

Biological activity of microalgae can be enhanced by manipulating the cultivation temperature and irradiance

Review Article

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Abstract: The escalating levels of antibiotic resistance among pathogenic bacteria and the side effects of chemotherapeutic drugs in use forced the efforts of scientists to search for natural antimicrobial and anticancer substances with novel structures and unique mechanism of action. Focusing on bioproducts, recent trends in drug research have shown that microalgae (including the cyanobacteria) are promising organisms to furnish novel and safer biologically active compounds. Many microalgal metabolites have been found to possess potent antibacterial, antifungal, antiviral, anticancer and antiinflammatory activities, as well as antioxidant, enzyme inhibiting and immunostimulating properties. In this paper, the studies on the biological activity of microalgae associated with potential medical and pharmaceutical applications are briefly presented. Attention is focused on the impact of cultivation temperature, irradiance and growth stage on the biomass accumulation, activity and pathways of cell metabolism and the possibilities of using these variable factors to increase the diversity and quantity of biologically active substances synthesized by microalgae.

Keywords: Antibacterial • Anticancer • Antifungal • Biologically Active Metabolites • Growth Stage

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1. Introduction

Microalgae are a large and heterogenous group of photoautotrophic microorganisms, including species from different phyla – Cyanophyta (blue-green algae, cyanoprokaryotes, cyanobacteria), Chlorophyta (green algae), Rhodophyta (red algae), Cryptophyta, Haptophyta, Pyrrophyta, Streptophyta, Heterokontophyta. Microalgae exhibit remarkable ecological plasticity, namely the ability to adapt to changing and frequently extreme environmental conditions such as temperature, light, salinity, pH and moisture, which defines their worldwide distribution [1,2]. To survive in a complex and competitive environment, these organisms have developed adaptive and defense strategies that are related to the synthesis of various,

some of which are unique, compounds from different metabolic pathways. Due to their extraordinary and diverse biosynthetic potential, and the possibility for controlled cultivation, microalgae are increasingly being used for biomass production and as a source of a vast range of valuable substances of industrial, ecological and pharmaceutical interest (reviewed by [3-5]).

2. Studies on the biological activity of microalgae associated with potential medical and pharmaceutical applications

A substance with antibacterial activity was first isolated from *Chlorella* in 1944. A fatty acid mixture, named "chlorellin" was shown to inhibit the growth of both

Gram-positive and Gram-negative bacteria [6]. Since then, microalgae have become the focus of extensive screening of extracts and metabolites with potent biological activities that could lead to discovery of useful natural pharmacological agents.

Among cyanobacteria, the most studied for their biological activities are *Arthrospira platensis*, *Aphanizomenon flos-aquae* and representatives of the genera *Anabaena*, *Fischerella*, *Hapalosiphon*, *Leptolyngbya*, *Nodularia*, *Nostoc*, *Oscillatoria*, *Phormidium*, *Synechococcus*, *Synechocystis*, *Stigonema*, *Tolypothrix* and *Symploca*. Biologically active metabolites in cyanobacteria belong to the groups of peptides, lipopeptides, fatty acids, polyketides, amides, lactones, alkaloids, terpenes, carbohydrates, amino acid derivatives, terpenoids, aromatic substances, etc. The majority of these metabolites are accumulated in cells but some are released into the medium. Examples of biologically active extracellular metabolites are: five antibacterial diterpenoids from *Nostoc commune*, one of which having also cytotoxic and molluscicidal activities [7] (Table 1), a brominated indole alkaloid of *Anabaena constricta* that possesses antimicrobial activity [8], antifungal peptides from *Tolypotrix byssoidea* [9], or the broad spectrum antibacterial and antifungal substance “parsiguine” excreted by *Fischerella ambigua*, collected from paddy fields [10]. Brazilian cyanobacterial isolates produce antimicrobial nonribosomal peptides [11]. Antimicrobial fatty acids and volatiles have been detected in lipophilic extracts from *Synechocystis* sp. [12]. The cell-free culture liquids, water and ethanol cellular extracts, fatty acid mixtures and polysaccharides from *Synechocystis* sp., *Gloeocapsa* sp. and *Nostoc entophyllum* inhibit the growth of selected pathogenic bacteria and fungus *Candida albicans*, with the exopolysaccharides having the strongest activity [13]. C-phycocyanin, a light-harvesting protein pigment, isolated from *Synechocystis* sp. and *Arthrospira fusiformis* has activity against *S. typhimurium* and *C. albicans* [13]. *Arthronema africanum* synthesizes high amounts of C-phycocyanin which shows antitumor action [Gardeva *et al.*, submitted for publication]. The C-PC from *Spirulina (Arthrospira) platensis* is known to have various biological activities and pharmacological properties, such as antibacterial [14], antifungal, antiviral [15] and anticancer [16] activities, anti-inflammatory, fibrinolytic [17], antidiabetic [18], anti-oxidant and free radical scavenging properties [19]. The isolated free fatty acids of *Gloeocapsa* sp. and *Synechocystis* sp. are found to have high activity against a human cervical carcinoma cell line (HeLa), with IC_{50} values lower than $15 \mu\text{g mL}^{-1}$ [20]. The acidic polysaccharide “nostoflan” has been isolated as an antiviral agent (against HSV-

1) from *Nostoc flagelliforme* [21]. A lectin isolated from the filamentous cyanobacterium *Oscillatoria agardhii* NIES-204 potently inhibits HIV replication in MT-4 cells [22]. The cosmopolitan freshwater cyanobacterium *Heteroleiblenia kuetzingii* produce intracellular and extracellular compounds, toxic to several mouse and fish cell lines [23]. Dolastatins (pseudopeptides) are an interesting group of biologically active metabolites, isolated from marine cyanoprobkaryotes, mainly from the genera *Lyngbya*, *Oscillatoria* and *Symploca* [24]. Dolastatins are the basis for the development of synthetic drug analogues having better pharmacological and pharmacokinetic properties in the treatment of different cancer types [25]. Largazole, the most powerful known natural inhibitor of class I histone deacetylases, is a cyclic depsipeptide isolated from *Symploca*. It shows remarkable *in vivo* anticancer and osteogenic activities [26].

Chlorophytic microalgae are also rich sources of substances with antimicrobial, antiviral, cytotoxic and immunostimulating activities (Table 1). El Semary *et al.* [27] have reported on the antibacterial activity of lipophilic extracts from *Chlorococcum* sp. and defined palmitic (hexadecanoic) acid as the active agent. The phenolic compounds and main pigments (β -carotene and chlorophyll) of *Chlorococcum himicola* cells show a dose-dependent negative effect on microbial growth [28]. Water and ethanol extracts from ten green microalgae of the genera *Chlorella*, *Desmococcus* and *Scenedesmus* exhibit significant antibacterial (against Gram-positive and Gram-negative bacteria) and antitumor (against four tumor cell lines) activities [29]. The authors have found variation in the activity among the strains from the same species and suggested this is due to strain differences or to the different physiological state of the cultures. The fatty acid mixtures from the Bulgarian isolates *Chlorella* sp. and *Coelastrella* sp. are shown to have significant activity against the HeLa cells [20]. Water, ethanol and hexane extracts and polysaccharide rich fraction of *Haematococcus pluvialis* and *Dunaliella salina*, widely used as carotenoid sources, are active against HSV-1 [30]. The antiviral activity of the ethanol extracts was linked to the presence of short-chain fatty acids, β -ionone, neophytadiene, phytol, palmitic and α -linolenic acids. The astaxanthin rich extract from *H. pluvialis* inhibit the growth of five cancer cell lines—HCT-116, HT-29, LS-174, WiDr and SW-480 [31]. Carotenoids from *Chlorella ellipsoidea* and *Chlorella vulgaris* also inhibit the proliferation of HCT-116 tumor cells with low values of the concentration, required for 50% inhibition (IC_{50} of 40.73 ± 3.71 and $40.31 \pm 4.43 \mu\text{g mL}^{-1}$, respectively) [32]. Violaxanthin, a pigment isolated from *Dunaliella tetriolecta*, has a dose-dependent

Table 1. Microalgae, active compounds which they produce, and biological activities and pharmacological properties of these compounds.

phylum/species	active compound	activity	reference
Cyanophyta			
<i>Nostoc commune</i>	diterpenoids	antibacterial, molluscicidal	[7]
<i>Anabaena constricta</i>	indole alkaloid	antimicrobial	[8]
<i>Tolypotrix byssoidea</i>	peptides	antifungal	[9]
<i>Fischerella ambigua</i>	"parsiguine"	antibacterial, antifungal	[10]
<i>Brazilian isolates</i>	nonribosomal peptides	antimicrobial	[11]
<i>Synechocystis</i> sp.	fatty acids, volatiles	antimicrobial	[12]
<i>Gloeocapsa</i> sp.	exopolysaccharide	antibacterial, antifungal	[13]
	fatty acid mixture	anticancer	[20]
<i>Synechocystis</i> sp.	exopolysaccharide	antibacterial, antifungal	[13]
	C-phycocyanin	antibacterial, antifungal	[13]
	fatty acid mixture	anticancer	[20]
<i>Spirulina platensis</i>	C-phycocyanin	antibacterial	[14]
		antifungal, antiviral	[15]
		anticancer	[16]
		anti-inflammatory, fibrinolytic	[17]
		antidiabetic	[18]
<i>Nostoc flagelliforme</i>	"nostoflan" (polysaccharide)	antiviral (HSV-1)	[21]
<i>Oscillatoria agardhii</i>	lectin	antiviral (HIV)	[22]
<i>Lyngbya</i> , <i>Symploca</i> , <i>Oscillatoria</i>	dolastatins (pseudopeptides)	anticancer	[24]
<i>Symploca</i>	largazole (cyclic depsipeptide)	anticancer, osteogenic	[26]
Chlorophyta			
<i>Chlorella</i>	"chlorellin" (fatty acid mixture)	antibacterial (Gram ⁺ and Gram ⁻)	[6]
<i>Chlorococcum</i> sp.	palmitic acid	antibacterial	[27]
<i>Chlorococcum himicola</i>	phenolic compounds, β -carotene, chlorophyll	antibacterial	[28]
<i>Chlorella</i> , <i>Desmococcus</i> , <i>Scenedesmus</i>	water and ethanol extracts	antibacterial, antitumor	[29]
<i>Chlorella</i> sp., <i>Coelastrella</i> sp.	fatty acid mixture	anticancer	[20]
<i>Haematococcus pluvialis</i> , <i>Dunaliella salina</i>	β -ionone, phytol, neophytadiene, palmitic acid, α -linolenic acid	antiviral (HSV-1)	[30]
<i>Haematococcus pluvialis</i>	astaxanthin	anticancer	[31]
	exopolysaccharide	immune stimulating	[35]
<i>Chlorella ellipsoidea</i> , <i>Chlorella vulgaris</i>	carotenoids	anticancer	[32]
<i>Dunaliella tetriolecta</i>	violaxanthin	anticancer	[33]
<i>Chlorella vulgaris</i>	glycoprotein	antimetastatic and immunopotentiating	[34]
Rhodophyta			
<i>Porphyridium</i> sp.	exopolysaccharide	antiretroviral	[36]
		antiinflammatory	[39]
<i>Porphyridium cruentum</i>	exopolysaccharide	anticancer	[37]
		antidiabetic	[38]
	B-phycoerythrin	antibacterial, antifungal	[13]
		antitumor	[41]
<i>Rhodella reticulata</i>	exopolysaccharide	antibacterial, antifungal	[13]
		antioxidant	[40]
		anticancer	[37]
<i>Gracilaria lemaneiformis</i>	R-phycoerythrin	free radicals scavenger, antitumor	[42]

Table 1. Microalgae, active compounds which they produce, and biological activities and pharmacological properties of these compounds.

Heterokontophyta			
<i>Phaeodactylum tricornutum</i>	hexadecenoic and hexadecatrienoic acids	antibacterial (Gram ⁺)	[43]
	eicosapentaenoic acid	antibacterial	[44]
<i>Cymbella</i> sp.	phlorotannins	antibacterial	[45]
<i>Haslea ostrearia</i>	marennine	antitumor	[46]
		antiviral, anticancer	[47]
<i>Melosira</i> , <i>Amphora</i> , <i>Phaeodactylum</i> , <i>Nitzschia</i>	aqueous extracts	anticancer, antithrombogenic	[48]
<i>Navicula directa</i>	"naviculan" (polysaccharide)	antiviral	[49]

suppressing effect on MCF-7 cancer cells even at a very low concentration ($0.1 \mu\text{g mL}^{-1}$) [33]. A glycoprotein prepared from *Chlorella vulgaris* culture supernatant exhibits protective activity against tumor metastasis and chemotherapy-induced immunosuppression in mice [34]. The sulfated exopolysaccharide of *Haematococcus lacustris* (*H. pluvialis*) is reported to have potent early innate immune stimulating activities by enhancing the expression of TNF- α , COX-2 and iNOS in murine macrophage cells [35].

The highly sulfated polysaccharides produced by the red microalgae *Porphyridium cruentum* and/or *Rhodella reticulata* are known to be active against pathogenic bacteria and the fungus *Candida albicans* [13], retroviruses [36], MCF-7 and HeLa human cell lines [37], and diabetes in mice [38] (Table 1). Their anti-inflammatory [39] and antioxidant potential [40] is also known. Minkova *et al.* [41] have established high selective *in vitro* activity of B-phycoerythrin from *Porphyridium cruentum* against the cells of myeloid Graffi tumor in hamsters. This biliprotein pigment has also a promising antibacterial and antifungal potential [13]. It has been reported that R-phycoerythrin of *Gracilaria lemaneiformis* can scavenge free radicals and exhibits antitumor activity [42].

Extracts from the marine diatom *Phaeodactylum tricornutum* exhibit antibacterial activity. Hexadecanoic and hexadecatrienoic fatty acids are responsible for growth inhibition of Gram-positive bacteria [43] (Table 1.), while the eicosapentaenoic fatty acid is active against a range of both Gram-positive and Gram-negative bacteria, including multi-resistant *Staphylococcus aureus* [44]. Similarly, organic and aqueous extracts from *Cymbella* sp. and the isolated phlorotannins show activity against six different species of pathogenic microorganisms [45]. Marennine, a hydrosoluble blue-green pigment synthesised and excreted by *Haslea ostrearia* has an antiproliferative effect on three solid tumor cell lines [46], and also exhibits antibacterial (*Vibrio aestuarianus*), antiviral (HSV1) and anticancer

activities [47]. Extracts from marine benthic diatoms of the genera *Melosira*, *Amphora*, *Phaeodactylum* and *Nitzschia* are reported to induce leukemia cell death and to contain inhibitors of blood platelet activation [48]. Naviculan, a sulfated polysaccharide isolated from the deep-sea water diatom *Navicula directa* has a broad antiviral spectrum against enveloped viruses [49].

Literature review and the representative examples listed in Table 1 show that the polysaccharides, fatty acids and pigments/pigment derivatives synthesized by microalgae from different phyla exhibit biological activity. However, when prepared from a variety of sources, each of these compounds shows a different potential and spectrum of activity. This is associated with the interspecies difference in the physicochemical characteristics of the compound, such as molecular weight, composition, chain conformation, as well as the specificity of the experimental test models. On the other hand, the production of certain bioactive compounds is determined by the taxonomic position of microalgae. Phycobiliproteins (phycoerythrins and phycocyanins), for example, are accessory light-harvesting protein pigments, characteristic of Cyanophyta, Rhodophyta and Cryptophyta. Cyanoprokaryotes are a source of structurally novel cyclic peptides and depsipeptides (reviewed in [50]). Phlorotannins are a type of tannins that is found in some Heterokontophyta algae. Only the diatom *Haslea ostrearia* is shown to synthesize the pigment marennine.

Some of the studies on biological activity have been conducted with microalgae collected directly from their natural habitat. Often this biomass is a mixture of several species, making uncertain the origin of each isolated natural product. Using cultured microalgae has several advantages over field collections. Controlled culture conditions allow investigation of species that cannot reach adequate density in nature; ensure purity of the unialgal biological material; provide a possibility for fast and repetitive biomass production, as well as an opportunity to explore and use the effect of cultivation conditions on biomass accumulation, its biochemical

composition, and most importantly, on the synthesis of bioactive metabolites.

3. Effect of cultivation temperature, irradiance and their interaction on the growth and metabolic activity of microalgae

The effects of environmental factors on the growth of microalgae, their physiological and metabolic responses are determined by biochemical processes under genetic control [51,52]. The main physical factors regulating cell growth and metabolic activity are temperature and light intensity.

The effect of temperature is associated mainly with changes in cellular structural components (especially lipids and proteins) and reaction rates. As a consequence of these primary effects, there are also secondary effects on metabolic regulatory mechanisms, specificity of enzyme reactions, cell permeability and cell composition [53]. Low temperatures lead to a decrease in enzyme activity, membrane fluidity and electron transfer in electron transport chains thus resulting in a decrease of photosynthesis, respiration and subsequently in the growth reduction of algal cultures [1]. To survive, cells trigger different mechanisms such as an increase in enzyme synthesis [54], restoring the fluidity of membranes by increasing the proportion of unsaturated to saturated fatty acids, an increase of cis-double bonds and shortening of fatty acid chains [55,56]. Reduction of algal growth at high temperatures is related to denaturation and degradation of some proteins [57], disturbed functions of cell membranes due to changes in their composition and physical state [58], reduced functionality of the photosynthetic machinery especially of photosystem II (PSII) [59], decreased RUBISCO activity and/or its activase and stimulated respiration [60].

Photosynthetically active radiation (light) is the driving force for photosynthesis. Changes in light intensity induce a physiological response, called photoacclimation, in the course of which algal cells undergo dynamic changes in their ultrastructure, composition, biophysical and physiological properties, to optimize light harvesting and energy utilization [61,62]. At low light intensities, cells maximize the use of available light by increasing cellular pigmentation [62-64]. To avoid the harmful effects of excess energy at high irradiance, cells decrease their chlorophyll and accessory pigment contents [62], increase the amount of photoprotective carotenoids [65], and accumulate storage compounds [61,66]. In addition to the downregulation of photosystem I (PSI) content,

some cyanobacteria have developed an interesting mechanism to decrease intracellular light energy by synthesizing effective "sun-blocking" pigments such as the indole-alkaloid scytonemin and myxoxanthophyll.

Although the interaction between light intensity and temperature plays an important role for algal growth and metabolic regulation, the knowledge of this interaction is limited. The influence of temperature on growth and biochemical composition depends on the irradiance level and *vice versa* [63,67-69]. For most algae, the combinations of high temperatures with high light intensities or low temperatures with high irradiance levels are unfavorable conditions at which negligible growth is observed. In general, changes in temperature and irradiance affect photosynthesis, thus altering carbon fixation and the allocation of carbon into different types of macromolecules. The effects of environmental factors, including temperature and light, as well as their cross-interactions on the biochemical composition of microalgae have been recently reviewed in Juneja *et al.* [70]. Species-specific responses of cultured algae are observed, indicating different metabolic regulation that may reflect differences in the growth conditions of the native habitats of each species.

4. Effects of cultivation temperature, irradiance and growth phase on the biological activity of microalgae

4.1 Effects of temperature, irradiance and their interaction on the cyanobacterial biological activity

The effects of the temperature and irradiance on the growth and synthesis of biologically active substances are best studied in the cyanoprokaryotes. The response to changing temperatures and light intensities is found to be species-dependent and even strain-specific. Some species produce the greatest amount of bioactive metabolites at optimal growth temperature and/or irradiance. The optimal temperature for growth and synthesis of toxins in *Planktothrix agardhii* is 25°C [71]. In *Microcystis aeruginosa* (Kützing, UTEX 2667) maximum growth and production of microcystins are reached at 26°C and light intensity of $2 \times 112 \mu\text{mol m}^{-2} \text{s}^{-1}$ [72]. Noaman *et al.* [73] report that the optimal temperature for growth and production of an antimicrobial substance in *Synechococcus leopoliensis* is 35°C. Extracellular filtrates from *Anabaena* sp. and *Calothrix* sp., cultivated at the most favorable conditions for growth of light intensity (90–100 μmol

photons $\text{m}^{-2} \text{s}^{-1}$) and temperature ($40 \pm 2^\circ\text{C}$) show the highest activity against the phytopathogens *Rhizoctonia bataticola* and *Pythium debaryanum* [74]. When the cultivation temperature is lowered, the inhibiting effect of *Anabaena* sp. filtrate on *R. bataticola* is weaker (at 27°C) or even absent (at 20°C). A similar effect of temperature decrease on the fungicidal activity of several *Anabaena* strains is described by Chaudhary *et al.* [75]. For two *Nodularia spumigena* strains [76] and *Microcystis aeruginosa* [77] the highest toxin concentration and the best growth are observed at the same irradiance. In *Spirulina* sp. isolated from Wadi El Natron lake, Egypt, the best growth is registered at 30°C and $48.4 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, which coincides with the maximum accumulation of some bioactive compounds (β -carotene and phycobiliproteins), while the content of total lipids is enhanced by the lower light intensity ($14.52 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) [78].

However, the conditions for optimal growth often do not coincide with the optimal conditions for the production of bioactive substances. Many authors have found that the optimal temperature for synthesis of biologically active metabolites is lower than the optimal growth temperature. Maximal concentration of cylindrospermopsin in *Cylindrospermopsis raciborskii* cultures is detected at 20°C but optimal growth is achieved at 35°C [79]. *Oscillatoria angustissima* and *Calothrix parietina* produce antibiotics, inhibiting the growth of natural isolates of cyanobacteria and green algae, several bacteria and fungi. High amounts of the antibiotic substances (70.9 and 82.5 mg g^{-1} biomass for *O. angustissima* and *C. parietina*, respectively) are synthesized at 25°C , while algal biomass, chlorophyll *a* and protein content increase with temperature increase up to 30°C [80]. Suboptimal growth temperatures (15 – 26°C) stimulate antibacterial activity and cytotoxicity against HeLa cells of the exopolysaccharides (EPS) from *Gloeocapsa* sp. The higher activity is related not only to increased quantity of high molecular weight polysaccharides, but also to qualitative changes in the EPS obtained under these conditions [81]. *Gloeocapsa* sp., grown at three different temperatures (15 , 34 and 38°C) under two light intensities (132 and $2 \times 132 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), has identical fatty acid profiles, but the relative content of individual fatty acids varies among cultivation conditions. The fatty acid mixtures, obtained at 15°C under both irradiance levels, contain higher amounts of alpha-linolenic and stearic acids compared to other samples and show highest activity against bacteria and HeLa cells [81]. Like *Gloeocapsa* sp., in *Phormidium* sp. the suboptimal growth temperature (30°C) is associated with growth retardance and considerable production of extracellular biologically active compounds

[82]. Novel compounds, the cyanothecamides A, B and C, are detected in *Cyanothece* PCC 7425 only when the strain is subjected to a heat shock at 37°C for 24 h [83]. Maximal toxin production at light intensity lower than that needed for maximal growth is also described. *Anabaena* strain 90 produces three types of bioactive peptides, namely microcystins, anabaenopeptilides and anabaenopeptines [84]. The highest peptide concentration is achieved after 13-day cultivation under light intensity of $23 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, while growth is better under higher irradiance and prolonged cultivation. The biomass of *Planktothrix agardhii* does not change significantly under different illumination levels, while the intracellular concentration of toxins is higher at lower light intensities (12 – $24 \mu\text{mol m}^{-2} \text{s}^{-1}$) [71]. A similar effect of irradiance on production of microcystin (MC) and nostophycin (NP) from *Nostoc* sp. strain 152 was observed by Kurmayer [85]. Both intra- and extracellular MC and NP concentrations are negatively correlated with irradiance. The antibacterial activity of water cellular extract and EPS from *Synechocystis* sp. R10, and the toxicity of its fatty acids against HeLa cells, are enhanced by lower light intensities that are less favorable for growth ($132 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). In contrast, the cytotoxicity of water extracts and culture liquids of *Synechocystis* sp. R10 is greater after cultivation at higher (doubled) light intensity [86]. In *Scytonema*, increasing irradiance levels gradually increases antibiotic production [87]. The increase of light intensity stimulates the secretion of polymeric substances from *Arthrospira platensis* [68] and improves its carotenoid content [88].

The combination of temperature and irradiance also significantly affects toxin content in cultures and the expression of biological activity of cyanobacteria. The highest content of extracellular toxin from *Nodularia spumigena* is detected at the highest values of both parameters (30°C , $80 \mu\text{mol m}^{-2} \text{s}^{-1}$) [89]. In contrast, for *Anabaena* sp. strains high temperatures (25 – 30°C) together with higher light intensity ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) reduces the amount of cellular toxins [90]. Low light intensity ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$) in combination with low temperature (10°C) significantly enhances the production of inhibitory metabolites by *Fischerella muscicolla* UTEX 1829 [91]. *Synechocystis* sp. R10, grown at 26 and 32°C (temperatures that are optimal under low light intensity and suboptimal at high light intensity) exhibits the strongest bioactivity [86].

4.2 Effects of growth phase on the biological activity of cyanoprokaryotes

The relationship between synthesis of biologically active substances and growth phase is also species-dependent.

Saker and Griffiths [79] have reported a considerable enhancement of cylindrospermopsin production and excretion in two isolates of *Cylindrospermopsis raciborskii* when entering stationary phase. Chaudhary *et al.* [75], showed that their oldest (4-week-old) *Anabaena* cultures incubated under continuous light and high temperature (40°C) had highest fungicidal and hydrolytic enzyme activity. *Anabaena laxa* synthesizes a cyclic peptide, responsible for fungicidal activity against *Pythium debaryanum*, and its production increases when the cyanobacterium reaches stationary phase (28d) [92]. Volk [93] reported changes in the content of exometabolites of the cyanobacterium *Nostoc insulare* with growth phase. During the exponential growth phase, a nontoxic metabolite is prevalent, while in the stationary phase the content of antimicrobial, cytotoxic metabolites increases. Aging is determined as an important factor for increasing EPS production by *Phormidium tenue* [94]. Maximal antimicrobial activity of *Synechococcus leopoliensis* [73] and *Oscillatoria* and *Calothrix* [80] is detected in the post-exponential growth stage. A more continuous cultivation of *Gloeocapsa* sp. results in increased antibacterial, antifungal and cytotoxic activities of its EPS [81]. For *Synechocystis* sp. R10, entering the stationary growth phase is related to a broader antimicrobial spectrum and enhanced activity of the intracellular water soluble metabolites against microbes and HeLa cells, while the substances excreted in the medium have a weaker antimicrobial potential in comparison to samples from the exponential phase [86]. The freshwater cyanobacteria *Oscillatoria* sp. BTCC/A0004 and *Scytonema* sp. TISTR 8208 release into the medium a pink pigment that inhibits the growth of the green microalgae *Chlorella fusca* and *Chlamydomonas reinhardtii*. The synthesis of this pigment occurs during active cell growth for both strains and it decreases with prolonged cultivation [95]. An extracellular substance from *Oscillatoria* sp. suppresses the green alga *Chlorella vulgaris* and this allelopathic action is stronger in the early growth stages of the cyanoprokaryote [96]. Linear dependence between the production rate of anabaenopeptides and microviridin I and growth rate of *Planktothrix agardhii* determines growth activity as being an important regulator of production of these bioactive oligopeptides [97].

4.3 Effects of cultivation temperature, irradiance and growth phase on biological activity of eukaryotic microalgae

The effect of temperature and/or irradiance and growth stage on the synthesis of bioactive metabolites in eukaryotic microalgae has also been studied, although

to a lesser extent. Light intensity is a determining factor for biosynthesis and accumulation of both EPS and phycobiliproteins in *Porphyridium purpureum* [98] where a direct linear dependence between biosynthesis of EPS and light intensity (from 1.3 g L⁻¹ at 120 μmol m⁻² s⁻¹ to 4.5 g L⁻¹ at 240 μmol m⁻² s⁻¹) was found. In contrast, a four- to ten-fold increase in each of the phycobiliprotein compounds (phycoerythrin, R-phyococyanin and allophyococyanin) occurred when light irradiance was reduced from 240 to 120 μmol m⁻² s⁻¹. *Haematococcus pluvialis* grown at low light intensity accumulates biomass rapidly, but no significant production of carotenoids in the growing cells is observed. Under stress conditions such as high temperature, high light intensity, salt stress or nutrient deficiency, cell proliferation is suppressed and resting spores (aplanospores) are formed, accompanied by intensive production of carotenoids (mainly astaxanthin) [99,100]. High temperature (38.5°C) and high light intensity (770 μmol m⁻² s⁻¹) favour the accumulation of β-carotene in *Dunaliella salina* [101]. Mendoza *et al.* [102] reported the induction of carotenogenesis and accumulation of polyunsaturated fatty acids in *Dunaliella salina* subjected to a suboptimal (18°C) growth temperature. Growth-limiting temperature (33°C) is found to stimulate carotenogenesis in *Muriellopsis* sp. as the lutein content per cell increases about sixfold compared to the optimal temperature (28°C) [103]. Sánchez *et al.* [104] studied the influence of interactions between irradiance and temperature on growth and lutein content of *Scenedesmus almeriensis*. Maximum biomass productivity was reached at 33°C and 1700 μmol m⁻² s⁻¹, while 44°C and 1233 μmol m⁻² s⁻¹ are the optimal conditions for lutein accumulation. In *Chlorella zofingiensis* strain CCAP 211/14, however, the optimal temperatures for accumulation of total carotenoids coincide with those for optimal growth (between 24°C and 28°C), with a maximum value at 24°C for astaxanthin and at 28°C for lutein. Astaxanthin is most abundant at light intensities optimal for cell growth (460 and 920 μmol photons m⁻² s⁻¹). In contrast, lutein content was about 2-fold higher in cells cultured at a lower (90 μmol photon m⁻² s⁻¹) irradiance. Lutein is the prevalent carotenoid during early stages of cultivation of *C. zofingiensis* (over 4 mg g⁻¹ dry weight), whereas astaxanthin accumulates progressively, to reach a maximum (1.5 mg g⁻¹ dry weight) in the late stationary phase [105]. Accumulation of polysaccharides in the microalga *Porphyridium cruentum* is highly stimulated in the late exponential to stationary phase [106]. In outdoor mass cultivation of *Parietochloris incisa*, which is considered the greatest plant producer of arachidonic acid (AA), light intensity of 250 μmol photons m⁻² s⁻¹ limits growth but favors accumulation of AA, while at high irradiance levels

(2 500 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) growth is stimulated but the AA content is low [107]. Solovchenko *et al.* [108] found that changes in lipid metabolism under the combined impact of lower light intensity and nitrogen deficiency are crucial for the synthesis of AA from that green microalga. At higher irradiances (200 and 400 $\mu\text{mol m}^{-2} \text{ s}^{-1}$) on complete medium, *Parietochloris incisa* displays a higher growth rate and an increase in the carotenoid content, especially that of β -carotene and lutein [109]. Compared to low light, high light conditions increase the proportion of PUFAs in the diatom *Thalassiosira pseudonana* [110]. In contrast, in the eustigmatophycean microalga *Trachydiscus minutus* the proportion of PUFAs decreases when light intensity and also temperature is increased [111]. Highest biomass and eicosapentaenoic acid (EPA) production by the diatom *Phaeodactylum tricornutum* is observed at temperatures of 21.5 to 23°C [112]. Unsaturated fatty acid biosynthesis can be stimulated by a number of environmental stresses, including low temperature stress. For example, the yields of PUFA and EPA increase by 120% in *Phaeodactylum tricornutum* when the temperature is changed from 25°C to 10°C for 12 h [113]. Similarly, the haptophycean *Pavlova lutheri* increases its relative EPA content from 20.3 to 30.3 M% and *Isochrysis galbana* has higher levels of α -linolenic acid and docosahexaenoic acid when the culture temperature is reduced to 15°C [114,115]. Cultivation of *Porphyridium cruentum* at low temperature (18°C) until reaching stationary growth stage results in accumulation of PUFAs (43.7% of total fatty acids), α - and γ -tocopherol (vitamin E) in cells [116]. An increase in PUFAs is expected as these fatty acids have good flow properties and are predominately used in the cell membrane to maintain fluidity during low temperatures. With aging however, the proportion of PUFAs in total lipids decreases in the marine haptophyte *Isochrysis galbana* [117], the green algae *Dunaliella salina* [118] and *Nannochloropsis oculata* [119], and some diatoms [110,120]. In *Nannochloropsis oculata*, α -tocopherol content depends on the nitrogen source and concentration and also on the growth phase [121]. Increased synthesis of α -tocopherol during the life cycle is probably due to an increased need for antioxidants in the process of cellular aging [122]. Exposure of *Dunaliella tertiolecta* to high light increases the production of ascorbic acid (Vitamin C) [123], known to have activity against cancer, atherosclerosis and as an immunomodulator. In the dinoflagellate microalga *Prorocentrum belizeanum*, the synthesis of a cytotoxic okadaic acid (OA) in cells is decoupled from the optimal growth conditions (25°C and 40 $\mu\text{mol m}^{-2} \text{ s}^{-1}$), as OA

overproduction is observed at higher temperature (28°C) and when both the temperature and the irradiance are low (18°C and 20 $\mu\text{mol m}^{-2} \text{ s}^{-1}$) [124].

5. Conclusion

Based on the literature reviewed, the potential and spectra of biological activities of microalgal species differ. The variety and quantity of the synthesized biologically active substances are species-specific, but depend largely on environmental temperature and light, and on age of the culture, being therefore amenable to manipulation by changing growth conditions. The influence of these factors on biological activity is indirect and is a consequence of tightly regulated physiological-biochemical changes that occur in the cells in response to various impacts [51,52,55,83,91,100]. Some species produce maximal amounts of biologically active metabolites under optimal growth temperatures and/or light intensities. However, the highest amounts and/or the widest variety of such metabolites are often produced under stress conditions. This is associated with the role of some bioactive compounds, synthesized by stress-altered metabolic pathways, in providing survival benefits to the cells and their adaptation to adverse and competitive environments [51,64,68,88,93,95,100,102,108,109,114]. The reduced accumulation of biomass under stress conditions can be overcome by biphasic cultivation – cells are first cultured under conditions optimal for growth and then subjected to the action of an appropriate stressor to induce accumulation of the respective valuable product. This technology is already used for the production of β -carotene from *Dunaliella salina* [125], astaxanthin from *Haematococcus pluvialis* [126] and polyunsaturated fatty acids from different microalgae [127], but it has the potential for obtaining other biologically active substances. Advancements in the accumulation of knowledge of the physiology, biochemistry and molecular biology of the bioactive substance-producing species is undoubtedly useful for the development of microalgae-based processes and technologies. Identification of the appropriate cultivation conditions for any particular strain-producer could be an efficient approach for obtaining larger quantities of biologically active metabolites, as well as for detection and characterization of new substances with high activity and a broader action spectrum.

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