Composition, cultivation and potential applications of *Chlorella zofingiensis* – A comprehensive review

Malihe Gorgich [a,b,*], António A. Martins [a], Teresa M. Mata [c], Nídia S. Caetano [a,b,*]

[a] *LEPABE - Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal*
[b] *CIETI, School of Engineering (ISEP), Polytechnic Institute of Porto (P. Porto), R. Dr. António Bernardino de Almeida, 431, 4249-015 Porto, Portugal*
[c] *INEGI-Institute of Science and Innovation in Mechanical and Industrial Engineering, R. Dr. Roberto Frias 400, 4200-465 Porto, Portugal*

Keywords:
Astaxanthin
Canthaxanthin
Lipid
Lutein
Stress factor
Zeaxanthin

A B S T R A C T

Chlorella zofingiensis has been in the center of attention due to its viability of cultivation, capability to yield various products, and applicability in various industrial sectors. Several research studies have evaluated this microalga as a bio-factory for carotenoids and lipids production, which are interesting compounds for industrial applications. The microalgae's robustness to various environmental and stress conditions such as light, temperature, salinity, and nutrients' deficiency/excess promotes its importance as a promising source of the mentioned valuable products. Hence, this study intends to review the most prominent contents of *C. zofingiensis* (i.e., lutein, astaxanthin, exopolysaccharides or EPS, lipids, etc.) and analyze the cultivation and stress conditions which directly influence the quality and quantity of the desired compounds. Upon review, discussions will be conducted to investigate the ability to combine stress factors to increase the contents of interest. Following an investigation over the composition and content induction approaches, several commercial applications of this microalga will be listed and explained, in particular, to obtain valuable products which have been discussed. These applications are chosen to cover multiple sectors, from medical and pharmaceutical, to food and feedstock, biofuel and energy, and wastewater treatment. The broad capability and robustness of *C. zofingiensis* make it a suitable investment to target more than one sector. Finally, it is discussed and presented a tentative design of a pipeline combining several applications to increase the economic viability of obtaining multiple products.

1. Introduction

Microalgae are photosynthetic microorganisms that convert sunlight, CO_2, and nutrients into biomass [1]. They can adapt to adverse environmental conditions, being found in almost all ecosystems where sunlight and water coincide, such as soils, ice, lakes, rivers, hot springs, and oceans [2]. Being flexible and easily adaptable to various environments has made them suitable cultures on non-arable land without competing with conventional human food production [2].

Microalgae species can be classified according to their target products, which in turn depends on biomass composition. They are microorganisms, either in mono or multicellular form, classified based on their pigment composition. While there are nine classes of taxonomy, in this research, the largest class is studied. This class also contains species of Chlorophyceae (green algae), Phaeophyceae (brown algae), Pyrrophyceae (dinoflagellates), Chrysophyceae (golden-brown algae), Bacillariophyceae (diatoms), and Rhodophyceae (red algae) [3].

Microalgae can accumulate different desirable compounds, which influence their growth rate and productivity. Phosphorus and nitrogen are important elements for microalgal growth. At the same time, Na, Mg, K, Ca, Mn, B, Mo, Zn, Co, and Fe can also be mentioned to influence the content and quantity of their biomass [1]. The microalgal biomass mainly comprises carbohydrates, proteins, and lipids and may contain smaller amounts of rare valuable substances such as antibiotics, carotenoids, and steroids [4]. It has been revealed that under different cultivation conditions, they can accumulate triacylglycerols or storage lipids in the cells, as well [5].

Chlorella zofingiensis (*C. zofingiensis*) is a eukaryotic green microalga [4] that belongs to the Chlorophyceae group [2]; however, there is a consensus that it can also be considered into the genus of *Muriella* [7], or

* Corresponding authors at: LEPABE - Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal.
E-mail addresses: up201306568@fe.up.pt (M. Gorgich), aamartins@fe.up.pt (A.A. Martins), nsc@isep.ipp.pt (N.S. Caetano).

Mychonastes [8], as well as *Chromochloris* [9]. It is estimated that there are almost 350 genera and more than 2650 living species identified in the Chlorophyceae class, with a large variety of shapes and forms, from free-swimming unicellular to non-flagellate unicells colonies [10]. *C. zofingiensis* is described in the literature as spherical shape cells, with a diameter ranging from 2 to 15 μm, without flagella, and with a cup or bowl-shaped chloroplast (Fig. 1) [6]. Cup-shaped chloroplasts have been observed in the cytoplasm of *C. zofingiensis* cells, with the size of almost half of the cell volume, containing scattered granules of starch, with the cell's nucleus located in the center. Observed in an oval shape, mitochondria are closely associated with chloroplast. There are also lipid bodies spreading in the cytoplasm [6].

These cells of *C. zofingiensis* can live either in saline or in freshwater, in both monocellular and colony forms [11]. Growing in either photoautotrophic, heterotrophic, or mixotrophic environments, *C. zofingiensis* uses the available dissolved carbon to grow [12]. Its life cycle can be generally represented in three stages: growing, ripening, and maturing, and dividing [13]. Briefly, in photoautotrophic cultivation, unfavorable stress factors such as intensive light, attenuate biomass production and algal growth. In response, the microalgal cells are mutated to produce more protection layers to shade the cells. This response stimulates the production of astaxanthin. Contrarily, in heterotrophic cultures, *C. zofingiensis* is fed mostly on organic carbon sources, which boost growth rate and contents (such as astaxanthin) [14]. In later sections, more details will be presented to discuss the effect of each individual or combined stress factor.

Despite being discovered early in the 1970s, *C. zofingiensis* had not been well-studied until the comprehensive research studies conducted by Del Campo et al. [15], who showed the potential of this microalga to accumulate significant amounts of valuable carotenoids and starch, and under certain conditions to also accumulate lipids [4]. Later investigations revealed the contents and highlighted their applications in various fields. The main objective of this study is to review the high-value contents of *C. zofingiensis*. In addition, it is purposed to highlight several applications in which the reviewed microalga can be invested to obtain valuable (co-)products.

First, a review of its main composition and cultivation influencing factors is presented to show the potential applications of this microalga. Section 2 contains details about the contents which can be obtained from the mass cultivation of *C. zofingiensis*. In the third section, cultivation systems and conditions will be presented, showing the robustness of this microalga, and describing how stress factors affect the quality and quantity of each mentioned content. Based on the discussed contents of interest and cultivation approaches, in Section 4, various applications are listed and categorized by industry sector, including medical and pharmaceutical, food and feed, cosmetics, biofuels, and wastewater treatment. For each application, supportive facts will be studied up to the scope of environmental engineering. Finally, in Section 5, the discussions will be wrapped up and concluded.

2. Composition

Since the discovery of *C. zofingiensis* early in the 1970s, it has been in the center of attention for numerous research studies due to the particular nutritional characteristics, relevant from pharmaceutical [16] to the food industry [17].

The composition of *C. zofingiensis* depends on factors, such as nutritional conditions, light intensity and regime, aeration, and temperature. Nevertheless, the main compounds of interest found in the *C. zofingiensis* cells are mentioned in several studies, including sustainable sources of bioactive metabolites, such as polyphenols, vitamins [17], lipids, and proteins [18]. This section is intended to review the three categories of compounds being found in *C. zofingiensis*, which are in the vast interest of markets, including lipids, carotenoids, and exopolysaccharides (EPS). In later sections, the application of the described contents will be discussed as well.

2.1. Carotenoids of C. zofingiensis

In particular, *C. zofingiensis* is a rich source of carotenoids, which are pigments from yellow to red color, belonging to a group of various isoprenoid pigments, the majority of which are formed by a 40-carbon chain conjugated by double bonds [6]. Synthesized through photosynthesis, these colored pigments are light-harvesting antennae of the chloroplasts that assist in transferring energy to/from the chlorophylls [19].

The most notable carotenoids available in *C. zofingiensis* are lutein, astaxanthin, canthaxanthin, and zeaxanthin (Fig. 1). Although primary keto-carotenoids, such as β-carotene (in the form of provitamin A carotenoid), can be obtained from the five major edible crops (such as corn, wheat, rice, potato, and cassava), none of these agricultural sources is suitable for the extraction of the aforementioned compounds for medical or pharmaceutical use, since they are grown for human food [20]. Besides, there are limitations for natural body uptake of keto-carotenoids, which highlight the demand for pharmaceutical products (Fig. 2).

With the chemical formula $C_{40}H_{56}O_2$ and 568.87 g/mol of molar mass, lutein is a main carotenoid in the light-harvesting antenna, having the appropriate structure to harvest light and to protect against photo-oxidation from the excess sunlight absorbed [21]. It is stored in the chloroplast during the early stages of cultivation [18]. In nature, other main sources of lutein are the marigold flowers, containing about 20 mg g^{-1}. Although lutein contents from algal species are less than 5 mg g^{-1}, microalgae present several advantages compared to other natural lutein

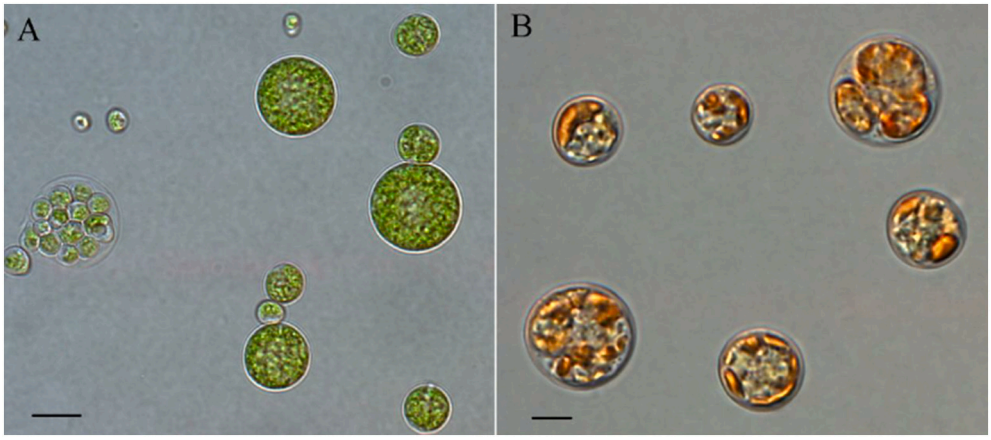

Fig. 1. C. zofingiensis cells; A) Normal growth; B) under stress factor. Bars: 5 μm (the image is adapted from [6]).

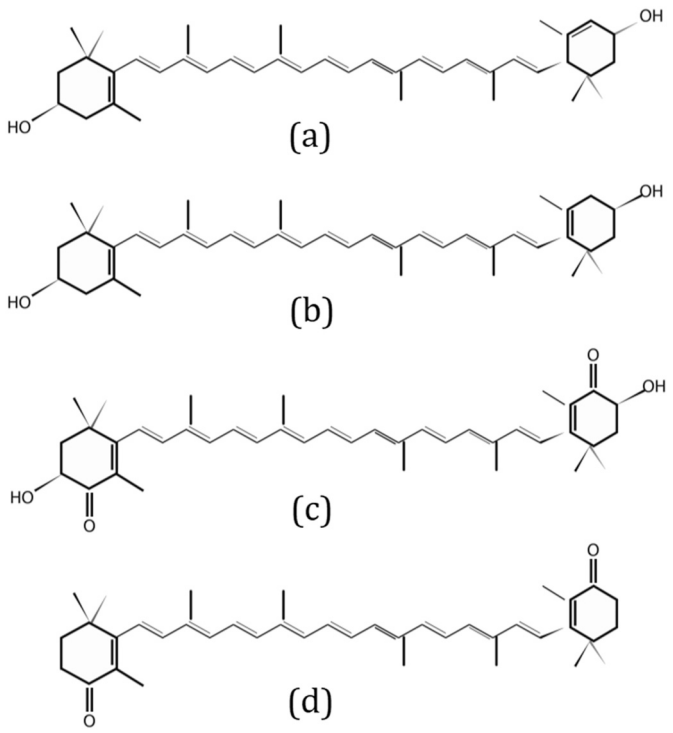

Fig. 2. Chemical structures of the *C. zofingiensis* carotenoids: (a) Lutein, (b) Zeaxanthin, (c) Astaxanthin, and (d) Canthaxanthin (image is adapted from [6]).

sources. For example, they grow fast, there is no need for arable land and can be harvested during the whole year [22].

Astaxanthin, with the chemical formula $C_{40}H_{52}O_4$ and 596.86 g mol^{-1} of molar mass, including cis-trans isomers, is classified into the oxygenated xanthophyll group of carotenoids. Structurally, each ionone ring of astaxanthin has hydroxyl and keto groups that contribute to its potential antioxidant activity [23]. It has been observed either in free form or esterified, respectively synthetic and natural astaxanthin [23]. As a secondary keto-carotenoid, astaxanthin is accumulated in high amounts in *C. zofingiensis* under specific growth conditions [24]. It is accumulated progressively in lipid bodies, outside the chloroplasts, during the various growth stages [25]. While its role in algal cells is not fully understood, it is reported to be involved in several mechanisms, including to control irradiation to cells' components (photo-protective filter), to prevent accumulation of oxygen radicals (antioxidant), to act as a hydrophobic layer to avoid water loss during either salinization or dehydration (dehydration controller) [26]. Briefly, it can be said that this pigment is responsible for protecting the algal cells against oxidative damage imposed by environmental conditions [15]. The listed secondary keto-carotenoids have an anti-oxidative role by suppressing the excessive reactive oxygen species and free radicals [27].

Zeaxanthin has chemical formula $C_{40}H_{56}O_2$ and 568.87 g mol^{-1} of molar mass and is one of the most common carotenoid alcohols, extremely limited in natural resources. Lutein and zeaxanthin have identical chemical structures and are isomers. Although *C. zofingiensis* cannot naturally produce zeaxanthin, it has been mutated (CZ-bkt1) to accumulate high amounts of this compound [20]. Canthaxanthin has the chemical formula $C_{40}H_{52}O_2$ and 564.82 g/mol of molar mass and is another type of keto-carotenoid stored in the form of free monoesters and diesters of the pigments [28]. Like astaxanthin, it is stored in lipid bodies outside the chloroplast [6] while it is mostly known for its strong antioxidant activity that is higher than that of β-carotene [29]. It also has been reported for immunomodulatory activity such as enhancing the proliferation and function of immune-competent cells.

2.2. Lipids

C. zofingiensis has long been the center of commercial attention for either human food, animal feed, or bioactive compounds. Additionally, there have been several investigations reporting the potential of studying microalgae for biodiesel production. Lipids, comprising both neutral and polar molecules, are biological compounds of fatty acids soluble in organic solvents. They can be found in both forms of saturated and unsaturated fatty acids. The difference between these two forms is associated with the presence of at least one or more double bonds [30].

Several investigations have highlighted that nutrient limitation (known as starvation) could significantly motivate metabolisms to increase the lipid accumulation in *C. zofingiensis*. The growth and accumulation of lipids in microalgae [31], as well as the composition and fatty acid profile of lipids [30], can also be affected by other factors, such as CO_2 aeration, temperature, and light exposure (in Section 3.2.2, more specific details related to each factor will be discussed). The economic viability of biofuels production via microalgae depends directly on biomass productivity (from algal biomass) and lipid synthetic productivity (from biorefinery procedure). From this perspective, *C. zofingiensis* can be seen as an ideal oil-rich microalga with the capacity to produce lipids at the highest level of productivity (compared to other microalgae species) due to the high lipid cell content and biomass production. Therefore, in commercial-scale algal oil production, it has been determined as a bioresource for the production and accumulation of lipids under controlled environmental conditions [32]. Depicted in Fig. 3, lipids from *C. zofingiensis* cultures mostly contain oleic acid (C18:1), linoleic acid (C18:2), and palmitic acid (C16:0) [33]. The fatty acids in *C. zofingiensis* cells mostly include 16–18 carbons with a maximum unsaturation degree of three. This characteristic makes it suitable to be used for biodiesel purposes.

2.3. Exopolysaccharides

Carotenoids and lipids are not the only attractive commercial product that can be obtained from *C. zofingiensis*. Other compounds such as exopolysaccharides (EPS) can also be obtained. Briefly, EPS are extracellular natural anticoagulant biopolymers (mainly consisting of a complex high-molecular-weight (HMW) mixture of biopolymers, including glucose, fucose, galactose, rhamnose, mannose, xylose, and other sugar derivatives [35]), which bind the microalgal cells together, and at the same time, protect cells against dewatering and toxic substances. Additionally, it has been reported to serve as energy and carbon

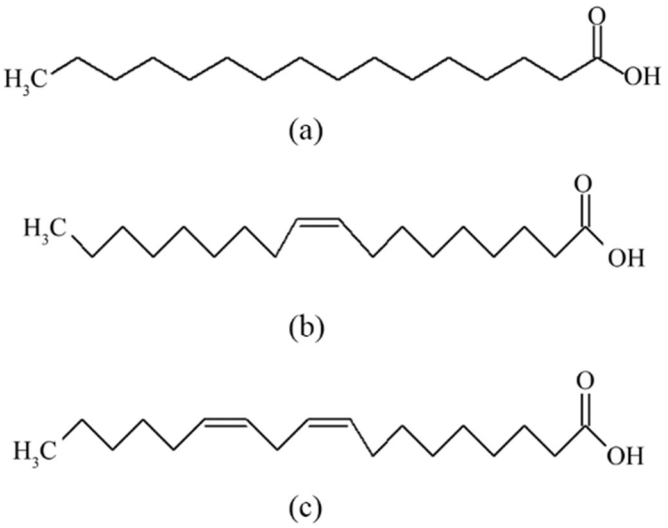

Fig. 3. Type of lipids found in *C. zofingiensis*; a) palmitic acid, b) oleic acid, and c) linoleic acid (the image is adapted from [34]).

sinks responding to stress [36]. Monosaccharides are the main compounds defining the EPS biological activities. By the order, mannose, rhamnose, and uronic acid are responsible compounds for the reaction with heavy metals, while rhamnose, together with fucose and *N*-acetylglucosamine, is responsible for flocculation. Finally, arabinose contributes to the aggregation of the algal colony [35].

Evaluations have shown that the radical scavenging activities of exopolysaccharide obtained from *C. zofingiensis* demonstrated against hydroxyl and DPPH (1,1-diphenyl-2-picrylhydrazyl) is dose-dependent. At the concentration of 1.0 mg·mL^{-1}, it showed 44.5% of hydroxyl radical scavenging effects, while by increasing the concentration to 3.0 mg/mL, the EPS extracts inhibited 71.5% of the DPPH radicals [35].

Although the production of EPS from microalgae is lower than commercially driven bacterial sources, especially for *C. zofingiensis*, the yield is less than other algal sources [35]. Still, comparing to marine bacteria, it is significantly higher [37]. Besides, it is an advantage for *C. zofingiensis* that the culture conditions can be modified (i.e., by adding glucose stress) to obtain EPS as a co-product along with others, cost-effectively [35].

3. Cultivation

First, the cultivation systems are reviewed in this section, covering natural ecosystems and synthetic containers to culture the studied microalga. The detailed study of stress factors to induce content requires in-depth investigation of the growing conditions. Followed by cultivation approaches, growing conditions will be studied to analyze the effects of each stress on the content and quality of the biomass. Considering the various cultivation conditions to which *C. zofingiensis* can be adapted, different cultivation systems can also be employed in its mass production.

3.1. Cultivation systems

Open systems, commonly referred to as outdoor cultivation, are widely employed since the late 1940s. Raceway ponds are the most well-known open cultivation systems for microalgae [4]. Commonly, in the mass production of microalgal products, pond dimensions of 0.5 m, 8 m, and 50 m in height, width, and length, respectively, are reported [38]. Up to 30 cm depth, *Chlorella* strains can absorb sunlight as a source of energy and organic or inorganic dissolved compounds to supply their nutrients demand [6]. Though easy to set up and low in maintenance cost, open ponds have several drawbacks, including low algal cells density, rapid water evaporation from the culture medium, culture contamination, and difficulty in temperature control [6].

Unlike open systems, closed systems provide controlled environments to address the mentioned disadvantages. Closed photobioreactors (PBRs) can be transparent materials with a large surface area/volume ratio [39]. Several PBRs are designed concerning requirements for several biomass applications. For commercial purposes, tubular and panel shapes are the most recommended for the cultivation of *C. zofingiensis* [6], with a typical volume from 15 to 30 L. With algal strains being cultured in a controlled medium and with sufficient light penetration (i.e., in photoautotrophic conditions), biomass productivity of *C. zofingiensis* is reported to be added up to 1.2 g·L^{-1}·day^{-1} [6]. However, drawbacks are reported for PBRs when cultures are scaled up, such as poor mixing, a buildup of dissolved oxygen, and high costs for construction and maintenance, which constitute some obstacles that need to be overcome when closed systems are intended [31].

Fermenters are widely employed when non-photosynthetic cultures are intended. Fed-batch is the most common method in the fermentation industry, which can attain a high cell density. Besides, controlled growth under aseptic conditions in fermenters increases the contents of the cultured cells by decreasing the loss of nutrients. Another advantage consists of elimination of the requirement for light and offers the possibility of increasing productivity. The application of fermenters in high glucose medium has been tested for astaxanthin production in heterotrophic cultures [24]. Although high astaxanthin yield (56 mg·L^{-1}) is reported by Liu et al. [40], these cultivation systems are not commercially viable due to their high construction and maintenance costs (for fermenters) and low industrial yield (0.5% of dry content [41]), particularly when large-scale production is intended [6]. In another study, Liu et al. [42] showed that the employment of a suitable fermenter controlled the growth of *C. zofingiensis* for specific contents like astaxanthin.

3.2. Cultivation conditions and target components

Several research studies showed that their cultures' conditions influence the contents of *Chlorella* species. For example, it has been shown that either individual or combined effects of different environmental [18] and nutritional conditions [43] can induce modifications of cell morphology and performance and motivate cultures to generate and accumulate different compounds [44]. This phenomenon can be observed based on the color changes of *C. zofingiensis*, that when cultivated under different conditions, can influence the algae cells to change from green to red or yellowish color [45], corresponding to the production of pigments [46].

Additionally, Huang et al. [20] indicated that it is possible to extract several targeted products, such as astaxanthin, zeaxanthin, lutein, and β-carotene simultaneously, by considering various cultivation modes using *C. zofingiensis*. In the following subsections, several cultivation modes are briefly studied.

3.2.1. Microalgae growth modes

The photoautotrophic growth mode is still the most common cultivation method employed commercially for *C. zofingiensis*. Under photoautotrophic conditions, this microalga showed similar behavior to other species belonging to the Chlorophyceae category, exhibiting both a high specific growth rate (about 0.04 h^{--1}) and high biomass concentration (over 7 g dry weight·/L^{-1}) [47]. Azaman et al. [18] refer that chlorophyll *a* is the main pigment found in *C. zofingiensis* grown under photoautotrophic conditions since most contents of chlorophyll *b* are converted to chlorophyll *a* through the process of collecting energy.

Mixotrophic growth is also well-known to enhance the production of pigments. Color changes of the culture are an indicator for the accumulation of colorful pigments [18]. The change in the number of pigments and several microscopic changes are tracked in the algal cells, such as morphological alterations. Under mixotrophic conditions, *C. zofingiensis* presents larger cells, reported to be double than those in autotrophic mode, approximately from 4 μm to 15 μm [48]. This change in the cell size justifies more contents, such as multiple chloroplasts in *Chlorella* species. This increased chloroplasts triggers the construction of larger cells to enhance the accumulation of glycolytic lipids in storage vesicles and photosynthetic carbon fixation [49]. Thus, under mixotrophic growth conditions, *C. zofingiensis* showed higher lipid and starch productivities and their accumulation in the cells [18]. Despite the several advantages such as higher growth rate [50] and productivity [51], [52], the mixotrophic growth mode has not been employed commercially due to the high operating costs in large-scale infrastructures [53].

A quick comparison between mixotrophic and photoautotrophic conditions reveals that the cells cultured under the mixotrophic conditions contain 80% less chlorophyll *a* and b contents compared with those cells cultured under photoautotrophic conditions [54]. In several studies, the advantages of photoautotrophic conditions compared to mixotrophic conditions are discussed concerning the commercial benefits of producing specific high-value compounds [55]; however, there are some disadvantages, such as low biomass concentration and a long cultivation period [55].

In contrast, few studies have analyzed the heterotrophic growth conditions for *C. zofingiensis* [6,24]. The most detailed one is the

research carried out by Li et al. [27], who concluded that phylogenetic relatives of *C. zofingiensis* could accumulate more lipid content under heterotrophic growth conditions. In the study conducted by Wang et al., emission of CO_2 has been mentioned as a drawback of heterotrophic culture mode, which can be overcome if the cultivation is carried out in mixotrophic conditions [56]. The same drawback of CO_2 emission has been pointed out by Liu et al. [40] for targeting *C. zofingiensis* as a biofuel resource. In addition, cultivation costs will be uneconomical since glucose demands more than 80% of the total medium cost. The discussed issues can be overcome in case the cultivation is carried out in mixotrophic conditions [56,57], and the demanded glucose is obtained from low-cost sources, such as industrial or agricultural waste or other cellulosic materials [57].

3.2.2. Stress conditions

In general, algal metabolism can be stimulated to increase the production of a target component by changing the environmental conditions [58]. Any changes in environmental factors, such as temperature, light, pH, and nutrient levels, affect cellular activities, influencing photosynthesis, growth rate, and cell composition [18]. In Fig. 4, cell contents are labeled in both favorable and stress conditions in which lipid is accumulated in the cell.

In commercial applications, to increase the productivity rate of a given product, stress conditions are widely employed to batch cultures. As an instance, two independent studies by Del Campo et al. [15] and Ip and Chen [45] demonstrated that the amount of astaxanthin and lutein in *C. zofingiensis* cultures increased when they were grown in the photoautotrophic mode under stress conditions (three folds higher when using intensive light irradiation). This phenomenon occurs because both lutein and astaxanthin play a defensive mechanism against environmental changes [4].

Table 1 summarizes the different types of stress conditions that can be applied to *C. zofingiensis* to enhance the yield of specific components. The details of each stress factor will be explained in further sub-sections.

Table 1
Stress conditions applied to *C. zofingiensis* and their effect on specific components.

Stress conditions	Stress imposition[a]		Effect	References
Temperature	↑		Growth rate decrease (>35 °C) Higher biomass productivity (up to 35 °C) Astaxanthin increase (33 °C)	[4], [59], [60]
	↓		Lutein increase (28 °C)	[59]
Light	↑		Lutein and astaxanthin increase	[15], [50]
	↓		Lipid and starch increase	[43,56,57], [61]
Nutrient excess/ deprivation	N	↑	Lutein increase	[15], [31]
		↓	Astaxanthin and lipid increase	[6], [63]
	Fe	↑	Astaxanthin increase	[6]
		↓	Decrease growth rate	[64]
	C	↑	Astaxanthin increase	[65], [24]
Salinity	↑		Growth rate decrease Astaxanthin increase (up to 0.2 M NaCl) Triacylglycerol increase	[15], [45], [6], [27], [66] [66]
Mixing and turbulence	↑ Suitable		Growth rate decrease Uniform temperature and light distribution	[67] [68,69] [4]
	↓		Light irradiance decrease	
Gas transfer	↑		Carbon increase, resulting in high lipid content	[4,6]
Glucose	↑		Astaxanthin increase	[23,24,73,74], [57], [72]
ROS	↑		Astaxanthin increase Lipid increase	[73], [74], [75] [76]
pH	↓		Increase astaxanthin	[6],

[a] The up-arrow direction indicates factor increase, and the downward direction indicates factor decrease.

3.2.2.1. Temperature. Temperature changes can be a potential source of stress for microalgae cultures, directly affecting cell size and structure. Increasing growth temperature stimulates carotenoids biosynthesis by increasing the enzymes involved in their production [4]. Based on van't Hoff's rule, biological reaction rate should double approximately with each 10 °C increase in temperature. Still, the Arrhenius equation is only applicable in a defined temperature range for each microalgae species. It is important to mention that this increase is nonlinear because when the maximum growth rate is reached, no more radiation or temperature can propel the crop to increase biomass production [4]. The experiments conducted by Lui et al. [6] revealed that while the microalga can grow at the temperature range of 20 to 30 °C, the best temperature for the astaxanthin high yield production is from 25 to 30 °C.

The temperature in microalgae cultures is directly related to sunlight. The theoretical conversion rate of light energy to chemical energy (through photosynthesis pathway) is around 31%, indicating that at least 69% is absorbed as heat, increasing the culture medium

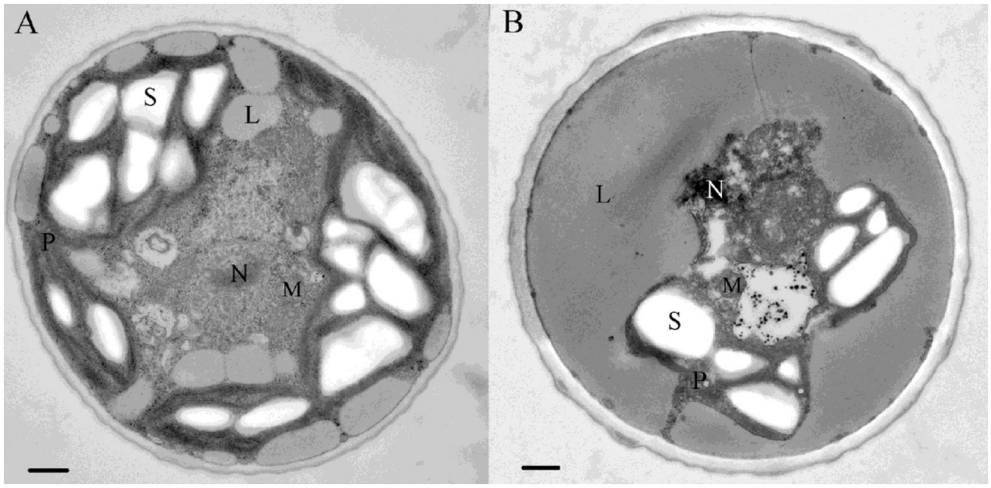

Fig. 4. *C. zofingiensis* cells; A) favorable, and B) stress conditions; lipid body (L); nucleus (N); mitochondria (M); plastid (P); starch granule (S). Scale of the black bars is 0.5 μm.

temperature [77]. In open systems, such as raceway ponds, temperature control is carried out naturally by the circulation of the culture media. In contrast, in closed systems, the temperature must be controlled to avoid increasing the culture's medium temperature, an important factor when designing PBRs [4].

There are few research studies on outdoor cultivation of *C. zofingiensis* during the high-temperature season. Huo et al. [59] exposed cultures to direct sunlight during the warm season, from June to August, in an outdoor photobioreactor located in southern China. As expected, microalgae cells absorbed light, and the temperature exceeded 45 °C in the outer tubular photobioreactors, reporting adverse effects on the growth rate. To cool down the installed photobioreactors to the range of 35 to 37 °C, they benefited from simple methods such as parasols, or more complicated, including polyethylene tube materials and mid-day atomization spray. In another study, Feng et al. [63] measured lipid contents of outdoor cultures, influenced by three factors: temperature, light intensity, and nitrogen deficiency. Focusing on the temperature, they concluded that the cultures exposed to a higher temperature (in their study, those cultivated in spring, compared to the ones studied in the autumn) reached higher biomass productivity (which might also be induced by higher irradiation). In the final reviewed work, the effect of temperature can be mentioned in the study of Zhu et al. [60], in which they clearly stated the contribution of robust growth of the microalgal cells (from 115 to 2046 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with the outdoor temperature (registered from 21.5 to 34.5 °C, respectively).

3.2.2.2. Light. In the previous section, it was discussed that photosynthesis is connected with both light and temperature. Considering the light effect, the pigments content, such as chlorophyll *a* and chlorophyll *b*, decreases in response to high light intensity during photosynthesis. It should be noted that the illumination intensity, the direction at which cultures are exposed to light, and finally, the light to dark cycle, induce various changes, not only in the microorganism content but also in cell morphology [4], which is called light stress. The research study conducted by George et al. [48] has demonstrated that light intensity influences the algal cell morphology, reporting that in cultures grown under 150 μmol photons $m^{-2} \cdot s^{-1}$ cells size tends to increase. Furthermore, Azaman et al. [18] reported that moderate light intensity (100 μmol photons $m^{-2} \cdot s^{-1}$) is sufficient to induce changes in the cellular level like what typically occurs in mixotrophic growth mode.

The photo-induction theory has been used to explain such an increase in two ways [78]. The first way attempts to explain the improvements of the volumetric production by associating it to the improved growth of the microorganism [79]. In contrast, the second way justifies the increases in the accumulation of cellular contents by associating it to the activity of carotenoid biosynthesis enzymes, stimulated by illumination [77]. Studies support the influence of light irradiance on the *C. zofingiensis* growth and the accumulation of cellular components. For instance, Del Campo et al. [15] reported that the change influences lutein and astaxanthin contents in light irradiance up to three folds. The experiment conducted by Feng et al. [64] expressed that the outdoor cultures exposed to higher irradiance (those cultivated in spring vs. the ones in autumn) stored more astaxanthin. More astaxanthin accumulation can be justified since secondary carotenoids serve as photoprotective agents [79]. Contrarily to high and moderate intensities, light deprivation can trigger pathways to change either the growth rate or contents in cultures.

Chen et al. [61] showed that compared to glucose-fed *C. zofingiensis* cultures exposed to light, dark cultivated ones can accumulate more lipids and starch. Detailed analysis showed that starch is accumulated more than lipids, which is consistent with the other experiments considering photoautotrophic conditions [62]. They justified that since lipids require less energy for cell utilization, they are less produced than starch [50]. Besides, considering cells replication as an energy-consuming process, microalgae cultures accumulate sufficient energy in the form of lipids before cell division. Chen et al. [61] reported that after replication, *C. zofingiensis* cells are more likely to consume lipids instead of starch to provide energy for cell division. Therefore, under stress conditions, when the cell growth rate is reduced, the cells store a high amount of lipids. Briefly, the light effect induces two different behaviors on *C. zofingiensis* cultures: on one side, by increasing light intensity, cell division is increased, leading to higher biomass yield, while on the other side, the ratio of lipid to starch contents is decreased since, unlike starch, the lipid is consumed faster [61]. The effect of light deprivation on the growth rate has been analyzed by Sun et al. [24], showing that for cultures fed with glucose and mannose, a specific growth rate of around 0.03 h^{-1} and cell density of 53 g dry weight L^{-1} could be obtained when the cultures are deprived of light.

The light intensity and light bandwidth are shown to influence the carotenoid production and accumulation rates of the microalgal content. Hang [4] reported that *C. zofingiensis* accumulates more carotenoids when it is exposed to white light. In addition, Voskresenskaya et al. [80] research study showed that a higher dry weight biomass production is obtained. The experience carried out by Sudibyo et al. [11] revealed that under 12 h of light/dark regime with less salinity in medium, red light presented the highest cell density, comparing to the blue and white lights. They concluded that since the red light carries less energy per photon, it provides a more stable energy source for the microalgal cells. Contrarily, they discussed the advantages of blue light for producing more dry weight of biomass, as the blue light stimulates the activity of the specific enzymes (ribulose-1, 5-bisphosphate carboxylase (Rubisco)) responsible for photosynthesis [11]. Therefore, higher lipids content can be accumulated. According to the distribution of pigments in *C. zofingiensis*, the blue and red lights are evaluated as suitable bandwidth for photosynthesis, in contrast with the green light, which inhibits the cells' growth [81].

3.2.2.3. Nutrient deprivation. The experiment carried out by Mujtaba et al. [82] revealed that nutrients deprivation could be seen as a common stress induction method, which significantly alters the accumulation rate of various valuable components, as well as their quality in *C. zofingiensis*. In this regard, the effect of nitrogen concentration in the form of nitrate has been widely studied. Briefly, under nitrogen deficiency conditions, *C. zofingiensis* shows high amounts of secondary ketocarotenoids [6]. The same trend is proven for compounds with a high carbon to nitrogen ratio, as they are reported to induce astaxanthin production [65]. The mentioned trend was observed when mixotrophic cultures were deprived of nitrogen and exposed to excess organic carbon substrates [24]. Contrarily, the maximum cellular level of lutein was recorded at high nitrate [15]. In a nutshell, nitrogen deprivation stimulates an increase in the astaxanthin content at the expense of lutein decrease. Contrarily, a high concentration of nitrogen can accumulate more lutein; however, astaxanthin's content is reduced significantly [15]. The reason can be explained by looking at the β-carotene hydroxylase (CHYb that is one gene Cz12g16080) and ketolase (BKT) presented in two genes; Cz13g13100 or BKT1 and Cz04g11250 or BKT2 genes possessed by the studied microalga. Since BKT1 is transcript abundant almost 30-fold more than BKT2 when up-regulated by nitrogen deficiency, it dominates the production of astaxanthin biosynthesis [20,83]. At the same time, lutein decrease is associated with: first, the down-regulation of lutein biosynthesis genes in case of nitrogen deficiency (lycopene ε-cyclase or LCYe, cytochrome P450 beta hydroxylase or CYP97A, and epsilon hydroxylase or CYP97C); and second, the increase in the up regulation of CHYb and BKT genes to convert carotenoid to astaxanthin [83].

Alongside keto-carotenoids, Feng et al. [63] reported that *C. zofingiensis* stored lipids, reaching a lipid content up to 65.8% of its dry weight when stressed with nitrogen deficiency outdoors flat bioreactors. In another research study [31], designed to investigate the effect of several micronutrients on lipid contents, the same authors

demonstrated that the *C. zofingiensis* highest lipid content (65.1%) was obtained under nitrogen deficiency. An increase in the TAG content was also reported (4.5 $mg.g^{-1}$) by Mulders et al. [84]. The same increase trend was again reported in the experiment by Zhu et al. [85] for the starch content followed by nitrogen deficiency.

Other micronutrients have also been taken into consideration for both the growth rate as well as the contents. The importance of iron ions was also examined in an experiment by Ip et al. [64] which showed that iron deficiency decreases the cells' growth. At the same time, the excess of this micronutrient boosts the astaxanthin content [6]. Wang et al. [56] studied the effect of iron on fatty acid and astaxanthin accumulation by adding 0.2 mM Fe^{2+} to the medium. The employed stress promoted the highest contents of astaxanthin (2.2 $mg{\cdot}g^{-1}$) and total fatty acids with 41.8% dry weight.

Unlike nitrogen and iron, phosphorus was not evaluated as a prominent nutrient for the production of secondary carotenoids. Either in the forms of $H_2PO_4^-$ or HPO_4^{2-}, with a concentration in the range of 0.04–0.33 $g{\cdot}L^{-1}$, no significant change has been observed neither in astaxanthin content nor in the culture's growth rate [6]. Perez-Garcia et al. [86] stated that PO_4 affects both the generation and the transformation of metabolic energy to promote the accumulation of metabolites; however, Feng et al. [31] showed that the lipid content of the cells grown in a PO_4- deficient medium was higher than the ones cultivated in PO_4-sufficient medium (44.7% vs. 33.5%). In an investigation targeting the effect of wastewater on *C. zofingiensis*, Zhao et al. [87] claimed that they did not see any significant biomass change induced by increasing the phosphorous content. However, they stated that the concentration of this micronutrient was decreased in the medium.

3.2.2.4. Reactive oxygen species. Reactive oxygen species (ROS) are chemically reactive chemical compounds with oxygen [88]. Experiments have shown a clear relationship between astaxanthin and ROS levels in heterotrophic *C. zofingiensis* cultures. Biologically, carotenogenesis in *C. zofingiensis* can be regulated via two signals: the alternative mitochondrial pathway, which includes ROS, and organic acids from the tricarboxylic acid cycle (TCA) [75]. This is justified by the biological response to ROS stress conditions, at which algal cells are willing to generate compounds to activate specific defense mechanisms.

In heterotrophic cultivation systems, ROS have been evaluated to induce astaxanthin biosynthesis in microbial cells, including microalgae [74], since algal cells tend to balance cellular ROS by enhancing antioxidative activities (producing more astaxanthin as a natural antioxidant) to neutralize the excessive toxic ROS [89,90]. In an experiment by Zhang et al. [73], they recorded intracellular ROS level coordinated with astaxanthin content in heterotrophic *C. zofingiensis* cells. They showed that the intracellular ROS level had been correlated directly with astaxanthin content. In cultures treated with cerulenin, an increase of cerulenin was accompanied by increased ROS level, with a similar trend to astaxanthin change. Comparably, in cultures treated with sesamol, where ROS level showed a negative correlation with sesamol concentration, the same pattern was observed for astaxanthin content [73]. Other researches have validated the same trend, that the presence of ROS compounds, the keto-carotenoids responsible antioxidant effect was increased to protect the cells. For instance, Ip and Chen [45] employed H_2O_2 0.1 mM to apply ROS stress, and they claimed an increase of astaxanthin yield from 9.9 (control sample) to 12.58 $mg{\cdot}g^{-1}$. Considering lutein content, Dong et al. [91] reported that the addition of 0.1 $mmol{\cdot}L^{-1}$ H_2O_2 and 0.01 $mmol{\cdot}L^{-1}$ NaClO (oxidative stress) enhanced lutein content from 1.75 to 1.95 $mg{\cdot}g^{-1}$. However, they did not discuss if the lutein accumulation might be induced by another stress factor imposed by adding 0.5 $mmol{\cdot}L^{-1}$ Fe^{2+} to the culture medium.

ROS has been evaluated as effective stress for enhancing the level of accumulated lipids in algal cells. Zhang et al. [92] have reported that ROS upregulates the genes responsible for lipid biosynthesis and accumulation of essential lipid metabolisms, including Fatty acids and triacylglycerol (TAG) synthesis. As the final instance, Onay [76] reported that by the increase of ROS, lipid contents of *C. zofingiensis* have increased while the carotenoid contents were recorded higher.

3.2.2.5. Salinity. Due to the ease of accessibility to saltwater sources in most parts of the world, greater attention has been paid to more robust cultures to certain levels of salinity. Del Campo et al. [15] observed a significant increase in the carotenoid accumulation influenced by salt stress. The stimulation was specifically distinctive for astaxanthin since the content was increased to 60% higher at 0.2 M NaCl than in freshwater cultures. However, it had adverse effects on the culture's growth. It is justified since salt stress increases the generation of intracellular ROS in *C. zofingiensis* cells [93]. Li et al. [27] analyzed the amount of astaxanthin in *C. zofingiensis* in response to several stress conditions, including salinity stress, revealing that NaCl stress regulates the transcription of carotenogenic genes. Salinity concentrations of more than 0.2 M suppress the growth by building up additional Na^+ in the cells and the hyperosmotic stress. It can be seen in the experiment by Ip et al. [45], who reported that the highest astaxanthin content was achieved at 0.08 M NaCl. In addition, they reported that for salinity concentrations in the range of 0.2 to 0.4 M, the growth rate reduction is significant, of around 30 to 50%, respectively [15].

Importantly, it must be considered that nitrogen metabolism is also severely affected by salt stress in *C. zofingiensis* [66]. In other words, the employment of multiple stress factors might be unnecessary as the cell growth pathway induced by nitrogen excess is suppressed. From the positive side, this highlights the importance of salinity to control the production (and accumulation) of the content of interests since the production pipeline can be configured in such a way to combine the stimulation of keto-carotenoids production induced by salinity together with nitrogen deprivation, and high irradiance. As an instance, Pelah et al. [94] showed that *C. zofingiensis* cultured under both salinity stress (2% NaCl) and nitrogen deprivation (0.1 $g{\cdot}L^{-1}$) and high light intensity (300 μmol photons.$m^{-2}{\cdot}s^{-1}$) accumulated significant amounts of canthaxanthin (8.5 $mg{\cdot}g^{-1}$ of dry biomass).

Lipid and astaxanthin are not the only compounds affected by the salinity, as it has been claimed that salt stress stimulated starch catabolism by decreasing its content. In a recent experiment, Mao et al. [66] re-evaluated optimal salt concentration to increase both Triacylglycerol (TAG) and astaxanthin production. Their analysis showed that 0.2 M NaCl could stimulate the algal cells to store the targeted products.

3.2.2.6. pH. Generally, several experiments have studied microalgae growth in pH in the range of 7 to 8.5 [4,63–65]. However, it has been observed that due to nutrients oxidation, pH increases gradually. To compensate the increase of pH to a comfortable range for cultures, the nutrient medium is adjusted to 6.5 [97] while *C. zofingiensis* tolerates a wide range of pH, from 5.5 to 8.5. Evaluation of accumulated astaxanthin shows that at pH 5.5, the highest astaxanthin content and yield is obtained [6]. In a study conducted by Chen et al. [98], the addition of pyruvate, citrate, and malic acid was carried out to evaluate the production of astaxanthin, which resulted in enhancement of the yield from 8.36 to 10.72 $mg{\cdot}L^{-1}$ (28.2%) by addition of 100 mM pyruvate.

Cells' nitrogen uptake has been under investigation for decreasing the pH of the medium. To compensate for the drop, it has been proposed either to control the medium pH (and maintain it at the range of tolerance) or by using micronutrient sources that are not associated with pH change. As an instance, the research by Chen et al. [99] suggested replacing the nitrate source with urea of the wastewater. They evaluated the concentrations at 3.81 $mmol{\cdot}L^{-1}$ demonstrated favorable effects in the cells' growth, while it did not affect the pH of the medium upon gradual uptake of nitrogen.

3.2.2.7. Mixing and turbulence. Proper mixing of microalgal cells in the culture medium guarantees uniform distribution of cells, light, and

nutrient concentration, and it helps eliminate the temperature excess [4]. However, improper mixing can cause cell damage due to shear stress [67]. Intense mechanical agitation (more than 300 rpm [100]) causes permanent damages to the cell membrane, which affects the number of cells and thus, decreases the biomass growth rate [101].

A significant decrease in the biomass growth rate was also reported in air-lift photobioreactors when the gas turbulence was above the optimum level [68] (note that the conducted research has been targeting *Chlorella* species in general). However, for species sensitive to mechanical pumping, gas mixing systems (i.e., bubble column systems) are preferable since they have been evaluated as causing fewer adverse effects [69].

3.2.2.8. Gas transfer. Organic carbon is an important source for almost all targeted components of *C. zofingiensis*. Providing a sufficient source of carbon to be absorbed assures commercially acceptable biomass yield [101]. Ip and Chen [14] investigated a light-independent cultivation system, in which they demonstrated that the compositions of organic compounds in the medium, especially the carbon to nitrogen ratio, stimulates carotenogenesis. When grown in photoautotrophic conditions, cultures mostly use inorganic carbon sources in various forms (CO_2 (aq), H_2CO_3, HCO_3^- and CO_3^{2-}) to perform photosynthesis [102]. Although some sources such as HCO_3^- can be easily absorbed by cells, they are a poor source of carbon, compared with CO_2. Consequently, CO_2-enriched air is commonly obtained by mixing nutrient gas with the feeding medium via either a high bubbling rate (tiny bubbles) with a low inlet pressure of CO_2 or a low bubbling rate with a high inlet pressure of CO_2 [68]. Several investigations showed that the productivity rate of *Chlorella* species increases about 7–10% when the proper level of CO_2 is injected to control both pH [68] and carbon source [53]. Too low concentration of CO_2 has been associated with inefficient photosynthesis, resulting in decreased biomass productivity, especially when organic carbon is also not present in the medium [6].

It is important to note that the injection of CO_2 into the culture influences the pH of the medium. Under high concentrations of CO_2, microalgal cells are inhibited since excess CO_2 is converted to H_2CO_3. Technically, there are mainly two methods to regulate the pH upon the injection of CO_2. The simplest way is to control the airflow by measuring pH. As soon as the acidity level crosses from the defined range, CO_2 injection is turned on or off to stabilize the pH of the medium [68]. The other way is to regulate the pH through a chemical reaction that involves adding a compound to keep the acidity level in the desired range. More explanation in this regard should be followed on the topic of pH stress.

3.2.2.9. Glucose. Among all research studies on stress factors, Sun et al. [24] reported that *C. zofingiensis* cultures can reach high cell concentration (up to 53 g L^{-1}) by employing a glucose-fed-batch fermentation strategy (regardless of the maintenance cost of fermenters). High biomass yield is not the only result since Liu et al. [57] reported the same trend for astaxanthin content when cultures are grown with glucose as the only carbon source. Besides, to reduce the cost required for providing inorganic carbon source, Liu et al. [72] showed that in heterotrophic cultures, feeding with a low-cost carbon source such as cane molasses can result in biomass production at (1.79 $g{\cdot}L^{-1}{\cdot}day^{-1}$) as well as astaxanthin production rate at (1.99 $mg{\cdot}L^{-1}{\cdot}day^{-1}$). According to Komor and Tanner [103], the cell components include an inducible hexose/H+ active glucose symporter, which enables the cell to uptake glucose from the medium in just a few minutes [70]. In the experiment carried out by Ip and Chen [14], they indicated that that higher glucose concentration in the medium would result in the higher forms of secondary carotenoids, observing the highest specific growth rate (0.031 h^{-1}) and the highest growth yield (0.44 $g{\cdot}g^{-1}$) at the concentration of 20 $g{\cdot}L^{-1}$ for glucose. Further increase of the glucose concentration (60 $g{\cdot}L^{-1}$) leads to decrease of specific growth rate as well as growth yield (0.024 h^{-1} and 0.32 $g{\cdot}g^{-1}$, respectively).

Through a detailed experiment, Liu et al. [71] studied the influence of both monosaccharides and disaccharides enriched media, finding that glucose, fructose, mannose, and sucrose have positive effects in increasing growth rate (0.03 h^{-1}) and astaxanthin content. At the same time, the same trend was not observed in media enriched with lactose and galactose. Additionally, they observed an almost 900% increase in lipid yield in heterotrophic cells fed with 30 $g{\cdot}L^{-1}$ of glucose. In another experiment by Zhang et al., it was discussed that among a set of glucose concentrations, the maximum amount for glucose resulting in high astaxanthin yield is reported to be 30 $g{\cdot}L^{-1}$ for *C. zofingiensis* cultured in heterotrophic condition [104].

In addition to the type of monosaccharide, the exposure duration has been studied as well. For instance, Zhang et al. [105] showed that glucose could increase its accumulation and fatty acid contents (by changing cellular lipid composition) while decreasing chlorophylls content. Interestingly, they observed that the increase of the astaxanthin only occurred within the first 24 h after the exposure (comparing with the control group). Moreover, after 196 h, it was observed that the content of astaxanthin in N-deprived cultures reached 0.58% of the dried weight, which was almost 27% more than the glucose-treated cultures.

To induce EPS production under mixotrophic conditions, BG-11 medium is recommended while glucose is added. With this suggestion, Zhang et al. stated that *C. zofingiensis* cells peaked (2.3 $g{\cdot}L^{-1}$) for biomass production on the 3rd day, while maximum EPS was observed at the peak a day later with 208.4 $mg{\cdot}L^{-1}$. In general, they reported the EPS production could reach 91.0 $mg{\cdot}g^{-1}$ dry cell weight [35] with the glucose stress.

4. *C. zofingiensis* applications

C. zofingiensis has received increasing attention commercially because of high-value products, including lipids [31,49], protein [106], and carotenoids [6]. In Table 2, the applications are classified by industry sector, briefly discussed in the following subsections.

4.1. Medical use

It was studied that carotenoids can be looked at as one of the objectives for culturing *C. zofingiensis*. This section, it is aimed to briefly highlight the necessity of carotenoids, to promote the importance of the studied microalgae to obtain this valuable product. In this regard, by considering a normal lifestyle, expected or unexpected exposure to free radical compounds has been increased via contaminants, chemicals, tobacco smoke, physiological stress, and ultraviolet (UV) radiation [115]. External sources and internal sources such as phagocytes can also increase the level of free radicals in the human body. Free radicals (i.e., hydroxyl and peroxyl radicals) are highly reactive compounds with an open side of oxygen ions that react with proteins and lipid membranes. They are produced in the body during normal metabolic reactions and processes that damage DNA. Oxidative damage is directly associated with aging, atherogenesis, macular degeneration, and cancer [4]. Carotenoids have an antioxidant role as they react with single oxygen, resulting in removing the oxidant and preventing other molecules or tissues from reacting with it instead [135]. Thus, neutralizing oxidants prevent them from producing more free radicals initiated by the reaction of poly-unsaturated fatty acids [1]. Epidemiological studies have shown that some carotenoids are effective agents to prevent several diseases, including cancer [136].

First, for lutein, there is evidence supporting its protective role against chronic diseases such as eye and heart diseases and those that compromise immune status [119]. Additionally, it is an essential compound preventing the development of cataracts and macular degeneration [89,90]. Lutein is known to hamper the progression of early atherosclerosis [120]. Several investigations have also discussed the positive effect of lutein in cell-mediated and humoral immune responses

Table 2
Applications of *C. zofingiensis* components in different industry sectors.

Sector	Compound	Application	Reference
Food	Astaxanthin	Colorant for salmon, carp, and red sea bream	[107], [108]
		Colorant and nutrient in the poultry industry	[20], [109]
	Lutein	Feed additives and colorants in poultry farming	[110], [111]
	Canthaxanthin	Food colorant for egg yolk and chicken skin	[6]
		Colorant in larval fish and shrimp	[6]
	Zeaxanthin	Feed additives and colorants in poultry farming and shellfish and salmon	[110], [111], [112], [113]
Medical and pharmaceutical	Astaxanthin	Decreasing effects of ultraviolet radiation	[4]
		Decreasing chance of cancer development	[112]
		Stimulating the production of immune T-cells	[114], [115]
		Increasing high-density lipoproteins (HDL)	[15], [116]
	Zeaxanthin	Reducing the risks of cataract	[117]
		Reducing the risk of cancer (colon cancer based on in vivo study)	[114]
		Nourishing of retinal cells	[118]
	Lutein	Protecting against chronic diseases (eye and heart diseases)	[119]
		Compromising immune status	[119]
		Preventing cataracts and macular degeneration	[114]
		hampering the progression of early atherosclerosis	[120]
		Stimulating humoral immune response	[121]
	EPS	Showing antitumor properties	[122], [123]
		Antiviral and immunomodulation activities	[124]
		bio-flocculants	[125]
		Protecting cells from antibiotics, toxic heavy metals, and phagocytosis	[123]
		Stabilizing and emulsifying	[125]
Cosmetics	Chlorophyll	Inhibiting lipid peroxidation to protect skin damages	[126]
	Astaxanthin	Skin and eye health promotion	[127]
		Tyrosinase inhibitory effect (skin whitening)	[128]
	Lutein	Skin and eye health promotion	[127]
		Colorant for lipstick, eye shadow, and face make-up	[129], [130]
	Zeaxanthin	Avoiding cells degradation	[131]
		Tyrosinase inhibitory effect (skin whitening)	[128]
Industrial	Lipid	Biodiesel	[60]
	Starch and cellulose	Bioethanol	[132]
	Wastewater treatment	Nitrogen and phosphorus absorption	[87], [133], [134]

in canines [121]. Zeaxanthin is another carotenoid found in *C. zofingiensis* responsible for reducing the risks of cataracts [117], cancer, and low-density lipoprotein oxidation [139]. Briefly, lutein and zeaxanthin play important roles for biological functioning, mostly referred to as essential compounds to feed retinal cells [119] and protect cells from age-related degeneration, especially retinal cells, against elderly blindness [131]. In vivo experiments have revealed that macular pigments in the human retina are significantly increased by including a daily dosage of lutein-zeaxanthin for eight weeks [114].

Astaxanthin, another keto-carotenoid from *C. zofingiensis*, has interesting applications for pharmaceutical and nutraceutical products [27]. In the form of provitamin A, astaxanthin is classified in the group of essential nutrients required for human health [117]. As a natural nutritional compound, it is mainly obtained through food supplements. Astaxanthin contains ten times more antioxidant levels of β-carotene and, comparably, 100 to 500 times more antioxidant capacity than Vitamin E. Several studies indicated that astaxanthin is a stronger antioxidant than other carotenoids, such as lutein [4]. As a strong antioxidant, it is widely used to decrease the harmful effects of ultraviolet radiation.

Additionally, it plays an important role against several types of cancers. In vivo studies have revealed the chance of colon cancer development has been significantly reduced in F344 rats fed with astaxanthin or canthaxanthin at a 500 ppm dose for 34 weeks [112]. Other medical applications reported include enhancement of the human immune system by stimulating the production of antibodies and increasing T-cells [115], increasing high-density lipoproteins [15], and adjusting LDL and HDL cholesterol levels in the blood [116]. While the excess of Vitamin A is toxic for humans, astaxanthin is a compound unwilling to change to Vitamin A, which is different from other carotenoids [23].

Astaxanthin is not the only component of *C. zofingiensis* under attention from a medical point of view since its EPS content is also commercially attractive. It has been reported that EPS compounds have potential biological activities [122], such as antitumor [123], antiviral and immunomodulation activities [124]. Besides, other medical applications of EPS have been investigated, including nontoxic [140], antioxidant [141], stabilizing and emulsifying [125], smoothening [36], antibiofilm [142], and bio-flocculants [125]. Finally, the research presented in [123] has mentioned that EPS protects cells from antibiotics, toxic heavy metals, and phagocytosis. It has been reported that EPS from microalgae sources is a promising alternative to chemotherapy. For instance, EPS extracted from *C. zofingiensis* showed inhibitory effects through *in vitro* studies on human colon cancer cell lines HCT8. More details are out of the scope of this study; however, it is presented in [35].

4.2. Food and feed

As described above, *C. zofingiensis* is a potential alternative source of carotenoids for medical purposes [143]. However, there are also applications for it in the food and feed industry [113]. Carotenoids can also be used as necessary ingredients in nutritional supplements for color purposes, such as natural color additives in human food and animal feed [144]. The importance of these products has increased significantly. As reported by the end of 2018, the market value of β-carotene and lutein reached $334 and $309 million, respectively, only for feedstock supplements applications [145]. Concerning the human diet, *C. zofingiensis* pigments have been studied to be used as both colorant [144] and flavor adjuvant in the food industry [146], including gum and toffee [113].

The second main use of astaxanthin has been listed as a feed additive for aquaculture [107] to provide the required pigment for the marine animal diet [108]. For example, astaxanthin is mainly used as a natural colorant in salmon, carp, and red sea bream diets. This natural pigment has also been employed in the poultry industry [20] as a rich source diet containing long-chain polyunsaturated fatty acids, including docosahexaenoic and eicosapentaenoic acids. Besides, it can provide protein,

microelement, vitamin and antioxidants source, and finally, a natural pigmentation agent for skin and egg yolks [109].

Canthaxanthin is employed as a food colorant for egg yolks and chicken skin. Besides, it is correlated with the increase in the content of Vitamin E in the liver when used in the poultry diet. In contrast with poultry, the marine industry is more interested in astaxanthin since it induces higher color intensity and better absorption by the digestive tract of salmonids and benefits the growth and survival of larval fish and shrimp [6]. Similar to astaxanthin and canthaxanthin, both zeaxanthin and lutein are looked at as interesting feed additives [110] and colorants in the feed industry (poultry farming) [111]. Zeaxanthin contains food additive color E161h (yellow-red pigment), widely used in both pharmaceutical and food industries [112]. Apart from using in the human diet as a colorant, zeaxanthin pigments produced by microalgae are also used in shellfish and salmon to increase the quality and color of the product [113].

4.3. Cosmetics

The trend towards a healthy lifestyle has increased the demand for cosmeceuticals containing natural algal compounds. As an initial step to prevent skin aging, moisturization helps preserve skin elasticity [127]. The benefits of astaxanthin and lutein have been re-evaluated on skin and eye health promotion *in vitro* and *in vivo* studies [127]. The experiment performed by Ariede et al. [129] revealed that *Chlorella* extracts could increase the smoothing and elasticity of the skin. Since microalgal-derived pigments, main astaxanthin, are non-toxic for skin, companies are willing to use them in sun protection and hair care products [113]. Protection by UV can be presented in two ways: reflection and absorption. Physically, radiation is reflected or blocked to avoid rays penetrating the skin (acts like a hat). At the same time, chemically, absorption compounds are used to filter specific wavelength from reaching cells. Oxybenzone is a chemical absorber widely used in sunscreen cream. It is determined to contain toxic compounds to marine organisms [147], and hence, it is banned in some regions (i.e., Hawaii). Meanwhile, biological absorbers which are environmentally friendly are gaining attention, including compounds (carotenoids) presented in the studied microalgae [126].

Melanin pigment is the reason for hair, skin, and eyes color. They are also responsible for the protection of the skin against UV damage. However, melanin overproduction is associated with regional skin color differences. The main enzyme regulating skin pigmentation is tyrosinase, in which high energy wavelength or ROS compounds convert it to melanin via oxidation [126]. Both astaxanthin and zeaxanthin show tyrosinase inhibitory effects [128]. That is why astaxanthin, as a biological compound, can be listed as an effective compound for skin whitening. Among all keto carotenoids available in *C. zofingiensis*, astaxanthin is widely used in sunscreen creams. The other carotenoids that are the most demanded algal pigment also have applications in cosmetics, including natural colorant in lipstick, eye shadow, and face make-up [129]. For instance, lutein is another carotenoid employed in the cosmetic industry as a natural colorant [130].

As the main natural layer of defense, a natural antioxidant is possessed by skin cells to cancel destabilization effects of skin exposed ROS [148]. But by aging, the antioxidant discharge level decreases, and therefore, the skin is affected more by the surrounding oxidative compounds [149]. Microalgae extracts, including chlorophyll and carotenoids, are known to inhibit lipid peroxidation from protecting skin damages [126].

4.4. Biofuels

The growing consumption of fossil fuels as an unsustainable energy resource is tightly correlated with many environmental problems [150]. The excessive consumption of fossil fuels is associated with multiple issues, including fuel demand, ecological degradation, and climatic change. Emission of CO, CO_2, SO_x, and NO_x into the atmosphere released from the combustion of fossil fuels aggregates greenhouse effects and leads to global warming [151]. Thus, finding a new renewable, environmentally friendly, and alternative energy source is inevitable. Biodiesel mainly obtained from vegetable oils such as soybeans showed to be a potential alternative to fossil fuels [116,117]. However, these are not sustainable sources of biodiesel as there is direct competition with edible feedstock used for human food and arable land used for agriculture crops. Concerning the sustainability of microalgal biofuel, the listed properties, including low land demands [154] and low nutrient requirements and the ability to fix CO_2 [133], have elevated the importance of *C. zofingiensis* as a viable feedstock for biofuel. The ability of this microalga to accumulate lipids opens a new scientific field and raises the possibility of using it as a cell factory for algal oil [57].

Many studies intended to increase the accumulation rate of neutral lipids, in particular, TAG in microalgal cells by changing culture conditions, such as nitrogen [155] or phosphorus deprivation [156]; however, the low biomass productivity remains the major limitation [157]. It has been reported in several studies that TAG produced by *C. zofingiensis* can be converted to biodiesel [57]. Nevertheless, the TAG content significantly depends on the culture's conditions [58]. *Chlorella* cultures can adapt to several cultivating conditions under different environmental stress factors to maintain their fast growth rate, up to 0.769 d^{-1}, while producing TAG [63]. Measurements have shown that the lipid content of *C. zofingiensis* can reach up to 52% of its dry biomass if cultured under indoor heterotrophic conditions [57]. Apart from the lipid content, the quality of biodiesel depends on the fatty acids profile. The lipids available in *C. zofingiensis* cells consist of fatty acids predominantly with C(16–18) with the maximum unsaturation degree of 3 [33], almost similar to traditional plant oils used in biodiesel.

As one of the sub-classes of biofuel, biodiesel is a mixture of fatty acid alkyl esters obtained from a transesterification process resulting from the reaction of triacylglycerols with either methanol or ethanol. The Fatty acid methyl esters (FAME) composition of *C. zofingiensis* derived biodiesel includes mostly C18:2 (octadecadienoic acid methyl ester) with the content ranging from 22.94% to 36.05%, C16:0 (palmitic acid methyl ester), C16:1 (palmitoleic acid methyl ester), C18:0 (stearic acid methyl ester), C18:2 and C18:3 (octadecatrienoic acid methyl ester), which accounted for 71.79–84.97% of total FAME [158]. It is important to note that the fatty acids with 16–18 carbon strains are considered adequate to be converted into qualified biodiesel, which properties of density, viscosity, flash point, and heating value comply with the specifications in the EN 14214 standard [60].

As mentioned before, culture's conditions can influence the quality of lipid. Compared to cultures from photoautotrophic cells, lipids from *C. zofingiensis* cultures grown in dark heterotrophic conditions are reported to present high content of oleic acid (C18:1), linoleic acid (C18:2), and palmitic oil (C16:0). These compounds can balance oxidative stability and low-temperature properties, which results in biodiesel with high quality [57].

Both starch and cellulose obtained from *C. zofingiensis* can be converted to fermentable sugars to obtain bioethanol; however, starch is more suitable for the saccharification procedure since amylase can be obtained easily in comparison to cellulose [132]. Besides, since the content of lignin and hemicellulose are very low in algal cells, hydrolyzation is easier to be performed. As discussed previously, starch accumulated in larger quantities (than lipid) in *C. zofingiensis* under the same cultivation conditions. Therefore, *C. zofingiensis* has been evaluated as suitable for bioethanol production, especially under nitrogen starvation conditions [159].

4.5. Wastewater treatment

Unicellular microalga, in particular *C. zofingiensis*, has been studied to be employed for wastewater treatment [134], aiming to accomplish nutrient removal while increasing lipid productivity for biodiesel.

Studies have demonstrated that the inorganic contents found in municipal wastewater decrease algal biomass production [160]. To this extent, several studies are suggesting the use of mixtures of artificial nutrient media with wastewater to ensure sufficient concentrations of carbon sources [125,126].

The addition of biogas slurry as a source of nutrients for microalgae growth is another possibility suggested by Zhou et al. [133]. They proposed to combine the mixed wastewater to microalgae *C. zofingiensis* cultures in PBRs. They focused on the performance of *Chlorella* to uptake nutrients required to increase the biomass and lipid productivity and to remove the excess of nutrients. Finally, they evaluated their proposal as a low-cost and environmentally-friendly method for large-scale algal cultivation [133].

Treatment of industrial wastewater is also under attention in many research studies. Most of them focus on the assessment of wastewater for providing microalgal nutrients, have demonstrated several disadvantages to the current systems for wastewater cultivation. However, in the presence of toxic heavy metals, *C. zofingiensis* cultures cannot perform nutrient uptake as expected [133]. Although heavy metals make a toxic contribution to these microalgae cells, there have been some solutions to isolate the algal cells away from toxic compounds. At the same time, they can still uptake nutrients from the effluent. Using extracellular polymers around algal cells [163] or immobilization of microalgae by polymers are some techniques reported to increase the treatment efficiency by enhancing the cell viability [62].

The study by Zhao et al. [87] analyses three microalgae species: *Chlorella vulgaris*, *C. zofingiensis*, and *Scenedesmus* sp. for synthetic wastewater treatment. They reported that all cultures showed positive results in their growth and nutrition uptake process. After 132 h of cultivation, *C. zofingiensis* absorbed nitrogen at a 99.1% ratio, with phosphorus assimilation at a 100% ratio. They also reported that the lipid productivity of *C. zofingiensis* exceeded one of the other two species by 4.56% and 31.00% for *Chlorella vulgaris* and *Scenedesmus* sp., respectively.

5. Conclusion

Microalgae are being looked more and more as new sustainable biofactories with several components with potential application in many industrial sectors. In a reserve direction, this section intends to wrap up the individual sections discussed in the presented study, but with a bottom-up view, it is intended to take a brief look at what has been discussed. First, looking back to Section 4, where the applications were discussed, *C. zofingiensis* was highlighted as a robust microalga for several sectors. It means by providing a suitable platform, it can be used in multiple industrial sectors simultaneously while various products of interest can be targeted. For instance, by setting up a correct design of bioreactors, wastewater can be (naturally and partially) treated, and the microalgal cells can accumulate lipids. For such design specifically, it is important to study the contents of the wastewater not only to target a viable product from *C. zofingiensis* but also to avoid exposing the cells to (possible) toxic compounds. In another design, a main product and co-product (such as astaxanthin as the main product and β-carotene as the co-product) can be obtained to satisfy the requirement for both pharmaceutical and feedstock sectors. The same chain can be utilized with suitable stress factors to obtain lipids together with lutein contents. The ability to target multiple products via engineering of the cultivation makes *C. zofingiensis* interesting for further researches. Nevertheless, the correct design of such a chain of bioreactors demands to fully understand the details of each target product and the required conditions to increase them in the cell.

Simply said, stress factors play an important role in this regard, which was covered in Section 3. Besides, there was also a discussion in which multiple stress factors were employed in the studied microalgae culture. While some stress factors may have positive synergic effects (intensive light and N-deficiency to obtain astaxanthin, for instance), other combinations may negatively neutralize each other (such as salinity together with nitrogen excess for growth rate). Additionally, to choose a correct cultivation system, a discussion has been presented in the first part of Section 3. Back to Section 2, where the cells compositions were discussed, critic readers may argue about details that might be common between several species. It should be reminded that reviewing the applications of *C. zofingiensis* without touching the studied microalgae compositions is absurd. Besides, special attention was given to present and discussing the findings related to *C. zofingiensis*. However, some references compared the efficiency of the studied microalga with other competitive species to prove the efficiency of their proposed approach.

Last but not least, through reviewing several experiments and observations, it can be concluded that *C. zofingiensis* has shown a promising potential to be considered in most of the listed commercial applications. Nevertheless, some aspects demand more research to reach the economic viability of the algal products at an industrial scale. In this regard, the applications of *C. zofingiensis* in cosmetic products can be highlighted since high-value co-products can be accumulated in reasonable quantities in the cultures. Another field that needs more attention is the production and extraction of EPS used for medical and biomedical purposes. Although our experiments have concluded low yield extraction of EPS, employment of stress conditions can motivate the production of the compound.

CRediT authorship contribution statement

Details of each author with their contribution in this paper:

Name of the author	Types of contribution
Malihe Gorgich	Conceptualization, Investigation, Writing - original draft
António A. Martins	Writing - review & editing
Teresa M. Mata	Conceptualization, Writing - review & editing
Nídia S. Caetano	Project administration, Supervision, Validation, Writing - review & editing

Declaration of competing interest

We, the authors of this manuscript, indicate no conflict of interests regarding to the preparation, submission, and publication of this paper. The paper is original and unpublished and is not being considered for publication elsewhere.

Acknowledgment

No funding source for acknowledgment.

Statement of informed consent

No conflicts, informed consent, or human or animal rights apply to this study.

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