activated T cells and NK cells produce IFN-γ, which is involved in immune-regulation, B cell differentiation, and antiviral activity [98]. It was shown that astaxanthin decreased bacterial load and gastric inflammation in mice infected with *Helicobacter pylori* by shifting the T-lymphocyte response from a Th1 response dominated by interferon-γ (IFN-γ) to a Th1/Th2 response dominated by IFN-γ and IL-4 [92]. Another study also indicated that astaxanthin modulated in vitro the lymphocytic immune response in primary cultured lymphocytes from the spleen of BALB/c mice and that it partly exerted its ex vivo immunomodulatory effects by increasing INF-γ and IL-2 production, which are the major Th1 cytokines involved in cellular immune activation of dendritic cells and monocytes, in the presence of LPS (a C cell-dependent mitogen) or Con A (concanavalin A, a T cell-dependent mitogen), without inducing cytotoxicity [90].

Dietary astaxanthin also heightened cell-mediated and humoral immune responses in dogs [93] and cats [91]. In dogs, it was shown that dietary astaxanthin enhanced cutaneous delayed-type hypersensitivity (DTH) response to intradermal challenge with a vaccine, as well as Con A-induced lymphocyte proliferation and natural killer (NK) cell cytotoxic activity in a dose-dependent manner. In addition, it increased concentrations of IgG, IgM, and B cell populations in a dose-dependent manner. Furthermore, this study showed that astaxanthin reduced DNA damage and acute-phase protein (CRP) production in dogs [93]. Similar results were obtained in cats to whom dietary astaxanthin enhanced DTH response to both specific (vaccine) and nonspecific (Con A) antigens. In addition, cats fed with astaxanthin have heightened PBMNC proliferation and natural NK cell cytotoxic activity. The leukocyte population of total T and T helper cells were also higher in cats fed with astaxanthin in a dose-dependent manner. Dietary astaxanthin finally increased concentrations of plasma IgG and IgM. In contrast to dogs [93], astaxanthin decreased the population of B cells in cats [91].

In human, a randomized double-blind, placebo-controlled study performed on young healthy female subjects reported that dietary astaxanthin extracted from *H. pluvialis* at doses from 2 to 8 mg/day for eight weeks enhanced both cell-mediated and humoral immune responses. Indeed, it stimulated T cell and B cell mitogen-induced lymphocyte proliferation, NK cell cytotoxic activity, IFN-γ, and IL-6 production, and lymphocyte function-associated antigen 1 (LFA-1) expression. The subjects fed with 2 mg/day of astaxanthin had a higher tuberculin response to the tuberculin DTH test (a reliable clinical test to assess in vivo T cell function) as compared to subjects un-supplemented. Furthermore, this study has shown that dietary astaxanthin decreased DNA damage biomarker and acute-phase protein [114] (Table 2).

2.5.7. Anti-Cancer Activities

Pharmacological or naturally occurring compounds inhibit the development of invasive cancer by preventing the initiation of carcinogenesis or by arresting or reversing processes of tumor progression [98]. Oxidative stress which leads to damage of DNA and inflammation play important roles in all stages of carcinogenesis and tumorigenesis [99]. The immunomodulatory, antioxidative, and anti-inflammatory activities of astaxanthin could influence the etiology of cancer [114]. Moreover, it was demonstrated in many in vitro and in vivo studies that astaxanthin reduces the tumorigenesis also through the inhibition of cell proliferation, cell cycle arrest, and the induction of cell apoptosis [126].

In male ICR mice, it was shown that astaxanthin administration had a chemopreventive effect on urinary bladder carcinogenesis induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (OH-BBN), as evidenced by the reduced incidences of preneoplastic lesions and neoplasms in the bladder. This effect was partly due to suppression of cell proliferation, as revealed by the decreased number/nucleus of silver-stained nucleolar organizer region (AgNOR) proteins, an index of cell proliferation, in the transitional epithelium exposed to OH-BBN. This study has also reported that the antiproliferative potential was greater for astaxanthin as compared to canthaxanthin [100].

It was also demonstrated that astaxanthin significantly reduced proliferation rates and inhibited breast cancer cell migration in breast epithelial cells in vitro [94]. In female BALB/c mice inoculated with a mammary tumor cell line, it was reported that astaxanthin extended tumor latency and delayed tumor growth, and modulated immune response when it is given before tumor initiation [101]. Astaxanthin was more active than β-carotene, lutein, and canthaxanthin in inhibiting mammary tumor growth in mice [102,114].

In male F344 rats, it was shown that astaxanthin has also chemopreventive effects on oral carcinogenesis induced by 4-nitroquinoline 1-oxide (4-NQO), as evidenced by the reduced incidences of preneoplastic lesions and neoplasms in the oral cavity. This tumor inhibitory potential is probably linked to the suppression of cell proliferation activity, as revealed by the decreased AgNOR protein number/nucleus [103]. STAT-3 is a cytosolic transcription factor involved in the activation of various genes implicated in tumor progression. In the 7, 12-dimethylbenz(a)anthracene (DMBA)-induced hamster buccal pouch (HBP) carcinogenesis model, it was reported that dietary astaxanthin prevented the development and progression of HBP carcinomas by inhibiting key events in JAK/STAT signaling especially STAT-3 phosphorylation and its subsequent nuclear translocation. Consequently, it downregulated the expression of STAT-3 target genes involved in cell proliferation, invasion, and angiogenesis [104].

Also, astaxanthin was found to exert antitumor activity through the enhancement of immune responses. NK cells have an impact on the immuno-surveillance capacity against tumors and virus-infected cells. It was shown that astaxanthin-supplementation significantly lowered tumor size and weight in BALB/c mice transplanted with methylcholanthrene-induced fibrosarcoma (Meth-A tumor) cells. This antitumor activity was correlated with higher cytotoxic T lymphocyte (CTL) activity and IFN-γ production by NK cells in tumor-draining lymph nodes (TDLN) and spleen cells [98].

Liver cancer is among the most common malignant tumors. Oval cells, which can differentiate into hepatocytes and biliary epithelial cells, and which lead to liver regeneration when mature hepatocytes are injured, can trigger hepatic cancer, especially when an irreversible block of the process of normal differentiation is disturbed. In experiments using cultured oval cells isolated from partial hepatectomized or diethylnitrosamine (DEN)-treated rats, it was reported that astaxanthin increased albumin expression, a marker of mature hepatocyte, indicating an intensive differentiating effect. Furthermore, it inhibited the proliferative activity of oval cells which was increased by these treatments, and gradually elevated acute-phase protein levels, especially fibrinogen [95]. Another in vitro study showed that astaxanthin can inhibit H22 mice hepatoma cell proliferation in dose-and time-dependent manners by promoting apoptosis via inhibition of the JAK/STAT3 signaling pathway. Astaxanthin induced cell necrosis and cell cycle arrest in G2 phase in vitro and in vivo in mice. In vivo, astaxanthin also exerted a significant anti-tumor activity in a dose-dependent manner [96]. In rat model, another study showed that astaxanthin attenuated oxidative stress, DNA damage, cell death, as well as preneoplastic foci formation, an early biomarker of hepatocarcinogenesis, induced by cyclophosphamide (CP), a cytotoxic alkylating agent, in liver. This study suggested that this chemopreventive effect is partly mediated through the Nrf2-ARE pathway, which restores antioxidant system activity [57].

Colon cancer is also one of the major causes of cancer mortality worldwide. Colonic crypt cell hyperproliferation has been suggested to play a significant role in colorectal cancer formation and development. Aberrant crypt foci (ACF) represent precursor lesions in colon cancer and are widely used as early markers of colon cancer risk. They are characterized by the increased size of the crypts, a thickened layer of epithelial cells, an increased pericryptal space, and an irregular lumen [99]. Using F344 rats, it was shown that dietary astaxanthin played several effects during the post-initiation phase of colon carcinogenesis initiated with azoxymethane (AOM), as revealed by a reduced incidence and multiplicity of neoplasms in the large intestine, an inhibited development of ACF, and a decrease in cell proliferation activity [103]. In 1, 2 dimethyl hydrazine (DMH)-induced colon carcinogenesis in rat, pre-treatment with astaxanthin markedly reduced the degree of histological lesions, ACF

development, and cell proliferation. The results of this study revealed that astaxanthin significantly reduced the decrease of colon enzymatic (SOD, CAT, GPx, and GR) and non-enzymatic (GSH, vitamins C, E, and A) antioxidants and the increase of lipid peroxidation markers (TBARS, hydroperoxide) in colon and plasma. Thus, it was suggested that the chemoprotective effects of astaxanthin against colonic preneoplastic progression were partly due to its antioxidant activity [99]. Another study showed that astaxanthin exhibited anti-inflammatory and anti-cancer effects by inducing apoptosis in DMH-induced rat colon carcinogenesis, through the modulation of the expressions of NFκB, COX-2, matrix metalloproteinases (MMP) 2/9, proliferating cell nuclear antigen (PCNA), extracellular signal-regulated kinase-2 (ERK-2) and Akt proteins, which are involved in colon carcinogenesis [67]. In male ICR mice, it was reported that dietary astaxanthin significantly inhibited the occurrence of colonic mucosal ulcers, dysplastic crypts, and colonic adenocarcinoma in inflammation-related colon carcinogenesis. It possibly acted by suppressing the expression of inflammatory cytokines, including NF-κB, TNF-α, and IL-1β, inhibiting proliferation, and inducing apoptosis in colonic adenocarcinomas induced by azoxymethane (AOM)/dextran sulfate sodium (DSS), colon carcinogens. Astaxanthin also significantly inhibited the development of DSS-induced colitis and lowered the expression of NF-κB, inflammatory cytokines, including IL-1β, IL-6, and COX-2. This study then concluded that astaxanthin probably suppresses colitis and colitis-related colon carcinogenesis in mice, partly through inhibition of proliferation and expression of inflammatory cytokines including NF-κB [66].

In a study performed on HCT-116 human colon adenocarcinoma cells, astaxanthin extracted from *H. pluvialis* was able to inhibit cell growth in a dose- and time-dependent manner, by arresting cell cycle progression through a decrease of cyclin D1, a G0/G1 phase-related cyclin, expression concomitantly to an increase of p53 and cyclin kinase inhibitors, including $p21^{WAF-1/CIP-1}$ and p27, expression in a dose-dependent manner. It also mediated its protective effect by promoting apoptosis, as demonstrated by the increased number of apoptotic cells and activation of caspase-3, through a down-regulation of the phosphorylation of Akt, a modification of the apoptosis promoter Bax/apoptosis suppressors, such as Bcl-2 and Bcl-XL, and an increase of the phosphorylation of p38, JNK, and ERK 1/2, apoptosis-related MAPK kinases implicated in apoptosis induction [97].

2.5.8. Effects on the Nervous System and Cerebral and Visual Functions

Ischemic stroke is a leading cause of death and disability across the world. Ischemia induces an imbalance of endogenous oxidants and antioxidants and overproduction of toxic free radicals. Reperfusion is correlated with the massive production of ROS and RNS. This oxidative stress may lead to brain tissue damage directly through membranous lipid peroxidation, protein, and DNA oxidation and indirectly via inflammation and apoptosis. A study performed in vitro demonstrated the neuroprotective effect of astaxanthin pretreatment on oxidative stress-induced toxicity in primary culture of cortical neurons, as shown by the attenuation of H_2O_2-induced cell viability loss and apoptosis and the restoration of the mitochondrial membrane potential (MMP). The results obtained in vivo revealed that astaxanthin pretreatment prevented cerebral ischemic injury induced by middle cerebral artery occlusion (MCAO) and reperfusion in rats. It dramatically diminished infarct volume and improved neurological-deficit in a dose-dependent manner. It was then concluded that this neuroprotective effect could be partly due to the antioxidant activities of astaxanthin [105].

Aging is a primary risk factor for the development of many diseases including neurodegeneration. It leads to numerous anatomical and physiological changes, especially in the brain, and can compromise cognitive functions including memory, attention, executive function, and perception. Among several molecular mechanisms involved in neurodegeneration increased inflammation, mitochondrial dysfunction, disrupted calcium homeostasis, and elevated oxidative stress within the brain are thought to play a major role. It was notably shown that astaxanthin crossed the blood–brain barrier and was detectable in the brain tissue. It could also mitigate neuropathy in normal aging and neurodegenerative disease by its anti-inflammatory, anti-apoptotic, and antioxidant effects, as well as its potential to promote or maintain neural plasticity [127].

Many central nervous system disorders, including multiple sclerosis (MS), Alzheimer's disease (AD), and Parkinson's disease (PD) result in the activation of microglial cells, the resident macrophages of the brain, which release immune mediators (such as NO• and ROS/RNS), pro-inflammatory cytokines (such as IL-6 and chemokines, considered as putative neurotoxins contributing in chronic neurodegeneration) [106]. For example, excessive IL-6 production by activated microglia was linked to many neurological disorders. In LPS- or stromal-derived factor (SDF)-1α-stimulated BV-2 murine microglial cells, it was reported that astaxanthin displayed inhibitory effects on several intermediates of the NF-κB cascade, such as IKKα/β, IκBα, and NF-κB p65 phosphorylation, as well as on ERK1/2 and mitogen- and stress-activated protein kinase 1 (MSK1). Consequently, it modulated IL-6 expression in these activated cells [107].

Parkinson's disease (PD) is known as a progressive, neurodegenerative movement disorder, characterized by the loss of nigrostriatal (substantia nigra) dopaminergic neurons. Among mechanisms responsible for this degeneration, oxidative stress, mitochondrial inhibition, and impairment of the ubiquitin-proteasome system are known to play important roles in the pathogenesis of PD and other neurodegenerative disorders [51,108]. In SH-SY5Y human neuroblastoma cells, it was observed that astaxanthin-pretreatment attenuated the toxicity induced by docosahexaenoic acid hydroperoxide (DHA-OOH) or 6-hydroxydopamine (6-OHDA), ROS-inducing neurotoxins. Moreover, it significantly inhibited apoptosis, intracellular ROS generation, and mitochondrial abnormalities (as evidenced by a reduced cytochrome C release and protein carbonyls and increased mitochondrial membrane potential (MMP)) occurred in these treated cells. The study then suggested that the neuroprotective effect of astaxanthin was dependent upon its antioxidant potential and mitochondria protection [108]. In PC12 rat cells, which are widely used as cellular models of PD as they share features with midbrain dopaminergic neurons, astaxanthin was found to exert neuroprotective effects on 1-methyl-4-phenylpyridinium ion (MPP^+)-induced oxidative stress by reducing the increased intracellular ROS production, suppressing NOX2 expression and increasing Nrf2 and HO-1 expression. Thus, astaxanthin suppressed MPP^+-induced oxidative stress in PC12 cells via the HO-1/NOX2 axis [51].

Alzheimer's disease (AD) is an age-related neurodegenerative disease characterized by the deposition of beta-amyloid peptide (Aβ) plaques and intracellular neurofibrillary tangles and loss of neurons in the brain. Several lines of evidence indicated that Aβ has a causal role in the development and progression of AD and may directly induce neuronal cell death. In a study based on human neuroblastoma SH-SY5Y cells, it was demonstrated that astaxanthin pretreatment significantly attenuated the $A\beta_{25\text{-}35}$-induced viability loss, apoptotic rate, and ROS production. In addition, it inhibited the $A\beta_{25\text{-}35}$-induced lowered membrane potential and decreased Bcl-2/Bax ratio. Astaxanthin could prevent the activation of p38MAPK kinase pathways induced by Aβ. Moreover, astaxanthin increased antioxidant enzyme HO-1 expression, which is correlated with its protective effect against $A\beta_{25\text{-}35}$-induced injury, in concentration- and time-dependent manners. Thus, it was suggested that astaxanthin could induce HO-1 expression through activation of the ERK1/2 signal pathway, thereby protecting cells from Aβ-induced oxidative cell death [109].

Neurogenesis and plasticity significantly decrease with age, a trend that also reflects a concomitant cognitive decline. Neural progenitor cells (NPCs) are self-renewing stem cells that can supply new neurons to the hippocampus and can replace degenerating cells and their age-related decrease [127]. In cultured NPCs, it was shown that astaxanthin pretreatment significantly inhibited H_2O_2-mediated apoptosis and induced cell growth in a dose-dependent manner via the inhibition of p38 signaling pathway and the activation of the MEK signaling pathway [110]. Another study revealed that astaxanthin increased proliferation and colony formation of NPCs in a dose- and time-dependent manner through the up-regulation of proliferation-related transcription factors (Rex 1, CDK1, and CDK2) coupled with overexpression of stemness genes products (OCT4, SOX2, Nanog, and KLF4). This improved proliferation was associated with significant activation of PI3K and its downstream mediators (p-Rac, p-c-Raf, p-MEK, p-ERK, p-Akt, p-mTOR, and p-Stat3) in a time-dependent manner. Thus, it was then suggested that astaxanthin could induce proliferation of NPCs via activation of

the PI3K and MEK signaling pathways and could improve stem cell potency via stemness acting signals [111]. In vivo studies performed on mice have shown that astaxanthin significantly shortened the latency of escaping onto the platform in the Morris water maze learning performance test [69,113], a spatial learning task mediated by the hippocampus which may reflect its activity in neurogenesis.

Age-related macular degeneration (AMD) is a major cause of irreversible vision loss in elderly people in the developed world. Pathologic damage to retinal pigment epithelial (RPE) cells is an early event in AMD and oxidative stress is thought to play a significant role in the development and the progression of age-related RPE cell degeneration, dysfunction, and loss. In a study based on ARPE-19 cells (a human RPE cell line), astaxanthin treatment was found to exert a protective effect against oxidative stress, as shown by the reduced H_2O_2-induced cell viability loss, apoptosis, and intracellular generation of ROS. Furthermore, it activated the Nrf2-ARE pathway by inducing Nrf2 nuclear localization. Consequently, the expression of Phase II enzymes NAD(P)H quinine oxidoreductase 1 (NQO1), HO-1, glutamate-cysteine ligase modifier subunit (GCLM), and glutamate-cysteine ligase catalytic subunit (GCLC) was significantly increased. It also inhibited the expression of H_2O_2-induced cleaved caspase-3 protein. The Nrf2-mediated upregulation of Phase II enzyme expressions involves the activation of the PI3K/Akt pathway which probably acts in the protective effect of astaxanthin on the ARPE-19 cells [112].

Retinal ganglion cell (RGC) death is a common feature of many ophthalmic disorders, such as glaucoma, optic neuropathies, and various retinovascular diseases such as diabetic retinopathy and retinal vein occlusions. The death of RGC may occur via a variety of mechanisms involving, among others, ROS, excitatory amino acids, NO, and apoptosis. In cultured rat RGCs, it was shown that astaxanthin inhibited the neurotoxicity induced by H_2O_2 or serum deprivation by reducing the intracellular oxidation induced by ROS via scavenging H_2O_2, $O_2\bullet^-$, and HO•. Furthermore, it decreased the radical generation induced by serum deprivation in mitochondria. When analyzed in vivo, the results showed that a diet containing astaxanthin reduced the retinal damage induced by intravitreal N-methyl-D-aspartate (NMDA) injection, reduced the expression of 4-HNE-modified protein and 8-OHdG in mice. Thus, the neuroprotective effects of astaxanthin against retinal damage is expected to be partly mediated via its antioxidant activities [49].

In an open-label clinical study conducted on healthy aged male subjects who complained of age-related forgetfulness, it was observed that supplementation of astaxanthin extracted from *H. pluvialis* at a dose of 12 mg/day for 12 weeks improved the performance of the CogHealth test (a cognitive function test specifically designed to detect changes in healthy or middle-impaired subjects at an early date) and on the P300 test (which is used to evaluate mental workload). The study suggested that astaxanthin can improve brain function including cognition, attention, memory, information processing, and resultant behaviors in older persons [40]. Similar results were obtained in a randomized double-blind, placebo-controlled clinical trial in which astaxanthin supplementation at doses of 6 or 12 mg/day for 12 weeks was found to improve performance of the CogHealth test, as well as the Groton Maze Learning Test (GMLT, which is a computer-based maze learning test for the assessment of learning) on healthy subjects aged from 45 to 64 years who complained of the same troubles [119] (Table 2).

On the other hand, in a randomized controlled trial on patients with non-advanced AMD, it was reported that dysfunction in the central retina could be improved by an oral administration of astaxanthin at a dose of 4 mg/day for 12 months in combination with other antioxidants, such as vitamins C and E, zinc, copper, lutein, and zeaxanthin. Indeed, multifocal electroretinogram response amplitude densities (RADs) for 0° to 5° of eccentricity from the fovea, which was reduced in patients with non-advanced AMD, were increased significantly after astaxanthin supplementation [42]. Work at visual display terminals (VDT) is reported to induce various visual problems such as eye strain, blurring, and diplopia, and have adverse effects on the visual system. For example, VDT workers have lower accommodation amplitude and critical flicker fusion (CFF) than non-VDT workers. In a randomized double-blind, placebo-controlled study, it was observed that the accommodation

amplitude of VDT workers was significantly improved when astaxanthin extracted from *H. pluvialis* was used in diet at a dose of 5 mg/day for four weeks [120] (Table 2).

3. *Haematococcus pluvialis*: Biological, Physiological, and Biochemical Aspects

Most of the clinical studies presented above concerned astaxanthin produced and extracted from the microalgae *H. pluvialis*, which is the most promising source of natural astaxanthin for human applications. This chapter presents the biological and physiological properties of this alga as well as the biotechnological aspects allowing the production of astaxanthin.

3.1. Taxonomy and Distribution

Haematococcus pluvialis Flotow (also known as *H. lacustris* (Girod-Chantrans) Rostafinski [128], *Sphaerella lacustris* (Girod) Wittrock or *Protococcus pluvialis* Kützing [129] is a species of unicellular green microalgae living in freshwater that was described by the German soldier and botanist Julius von Flotow in 1844 [130]. We owe the first description of its biology and life cycle to the American botanist Tracy Elliot Hazen in 1899 [129]. From a taxonomic point of view, this eukaryotic organism belongs to the kingdom *Plantae*, phylum *Chlorophyta*, class *Chlorophyceae*, order *Volvocales* (also known as *Chlamydomonadales*), and family *Haematococcaceae* [128]. It is ubiquitous and colonizes mainly temporary stagnant water reservoirs (such as pools, ponds, tanks, etc.), whether natural or artificial. It has been isolated in most continents: Europe (from Norway to Spain), America (Mexico, Québec, Argentina, and Brazil), Asia (China, Taiwan, and India), Oceania (New Zealand and Hawaiian Islands) [128], and Africa (South Africa) [131,132], in a wide variety of environmental and climatic conditions [133] such as freshwater fishpond in Romania [134], rock bowls filled with melted snow in Norway [135], brackish water on coastal rocks in Russia [136], a dried fountain in Bulgaria [137], or even on rooftop of a building in South Korea [138].

Some strains are conserved in national microalgae banks and their genome has been sequenced. Among them, strains NIES-144 (National Institute for Environmental Studies, Tsukuba, Japan), CCAP 34/7, CCAP 34/8 (Culture Collection of Algae and Protozoa, Scottish Marine Institute, Oban, Scotland, UK), K-0084 (Scandinavian Culture Center for Algae and Protozoa, University of Copenhagen, Denmark), UTEX 16 (Culture Collection of Algae, University of Texas, Austin, USA) and SAG 34-1 (Culture Collection of Algae, University of Göttingen, Germany) are frequently used for research and production purposes.

3.2. Morphology and Life Cycle

There is a cellular polymorphism during the life cycle of *H. pluvialis* that allows it to adapt to adverse environmental conditions. Among them, we can distinguish macrozooids (or zoospores), palmelloids (or coccoids), hematocysts (or aplanospores) (Figure 4), and microzooids [5].

The macrozooid state is characterized by spherical, ellipsoidal, or pear-shaped green vegetative cells of about 8 to 20 µm in diameter (Figure 4a). Cells are motile due to two flagella of equal length emerging from the anterior end. The chloroplast is cup-shaped with numerous, scattered pyrenoids [133]. They also exhibit a distinct, multilayer, voluminous, and transparent extracellular matrix of variable thickness which is characteristic of volvocalean motile cells. It is consisting of a median tripartite crystalline layer surrounded by two layers of the gelatinous matrix. Genus-specific fine radiating strands emanate from the protoplast and penetrate the extracellular matrix [139]. This type of cell predominates under favorable growth conditions and represents the fast-growing vegetative cells which may divide into two to eight daughter cells through mitosis [5].

When environmental or culture conditions become less favorable (high light, nutrient starvation, etc.), macrozooids lose their flagella, their protoplast rounds off and increases in size and they develop into non-motile vegetative coccoid cells (Figure 4b,c). This palmelloid stage is characterized in particular by the presence of secretory vesicles, localized in the periphery of the nucleus and near the cytoplasmic membrane, which contributes to the formation of a primary cellulosic

wall consisting in β-1-4-glycosidic linkages [139]. Once culture conditions improve, the coccoid cells will quickly divide into thirty-two daughter flagellated cells retained inside the mother cell wall [140]. On the contrary, if unfavorable environmental or culture conditions extend, the coccoid cells may begin to undergo structural transformations at an intermediate stage (Figure 4d,e) and become greenish-orange due to the production of secondary carotenoids, including astaxanthin, in response to stress. The β-carotene, already presents or newly synthetized in chloroplasts, is the precursor in the biosynthesis of astaxanthin [141]. The process continues in the cytosol inside of lipid droplets which appear in the perinuclear space [142]. Starch grains also appear around pyrenoids and partial thylakoid degradation is observed [140].

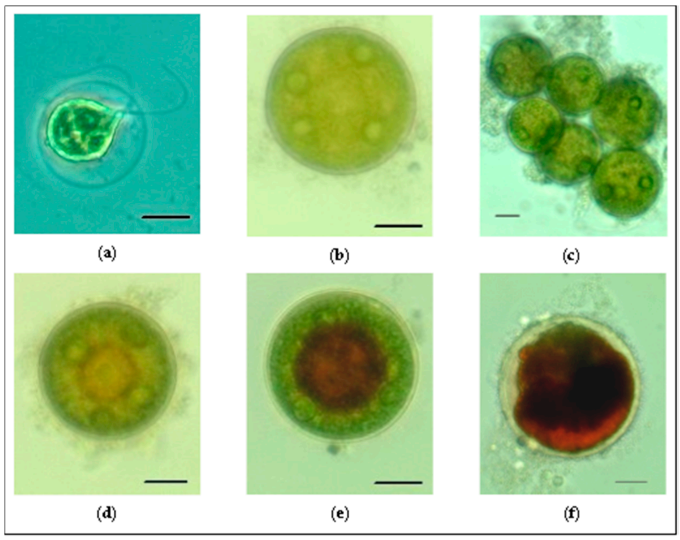

Figure 4. Light micrographs of *H. pluvialis* cells. (**a**) zoospore, phase contrast; (**b**,**c**) palmella cells; (**d**,**e**) intermediate cells, bright field, scale bar = 10 μm; (**f**) mature aplanospores, bright field, scale bar = 20 μm. The *H. pluvialis* strain used here was isolated from Reunion Island (*Photographs: SJ*).

This third intermediate state is followed by the cessation of cell division and the development of red aplanospores (Figure 4f), or hematocysts, which are non-motile spores with thick and rigid cell walls [5]. During this stage, a trilaminar sheath develops between the primary wall and the cytoplasmic membrane followed by a secondary wall, consisting mostly of mannan [5,139], while the extracellular matrix breaks down partially [139]. Concomitantly, the number of oil droplets containing astaxanthin increases in the cytoplasm, whereas chlorophyll biosynthesis decreases and chloroplasts degrade [140,142]. However, aplanospores maintain a photosynthetic activity and cells continue to grow in size and weight [5,143–145]. At the mature stage of aplanospores, the extracellular matrix and the primary wall are disintegrated. At this stage, the cell wall consists of 70% carbohydrates, mainly in mannose. Notably, it also contains 3% of acetolysis-resistant sporopollenin-like materials, called algaenan [139]. When environmental or culture conditions return to optimal, aplanospores may germinate to form macrozooids, and cells initiate a new vegetative growth cycle [5].

Microzooids are gametes, small cells (<10 μm in diameter) with high-speed motility. Sexual reproduction is rare in *H. pluvialis* but after exposure to extreme conditions such as freezing, desiccation, or nutrient starvation, followed by favorable conditions, gametogenesis may occur and the aplanospores will then develop into gametocysts containing up to 64 gametes [5].

3.3. Carotenogenesis in H. pluvialis

In higher plants, carotenoids are synthesized from isopentenyl pyrophosphate (IPP), the molecular precursor of isoprenoids, which is produced either via the mevalonate pathway in the cytosol or via the 1-deoxy-D-Xylulose-5-phosphate (DOXP) pathway in the chloroplast [146].

In green algae *H. pluvialis*, IPP is derived exclusively from the DOXP pathway [147,148] (Figure 5). IPP is isomerized by the isopentenyl pyrophosphate isomerase (IPI) into dimethylallyl diphosphate (DMAPP). DMAPP is alkylated with IPP by geranyl pyrophosphate synthase (GPPS) to form geranyl pyrophosphate (GPP), which, with IPP alkylations, form farnesyl pyrophosphate (FPP) by FPP synthase (FPPS), then geranylgeranyl pyrophosphate (GGPP, 20 carbon atoms) by GGPP synthase (GGPPS) [5,148,149].

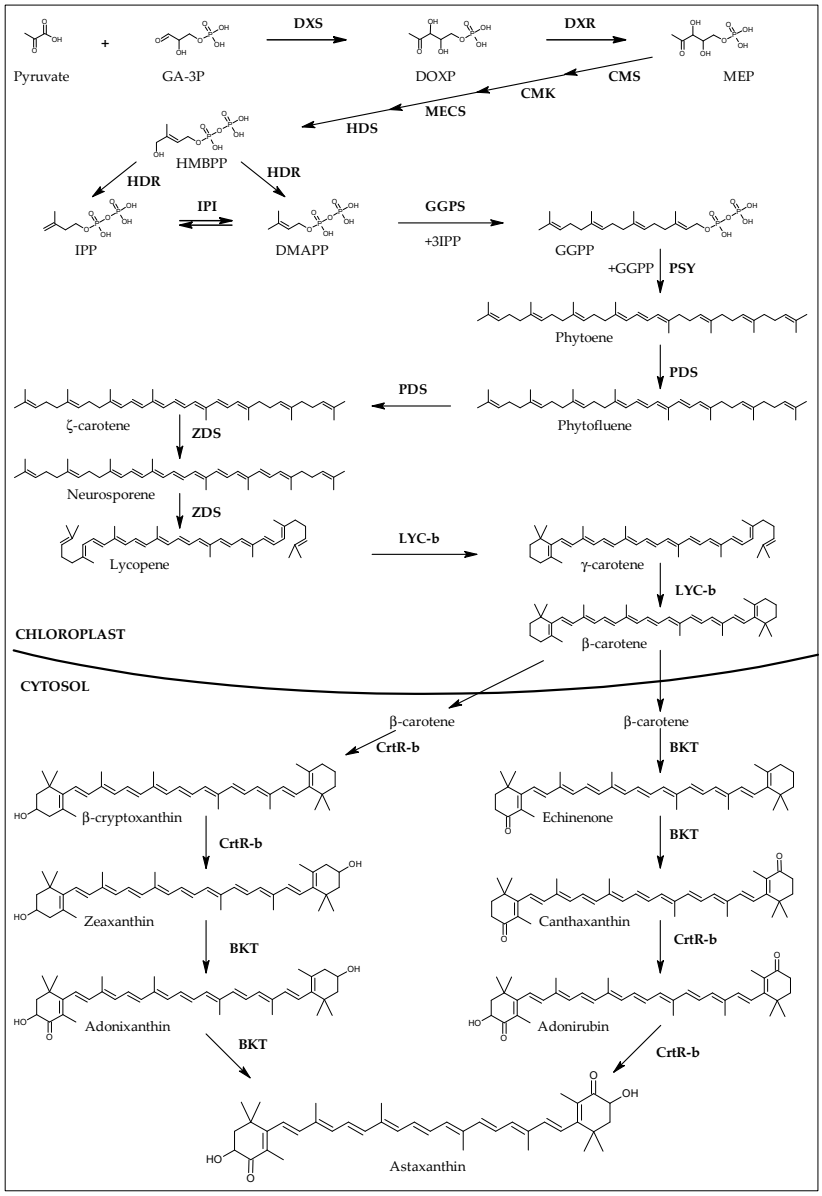

Figure 5. Overall pathway for astaxanthin biosynthesis in *H. pluvialis* [5,148,149]. Abbreviations: GA-3P, D-glyceraldehyde-3-phosphate; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; DOXP, 1-deoxy-D-xylulose-5-phosphate; DXR, 1-deoxy-D-xylulose-5-phosphate reducto-isomerase; MEP, 2-C-methyl-D-erythritol-4-phosphate; CMS, 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase; CMK, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; MECS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS, 1-hydroxy-2-methyl-2-butenyl 4-diphosphate synthase; HMBPP; 1-hydroxyl-2-methyl-2-butenyl-4-diphosphate; HDR, 1-hydroxy-2-methyl-2-butenyl 4-diphosphate reductase; IPP, isopentenyl pyrophosphate; IPI, isopentenyl pyrophosphate isomerase; DMAPP, dimethylallyl pyrophosphate; GGPS, geranylgeranyl pyrophosphate synthase; GGPP, geranylgeranyl pyrophosphate; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; LYC-b, lycopene β-cyclase; BKT, β-carotene ketolase; CrtR-b, β-carotene 3,3′-hydroxylase.

The first step of carotenoids biosynthesis is catalyzed by the enzyme phytoene synthase (PSY) in chloroplasts. It catalyzes the condensation of two GGPP, to form phytoene (40 carbon atoms), the precursor of all carotenoids [5,148]. Next, the enzyme phytoene desaturase (PDS) convert phytoene (colorless) into phytofluene (yellow) then ζ-carotene, which is subsequently converted into neurosporene (orange), then lycopene (red) by ζ-carotene desaturase (ZDS) through several desaturation reactions. These dehydrogenation steps lengthen the series of conjugated carbon–carbon double bonds which constitute the basis of the chromophore of carotenoids [5,148]. With flavine adenine dinucleotide (FAD) as a cofactor, these enzymes require the plastid terminal oxidase enzyme (PTOX) and the plastoquinone pool as electron acceptors [150,151]. The lycopene β-cyclase (LCY-b) will then catalyze in the chloroplast two β-cyclization reactions at each end of the lycopene molecule to form β-carotene [148].

The β-carotene ketolase (BKT) will add a keto group at carbon 4 of one or both β-carotene rings to form echinenone and canthaxanthin, respectively. Because BKT activity is only located in cytoplasmic lipid bodies, a transport of intermediate carotenoids from the chloroplast to the cytoplasm is required [152]. Finally, canthaxanthin is hydroxylated at carbon 3 of rings by the β-carotene hydroxylase (CrtR-b) to form adonirubin, then astaxanthin. In an alternative pathway, CrtR-b adds hydroxyl groups at β-carotene to form β-cryptoxanthin, then zeaxanthin, which will be finally converted into astaxanthin by BKT through the adonixanthin formation [5,148,153].

In *H. pluvialis*, the greater part of the astaxanthin is esterified with fatty acids. A recent study showed that this esterification step occurred in the endoplasmic reticulum and was probably catalyzed by diacylglycerol acyltransferases (DGATs). This study also suggested that the conversion of β-carotene in astaxanthin partly occurs in the endoplasmic reticulum [154].

3.4. Physiological Role of Astaxanthin Accumulation

In a normal way, photosynthesis generates reactive oxygen species (ROS) in chloroplasts which are neutralized by an endogen antioxidative system composed of superoxide dismutase, catalase, and peroxidase enzymes [155]. However, under adverse environmental or culture conditions (like high luminosity, high salinity, nutrient starvation, heavy metals, etc.), the balance between generation and detoxification of ROS is broken and leads to oxidative stress [5].

In *H. pluvialis*, the early oxidative stress response involves the up-regulation of the anti-oxidative enzymatic system. When stressful conditions persist, this response is replaced by the biosynthesis and accumulation of astaxanthin, which can be interpreted as a long-term defense mechanism [156].

Astaxanthin seems to acts as a screen that partially filters the penetration of light into chloroplasts, in particular in the blue field, reducing photo-oxidative damage of the photosynthetic apparatus caused by excess light [157]. A recently published work showed that lipid droplets containing astaxanthin in green–red cyst cells will freely and rapidly migrate from the center to the periphery of the cell, through chloroplast, in response to intense light. This phenomenon was found to be reversible in the dark. This study also indicated that this photo-response dynamic does not concern the other pigments present in the cells, namely chlorophylls, lutein, and β-carotene [158]. The astaxanthin biosynthesis was found to consumes approximately 10% of the molecular oxygen produced by photosynthesis during stress via two distinct ways. On the one hand, the formation of astaxanthin requires oxygen atoms. On the other hand, the conversion of molecular oxygen into water by plastid terminal oxidase (PTOX) uses electrons derived from desaturation steps of carotenogenesis (see the previous chapter) via the photosynthetic plastoquinone pool [159]. Finally, astaxanthin acts as a powerful antioxidant in the cellular prevention of oxidative stress by quenching or scavenging ROS such as superoxide anion radicals (O_2^-) or singlet oxygen ($^1O_2$) and other free radicals [160–162].

3.5. Biochemical Composition of H. pluvialis Biomass

The biochemical composition of *H. pluvialis* is very variable and is highly dependent on the origin of the strain, its life-cycle stage, and the environmental or culture conditions. Astaxanthin biosynthesis

and accumulation are associated with changes in primary metabolism pathways, which are summed up overall by an increase in the levels of carbohydrates and fatty acids, and a decrease in that of nucleotides and amino acids (excepted for glutamic acid and reduced glutathione) [163].

In vegetative stages of culture, in which macrozooids and coccoid cells are predominant, the biomass can be relatively rich in proteins and can represent up to 40% of dry weight [164,165]. In the red stage of culture, when hematocysts are predominant, the protein content can decrease up to around 14% depending on the stress conditions [138,164,165]. In these cells, the total amino acid content is composed of 46% of essential amino acids and glutamic acid is predominant [138].

The total carbohydrate content of green vegetative cells is comprised of 22–39% of total dry weight [164–166], but it can reach up to 64% depending on the strain and the culture media [137]. In hematocysts, the carbohydrate content significantly increases between 35% to 49% [138,165] and this content can be even higher (from 63% [166] to 74% [164]) under nitrogen starvation. In these cells, D-glucose was the main monosaccharide [138]. Several studies also suggested that, under stress conditions, the carbohydrate content increased in the form of starch which is then partially degraded possibly to support fatty acid synthesis [166].

The lipid content of the algae varies between 8% to 18% of the total dry weight in the green vegetative cells [137,164,167,168]. In hematocysts, this content can increase up to 46% of the total dry weight [138,164,167,168]. In vegetative cells, the lipid profile is variable and depends on the strain and culture conditions. While some publications report that glycolipids are the main class of lipids, followed by phospholipids and neutral lipids [20,169], another study suggests that neutral lipids are majoritary [168]. In any case, the neutral lipids become the majoritary lipid class under stress conditions such as high light or nitrogen deprivation [20,168,169]. This is linked to the synthesis of triacylglycerols (TAG) which are accumulated in cytoplasmic lipid globules [20,169]. The fatty acids profile is also very variable but palmitic ($C_{16:0}$), linoleic ($C_{18:2,\ n-6}$), and linolenic ($C_{18:3,\ n-3}$) acids are found to be the main fatty acids in vegetative cells and can reach up to 70% of total fatty acids. Under stress conditions, the oleic acid ($C_{18:1,\ n-9}$) content increases strongly, the saturated and other monounsaturated fatty acid contents also are higher, whereas polyunsaturated fatty acid contents decrease [170]. This phenomenon is also observable in the fatty acid profile of neutral lipids, including TAG, which is mainly composed of palmitic, oleic, linoleic, and linolenic acids. Oleic acid, which is not the predominant fatty acid in vegetative cells, becomes the majority under stress conditions, to represent about 30% of total fatty acids content in neutral lipids, while polyunsaturated fatty acid contents overall decrease [20,169]. The synthesis of TAG accompanying the encystment involves that of de novo fatty acids consisting mostly of oleic, palmitic, and linoleic acids. Some studies have shown that the enhanced accumulation of oleic acid is linearly correlated with that of astaxanthin and suggested that an accumulation of a pool of TAG may be a prerequisite for the initiation of fatty acid-esterified astaxanthin accumulation in lipid droplets [169]. In glycolipids, which are mainly composed of linoleic and linolenic acids in vegetative cells, the transformation in hematocysts is accompanied by an increase of polyunsaturated fatty acids, especially linolenic and hexadecatetraenoic ($C_{16:4,\ n-3)}$ acids which can represent about 60% of total fatty acids of glycolipids. In vegetative cells, phospholipids are mainly composed of palmitic and linoleic acids which can represent more than 50% of the total fatty acids of this fraction, followed by arachidonic ($C_{20:4,\ n-6}$) and oleic acids. Under stress conditions, the linolenic acid content increases, while the linoleic and arachidonic acids contents decrease [20,169].

In vegetative green cells, the chlorophyll (a and b) content is about 1.8% of total biomass dry weight, whereas that of carotenoids is generally comprised between 0.33% and 0.5% [134,171], corresponding to an approximative mass ratio carotenoids/chlorophylls comprised between 0.18 and 0.28. In these cells, primary carotenoids are functional and structural components of the photosynthetic apparatus [148]. Among them, lutein is the main carotenoid and can represent more than 50%, even 70% of total carotenoids. Other primary carotenoids are significantly present such as neoxanthin, β-carotene, and violaxanthin, which is a xanthophyll cycle pigment and each of them represents from 8% to 13% of total carotenoids content [172]. To a lesser extent, other xanthophyll cycle carotenoids such

as antheraxanthin and zeaxanthin are also produced by the microalgae [144]. In aplanospores, the chlorophyll content decreased to about 0.5% of dry weight [134,171] while the carotenoid content increased, especially that of secondary carotenoids such as astaxanthin, which are accumulated as a response to stress (high light, nutrient starvation, etc.) and can reach more than 4% of the biomass dry weight [144,173,174]. Thereby, the ratio carotenoids/chlorophyll may increase up to 8 in some cases. Astaxanthin represents then from 80% to 95% of total carotenoids weight [19,144,172,175,176]. Other carotenoids are present to a much lesser extent such as primary carotenoids lutein, β-carotene, neoxanthin, violaxanthin [144], and secondary carotenoids canthaxanthin and echinenone [172,175].

In *H. pluvialis*, almost all astaxanthin is esterified with one or two fatty acids to form astaxanthin monoesters, which is usually the majoritary, and astaxanthin diesters. Free astaxanthin accounts for only 1% or 2% of total carotenoids [19,144]. The main fatty acids in astaxanthin monoesters are palmitic, oleic and linoleic acids [20]. In this microalgae, only the optical isomer (3S,3'S) exists and the all-*trans* (all-E) configurational isomer represents greater than 86 %, even though a small amount of *cis*-astaxanthin may be present [19].

3.6. Cultivation of H. pluvialis for Astaxanthin Production

3.6.1. Cultivation Process

H. pluvialis can be cultivated from laboratory to industrial scale to produce astaxanthin (Table 3). Most of the cultures are carried out under photo-autotrophic conditions for which light is the source of energy to achieve photosynthesis into thylakoids of chloroplasts, and incorporating mineral carbon (CO_2) into organic matter. However, *H. pluvialis* is one of the few microalgae that can be cultivated under heterotrophic (without light) and mixotrophic (with light exposure) conditions when an organic source of carbon, like acetate, is present in the medium.

At laboratory scale, *H. pluvialis* can be cultivated in conventional systems used for microalgae cultivation, including Petri dishes, microtubes, microplates, test tubes, flasks, bubbling or airlift columns, tubular or flat type photobioreactors, bags, fermenters, or open ponds [177–184]. At the industrial scale, photo-autotrophic cultivation of *H. pluvialis* is carried out either in open raceway ponds, wherein the culture medium is agitated using a paddlewheel type agitation process or in closed photobioreactor consisting of a translucent loop with variable configurations (tubular, cylindrical, flat panels, etc.) equipped with a circulation pump for the culture medium [144,171,174,185–187]. Due to the fragility of green vegetative cells and the relatively low cell concentration in the medium, only a low level of mixing is required at the start of cultivation to allow sufficient availability of light and nutrients, while at high cell concentration, the mixing must be increased to overcome the phenomenon of light limitation due to mutual shading of cells [172].

Many nutrient medium recipes have been developed for the cultivation of microalgae, among which the Bold Basal Medium (BBM) [188] or the modified BBM enriched in nitrogen, and the BG-11 medium [189] are currently used for *H. pluvialis* cultivation. Recently, it was shown in two-step cultivation that BBM reached higher biomass productivity as compared to BG-11 during the first vegetative growth stage, whereas a higher astaxanthin content was obtained using the nitrogen starved BG-11 medium [190]. The National Institute for Environmental Studies (NIES) medium is also well appropriated for astaxanthin production [182] as well as other media that have been specifically developed to optimize biomass and astaxanthin productivity such as the Optimal *Haematococcus* Medium (OHM) [191]. CO_2-injection can also be added into the medium to improve biomass productivity.

Photo-autotrophic culture is usually carried out in two stages: a so-called green stage during which culture conditions are optimized to improve vegetative growth, followed by a so-called red stage where astaxanthin is accumulated in cells upon exposure to stress conditions. In the case of photo-oxidative stress, photo-inhibition may occur at the beginning of the red stage which may also cause cell death. It was shown that motile cells were more sensitive to photo-inhibition than non-motile cells [143,145,172].

It was therefore suggested that strains with predominant palmelloid cells during the green stage have an advantage over those whose flagellated cells are predominant [145]. The initial biomass density for the red stage is also an important parameter to improve astaxanthin productivity. A low cell density led to significant photo-inhibition, whereas high cell density limited access to light and therefore photosynthesis. It was reported that an initial biomass concentration of about 0.8 $g.L^{-1}$ was optimal and allowed to reach astaxanthin productivity during the red stage of about 17 $mg.L^{-1}.d^{-1}$ in outdoor photobioreactors (9,1 when the green indoor period was taken into consideration) [180]. The maximum overall astaxanthin productivities reported in the literature were about 18 $mg.L^{-1}.day^{-1}$ in a laboratory scale [179] and 10 $mg.L^{-1}.day^{-1}$ (of total carotenoids) outdoor [174]. However, the development of innovative culture systems and strategies is expected to further enhance astaxanthin productivities. For example, in some strains of *H. pluvialis*, flagellate cells can synthesize astaxanthin [192]. Therefore, a one-step culture process wherein the flagellate stage would be maintained can be another strategy to produce astaxanthin. It was shown that this method allowed researchers to achieve overall astaxanthin productivity of 21 $mg.L^{-1}.day^{-1}$ in laboratory conditions [193] and 8 $mg.L^{-1}.day^{-1}$ outdoor [186]. More recently, at a laboratory scale, astaxanthin productivity of more than 45 $mg.L^{-1}.day^{-1}$ was achieved with a newly microreactor system [194]. In addition, some studies reported that *H. pluvialis* can be cultivated with an immobilized biofilm method using multilayer PBRs. This method allowed researchers to reach astaxanthin productivities of about 160 $mg.m^{-2}.day^{-1}$ in a two-phase culture [195] and 390 $mg.m^{-2}.day^{-1}$ in a one-phase culture [196].

Heterotrophic cultivations occur in darkness with an organic source of carbon, while mixotrophic cultivation requires light and organic and inorganic sources of carbon. Culture media for heterotrophic and mixotrophic cultivations, such as Kobayashi basal medium [197], are quite similar to those used for photo-autotrophic conditions but they usually contain acetate or yeast extracts as an organic carbon source. Culture systems used for photo-autotrophic cultivations can also be employed for heterotrophic and mixotrophic cultivations. In heterotrophy, the growth rate of *H. pluvialis* is generally lower than in photo-autotrophy, and astaxanthin can represent up to 0.5% of the total biomass dry weight [198]. In mixotrophy, the growth rate is higher than in heterotrophy. In this cultivation mode, the final cell densities can reach between 0.9 and 2.65 $g.L^{-1}$ and contain from 1% to 2% of astaxanthin [156,198–200].

An innovative process for the cultivation of the microalgae was recently proposed and it consisted of heterotrophic cultivation, followed by an acclimation period under moderate illumination before induction of the astaxanthin biosynthesis phase under photoautotrophic conditions. The resulting product showed an astaxanthin productivity of 10.5 $mg.L^{-1}.day^{-1}$ during the induction phase [201]. Similar levels of productivity could also be reached under mixotrophic conditions with the addition of potassium as an inducing agent [202]. More recently, a study developed a strategy to enhance effectively astaxanthin and lipid production in *H. pluvialis* under mixotrophic conditions and low light by the addition of glycerol as an organic carbon source [203].

Anyway, in the choice of cultivation processes to be implemented at the industrial scale, the environmental impact must be considered since the cultivation stage represents a significant part of the consumption of energy and inputs required for the production of natural astaxanthin. It is highly dependent on the system design, its location, its energy demand, the sources of energy and inputs used, and the recycling capacity. For example, it was recently shown that, even if astaxanthin yields are higher, systems which required artificial light such as Flat Panel Airlift (GWP) or Green Wall Panel (GWP) photobioreactors may have a higher environmental impact than those located outdoor under natural light such as Unilayer Horizontal Tubular (UHT) photobioreactor [204].

3.6.2. Impact of Physicochemical Factors on Growth and Productivity

Several physicochemical factors affected the growth and life-cycle of *H. pluvialis* and they have to be considered when cultivating this microalga to produce astaxanthin optimally. The most fundamentals are light intensity and quality, temperature, pH of the medium, nutrient availability, and presence of stress-inducing compounds.

Table 3. Cultivation of *H. pluvialis* in various types of photobioreactors and scales.

Photobioreactor Type	Culture Volume (L)	Culture Conditions	Green Stage Biomass Productivity ($g.L^{-1}.d^{-1}$)	Red Stage Biomass Productivity ($g.L^{-1}.d^{-1}$)	Astaxanthin Content (% Dry Weight)	Astaxanthin Productivity ($mg.L^{-1}.day^{-1}$)	Ref.
airlift column	30	autrotrophy, batch, indoor	0.03	0.01	2.7	0.44 (b)	[172]
bioreactor	2.5	mixotrophy, fed-batch, indoor	-	0.137	2–2.3	3.2 (a)	[200]
tubular PBR/open pond	25 000	autrotrophy, batch, outdoor	0.036–0.052	-	2.8–3	-	[185]
Fermentor/vessel	2.5	heterotrophy/autrotrophy, fed-batch/batch, indoor	0.275	0.05	2	4.4 (b)	[177]
flasks/Tubular PBR	50	autrotrophy, batch, indoor/outdoor	-	0.05	3.6	7.2 (c, d)	[144]
bubbling column	1.8	autrotrophy, continuous, chemostat, indoor	-	0.6	0.8	5.6 (a)	[178]
airlift tubular PBR	55	autrotrophy, batch, outdoor	-	0.41	1.1	4.4 (a)	[171]
bubbling column	55	autrotrophy, batch, outdoor	-	0.06	0.2	0.12 (a)	[171]
bubbling column	1.8	autrotrophy, continuous, indoor	0.58	-	-	-	[187]
airlift Tubular PBR	220	autrotrophy, continuous, outdoor	0.68	-	-	-	[187]
bubbling column	1	autrotrophy, fed-batch/batch, indoor	0.36	0.14	3.6	12 (b)	[179]
Airlift column	1	autrotrophy, fed-batch/batch, indoor	0.4	-	-	18 (b)	[179]
bubbling column	1.8	autrotrophy, continuous, chemostat, indoor	-	1.9	1.1	21 (a)	[193]
bubbling column	1.8	autrotrophy, continuous, chemostat, indoor	-	0.7	1	8 (a)	[186]
airlift tubular PBR	50	autrotrophy, continuous, chemostat, outdoor	-	0.6–0.7	1.34	8 (a)	[186]
bubbling column	0.5	autrotrophy, batch, indoor	0.5	0.21	4	11.5 (TC) (b)	[174]
flat panel/tubular PBRs	2 000	autrotrophy, batch, outdoor	0.37	0.21	3.8	10.1 (TC) (b,c)	[174]
open circular pond	3	autrotrophy, batch, indoor	-	0.15	2.8	4.3 (a)	[183]
flat type PBR	1	autrotrophy, fed-batch, indoor	0.33	-	4.8	14 (b)	[182]
bag type PBR	6	autrotrophy, batch, indoor	-	0.047	-	1.4 (a)	[181]
bubbling column	0,6	autrotrophy, batch, indoor/outdoor	-	0.58	2.7	17.1 (c, d)	[180]
immobilized biofilm	160 cm^2	autrotrophy, batch, indoor	-	6.8 $g.m^{-2}.d^{-1}$	2.2	164.5 $mg.m^{-2}.d^{-1}$ (d)	[195]
microreactor	0.1 10^{-3}	autrotrophy, batch, indoor	-	-	-	45.25 (b)	[194]
bubbling column	1	heterotrophy/autrotrophy, batch, indoor	-	0.3	2.37	10.5 (c)	[201]
bubbling column	0.05	mixotrophy, batch, indoor	0.22–0.35	-	-	10.2 (b)	[202]
bottle/airlift PBR	6.5	autotrophy, batch, indoor	-	0.16	4.94	7.8 (d)	[184]

(a) Obtained from one-step culture process. (b) Obtained from two-step culture process. Productivity value is calculated based on total culture time. (c) Induction of astaxanthin was performed outdoor; astaxanthin productivity was not corrected for dark hours. (d) Obtained from two-step culture process. Productivity value is calculated based on time of the red stage only. Abbreviations: PBR, photobioreactor; TC, total carotenoids.

Irradiance, Temperature, and pH of the Media

The vegetative growth of *H. pluvialis* is greatly improved under moderate irradiances. Optimum irradiance, which produced the maximum specific growth rate varied depending on the strain, temperature, cell density, and culture mode in the range of 20 to 90 µmol of photons.$m^{-2}.s^{-1}$ [5,172,205,206]. Above this optimum irradiance, a photo-saturation phenomenon will progressively occur, which will inhibit the vegetative growth and induces astaxanthin synthesis during the development of green vegetative cells into aplanospores [5]. This phenomenon is particularly important for light intensities higher than 150–170 µmol of photons.$m^{-2}.s^{-1}$ [164,207], but it was reported that the optimum irradiance for astaxanthin accumulation is situated between 1500 and 2000 µmol of photons.$m^{-2}.s^{-1}$ [208]. However, exposure to excessive light intensity increased cell mortality [143]. High light is considered as the most effective environmental factor to promote astaxanthin synthesis [209]. The light quality was also shown to impact biomass productivity and astaxanthin accumulation. For example, it was reported that red light from Light Emitting Diodes (LED) increased the cell growth of *H. pluvialis*, while blue light enhanced astaxanthin biosynthesis [210].

The optimal temperature for the growth of *H. pluvialis* varies depending on the strain and culture conditions in a range from 15 to 35 °C [137,205,206,211,212]. At higher than optimal temperatures, vegetative growth is inhibited while astaxanthin synthesis is induced [211]. Then, vegetative cells developed into large aplanospores, which may improve biomass productivity [205]. However, excessive temperatures will increase cell mortality [212,213]. It was also shown that cultures performed at higher temperatures may improve harvesting and utilization of the impinging light due to photo-acclimation, bringing optimum irradiance from 49 µmol of photons.m^{-2}.s^{-1} at 20 °C to 90 µmol of photons.m^{-2}.s^{-1} at 27 °C [205]. In another study, temperatures between 25 and 28 °C with an irradiance of 130 µmol of photons.m^{-2}.s^{-1} have allowed reaching a specific growth rate of 0.054 h^{-1} [206].

Several studies also reported that *H. pluvialis* was able to proliferate within a wide range pH comprised between 4 and 9 without a significant impact on growth, whereas cell propagation significantly decreased at pH 10 and stopped completely at pH 3. A recent study also suggested that an acidic cultivation could prevent contamination of the culture by the lethal fungus *Paraphysoderma sedebokerensis* [214]. Another study showed that higher biomass productivity was achieved under photo-autotrophic conditions in cultures buffered at pH 6.3 compared to those unbuffered media [215]. However, most studies indicated that, under photo-autotrophic or mixotrophic conditions, a medium at pH 7 was optimal for cell growth [172,216]. In mixotrophic cultures, the biomass yield was severely reduced at pH 6 and pH 9 while there was no growth at pH 5. Furthermore, astaxanthin production was higher at pH 7 as compared to any other pH tested under stress conditions [216]. In heterotrophic cultures, pH 8 was reported to be optimal for biomass production [177].

Nutrients Availability

The vegetative growth was improved when nitrogen concentration in the culture medium between was set between 5 to 10 mM [212,217]. Under lower nitrogen concentrations (from 1.5 to 5 mM), the growth rate was reduced [164] but the synthesis and accumulation of astaxanthin were enhanced [178]. Vegetative cells developed into reddish palmella cells and aplanospores [212]. Below 1.5 mM nitrogen, algal growth was severely limited and astaxanthin synthesis and accumulation were greatly stimulated [143] while chlorophyll content dropped [173]. However, this strong limitation of nitrogen availability negatively affected biomass concentration and as a consequence the astaxanthin productivity [178]. Under high irradiances, astaxanthin productivity was maximized at a nitrogen concentration in the feed medium which was comprised between 2.7 and 2.9 mM [178,207]. Additionally, it was shown that ammonium chloride was the best nitrogen-source for vegetative growth followed by nitrate and urea, but the cells did not survive during high irradiance treatment contrarily to culture performed using nitrate as a nitrogen source [207].

The optimal phosphate concentration in the feed medium for vegetative growth was observed around 0.5 mM. Higher concentration induced the formation of reddish palmella cells and aplanospores [212]. On the contrary, starvation of phosphate stopped cell division and enhanced astaxanthin accumulation in the cell [164] while chlorophyll content was not impacted [173].

A carbon source is necessary to obtain high productivity biomass and astaxanthin. The introduction of CO_2 or organic sources of carbon, such as acetate, altered the C/N balance, creating a relative nitrogen deficiency which stimulated astaxanthin accumulation and cyst formation [218,219]. In photo-autotrophic cultures, the addition of CO_2 at a level comprised between 1.5% and 5% in air has a beneficial effect on vegetative growth [217,218,220] and astaxanthin accumulation under stress, which resulted in a higher overall astaxanthin productivity. However, an excess of CO_2 up to 10% or 20% in air was deteriorative for growth, photosynthesis, and carbon assimilation [220]. A recent study was showed that industrial flue gas can be used for *H. pluvialis* cultivation under outdoor autotrophic conditions at the pilot scale [221]. An organic carbon source is required to cultivate the algae in mixotrophic conditions. Growth rates and accumulation of astaxanthin were enhanced by the addition of acetate at concentrations ranging from 1 to 30 mM [212,219]. The concentrations of acetate above 45 mM triggered a morphological change of vegetative cells into cyst cells [222]. However,

concentrations higher than 60 mM caused growth inhibition [219]. Malonate could also be used to improve the growth rate, but it became toxic at high concentrations [219]. A recent study also showed that highest astaxanthin concentration and productivity could be obtained with addition of potassium acetate, as compared to sodium acetate, magnesium acetate, or sodium hydrogen carbonate [202].

Stress-Inducing Agents

The addition of NaCl in the culture medium was found to stimulate the formation of palmella cells and aplanospores [212]. The effect of NaCl at a concentration of 40 mM was nearly as effective as exposure to high irradiances [172]. However, even if NaCl addition at a concentration ranging from 100 to 140 mM induced a massive accumulation of astaxanthin, it produced a complete cessation of growth and a high rate of cell mortality [143,164]. When salinity was set above 170 mM, *H. pluvialis* completely stopped growing [212]. Under mixotrophic or heterotrophic conditions, the addition of NaCl in the medium at a concentration comprised between 17 and 85 mM accelerated encystment and enhanced astaxanthin formation in cyst cells. However, as under photo-autotrophic conditions, high concentrations of NaCl caused cell mortality [216,223]. Another study reported that young cultures were more sensitive to a high concentration of NaCl than older cultures [216]. In heterotrophic cultures, the addition of $MgCl_2$ at a concentration of 10 mM was found to be more effective than NaCl for the formation of astaxanthin [223].

Morphological change of vegetative cells into cyst cells and astaxanthin synthesis could be induced by the addition of Fe^{2+} at concentrations around 0.4 mM [143,222]. Addition of Fe^{2+} catalyzed a Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow OH^- + HO\cdot + Fe^{3+}$), which then produced hydroxyl radicals and then enhanced carotenoid biosynthesis [222].

These parameters can be combined to improve biomass and/or astaxanthin productivity. For example, a high carotenoid productivity (25 $mg.L^{-1}.day^{-1}$ at the red stage, overall rate 13.9 $mg.L^{-1}.day^{-1}$) was obtained indoors when green biomass was exposed to a combination of high light and nitrogen deprivation [174]. It was found that nitrogen starvation inhibited cell division and chlorophyll biosynthesis, promoted chlorophyll b degradation, PTOX activity, cyclic electron transport, and favored respiration over photosynthesis, while strong light had the effect to activate xanthophyll cycle and carotenogenesis [224].

Microbial Contamination

It was found that astaxanthin productivity and long-term sustainability of *H. pluvialis* culture can be compromised by the development of microbial contaminants, including cyanobacteria, other microalgae, fungal parasites, and zooplankton predators like amoebas, ciliates, and rotifers [5]. Among these contaminants, the fungal parasite *Paraphysoderma sedebokerensis* was found to be one of the more feared for *H. pluvialis* culture [225–228]. Infections can be limited by the addition of lectins, which inhibit recognition between the parasite and host cells [226] or by the cultivation under acidic conditions (pH 4) [214].

3.7. Downstream Processing

3.7.1. Harvesting of *H. pluvialis* Biomass

Once the maturation stage of hematocysts is reached, biomass is separated from the culture medium and concentrated during the harvesting processes [5]. The harvesting technologies generally involve mechanical (gravity sedimentation, flotation, filtration, and centrifugation), chemical (coagulation/flocculation), biological (bio-flocculation), and to a lesser extent, electric-based methods (electrophorese) [1,229]. Among them, gravitational settling, as a preliminary step to thicken algal suspension into a slurry (consisting in 2% to 7% of the Total Suspended Solids (TSS)) followed by centrifugation, which allows the cell suspension to concentrate up to 25% TSS, are conventionally applied on a large scale for the harvest of *H. pluvialis* biomass [1,5,230–233]. It was shown using

various microalgae including *H. pluvialis* that with the combination of a preliminary step including sedimentation and disk-stack centrifugation at 13,000× *g* that biomass could be efficiently recovered by at least 95% [1]. Centrifugation is a rapid process that is not associated with cell composition changes and contamination. However, it is an energy-intensive step that limits its applicability only for the recovery of high-value products [1,229] because it may represent about 20–30% of the total production costs [1]. In a recent study, an electro-coagulation floatation (ECF) technique, based on foam-floatation dewatering which is a method widely used in wastewater treatment, was applied for harvesting *H. pluvialis*. The results showed that this technique can be used alone or in combination with gravitational settling and centrifugation to significantly improve the recovery of the algal biomass [234,235].

3.7.2. Drying of *H. pluvialis* Biomass

To preserve *H. pluvialis* biomass, a drying process is often applied to eliminate moisture for preventing the degradation of astaxanthin [1,233,236] and to extend its shelf life [237]. The drying methods that have been used for microalgae include spray drying, drum drying, freeze-drying, and sun-drying [238,239]. Among them, the most effective and frequently used methods to dry high-value products such as astaxanthin was spray drying [1,233,236,238–240]. This technology can reduce the moisture content in biomass by up to 5% [232]. However, it generates a high operation cost due to the requirement of high working temperature and it can cause a significant deterioration of some algal components, such as pigments [239,240]. Freeze-drying, or lyophilization, is a milder drying method that is widely used for drying microalgae at a laboratory scale. However, this technique is more expensive at the industrial scale, partly due to the requirement of freeze biomass [235,236,239]. Nevertheless, considering the *H. pluvialis* biomass stability and astaxanthin recovery efficiency, it was demonstrated that freeze-drying, followed by vacuum-packed storage at 20 °C, was the most profitable method by comparison with spray-drying to ensure a longer shelf life of astaxanthin from *H. pluvialis* [241].

3.7.3. Pretreatment of *H. pluvialis* Biomass

For efficient recovery of astaxanthin from the rigid structure of the hematocyst cell, a pretreatment step is required to disrupt the thick sporopollenin cell wall of both wet and dried biomass and to release astaxanthin in the medium. Conventional pretreatments generally involve mechanical methods such as physical disruption through pressing, bead milling and grinding, ultrasound, autoclave, or homogenization [242,243]. Such technologies are energy-intensive and are better operated at high cell density preparations. For enhanced extraction, these methods are often used in combination with chemical methods or pretreatments, such as acid, alkali, and enzymatic actions [243,244]. Mechanical pre-treatment methods including grinding [195,245], bead beating [246], French press and screw press have been reported to be useful to break the cell-walls of *H. pluvialis* [247] and they are widely used at commercial scale [230,231,242,248]. Different cell disruption processes have been assessed for recovering astaxanthin from encysted cells of *H. pluvialis* with acetone extraction. In particular, it was demonstrated that mechanical disruption in a high-pressure homogenizer or autoclave treatment (30 min, 121 °C) was more efficient concerning extraction and availability than other methods such as treatment with acid (HCl), alkali (NaOH), enzymes (protease K and driselase mixture) and spray drying [249]. High-pressure homogenizers are widely used to disrupt *Haematococcus* cells for use as fish feed [249]. Some experiments were also carried out using cryogenic grinding (−170 °C) of the dried biomass of *H. pluvialis* to extract astaxanthin with butylated hydroxytoluene [250], but this method cannot be considered for large-scale commercial use [239]. Extraction can also be improved by chemical pretreatment used alone. For example, up to 96% of astaxanthin could be successfully recovered from the treatment of *H. pluvialis* using 1–2 N hydrochloric acid pretreatment (5–10 min at 70 °C) without homogenization, followed by acetone extraction [251]. Ionic liquids (ILs) have low vapor pressure, low melting point, high thermal stability, and recyclability [252]. They can be used in

pretreatment to improve the extraction yield by organic solvents [253]. A recent study reported that the ionic liquid 1-ethyl-3-methylimidazolium (Emim) associated with the anions HSO_4^-, $CH_3SO_3^-$, and $(CF_3SO_2)_2^-$ has a high efficiency for the pretreatment of *H. pluvialis* before the separation of the astaxanthin/lipid by hexane. Under optimized conditions, i.e., 6.7% (v/v) in water, 30 °C, 60 min, at least 99% of astaxanthin was successfully recovered [254]. It was shown that ILs can be recovered and reused at least for three times without any regeneration or other treatment required. ILs are then seen as promising alternative for astaxanthin extraction as compared to other pretreatment techniques, such as pulsed electric field, ultra sounds, high-pressure microfluidization [235].

Once the cell walls are disrupted, the astaxanthin must be rapidly recovered within few hours, and lysed cells must be minimally exposed to the light condition to prevent the degradation of light-sensitive astaxanthin pigment [235].

3.7.4. Recovery of Astaxanthin from *H. pluvialis*

The conventional extraction methods for the recovery of astaxanthin from *H. pluvialis* biomass use organic solvents. The type of solvent used is highly dependent on the industrial application due to its potential hazard and toxicity. For instance, food or pharmaceutical products require milder recovery methods [235]. Thermal and/or chemical stresses generated during the extraction process can significantly affect the qualities of the extracted astaxanthin, including anti-oxidative activity, bioavailability, and purity. Therefore, mild operating conditions, including adequate temperature control and the use and minimization of less-toxic chemicals, should be duly taken into consideration [247]. Relatively mild organic solvents such as acetone, ethyl acetate, and ethanol are effective and are preferred for the recovery of astaxanthin from the biomass of *H. pluvialis* [247]. As already seen in the previous chapter, a mechanical and/or chemical pre-treatment can improve the extraction yields. Solvents can also be pressurized to improve the yield and the recovery time of astaxanthin. Pressurized liquid extraction (PLE) was investigated using hexane or ethanol as extracting solvents. The results suggested that the extraction yields were higher with ethanol at high temperatures (200 °C) than with hexane. However, the antioxidant activities of the extracts decreased at high temperature [245,247,255]. Solvent extraction could also be assisted by ultrasound to improve the astaxanthin recovery yields. High-power ultrasound generates intensive microbubbles in a liquid medium, which grow and collapse violently, creating a phenomenon called cavitation. The implosion generates a shock wave with powerful energy to disrupt cell walls, and thus allows a greater penetration and diffusion of the solvent into the cell [235,243]. Extraction yields of approximatively 27.6 $mg.g^{-1}$ of dried powder were obtained under optimal extraction conditions, including 48% ethanol in ethyl acetate with a liquid-to-solid ratio of 20:1 (mL per g) and extraction for 16 min at 41.1 °C under ultrasonic irradiation of 200 W [256,257]. However, this method is hardly applicable to large-scale applications [243].

As an alternative to conventional organic solvents, supercritical CO_2 ($ScCO_2$)-based extraction has been widely used in the astaxanthin recovery process with high efficiency and speed, as well as greater sustainability [235,247]. Ethanol is often used as a polar co-solvent to improve solubility and selectivity of astaxanthin in CO_2 [235,258–261]. It was shown that Sc-CO_2 and 20% (v/v) ethanol under low-pressure conditions (8 MPa) at 55 °C for 15 min allowed to obtain a recovery of astaxanthin of 98.3% [262]. Without co-solvent, and because of the low solubility of astaxanthin in CO_2, high extraction pressures were required for efficient astaxanthin recovery from *H. pluvialis* [261]. A study showed that a pressure of about 40 MPa could enhance significantly the astaxanthin recovery yield rather than utilizing co-solvents [235,263]. As for conventional extraction methods, the recovery efficiency and cost of the $ScCO_2$ extraction could be improved using pre-treatment consisting of a mechanical cell wall disruption for example [246,247,264]. $ScCO_2$ extraction of astaxanthin was also performed and was compared to acetone extraction. The biomass was previously freeze-dried and ground with a ball mill. The highest recovery of carotenoids (92%) was obtained at a pressure of 300 bar and the temperature of 60 °C, using ethanol as a co-solvent [246,247].

More innovative methods have been developed but their application at a large scale remains to be demonstrated. A greener recovery approach using common vegetable oils such as soybean, corn, grapeseed, and olive to extract astaxanthin directly from culture medium has been proposed. The culture medium was mixed with oils and astaxanthin was extracted from the cell to oil phase by hydrophobic interactions. Then, oil extracts were separated by gravity settling. This method allowed researchers to reach recovery yields of about 88% and could even reach up to 93.9% with olive oil [235,257,265]. Another innovative process is based on the combination of supramolecular solvents (SUPRAS), nanostructured liquids generated from amphiphiles (e.g., carboxylic acids, alkanols, alkyl sulfates, and alkyl phenols), and nanostructurated lipids carriers (NLCs). This nanosystem resulted in a high extraction efficiency and a simultaneous encapsulation and stabilization of the extracts (the antioxidant activity was preserved during 180 days at 4 °C) [235,266].

A possible process for the production of marketable astaxanthin from *H. pluvialis* cultivation is recapitulated in Figure 6.

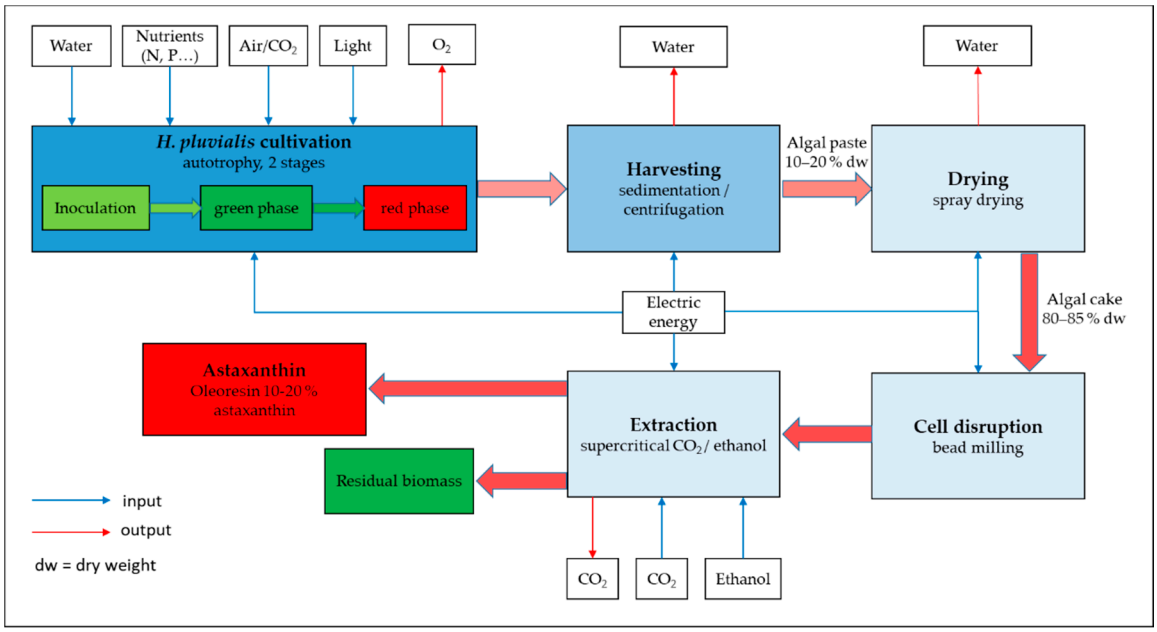

Figure 6. Flow chart of a possible astaxanthin production process ([1,204]).

3.8. Economic Potential of H. pluvialis-Derived Astaxanthin

As mentioned in the introduction of this review, *H. pluvialis*-derived astaxanthin occupies only a small part of the global astaxanthin market and, due to its relatively high production cost, is mainly dedicated to the dietary supplement market [1]. This market, estimated approximatively at USD 123 billion in 2019, is projected by some economical analysts to expand at a compound annual growth rate (CAGR) of 8.2% to reach USD 230 billion in 2027 [267]. Considering the growing evidence of its nutritional values, the *H. pluvialis*-derived astaxanthin market should follow this dynamism and occupy a representative place in the global dietary supplement market in the coming years.

Another dynamic market could open up for *H. pluvialis*-derived astaxanthin, that of functional foods. Indeed, astaxanthin as a food ingredient may have, on the one hand, technological functionalities due to its colorant property and its antioxidant activity which can protect functional foods during processing and storage. On the other hand, the biological activities of astaxanthin provide functional food with its biological functionalities [35]. The global functional foods market size was estimated at USD 161 billion in 2018 and it is anticipated to register a CAGR of 7.9% until 2025 [268] (GVR 2019). This market would therefore represent a great economic opportunity for H. pluvialis-derived astaxanthin when the regulatory obstacles to its access were removed.

4. Conclusions

As discussed in this review, the cultivation of *H. pluvialis* for the bioproduction of natural astaxanthin is widely studied worldwide and it is already being applied on a commercial scale with a good apparent economic viability. However, the cost of production of astaxanthin remains high and restricts its applications for a product with high added value, such as food supplements and pharmaceuticals. To reach lower value-added markets, such as human food and animal feed, research efforts need to focus on improving the productivity of biomass and astaxanthin through a deeper knowledge of the physiology of the microalgae, as well as some innovation in the cultivation process. Because climatic and environmental conditions can have a significant impact on biomass and astaxanthin productivities, the location of future commercial-scale culture systems should be chosen wisely, preferably in sunny and temperate areas. Moreover, to cultivate strains already adapted to climatic conditions and to avoid the introduction of foreign strains, the search for local strains, possibly more productive, seems desirable. To limit the ecological impact and to improve the economic profitability of this activity, research should be carried out on the use of more sustainable and cheaper inputs, as well as on less energy-consuming processes.

Alongside these technical aspects, the regulatory issues can constitute a real obstacle to a wider commercial development of natural astaxanthin. Indeed, despite the growing number of studies demonstrating its beneficial effects on health and safety, regulatory authorities, particularly in the United States and Europe, do not yet approve its more global use in food, as a natural coloring agent and/or antioxidant, nor in functional foods. In addition, in the case of food supplements, no health claim has been validated to date for astaxanthin by these regulatory authorities. To knock down these barriers, research on the biological activities and catabolism of astaxanthin must be continued, in particular through larger clinical studies, and additional toxicological studies must be carried out to confirm the total safety of the astaxanthin produced from the culture of *H. pluvialis*. It would also be interesting to carry out clinical trials on the effects of astaxanthin on diseases such as diabetes and cancer for which too few studies in humans are available.

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