Article

Preliminary Evaluation of Watermelon Liquid Waste as an Alternative Substrate for Microalgae Cultivation: A Circular Economy Approach to the Production of High-Value Secondary Products by *Chlorella vulgaris*, *Scenedesmus* sp., *Arthrospira platensis*, and *Chlamydomonas pitschmanii*

Paolina Scarponi [1,*], Lorenzo Fontana [1], Francesco Bertesi [1], Veronica D'Eusanio [2], Lorenzo Tassi [2] and Luca Forti [1]

1   Department of Life Sciences, University of Modena and Reggio Emilia, Via G. Campi 103, 41125 Modena, Italy; luca.forti@unimore.it (L.F.)
2   Department of Chemical and Geological Sciences, University of Modena and Reggio Emilia, Via G. Campi 103, 41125 Modena, Italy
*   Correspondence: p.scarponi@unimore.it

Abstract: In Italy, watermelon cultivation spans 9510 hectares, with production levels largely influenced by seasonal market demand. As a result, surplus watermelon left unsold by September often remain in the fields, where they decompose naturally and go to waste. A chemical analysis of the watermelon liquid fraction waste (WW) indicates a high carbohydrate concentration, highlighting the potential for biotechnological valorization of this waste stream, converting it into lipids or exopolysaccharides (EPSs). This study investigates the feasibility of utilizing WW as an alternative growth substrate for microalgae, aligning with circular economy principles and advancing sustainable agricultural practices. By repurposing agricultural byproducts, this research supports biorefinery objectives, aiming to convert biomass into high-value secondary products, including biofuels, pigments, and nutraceuticals. *Scenedesmus* and *Chlorella* strains demonstrated promising growth and adaptability in WW, achieving biomass yields of $0.95 \pm 0.07 \text{ g L}^{-1}$ and $0.37 \pm 0.02 \text{ g L}^{-1}$, respectively, with a significant EPS production observed as medium gelation. Although lipid accumulation was limited in this case by the WW substrate, the lipid profiles of both strains were distinctively altered, notably lacking linolenic acid.

Keywords: microalgae; *Chlorella vulgaris*; *Scenedesmus* sp.; watermelon liquid fraction waste; lipid; exopolysaccharides; circular economy

1. Introduction

Italian watermelon production accounts for approximately 2.2% of the total agricultural land used for vegetable cultivation, with the harvest season mainly concentrated from July to September. Due to market demand fluctuations, substantial volumes of watermelons often remain in the fields as waste [1]. Typically, this waste decomposes naturally under aerobic conditions; however, anaerobic decomposition can release ecotoxic compounds that may disrupt native soil microflora [2]. In aerobic decomposition, microorganisms break down organic matter in the presence of oxygen, which generally results in the production of carbon dioxide, water, and heat. This process is relatively eco-friendly, as it produces fewer harmful byproducts and can contribute to soil health by enriching it with organic matter and nutrients. In contrast, anaerobic decomposition occurs in environments lacking oxygen, and, under these conditions, different microorganisms metabolize organic matter, producing methane, hydrogen sulfide, and other volatile organic compounds. In particular, methane, a potent greenhouse gas, contributes to atmospheric warming, making anaerobic

decomposition an environmental concern if large volumes of watermelon waste decompose under low-oxygen conditions. Additionally, the accumulation of anaerobic byproducts can disrupt local soil ecology, affecting plant growth and microbial diversity.

Although integrating watermelon waste into a circular economy framework holds promise, knowledge about its treatment and reuse remains limited. Watermelon liquid fraction waste (WW) has shown to contain high levels of carbohydrates, vitamins, polyphenols, and water (Table 1) [3–5], indicating potential as a substrate in biotechnological processes, such as microbial and microalgal fermentation, to produce biomass for bioenergy. Specifically, with a carbon-to-nitrogen (C/N) ratio of 21.59%, WW may favor lipid accumulation in microalgal biomass, further enhancing its bioenergy applications [2].

Table 1. Chemical–physical characterization of WW.

Citric Acid (g% mL^{-1})	**0.09 $\pm$ 0.00**
Soluble solid ($^{\circ}$Brix)	2.3 $\pm$ 0.1
pH	4.83–5.40
Polyphenols (mg L^{-1})	37.6 $\pm$ 0.58
DPPH (mg$_{\text{Trolox eq.}}$ 100 g$_{\text{dry weight}}$$^{-1}$)	150–160
DPPH (%)	41.5 $\pm$ 1.8
Moisture (%)	92–93
Protein (%)	3.53–4.85
Lipid (%)	1.50–3.13
Carbohydrate (%)	39.46–55.52
Ash (%)	5.85–7.48
Vitamin A (mg g$_{\text{db}}$$^{-1}$)	0.33–0.39
Vitamin C (mg g$_{\text{db}}$$^{-1}$)	0.06–0.07
Tiammin (mg g$_{\text{db}}$$^{-1}$)	0.01
Riboflavin (mg g$_{\text{db}}$$^{-1}$)	0.0005–0.0009
Niacin (mg g$_{\text{db}}$$^{-1}$)	0.0008–0.0011
Total Polyphenolic Content (TPC) (mg $_{\text{a. clorogenic eq.}}$ Kg$_{\text{wb}}$$^{-1}$)	341.15 $\pm$ 3.74
Citrullin (mg$_{\text{citrulline}}$% g$_{\text{db}}$$^{-1}$)	60–80
Total Flavonoid Content (TFC) (mg$_{\text{quercitin eq.}}$% g$_{\text{db}}$$^{-1}$)	280–350

db = dry basis; wb = wet basis.

Previous studies have demonstrated WW's effectiveness in supporting biomass production with organisms like *Aspergillus niger* and *Mucor* sp., indicating its potential for applications in animal feed [6], biohydrogen synthesis, lipid production [7], bioethanol [8], polyhydroxyalkanoates [2], and fungal biomass production [9]. However, these studies have primarily focused on bacterial and fungal fermentation, leaving the potential of microalgae for WW biodegradation largely unexplored. Microalgae species such as *Chlorella* sp., *Scenedesmus* sp., *Chlamydomonas* sp., and *Arthrospira* sp. are well known for their ability to treat organic waste within a circular economy framework. These species offer advantages in bioremediation, lipid synthesis for third-generation biofuels, and high protein content [10–12]. This study aims to evaluate the potential of these microalgae species in treating watermelon wastewater by assessing their biomass production and the accumulation of high-value secondary products. The ultimate goal is to integrate this biotreatment into local wastewater management systems, particularly to benefit small-scale farmers in Italy, where these wastes are discharged in land disposals, limiting the land cultivation. In addition, the small microalgae treatment plant construction near the watermelon cultivation land could positively influence the agricultural production, with the waste treatment, land use for other superior plant cultivation, and microalgae biomass production, creating new income for watermelon cultivators.

2. Materials and Methods

2.1. Microalgae Strains

The following microalgae strains were obtained from the Algal Collection University Federico II (ACUF)—Naples, Italy: *Chlorella vulgaris* Beij 863, *Scenedesmus* sp. 145, *Chlamydomonas pitschmannii* Ettl 292, and *Arthrospira platensis* (commonly known as Spirulina). The strains were maintained at room temperature (25 °C) under continuous agitation at 150 rpm, exposed to white light irradiation (1364 lux), and supplied with air via a Sera air 275 R plus air pump (140 mbar pressure, 4W, 275 L h^{-1}, Heinsberg, Germany). The growth medium used was Bold's Basal Medium with vitamins (BBM+V), which was prepared by adding the following components: 10 mL of $NaNO_3$ (25 g L^{-1}), $CaCl_2 \cdot 2\ H_2O$ (2.5 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (7.5 g L^{-1}), $K_2HPO_4 \cdot 3H_2O$ (7.5 g L^{-1}), KH_2PO_4 (17.5 g L^{-1}), and NaCl (2.5 g L^{-1}). Additionally, 1 mL of a aqueous vitamin mix solution (thiamine 0.1 g 100 mL^{-1}, biotin 25×10^{-6} g 100 mL^{-1}, vitamin B12 15×10^{-6} g 100 mL^{-1}) and 6 mL of a metal solution ($FeCl_3 \cdot 6H_2O$ 0.097 g L^{-1}, $MnCl_2 \cdot 4H_2O$ 0.041 g L^{-1}, $ZnCl_2$ 0.005 g L^{-1}, $CoCl_2 \cdot 6H_2O$ 0.002 g L^{-1}, $Na_2MoO_4 \cdot 2H_2O$ 0.004 g/L) were added. All media components and reagents were purchased from CARLO ERBA Reagents (Cornaredo, Italy), or Merck KGaA (Darmstadt, Germany).

2.2. Watermelon Liquid Fraction Waste (WW)

Watermelon waste was recovered by an agricultural center in Italy (Modena) as reported by Maletti et al. [13]. The liquid fraction of watermelon waste pulp was recovered through centrifugation at 4500 rpm for 10 min (Neya 16 XS, Remi Elektrotechnik LTD., Mumbai, India). Following centrifugation, the liquid fraction was filtered using Whatman paper filters. Prior to use, the liquid fraction was autoclaved to eliminate any fungal or bacterial contaminants. The WW autoclaved chemical characteristics were reported in a study conducted by Maletti et al. [13], where the total soluble carbohydrates resulted in 32.6 $\pm$ 1.2%. An elementary composition (C and N) was performed using an Organic Elemental Analyzer (FLASH2000, configuration CHNS, Thermos Fisher Scientific, Waltham, MA, USA) using He as a carrier and reference with flows of 140 mL min^{-1} and 100 mL min^{-1}, respectively. Oxygen flux, for combustion, was set to 250 mL min^{-1} for 3 s. The oven temperature was set at 950 °C. When the oxygen was injected, the temperature was 1800 °C, and the gasified sample was analyzed using a GC column (PTFE: 2 m 6 $\times$ 5 mm) with a TCD detector (65 °C). An amount of 1–2 mg of dry matter (WW diluted 1:10 in BBM+V) was used as the sample. The total time run was 720 s. The K factor was applied as a calibration method using 2,5-Bis(5-tert-butyl-2-benzo-oxazol-2-yl) (BBOT) as standard. The same analysis was performed on liquid fraction after microalgae proliferation.

2.3. Experimental Set Up

The experiment was carried out in the laboratory of Modena and Reggio Emilia University (Modena, Italy). WW was used as a substrate for microalgae cultivation at a 1:10 dilution ratio, with the BBM+V medium used to dilute the waste. The inoculum concentrations were as follows: *Chlorella* at 1.04 $\pm$ 0.00 million cells mL^{-1} and 0.03 $\pm$ 0.00 g L^{-1}; *Scenedesmus* at 1.08 $\pm$ 0.34 million cells mL^{-1} and 0.04 $\pm$ 0.00 g L^{-1}; *Chlamydomonas* at 2.78 $\pm$ 0.00 g L^{-1}; and *Spirulina* at 14.37 $\pm$ 0.00 g L^{-1}. Continuous mechanical agitation (150 rpm), light irradiation (2500 lux), and air bubbling (275 L h^{-1}) were applied throughout the experiment. The batch test was conducted over 7 days, corresponding to the stationary phase observed under control conditions (BBM+V). A negative control was performed using only WW diluted 1:10 without any microalgae inoculum. An additional negative control involved autoclaved WW diluted 1:10 with BBM+V, tested with and without aeration for 7 days. At the end of the experiment, no changes in medium density or pH were detected.

To evaluate the necessity of waste sterilization, non-sterilized WW was used at a 1:10 dilution as a substrate for the microalgae strains under aerobic conditions. In this case,

biological contamination by mold was observed within 24 h, with the formation of black mold colonies and the subsequent death of the microalgae strains.

2.4. Biomass Monitoring

The daily monitoring of microalgae growth was conducted through cell count analysis using a Bürker chamber and dry weight measurements. The growth rate was calculated with Equation (1), as follows:

$$Growth\ rate\ \left(\mu^{-1}\right) = \frac{Ln\ X_f - LnX_i}{t_f - t_i} \tag{1}$$

At the end of the experiment, total lipid quantification was performed on lyophilized biomasses using the Bligh and Dyer methodology [14] (Heto LyoLab 3000, Analitica de Mori, Milan, Italy). The pH at the end of the test was measured with a litmus test. Chlorophyll a, chlorophyll b, and carotenoids were quantified following the Costache et al. [15] methodology.

For pigment extraction, 1 mL of microalgae biomass sample was treated with acetone as the solvent, with the extraction carried out at 40 °C for 5 min. The resulting liquid fraction was then analyzed at 662 nm, 645 nm, and 470 nm using a UV-VIS spectrophotometer (Jasco V-770, Cremella (LC), Italy). The quantification of pigments was calculated using Equations (2)–(4).

$$Chlorophyll\ a = 11.75 \cdot Abs_{662} - 2.350 \cdot Abs_{645} \tag{2}$$

$$Chlorophyll\ b = 18.61 \cdot Abs_{645} - 3.960 \cdot Abs_{662} \tag{3}$$

$$Carotenoids = 1000 \cdot Abs_{470} - 2.270 \cdot Chl\ a - 81.4\ Chl\ b/227 \tag{4}$$

To characterize the lipid storage in microalgae biomass, the lipids extracted from microalgae biomasses were transesterified following the method performed by Jayakumar et al. [16]. Dried lipids were used as samples and diluted with a KOH solution (0.4% *v/v* in methanol) at a ratio of 0.5:1 (KOH solution/lipid). The solution was heated at 55 °C for 3 h. Afterward, 1 mL of hexane and 2 mL of water were added to the solution, which was then vortexed for 30 s. Following the separation phase, the hexane layer containing the transesterified lipids was recovered, dried, and stored at 4 °C.

The fatty acid methyl esters (FAMEs) were analyzed with an Agilent 7890B Gas Chromatograph equipped with a 25 m × 0.2 mm ID, a column with a 0.5 μm film thickness coated with a (5%)-diphenyl-(95%)-dimethylpolysiloxane copolymer as the stationary phase. The sample (1 μL) was manually injected in split mode (1:50), the injector temperature was set to 250 °C with a purge flow rate of 3 mL min^{-1}, and helium was used as the carrier gas, with a flow rate of 1.2 mL min^{-1} through the column. The column temperature was held at 130 °C for 2 min, then increased by 10 °C min^{-1} to 230 °C, where it was held for 2 min.

The column effluent was introduced into the ion source of an Agilent 5977B GC/MSD (Agilent Technologies, Santa Clara, CA, USA). The transfer line and ion source temperatures were 260 °C and 230 °C, respectively. Ions were generated by a 70 eV electron beam with an emission current of 35 μA. The acceleration voltage was applied after a solvent delay of 240 s, and the detector voltage ranged from 1500 to 2000 V.

3. Results

3.1. Biomass Growth

Biomass monitoring (Figure 1, Table 2) revealed a significant increase in biomass production for *Scenedesmus* and *Chlorella* when cultivated with WW as a substrate. In contrast, *Spirulina* and *Chlamydomonas* showed inhibited growth. This disparity in biomass production could likely be attributed to WW's high carbohydrate concentration, which seems to selectively affect certain microalgal strains. The tolerance to high sugar levels appears strain-dependent, with *Scenedesmus* and *Chlorella* demonstrating greater adaptability.
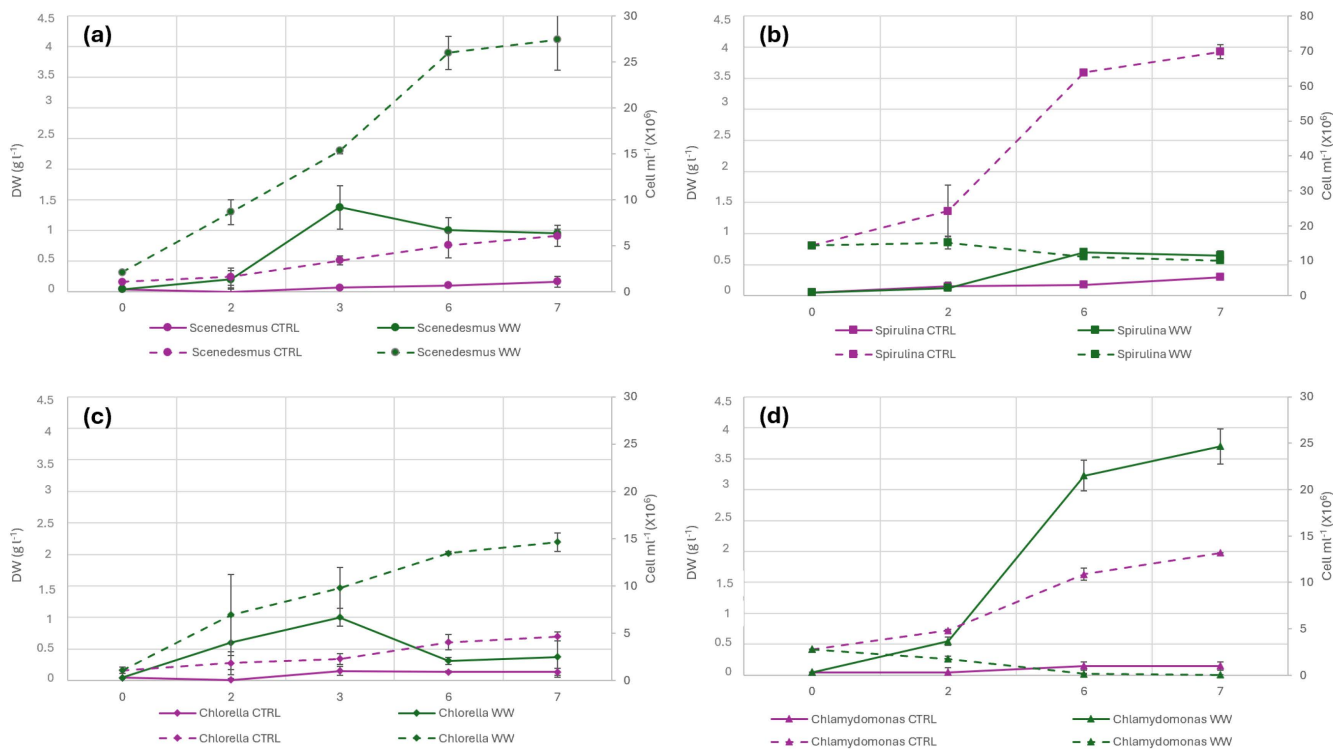
Figure 1. Cell count (dot line) and dry weight (continuous line) of (**a**) *Scenedesmus*, (**b**) *Spirulina*, (**c**) *Chlorella*, and (**d**) *Chlamydomonas* in control (CTRL) and experimental conditions (WW).

Table 2. Dry weight, cell count, and growth rate (μ) at the end of experimental tests for *Scenedesmus*, *Chlorella*, *Spirulina*, and *Chlamydomonas* in control (CTRL) and experimental (WW) conditions.

		Dry Weight (g L^{-1})	Cell Count (Million Cell mL^{-1})	μ (d^{-1})
Scenedesmus	CTRL	0.16 ± 0.09	6.07 ± 1.16	0.23 ± 0.02
	WW	0.95 ± 0.07	21.38 ± 3.36	0.40 ± 0.02
Chlorella	CTRL	0.14 ± 0.05	4.67 ± 0.49	0.21 ± 0.03
	WW	0.37 ± 0.02	14.62 ± 1.00	0.37 ± 0.04
Spirulina	CTRL	0.30 ± 0.00	69.75 ± 2.00	22 ± 0.00
	WW	0.65 ± 0.07	10.08 ± 0.12	0.00 ± 0.00
Chlamydomonas	CTRL	0.15 ± 0.07	13.17 ± 0.00	0.22 ± 0.00
	WW	3.70 ± 0.28	0.06 ± 0.04	0.00 ± 0.09

As reported by Chiranjeevi et al. [17], excess glucose can enhance biomass production in specific microalgae strains by entering various metabolic pathways such as the Embden–Meyerhof pathway, pentose phosphate pathway, and tricarboxylic acid cycles, through direct diffusion across the cell membrane. These processes provide higher energy yields, in the form of ATP and NADPH, than photosynthesis alone.

The strain-specific capacity for sugar metabolism has been widely studied, including Chandra et al. [18], Chairsilp et al. [19], Huang et al. [20], and Silaban et al. [21], who studied pure microalgal cultures or co-cultures grown on different sugars. Notably, Andreeva et al. [22] examined the growth performance of *Chlorella vulgaris*, *Arthrospira platensis* (Spirulina), and *Dunaliella salina* when fructose, sucrose, glucose, and maltose were used as substrates at concentrations ranging from 0 g/L to 5 g/L. Their findings highlighted *Chlorella*'s strong ability to tolerate high sugar concentrations. In contrast, *Spirulina* was inhibited by concentrations of maltose, sucrose, and fructose exceeding 1 g/L, although it showed some tolerance to glucose, albeit with a lower biomass density compared to the

control condition. *Dunaliella*, on the other hand, was inhibited by the addition of any added sugars compared to the control condition.

3.2. Pigment Quantification

Pigment quantification in *Scenedesmus* and *Chlorella* biomasses (Figure 2) did not differ significantly between control and experimental conditions. A similar outcome was reported by Chandra et al. [20], where the glucose addition increased biomass production without affecting pigment storage. The increase in medium density did not permit the correct sampling of microalgae biomass for *Scenedesmus* and *Chlorella* strains; for this reason, higher standard deviation was observed during this analysis. Specifically, increased medium density and gel formation observed during cultivation with WW in the medium led to biomass aggregation, making homogeneous sampling difficult for chlorophyll analysis. Such aggregation effects were also reported by Andreeva et al. [22], where sugar additions caused cell adhesion and aggregation, complicating mixing and sampling.
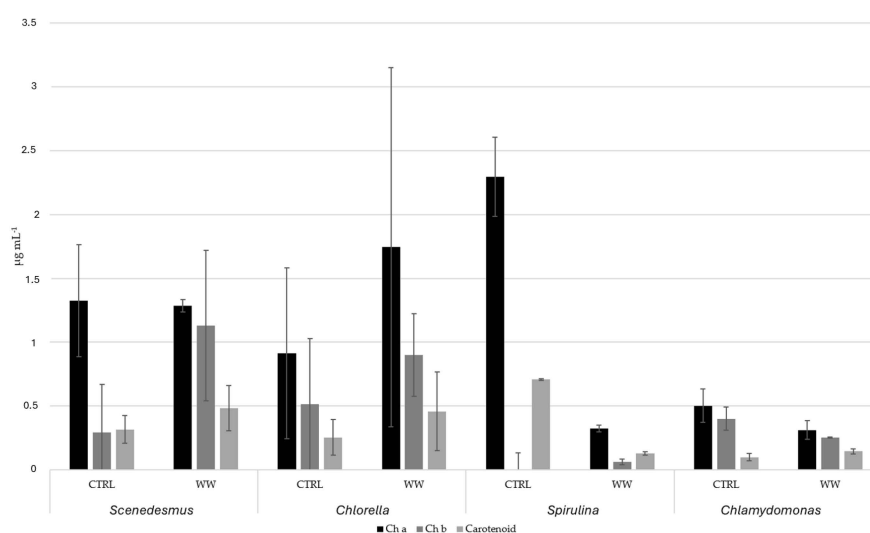

Figure 2. Chlorophyll a (Ch a), Chlorophyll b (Ch b), and Carotenoid storage in *Scenedesmus*, *Chlorella*, *Spirulina*, and *Chlamydomonas* in control (CTRL) and experimental conditions (WW).

Despite these challenges, the use of WW as a substrate for both *Scenedesmus* and *Chlorella* resulted in increased chlorophyll b content, likely due to self-shading effects. In contrast, *Spirulina* and *Chlamydomonas* biomasses showed comparable or reduced pigment storage relative to control conditions. However, the application of these strains was deemed unsuitable due to the growth inhibition observed during biomass monitoring.

Overall, pigment quantification, particularly chlorophyll content increase, was used as a growth parameter, as it allowed for the correlation of CO_2 fixation with sunlight as the energy source. Typically, higher sugar concentrations impacted chlorophyll levels and mixotrophic growth productivity, with glucose specifically influencing CO_2 fixation and altering the photosynthetic pathway, leading to changes in the pigment system. While this effect could be mitigated by using light–dark cycles, some microalgae strains exhibited negative effects on photosynthesis when glucose was added. This result highlights the simultaneous operation of organic carbon-dependent respiration and the photosynthetic process in mixotrophic metabolism [23,24].

3.3. Secondary High Value Products Storage

3.3.1. Lipid Quantification and Characterization

Lipid storage in microalgal biomass under control conditions was 20.06 ± 0.04% and 33.51 ± 8.16% for *Scenedesmus* and *Chlorella*, respectively, while lipid storage levels decreased to 13.54 ± 5.49% and 20.33 ± 0.70%, respectively, when cultivated on WW. Typically, these strains exhibit lipid storage levels of approximately 30% for *Scenedesmus* and

20% for *Chlorella* [25]. The observed decrease in lipid storage may indicate a metabolic shift in carbon flow biosynthesis pathways, as suggested by Liu et al. [26]. While the addition of CO_2 usually directs the carbon flow towards lipid biosynthesis, when these microalgae strains were in symbiotic interaction with bacteria, other high value bioproducts, such as exopolysaccharides, were instead produced. Interestingly, *Spirulina* and *Chlamydomonas* strains yielded noteworthy results. While yeast and mold contamination limited microalgae growth, the harvested biomass still demonstrated substantial lipid accumulation. *Spirulina* exhibited lipid levels of $11.25 \pm 5.30\%$ and $24.05 \pm 3.70\%$ under control and experimental conditions, respectively. *Chlamydomonas* showed lipid contents of $26.92 \pm 5.44\%$ and $24.33 \pm 2.26\%$ under control and experimental conditions, respectively. Lipid accumulation in control conditions was consistent with literature values for both *Spirulina* (11.24% to 36.36% [10,27]) and *Chlamydomonas* (~20% [28,29]). The increased lipid content observed in WW-supplemented cultures may be attributed to microbiological contamination, potentially influencing total lipid quantification and characterization. It is important to note that the contamination encountered in this experiment differed from that observed in non-sterilized WW systems, highlighting the need for further investigation to elucidate the role of microbial interactions on lipid accumulation.

Table 3 and Figure 3 provide a detailed characterization of the lipid fraction. Significant differences in lipid storage were observed when WW was used as a substrate compared to control conditions. The percentage of saturated fatty acids in *Spirulina* under control conditions ranged from 11.24% to 37.09% [10,27], which is lower in comparison to the values measured during the tests. This difference could be attributed to variations in experimental conditions. Furthermore, when WW was used as a substrate, the composition of polyunsaturated, saturated, and monounsaturated fatty acids was significantly influenced by microbial contamination. *Chlamydomonas* showed differences in lipid composition between control and WW conditions compared to literature values. The percentage of saturated fatty acids was higher than previously reported values of 1.14–23.1% under control conditions and 9.76–29.3% under stress conditions [29,30]. The percentages of saturated and mono- or polyunsaturated fatty acids for *Chlorella* and *Scenedesmus* under control conditions were consistent with literature values (>40% and <50%, respectively [25]). Notably, both *Spirulina* and *Chlorella* increased their saturated fatty acid content when cultivated on agro-waste substrates. In contrast, *Scenedesmus* and *Chlamydomonas* showed an increase in monounsaturated lipids and a decrease in polyunsaturated lipids when WW was used as a substrate. Interestingly, monounsaturated lipids were absent under control conditions for these strains.

Table 3. Polyunsaturated, saturated, and monounsaturated lipid percentage in *Spirulina*, *Chlamydomonas*, *Chlorella*, and *Scenedesmus* biomasses.

	Spirulina		*Chlamydomonas*		*Chlorella*		*Scenedesmus*	
	CTRL	WW	CTRL	WW	CTRL	WW	CTRL	WW
Polyunsaturated (%)	27.68 ± 0.47	2.19 ± 2.44	58.78 ± 0.04	12.48 ± 4.21	75.15 ± 9.26	0.86 ± 0.99	69.80 ± 5.21	3.60 ± 0.47
Saturated (%)	53.53 ± 0.06	71.14 ± 0.01	41.22 ± 0.04	52.30 ± 17.69	24.85 ± 9.26	82.55 ± 4.42	30.20 ± 5.22	50.84 ± 7.97
Monounsaturated (%)	14.24 ± 0.17	15.05 ± 0.05	0.00 ± 0.00	34.32 ± 13.27	0.00 ± 0.00	11.26 ± 2.59	0.00 ± 0.00	36.80 ± 6.08
Other (%)	4.55 ± 0.35	11.62 ± 2.39	0.00 ± 0.00	0.90 ± 0.21	0.00 ± 0.00	5.33 ± 1.92	0.00 ± 0.00	8.76 ± 1.19

Figure 3 illustrates a significant increase in stearic acid in the lipid fraction of *Spirulina*, *Chlorella*, *Scenedesmus*, and *Chlamydomonas* when cultivated on WW. Additionally, the WW usage influenced the synthesis of linolenic acid in *Chlamydomonas*, *Chlorella*, and *Scenedesmus* lipid fractions. This linoleic acid concentration is particularly relevant for biodiesel production, as the fatty acid composition aligns with European standards (EN14214 [31]). However, the test results indicated some challenges regarding lipid and biomass production.

The fatty acid profile obtained in this study is consistent with previous research by Davani et al. [11], Ramírez-Rodrigues et al. [28], Pascoal et al. [32], and Mathimani et al. [26] for *Chlorella*, *Scenedesmus*, *Chlamydomonas*, and *Spirulina* under control conditions. When WW was used as a substrate, the lipid composition was significantly altered due to the waste

addition. As reported by Sharma et al. [33], the use of wastewater and the establishment of microalgae–bacteria consortia led to the increased presence of fatty acids such as palmitic, myristic, lauric, docosahexaenoic, and oleic acids. In particular, synthetic media tend to promote the production of polyunsaturated fatty acids, while waste substrates and consortia formation favor saturated fatty acids, primarily palmitic acid (<40%).
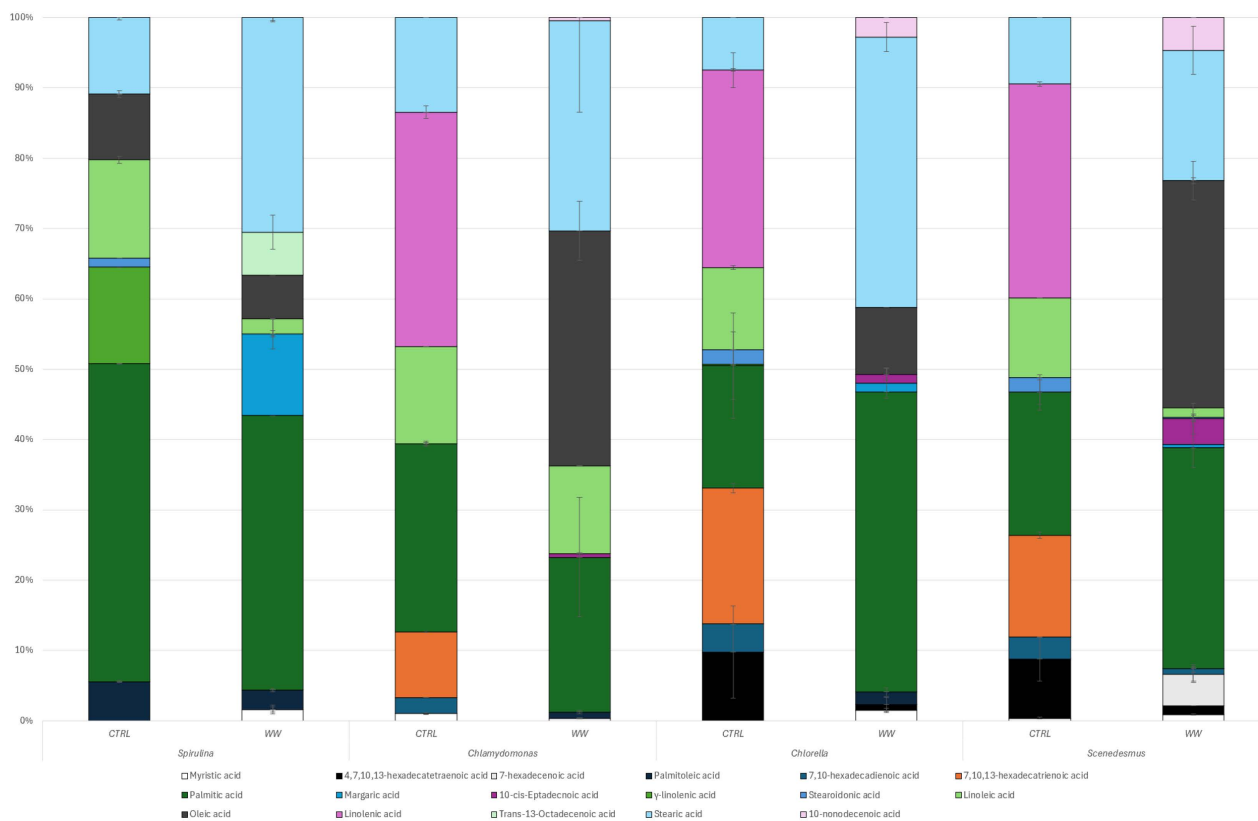

Figure 3. Fatty acid percentage in *Spirulina*, *Chlamydomonas*, *Chlorella*, and *Scenedesmus* biomasses.

The increase in monounsaturated fatty acids under experimental conditions is noteworthy for biodiesel production, as these fatty acids improve oxidative stability and cold flow properties [3]. However, changes in lipid composition could also be linked to oxidative stress, as noted in Pascoal et al. [32]. This stress, influenced by light intensity and temperature, can lead to a decrease in unsaturated lipid content, particularly gamma-linolenic, alpha-linolenic, and linolenic acids.

However, the low lipid storage in microalgae biomass detected under WW conditions could limit its integration for biofuel production. At the same time, extracts of microalgae could be applied as cosmetic ingredients or applied in feed or food sectors associated with their nutritional property, as for *C. reinharditii*, which is recognized as GRAS in the EU [34–36].

3.3.2. Medium Jellification

The cultivation of *Scenedesmus* and *Chlorella* on WW resulted in a pH increase from five to seven, along with a noticeable change in medium density (Figure 4). This density increase is likely attributed to the production of exopolysaccharides (EPSs). The combination of high carbon concentration, nitrogen limitation, and pH rises, suggesting a metabolic shift in *Scenedesmus* and *Chlorella*, favoring EPS synthesis over lipid storage. Under conditions of excess carbon availability and nitrogen starvation, the metabolic pathway shifts from lipid synthesis to EPS production [37,38]. Many microalgae species, such as *Botryococcus braunii* [39], *Chlamydomonas* [40], and *Chlorella* sp. [41], are known to produce EPS, which forms a protective gel matrix (mucilage) around the cells. This matrix can enhance cell

defense against environmental stressors like high light intensity and desiccation, as well as facilitating nutrient fixation [42]. The most common strategy to induce EPS synthesis in microalgae involves limiting essential nutrients such as nitrogen, phosphorus, or sulfur in the culture media. The resulting imbalance in the carbon-to-nitrogen ratio (C/N) impacts carbon metabolism, driving EPS production [43,44]. During the experiment, the initial percentage of N, C, and H, $1.58 \pm 0.00\%$, $34.86 \pm 0.01\%$, and $6.55 \pm 0.06\%$, respectively, in WW 1:10 diluted in BBM+V, showed the unbalanced C/N that could positively influence the EPS synthesis. Depending on the strain and conditions, EPS synthesis can occur during either the growth phase or the stationary phase [39]. External factors such as irradiance, salinity, and temperature also play a role, with high continuous light promoting EPS release [45].
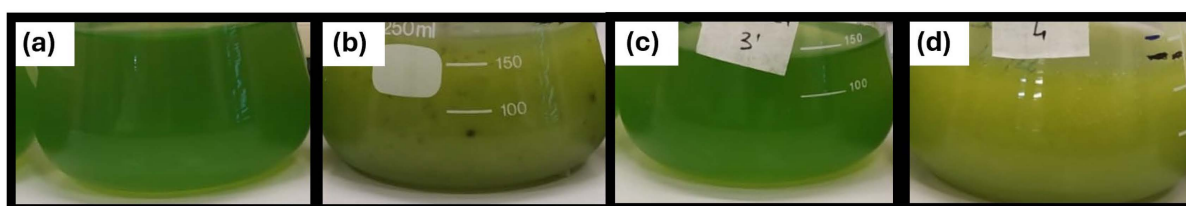

Figure 4. Medium jellification associated with EPS synthesis in *Scenedesmus* and *Chlorella* control conditions (**a**,**c**) and experimental conditions (WW) (**b**,**d**) at the end of the test.

WW, being rich in carbohydrates, likely served as a substrate for microalgae growth. The limited nitrogen uptake created an imbalance in the C/N ratio due to the abundance of carbon, which may have triggered a metabolic shift favoring both lipid storage and EPS production [46,47]—both lipid and EPS synthesis typically occur when the microalgae culture enters the stationary or death phase, when nitrogen starvation limits further proliferation [48].

During the cultivation of *Scenedesmus* and *Chlorella* on WW, an increase in medium density was observed, possibly due to the secretion of organic compounds by the microalgal biomass. In contrast, the negative control, which showed environmental mold contamination, did not exhibit any change in medium density, further supporting the link between density increase and microalgal secretion. The limited nitrogen availability created a C/N imbalance, potentially triggering both high lipid storage and EPS production [46,47].

A critical challenge in microalgal EPS production is achieving high biomass productivity and EPS yields within a reasonable timeframe. In strains where EPS production is induced by nutrient imbalance, growth rates can be reduced due to nitrogen depletion's impact on protein synthesis and cell proliferation [42]. For this reason, EPS production is often achieved in batch or semi-continuous systems [48]. Data from this experiment suggest that *Scenedesmus* and *Chlorella* are promising strains for producing high EPS concentrations, as demonstrated by their high dry weight and growth rates of 0.95 ± 0.07 g L^{-1}, 0.37 ± 0.02 g L^{-1}, 0.40 ± 0.02 d^{-1}, and 0.37 ± 0.04 d^{-1}, respectively. Further characterization of the gel matrix and quantification of the released compounds could improve the integration of waste treatment processes, such as using WW as an alternative substrate for EPS production.

Regarding the difference in EPS production and lipid storage detected during this experiment, a possible response about the switch metabolic synthesis switch could be attributed to the substrate used in these tests. Regarding microalgae, the influence of carbon source on the metabolic pathway was reported. Glucose and saccharose were converted by the Embden–Meyerhof Pathway (EMP) and tricarboxylic acid (TCA) cycle to produce ATP. When the unbalanced medium was applied, biomass proliferation was limited, and, at the same time, cells used the excess of sugars to produce ATP. The ATP synthesized was used to produce molecular structures (pigments, enzyme) or macromolecules (EPS or lipid). When glucose and saccharose were abundant in the medium, EPS synthesis could be enhanced [49]. On the other hand, a lipid synthesis increase was reported when

green microalgae, as *Chlorella* strains, were cultivated under stress conditions such as glucose excess, nitrogen starvation, and darkness [50]. Some authors suggested that the lipid accumulation was enhanced by growth conditions and the type of carbon applied as substrate that influenced the carbon flux towards EPS synthesis or lipid storage [49–52]. All observations suggested that a possible central carbon metabolism alteration, using genetic engineering, could switch EPS production and lipid synthesis, in particular when the acyl-CoA and glycerol-3-phosphate concentration was increased, which are fundamentally molecular for the lipid synthesis. However, the exact mechanism was still unclear, and several researchers are analyzing the transcriptome and genome in order to understand how to "channel" the carbon flux to the production of specific secondary high value products [50].

4. Conclusions

Integrating watermelon waste into a circular economy framework presents a viable alternative to traditional land disposal, which could help reduce land pollution while providing a carbon-rich substrate for microalgae cultivation. The results demonstrate that *Scenedesmus* and *Chlorella* strains successfully utilized watermelon waste as a substrate, achieving notable biomass yields. In contrast, *Spirulina* and *Chlamydomonas* experienced inhibited growth, likely due to the high sugar concentrations in the waste, which some strains cannot tolerate.

Scenedesmus and *Chlorella* exhibited the highest biomass production on watermelon waste, with yields of 0.95 ± 0.07 g L^{-1} and 0.37 ± 0.02 g L^{-1}, respectively, and growth rates of 0.40 ± 0.02 d^{-1} and 0.37 ± 0.04 d^{-1}, respectively. The high sugar content and unbalanced C/N ratio in watermelon substrate influenced both chlorophyll and lipid levels in microalgae biomass. However, lipid storage was lower than the control condition, though the lipid profile shifted with the substrate change, notably showing the absence of linolenic acid. However, the lipid productivity resulted low for the biofuels production, likely due to EPS synthesis in the medium, which led to medium gelation. EPS synthesis was induced by the unbalanced C/N ratio, a condition created using watermelon waste as substrate. The results of this study highlight a potential new application of watermelon waste in the circular economy, offering a novel in situ waste treatment for farmers.

Future research will focus on medium optimization, understanding what kind of substrate enhances the EPS synthesis for *Scenedesmus* and *Chlorella* strains, optimize the light irradiation, and scale up the system. In addition, deeply microalgal EPS characterization was required to understand their application and economic value.

Author Contributions: Conceptualization, P.S. and L.F. (Luca Forti); Investigation, L.F. (Lorenzo Fontana) and F.B.; Data curation, P.S., V.D. and L.F. (Luca Forti); Writing—original draft preparation, P.S. and V.D.; Writing—review and editing, P.S., L.T. and L.F. (Luca Forti); Supervision, L.F. (Luca Forti); Funding acquisition, L.F. (Luca Forti). All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in this article, and further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Pardossi. *Orticoltura: Principi e Pratica*; Edagricole: Milano, Bologna, 2018.
2. Awasthi, M.K.; Kumar, V.; Yadav, V.; Sarsaiya, S.; Awasthi, S.K.; Sindhu, R.; Binod, P.; Kumar, V.; Pandey, A.; Zhang, Z. Current state of the art biotechnological strategies for conversion of watermelon wastes residues to biopolymers production: A review. *Chemosphere* **2021**, *290*, 133310. [CrossRef] [PubMed]
3. Wang, J.; Shi, Y.; Zhang, H.; Deng, X.; Wang, Y.; Ma, Y.; Zhao, X.; Zhang, C. Characterization and Comparison of Unfermented and Fermented Seed-Watermelon Juice. *J. Food Qual.* **2018**, *2018*, 4083903. [CrossRef]
4. Zia, S.; Khan, M.R.; Khaneghah, A.M.; Aadil, R.M. Characterization, bioactive compounds, and antioxidant profiling of edible and waste parts of different watermelon (*Citrullus lanatus*) cultivars. In *Biomass Conversion and Biorefinery*; Springer: Berlin/Heidelberg, Germany, 2023; pp. 1–13. [CrossRef]
5. Fundo, J.F.; Miller, F.A.; Garcia, E.; Santos, J.R.; Silva, C.L.M.; Brandão, T.R.S. Physicochemical characteristics, bioactive compounds and antioxidant activity in juice, pulp, peel and seeds of Cantaloupe melon. *J. Food Meas. Charact.* **2017**, *12*, 292–300. [CrossRef]
6. Ekloh, E.; Yafetto, L. Fermentation and valorization of watermelon (*Citrullus lanatus*) rind wastes into livestock feed using Aspergillus niger and Mucor sp. *Sci. Afr.* **2023**, *23*, e02035. [CrossRef]
7. Greses, S.; Tomás-Pejó, E.; González-Fernández, C. Short-chain fatty acids and hydrogen production in one single anaerobic fermentation stage using carbohydrate-Rich food waste. *J. Clean. Prod.* **2020**, *284*, 124727. [CrossRef]
8. Kassim, M.A.; Hussin, A.H.; Meng, T.K.; Kamaludin, R.; Zaki, M.S.I.M.; Zakaria, W.Z.E.W. Valorisation of watermelon (*Citrullus lanatus*) rind waste into bioethanol: An optimization and kinetic studies. *Int. J. Environ. Sci. Technol.* **2021**, *19*, 2545–2558. [CrossRef]
9. Hashem, A.H.; Suleiman, W.B.; Abu-Elreesh, G.; Shehabeldine, A.M.; Khalil, A.M.A. Sustainable lipid production from oleaginous fungus *Syncephalastrum racemosum* using synthetic and watermelon peel waste media. *Bioresour. Technol. Rep.* **2020**, *12*, 100569. [CrossRef]
10. Davani, L.; Terenzi, C.; Tumiatti, V.; De Simone, A.; Andrisano, V.; Montanari, S. Integrated analytical approaches for the characterization of Spirulina and Chlorella microalgae. *J. Pharm. Biomed. Anal.* **2022**, *219*, 114943. [CrossRef]
11. Faruque, M.O.; Hossain, M.M.; Razzak, S.A. Photoautotrophic Cultivation, Lipid Enhancement, and Dry Biomass Characterization of Microalgae Scenedesmus dimorphus for Bioenergy Application. *Arab. J. Sci. Eng.* **2023**, *48*, 16263–16280. [CrossRef]
12. Kumar, C.P.; Sylas, V.; Mechery, J.; Ambily, V.; Kabeer, R.; Sunila, C. Phycoremediation of cashew nut processing wastewater and production of biodiesel using *Planktochlorella nurekis* and *Chlamydomonas reinhardtii*. *Algal Res.* **2022**, *69*, 102924. [CrossRef]
13. Maletti, L.; D'Eusanio, V.; Lancellotti, L.; Marchetti, A.; Pincelli, L.; Strani, L.; Tassi, L. Candying process for enhancing pre-waste watermelon rinds to increase food sustainability. *Futur. Foods* **2022**, *6*, 100182. [CrossRef]
14. Manirakiza, P.; Covaci, A.; Schepens, P. Comparative study on total lipid determination using Soxhlet, Roese-Gottlieb, Bligh & Dyer, and Modified Blight & Dyer extraction methods. *J. Food Compos. Anal.* **2001**, *14*, 93–100.
15. Costache, M.; Campeanu, G.; Neata, G. Studies concerning the extraction of chlorophyll and total carotenoids from vegetables. *Rom. Biotechnol. Lett.* **2012**, *17*, 7702–7708.
16. Jayakumar, S.; Bhuyar, P.; Pugazhendhi, A.; Rahim, M.; Maniam, G.; Govindan, N. Effects of light intensity and nutrient on the lipid content of marine microalga (diatom) *Amphipropra* sp. for promising biodiesel production. *Sci. Total Environ.* **2021**, *768*, 145471. [CrossRef]
17. Chiranjeevi, P.; Mohan, S.V. Critical parametric influence on microalgae cultivation towards maximizing biomass growth with simultaneous lipid productivity. *Renew. Energy* **2016**, *98*, 64–71. [CrossRef]
18. Chandra, R.; Rohit, M.; Swamy, Y.; Mohan, S.V. Regulatory function of organic carbon supplementation on biodiesel production during growth and nutrient stress phases of mixotrophic microalgae cultivation. *Bioresour. Technol.* **2014**, *165*, 279–287. [CrossRef]
19. Cheirsilp, B.; Torpee, S. Enhanced growth and lipid production of microalgae under mixotrophic culture condition: Effect of light intensity, glucose concentration and fed-batch cultivation. *Bioresour. Technol.* **2012**, *110*, 510–516. [CrossRef]
20. Huang, Y.; Luo, L.; Xu, K.; Wang, X.C.; Huang, Y.; Luo, L.; Xu, K.; Wang, X.C.; Huang, Y.; Luo, L.; et al. Characteristics of external carbon uptake by microalgae growth and associated effects on algal biomass composition. *Bioresour. Technol.* **2019**, *292*, 121887. [CrossRef]
21. Silaban, A.; Bai, R.; Gutierrez-Wing, M.T.; Negulescu, I.I.; Rusch, K.A. Effect of organic carbon, C:N ratio and light on the growth and lipid productivity of microalgae/cyanobacteria coculture. *Eng. Life Sci.* **2013**, *14*, 47–56. [CrossRef]
22. Andreeva, A.; Budenkova, E.; Babich, O.; Sukhikh, S.; Dolganyuk, V.; Michaud, P.; Ivanova, S. Influence of Carbohydrate Additives on the Growth Rate of Microalgae Biomass with an Increased Carbohydrate Content. *Mar. Drugs* **2021**, *19*, 381. [CrossRef]
23. Orús, M.; Marco, E.; Martínez, F. Suitability of Chlorella vulgaris UAM 101 for heterotrophic biomass production. *Bioresour. Technol.* **1991**, *38*, 179–184. [CrossRef]
24. Marquez, F.J.; Sasaki, K.; Kakizono, T.; Nishio, N.; Nagai, S. Growth characteristics of Spirulina platensis in mixotrophic and heterotrophic conditions. *J. Ferment. Bioeng.* **1993**, *76*, 408–410. [CrossRef]

25. Mathimani, T.; Sekar, M.; Shanmugam, S.; Sabir, J.S.; Chi, N.T.L.; Pugazhendhi, A. Relative abundance of lipid types among Chlorella sp. and Scenedesmus sp. and ameliorating homogeneous acid catalytic conditions using central composite design (CCD) for maximizing fatty acid methyl ester yield. *Sci. Total. Environ.* **2021**, *771*, 144700. [CrossRef]

26. Liu, X.; Ji, B.; Li, A. Enhancing biolipid production and self-flocculation of Chlorella vulgaris by extracellular polymeric substances from granular sludge with CO_2 addition: Microscopic mechanism of microalgae-bacteria symbiosis. *Water Res.* **2023**, *236*, 119960. [CrossRef]

27. Ramírez-Rodrigues, M.M.; Estrada-Beristain, C.; Metri-Ojeda, J.; Pérez-Alva, A.; Baigts-Allende, D.K. *Spirulina platensis* Protein as Sustainable Ingredient for Nutritional Food Products Development. *Sustainability* **2021**, *13*, 6849. [CrossRef]

28. Devadasu, E.; Subramanyam, R. Enhanced Lipid Production in Chlamydomonas reinhardtii Caused by Severe Iron Deficiency. *Front. Plant Sci.* **2021**, *12*, 615577. [CrossRef]

29. Qari, H.A.; Oves, M. Fatty acid synthesis by *Chlamydomonas reinhardtii* in phosphorus limitation. *J. Bioenerg. Biomembr.* **2020**, *52*, 27–38. [CrossRef]

30. Pascoal, P.V.; Ribeiro, D.M.; Cereijo, C.R.; Santana, H.; Nascimento, R.C.; Steindorf, A.S.; Calsing, L.C.G.; Formighieri, E.F.; Brasil, B.S.A.F. Biochemical and phylogenetic characterization of the wastewater tolerant Chlamydomonas biconvexa Embrapa|LBA40 strain cultivated in palm oil mill effluent. *PLoS ONE* **2021**, *16*, e0249089. [CrossRef]

31. *EN 14214*; Liquid Petroleum Products—Fatty Acid Methyl Esters (FAME) for Use in Diesel Engines and Heating Applications—Requirements and Test Methods. European Committee for Standardization: Brussels, Belgium, 2019.

32. Sharma, J.; Kumar, V.; Kumar, S.S.; Malyan, S.K.; Mathimani, T.; Bishnoi, N.R.; Pugazhendhi, A. Microalgal consortia for municipal wastewater treatment–Lipid augmentation and fatty acid profiling for biodiesel production. *J. Photochem. Photobiol. B Biol.* **2020**, *202*, 111638. [CrossRef]

33. Mathimani, T.; Uma, L.; Prabaharan, D. Optimization of direct solvent lipid extraction kinetics on marine trebouxiophycean alga by central composite design—Bioenergy perspective. *Energy Convers. Manag.* **2017**, *142*, 334–346. [CrossRef]

34. Babiak, W.; Krzemińska, I. Extracellular Polymeric Substances (EPS) as Microalgal Bioproducts: A Review of Factors Affecting EPS Synthesis and Application in Flocculation Processes. *Energies* **2021**, *14*, 4007. [CrossRef]

35. Cicci, A.; Scarponi, P.; Cavinato, C.; Bravi, M. Microalgae production in olive mill wastewater fractions and cattle digestate slurry: Bioremediation effects and suitability for energy and feed uses. *Sci. Total. Environ.* **2024**, *932*, 172773. [CrossRef] [PubMed]

36. European Commission. CosIng-Cosmetics Ingredients. 2024. Available online: https://ec.europa.eu/growth/tools-databases/cosing/ (accessed on 29 October 2024).

37. SpecialChem. INCI Database Directory. 2024. Available online: https://cosmetics.specialchem.com/inci-names (accessed on 29 October 2024).

38. Delattre, C.; Pierre, G.; Laroche, C.; Michaud, P. Production, extraction and characterization of microalgal and cyanobacterial exopolysaccharides. *Biotechnol. Adv.* **2016**, *34*, 1159–1179. [CrossRef]

39. Lupi, F.; Fernandes, H.; Tomé, M.; Sá-Correia, I.; Novais, J. Influence of nitrogen source and photoperiod on exopolysaccharide synthesis by the microalga *Botryococcus braunii* UC 58. *Enzym. Microb. Technol.* **1994**, *16*, 546–550. [CrossRef]

40. Kroen, W.K.; Rayburn, W.R. Influence of growth status and nutrient on extracellular polysaccharide synthesis by the soil alga Chlamydomonas mexicana (*Chlorophyceae*). *J. Phycol.* **1984**, *20*, 253–257. [CrossRef]

41. Brányiková, I.; Maršálková, B.; Doucha, J.; Brányik, T.; Bišová, K.; Zachleder, V.; Vítová, M. Microalgae—Novel highly efficient starch producers. *Biotechnol. Bioeng.* **2010**, *108*, 766–776. [CrossRef]

42. Laroche, C. Exopolysaccharides from Microalgae and Cyanobacteria: Diversity of Strains, Production Strategies, and Applications. *Mar. Drugs* **2022**, *20*, 336. [CrossRef]

43. Kumar, A.S.; Mody, K.; Jha, B. Bacterial exopolysaccharides—A perception. *J. Basic Microbiol.* **2007**, *47*, 103–117. [CrossRef]

44. Otero, A.; Vincenzini, M. Nostoc (*Cyanophyceae*) goes nude: Extracellular polysaccharides serve as a sink for reducing power under unbalanced C/N metabolism. *J. Phycol.* **2004**, *40*, 74–81. [CrossRef]

45. Han, P.-P.; Shen, S.-G.; Wang, H.-Y.; Sun, Y.; Dai, Y.-J.; Jia, S.-R. Comparative metabolomic analysis of the effects of light quality on polysaccharide production of cyanobacterium *Nostoc flagelliforme*. *Algal Res.* **2015**, *9*, 143–150. [CrossRef]

46. Arad, S.; Friedman, O.; Rotem, A. Effect of nitrogen on polysaccharide production in a *Porphyridium* sp. *Appl. Environ. Microbiol.* **1988**, *54*, 2411–2414. [CrossRef] [PubMed]

47. Li, S.; Ji, L.; Chen, C.; Zhao, S.; Sun, M.; Gao, Z.; Wu, H.; Fan, J. Efficient accumulation of high-value bioactive substances by carbon to nitrogen ratio regulation in marine microalgae *Porphyridium purpureum*. *Bioresour. Technol.* **2020**, *309*, 123362. [CrossRef] [PubMed]

48. Raungsomboon, S.; Chidthaisong, A.; Bunnag, B.; Inthorn, D.; Harvey, N.W. Production, composition and Pb^{2+} adsorption characteristics of capsular polysaccharides extracted from a cyanobacterium *Gloeocapsa gelatinosa*. *Water Res.* **2006**, *40*, 3759–3766. [CrossRef] [PubMed]

49. Soanen, N.; Da Silva, E.; Gardarin, C.; Michaud, P.; Laroche, C. Improvement of exopolysaccharide production by *Porphyridium marinum*. *Bioresour. Technol.* **2016**, *213*, 231–238. [CrossRef] [PubMed]

50. Vo, H.N.P.; Ngo, H.H.; Guo, W.; Liu, Y.; Chang, S.W.; Nguyen, D.D.; Zhang, X.; Liang, H.; Xue, S. Selective carbon sources and salinities enhance enzymes and extracellular polymeric substances extrusion of *Chlorella* sp. for potential co-metabolism. *Bioresour. Technol.* **2020**, *303*, 122877. [CrossRef]

51. Tan, K.W.M.; Lee, Y.K. The dilemma for lipid productivity in green microalgae: Importance of substrate provision in improving oil yield without sacrificing growth. *Biotechnol. Biofuels* **2016**, *9*, 255. [CrossRef]

52. Johnson, X.; Alric, J. Central Carbon Metabolism and Electron Transport in Chlamydomonas reinhardtii: Metabolic Constraints for Carbon Partitioning between Oil and Starch. *Eukaryot. Cell* **2013**, *12*, 776–793. [CrossRef]