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Microalga *Chlamydomonas reinhardtii* cultivation and biomass processing optimization for biological activity improvement

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ABSTRACT

Chlamydomonas reinhardtii is a unicellular green alga with promising biotechnological potential. This study systematically examined the interdependence between post-cultivation processing and chemical composition and food-relevant bioactivity of *C. reinhardtii*. After cultivation optimization, we determined pigment, protein, and lipid content and assessed biological activity through various in vitro assays and in vivo zebrafish toxicity testing. Results showed strong antioxidative, antidiabetic, and antimicrobial properties, particularly when biomass was dried at temperatures below 60 °C. This indicates that important metabolites, such as fatty acids and terpenoids, are better preserved during oven-drying than lyophilization. Bioactive compounds were detected by Q-TOF and linked with observed activities. This is the first comprehensive study combining cultivation optimization, processing methods, and bioactivity evaluation of *C. reinhardtii*. Our findings show that *C. reinhardtii* could be used to develop functional food and pharmaceutical products, and that preservation of bioactive compounds is critical for the greatest efficacy and product quality.

1. Introduction

Microalgae are a group of predominantly unicellular photosynthetic microorganisms with various aquatic and terrestrial habitats [1]. Due to the continuous growth of the world population and the significant negative impacts of climate change on the availability and use of terrestrial resources for food production, recent studies suggest that microalgae could be a sustainable alternative to conventional food sources [2]. Their ability to be cultivated in smaller uncultivable areas with the use of brackish, sea, or wastewater offers great advantages compared to traditional crops. Furthermore, microalgal metabolites have been shown to possess greater biological activity than those of plant origin [3–5]. Increased consumers' awareness of food quality has encouraged research and development of new products that will, besides their nutritional value, contribute to the prevention of various health conditions, such as type 2 diabetes, hypertension, neurodegenerative diseases, obesity, and/or hematological malignancies [6,7]. In that sense, microalgae, as a whole biomass or purified extracts/fractions, represent a great source of nutrients and bioactive compounds.

Biotechnological production of microalgae involves various processes to ensure its effectiveness and profitability. The microalgae production process usually starts with selecting an appropriate microalgal strain and optimizing cultivation parameters, such as medium composition, light intensity and exposure time, temperature, and pH. Specifically, medium composition and light conditions are among the main parameters impacting photosynthetic growth of microalgae [8]. For example, cultivation in an inadequately composed medium can cause alterations in biochemical pathways and the production of undesirable microalgal metabolites [9]. In addition, during the late exponential growth phase, when nutrient availability becomes limited, the applied

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type of light becomes the prevalent parameter controlling the synthesis and accumulation of bioactive compounds [10]. Moreover, artificial lighting, such as fluorescence lamps or light-emitting diodes (LEDs), is mainly being used [11]. However, LEDs have lately been emerging as more efficient and economical light sources for the indoor cultivation of microalgae [12].

After cultivation, it is crucial to optimize biomass processing techniques, such as drying, as well as extraction and purification of target compounds, to ensure successful production of microalgae with the lowest possible production costs [13]. Some of the most valuable microalgal compounds investigated so far are pigments, lipids, proteins, polyunsaturated fatty acids, vitamins, and minerals, among which pigments (chlorophylls, carotenoids, and phycobiliproteins) exhibit one of the greatest potentials to be used as antioxidants and antibacterial compounds in nutraceuticals and pharmaceuticals [14]. To date, *Arthrospira* (*Spirulina*) and *Chlorella* species are the two most utilized microalgae in the food and pharmaceutical industries due to their rich nutritional profiles and health benefits [15,16]. In recent years, green unicellular microalga *Chlamydomonas reinhardtii*, primarily used as a model organism in molecular biology, has been studied as a natural source of pigments, lipids, proteins, polysaccharides, and other compounds with potential biological activity [5,17]. Also, it has well-characterized physiology and can be easily genetically manipulated, unlike other industrial microalgal species, e.g., *Chlorella*, which represents a major advantage for applied biotechnological development [18]. This enables refined expression of complex pathways and provides an extensive understanding of various processes (e.g., photosynthesis, nitrogen metabolism, respiration, biosynthetic pathways of lipids, carotenoids, etc.) [19]. Moreover, *C. reinhardtii* has been successfully engineered for the production of various products, such as biohydrogen, high-value proteins (e.g., vaccines, antibodies, and bioactive peptides), and other metabolites (e.g., carotenoids, lipids, terpenoids, and starch), which has not yet been achieved in other algal species [5]. In addition, *C. reinhardtii* is classified as a GRAS (Generally Recognized as Safe) organism, meaning that this microalga is safe for consumers [20]. However, studies investigating the bioactive properties of *C. reinhardtii*, especially in correlation with biomass processing conditions, are still scarce [21–23].

Therefore, this study aimed to determine optimal cultivation conditions (media composition and the type of illumination) of microalga *C. reinhardtii* in a small- and large-scale system and determine the optimal biomass drying method that will potentially improve the chemical composition and biological activity of this microalga. Due to the complexity of this study, a schematic workflow is provided in Supplementary data (Fig. S1). The results presented in this paper give a new perspective on the potential use of microalga *C. reinhardtii* in developing novel natural-based nutraceutical and pharmaceutical products.

2. Materials and methods

2.1. Chemicals

A full list of chemicals used in this study is provided in the Supplementary data.

2.2. Optimization of C. reinhardtii growth conditions

Axenic microalgal strain *Chlamydomonas reinhardtii* 11/32A used in this study was purchased from the Culture Collection of Algae and Protozoa (CCAP, Scotland, UK). Microalga *C. reinhardtii* was cultivated in 100 mL Erlenmeyer flasks with a working volume of 30 mL and in triplicate to determine optimal growth parameters. Two types of lights (warm white light – WWL and the combination of red and blue light – RBL) and three different media (BBM, 3N-BBM+V, and Jaworski medium [24]) were used. The compositions of media are given in Supplementary data (Table S1). Other growth parameters included the temperature of 25 °C, media pH of 7 ± 0.2, continuous orbital shaking (100 min^{-1}), and continuous illumination (24:0, light:dark) of 3800 lx. During the 14-day cultivation of the microalga in different media, cell growth was monitored every 2–3 days using a Thoma cell counting chamber (Graticules Optics, UK). The cell density was also determined spectrophotometrically by measuring absorbance at 680 nm using an Infinite 200 PRO spectrophotofluorimeter (Tecan, Austria). All measurements were performed in triplicate.

After determining optimal growth parameters, the microalgal culture was scaled up. To obtain greater amounts of *C. reinhardtii* biomass, the inoculum was prepared in a 2 L bioreactor and used for setting up the cultivation in a 20 L bioreactor. In the bubble column bioreactor, *C. reinhardtii* was cultivated at 25 °C and aerated with an air flow of 150 L h^{-1} until the cells reached the late exponential phase and then further processed to obtain dry microalgal biomass.

2.3. Drying of the microalgal biomass

Before drying, cells were centrifuged for 10 min at 4200 rpm using an Eppendorf 5804 R centrifuge (Hamburg, Germany) and washed twice with deionized water. The concentrated biomass was then either dried at 40, 50, 60, 80, or 100 °C using a ThermoFisher Scientific drying oven (Heratherm™ Advanced Protocol Ovens, 230VAC 60 Hz model, Waltham, Massachusetts, USA) or lyophilized using Christ Freeze Dryer (Alpha 1–4 LSC model, Osterode, Germany). Dry biomass was homogenized using a bead mill homogenizer (Tehtnica Millmix 20, Domel d.o.o., Železniki, Slovenia) to obtain biomass with similar-sized particles (< 1 mm).

2.4. Preparation of C. reinhardtii fractions

Extracts were prepared by mixing 100 mg of either dried (40, 50, 60, 80, and 100 °C) or lyophilized biomass with methanol (MeOH) and dichloromethane (DCM) solvent solution (1:1, *v/v*), followed by sonification in an ultrasonic bath (Bandelin, Sonorex digiplus 560 W, Berlin, Germany) at 100 % frequency for 15 min. Four extractions were performed in total and, between each extraction, the supernatants were collected and filtered through a 0.45 μm PTFE syringe filter. After the extraction was completed, the extracts were mixed with C18 powder. The solvent solution was evaporated from the collected supernatants in a stream of liquid nitrogen (5.0, Messer, Croatia) to obtain dry extracts that were subjected to fractionation.

Extracts were fractioned using solid-phase extraction (SPE) and a C18 cartridge (Agilent Bond Elut, Santa Clara, CA, USA). The cartridge was conditioned with methanol and water, followed by fractionation of the obtained extracts using solvents of different polarity in the following order: water, water:methanol (1:1, *v/v*), methanol, dichloromethane. Fractions collected using water and water:methanol solution were not analyzed, whereas methanol (F3) and dichloromethane (F4) fractions were collected, vaporized, and stored until evaluation of their biological properties [25,26]. Before investigating their biological activity, dry F3 fractions were dissolved in methanol and F4 fractions in DMSO to prepare concentrations that would be further diluted using appropriate solutions.

2.5. Chemical analyses of C. reinhardtii

To investigate the impact of specific drying treatment on the chemical properties of prepared microalgal biomasses, total protein and pigment contents were determined spectrophotometrically. The total lipid and fatty acid content were analyzed using gas chromatography (Agilent 8890 gas chromatograph, Santa Clara, CA, USA). *C. reinhardtii* fractions were analyzed using a Q-TOF system.

2.5.1. Total protein content

Total protein content was determined using the Lowry method [27]

with modifications. Firstly, 20 mg of dried or lyophilized biomass was mixed with Mili-Q water (pH = 12) in a volumetric flask (final volume 10 mL). The flasks were placed in an ultrasonic bath (40 °C; 60 % frequency) and sonicated under alkaline conditions for an hour. Then, the mixtures were centrifuged at 4200 rpm for 10 min, followed by the collection of the supernatants and cooling to room temperature. Eventually, the Lowry solution was added to the cooled supernatants, which were then incubated for 15 min at room temperature. Next, 100 μL of 1.0 N Folin-Ciocalteu's phenol reagent was added, and the supernatants were incubated for 30 min at room temperature. After the second incubation, the absorbance was measured at 750 nm. In addition, bovine serum albumin (BSA) was used as a standard to determine the possible presence of interfering agents in the sample matrix.

2.5.2. Photosynthetic pigments

Photosynthetic pigments (carotenoids, chlorophylls *a* and *b*, pheophytins *a* and *b*) present in the dried and lyophilized biomasses were detected and quantified using the spectrophotometric method described by Babadi et al. [28], with minor modifications. The extraction of pigments started by weighing 10 mg of the biomass and mixing it with 2 mL of methanol. The samples were placed in an ultrasonic bath for 5 min at 100 % frequency and then centrifuged (10,000 rpm) for 5 min. The supernatants were collected and the pellets were used for another extraction. The described extraction procedure was repeated until colorless pellets were obtained. The quantification of the mentioned pigments from the collected supernatants was conducted spectrophotometrically and calculated using the equations previously reported by [29].

2.5.3. Determination of total lipids and fatty acids analysis

Total lipid contents and fatty acid analyses of the biomasses were conducted using gas chromatography (GC). Extraction of total lipids and lipid transmethylation was carried out as described in the paper of Martić et al. [26], with minor modifications. Briefly, in each vial with 10 mg of the dried or lyophilized biomass, 20 μL of chloroform, 200 μL of chloroform:methanol solvent mixture (2:1, *v/v*), and 300 μL of 0.6 M HCl diluted in methanol were added. Afterward, the vials were vortexed and placed in a water bath preheated to 85 °C for an hour, followed by cooling at room temperature for 15 min and hexane addition (1 mL). After an additional 1-h incubation, the top phases containing methyl esters were collected from each sample and mixed with 5 μL of pentadecane, an analytical internal standard.

The determination of fatty acid methyl esters (FAMEs) was conducted using a gas chromatograph (Agilent 8890, Santa Clara, CA, USA) equipped with a flame ionization detector (FID). In addition, the GC was supplied with a fused silica capillary column (DB-WAX Ultra I (123-7063UI-INT), 60 m × 0.320 mm × 0.50 μm; Agilent, Santa Clara, CA, USA), an autoinjector, and OpenLab CDS software. The injected sample volume was 1 μL and helium was used as a carrier gas. Initially, the oven temperature was 80 °C/1 min, followed by an increase to 175 °C (25 °C/min) and then 185 °C (10 °C/min). After 5 min, the temperature was raised to 235 °C (15 °C/min) and remained for 5 min. The final temperature was 250 °C, increased at a 15 °C/min rate, and kept at that temperature for 27 min. The obtained data were analyzed using the software, whereas FAME identification was conducted by internal calibration and utilization of FAME Standard Calibration Mix C8:0-C24:0.

2.5.4. LC/Q-TOF analysis of C. reinhardtii fractions

Liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) was performed using a Quadrupole Time-of-Flight system (Q-TOF) (6546 LC/Q-TOF, Agilent, Santa Clara, CA, USA). Q-TOF equipped with a Dual Jet Stream electrospray ionization source (Dual AJS ESI, Agilent, Santa Clara, CA, USA) was connected to an ultra-high-performance liquid chromatography (UHPLC) instrument (1290 Infinity, Agilent, Santa Clara, CA, USA). Sample separation occurred on a ZORBAX Eclipse Plus C18 RRHD column (2.1 × 50 mm, 1.8 μm, Agilent, Santa Clara, CA, USA) under the conditions described in Vadlja et al. [30]. The data acquisition process was managed through Agilent MassHunter Acquisition Software (version 10.1), and the resulting raw data were processed using MassHunter Qualitative Software (version 10.0). The processed data were then exported in mgf format for further bioinformatics-based compound identification.

2.6. Biological activities of microalga C. reinhardtii

2.6.1. In vitro determination of antioxidative activity

The antioxidant activity of *C. reinhardtii* methanolic extracts, as well as methanolic (F3) and dichloromethane (F4) fractions, was determined in vitro using 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), 2,2-diphenyl-1-picrylhydrazyl-hydrate (DPPH), ferric reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC) assays [31]. Spectrophotometric measurements of ABTS, DPPH, and FRAP assays were performed in triplicate using a Spectramax ABS plus (Molecular Devices, San Jose, CA, USA) microplate reader. Fluorometric measurements of the ORAC assay were conducted using an Infinite 200 PRO spectrophotofluorimeter (Tecan, Austria). All measurements were performed in triplicate, and the results are shown as mean ± standard deviation. The assays were performed as described in Radman et al. [25].

2.6.2. Determination of alpha-amylase inhibitory activity

The antidiabetic potential of fractions was determined by the slightly modified Caraway-Somogyi iodine/potassium iodide (IKI) method [32]. Dilutions of *C. reinhardtii* samples and positive control (acarbose), as well as α-amylase solution (0.5 mg/mL) and 0.05 % starch solution, were prepared in a 10 mM phosphate buffer (pH 6.9). In a 96-well microplate, 25 μL of the sample or acarbose and 50 μL of the enzyme solution were mixed and the microplate was incubated for 10 min at 37 °C. After preincubation, 50 μL of starch solution was added to the wells to start the reaction and incubated for another 15 min at 37 °C. The reaction was terminated by adding 25 μL of 1 M HCl, followed by iodine-potassium iodide solution addition (100 μL) and absorbance reading at 630 nm. The assay was performed in triplicate.

2.6.3. Determination of pancreatic lipase inhibitory activity

Pancreatic lipase inhibition was investigated using a colorimetric assay as described in Baković et al. [33], with slight modifications. Briefly, each sample was mixed with the enzyme (pancreatic lipase) and preincubated at 37 °C for 10 min. Afterward, the reaction was initiated by adding a substrate (*p*-Nitrophenyl palmitate; PNPP) mixture and monitored by measuring the absorbance at 410 nm. Orlistat, a pancreatic lipase inhibitor, was used as a positive control.

2.6.4. Determination of acetylcholinesterase inhibitory activity

Inhibition of acetylcholinesterase (AChE) in the presence of *C. reinhardtii* fractions was examined using the Ellman method [34] with slight modifications. Briefly, 20 μL of fractions were mixed with a reaction mixture containing 0.3 mM DTNB (with or without enzyme) and 50 mM Tris buffer (pH 8). After a 15-min incubation at 37 °C, 15 nM acetylthiocholine iodide (substrate) was added. Subsequently, the substrate hydrolysis was spectrophotometrically observed for 20 min at 412 nm. Tacrine was used as a positive control and the assay was performed in triplicate. Since DMSO may affect the efficiency and catalytic activity of acetylcholinesterase, F4 samples were not analyzed [35].

2.6.5. Determination of antimicrobial properties

The antimicrobial properties of the samples were investigated using the standardized broth microdilution method issued by the Clinical and Laboratory Standards Institute [36]. The bacterial panel consisted of three Gram-negative bacteria (*Escherichia coli* 12241, *Escherichia coli* MG1665, and *Klebsiella pneumoniae* ATCC 700608) and a Gram-positive bacterium (*Staphylococcus aureus* ATCC 6538) from the collection of the

Laboratory for Aquaculture Biotechnology. Antimicrobial tests were performed in triplicate in 96-well plates with sample concentrations ranging from 0.1 to 50 μg/mL. The antibiotic chloramphenicol was used as a positive and sterile medium as a negative control. After setting up the experiment, the microtiter plate with samples and bacteria was incubated for 24 h at 35 °C. The next day, absorbance was measured at 600 nm. The results were evaluated and expressed as the minimum inhibitory concentration (MIC), which indicates the lowest concentration of the sample that inhibited the growth of bacteria.

2.7. Zebrafish Embryotoxicity test (ZET)

Zebrafish *Danio rerio* (wild-type WIK strain) was obtained from the European Zebrafish Resource Center (Karlsruhe Institute of Technology, Germany). Zebrafish adults were maintained and spawned under controlled laboratory conditions, as described by Babić et al. [37]. To assess the potential toxicity of *C. reinhardtii* fractions, ZET was conducted according to the OECD [38]. Briefly, the experiment was set up in a 24-well plate, using 10 embryos in three replicas, resulting in a total of 30 embryos per tested concentration. The concentrations of fractions ranged from 12.5 to 300 μg/mL. Artificial water served as a negative control. Exposed embryos were incubated (Innova 42, New Brunswick, Canada) for 120 h at 26 °C. The effects of the samples on the zebrafish survival and development were observed using an inverted microscope (Olympus CKX41) supplied with a digital camera (Leica EC3) and LAS EZ 3.2.0 software.

2.8. Statistical evaluation of data

Statistical analyses and graphical presentation of the data were conducted using the GraphPad Prism 8.0 program (GraphPad Software Inc., San Diego, CA, USA). All results were expressed as means ± standard deviations (SD). Before the determination of median inhibitory concentration (IC_{50}), data were subjected to logarithmic transformation. One-way analysis of variance (ANOVA) and Tukey's post hoc test were performed to examine the significance of the difference between treatments. When the assumption of normality was violated, the Kruskal-Wallis test was performed. Distinctions between the samples were considered significant if the *p*-value was 0.05 or less. Processed data obtained after LC/Q-TOF analysis and bioinformatics-based compound identification were used for exploratory analyses and Spearman's rank correlation tests using R v.4.4.3 [39].

3. Results

3.1. Microalgal growth rates and optimization of growth parameters

Comparison of *C. reinhardtii* growth curves for different cultivation conditions is presented in Supplementary data (Fig. S2). Regardless of the media composition, WWL stimulated the growth of *C. reinhardtii* cells. The highest density and specific growth rate of cells were detected using 3N-BBM+V medium and WWL (2.67×10^6 cells/mL and 1.89 ± 0.10 day^{-1}), followed by the same medium and RBL (1.97×10^6 cells/mL and 1.40 ± 0.04 day^{-1}). Cultivation in unfortified BBM medium resulted in comparable cell density (WWL: 1.79×10^6 cells/mL and RBL: 1.67×10^6 cells/mL), with more significant ($p < 0.05$) differences observed in specific growth rate (WWL: 1.69 ± 0.10 day^{-1} and RBL: 1.29 ± 0.11 day^{-1}). No significant differences were observed between the WWL and RBL during the cultivation of *C. reinhardtii* in the Jaworski medium, which also resulted in the lowest cell density and specific growth rate. Therefore, further cultivation was conducted in 3N-BBM+V medium.

3.2. Total pigment content

Pigments detected in *C. reinhardtii* biomasses after drying under different conditions are shown in Fig. 1. The concentrations of chlorophylls *a* (Fig. 1a) and *b* (Fig. 1b) and total carotenoids (Fig. 1c) detected in the biomass that was either dried at 40 °C or lyophilized were significantly higher compared to other treatments. Pheophytins, chlorophyll derivatives, were detected in all samples (Fig. 1d). However, they are predominantly pheophytins *a* (Fig. 1e) since pheophytin *b* was detected only in biomasses dried at 60 and 80 °C (Fig. 1f).

3.3. Total protein content

The highest protein content was observed in *C. reinhardtii* biomass that was either lyophilized (38.62 ± 2.94 %) or dried at 40 °C (34.45 ± 2.07 %), followed by 50 °C (26.20 ± 2.21 %). Higher temperatures (60, 80, and 100 °C) resulted in a decline of the protein content in analyzed dry biomasses, with obtained values being 10.05 ± 2.57 %, 9.45 ± 1.32 %, and 15.25 ± 0.61 %, respectively.

3.4. Total lipid content and fatty acid analysis

The total lipid contents of *C. reinhardtii* biomasses are shown in Table 1. The total lipid contents of the analyzed samples were not notably different. However, ratios of saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids depended on the applied drying method. The highest total lipid and PUFA contents were detected in the biomass dried at 80 °C. On the contrary, the lowest total lipid contents were observed in lyophilized biomass and the biomass dried at 40 °C. Also, the most notable difference in the MUFA and PUFA contents can be seen between those two samples. Precisely, PUFA content was approximately two times higher in the lyophilized biomass than in the biomass dried at 40 °C. An opposite effect was observed in their MUFA contents, with 40 °C having a 4-fold higher MUFA percentage. Overall, SFAs or PUFAs were the main types of fats in all tested samples.

In total, 24 fatty acids (FAs) were detected. Out of all observed FAs, only five were MUFAs, and the others were SFAs and PUFAs. Palmitic acid (C16:0) was the most prevalent SFA in all *C. reinhardtii* samples, with the highest percentages (> 40 %) in the 50 °C and lyophilized samples. A MUFA with the highest proportion in all analyzed samples was C18:1 (cis 9, trans 9), and gamma-linolenic acid (C18:3 (cis 6, 9, 12)) among PUFAs. In addition, docosahexaenoic acid (C22:6 (cis 4, 7, 10, 13, 16, 19); omega-3 FA) and linoleic acid (C18:2 (cis 9, 12); omega-6 FA) are PUFAs only detected in 50 °C and lyophilized samples. Contrarily, linolenic (C18:3 (cis 9, 12, 15)) and docosadienoic (C22:2 (cis 13, 16)) acids are PUFAs detected in all samples, besides 50 °C and lyophilized. A complete list of detected FAs is provided in Supplementary data (Table S2).

3.5. LC/Q-TOF analysis

C. reinhardtii F3 and F4 fractions were subjected to LC/Q-TOF analysis to determine compounds present in fractions and analyze their reported bioactive properties within the COCONUT base (Supplementary data; Table S3).

In total, 683 compounds were detected in F3 and 44 in F4 fractions. Among them, the 40 °C F3 contained the highest number of detected compounds (208). Strikingly, the F4 sample with the highest number of detected compounds (14) was the one prepared from the biomass that was dried at 50 °C. Out of all detected compounds, 102 distinct compounds were observed in F3 fractions and 9 in F4 fractions. These results show that the number of distinct compounds detected in F3 fractions was approximately 10 times higher compared to the F4 fractions. Predominant groups of distinct compounds observed in F3 and F4 fractions are shown in Fig. 2.

In the analyzed samples, the most abundant lipid compounds were long-chain fatty acids, mainly octadecenoic acids, and conjugated fatty acids, which are a combination of PUFA's positional and geometric isomers with conjugated double bonds [40]. Glycero(phospho)lipids

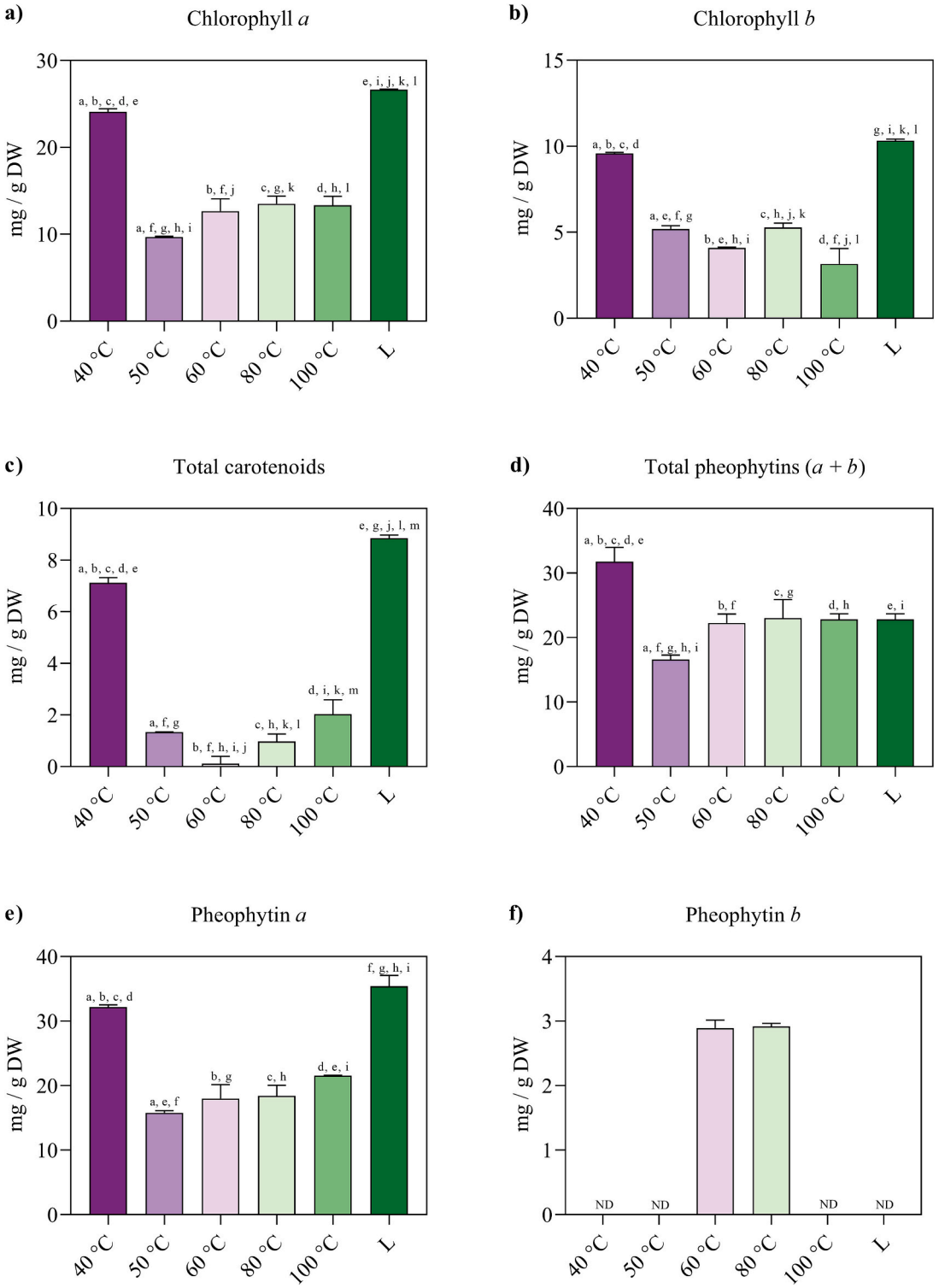

Fig. 1. Pigments (Chlorophyll *a* (**a**), Chlorophyll *b* (**b**), total carotenoids (**c**), total pheophytins (**d**), pheophytin *a* (**e**) and pheophytin *b* (**f**)) detected in *C. reinhardtii* biomass dried at 40, 50, 60, 80, and 100 °C or lyophilized (L). The same letters above columns symbolize significant differences between the tested conditions ($p < 0.05$). *ND – not detected.*

were mainly detected in *C. reinhardtii* fractions obtained from the biomass dried at 40 or 50 °C, showing their thermolabile nature at high temperatures. Similar observations were made for octadecanoids and eicosanoids. Octadecanoids are one of the main classes of phytohormones regulating stress signaling processes [41], which were also predominantly observed in the low-temperature (40–60 °C) oven-dried biomass. Stigmasterol (CNP0177659) and ergosterol (CNP0168945) were the only steroids observed in all F3 fractions, with potential antidiabetic and antilipidemic properties.

Besides fatty acids and their derivatives, terpenoids, mainly carotenoids and sesquiterpenoids, were the second most prevalent class of compounds. In the analyzed samples, the most abundant carotenoids were β-carotene (CNP0310085) and diatoxanthin (CNP0214183). After carotenoids, the most abundant terpenoids were sesquiterpenoids,

Table 1
Total lipid content and the ratios of SFAs, MUFAs, and PUFAs detected in *C. reinhardtii* biomass dried at 40, 50, 60, 80, and 100 °C or lyophilized – L. The results are expressed as percentages of dry weight.

Sample	Total lipid content ± SD [%]	SFA ± SD [%]	MUFA ± SD [%]	PUFA ± SD [%]
40 °C	4.37 ± 0.97	46.46 ± 9.31	32.72 ± 7.27	20.82 ± 16.58
50 °C	5.25 ± 0.03	46.93 ± 0.25	10.29 ± 0.12	42.78 ± 0.36
60 °C	4.49 ± 0.01	27.21 ± 0.05	26.95 ± 0.00	45.84 ± 0.05
80 °C	5.67 ± 0.00	26.57 ± 0.03	27.14 ± 0.16	46.29 ± 0.13
100 °C	5.16 ± 0.18	21.71 ± 5.35	25.51 ± 1.52	52.78 ± 3.83
L	4.24 ± 0.18	45.82 ± 0.14	7.65 ± 0.22	46.53 ± 0.35

compounds with three isoprene units known to exhibit various biological activities (e.g., antidiabetic, antimicrobial, and cytotoxic) [42]. However, two terpenoid compounds were also observed in *C. reinhardtii* F4 fractions. One of them is parahigginol B (CNP0167282), a sesquiterpenoid with the prediction of antilipidemic properties, detected in all *C. reinhardtii* F4 fractions besides the lyophilized one. The second one is didrovaltrate (CNP0293056), a monoterpenoid observed only in the 50 °C F4 sample. In addition, both detected terpenoids exhibit potential antimicrobial properties. Phloroglucinol (CNP0274318), another compound observed only in F4 fractions, is an algal metabolite known for its antioxidant, antidiabetic, antimicrobial, and cytoprotective properties [43]. Overall, a noticeable decline in detected terpenoids was observed with an increase in the drying temperature of *C. reinhardtii* biomass.

Besides fatty acids and terpenoids, other identified classes of compounds were steroids, mainly ergosterols, and a polyketide Heterocornol F (CNP0072420). Thermolide C (CNP0354905) was the only macrolide in the analyzed samples, specifically the 40 °C F3 sample.

Bioactivity predictions, namely antioxidative, antidiabetic, antilipidemic, antimicrobial, and neuroprotective properties, were compared to the results of the conducted in vitro assays. Significant ($p < 0.05$) correlations were observed between the obtained experimental data and the number of detected unique compounds with the prediction of having antidiabetic and/or antimicrobial properties. In the case of antidiabetic properties, a moderate positive correlation ($\rho = 0.61$; $S = 85.75$) was observed between the obtained quantitative data and antidiabetic activity predictions of the microalgal compounds. The number of detected unique compounds with the prediction of antimicrobial activity negatively correlated with the MIC results acquired for *E. coli* 12241 and *E. coli* MG1665. The correlation in both cases was moderate, with rho values of −0.68 ($S = 369.3$) and −0.64 ($S = 360.1$), respectively. On the contrary, a positive correlation ($\rho = 0.71$; $S = 64.54$) was observed between *K. pneumoniae* MIC values and the number of detected compounds with antimicrobial predictions.

Overall, the results of Q-TOF analysis indicate that *C. reinhardtii* has a biological potential dependent on the applied post-cultivation processing method. However, the results of enzymatic assays conducted to assess the biological potential of *C. reinhardtii* suggest that this microalga has a greater potential which is not reflected in the available NP databases.

3.6. Biological activities of microalga C. reinhardtii

3.6.1. Antioxidative properties

Methods used to investigate the antioxidative properties of *C. reinhardtii* samples were based on different mechanisms. Specifically, the principle of the ORAC assay is based on the hydrogen atom transfer, whereas the FRAP method is based on the electron transfer. Mechanisms of ABTS and DPPH tests are the combination of the two already mentioned methods and are therefore also called mixed tests [31]. The results for F3 fractions (Fig. 3) show that the highest antioxidative activity, except for ORAC, was observed in the samples obtained from the biomass dried at 40 °C or lyophilized.

The differences among the samples are most prominently observed in results for ABTS (Fig. 3a) and DPPH (Fig. 3b), in which the highest antioxidative activities for the 40 °C F3 fraction were 284.49 ± 18.76 mg TE/g fraction and 328.61 ± 37.13 mg AAE/g fraction, respectively. In comparison, the lyophilized F3 fraction exhibited antioxidative activities of 305.26 ± 10.37 mg TE/g fraction (ABTS) and 220.73 ± 9.43 mg AAE/g fraction (DPPH). The FRAP assay results (Fig. 3c) indicate that the drying method of *C. reinhardtii* biomass does not cause notable differences among F3 fractions. Differences between the F3 fractions were more distinguishable using ORAC (Fig. 3d), according to which the lyophilized F3 fraction exhibited significantly lower ($p < 0.0001$) activity in comparison to the 40 °C and 50 °C F3 fractions. The lowest antioxidative activities were observed in the F3 fractions prepared from the biomasses dried at higher temperatures (80 and 100 °C), regardless of the used assay.

The results acquired for F4 fractions (Fig. 4) also show that the samples obtained from biomasses that were either dried at lower temperatures (40, 50, and 60 °C) or lyophilized have the highest antioxidative potential. Contrary to the F3 fractions, more pronounced differences in results were observed among all four assays. The results obtained after conducting ABTS (Fig. 4a) and DPPH (Fig. 4b) assays suggest that the 40 °C F4 sample has the highest antioxidative potential (159.58 ± 4.50 mg TE/g fraction and 326.43 ± 20.66 mg AAE/g fraction, respectively). FRAP results (Fig. 4c) show significantly higher antioxidative activity of the lyophilized F4 sample ($p < 0.0001$) in comparison to the other F4 fractions. On the contrary, the results obtained using ORAC (Fig. 4d) were not as striking as the FRAP results, but also suggest that the lyophilized F4 sample has the highest antioxidative activity.

3.6.2. Antidiabetic and antilipidemic properties

Results of the determination of antidiabetic and antilipidemic properties of *C. reinhardtii* fractions are presented in Fig. 5. All tested fractions caused inhibition of alpha-amylase, with F3 fractions exhibiting greater antidiabetic potential (Fig. 5a) compared to the F4 fractions (Fig. 5b). IC_{50} values obtained for all F3 fractions were lower than 100 μg/mL, with 50 °C F3 having the lowest IC_{50} value of 67.76 ± 2.71 μg/mL and the highest antidiabetic potential among all tested fractions. In the case of F4 fractions, the lowest IC_{50} value of 145.73 ± 1.65 μg/mL was obtained for 40 °C, followed by 60 °C (180.63 ± 1.75 μg/mL). Overall, the alpha-amylase inhibition rate of the F4 fractions declined as the drying temperature of the collected biomass increased, especially when temperatures above 60 °C were used.

C. reinhardtii fractions also inhibited the activity of pancreatic lipase. Among F3 fractions, the sample with the highest antilipidemic potential was the one prepared using biomass dried at 60 °C (IC_{50} = 138.87 ± 20.37 μg/mL) (Fig. 5c). Moreover, two F3 fractions (40 °C and lyophilized) and the lyophilized F4 fraction caused lipase inhibition at higher tested concentrations (>250 μg/mL), with the maximum inhibition of 18.46 %, 10.15 %, and 22.05 %, respectively. Overall, higher IC_{50} values were obtained for most F4 fractions (Fig. 5d), indicating higher antilipidemic potential of *C. reinhardtii* F3 fractions.

3.6.3. Neuroprotective properties

Neuroprotective activities of F3 samples were not significantly different, with IC_{50} values ranging from 573.60 ± 58.62 to 786.97 ± 109.51 μg/mL. The only exception was 40 °C, the sample with the lowest neuroprotective activity. The latter had a significantly higher ($p < 0.0001$) IC_{50}, which was approximately two times higher (1434.67 ± 128.81 μg/mL) than in other samples.

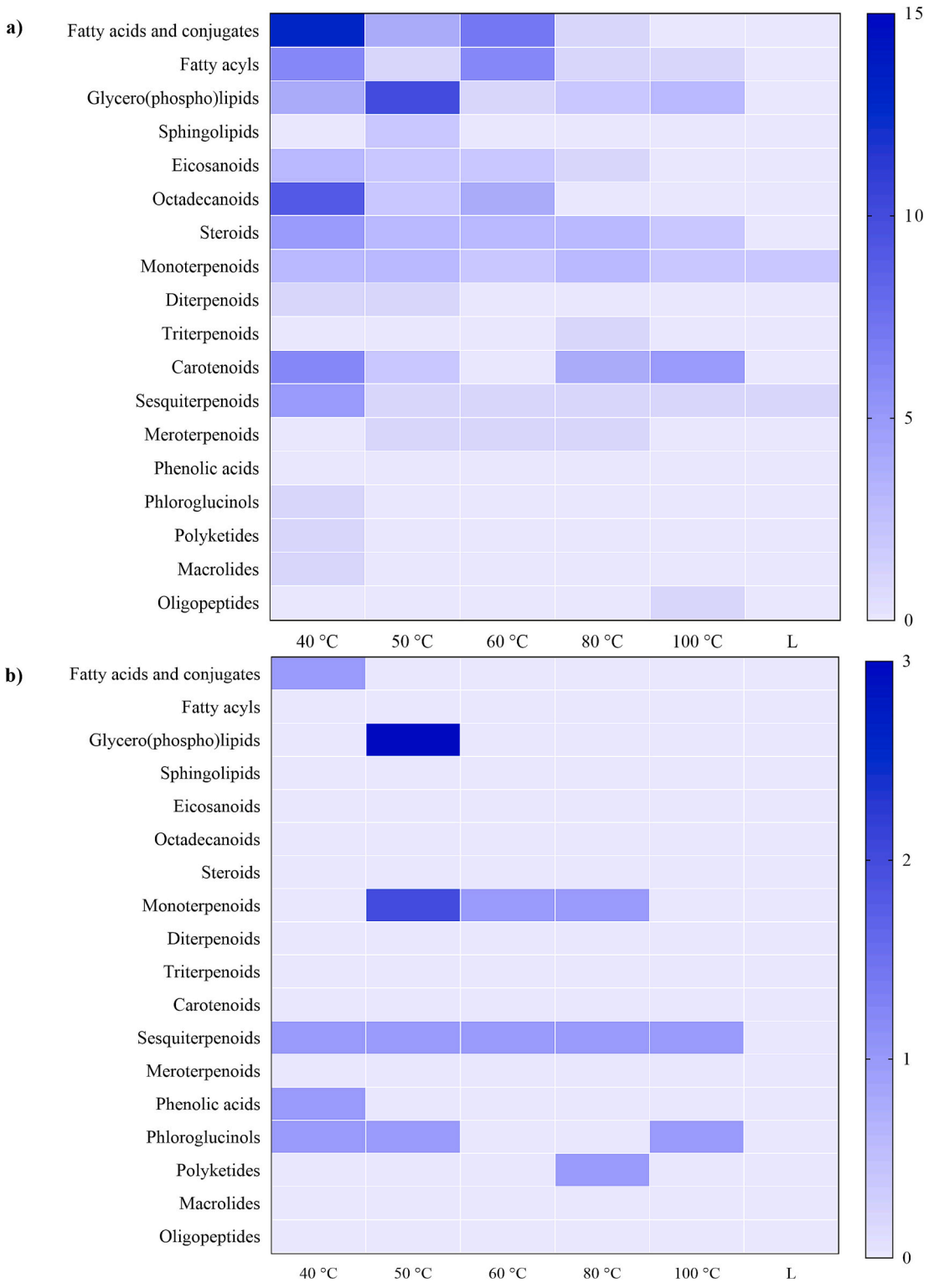

Fig. 2. Heatmaps showing the impact of the drying method (40, 50, 60, 80, and 100 °C or lyophilization – L) on the number of distinct compounds observed in *C. reinhardtii* F3 fractions (**a**) and F4 fractions (**b**). Color density indicates the number of specific compounds observed in *C. reinhardtii* samples.

3.6.4. Antimicrobial properties

Both F3 and F4 fractions had an antimicrobial effect on the tested bacteria, but at different concentrations. In most cases, *C. reinhardtii* fractions expressed higher antimicrobial effects on the Gram-negative bacteria (minimal MIC value was 0.10 μg/mL) than on the Gram-positive bacteria (minimal MIC value was 0.78 μg/mL). In addition, in the case of *E. coli*, lower MICs were determined in the MG1665 strain, except for the 50 °C F3 fraction (Table 2). Notable effects were also observed on *K. pneumoniae*, with MIC values ranging from 0.10 to 0.78 μg/mL. Overall, F3 fractions mainly resulted in lower MIC values than the F4 fractions.

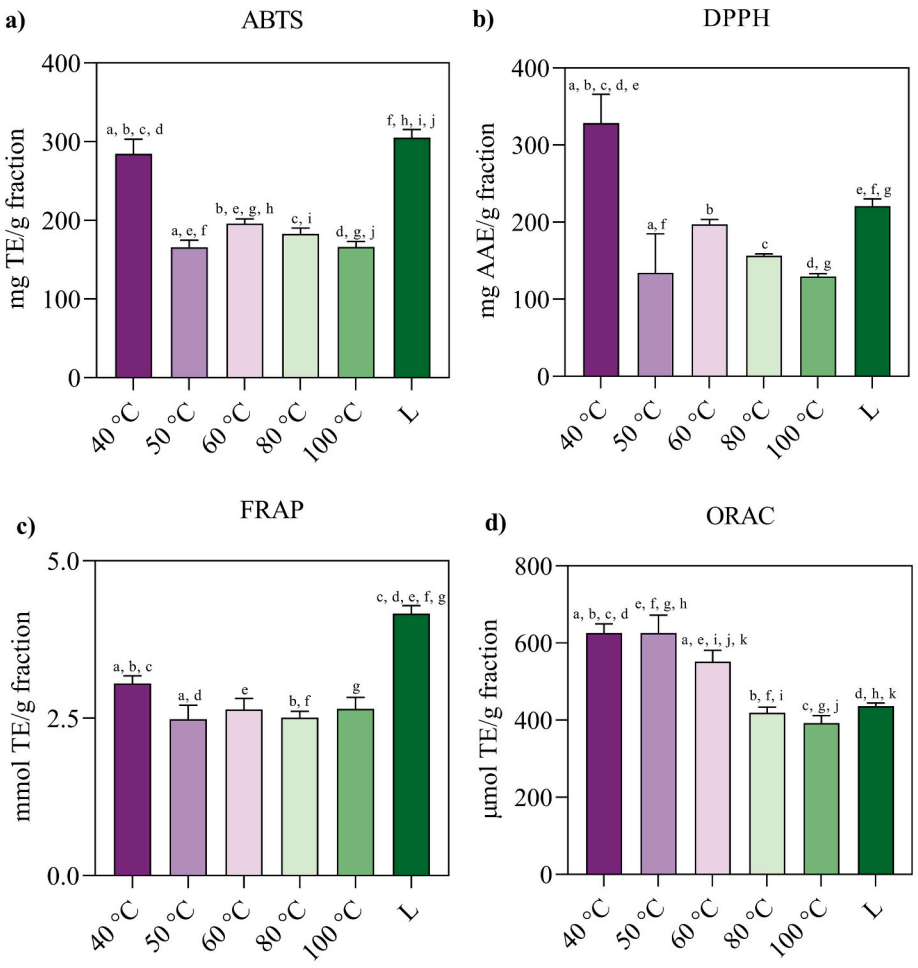

Fig. 3. Antioxidative properties of *C. reinhardtii* F3 fractions, obtained from the biomass dried at 40, 50, 60, 80, and 100 °C or lyophilized – L, determined using different methods: **a)** ABTS, **b)** DPPH, **c)** FRAP, and **d)** ORAC. The same letters above columns symbolize significant differences between the F3 fractions ($p < 0.05$).

3.7. Zebrafish embryotoxicity test (ZET)

No adverse effects on zebrafish survival and development were observed following exposure to the 40 °C F3 fraction or any of the F4 fractions, even at the highest tested concentration of 300 µg/mL. In contrast, exposure to other F3 fractions exhibited a dose-dependent increase in developmental toxicity. Specifically, after 120 h of exposure of embryos to the F3 fractions at concentrations above 150 µg/mL, 100 % mortality was observed. At 150 µg/mL, all F3 fractions, except for 40 °C, caused malformations in all embryos. Concentrations of F3 samples (besides 40 °C) below 150 µg/mL also caused malformations, mainly scoliosis, pericardial edema, and yolk sac abnormalities, with varying percentages that declined proportionally with the tested concentration. These adverse effects were not observed at the following concentrations of the 50 °C, 60 °C, 80 °C, 100 °C, and lyophilized F3 samples: 45, 80, 60, 40, and 35 µg/mL, respectively. Zebrafish embryos in the control group (artificial water) developed normally, without any negative effects observed 120 h post-fertilization.

4. Discussion

The biotechnical potential of microalgae largely depends on cultivation and post-cultivation processing conditions, which significantly affect biomass yield and chemical composition [44,45]. In previous studies, *C. reinhardtii* has been cultivated under various cultivation conditions (e.g., different media compositions, cultivation systems, pH, temperature, and light type/intensity), without comparing the effects of these parameters on microalgal productivity. Moreover, in most of these studies, different *C. reinhardtii* strains were used, making the comparison of biomass yield and productivity even more challenging. Consequently, all these difficulties resulted in an inability to provide a straightforward answer on which parameters yield the greatest *C. reinhardtii* productivity. Therefore, we began this study by investigating different media compositions and illumination types to determine optimal cultivating conditions of *C. reinhardtii*. Cultivation in 3N-BBM+V medium and under white light (LED) resulted in the highest cell concentration and specific growth rate. Enhanced performance was attributed to higher nitrogen and phosphorus content, which are essential for algal metabolism and growth [46]. Although the modified BBM medium contains three times more nitrogen than standard BBM, this does not explain the lower growth observed in Jaworski medium. One of the possible reasons might be light limitation that occurs at high cell densities during photoautotrophic cultivation [47]. Moreover, nitrogen or phosphorus insufficiency was proven to cause inhibition of *C. reinhardtii* growth [48]. Since 3N-BBM+V contains a higher concentration of phosphorus (provided as orthophosphate) than Jaworski medium, the observed higher growth rates suggest that phosphorus concentration plays a key role. This was expected, as phosphorus is essential for cell metabolism, being a component of ATP, nucleic acids, and phospholipids [49]. In addition, we also observed that white LEDs result in a higher cell density and specific growth rate of *C. reinhardtii* cells compared to the red-blue light. While the cultivation of this strain has not been studied under these lighting conditions, similar findings have been reported for other microalgae. Hashmi et al. [50] observed enhanced growth of

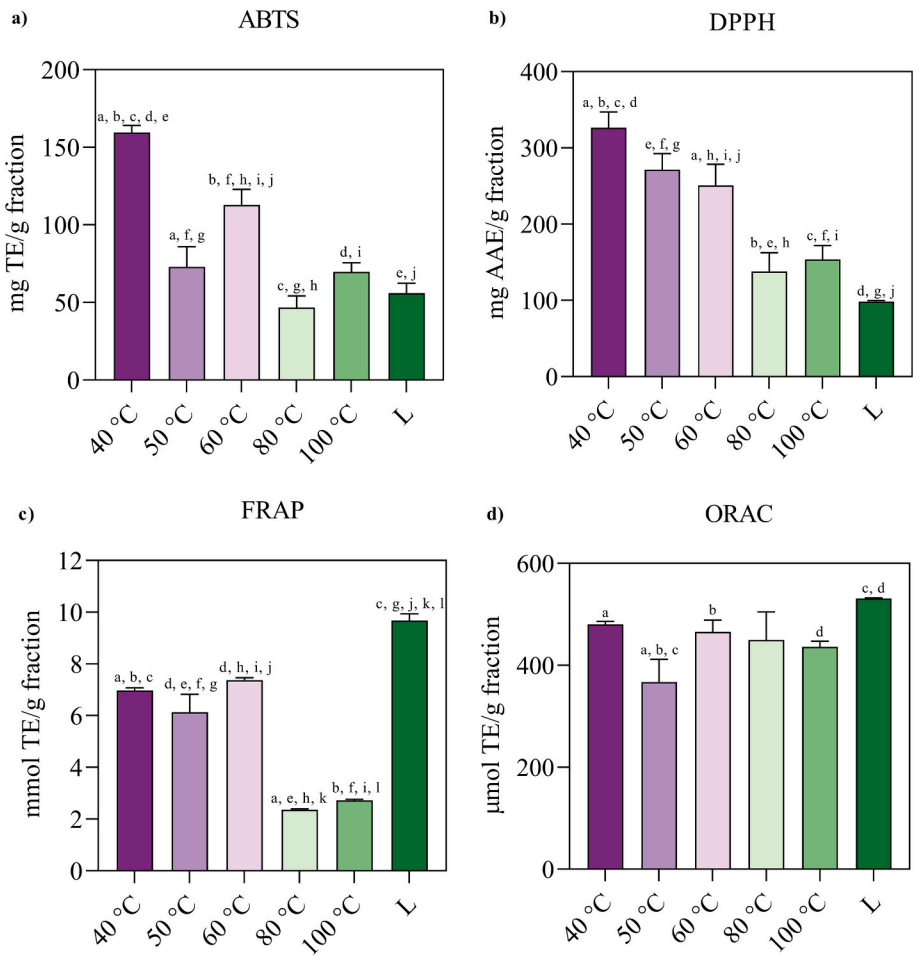

Fig. 4. Antioxidative properties of *C. reinhardtii* F4 fractions, obtained from the biomass dried at 40, 50, 60, 80, and 100 °C or lyophilized – L, determined using different methods: **a)** ABTS, **b)** DPPH, **c)** FRAP, and **d)** ORAC. Results are expressed as mean ± SD. The same letters above columns symbolize significant differences between the F4 fractions ($p < 0.05$).

Chlorococcum sp. under white light, while Hultberg et al. [51] reported nearly two-fold higher biomass in *Chlorella vulgaris* cultivated under white light than colored light. However, several studies have also reported that blue or red light enhances cell density and specific growth rate in comparison to the use of only white light [50,52–57]. In addition, researchers have also evaluated the influence of blue and red light independently, as well as white light. For example, de Mooij et al. [58] showed a higher growth rate of *C. reinhardtii* CC-1690 cultivated under red or blue light than warm white light. Similarly, Li et al. [10] demonstrated temperature-dependent responses to light color in the CC-125 strain. On the contrary, Bialevich et al. [59] reported no significant effect of the light type on the growth of *C. reinhardtii* (CC–1690, wild type) cells. These inconsistencies are likely a result of differences in strain and cultivation conditions. Therefore, strain-specific optimization is essential before scaling up cultivation.

Following cultivation, drying/dewatering of the biomass is one of the most important downstream steps that significantly impacts the quality and profitability of the final product [60]. Biomass drying before solvent extraction is essential since excess moisture can reduce extraction efficiency [61]. Common drying techniques include sun drying, oven drying, freeze drying (lyophilization), spray drying, and fluidized bed drying [21]. Among these, oven drying, typically conducted at 40–70 °C, is still one of the most commonly used methods for drying microalgal biomass [62]. Although lyophilization offers superior preservation of thermolabile compounds, it is expensive compared to conventional drying techniques [21].

In this study, *C. reinhardtii* biomass was dried using oven drying at various temperatures and lyophilization to assess the impact of drying methods on its chemical composition and bioactivity. Lyophilized biomass contained higher amounts of proteins, PUFAs (especially C18:2, C20:2, and DHA), chlorophylls, carotenoids, and pheophytin *a* compared to the oven-dried biomass. However, biomass dried at 40 °C exhibited a comparable profile to lyophilized material, indicating that low-temperature drying effectively preserves thermo-degradable compounds. Similar results were presented in studies investigating the impact of the applied drying technique on other green microalgae, such as *Tetraselmis subcordiformis* [60], *Scenedesmus obliquus* [63], and *Chlorella* sp. [64], which all reported superior retention of bioactive compounds following lyophilization or low-temperature oven drying. Also, Karmakar et al. [65] showed that lyophilization preserves *Chlorella vulgaris* bioactive compounds suspected of thermal degradation.

After drying, we prepared fractions from each *C. reinhardtii* biomass to investigate whether the applied drying treatments caused differences in the chemical composition and bioactive properties. Methanol and dichloromethane were used since they were shown to be the most suitable solvents for the extraction of bioactive compounds from complex matrices, such as algae, due to their polarity and ability to disintegrate cell membranes more efficiently than other commonly used solvents [60].

Firstly, Q-TOF analysis was conducted to identify bioactive compounds in F3 and F4 fractions, and analyze their bioactive properties within the COCONUT base. Greater quantities of compounds were detected in F3 than in F4 fractions, with the diversity and quantity of compounds declining as the drying temperature increased. Regardless of

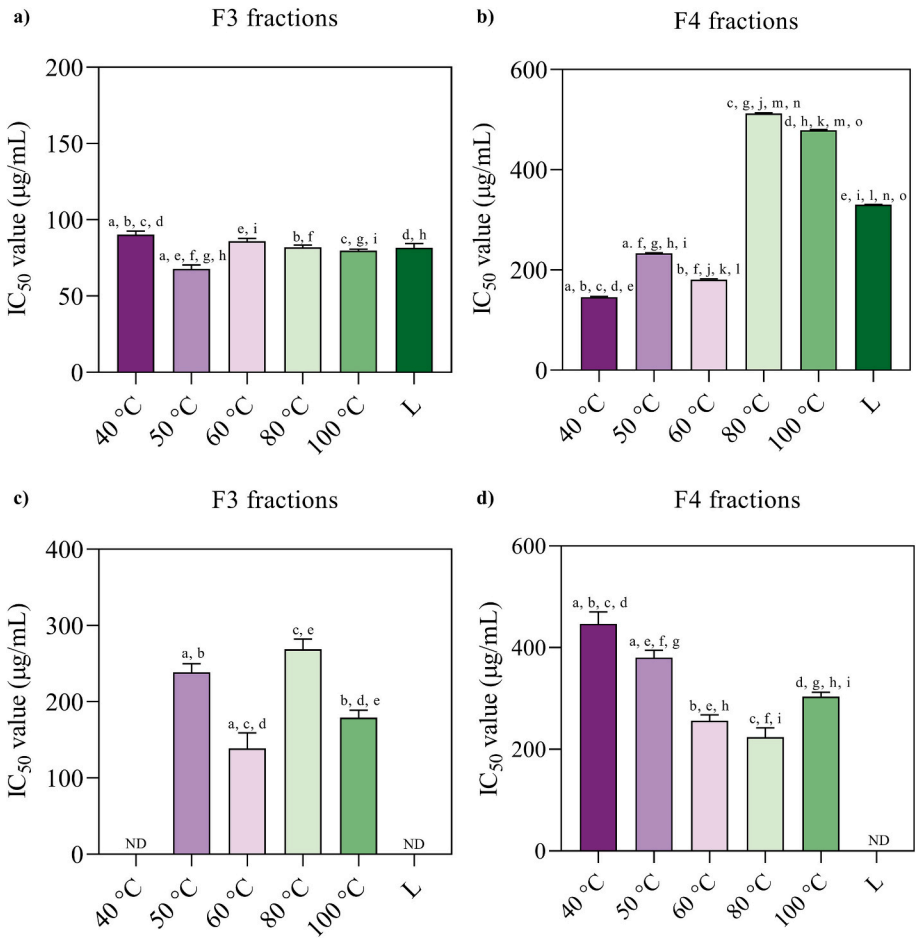

Fig. 5. Antidiabetic (**a** and **b**) and antilipidemic (**c** and **d**) activity of *C. reinhardtii* F3 and F4 fractions, obtained from the biomass dried at 40, 50, 60, 80, and 100 °C or lyophilized – L. Results are expressed as IC_{50} (mean ± SD) of the microalgal fractions. The same letters above columns symbolize significant differences between the tested fractions ($p < 0.05$). *ND – not detected.*

Table 2
Antimicrobial activities of *C. reinhardtii* F3 and F4 fractions obtained from the biomass dried at 40, 50, 60, 80, and 100 °C or lyophilized – L. The results are expressed as MIC values (µg/mL).

Fraction and condition		MIC values (µg/mL)			
		S. aureus	*K. pneumoniae*	*E. coli* 12,241	*E. coli* MG1665
F3 fraction	40 °C	1.56	0.39	0.39	0.10
	50 °C	3.13	0.78	0.20	0.39
	60 °C	3.13	0.78	0.78	0.78
	80 °C	0.78	0.78	0.20	0.10
	100 °C	3.13	0.39	50.00	0.20
	L	1.56	0.78	0.39	0.10
F4 fraction	40 °C	3.13	0.39	50.00	0.78
	50 °C	6.25	0.39	0.78	0.39
	60 °C	3.13	0.10	1.56	0.78
	80 °C	25.00	0.10	50.00	0.78
	100 °C	3.13	0.20	50.00	1.56
	L	50.00	0.39	50.00	0.78

these variations, detected compounds can be classified into two groups: fatty acids and their derivatives, and terpenoids. The most abundant fatty acid derivatives were glycero(phospho)lipids, eicosanoids, and steroids. Glycero(phospo)lipids are complex lipids consisting of a glycerol base linked to fatty acid molecules (and a phosphate group) that have a role in membrane formation, intracellular signaling paths, and energy storage, and are considered a potential therapeutic agent for cancer or metabolic disorders [66,67]. Eicosanoids, lipid-based signaling molecules derived from 20-carbon PUFAs [68], have been identified as anti-inflammatory and antimicrobial agents [69]. Since the solubility of steroids depends on their structure, their presence in the samples depends on the solubility of solvents used during extraction and fractionation processes [70]. In addition, stigmasterol and ergosterol are the most common phytosterols and are usually extracted using a two-phase solvent system [71]. Terpenoids are natural compounds derived from terpenes, compounds with a structure consisting of isoprene units [72]. Carotenoids, also known as tetraterpenoids, are natural pigments consisting of two classes: carotenes, compounds consisting only of carbon and hydrogen atoms, and xanthophylls, which also include hydroxyl groups [73]. Overall, 40 °C and 50 °C F3 samples had the greatest bioactive potential based on the number of detected compounds with predicted bioactivity. To confirm or deny these findings, a series of in vitro and in vivo assays were conducted.

The antioxidative properties of the analyzed samples varied depending on the applied drying technique and the assay used to evaluate antioxidative properties. Consistently across both F3 and F4 fractions, drying the biomass at higher temperatures (80 °C and 100 °C) resulted in a marked decline in antioxidant activity, irrespective of the assay employed. These findings underscore the critical role of biomass processing conditions in preserving the bioactive properties of *C. reinhardtii* fractions. Since the mechanisms of the used assays are based on different principles, the results differed. The highest antioxidative activity was observed in the 40 °C and lyophilized F3 samples according to the ABTS and DPPH methods. In the case of FRAP and ORAC methods, these two samples also had the best activity, but

differences between the samples were not as straightforward, suggesting that these assays may not be suitable for this type of sample. Also, a similar conclusion was previously reported, with one of the main issues being the limited solubility of the samples in the reaction mix. In particular, organic solvents, including methanol and DMSO, have been shown to influence the rate of the kinetic reaction used in ORAC assays [74]. In general, our results favor oven drying at lower temperatures (below 60 °C) to preserve antioxidative properties of *C. reinhardtii*. Similar results were also obtained for *Spirulina platensis* [62,75]. In addition, our results suggest that mixed tests, such as ABTS and DPPH, are more suitable for evaluating antioxidative properties of microalgal extracts or fractions. Our indications are supported by other studies that also recommend using these two assays [76,77].

The lowest IC_{50}, determined after measuring alpha-amylase inhibition, was observed in the 50 °C F3 sample, likely due to the presence of antidiabetic compounds such as sphingolipids (crucigasterin 277 (CNP0262292) and obscuraminol A (CNPO277279)). Nevertheless, notable results were also obtained for other F3 fractions, with IC_{50} values ranging between 80 and 90 μg/mL, presumably due to the presence of fatty acids and carotenoids [78]. F4 fractions exhibited higher IC_{50} values, indicating their lower antidiabetic potential. Also, samples dried at high temperatures (80 and 100 °C) resulted in an approximately two-fold higher IC_{50} than those dried at lower temperatures ($\leq$ 60 °C). Despite the lack of studies investigating the antidiabetic activity of microalgae, terpenoids and steroids observed in several microalgal species (e.g., *Nannochloropsis* oculata, *Chlorella vulgaris*, *Spirulina platensis*, *C. reinhardtii*) are considered as main contributors to their antidiabetic properties [79,80]. IC_{50} values for pancreatic lipase inhibition (179.07–268.67 μg/mL for F3 and 223.53–446.85 μg/mL for F4 fractions) showed lower antilipidemic than antidiabetic potential of the fractions, indicating that the post-cultivation processing of the biomass has a greater impact on the antilipidemic properties. To our knowledge, studies investigating antilipidemic properties of microalgae using in vitro assays are very scarce. Still, some microalgae (e.g., *Spirulina platensis*, *Chlorella vulgaris*, *Isochrysis galbana*, *Parachlorella beijerinckii*, *Dunaliella*, *Porphyridium*, and *Nannochloropsis oculata*) have shown hypolipidemic properties in different animal models, such as mice, rats, rabbits, chickens, and bucks, presumably due to the presence of EPA, DHA, γ-linolenic acid, and pigments such as phycocyanin, phycocyanobilin, bilirubin, and biliverdin [81,82].

Among the analyzed bioactive properties, F3 fractions seem to have the lowest neuroprotective potential. Samples with the highest neuroprotective potential were those prepared from the biomass dried at 60 °C or lyophilized. Still, according to the classification of natural extracts/fractions based on their potency to inhibit acetylcholinesterase, *C. reinhardtii* F3 fractions are considered low potency inhibitors ($200 < IC_{50} < 1000$ μg/mL) [83]. Neuroprotective properties of several microalgal species, e.g., *Nitzschia amabilis*, *Tisochrysis lutea*, *Dunaliella salina*, *Chlorella vulgaris*, *Spirulina platensis*, *Haematococcus lacustris*, have been investigated, with results suggesting that carotenoids may be responsible for their activity [84]. However, none of those studies used the extraction and fractionation conditions presented in this paper, indicating that these methods may not be suitable for extracting compounds with neuroprotective properties, such as astaxanthin or lutein [85].

C. reinhardtii fractions inhibited the growth of all four tested bacteria, but the results varied based on the analyzed bacterial strain or sample. While F3 fractions exhibited higher antimicrobial activity than F4 fractions, this was not the case for *K. pneumoniae*, where MIC values for F4 fractions were lower than for F3 fractions. Unexpectedly, a positive correlation was observed between *K. pneumoniae* MIC values and the number of detected compounds with potential antimicrobial properties, indicating that the greater number of compounds does not enhance the antimicrobial effect in the case of this bacterium. Moreover, *C. reinhardtii* fractions caused greater growth inhibition of Gram-negative than Gram-positive bacteria, regardless of the applied drying treatment and at very low concentrations, indicating their exceptional antimicrobial potential. Overall, the observed differences in MIC values can be attributed to the differences in their cell wall structures and the activity of efflux pumps, transport proteins positioned in the bacterial cytoplasmic membrane regulating the transport of toxic substances [86,87]. Previously, the antimicrobial activity of *C. reinhardtii* extracts was tested in several studies using the disk diffusion method, indicating potent antimicrobial activities against Gram-positive and Gram-negative bacteria [88,89]. This study extended these findings by determining MIC values for *C. reinhardtii* fractions. Besides, many microalgae exhibit antimicrobial properties, including *Chlorella vulgaris*, *Isochrysis galbana*, *Scenedesmus* sp., *Dunaliella salina*, *Cosmarium* sp., *Coccomyxa onubensis*, and *Spirulina platensis* [90–92].

ZET assay is a widely used method to evaluate the developmental and acute toxicity of substances, including microalgal samples. It assesses the effects on zebrafish embryos and larvae, providing insights into potential teratogenic and lethal outcomes. While in vivo studies using the ZET method to assess the toxicity of *C. reinhardtii* are still scarce, existing research on other microalgal species offers valuable context. For example, chloroform extracts of *Plectonema* and *Tolypothrix* have been reported to cause developmental abnormalities in zebrafish embryos at concentrations of 50 and 100 μg/mL [93]. Similarly, cyanotoxins derived from *Nostoc* sp., *Desertifilum tharense*, and *Anagnostidinema amphibium* induced pericardial edema, and tail and spine bending [94]. Indole alkaloids extracted from the *Fischerella* genus were also shown to be toxic for zebrafish embryos [95], as well as amphidinols from the microalga *Amphidinium carterae* [77]. However, bioactive exopolysaccharides from *Porphyridium cruentum* (*purpureum*) were not toxic at lower tested concentrations, but caused mortality at higher concentrations [96]. These findings underscore the importance of evaluating the toxicity of microalgal samples to ensure their safety for potential applications.

To our knowledge, in vivo studies of *C. reinhardtii* fractions on zebrafish embryos have not been conducted until now. Alvarez et al. [97] investigated the impact of *C. reinhardtii* after injection into *D. rerio* embryos, showing no significant inflammation. Our results showed that some of the F3 fractions (50–100 °C and lyophilized) caused developmental abnormalities at 150 μg/mL, indicating that these samples may also contain toxins. This result is somewhat expected since it was shown that the production of algal toxins may vary among strains [98]. In addition, microalgae are capable of producing polyketide-derived compounds which can cause malformations and death in fish [99]. Moreover, microalgae can also produce toxins with peptide, alkaloid, and alkyl-phenol structures [100]. Some of these compounds were detected in *C. reinhardtii* fractions, mainly those prepared from the over-dried biomass. However, one of the most biologically potent samples (40 °C F3) caused no abnormalities at the highest tested concentrations, showing that the drying of the biomass at 40 °C may be the most appropriate drying method for *C. reinhardtii* biomass.

Overall, our results indicate that low-temperature drying (< 60 °C) more effectively preserves key *C. reinhardtii* bioactive compounds, such as fatty acids and terpenoids, presumably due to shorter drying and reduced oxidative degradation. In general, freeze-drying is considered optimal for preserving bioactive compounds and minimizing thermal degradation [101]. However, longer drying times compared to low-temperature drying (< 60 °C) and extended exposure of oxidation-prone compounds, such as unsaturated fatty acids and terpenoids, to oxygen and moisture could potentially lead to degradation of bioactive compounds in lyophilized biomass and reduced bioactivity. To enhance the clarity of our results, we provided two radar charts showing the relationship between drying parameters, the number of detected compounds, bioactive properties, and toxicity of *C. reinhardtii* fraction (Fig. S3).

5. Conclusions

Results presented in this systematic paper provide valuable insight into the biological potential of microalga *C. reinhardtii* and the possibility for enhancing its properties through processing optimization. After the cultivation in optimized conditions, which include the 3N-BBM+V medium and warm white LED light, the chemical and biological properties of *C. reinhardtii* fractions prepared from differently processed biomasses were analyzed. Overall, lower drying temperatures (< 60 °C) are more effective in preserving the bioactive properties of *C. reinhardtii* in comparison to high-temperature drying or lyophilization. *C. reinhardtii* fractions demonstrated notable antidiabetic, antimicrobial, antioxidative, and antilipidemic properties. This drying method is also simpler and cheaper because it does not require the use of expensive vacuum systems and long drying times and is, consequently, more cost-efficient for the industrial production of functional foods and other products with *C. reinhardtii*. Our findings also present a great potential for competitive funding opportunities, particularly those supporting research within biotechnology and development of sustainable industrial processes. Overall, based on the results presented in this paper, after conducting various in vitro and in vivo assays, *C. reinhardtii* has a promising potential to be used in novel nutraceutical and pharmaceutical products. In addition, it is important that in future studies we identify additional fractions, investigate their bioavailability, and determine how functional microalgal compounds are metabolized in humans.

CRediT authorship contribution statement

Tamara Vujović: Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Sanja Babić Brčić:** Writing – review & editing, Methodology, Investigation. **Tina Paradžik:** Writing – review & editing, Methodology, Investigation. **Marija Miloš:** Methodology, Investigation. **Maja Galić Perečinec:** Methodology, Investigation. **Lucija Vranjković:** Methodology, Investigation. **Antonio Starčević:** Methodology, Investigation. **Krunoslav Bojanić:** Formal analysis. **Rozelindra Čož-Rakovac:** Resources, Funding acquisition. **Mirela Ivančić Šantek:** Supervision. **Ivančica Strunjak-Perović:** Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: There are no additional relationships or activities to declare. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2025.104354.

Data availability

Data will be made available on request.

References

[1] T. Huarancca Reyes, C. Chiellini, E. Barozzi, C. Sandoval, C. Echeverría, L. Guglielminetti, Exploring the physiological multiplicity of native microalgae from the Ecuadorian Highland, Italian lowland and indoor locations in response to UV-B, Int. J. Mol. Sci. 24 (2023), https://doi.org/10.3390/ijms24021346.
[2] M. Kaur, S. Bhatia, U. Gupta, E. Decker, Y. Tak, M. Bali, V.K. Gupta, R.A. Dar, S. Bala, Microalgal bioactive metabolites as promising implements in nutraceuticals and pharmaceuticals: inspiring therapy for health benefits, Phytochem. Rev. (2023), https://doi.org/10.1007/s11101-022-09848-7. Springer Netherlands.
[3] S. Ramos-Romero, J.R. Torrella, T. Pagès, G. Viscor, J.L. Torres, Edible microalgae and their bioactive compounds in the prevention and treatment of metabolic alterations, Nutrients 13 (2021) 1–16, https://doi.org/10.3390/nu13020563.
[4] B. Rana, M. Bhattacharyya, B. Patni, M. Arya, G.K. Joshi, The realm of microbial pigments in the food color market, Front. Sustain. Food Syst. 5 (2021) 1–14, https://doi.org/10.3389/fsufs.2021.603892.
[5] A. Masi, F. Leonelli, V. Scognamiglio, G. Gasperuzzo, A. Antonacci, M.A. Terzidis, Chlamydomonas reinhardtii: a factory of nutraceutical and food supplements for human health, Molecules 28 (2023) 1–22, https://doi.org/10.3390/molecules28031185.
[6] M. Silva, F. Kamberovic, S.T. Uota, I.M. Kovan, C.S.B. Viegas, D.C. Simes, K. N. Gangadhar, J. Varela, L. Barreira, Microalgae as potential sources of bioactive compounds for functional foods and pharmaceuticals, Appl. Sci. 12 (2022), https://doi.org/10.3390/app12125877.
[7] T. Vujović, T. Paradžik, S. Babić Brčić, R. Piva, Unlocking the therapeutic potential of algae-derived compounds in hematological malignancies, Cancers (Basel) 17 (2025), https://doi.org/10.3390/cancers17020318.
[8] B. Chakraborty, K. Gayen, T.K. Bhowmick, Transition from synthetic to alternative media for microalgae cultivation: a critical review, Sci. Total Environ. 897 (2023), https://doi.org/10.1016/j.scitotenv.2023.165412.
[9] R. Dixit, S. Singh, M.K. Enamala, A. Patel, Effect of various growth medium on the physiology and De novo lipogenesis of a freshwater microalga Scenedesmus rotundus-MG910488 under autotrophic condition, Clean Technol. 4 (2022) 733–751, https://doi.org/10.3390/cleantechnol4030045.
[10] X. Li, S. Slavens, D.W. Crunkleton, T.W. Johannes, Interactive effect of light quality and temperature on Chlamydomonas reinhardtii growth kinetics and lipid synthesis, Algal Res. 53 (2021) 1–25, https://doi.org/10.1016/j.algal.2020.102127.
[11] P.S.C. Schulze, H.G.C. Pereira, T.F.C. Santos, L. Schueler, R. Guerra, L.A. Barreira, J.A. Perales, J.C.S. Varela, Effect of light quality supplied by light emitting diodes (LEDs) on growth and biochemical profiles of Nannochloropsis oculata and Tetraselmis chuii, Algal Res. 16 (2016) 387–398, https://doi.org/10.1016/j.algal.2016.03.034.
[12] H.-H. Lee, Q. Xing, J.-S. Park, H. Lee, C. Yarish, J.K. Kim, Effects of different artificial photosynthetically active radiation (PAR) sources and intensity on the growth and nutrient uptake in Ulva prolifera and Neopyropia yezoensis, Algal Res. 72 (2023) 103151, https://doi.org/10.1016/j.algal.2023.103151.
[13] E.S.J. Thoré, K. Muylaert, M.G. Bertram, T. Brodin, Microalgae, Curr. Biol. 33 (2023) R91–R95, https://doi.org/10.1016/j.cub.2022.12.032.
[14] H. Sun, Y. Wang, Y. He, B. Liu, H. Mou, F. Chen, S. Yang, Microalgae-derived pigments for the food industry, Mar. Drugs 21 (2023), https://doi.org/10.3390/md21020082.
[15] I. Barkia, N. Saari, S.R. Manning, Microalgae for high-value products towards human health and nutrition, Mar. Drugs 17 (2019) 1–29, https://doi.org/10.3390/md17050304.
[16] H.S. Mostafa, M.M. Hashem, Microalgae as a source of carotenoids in foods, obstacles and solutions, springer, Netherlands (2024), https://doi.org/10.1007/s11101-024-10039-9.
[17] M.A. Scaife, G.T.D.T. Nguyen, J. Rico, D. Lambert, K.E. Helliwell, A.G. Smith, Establishing Chlamydomonas reinhardtii as an industrial biotechnology host, Plant J. 82 (2015) 532–546, https://doi.org/10.1111/tpj.12781.
[18] N.T. Tran, R. Kaldenhoff, Achievements and challenges of genetic engineering of the model green alga Chlamydomonas reinhardtii, Algal Res. 50 (2020) 101986, https://doi.org/10.1016/j.algal.2020.101986.
[19] M.J. Torres, C.M. Bellido-Pedraza, A. Llamas, Applications of the microalgae Chlamydomonas and its bacterial consortia in detoxification and bioproduction, Life 14 (2024), https://doi.org/10.3390/life14080940.
[20] L. Stoffels, A. Finlan, G. Mannall, S. Purton, B. Parker, Downstream processing of *Chlamydomonas reinhardtii* TN72 for recombinant protein recovery, Front. Bioeng. Biotechnol. 7 (2019) 1–13, https://doi.org/10.3389/fbioe.2019.00383.
[21] L. Brennan, P. Owende, Biofuels from microalgae-a review of technologies for production, processing, and extractions of biofuels and co-products, Renew. Sust. Energ. Rev. 14 (2010) 557–577, https://doi.org/10.1016/j.rser.2009.10.009.
[22] K. Ma, L. Deng, H. Wu, J. Fan, Towards green biomanufacturing of high-value recombinant proteins using promising cell factory: Chlamydomonas reinhardtii chloroplast, Bioresour. Bioprocess. 9 (2022), https://doi.org/10.1186/s40643-022-00568-6.
[23] C.A.D. Arias, C.F.M. de Oliveira, J.V.D. Molino, L.S. Ferreira-Camargo, M. C. Matsudo, J.C.M. de Carvalho, Production of recombinant biopharmaceuticals in Chlamydomonas reinhardtii, Int. J. Plant Biol. 14 (2023) 39–52, https://doi.org/10.3390/ijpb14010004.
[24] CCAP, List of Media Used to Maintain Strains at CCAP, and Other Growth Media. https://www.ccap.ac.uk/index.php/media-recipes/, 2025.

[25] S. Radman, L. Čižmek, S. Babić, A.M. Cikoš, R. Čož-Rakovac, S. Jokić, I. Jerković, Bioprospecting of less-polar fractions of Ericaria crinita and Ericaria amentacea: developmental toxicity and antioxidant activity, Mar. Drugs 20 (2022), https://doi.org/10.3390/md20010057.
[26] A. Martić, L. Cižmek, T. Ul'yanovskii, N.V. Paradžik, L. Perković, G. Matijević, T. Vujović, M. Baković, S. Babić, D.S. Kosyakov, et al., Intra-species variations of bioactive compounds of two *Dictyota* species from the Adriatic Sea: antioxidant, and neuroprotective potential, Antioxidants 12 (2023) 857, https://doi.org/10.3390/antiox12040857.
[27] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275, https://doi.org/10.1016/s0021-9258(19)52451-6.
[28] F. Eghbali Babadi, P. Boonnoun, K. Nootong, S. Powtongsook, M. Goto, A. Shotipruk, Identification of carotenoids and chlorophylls from green algae Chlorococcum humicola and extraction by liquefied dimethyl ether, Food Bioprod. Process. 123 (2020) 296–303, https://doi.org/10.1016/j.fbp.2020.07.008.
[29] H.K. Lichtenthaler, Chlorophylls and carotenoids: pigments of photosynthetic biomembranes, Methods Enzymol. 148 (1987) 350–382, https://doi.org/10.1016/0076-6879(87)48036-1.
[30] D. Vadlja, M. Bujak, R. Čož-Rakovac, M. Roje, L. Čižmek, A. Horvatić, E. Svetličić, J. Diminić, S. Rakovac, D. Oros, J. Zucko, A. Starcevic, Bioprospecting for microorganisms in Peloids—extreme environment known for its healing properties, Front. Mar. Sci. 9 (2022) 1–12, https://doi.org/10.3389/fmars.2022.822139.
[31] I.G. Munteanu, C. Apetrei, Analytical methods used in determining antioxidant activity: a review, Int. J. Mol. Sci. 22 (2021), https://doi.org/10.3390/ijms22073380.
[32] I. Lazarova, G. Zengin, O. Bender, D. Zheleva-Dimitrova, S. Uysal, R. Ceylan, R. Gevrenova, A. Aktumsek, M. Acar, M. Gunduz, A comparative study of Bulgarian and Turkish Asphodeline lutea root extracts: HPLC-UV profiles, enzyme inhibitory potentials and anti-proliferative activities against MCF-7 and MCF-10A cell lines, J. Funct. Foods 15 (2015) 254–263, https://doi.org/10.1016/j.jff.2015.03.032.
[33] M. Baković, L. Perković, G. Matijević, A. Martić, T. Vujović, S. Ekić, M. Fumić, S. Jurić, R. Čož-Rakovac, M. Roje, S. Jokić, I. Jerković, Bioprospecting of five *Ocimum* sp. cultivars from Croatia: new potential for dietary and dermatological application with embryotoxicity tests, Pharmaceuticals 16 (2023) 1–19, https://doi.org/10.3390/ph16070981.
[34] M. Mathew, S. Subramanian, In vitro screening for anti-cholinesterase and antioxidant activity of methanolic extracts of ayurvedic medicinal plants used for cognitive disorders, PLoS One 9 (2014) 1–7, https://doi.org/10.1371/journal.pone.0086804.
[35] A. Kumar, T. Darreh-Shori, DMSO: a mixed-competitive inhibitor of human acetylcholinesterase, ACS Chem. Neurosci. 8 (2017) 2618–2625, https://doi.org/10.1021/acschemneuro.7b00344.
[36] Clinical and Laboratory Standards Institute (CLSI), M07: clinical and laboratory standards institute methods for dilution antimicrobial susceptibility tests for Bacteria that grow aerobically, Clin. Lab. Stand. Inst. (2018) 91.
[37] S. Babić, L. Čižmek, A. Maršavelski, O. Malev, M. Pflieger, I. Strunjak-Perović, N. T. Popović, R. Čož-Rakovac, P. Trebše, Utilization of the zebrafish model to unravel the harmful effects of biomass burning during Amazonian wildfires, Sci. Rep. 11 (2021) 1–12, https://doi.org/10.1038/s41598-021-81789-1.
[38] OECD, Test No. 236: Fish Embryo Acute Toxicity (FET) Test., OECD Guidel. Test. Chem. Sect. 2, OECD Publ, 2013, pp. 1–22. http://www.oecd-ilibrary.org.
[39] R Core Team, R: A Language and Environment for Statistical Computing. https://www.r-project.org/, 2024.
[40] T. Aziz, A. Sarwar, Z. Daudzai, J. Naseeb, J.U. Din, U. Aftab, A. Saidal, M. Ghani, A.A. Khan, N.A.Z. Sumaira, M. Shahzad, C.U.I. Haiyang, L. Lin, Conjugated fatty acids (Cfas) production via various bacterial strains and their applications. a review, J. Chil. Chem. Soc. 67 (2022) 5445–5452, https://doi.org/10.4067/S0717-97072022000105445.
[41] A. Quaranta, J. Revol-Cavalier, C.E. Wheelock, The octadecanoids: an emerging class of lipid mediators, Biochem. Soc. Trans. 50 (2022) 1569–1582, https://doi.org/10.1042/BST20210644.
[42] S.A. Riyadi, A.A. Naini, U. Supratman, Sesquiterpenoids from Meliaceae Family and their biological activities, Molecules 28 (2023) 4874, https://doi.org/10.3390/molecules28124874.
[43] D. Pádua, E. Rocha, D. Gargiulo, A.A. Ramos, Bioactive compounds from brown seaweeds: phloroglucinol, fucoxanthin and fucoidan as promising therapeutic agents against breast cancer, Phytochem. Lett. 14 (2015) 91–98, https://doi.org/10.1016/j.phytol.2015.09.007.
[44] I.Y. López-Pacheco, V.G. Ayala-Moreno, C.A. Mejia-Melara, J. Rodríguez-Rodríguez, S.P. Cuellar-Bermudez, R.B. González-González, K.G. Coronado-Apodaca, L.I. Farfan-Cabrera, G.M. González-Meza, H.M.N. Iqbal, R. Parra-Saldívar, Growth behavior, biomass composition and fatty acid methyl esters (FAMEs) production potential of Chlamydomonas reinhardtii, and Chlorella vulgaris cultures, Mar. Drugs 21 (2023), https://doi.org/10.3390/md21080450.
[45] V. Põjo, T. Tavares, F.X. Malcata, Processing methodologies of wet microalga biomass toward oil separation: an overview, Molecules 26 (2021) 1–23, https://doi.org/10.3390/molecules26030641.
[46] B. Zhang, K. Ogden, Nitrogen balances and impacts on the algae cultivation-extraction-digestion-cultivation process, Algal Res. 39 (2019) 101434, https://doi.org/10.1016/j.algal.2019.101434.
[47] Q. Wu, L. Guo, Y. Wang, Y. Zhao, C. Jin, M. Gao, Z. She, Phosphorus uptake, distribution and transformation with Chlorella vulgaris under different trophic modes, Chemosphere 285 (2021) 131366, https://doi.org/10.1016/j.chemosphere.2021.131366.
[48] L. Yang, J. Chen, S. Qin, M. Zeng, Y. Jiang, L. Hu, P. Xiao, W. Hao, Z. Hu, A. Lei, J. Wang, Growth and lipid accumulation by different nutrients in the microalga *Chlamydomonas reinhardtii*, Biotechnol. Biofuels 11 (2018) 1–12, https://doi.org/10.1186/s13068-018-1041-z.
[49] M.A. Yaakob, R.M.S.R. Mohamed, A. Al-Gheethi, R.A. Gokare, R.R. Ambati, Influence of nitrogen and phosphorus on microalgal growth, biomass, lipid, and fatty acid production: an overview, Cells 10 (2021) 10–20.
[50] Z. Hashmi, J. Zaini, R. Abdullah, M.S.A. Bakar, M.R. Bilad, Effects of light color on growth, nutrient uptake, and harvesting of the indigenous strain of Chlorococcum sp, Bioresour. Technol. Rep. 28 (2024) 101966, https://doi.org/10.1016/j.biteb.2024.101966.
[51] M. Hultberg, H.L. Jönsson, K.J. Bergstrand, A.S. Carlsson, Impact of light quality on biomass production and fatty acid content in the microalga Chlorella vulgaris, Bioresour. Technol. 159 (2014) 465–467, https://doi.org/10.1016/j.biortech.2014.03.092.
[52] P. Das, W. Lei, S.S. Aziz, J.P. Obbard, Enhanced algae growth in both phototrophic and mixotrophic culture under blue light, Bioresour. Technol. 102 (2011) 3883–3887, https://doi.org/10.1016/j.biortech.2010.11.102.
[53] C.L. Teo, M. Atta, A. Bukhari, M. Taisir, A.M. Yusuf, A. Idris, Enhancing growth and lipid production of marine microalgae for biodiesel production via the use of different LED wavelengths, Bioresour. Technol. 162 (2014) 38–44, https://doi.org/10.1016/j.biortech.2014.03.113.
[54] D. Sharmila, A. Suresh, J. Indhumathi, K. Gowtham, N. Velmurugan, Impact of various color filtered LED lights on microalgae growth, pigments and lipid production, Eur. J. Biotechnol. Biosci. 6 (2018) 1–7. www.biosciencejournals.com.
[55] B. Lv, Z. Liu, Y. Chen, S. Lan, J. Mao, Z. Gu, A. Wang, F. Yu, X. Zheng, H. E. Vasquez, Effect of different colored LED lighting on the growth and pigment content of Isochrysis zhanjiangensis under laboratory conditions, J. Mar. Sci. Eng. 10 (2022), https://doi.org/10.3390/jmse10111752.
[56] S.V. Shah, B.Y. Lamba, R. Sharma, A.K. Tiwari, A. Parveen, V. Kumar, Effect of different light on microalgae growth and its integration with carbon dioxide sequestration technologies with emphasis on biofixation by microalgae, Mater Today Proc. 73 (2023) 92–99, https://doi.org/10.1016/j.matpr.2022.09.370.
[57] Y. Sun, J. Zhang, J. Xu, J. Cao, Y. Li, The effects of different LED lights on the Main nutritional compositions of Isochrysis zhanjiangensis, Fishes 8 (2023) 1–9, https://doi.org/10.3390/fishes8030124.
[58] T. de Mooij, G. de Vries, C. Latsos, R.H. Wijffels, M. Janssen, Impact of light color on photobioreactor productivity, Algal Res. 15 (2016) 32–42, https://doi.org/10.1016/j.algal.2016.01.015.
[59] V. Bialevich, V. Zachleder, K. Bišová, The effect of variable light source and light intensity on the growth of three algal species, Cells 11 (2022), https://doi.org/10.3390/cells11081293.
[60] H. Aljabri, M. Cherif, S.A. Siddiqui, T. Bounnit, I. Saadaoui, Evidence of the drying technique's impact on the biomass quality of Tetraselmis subcordiformis (Chlorophyceae), Biotechnol. Biofuels Bioprod. 16 (2023) 1–11, https://doi.org/10.1186/s13068-023-02335-x.
[61] T. Wang, L. Zhu, L. Mei, H. Kanda, Extraction and separation of natural products from microalgae and other natural sources using liquefied dimethyl ether, a green solvent: a review, Foods 13 (2024), https://doi.org/10.3390/foods13020352.
[62] A. Hernández, M. González-Moya, A. Márquez, L. Acevedo, Review microalgae drying: a comprehensive exploration from conventional air drying to microwave drying methods, Future Foods 10 (2024), https://doi.org/10.1016/j.fufo.2024.100420.
[63] C.Y.B. de Oliveira, T.L. Viegas, R.G. Lopes, H. Cella, R.S. Menezes, A.T. Soares, N. R. Antoniosi Filho, R.B. Derner, A comparison of harvesting and drying methodologies on fatty acids composition of the green microalga Scenedesmus obliquus, Biomass Bioenergy 132 (2020) 105437, https://doi.org/10.1016/j.biombioe.2019.105437.
[64] M. Amin, P. Chetpattananondh, C.-K. Cheng, S.K. Sami, M.N. Khan, Drying characteristics and impacts on quality of marine *Chlorella* sp. biomass and extracts for fuel applications, J. Environ. Chem. Eng. 9 (2021) 106386, https://doi.org/10.1016/j.jece.2021.106386.
[65] D. Karmakar, Kinetic study and modelling of drying of *Chlorella vulgaris*, Heliyon 10 (2024) e38441, https://doi.org/10.1016/j.heliyon.2024.e38441.
[66] D.R. Voelker, Glycerolipid Structure, Function, and Synthesis in Eukaryotes, 2nd ed, Elsevier Inc., 2013, https://doi.org/10.1016/B978-0-12-378630-2.00510-7.
[67] D.J. Stephenson, L.A. Hoeferlin, C.E. Chalfant, Lipidomics in translational research and the clinical significance of lipid-based biomarkers, Transl. Res. 189 (2017) 13–29, https://doi.org/10.1016/j.trsl.2017.06.006.
[68] A.S. Salsinha, R. Socodato, J.B. Relvas, M. Pintado, Chapter 3 - the pro- and antiinflammatory activity of fatty acids, in: M. Pintado, M. Machado, A. M. Gomes, A.S. Salsinha, L.M. Rodríguez-Alcalá (Eds.), Bioact. Lipids, Elsevier, 2023, pp. 51–75, https://doi.org/10.1016/C2020-0-01077-7.
[69] A.E.F. Sheppe, M.J. Edelmann, Roles of eicosanoids in regulating inflammation and neutrophil migration as an innate host response to bacterial infections, Infect. Immun. 89 (2021), https://doi.org/10.1128/IAI.00095-21.
[70] M.M. Stevens, A.R. Honerkamp-Smith, S.L. Keller, Solubility limits of cholesterol, lanosterol, ergosterol, stigmasterol, and β-sitosterol in electroformed lipid vesicles, Soft Matter 6 (2010) 5882–5890, https://doi.org/10.1039/c0sm00373e.
[71] L. Huang, Y. Cao, H. Xu, G. Chen, Separation and purification of ergosterol and stigmasterol in Anoectochilus roxburghii (wall) Lindl by high-speed counter-current chromatography, J. Sep. Sci. 34 (2011) 385–392, https://doi.org/10.1002/jssc.201000577.

[72] A. Masyita, R. Mustika Sari, A. Dwi Astuti, B. Yasir, N. Rahma Rumata, T. Bin Emran, F. Nainu, J. Simal-Gandara, Terpenes and terpenoids as main bioactive compounds of essential oils, their roles in human health and potential application as natural food preservatives, Food Chem. X 13 (2022) 100217, https://doi.org/10.1016/j.fochx.2022.100217.
[73] S.E. Thomas, E.J. Johnson, Xanthophylls, Adv. Nutr. 9 (2018) 160–162, https://doi.org/10.1093/advances/nmx005.
[74] J.R.B. Carvalho, A.N. Meireles, S.S. Marques, B.J.R. Gregório, I.I. Ramos, E.M. P. Silva, L. Barreiros, M.A. Segundo, Exploiting kinetic features of ORAC assay for evaluation of radical scavenging capacity, Antioxidants 12 (2023) 1–14, https://doi.org/10.3390/antiox12020505.
[75] F. de F. Neves, M. Demarco, G. Tribuzi, Drying and quality of microalgal powders for human alimentation, in: Microalgae - From Physiol. to Appl, IntechOpen, 2019, https://doi.org/10.5772/intechopen.89324.
[76] N. Coulombier, T. Jauffrais, N. Lebouvier, Antioxidant compounds from microalgae: a review, Mar. Drugs 19 (2021), https://doi.org/10.3390/md19100549.
[77] N. Yang, Q. Zhang, J. Chen, S. Wu, R. Chen, L. Yao, B. Li, X. Liu, R. Zhang, Z. Zhang, Study on bioactive compounds of microalgae as antioxidants in a bibliometric analysis and visualization perspective, Front. Plant Sci. 14 (2023) 1–15, https://doi.org/10.3389/fpls.2023.1144326.
[78] K. Tamel Selvan, J.A. Goon, S. Makpol, J.K. Tan, Therapeutic potentials of microalgae and their bioactive compounds on diabetes mellitus, Mar. Drugs 21 (2023), https://doi.org/10.3390/md21090462.
[79] A.M. Abo-Shady, S.F. Gheda, G.A. Ismail, J. Cotas, L. Pereira, O.H. Abdel-Karim, Antioxidant and antidiabetic activity of algae, Life 13 (2023) 1–26, https://doi.org/10.3390/life13020460.
[80] S. Sahoo, M. Samantaray, M. Jena, M. Gosu, P.P. Bhuyan, D. Shin, B. Pradhan, In vitro and in silico studies to explore potent antidiabetic inhibitor against human pancreatic alpha-amylase from the methanolic extract of the green microalga *Chlorella vulgaris*, J. Biomol. Struct. Dyn. 42 (2024) 8089–8099, https://doi.org/10.1080/07391102.2023.2244592.
[81] C. Zhao, Y. Wu, C. Yang, B. Liu, Y. Huang, Hypotensive, hypoglycaemic and hypolipidaemic effects of bioactive compounds from microalgae and marine micro-organisms, Int. J. Food Sci. Technol. 50 (2015) 1705–1717, https://doi.org/10.1111/ijfs.12860.
[82] F. Nasirian, H. Sarir, N. Moradi-Kor, Antihyperglycemic and antihyperlipidemic activities of Nannochloropsis oculata microalgae in Streptozotocin-induced diabetic rats, Biomol. Concepts 10 (2019) 37–43, https://doi.org/10.1515/bmc-2019-0004.
[83] T.C. Dos Santos, T.M. Gomes, B.A.S. Pinto, A.L. Camara, A.M. De Andrade Paes, Naturally occurring acetylcholinesterase inhibitors and their potential use for Alzheimer's disease therapy, Front. Pharmacol. 9 (2018) 1–14, https://doi.org/10.3389/fphar.2018.01192.
[84] K. Odenthal, E. Nunes, N. Nunes, T. Fernandes, I.A. Fernandes, M.A.A. Pinheiro de Carvalho, Microalgae and cyanobacteria as natural sources of antioxidant enzymes and enzyme inhibitors for Alzheimer's and diabetes, Algal Res. 82 (2024), https://doi.org/10.1016/j.algal.2024.103610.
[85] J.Y. Lee, C.Y. Wong, R.Y. Koh, C.L. Lim, Y.Y. Kok, S.M. Chye, Natural bioactive compounds from macroalgae and microalgae for the treatment of Alzheimer's disease: a review, Yale J. Biol. Med. 97 (2024) 205–224, https://doi.org/10.59249/JNKB9714.
[86] S.N. Vahdati, H. Behboudi, S. Tavakoli, F. Aminian, R. Ranjbar, Antimicrobial potential of the green microalgae isolated from the Persian Gulf, Iran. J. Public Health 51 (2022) 1134–1142, https://doi.org/10.18502/ijph.v51i5.9428.
[87] N. Kadeřábková, A.J.S. Mahmood, D.A.I. Mavridou, Antibiotic susceptibility testing using minimum inhibitory concentration (MIC) assays, NPJ Antimicrob. Resist. 2 (2024) 37, https://doi.org/10.1038/s44259-024-00051-6.
[88] K.P. Renukadevi, P.S. Saravana, J. Angayarkanni, Antimicrobial and antioxidant activity of Chlamydomonas reinhardtii sp, Int. J. Pharm. Sci. Res. 2 (2011) 1467–1472, https://doi.org/10.13040/IJPSR.0975-8232.2(6).
[89] H.A. Ghaidaa, H.Z. Neihaya, Z.M. Nada, M.A. Amna, The biofilm inhibitory potential of compound produced from Chlamydomonas reinhardtii against pathogenic microorganisms, Baghdad Sci. J. 17 (2020) 34–41, https://doi.org/10.21123/bsj.2020.17.1.0034.
[90] A.M.E. Abdel-Moneim, M.T. El-Saadony, A.M. Shehata, A.M. Saad, S.A. Aldhumri, S.M. Ouda, N.M. Mesalam, Antioxidant and antimicrobial activities of *Spirulina platensis* extracts and biogenic selenium nanoparticles against selected pathogenic bacteria and fungi, Saudi Aust. J. Biol. Sci. 29 (2022) 1197–1209, https://doi.org/10.1016/j.sjbs.2021.09.046.
[91] F. Alsenani, K.R. Tupally, E.T. Chua, E. Eltanahy, H. Alsufyani, H.S. Parekh, P. M. Schenk, Evaluation of microalgae and cyanobacteria as potential sources of antimicrobial compounds, Saudi Pharm. J. 28 (2020) 1834–1841, https://doi.org/10.1016/j.jsps.2020.11.010.
[92] S.M. Little, G.N.A. Senhorinho, M. Saleh, N. Basiliko, J.A. Scott, Antibacterial compounds in green microalgae from extreme environments: a review, Algae 36 (2021) 61–72, https://doi.org/10.4490/algae.2021.36.3.6.
[93] J.P. Berry, M. Gantar, P.D.L. Gibbs, M.C. Schmale, The zebrafish (Danio rerio) embryo as a model system for identification and characterization of developmental toxins from marine and freshwater microalgae, Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 145 (2007) 61–72, https://doi.org/10.1016/j.cbpc.2006.07.011.
[94] F.R. Jacinavicius, V. Geraldes, K. Fernandes, C.M. Crnkovic, W.A. Gama, E. Pinto, Toxicological effects of cyanobacterial metabolites on zebrafish larval development, Harmful Algae 125 (2023) 102430, https://doi.org/10.1016/j.hal.2023.102430.
[95] K. Walton, M. Gantar, P.D.L. Gibbs, M.C. Schmale, J.P. Berry, Indole alkaloids from Fischerella inhibit vertebrate development in the zebrafish (Danio rerio) embryo model, Toxins (Basel) 6 (2014) 3568–3581, https://doi.org/10.3390/toxins6123568.
[96] Y. Risjani, N. Mutmainnah, P. Manurung, S.N. Wulan, Yunianta, Toxic and stimulates immune response against Vibriosis: the assessment using zebrafish and white shrimp *Litopenaeus vannamei*, Mar. Drugs 19 (3) (2021) 133.
[97] M. Alvarez, N. Reynaert, M.N. Chávez, G. Aedo, F. Araya, U. Hopfner, J. Fernández, M.L. Allende, J.T. Egaña, Generation of viable plant-vertebrate chimeras, PLoS One 10 (2015) 1–12, https://doi.org/10.1371/journal.pone.0130295.
[98] A.M.N. Caruana, Z. Amzil, Microalgae and Toxins, Elsevier Inc., 2018, https://doi.org/10.1016/B978-0-12-811405-6.00013-X.
[99] X. Wang, M. Fon, A.J.C. Andersen, A. Solhaug, R.A. Ingebrigtsen, I.A. Samdal, S. Uhlig, C.O. Miles, B. Edvardsen, T.O. Larsen, Insights into the nature of ichthyotoxins from the *Chrysochromulina leadbeateri* blooms in northern Norwegian fjords, Harmful Algae 137 (2024) 1–32, https://doi.org/10.1016/j.hal.2024.102681.
[100] H. Katircioğlu, B.S. Akin, T. Atici, Microalgal toxin(s): characteristics and importance, African, J. Biotechnol. 3 (2004) 667–674.
[101] A.A. de L. Santos, G.F. Leal, M.R. Marques, L.C.C. Reis, J.R. de J. Junqueira, L. L. Macedo, J.L.G. Corrêa, Emerging drying technologies and their impact on bioactive compounds: a systematic and bibliometric review, Appl. Sci. 15 (2025) 1–32, https://doi.org/10.3390/app15126653.