Journal of Medicinal Plants and By-products

Original Article

A Critical Review on Nutrients Requirements, Morphology, Environmental Factors and Applications of Chlorella Vulgaris and Chlorella Zofingiensis

Mojtaba Maleki Rad[1], Arman Nik Ardestani[1] and Maryam Abbasi[1*]

[1]*Faculty of Civil, Water and Environmental Engineering, Shahid Beheshti University, Tehran, Iran*

[*]Corresponding author: Email Address: mary_abbasi@sbu.ac.ir

Article History: Received: 17 April 2023/Accepted in revised form: 13 October 2023

ABSTRACT

Understanding the importance of microalgae in the health of humanity accelerate the progress in microalgae production through investigation and industrial production as an alternative for chemical and unhealthy productions. In this regard, green microalgae, Chlorella vulgaris and Chlorella zofingiensis have extensive applications representing their importance. Both chlorellae can be cultured in indoor environments and outdoors by remarkable adaption to the new altered condition. However, the growth rate is fast in three culture modes, including phototrophic, heterotrophic and mixotrophic. These robust biotechnological traits attract them more for researchers and engineers. There are some models and plans to increase the efficiency of products that, economically and technologically, must be feasible and plausible. In this study, we discussed investigations on scaling up the plants and their challenges. To cultivate and raise the growth rate of microalgae, knowledge of the nutrient requirements, the procedure of growth, and restrictions are necessary. Therefore, this study focused on comprehensive review about two widely-used and industrial applicable microalgae including Chlorella vulgaris and Chlorella zofingiensis. The differences and similarities in morphology, growth factors were assessed to help decision-makers and researches in this field.

Keyword: Chlorella vulgaris, Chlorella zofingiensis, Morphology, Cultivation factors, Nutrients, Applications

INTRODUCTION

Microalgae are unicellular or multicellular plant-like creatures that grow with little water, nutrients, or carbon dioxide and may absorb solar energy to utilize photosynthesis process as a means of energy acquisition [1]. Over the previous 3.5 billion years, these ancient eukaryotes have steadily developed from bacterial cyanobacteria and continue to demonstrate their flexibility by acclimating to varied harsh conditions [2]. As a result of the steady development and structural similarities between microalgae and plants, microalgae are assumed to be plant progenitors [3].

A variety of high-value compounds are manufactured by microalgae, such as carotenoids, phycobilins, polysaccharides, vitamins, and sterols, and unsaturated fatty acids (such as omega 3) have made these microorganisms attractive for different industries. Microalgae have a greater rate of polyunsaturated fatty acid synthesis than fish. Furthermore, due mainly to fish nutrition, fish oil might be severely polluted with mercury. As a result, microalgae are regarded as an excellent alternative for aquaculture industries particular fish farming. Saturated and monounsaturated fatty acids are also found in microalgae. Unlike long-chain polyunsaturated fatty acids, these lipids are hydrocarbons having 14-20 carbon and are primarily used in the manufacturing of biodiesel. As a result, microalgae are regarded as a valuable source of green energy with little pollution and high efficiency [3, 4]. Therefore, it is commonly acknowledged that, in order to further boost the development of the microalgae industry, researchers from both academia and industry should concentrate

Chlorophycea are an important class of microalgae known for their high rate of biomass production. Chlorophycea includes multiple genera, including Chlorella, Ankistrodesmus, Scenedesmus, Chlamydomonas, Botryoccocus, and Dunaliella [1]. Chlorella has been identified as the most potential source of biofuel precursors, as well as high-value pharmaceutical and nutritional pigments and cartenoids [7]. In this regard, some famous Chlorella species have been utilized more than others in many sectors, making them recognized as a

global demand due to their remarkable properties such as good nutritional value, pharmaceutical applications, and simplicity of cultivation. *C. vulgaris* and *C. zofingiensis* are eukaryote freshwater unicellular green microalga and they demonstrate their adaptability by acclimating to a variety of challenging situations. The majority of the C.Vulgaris cell is composed of protein (51-58%) with the remainder dedicated to carbohydrate, lipid, and other valuable biocomponds such as vitamins and antioxidants.[5, 6]. The majority of C.zofingiensis cells are made up of lipids and polysaccharides, with the rest containing valuable bioactive components, similar to C.vulgaris. [7, 8]. Although both *C. vulgaris* and *C. zofingiensis* belong to the same class and same genus of microalgae, their morphology, necessary environmental conditions, and products can be different. In this regard, all the environmental parameters, nutrient requirements and the cell and its properties are critical for efficient cultivation and maximum growth rate. Although the morphology of C. vulgaris has been extensively examined, the morphology of C. zofingiensis has not been widely studied. Moreover, there is no comprehensive review on the morphology of both chlorella microalgae including cell shape, reproduction process, and similarities and differences in important organelles such as cell wall, nucleus, mitochondrion, and chloroplast, which is essential for genetic engineering and extraction methods. It is also necessary to assess and compare present and prospective commercial and industrial applications of these chlorella microalgae as well as environmental requirements, nutrient materials and their optimum concentrations.

The aim of present study was to suggest and categorize similarities and differences of these two microalgae in different areas. Morphological of both microalgae particularly important organelles such as cell wall, nucleus, mitochondrion and chloroplast were discussed. Environmental requirements reported included light, temperature, PH value and salinity. Moreover, suitable range for efficient cultivation for each environmental requirements for both microalgae were addressed. The effect of different nutrients such as carbon, nitrogen and phosphorus separately, combination of nitrogen, phosphorous and sulfur and cultivation mode were fully investigated. In addition, combination of environmental requirements and nutrients were also studied. Finally, products and industrial applications of each microalgae according to its specific properties and bio compounds were discussed.

Morphology

Several *Chlorella* strains have been investigated in the literature; however, *Chlorella* classification has been considered problematic due to insufficient morphological information. Therefore, the ultrastructure of *Chlorella* strains has been broadly investigated in recent years [9, 10].

Chlorella species are divided into four major types, Based on cell shape. These four types are known as:

1) Spherical cells (which ratio of two axes are equal to one)
2) Ellipsoidal shape (ratio of longer axe to shorter axe is between 1.45 and 1.60)
3) Some species are both spherical and ellipsoidal, and
4) Some of the other species are globular to sub-spherical. Also, these species are no-motile and without flagella [10-12].

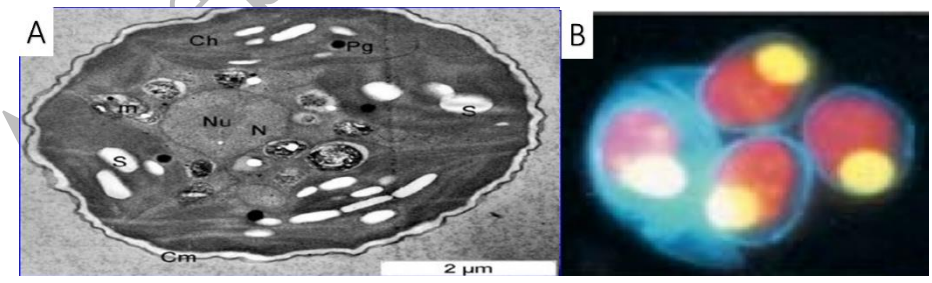

Fig. 1 A) C. zofingiensis ultrastructure [2]; B) Daughter cells leave mother cell after maturation while cell wall of mother cell rupture [11].

Almost all of the *Chlorella* species have no sexual life and reproduce asexually. They use an asexual atmosphere reproduction method and contain three steps: growth, ripening and division [10, 12-14]. In which daughter cells called autosphore grows in autosphorangium inside mother cells. Their capacity is 3,4,8,16,32 per autosphorangium. When these daughter cells (autosphores) mature enough, they will be released via the rupture of mother cells [9-11, 13]. Fig. 1C shows this reproduction method.

C. vulgaris is 2-10µm in cell diameter and spherical to ellipsoidal in cell shape [2, 10, 15]. *C. zofingiensis* is varied 2-15µm in diameters and spherical in cell shape [14]. Fig. 1B shows the ultrastructure of *C. vulgaris* and Fig. 1A *C. zofingiensis.*

Cell wall

The cell wall is responsible for retaining cell integrity and responsible for nutrients and other material transparent and out of the cell. It is also considered as a defensive line against harsh and unsuitable environmental conditions and invaders [2, 16]. The average thickness of *Chlorella* species cell wall thickness is 17-20nm, but the cell wall's size, structure, components, and properties can vary from one species to another [2, 16-19]. These differences in the cell wall can be seen even in strains of one species, making morphological investigation of microalgae cell walls tough [20].

C. zofingiensis cell wall has a rough and irregular surface covered by narrow and subtle ribs. Fig. 2A also shows the tail-like structure and inseparable membrane between two *C. zofingiensis* cells known as mucilage. *C. vulgaris* also has an electron-dense thin cell wall [2]. Fig. 2B and Fig. 2C indicate Cell wall of *C. vulgaris* consists of 2 distinct components. The inner component is microfibrillar, and the outer component is trilaminar. It is also reported that the outer component of *C. vulgaris* cell wall is resistant to enzyme attacks [19].

Takeda [17] experiments, introduced an index based on cell wall composition [17]. Polysaccharides are critical compositions of the cell wall. Polysaccharides are polymers of monosaccharaides that bond together by glycosidic bonds made of polysaccharides [10, 18, 21]. Therefore, (Takeda 1991) experiments suggested that *chlorella* sugar-based cell walls are classified into two major types; glucose-mannose type and glucosamine type. This research suggested that *C. vulgaris* rigid cell wall composition is just glucosamine, While *C. zofingiensis* rigid cell wall is made of 20-30% Monos, and the rest is Glucose. This research also utilizes ruthenium red in order to visualize whether acid polysaccharides exist or not. Fig. 3 suggests this classification as *C. vulgaris* is (2.1.2) and *C. zofingiensis* is (1.2.2)

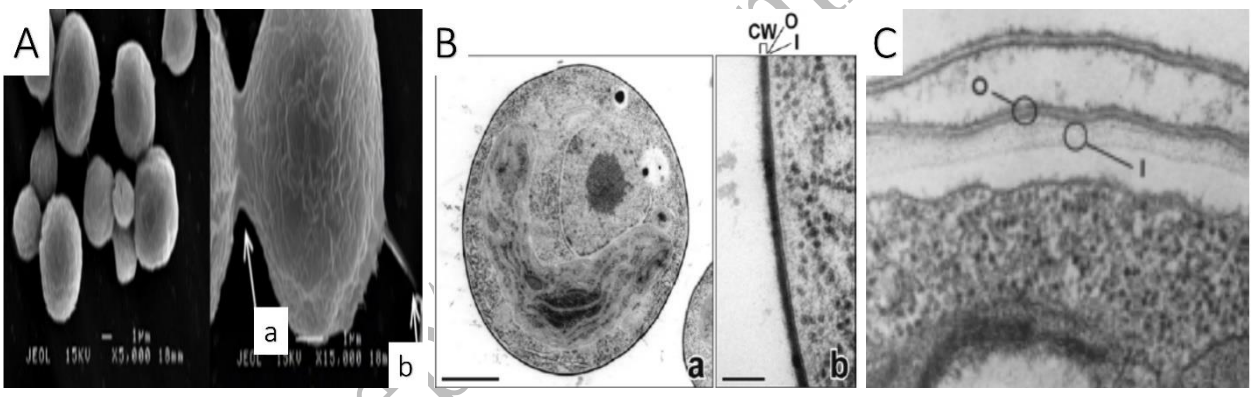

Fig. 2 A) scanning electron micrographs of C. zofingiensis. Arrows in C. zofingiensis indicate the tail-like structure and mucilage[11]; B) Transmission electron micrograph of a) Chlorella vulgaris b) C. vulgaris cell wall with higher magnification. The inner surface of the plasma membrane, O outer surface of the plasma membrane (scale bar is 1µm in and C) component (I) is microfibrillar, and the outer emnpound (O) is trilaminar [19].100 nm in b [16];

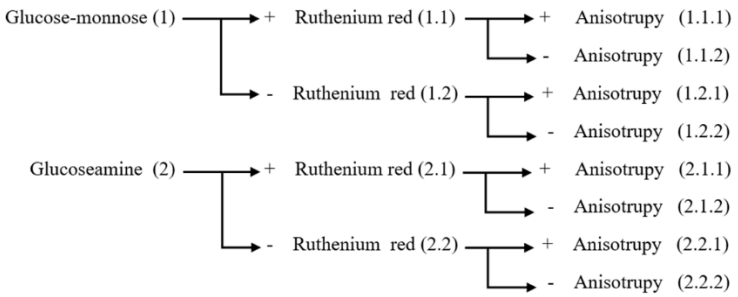

Fig. 3 Classification of the cell wall of *Chlorella* specie microalgae [17].

Cytoplasm

The cytoplasm is a group of particles in different sizes and shapes suspended in a gel-like liquid medium covering the whole space between the nucleus and cell wall [22, 23]. The cytoplasm is a workshop of cells, and so many substances are synthesized and broken down. These reactions start with protein synthesis, and protein synthesis begins with mRNA (messenger RNA) cooperating with ribosome [23]. The cytoplasm contains different particles known as an organelle, and each organelle is responsible for different tasks in the cell that impotent organelles in microalgae include mitochondrion, chloroplast, nucleus, Golgi apparatus, endoplasmic reticulum, and lysosome [24].

Nucleus

The most extensive organelle in both microalgae is the nucleus. As presented by Fig. 1A and 1 B nucleus of both *C. zofingiensis* and *C. vulgaris* is located in the center of the cell. The nucleus acts as a library and keeps genetic information of the cell through DNA, and almost all of the genetic information of an individual is kept and secured in the DNA [24]. Besides genetic materials, other actions such as DNA replication, transcription, and RNA processing occur in the nucleus [24]. gene expression is one of these duties, expressing through unlocking the particular genetic information and producing messenger molecules that later act on different organelles in the cytoplasm [23].

One of these messenger molecules synthesized in the nucleus is ribosomal RNA (RRNA). RRNAs are produced in the nucleus and then assembled into the ribosome. The nucleolus is the part that is responsible for that. According to different taxonomic and phylogenic investigations based on RRNA, *Chlorella* species are categorized into two major groups: *Trebouxiophyceae* and *Chlorophyceae*; also, both classes are sisters. *C.* vulgaris belongs to *Trebouxiophyceae* [15, 25], and *C. zofingiensis* belongs to class *Chlorophyceae* [25].

Mitochondrion

The mitochondrion is often called the "energy factory" or powerhouse of the cell due to its duty to produce Adenosine Triphosphate (ATP), an energy-rich molecule and considered a universal energy source biologic reactions are produced in the respiration process. During this process, nutrients such as Glucose break down, and ATP is synthesized. In addition, other by-products such as water and carbon dioxide are also produced. The mitochondrion also plays other vital roles such as programmed cell death and steroid production in the cell [23, 24].

Mitochondrion consists of two membranes; outer membrane and inner membrane. The outer membrane surrounds the whole organelle and contains several holes, which allow small molecules such as water molecules in and out. This membrane is made of an equal ratio of both proteins and phospholipids. On the other hand, the inner membrane is poorly permeable and surrounds internal space, known as Matrix, and contains most mitochondrial proteins such as mitochondrial DNA (miDNA) and RNA, including ribosomes. The inner membrane also is made of three times more protein than phospholipids [2, 23].

Although the containment and roles of the mitochondrion are similar to each other in microalgae, the shapes of the mitochondrion might be different species from each other. *C. zofingiensis* mitochondrion is oval-shaped, and *C. vulgaris* mitochondrion is reported to be the elongated shape or branching in some specific samples [14, 26].

Chloroplast

Chloroplast is one of the most critical organelles in *Chlorella* microalgae, and both *C. vulgaris* and *C. zofingiensis* contain cup-shaped chloroplast [11, 15, 27]. In addition, photosynthesis reactions take place in this organelle [24]. Chloroplast is a double membrane composed of phospholipids and called an envelope. The outer membrane is penetrable to ions and metabolites, and the inner has different roles: protein transport and starch granules. These granular are basically made of amylose and amylopectin, which can be formed inside the chloroplast [2].

One part of the chloroplast is the pyrenoid located in the chloroplast and surrounded by the starch [9, 11, 27]. A high level of the enzyme ribulose-1,5-biphosphate carboxylase oxygenase (commonly known as Rubisco) responsible for carbon dioxide fixation can be found in the pyrenoid [2]. However, this part of the chloroplast is not observed in all species of the *Chlorella* microalgae. *C. vulgaris* has ellipsoidal to spherical pyrenoid, whereas *C. zofingiensis* does not have any pyrenoid [11, 27].

Environmental Factors

Light

Light is one of the essential factors of microalgae photoautotrophic growth, and chlorophyll chemical synthesis depends on light intensity [3, 14]. When a chlorophyll molecule from the antenna complex is excited, the energy is rapidly sent out from one molecule to another through a resonance energy transfer process until it finally reaches a suitable pair of chlorophyll molecules from the photochemical reaction center. Thus, each antenna complex acts like a natural pipe that collects light energy and directs it to a particular site to be efficiently utilized [28].

Several studies have reported an optimum range of light intensity for microalgae efficient growth [14, 29]. 100-150 $\mu E.m^{-2}. s^{-1}$ ($\mu E\ m^{-2}\ s^{-1} \equiv \mu mol\ m^{-2}\ s^{-1)}$ was suggested for optimum constant light intensity for *chlorella* species. Below the suitable range, light scarcity leads to photo limitation, resulting in a slow growth rate [14]. Indeed, low light intensity leads to low CO_2 fixation because light products such as ATP and Nicotinamide adenine dinucleotide phosphate (NADPH), which use in the Calvin–Benson cycle, can promote carbon fixation by Rubisco [30]. 250 mg L^{-1} day^{-1}in *C. vulgaris* biofixation rate is reached at a 12-h light/dark regimen [31]. Also, Orosa et. al found that only a trace amount of astaxanthin (below 0.08 mg g^{-1} dry weight) was synthesized Under low light intensity on *C. zofingiensis* [32].

In photo limitation mode, increasing light intensity will result in higher biomass productivity [3]. Above this intensity range (High light intensity), considered light stress, no longer depends on light energy [3]. Light stress occurs in the saturation range between the above optimum range and below the saturation value.

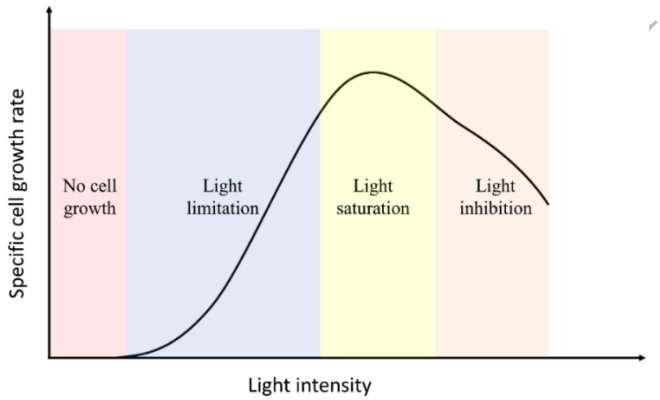

Fig. 4 Effect of light intensity on microalgae growth [33].

Exceeding saturation value may cause photo-inhibition, and in this case, increasing light intensity will result in a growth rate reduction. Light stress will increase lipid and fatty acid accumulation and intracellular reactive oxygen species (ROS) due to excess available energy in microalgae cells. ROS includes oxygen, superoxide anions, hydroxide radicals, single oxygen, and hydrogen peroxide, which generally are produced in the cell due to photosynthetic respiration activities, cellular metabolism of microalgae, and other living cells. ROS are highly reactive due to being free radical and tend to react with everything to become stable [3, 34]. Under normal conditions, ROS levels are maintained low and stable in the cell. A low amount of ROS is critical and acts as key signalling molecules in biosynthesis and bioreaction of cell metabolism; however, the high amount is highly lethal and can react with every cell compound and specific proteins [34].

In response to ROS production due to light stress, mRNA level of secondary carotenoids and genes increases in microalgae, leading to biosynthesis of specific keto-carotenoids out of β-carotene. This is a regular defensive mechanism to defend the cell against ROS high production. *C. zofingiensis* most produced carotenoids are zaexanthin and astaxanthin, which has long-chain carbon (over 40 carbon atoms) for controlling ROS level in microalgae [3, 14]. Whereas, *C. vulgaris* contained only a small amount of violaxanthin compared to the total content of carotenoids in light stress [35].

Excess photons lead to impinging on chloroplast that the accumulation of astaxanthin in cytoplasmic lipid bodies functions as a sunscreen and leads to reduced photodamage. Some studies have considered light intensity as the critical factor to achieve the maximum astaxanthin content. In this regard, Liu, Sun, Gerken, Liu, Jiang and Chen

[14] reported that astaxanthin achieved in heterotrophic cultures is below 2 $mg \cdot g^{-1}$ [14]. In contrast, in autotrophic cultures with high light intensity, the astaxanthin reached up to 6 $mg \cdot g^{-1}$(DW).

Saturation value is highly dependent on reactor cultivation conditions and other parameters. it is noteworthy to mention that, due to the shading effect (increasing cell density of external layer cells lead to prevent light from reaching internal cells and results in decreases in growth rate in the growth phase, constant light intensity is not a suitable option for efficient growth and biomass production. However, a stepwise increase of light intensity to saturation value can successfully require extra carbon source demand [3, 36].

Introducing another parameter that includes cell density is necessary in order to improve microalgae cultivation efficiency. This parameter is introduced as specific light intensity per cell ($\mu E.g.ds.s^{-1}$). According to this parameter, cell density enhancement will lead to effective light intensity reduction [37]. Therefore, increasing light intensity will be required in every stage of cultivation to reach maximum efficient growth [29].

Sunlight is mainly utilized in outdoor cultures and specifically open ponds. These open ponds mostly use sunlight as their light source. Utilizing sunlight as a light source has several disadvantages. One of the most significant drawbacks of sunlight is that in some warm areas, the sun's light intensity reaches the photo-inhibition range; specifically, in some tropical areas, it reaches 10^5 lux, which is not suitable for growth rate [28].

Due to the disadvantages of open systems and natural light sources, artificial lighting sources such as light-emitting diodes (LED) and optical fibers are used for indoor systems that are more controllable than sunlight. Visible light (400-750 nm) can supply the required energy for photosynthetic reactions [28, 38].

Higher lipid content and faster growth rates can be reached by stepping up light irradiance with red and white LED lamps [34]. Zhao *et. al* also showed that red light includes the optimal light wavelength for microalgae growth, biogas upgrading, and digested nutrient decrement [39].

del Pilar Sánchez-Saavedra, Sauceda-Carvajal, Castro-Ochoa and Molina-Cárdenas [40] investigated the effect of other artificial carbon sources, Blue, green, yellow, and white lights on *C. vulgaris* [41]:

- Blue light: increasing the biomass productivity, saturated fatty acids, and lipid production
- Yellow light: Chlorophyll a and carotenoid levels reached high values, and big cell size resulted
- Green light: Chlorophyll a and carotenoid levels reached higher values and higher organic dry weight (ODW) values
- White light: Protein content was significantly higher

Temperature

Microalgae can carry out cellular division, photosynthesis and produce more biomass production in the range of temperature between 15 to 30 °C, which stepwise enhancement has positive effects on enzymatic activities. However, to find the optimal growth, many studies in the various range have been conducted.

Serra-Maia, Bernard, Gonçalves, Bensalem and Lopes [41] investigated the optimal growth rate of *C. vulgaris* under Hinshelwood and Bernard-Rémond models and found that the viable growth rate and the mortality rate increase linearly and parabolically (18 °C to 30 °C) respectively, which 23°C is the optimal point of the net growth rate curve [42]. The range of 24°C to 28°C has been considered the optimal growth temperature of *C. zofingiensis* [3]. Further, Carotenoid accumulation is practically related to the temperature in which the highest lutein and astaxanthin production are observed at 28°C and 24°C in the cells of *C. zofingiensis*, respectively [3, 14].

A wide fluctuation in temperature and irradiance affects the growth of *chlorella* that generally occurs in the outdoor cultivation systems, which are influenced by the solar flux. Due to the hardness in controlling the water temperature (Culture temperature), low growth rates were reported for outdoor cultivation compared to indoor cultivation, whereas the photobioreactor (PBR) system provides entirely controllable conditions, which result in higher biomass and lipid production [42-45].

In Feng, Deng, Fan and Hu [43], first samples cultivated indoor culture to reach exponential phase then transferred into flat plate photobioreactors outdoor (the maximum irradiance and air temperatures were 1800 mol m^{-2} s^{-1} and 12°C _30°C, respectively) [44]. In the end, the highest specific growth rate and lipid production rate reached 0.362 $days^{-1}$ and 26.6 mg L^{-1} day^{-1}, respectively. These results agree with the capability of cultivation of

C. zofingiensis under outdoor conditions as same as other studies report the capability of both unicellular *chlorellae* to adapt and cultivate in outdoor systems [42, 44].
In the photoautotrophic process, the highest activity of Rubisco was achieved at 15 and 20°C in the Calvin cycle. In the heterotrophic process, the citrate synthase enzyme activity for assimilating the organic carbon in the tricarboxylic acid (TCA) cycle was obtained at a higher temperature [41, 46]. Nevertheless, generally, the temperature exceeding the optimal (i.e., 35°C) leads to enzymes' inactivation and denaturation. Consequently, the functionalities of the enzymes can effectively cause killing a fraction of the cells.
The atmosphere temperature can exceed 40°C in some countries, and this phenomenon leads to restraining the CO_2 fixation rate, more photosynthetic activity, and dissolve more oxygen concentration by oxygenase of Rubisco, but if temperature decline back to 25°C the remaining viable cells are susceptible to grow again [41, 47]. To prevent this phenomenon, shading and atomization spray can effectively reduce the photobioreactor temperature at 4–7 °C [28, 48].
Temperature stress has been shown to enormously increase ROS production, leading to an increase in antioxidants as Patel, Choi and Sim [3] reported that the antioxidant activity of *C. vulgaris* was doubled at 30°C [3]. Nonetheless, causing progressive oxidative destruction and cell death is due to increased ROS production in the cell [41].
Higher temperature (from 20 to 38 °C) inhibits the starch biosynthesis and inversely stimulate sucrose formation, which accelerates the conversion of starch to sucrose during both photosynthetic CO_2 fixation and dark respiration as Nakamura and Miyachi [49] showed at the presence of *C. vulgaris*, C-starch was degraded in spinach chloroplasts in the dark by the catalysis of amylases and polyglucan phosphorylase [50].
A harsh condition such as high temperature has a progressive effect on lipid accumulation as an energy reserve, leading to increased mortality and a decrease in the population of viable cells. Hence, the highest lipid content was obtained at 25 °C rather than 30 °C [46]. Undergoing heat stress results in the prevention of excitation energy transfer from Chl_a to Chl_b, which Subsequently leads to the accumulation of energy storage and less growth rate [41]. Besides that, high temperature leads to protein degradation inside cells and cell mortality; however, low-temperature synthesis of protein. Also, according to a study by Zhang, Gao, Guo, Wang, She, Gao, Zhao, Jin and Wang [46], Changes in carbohydrate content are not related to temperature variation [50].
Despite mentioned studies, some investigations report different results. Saad, Selahi, Zoromba, Mekki, El-Bana, Dosoky, Nobles and Shafik [50] claimed that 37 °C is the optimum temperature for growing *C. vulgaris* under different nitrogen conditions [51]. Also James, Al-Hinty and Salman [51] claimed that increasing temperature from 20 to 38 °C in cells grown arise the activity of Rubisco in *C. vulgaris* [52].
Low temperatures are often introduced as photo-inhibition due to the reduction of metabolic enzyme activities. The photosynthesis system (PS) can process fewer photons than at higher temperatures; also, low temperatures restrict cell growth by decreasing the fluidity of the cell membranes [42, 50]. the growth of *C. zofingiensis* in South China and Canada was limited to a certain extent because of the low temperature in winter [37].
In this regard, Gong and Bassi [42] investigated the lutein content in *C. vulgaris* at low temperature (0 °C to 14°C) and found that at lower temperatures as the irradiance get stronger, or as the light, hours get longer (from 14 hours to 20 hours) the lutein content decrease, because the cell become heavier and wall thickness increase. Whereas the higher temperature reduces the level of inhibition, enlarges the PS pool, which causes increasing the total pigment content and specific lutein content [43].

Adsorption and uptake of high concentrations of heavy metals are significant microalgae abilities, making them applicable for industrial effluents treatment. Mehta and Mehta, Singh and Gaur [52] confirmed that the rate of Cu^{2+}(extremely toxic) and Ni uptake by *C. vulgaris* increased from 6 to 25°C [53]. In contrast, the highest adsorption was achieved at 38°C and was not significantly affected by slight fluctuations in temperature.

pH

pH is one of the parameters determining the microalgae's performance to intracellular product formation and microalgae growth [53]. The consumption of nitrate and the degradation of the organic acids and photosynthesis influence the dynamics of pH on culture [54].

Some types of microalgae, such as spirulina, grow in high alkalinity; whereas, *C. vulgaris* tolerate low pH levels (from 3.0) better than high levels of pH (i.e., 11) [3, 41]. Ni^{+} adsorption at pH 3.5 and 5.5 by *C. vulgaris* agree to this point [55]. Table 1 shows the effect of pH on *Chlorella* growth.

The optimum pH range for the growth of *C. zofingiensis* is 5.5-8.5, which low pH is more appropriate for lipids and astaxanthin accumulation as far as maximum astaxanthin could be reached at pH 5.5 [37]. Moreover, Low pH is considered a stressed condition that leads to producing ROS. Likewise, antioxidants such as superoxide dismutase (SOD), catalase (CAT) are produced to prevent the hazardous effect of accumulated oxidizing agents [5].

Table 1 Different aspects of *Chlorella* cultivation under pH values.

Type	Strategy	Observations / Comments	Results /Solutions	Ref
vulgaris	Ammonium in the form of NH_4Cl as nutrient	Decline in pH	Adding sodium bicarbonate	Panahi, Khosroushahi, Sahebkar and Heidari [5]
vulgaris	Using only BG-11 broth for cultivation	Diminution in pH	Adding copolymer Polyacrylate polyalcohol (PP) increased the pH	[62]
vulgaris	Ni uptake	The carrier molecules were denatured at acidic pH and pH >7 precipitation as hydroxides.	Increased Ni uptake with an increase in pH from 3.5 to 6.8.	[55]
vulgaris	Hydrogen production	pH controls the degradation of carbon sources. Variable pH changes the metabolic pathways, which decrease the hydrogen yield.	The optimal pH is 8	[4, 39, 63]
vulgaris	Cu^{2+} maximal adsorption and maximal uptake rate (during 30 min)	The uptake reduction at pH>7 due to its precipitation as insoluble hydroxide salts	Adsorption at pH 4.5 and uptake at pH 6.0 is maximal	[52, 64]
vulgaris	Heterotrophically biomass growth and enhancement of lipid content under sulphur limitation	lipid content did not significantly change the variable pH. The best performance of biomass growth was received in pH 6.5 and 7.0. The maximum utilization of carbon per DW and the minimum consumption of sulphur obtained at the same time in the range of pH 7.0-7.5	Optimal pH 7.5	[53]
zofingienesis	pH control with: a. 5–6% CO2 aeration in the day b. Adding HAC with the speed of 1–2 mL per hour	a. Decreasing the average culture pH to 6.5–6.7 b. The pH Instantly increased from 6.6 to 9.5	The removal rates of TN and PO^{3-}_{4} respectively: a. 97.5% and 51.7% b. 79.6% and 42.0%	[61]
zofingienesis	pH control	Adjusted to pH four during 24 days.	5.9 (mg astaxanthin g^{-1} of dry weight)	[32]

The pH of microalgal culture increases gradually during the cultivation period as inorganic carbon uptake by microalgae. High pH (i.e., above 10) has been considered an obstacle of scale-up of tubular PBRs also due to

its toxic properties can disrupt the microalgae growth [56, 57]. On the other hand, high pH has some following significant advantages:

- Removing of ammonia via its volatilization and phosphorus by its precipitation (as $Ca_3(PO_4)_2$) which cause nutrient remediation [45, 53, 58]
- Sterilization of microalgae biomass in wastewater treatment like restricting the coliforms [53, 59]
- Inducing the flocculation by increasing pH between 9.5 to 11(Magnesium hydroxide induced flocculation at pH 9.7)
- VFAs like acetate can be consumed as an organic carbon source at high pH due to *Chlorella*'s ability to grow under heterotopic and autotrophic mediums [60].

Fluctuation is one of the most effective occurrences in microalgae growth. This phenomenon takes place when pH value rapidly changes in a wide range. Therefore, microalgae cannot adapt to the new condition, which causes a higher lag phase and cell mortality rate. In this regard, controlling pH results in higher biomass productivity as far as the highest biomass productivity could be obtained in complete pH control [53]. In contrast, irregular control of pH enhances the lipid content in the cell [60]. In this regard, two pH regulation CO_2 and HAC (as mentioned in table 1) were utilized in *C. zofingiensis* cultivation by Huo, Wang, Zhu, Zhou, Dong and Yuan [61] which pH was kept more stable during cultivation via CO_2 regulation compared to HAC regulation also resulted in higher biomass production, protein, and sugar content

Salinity

Salinity is a critical environmental factor for microalgae cultivation, influencing microalgae' physical and chemical reactions and structure [65]. Microalgae species are different in their salinity tolerance [66]. *Chlorella* species are among the most active microalgae due to their ability to survive in a wide salinity range [67].

The range of salinity tolerance for *C. vulgaris* is <0.5 M NaCl, whereas for *C. zofingiensis* is lower than 0.1M NaCl. In this range, the microalgae growth rate is not significantly influenced by salinity. In other words, salinity concentration in this range is not considered salt stress [14, 66]. More than these ranges, salt concentration may lead to a chain of the phenomenon that results in cellular fragmentation, slowing down cell multiplication, lysis, and may finally cell destruction [65, 67].

The first phenomenon is the limitation of microalgae's capability to synthesize chlorophylls and also binding proteins. Degradation of protein and microalgae limitation for binding new ones which can cause protein deficiency decrease intracellular enzyme activities and photosynthesis reactions export and import of ions by ATP (ATP is known as a carrier of energy, makes direct and indirect Na^+ export from the cell) more convenient. Furthermore, other means may lead to different changes in membrane permeability [65, 67, 68].

The second phenomenon is converting these microalgae colours from green to yellow, which occurs due to a significant decrease in chlorophyll (a) content. Therefore, the destruction of chlorophyll of microalgae in salt stress can be considered the main reason for the colour conversion of microalgae [65, 67]. a low concentration of deicing salt (the mineral form of NaCl) shows no significant effect on *C. vulgaris* growth rate up to 2 g L^{-1}, but a higher concentration of that salt, specifically higher than four g L^{-1}, increases inhibitory growth rate to 60-80%. These results are similar to organic solvents such as ethanol and methanolBarahoei, Hatamipour and Afsharzadeh [67]. Alternatively, Del Campo, Rodriguez, Moreno, Vargas, Rivas and Guerrero [69] indicated that 0.2 M and 0.4 M NaCl could affect both growth rate and maximal standing-cell density by about 30% and 50%, respectively [70].

Even if microalgae adapt to their environment, longer lag phases cause lower growth rates and dry weight reduction. The lag phase is the first stage of the microalgae growth rate. In this stage, microalgae adapt to their environment, and no multiplication and reproduction occur this stage by microalgae. This stage comprises three steps: 1. turgor restoration; 2. cell membrane adjustment to allow the absorption and also ion exchange and; 3. induction of the accumulated stress proteins and glycerol as osmo-protective compatible solutes, which is produced photosyntheticallyBarahoei, Hatamipour and Afsharzadeh [67].

Salt stress increases the level of secondary carotenoids such as astaxanthin and canthaxanthin and other anti-oxidative compounds such as polyphenols in *C. zofingiensis* and β-carotene in *C. Vulgaris* [69, 70]. Increasing ROS and free radicals' levels due to salt stress may activate a defensive mechanism of microalgae, producing and

accumulating different carotenoids such as β-carotene in *C. Vulgaris* for quenching free radicals and reducing dangerous ROS levels [66, 68, 71]. The reason is that salt stress can increase the transcript of the BKT gene (carotenoid ketolase) for increasing the production of carotenoids as a defensive mechanism to protect the cell [38, 70].

Another defensive mechanism of microalgae that may activate in the saline environment is lipid accumulation and increasing rate of lipid bio-synthesis because of oxidative stress in the cell due to high salinity [14, 36]. It can be concluded that salt stress makes microalgae a perfect choice for biodiesel production at the expense of growth rate.

Salinity tolerance of *C. vulgaris* is also dependent on the type of salts. Based on the study by Imaizumi, Nagao, Yusoff, Taguchi and Toda [37], modifying BG-11 culture by removing Na ion in BG-11 and substituting other ions such as magnesium and calcium salt tolerance of *C. vulgaris* and salinity removal capability are increased [28]. Also, according to the investigation conducted by Bar, Rise, Vishkautsan and Arad [72], ammonia removal improved using KCl instead of NaCl when using *C. vulgaris* for saline wastewater [73].

Nutrient

The nutrients in medium content and manufacturing conditions can determine the quality and health of the final products. In this regard, macronutrients such as C, N, P, and S have more effect rather than trace elements and minerals, so we concentrate more on macronutrients [73]. Photo-autotrophic, heterotrophic and mixotrophic are cultivations modes, and each of them has advantages and disadvantages, which knowing them can help us choose a suitable strategy to produce biomass and valuable chemicals through biorefinery processes. In the end, finding cost-effective and suitable nutrient resources for both *chlorella* based on their potential are studied.

Quality and Quantity of Nutrients

Carbon

Microalgae is capable of using organic carbon sources such as sugar and acetate, so some cheap and accessible carbon sources such as glucose, fructose, sucrose, malt extract and lactose were investigated by several studies on both *Chlorella*. Among these carbon sources, glucose has been chosen as the best carbon and energy source for cell growth [74, 75]. The initial glucose concentration of 5 g L^{-1} is the optimal concentration for biomass productivity, and if the stationary phase reaches 40 g L^{-1}, an inhibiting effect occurs that leads to intracellular lipid formation in *C. vulgaris* cultivation. [76-78]. By contrast, high initial glucose concentration positively enhances the astaxanthin content as it provides more carbon precursors entering the astaxanthin biosynthetic pathway. Liu, Sun, Gerken, Liu, Jiang and Chen [14] reached the highest astaxanthin content at 30 g L^{-1} glucose concentration [14]. Also, Sun, Wang, Li, Huang and Chen [74] achieved the highest biomass and astaxanthin concentration in heterotrophic growth of *C. zofingiensis* by applying fed-batch fermentation at 50 g L^{-1} glucose concentration [75].

The present form of carbon in phototrophic cultivation typically is CO_2, and many studies have been conducted to find the suitable concentration of CO_2, especially for these green microalgae, CO_2 is a mandatory element. Three ranges, 1-5%, 5-15%, or upper, have been an interesting subject for researchers. Mostly 1-5% has been considered as the appropriate concentration to reach the maximal microalgal growth [79]. Imaizumi investigated the range from 0.03% to 60% CO2, and 2.5% CO_2 was the optimum growth rate for *C. vulgaris*. Whereas, Hulatt and Thomas [80] reported that the growth rate in 12% CO_2 was significantly higher than in 4% CO_2 that could be interpreted through the CO_2:O_2 ratio, which remarkably influences the growth rate [81]. It is noteworthy that gradual enhancement even up to high levels such as 60% can reach high biomass productivity because microalgae have enough time to adapt to new conditions [81].

The deficiency of CO_2 such as 0.03% CO_2 (V/V) exists in the air is not sufficient either for photosynthetic or even mixotrophic culture [81, 82]. High CO_2 levels (10–25% or even more) are more suitable for lipid production than biomass production. Also, high CO_2 levels can acidify the cultivation medium because in the ionic equilibrium between carbon dioxide and HCO_3^-, the carbon, uptake through the enzyme carbonic anhydrase of CO_2 to form HCO_3^- which results in reducing the PH in culture due to reducing the hydroxyl ion (OH^-) [30].

Consequently, if the PH drops lower than the optimum range, an impressively decline occurs in microalgae's growth rate.

Carbon dioxide capture efficiency depends on the cultivation system mode, algal physiology, O_2 balance, environmental factors, and C/N/P ratio, representing the nutrient medium [30, 79]. The C/N/P ratio is critical in microalgae production in terms of high-value products, biomass concentration, and lipids, which are explained more in table 2 for both chlorellae [82]. The present challenges in proving sufficient CO_2 and new methods such as bacteria are provided in supplement (page S1).

Cultivation Modes

Water and nutrients are mandatory either for Photoautotrophic or heterotrophic cultivation to produce biomass rich in lipids, protein and high-value production; however, for Photoautotrophic growth, light and inorganic carbon such as CO_2 is compulsory, too. Some microalgae species such as chlorella can grow in a mixotrophic mode, which heterotrophic metabolism can aid in resolving the carbon source shortage in the culture medium.

Heterotrophic cultivation is more attractive than autotrophic cultivation for the following reasons: no cost of illumination, higher metabolite concentrations, high nutrient recovery efficiency, and bioremediation of textile wastewater (Table 2) [76, 83]. Also, mixotrophic cultivation has several advantages compared to both cultivation, such as; more extended exponential growth phase, switching from heterotrophic to autotrophic mode and protection from photo-oxidative damage by accumulating oxygen in enclosed photobioreactors [84]. In this regard, Liu first heterotrophically grow the C. zofingiensis cells to accumulate biomass, then the grown cells are set as the subject in photoautotrophic growth for astaxanthin biosynthesis [10]. Moreover, Liu, Sun, Gerken, Liu, Jiang and Chen [14] proposed that *C. zofingiensis* cultivated in fermenters then transferred to PBRs with high light conditions for astaxanthin induction, resulting in an enhancement in astaxanthin production by three-fold. More advantages of mixotrophic cultivation are provided in the supplement (Page S1).

Nitrogen (N)

Nitrogen is a substantial and notable element in the production of protein and nucleic acid. The formation of lipid and photosynthetic ability of algae depends on N abundance [43, 85]. Further, Proteins, lipids, and other components are stored in the microalgae biomass instead of polysaccharides when sufficient N is in culture [85]. In contrast, whether in high or low concentration, nitrogen stress affects the synthesis of amino acids, carbon fixation, cellular energy, photosynthesis rates, and transcript levels of genes involved in metabolism [86].

High N concentrations have some disadvantages; a) prohibit the activities of the pertinent enzymes in the cultivation process, b) dropping off the actual growth and metabolism rate [87], c) declining the electron transport rate (ETR) between PSI and PSII, d) making cell sizes bigger, e) showing lower photosynthetic efficiency [45], f) declining the lipid accumulation especially amount of C18:1 [76, 87]. In addition, the reduction in Actual quantum yield due to high nutrients leads to the formation of algae assimilating forces such as NADPH, ATP and carbon fixation.

On the other hand, due to N starvation, a reduction in the photochemical efficiency of PSII and reaction centers (RCs) also occur a reduction in Maximum quantum yield, which cause the carbon source to utilize to lipid accumulation of polysaccharides and certain oils in both *chlorella* instead of using for the synthesis of protein and carbohydrate [3, 88]. In this regard, Huang, Lou, Luo and Wang [85] investigated the range one up to 8 mgN L^{-1} under stable cultivation in all P-constant concentration groups and showed that the synthesis of carbohydrates in *C. vulgaris* is nitrogen-dependent [86]. With increasing N, a significant reduction has been observed in carbohydrate content.

As mentioned above, the protein production rate decreases; However, after a deprivation period, *C. vulgaris* can consume more nitrate and produce more proteins up to 44.3% than normal conditions [89]. In addition, the results of Feng, Deng, Fan and Hu [43] demonstrated that the lipid content and carotenoids of *C. zofingiensis* were higher than some other *Chlorella* species, such as *C. vulgaris* under N limitation [44].

In response to nutrient starvation conditions, *C. zofingiensis* accumulates triglycerides (TAG) and astaxanthin (Fig. 5F and 5G). Enhancing the de novo synthesized TAG accumulation via the expression level of ACCase or/and carbohydrates reduce the polar lipid content such as monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG). These polar lipids have been known as the main lipid components of the chloroplast

membrane [8, 88, 90]. Determining the carbon flux into TAG and the last step of TAG synthesis is catalyzed by Diacylglycerol acyltransferase (DGAT) [91]. Acetyl-CoA carboxylase (ACCase) and DAGTs play essential roles in TAG synthesis, as well as Phytoene synthase (PSY) and β-carotene ketolase (BKT) that play important roles in astaxanthin synthesis [8, 88]. PSY is the first gene in carotenoid biosynthesis, and BKT is the last gene in astaxanthin biosynthesis. Consequently, the up-regulation of PSY and BKT directly correlates with astaxanthin accumulation which is substantially present in the three forms, free, mono- and di-ester under stress conditions [14, 91, 92].

Many studies have been reported different TFA content concerning their conditions. Studies confirmed that N limitation augments lipid productivity and develops the lipid profile for biodiesel of both *C. vulgaris* and *C. zofingiensis* [49, 76, 90]. Under N limitation, oleic acid (C18:1) is dominant in the (Fatty Acid Methyl Ester) FAME profile that can be produced due to conversion of C18:0 to C18:1 via the stearoyl-acyl carrier protein desaturase gene [42]. Sakarika has obtained similar results under batch heterotrophic cultivation of *C. vulgaris*, Zhu, Takala, Hiltunen and Wang [84] by recycling harvest water and Liu, Mao, Zhou and Guarnieri [90] by applying the semi-continuous culture of *C. zofingiensis*.

Meanwhile, C18:2 and C18:1 were prevailing fatty acids in TAG under N and S starvation conditions [88]. The highest C18:2 at around 44% of the total FAMEs achieved by Lam and Lee [93] and reduction of C16:0 has verified about by all researches [94]. The lipids, mainly comprising TAG, can be refined into biodiesel via transesterification (alcoholysis) reactions, as shown in Fig. 5H [43, 61].

Phosphorus (P)

Microalgae can assimilate phosphate as polyphosphate granules in insoluble form and use phosphorus as inorganic orthophosphate (PO_4^{3-} such as $H_2PO_4^-$ or HPO_4^{2-}) by an active energy process for the synthesis of essential biomolecules such as nucleic acids, pigments, ATP and phospholipids [53, 76, 85, 86]. Besides, the production of metabolic enzymes, ribosomes, and rRNA depends on protein synthesis [3, 86]. Hence, the metabolic synthesis of DNA, RNA, intermediate metabolic products, and lipid production can be influenced [85]. Huang, Lou, Luo and Wang [85] confirms that under low P levels (P<0.4 $mg{\cdot}L^{-1}$), lipid accumulation was increased with stepping up P addition in N-constant groups of *C. vulgaris* cultivation. However, Li, Sun, Sun, Tang, Turaib, Wang, Cheng, Deng and Zhang [87] reported that significant enhancement (28.52mg L^{-1}) has a reverse influence and leads to reduction; ETR, the photosynthetic capacity, biomass and lipid accumulation [88]. Whereas, Mirizadeh, Nosrati and Shojaosadati [86] investigated the range of 40–330 mgP $\cdot L^{-1}$ and claimed no remarkable variation in specific growth rate, final cell density and astaxanthin content on C. zofingiensis cultivation [87].

Lower phosphorus concentrations lead to:

- Decrease in the Maximum quantum yield [87]
- support the cell duplication through declining the phospholipid or nucleic acid synthesis rate [76]
- A rapid reduction in the ETR causes a reduction in the efficiency of the Calvin cycle and the regeneration of NADPH. Therefore, the slow formation of NADPH leads to the reduction of chlorophyll because ADP and NADP+ are the electron acceptor molecules in the photosynthesis consumed by proteins, nucleic acids, and carbohydrates [86, 87, 90]
- increasing the total saturated fatty acids (SAFA) [57, 76].

Combination of N, P, S

The effect of nutrients is vital for understanding their role in the cultivation process, and the combination is significant to find suitable concertation in terms of quality and quantity of final biomass content. Some examples in table 2 investigate the effect of sulfur, phosphorus, and nitrogen combinations on nutrient removal efficiency and cellular component [76, 85]. In most cases, N and P add nitrate (NO_3^-) and phosphate (PO_4^{3-}) to the medium. Hence, BG-11 is a common medium due to containing $NaNO_3$ and K_2HPO_4 as supplying resources of nitrogen and phosphorus. In this regard, Feng, Deng, Fan and Hu [43] investigated the concentrations of $NaNO_3$ and $K_2HPO_4.3H_2O$ (ranged 0 g L^{-1}_6.0 g L^{-1}) on *C. zofingiensis* cultivation. In this study, the maximum biomass production rate (270 mg L^{-1} day^{-1}) and the highest μ_{max} (2.15 day^{-1}) were achieved when the concentrations of

$NaNO_3$ and $K_2HPO_4.3H_2O$ were 1.0 g L^{-1} and 0.01 g L^{-1}, respectively. Moreover, Apter et. al suggested that using sodium bicarbonate to the basal media helps to fixation of dissolved CO_2 and, consequently, produces more biomass production [3].

Feng, Deng, Fan and Hu [43] reported that the lipid productivity (44.7 mg L^{-1} day^{-1}) under P deficiency in *C. zofingiensis* cultivation was obviously lower than a medium under N deficiency mode (68.1 mg L^{-1} day^{-1}) because, under P deficiency mode, much lower biomass concentration and biomass productivity can be obtained [44]. Furthermore, the highest lipid production rate (87.1 mg $L^{-1}day^{-1}$) was achieved under nitrogen deficiency in *C. zofingiensis* cultivation which can be applied for astaxanthin production reported by Mirizadeh study. More investigations and reports about the combination of macronutrients are provided in the supplement (page S1) [86].

Sulphur adds to the medium in the form of MgSO4·7H2O, significantly increasing the final biomass concentration. Sakarika and Kornaros [76] reached the stationary phase on the 8th day with the maximum biomass generation of 2.69 gDW L^{-1} during S limitation in *C. vulgaris* cultivation [77]. Furthermore, Mao, Wu, Sun, Zhang and Chen [88] indicates that the highest TAG production rate (52.4 mg $L^{-1}d^{-1}$) was achieved under S starvation [89]. However, the highest astaxanthin productivity (0.62 mg $L^{-1}d^{-1}$) was obtained under N starvation at 16°C in C. zofingiensis photoautotrophic cultivation because N starvation triggers the up-regulation PSY and BKTs to a greater extent in comparison to S starvation.

Wastewater as a Nutrient Source

Many authors consider wastewater a rich source of nutrients and a sustainable, low-cost alternative for microalgae cultivation [44, 83]. Microalgae biomass with wastewater as a nutrient source is usually used as biofertilizers or transformed into biofuels because of its unsterile conditions. Mainly the exist of contamination in culture medium related to use the open pond systems instead of PBR or wastewater as a culture which accompanied with: a) Heavy metals, such as; cadmium, mercury, zinc, copper, aluminium, chromium, and nickel, b) Viruses, protozoa and Bacteria (such as coliforms) constitute the main pathogenic microorganisms, c) Presence of common hazardous chemicals such as PAHs, BDEs, TBT and emerging chemicals such as triclosan and diclofenac [44, 89, 94-96].

Generally, to prevent contaminating of culture, some strategies are remarked such as; Sterilization of the culture by autoclaving, Using the synthetic medium instead of observed medium because of wastewater priorities of daily plant operations (Fluctuation) and using membrane photobioreactor (i.e., 0.45 µm air filter). However, these are not cost-effective to scale up and end to laboratory application [60, 81]. In this regard, Yen, Hsu and Chen [81] suggested using piggery wastewater as a nutrient source and NaClO for sterilization, which is cost-efficient (Table 2) [82]. Using NaClO is more cost-effective than other methods; more information is provided in the supplement (page S1-S2).

The sterilization cost of contamination is not the only issue in this case, but organic shock loads and high nutrients are mattered, which leads to protein synthesis inhibition and reducing Carbohydrates through converting to lipids [85, 97]. In this case, the dilution can be effective to prevent overloading the nutrient in media. Liu et. al investigated a semi-continuous culture with three nitrogen concentrations of 5, 10, and 20 mg L^{-1} and three dilution rates of 0.25, 0.5 and 0.75 day^{-1} [90]. Consequently, the nitrogen concentration of 10 mg L^{-1} with the dilution rate of 0.5 day^{-1} reached the highest productivities of TAG (297 mg L^{-1} day^{-1}) and astaxanthin (3.3 mg L^{-1} day^{-1}) in *C. zofingiensis*. The main issue is that dilution of piggery wastewater requires plenty of clean water, but Recycling harvest water can be a better solution (As mentioned in table 2). Indeed, Recycling harvest water can save 30% of the total water input and be suitable for nutrient recovery in appropriate concentrations [83]. There are many investigations to recovery essential nutrients through low-cost organic to produce high-value products and increase biomass productivity; this section is provided in supplement (page S2-S3).

Combination of Environmental Factors and Nutrients

The combination of nutrients and environmental factors has been an interesting object for researchers due to its influential role in microalgae growth, especially under multi-stress conditions. In this regard, Orosa, Valero, Herrero and Abalde [32] investigated astaxanthin and lipid accumulation under N deprivation and high light (350 µmol photons m^{-2} s^{-1}), which resulted in increasing C18:1 up to 51.6% of TFAs through conversion of C18:0 to

C18:1 via the stearoyl-acyl gene also the ratio of astaxanthin reached up to 80% of total secondary carotenoids in *C. zofingiensis* cultivation [34].

Mirizadeh, Nosrati and Shojaosadati [86] investigated the synergistic effect of nitrogen and NaCl concentration on the lipid productivity by response surface methodology (RSM) for two days [87]. Both nutrient limitation and high salinity led to the lower chlorophyll and protein contents in *C. vulgaris*, and coupling them enhanced the production of ROS, which caused damage to lipid biosynthesis enzymes. Also, the salinity levels (up to 10 g L^{-1}) under N starvation do not benefit lipid accumulation.

Table 2 The effect of some investigated strategies on both *chlorella* in terms of optimization, bioremediation and production of valuable products.

Type of *chlorella*	Strategy	Comments / Condition	Results / Observations	Ref
vulgaris	Using in soilless tomato to save mineral nutrients	Duration: 152 days This study proved that using microalgae for this purpose is more suitable than soil. Less cost and harmful effects are the benefits of microalgae compared to mineral fertilizers	Increasing; vitamin C content, minerals content (P, Na and Mg), quality through sugar and carotenoid content, chlorophyll and photosynthesis rate, dry matter and fruit volume	[98]
vulgaris	The various ratio of C, N, P for optimal growth	a. stable cultivation b. wastewater treatment with CO2 supply	a. N:P →7.58:1 b. C: N:P → 106:16:1	[53, 85]
vulgaris	Comparing the nutrients removal from municipal wastewater in photobioreactor supplied with CO2-enriched air (5% CO2 v/v) to the normal air (0.03% CO2 v/v)	Removal has reached the maximum in the air (5% CO2 v/v) at 25ºC temperature 14 /10 hour of light and dark regime For 7 days	Removal percentages: (sCOD):84.6% NH_4^- N:88% NO_3^- N:72% PO_4^- P:92.8%	[53]
vulgaris	An algal membrane photobioreactor to remove the secondary effluent	at three days, HRT	The removal percentages of NO^{3-} and PO_4^{3-} were 53% and 89%, respectively.	[99]
vulgaris	Use of nitrogen and some trace elements such as Mn and Ca	the trace elements mostly participate in enzyme reactions.	Lipid production reached threefold	[3, 100]
vulgaris	membrane flat plate photobioreactor	removals after 24 hours under 300 µmol m^{-2} s^{-1} light irradiance 24/0 hour of light and dark regime	nitrate: 57% and phosphate: 43%	[89]
vulgaris	Synergistic effects and optimization of nitrogen and phosphorus concentrations	N and P concentrations ranged 0-56 and 0-19 mg L^{-1}, respectively.	Completely removing of nitrogen and phosphorus, Final biomass concentration: 1.58 mg L^{-1}	[101]
vulgaris	The treatment of urban wastewater in high rate algal/bacterial tubular photobioreactors	containing 81mg O_2/L of COD, 24.7mg N/L, and 2.1mg P/L under a light intensity of 2000µE/m_2 s and 5% CO_2 flue gas	The biomass productivity elevated from 10g TSS/m^2 day to 20g TSS/m^2 day also N and P removal enhanced from 63% to 95%, and 81% to 95%, respectively.	[102]

vulgaris	The effect of nutrient concentration with salt stress on lipid productivity , biomass concentration and removal efficiencies	2.6 mg L^{-1} of nitrogen, 6.3 g L^{-1} of NaCl synthetic medium HRT: 2 days pH was adjusted to 6.8	Removal percentages COD:80.5%, TN:70% and TP:78% lipid productivity: 80 mg L^{-1} day^{-1} biomass concentration: 4.9 g L^{-1}	[86]
vulgaris	The effect of N concentration with P constant level (N mode), P concentration with N constant (P mode) and combination of these modes on algal photosystem	The nitrogen mode has the most evident effect on lipid production, and the combination of N and P modes mode has minor effects on the increased lipid production	The highest lipid content was obtained at 0.25N, and the highest lipid production achieved at 0.5N in N mode (N=247.06 mg L^{-1})	[83]
vulgaris	Evaluating the lipid production under S, P and N limitation in heterotrophic cultivation	S limitation has the most significant effect on lipid accumulation among these three factors; however, N and P combined more. (40mM NO^{3-} and 5mM PO_4^{3-})	Lipid content under the combination of P, N: 57.25% g gDW^{-1} Under S limitation: 53.43±3.93% g gDW^{-1}	[95]
vulgaris	Comparing of cheaper and readily available carbon sources (glucose, fructose, sucrose and malt extract) and optimal concentration	Sucrose produced the highest hydrogen yield in comparison to other carbon sources. Besides that, malt extract is more cost-effective due to its low cost, which can be considered as a cheap source.	5 g L^{-1} concentration of each carbon source cause maxim hydrogen yield production and was the optimal concentration for amounts of lipid, carbohydrate, and proteins	[77, 78]
vulgaris	Antioxidant activity and production of some photosynthesis pigments under the various amount of selenium.	The effect of following concentration selenium on peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD). a. selenium (<75 mg L^{-1}) b. selenium (>100 mg L^{-1})	a. Positive effect b. Negative effect	[103]
zofingiensis	The effect of iron on astaxanthin accumulation and cell growth	Fe^{2+} acting as a generator of hydroxyl radical, stimulating carotenogenesis, and helping to BKT enzyme (carotenoid ketolase) performance	Further cell growth and biosynthesis of astaxanthin	[75]
zofingiensis	higher C/N ratio	The extra carbon enters into the carotenoid biosynthetic pathway, or N deficiency leads to astaxanthin accumulation.	a C/N ratio of 180 increases in astaxanthin content as compared to a C/N ratio of 30.	[44]
zofingiensis	The percentage of removal in Different sterilization pathway	Light intensity:230 ± 20 µmol m^{-2} s^{-1}Duration:10 days Indoor cultivation	The percentage of removal for COD, TN and TP were, respectively: a.79.84%,82.70%,98.17%	[45]

		Piggery wastewater as a nutrient resource a. autoclaving the wastewater b. pretreating via NaClO	b.78.29%,84.49%,95.26%	
zofingiensis	Comparison between 1. R (recirculated water systems) and 2. NR in fed-batch cultivation	200 µmol photon m^{-2} s^{-1} 5% CO^2 The whole cultivation period was 288 hours was divided into four stages, and the half of cultured liquid was taken out from the PBRs in each stage	The percentage of removal for COD, TKN and TP were, respectively: 1. 85.05%, 93.64%,98.45% 2. 77.61%,90.08%,93.16%	[104]
zofingiensis	Using harvest water of piggery wastewater in a different mode of N and P deficiency.	Using: a. 50% harvest water with sufficient nutrient b.100% harvest water with the starvation of both nutrients c. 100% Harvest water with N deficiency and P sufficient Duration:^ days	a.The highest biomass productivity. b. The highest lipid content and FAME yields c. The highest Biodiesel productivity 20.66 mg L^{-1} day^{-1}	[83]
zofingiensis	Assess the effects of Using the 3-methyladenine (3-MA) as an autophagy inhibitor on cell growth, astaxanthin and fatty acid production under nitrogen starvation	The remarkable enhancement in cellular ROS and PSY expression. Increasing the content of polyunsaturated and decreasing the content of saturated, monounsaturated fatty acids. Cultured in photobioreactor at 25 °C with 1.5% CO2.	1. 1. Not significant change in net growth 2. More TFA with enhancing the content of polyunsaturated fatty acids 3. Enhancing the accumulation of astaxanthin	[8]
zofingiensis	Using cerulenin as *an* inhibitor of de novo fatty acid biosynthesis to promote astaxanthin accumulation.	Cerulenin blocked the de novo fatty acid biosynthesis, and this reduction elevates the NADPH. Likewise, the molecular oxygen serves as the electron acceptor to offset insufficient NADP+ supply resulting in the generation of (ROS).	1. Astaxanthin content was reaching up to 40%. 2. Drastic decrease in TAG	[90, 105]

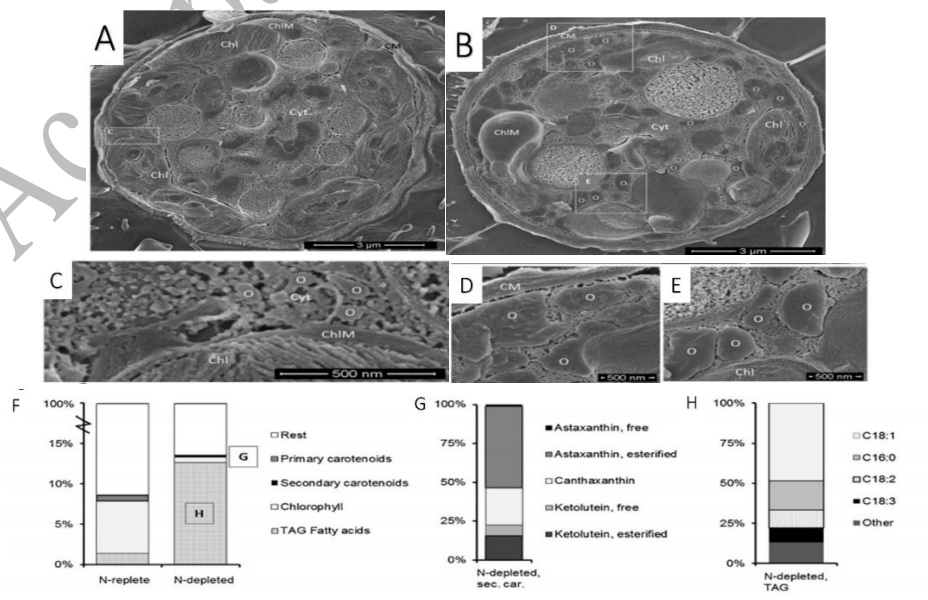

Fig. 5 SEM micrograph represents an altered content of *C. zofingiensis* under depleted nitrogen [97].

Products and Industrial Applications

Chlorella microalgae are one the most applicable microorganisms. Their applications can vary from human dietary to wastewater, heavy metal bioremediation, and biofuel production. They are considered single-cell producers of different proteins, fatty acids, and carotenoids. Indeed, *C. vulgaris* have been proposed as a protein source and *C. zofingiensis* as a carotenoid potential to solve the probabilistic famine due to the growing global population and disease [28, 106].

C. vulgaris used in cosmetic industries and is known as a food nutrient source due to its extractable proteins [9]. The production of carotenoids like β-carotene makes the *C. vulgaris* acts as a pro-vitamin A. Moreover, *C. vulgaris* utilize in food preservation due to the presence of synthetic antioxidants such as Butylated hydroxyanisole (BHA) and Butylated hydroxytoluene (BHT) [107].

C. zofingiensis represents a unique model system for primary and secondary carotenoids, which among secondary carotenoids, astaxanthin, a red ketocarotenoid, plays a significant role to promote its value. Further, *C. zofingiensis* has potent anti-oxidative activity and pigmentation function, showing the highest microalgae value. It applies in food supplements, food additives, nutraceuticals in cancer prevention and pharmaceutical industries [8, 69, 88, 108].

The price of astaxanthin in the market is around $12,000.00 per kilogram which the purity of astaxanthin has been considered a significant factor for industrialization. Indeed, its ratio to the total carotenoids is the main subject of determining the price in the market. The natural astaxanthin found in algae is essentially in esterified form with hydroxyl groups and fatty acids such as palmitic, stearic, and oleic acids; however, synthetic astaxanthin is the free form [14].

The applications of both *chlorella* that elucidate in table 3 encourage companies to industrialize both *C. vulgaris* and C. zofingienesis However, factors such as the risk of contamination, high production cost, and complex production process of algae products are expected to hinder the growth of the global *chlorella* market [106]. Some famous and certified companies are widely working on producing these unicellular. The information about these companies is provided in the supplement (page S3).

Table 3 Some applications of *chlorella* for industrialization purposes.

Type of *Chlorella*	Type of application	Application /Comments	Ref
vulgaris	Food	Producing β-carotene acts as pro-vitamin A and may be used as a natural food colour	[28]
vulgaris	Nutraceutical supplements	highly digestible proteins food additives Algal meal of *C. vulgaris* as a fish feed ingredient	[109]
zofingienesis	Human health	Producing astaxanthin leads to 1. Heart heath: improving blood levels of LDL and HDL 2. Enhancing the immune system levels help to raise the production of antibodies 3. Suitable potential of antioxidant activity and scavenging free radicals so result in reducing DNA damage	[110, 111]
zofingienesis	feed supplement for aquaculture (marine industry)	Producing astaxanthin leads to grow of larval fish and shrimp also to pigment ornamental fish	[112]
zofingienesis	Producing biofuel (environmentally friendly fuel)	1. Biogas: In anaerobic digestion, microalga biomass can be the type of digestion 2. Biodiesel: an alternative non-petroleum, which produce by transesterification of triglycerides or by esterification of free fatty acids 3. Bioethanol and Hydrogen: microalga biomass can provide high levels of carbon compounds, and it is appropriate for fermentation	[28, 81]

CONCLUSION

Despite the fact that there are over 200,000 different species of microalgae, only a few of these species, have been recognized suitable for human consumption and commercial cultivation. C. vulgaris and C. zofingiensis are among these species due to their bioactive compounds. These microalgae must be investigated in three aspects: morphology, environmental needs, and required nutrients, in order to improve their commercial culture capacity. Microalgae growing rate is highly depended on environmental requirements and necessary nutrients. Genetic engineering for enhancing productivity, the bioactive components and products of each microalga is depended on morphological and structural properties. Since both Chlorella microalgae belong to the same genus, their cell shape, nucleus location, and chloroplast shape and location are identical. Despite these similarities, several cell elements, such as cell wall materials and cell wall structure, as well as the position and shape of the mitochondrion, are different. Although the morphology of C. vulgaris has been extensively examined, the morphology of C. zofingiensis has not been widely studied. Therefore, further investigations are required to fully understand C. zofingiensis morphology. The PH value must also be kept between 5-7. Lower or higher ph values will result in enzymatic problems in the cell and, ultimately, low growth rate.

Both C. vulgaris and C. zofingiensis are dependent on specified light intensity, temperature, and salinity ranges. Abiotic stresses occur in microalgae above certain levels, and these stresses increase the formation of specific hazardous molecules known as ROS. The production of these compounds may result in the growth suppression of microalgae. As a result, these microalgae trigger a defense mechanism that involves the creation of certain enzymatic and non-enzymatic antioxidants. Lower than these ranges, however, the rate of growth would be limited.

Aside from environmental factors and structural features of both chlorella, finding a cheap and rich source that contains essential nutrients with the lowest contamination is a novel challenging issue. Based on this review, we aimed to study recent advancements and challenge researchers to identify methods and alternative solutions; however, more research is required to identify a cost-effective and indigenous nutrient source. Moreover, due to varying environmental conditions and the combination of nutrients in different growing techniques, determining a defined range of optimum concentration for distinct nutrients is not quite feasible.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

REFERENCES

1. Silva J., Alves C., Pinteus S., Reboleira J., Pedrosa R., Bernardino S. Chlorella, in Nonvitamin and nonmineral nutritional supplements. 2019; Elsevier, 187-193.
2. Safi C., Zebib B., Merah O., Pontalier P-Y., Vaca-Garcia C. Morphology, composition, production, processing and applications of Chlorella vulgaris: A review. Renewable and Sustainable Energy Reviews. 2014; 35: 265-278.
3. Patel A.K., Choi Y.Y., Sim S.J. Emerging prospects of mixotrophic microalgae: Way forward to sustainable bioprocess for environmental remediation and cost-effective biofuels. Bioresource Tech. 2020; 300: 122741.
4. Kosourov S., Seibert M., Ghirardi M.L. Effects of extracellular pH on the metabolic pathways in sulfur-deprived, H2-producing Chlamydomonas reinhardtii cultures. Plant and Cell Physiology. 2003; 44(2): 146-155.
5. Panahi Y., Khosroushahi A.Y., Sahebkar A., Heidari H.R. Impact of Cultivation Condition and Media Content onChlorella vulgaris Composition. Advanced Pharmaceutical Bulletin. 2019; 9(2): 182.
6. Azizi S., Bayat B., Tayebati H., Hashemi A., Pajoum Shariati F. Nitrate and phosphate removal from treated wastewater by Chlorella vulgaris under various light regimes within membrane flat plate photobioreactor. Environmental Progress & Sustainable Energy. 2021; 40(2): e13519.
7. Liu J., Batch Cultivation for Astaxanthin Analysis Using the Green Microalga Chlorella zofingiensis Under Multitrophic Growth Conditions, in Microbial Carotenoids. 2018; Springer. 97-106.
8. Zhang Z., Sun D., Cheng K-W., Chen F. Inhibition of autophagy modulates astaxanthin and total fatty acid biosynthesis in Chlorella zofingiensis under nitrogen starvation. Bioresource Tech. 2018; 247: 610-615.
9. Liu J. Chen F. Biology and industrial applications of Chlorella: advances and prospects. Microalgae Biotech. 2014; 1-35.

10. Phukan M.M., Chutia R.S., Konwar B., Kataki R. Microalgae Chlorella as a potential bio-energy feedstock. Applied Energy. 2011; 88(10): 3307-3312.
11. Azaman S.N.A. Morphological, biochemical and transcriptomic characterisation of Chlorella sorokiniana and Chlorella zofingiensis during normal and stress conditions. Uni. Sheffield. 2016.
12. Iwamoto H. Industrial production of microalgal cell-mass and secondary products-major industrial species. Handbook of microalgal culture: Biotech. and Applied Phycology. 2004; 255: 263.
13. Yamamoto M., Fujishita M., Hirata A., Kawano S. Regeneration and maturation of daughter cell walls in the autospore-forming green alga Chlorella vulgaris (Chlorophyta, Trebouxiophyceae). J. Plant Res. 2004; 117(4): 257-264.
14. Liu J., Sun Z., Gerken H., Liu Z., Jiang Y., Chen F. Chlorella zofingiensis as an alternative microalgal producer of astaxanthin: biology and industrial potential. Marine drugs. 2014; 12(6): 3487-3515.
15. Maruyama I., Nakao T., Shigeno I., Ando Y., Hirayama K. Application of unicellular algae Chlorella vulgaris for the mass-culture of marine rotifer Brachionus, in Live Food in Aquaculture. Springer. 1997; 133-138.
16. Yamamoto M., Kurihara I., Kawano S. Late type of daughter cell wall synthesis in one of the Chlorellaceae, Parachlorella kessleri (Chlorophyta, Trebouxiophyceae). Planta. 2005; 221(6): 766-775.
17. Takeda H. Sugar composition of the cell wall and the taxonomy of Chlorella (Chlorophyceae) 1. J. Phycology. 1991; 27(2): 224-232.
18. Yuan Q., Li H., Wei Z., Lv K., Gao C., Liu Y., Zhao L. Isolation, structures and biological activities of polysaccharides from Chlorella: A review. Int J. Biological Macromolecules. 2020.
19. Atkinson j.A., Gunning B., John P. Sporopollenin in the cell wall of Chlorella and other algae: ultrastructure, chemistry, and incorporation of 14 C-acetate, studied in synchronous cultures. Planta. 1972; 107(1): 1-32.
20. Yamada T. Sakaguchi K. Comparative studies on Chlorella cell walls: induction of protoplast formation. Archives of Microbio. 1982; 132(1): 10-13.
21. Pignolet O., Jubeau S., Vaca-Garcia C., Michaud P. Highly valuable microalgae: biochemical and topological aspects. J. Industrial Microbio. Biotech. 2013; 40(8): 781-796.
22. Dashek W.V. Harrison M. Plant cell biology. Series TitlDashek, W.V. and M. Harrison. Plant cell biology of Message. Plant Cell Biology of List [Content Type] 2006 Post Cited]. Sci Publishers..e. 2006.
23. Carlson B.M. Chapter 1 - Cells, in The Human Body, B.M. Carlson, Editor. 2019; Academic Press, 1-25.
24. Queiroz M.I., Vieira J.G., Maroneze M.M. Morphophysiological, structural, and metabolic aspects of microalgae, in Handbook of Microalgae-Based Processes and Products. 2020; Elsevier, 25-48.
25. Huss V.A., Frank C., Hartmann E.C., Hirmer M., Kloboucek A., Seidel B.M., Wenzeler P., Kessler E. Biochemical taxonomy and molecular phylogeny of the genus Chlorella sensu lato (Chlorophyta). J. Phycology. 1999; 35(3): 587-598.
26. Popova A., Comparative characteristic of mitochondria ultrastructural organization in Chlorella cells under altered gravity conditions. Advances in Space Research. 2003; 31(10): 2253-2259.
27. Li T., Xu J., Gao B., Xiang W., Li A., Zhang C. Morphology, growth, biochemical composition and photosynthetic performance of Chlorella vulgaris (Trebouxiophyceae) under low and high nitrogen supplies. Algal Res. 2016; 16: 481-491.
28. Costa J.A.V., de Morais M.G. An open pond system for microalgal cultivation, in Biofuels from algae. Elsevier. 2014; 1-22.
29. Imaizumi Y., Nagao N., Yusoff F.M., Kurosawa N., Kawasaki N., Toda T. Lumostatic operation controlled by the optimum light intensity per dry weight for the effective production of Chlorella zofingiensis in the high cell density continuous culture. Algal Res. 2016; 20: 110-117.
30. Morales M., Sánchez L., Revah S. The impact of environmental factors on carbon dioxide fixation by microalgae. FEMS Microbiology Letters. 2018; 365(3): fnx262.
31. Sydney E.d., Da Silva T., Tokarski A., Novak A.d., De Carvalho J., Woiciecohwski A., Larroche C., Soccol C. Screening of microalgae with potential for biodiesel production and nutrient removal from treated domestic sewage. Applied Energy. 2011; 88(10): 3291-3294.
32. Orosa M., Valero J., Herrero C., Abalde J. Comparison of the accumulation of astaxanthin in Haematococcus pluvialis and other green microalgae under N-starvation and high light conditions. Biotech Letters. 2001; 23(13): 1079-1085.
33. Zhang X. Microalgae removal of CO2 from flue gas. IEA Clean Coal Centre, UK. 2015.
34. Metsoviti M.N., Papapolymerou G., Karapanagiotidis I.T., Katsoulas N. Effect of light intensity and quality on growth rate and composition of Chlorella vulgaris. Plants. 2020; 9(1): 31.
35. Koch M., Kniggendorf A.K., Meinhardt-Wollweber M., Roth B. In vivo determination of carotenoid resonance excitation profiles of Chlorella vulgaris, Haematococcus pluvialis, and Porphyridium purpureum. J. Raman Spectroscopy. 2018; 49(3): 404-411.

36. Oncel S.S., Imamoglu E., Gunerken E., Sukan F.V. Comparison of different cultivation modes and light intensities using mono-cultures and co-cultures of Haematococcus pluvialis and Chlorella zofingiensis. J. Chem. Tech. Biotech. 2011; 86(3): 414-420.
37. Imaizumi Y., Nagao N., Yusoff F.M., Taguchi S., Toda T. Estimation of optimum specific light intensity per cell on a high-cell-density continuous culture of Chlorella zofingiensis not limited by nutrients or CO2. Bioresource Tech. 2014; 162: 53-59.
38. Li Y., Huang J., Sandmann G., Chen F. high-light and sodium chloride stress differentially regulate the biosynthesis of astaxanthin in chlorella zofingiensis (chlorophyceae) 1. J. Phycology. 2009; 45(3): 635-641.
39. Zhao Y., Wang J., Zhang H., Yan C., Zhang Y. Effects of various LED light wavelengths and intensities on microalgae-based simultaneous biogas upgrading and digestate nutrient reduction process. Bioresource Tech. 2013; 136: 461-468.
40. del Pilar Sánchez-Saavedra M., Sauceda-Carvajal D., Castro-Ochoa F.Y., Molina-Cárdenas C.A. The use of light spectra to improve the growth and lipid content of Chlorella vulgaris for biofuels production. BioEnergy Research. 2020; 13(2): 487-498.
41. Serra-Maia R., Bernard O., Gonçalves A., Bensalem S., Lopes F. Influence of temperature on Chlorella vulgaris growth and mortality rates in a photobioreactor. Algal Res. 2016; 18: 352-359.
42. Gong M. Bassi A. Investigation of Chlorella vulgaris UTEX 265 cultivation under light and low temperature stressed conditions for lutein production in flasks and the coiled tree photo-bioreactor (CTPBR). Applied biochemistry and biotechnology. 2017; 183(2): 652-671.
43. Feng P., Deng Z., Fan L., Hu Z. Lipid accumulation and growth characteristics of Chlorella zofingiensis under different nitrate and phosphate concentrations. J. Bioscience and Bioengineering. 2012; 114(4): 405-410.
44. Bechet Q., Shilton A., Fringer O.B., Munoz R., Guieysse B. Mechanistic modeling of broth temperature in outdoor photobioreactors. Environmental Sci. Tech. 2010; 44(6): 2197-2203.
45. Zhu L., Wang Z., Takala J., Hiltunen E., Qin L., Xu Z., Qin X., Yuan Z. Scale-up potential of cultivating Chlorella zofingiensis in piggery wastewater for biodiesel production. Bioresource Tech. 2013; 137: 318-325.
46. Zhang Z., Gao P., Guo L., Wang Y., She Z., Gao M., Zhao Y., Jin C., Wang G. Elucidating temperature on mixotrophic cultivation of a Chlorella vulgaris strain: Different carbon source application and enzyme activity revelation. Bioresource Tech. 2020; 314: 123721.
47. Manhaeghe D., Michels S., Rousseau D.P., Van Hulle S.W. A semi-mechanistic model describing the influence of light and temperature on the respiration and photosynthetic growth of Chlorella vulgaris. Bioresource Tech. 2019; 274: 361-370.
48. Huo S., Wang Z., Zhu S., Shu Q., Zhu L., Qin L., Zhou W., Feng P., Zhu F., Yuan Z. Biomass accumulation of Chlorella zofingiensis G1 cultures grown outdoors in photobioreactors. Frontiers in Energy Res. 2018; 6: 49.
49. Nakamura Y. Miyachi S. Effect of temperature on starch degradation in Chlorella vulgaris 11h cells. Plant and Cell Physiology. 1982; 23(2): 333-341.
50. Saad M.G., Selahi A., Zoromba M.S., Mekki L., El-Bana M., Dosoky N.S., Nobles D., Shafik H.M. A droplet-based gradient microfluidic to monitor and evaluate the growth of Chlorella vulgaris under different levels of nitrogen and temperatures. Algal Res. 2019; 44: 101657.
51. James C., Al-Hinty S., Salman A. Growth and ω3 fatty acid and amino acid composition of microalgae under different temperature regimes. Aquaculture. 1989; 77(4): 337-351.
52. Mehta S., Singh A., Gaur J. Kinetics of adsorption and uptake of Cu2+ by Chlorella vulgaris: influence of pH, temperature, culture age, and cations. J. Environmental Sci and Health, Part A. 2002; 37(3): 399-414.
53. Chaudhary R., Tong Y.W., Dikshit A.K. Kinetic study of nutrients removal from municipal wastewater by Chlorella vulgaris in photobioreactor supplied with CO2-enriched air. Environmental Tech. 2018.
54. Huo S., Chen Y., Liu Y., Zhu Y., Peng G., Dong R. Experiment on microalgae cultivation in BG11 nutrient solution adding biogas slurry. Transactions of the Chinese Soci Agric Engin. 2012; 28(8): 241-246.
55. Mehta S., Tripathi B., Gaur J. Influence of pH, temperature, culture age and cations on adsorption and uptake of Ni by Chlorella vulgaris. European J. Protistology. 2000; 36(4): 443-450.
56. Skowroński T., Szubińska S., Pawlik B., Jakubowski M., Bilewicz R., Cukrowska E. The influence of pH on cadmium toxicity to the green alga Stichococcus bacillaris and on the cadmium forms present in the culture medium. Environmental Pollution. 1991; 74(2): 89-100.
57. Morweiser M., Kruse O., Hankamer B., Posten C. Developments and perspectives of photobioreactors for biofuel production. Applied Microbiology and Biotech. 2010; 87(4): 1291-1301.
58. Shilton A., Pond treatment Tech. 2006.
59. Vandamme D., Foubert I., Fraeye I., Meesschaert B., Muylaert K. Flocculation of Chlorella vulgaris induced by high pH: role of magnesium and calcium and practical implications. Bioresource Tech. 2012; 105: 114-119.

60. Cho H.U., Kim Y.M., Choi Y-N., Xu X., Shin D.Y., Park J.M. Effects of pH control and concentration on microbial oil production from Chlorella vulgaris cultivated in the effluent of a low-cost organic waste fermentation system producing volatile fatty acids. Bioresource Tech. 2015; 184: 245-250.
61. Huo S., Wang Z., Zhu S., Zhou W., Dong R., Yuan Z. Cultivation of Chlorella zofingiensis in bench-scale outdoor ponds by regulation of pH using dairy wastewater in winter, South China. Bioresource Tech. 2012; 121: 76-82.
62. Memon A.R. Memon M.J. Process pH control and improvised cell cultivation during copolymer-induced pelagic cultivation of benthal Chlorella vulgaris. Environmental Progress & Sustainable Energy. 2020; 39(3): e13347.
63. Skjånes K., Knutsen G. Källqvist T., Lindblad P. H2 production from marine and freshwater species of green algae during sulfur deprivation and considerations for bioreactor design. Int J. hydrogen Energy. 2008; 33(2): 511-521.
64. Darimont A. Frenay J. Metals in aqueous solutions in: biosorption of heavy metals. Metals in aqueous solutions in: biosorption of heavy metals. 1989: 65-79.
65. Li F., Wu G., Hu S., Fan Z., Gao Q. Growth behavior and physiological characteristics of Chlorella vulgaris in the presence of deicing salt. Procedia Environmental Sci. 2013; 18: 20-25.
66. Ru I.T.K., Sung Y.Y., Jusoh M., Wahid M.E.A., Nagappan T., Chlorella vulgaris: A perspective on its potential for combining high biomass with high value bioproducts. Applied Phycology. 2020; 1(1): 2-11.
67. Barahoei M., Hatamipour M.S., Afsharzadeh S. Direct brackish water desalination using Chlorella vulgaris microalgae. Process Safety and Environmental Protection. 2021; 148: 237-248.
68. Ali H.E.A., El-fayoumy E.A., Rasmy W.E., Soliman R.M., Abdullah M.A. Two-stage cultivation of Chlorella vulgaris using light and salt stress conditions for simultaneous production of lipid, carotenoids, and antioxidants. J. Applied Phycology. 2021; 33(1): 227-239.
69. Del Campo J., Rodriguez H., Moreno J., Vargas M., Rivas J., Guerrero M. Accumulation of astaxanthin and lutein in Chlorella zofingiensis (Chlorophyta). Applied microbiology and biotechnology. 2004; 64(6): 848-854.
70. Pelah D., Sintov A., Cohen E. The effect of salt stress on the production of canthaxanthin and astaxanthin by Chlorella zofingiensis grown under limited light intensity. World J. Microbiology and Biotech. 2004; 20(5): 483-486.
71. Chew K.W., Chia S.R., Show P.L., Yap Y.J., Ling T.C., Chang J-S. Effects of water culture medium, cultivation systems and growth modes for microalgae cultivation: A review. J. the Taiwan Institute of Chem Engin. 2018; 91: 332-344.
72. Bar E., Rise M., Vishkautsan M., Arad S.M. Pigment and structural changes in Chlorella zofingiensis upon light and nitrogen stress. J Plant Physiology. 1995; 146(4): 527-534.
73. Rzymski P., Budzulak J., Niedzielski P., Klimaszyk P., Proch J., Kozak L., Poniedziałek B. Essential and toxic elements in commercial microalgal food supplements. J Applied Phycology. 2019; 31(6): 3567-3579.
74. Sun N., Wang Y., Li Y-T., Huang J-C., Chen F. Sugar-based growth, astaxanthin accumulation and carotenogenic transcription of heterotrophic Chlorella zofingiensis (Chlorophyta). Process Biochemistry. 2008; 43(11): 1288-1292.
75. Wang Y., Liu Z., Qin S. Effects of iron on fatty acid and astaxanthin accumulation in mixotrophic Chromochloris zofingiensis. Biotech Letters. 2013; 35(3): 351-357.
76. Sakarika M. Kornaros M. Kinetics of growth and lipids accumulation in Chlorella vulgaris during batch heterotrophic cultivation: Effect of different nutrient limitation strategies. Bioresource Tech. 2017; 243: 356-365.
77. Devgoswami C.R., Kalita M., Talukdar J., Bora R., Sharma P. Studies on the growth behavior of Chlorella, Haematococcus and Scenedesmus sp. in culture media with different concentrations of sodium bicarbonate and carbon dioxide gas. African J. Biotech. 2011; 10(61): 13128-13138.
78. Rashid N., Lee K., Han J-i., Gross M. Hydrogen production by immobilized Chlorella vulgaris: optimizing pH, carbon source and light. Bioprocess and biosystems engineering. 2013; 36(7): 867-872.
79. Chu W-L. Strategies to enhance production of microalgal biomass and lipids for biofuel feedstock. European J Phycology. 2017; 52(4): 419-437.
80. Hulatt C.J., Thomas D.N. Productivity, carbon dioxide uptake and net energy return of microalgal bubble column photobioreactors. Bioresource Technology. 2011; 102(10): 5775-5787.
81. Yen H-W., Hsu C-Y., Chen P-W. An integrated system of autotrophic Chlorella vulgaris cultivation using CO2 from the aerobic cultivation process of Rhodotorula glutinis. J. the Taiwan Institute of Chem Engin. 2016; 62: 158-161.
82. Sun H., Ren Y., Mao X., Li X., Zhang H., Lao Y., Chen F. Harnessing C/N balance of Chromochloris zofingiensis to overcome the potential conflict in microalgal production. Communications biology. 2020; 3(1): 1-13.
83. Zhu L., Takala J., Hiltunen E., Wang Z. Recycling harvest water to cultivate Chlorella zofingiensis under nutrient limitation for biodiesel production. Bioresource Tech. 2013; 144: 14-20.
84. Kröger M., Müller-Langer F. Impact of heterotrophic and mixotrophic growth of microalgae on the production of future biofuels. Biofuels. 2011; 2(2): 145-151.
85. Huang Y., Lou C., Luo L., Wang X.C. Insight into nitrogen and phosphorus coupling effects on mixotrophic Chlorella vulgaris growth under stably controlled nutrient conditions. Sci. the Total Environment. 2021; 752: 141747.

86. Mirizadeh S., Nosrati M., Shojaosadati S.A. Synergistic effect of nutrient and salt stress on lipid productivity of Chlorella vulgaris through two-stage cultivation. BioEnergy Res. 2020; 13(2): 507-517.
87. Li P., Sun X., Sun X., Tang J., Turaib A., Wang X., Cheng Z., Deng L., Zhang Y. Response of lipid productivity to photosynthesis of Chlorella vulgaris under various nutrient stress modes. J. Renewable and Sustainable Energy. 2020; 12(5): 056102.
88. Mao X., Wu T., Sun D., Zhang Z., Chen F. Differential responses of the green microalga Chlorella zofingiensis to the starvation of various nutrients for oil and astaxanthin production. Bioresource Tech. 2018; 249: 791-798.
89. Xie T., Xia Y., Zeng Y., Li X., Zhang Y. Nitrate concentration-shift cultivation to enhance protein content of heterotrophic microalga Chlorella vulgaris: Over-compensation strategy. Bioresource Tech. 2017; 233: 247-255.
90. Liu J., Mao X., Zhou W., Guarnieri M.T. Simultaneous production of triacylglycerol and high-value carotenoids by the astaxanthin-producing oleaginous green microalga Chlorella zofingiensis. Bioresource Tech. 2016; 214: 319-327.
91. Xu Y., Falarz L., Chen G. Characterization of type-2 diacylglycerol acyltransferases in the green microalga Chromochloris zofingiensis. J Agric Food Chem. 2018; 67(1): 291-298.
92. Chen J., Jiang X., Wei D. Effects of urea on cell growth and physiological response in pigment biosynthesis in mixotrophic Chromochloris zofingiensis. J. Applied Phycology. 2020; 32(3): 1607-1618.
93. Lam M.K., Lee K.T. Potential of using organic fertilizer to cultivate Chlorella vulgaris for biodiesel production. Applied Energy. 2012; 94: 303-308.
94. Chew K.W., Chia S.R., Show P.L., Ling T.C., Arya S.S., Chang J.-S. Food waste compost as an organic nutrient source for the cultivation of Chlorella vulgaris. Bioresource Technology. 2018; 267: 356-362.
95. Alcántara C., de Godos I., Muñoz R. Chapter 11 - Wastewater treatment and biomass generation with algae, in Wastewater Treatment Residues as Resources for Biorefinery Products and Biofuels, J.A. Olivares, *et al*., Editors. 2020; Elsevier, 229-254.
96. Gardner M., Comber S., Scrimshaw M.D., Cartmell E., Lester J., Ellor B. The significance of hazardous chemicals in wastewater treatment works effluents. Sci the Total Environment. 2012; 437: 363-372.
97. Mulders K.J., Weesepoel Y., Bodenes P., Lamers P.P., Vincken J-P., Martens D.E., Gruppen H., Wijffels R.H. Nitrogen-depleted Chlorella zofingiensis produces astaxanthin, ketolutein and their fatty acid esters: a carotenoid metabolism study. J. Applied Phycology. 2015; 27(1): 125-140.
98. Aydoner Coban G., Dasgan H., Akhoundnejad Y., Ak Cimen B. Use of microalgae (Chlorella vulgaris) to save mineral nutrients in soilless grown tomato. in XXX International Horticultural Congress IHC2018: II International Symposium on Soilless Culture and VIII Int. 2018; 1273.
99. Gao F., Yang Z-H., Li C., Zeng G-M., Ma D-H., Zhou L. A novel algal biofilm membrane photobioreactor for attached microalgae growth and nutrients removal from secondary effluent. Bioresource Tech. 2015; 179: 8-12.
100. Morschett H., Freier L., Rohde J., Wiechert W., von Lieres E., Oldiges M. A framework for accelerated phototrophic bioprocess development: integration of parallelized microscale cultivation, laboratory automation and Kriging-assisted experimental design. Biotechnology for Biofuels. 2017; 10(1): 1-13.
101. Alketife A.M., Judd S., Znad H. Synergistic effects and optimization of nitrogen and phosphorus concentrations on the growth and nutrient uptake of a freshwater Chlorella vulgaris. Environmental Technology. 2017; 38(1): 94-102.
102. Arbib Z., Ruiz J., Álvarez-Díaz P., Garrido-Pérez C., Barragan J., Perales J.A. Effect of pH control by means of flue gas addition on three different photo-bioreactors treating urban wastewater in long-term operation. Ecological Engineering. 2013; 57: 226-235.
103. Sun X., Zhong Y., Huang Z., Yang Y. Selenium accumulation in unicellular green alga Chlorella vulgaris and its effects on antioxidant enzymes and content of photosynthetic pigments. PLoS One. 2014; 9(11): e112270.
104. Yang K., Qin L., Wang Z., Feng W., Feng P., Zhu S., Xu J., Yuan Z. Water-saving analysis on an effective water reuse system in biodiesel feedstock production based on Chlorella zofingiensis fed-batch cultivation. Water Sci.Tech. 2015; 71(10): 1562-1568.
105. Sun D., Zhang Z. Reactive oxygen species derived from NADPH oxidase regulate astaxanthin and total fatty acid accumulation in Chromochloris zofingiensis. J. Applied Phycology. 2021; 33(2): 819-827.
106. Heredia-Arroyo T., Wei W., Ruan R., Hu B. Mixotrophic cultivation of Chlorella vulgaris and its potential application for the oil accumulation from non-sugar materials. Biomass and Bioenergy. 2011; 35(5): 2245-2253.
107. Rodriguez-Garcia I., Guil-Guerrero J.L. Evaluation of the antioxidant activity of three microalgal species for use as dietary supplements and in the preservation of foods. Food Chem. 2008; 108(3): 1023-1026.
108. Chen T., Wei D., Chen G., Wang Y., Chen F. Employment of organic acids to enhance astaxanthin formation in heterotrophic Chlorella zofingiensis. J. Food Processing and Preservation. 2009; 33(2): 271-284.
109. Enyidi U.D. Chlorella vulgaris as protein source in the diets of african catfish Clarias gariepinus. Fishes. 2017; 2(4): 17.

110. Yoshida H., Yanai H., Ito K., Tomono Y., Koikeda T., Tsukahara H., Tada N. Administration of natural astaxanthin increases serum HDL-cholesterol and adiponectin in subjects with mild hyperlipidemia. Atherosclerosis. 2010; 209(2): 520-523.
111. Jyonouchi H., Zhang L., Tomita Y. Studies of immunomodulating actions of carotenoids. II. astaxanthin enhances in vitro antibody production to T-dependent antigens without facilitating polyclonal B-cell activation. 1993.
112. Tamaru C.S., Ako H. Using commercial feeds for the culture of freshwater ornamental fishes in Hawaii. in UJNR AQUACULTURE PANEL SYMPOSIUM. 1999.