sustainability

MDPI

Article

Revolutionizing Agriculture: Leveraging Hydroponic Greenhouse Wastewater for Sustainable Microalgae-Based Biostimulant Production

María Álvarez-Gil [1,*], Mario Blanco-Vieites [1], David Suárez-Montes [1], Víctor Casado-Bañares [1], Jesús Fidel Delgado-Ramallo [1] and Eduardo Rodríguez [2,*]

[1] Neoalgae Micro Seaweed Products SL, Calle Carmen Leal Mata, 191, 33211 Gijon, Spain; marioblancovieites@gmail.com (M.B.-V.); dsuarezmon@gmail.com (D.S.-M.); vcasado@neoalgae.es (V.C.-B.); fdelgado@neoalgae.es (J.F.D.-R.)

[2] Department of Construction and Manufacturing Engineering, University of Oviedo, Pedro Puig Adam, s/n, 33203 Gijon, Spain

* Correspondence: malvarez@neoalgae.es (M.Á.-G.); eduardo@uniovi.es (E.R.); Tel.: +34-984-04-12-66 (M.Á.-G.)

Abstract: The current intensification in agricultural pressure has resulted in the addition of excessive amounts of nutrients. While hydroponic systems have become an agricultural tool to reduce this nutrient addition, the produced nutrient-rich drainage solution, which has a negative effect in water bodies, is still a barrier that needs to be overcome. A promising alternative for nutrient recovery is the cultivation of microalgae, which require a significant quantity of nutrients for their growth. Furthermore, their biostimulant properties enhance the circularity and sustainability approach. This study evaluates microalgae growth with a hydroponic drainage solution and the use of the resulting biomass as a source for biostimulant. Three microalgae strains were tested (*Desmodesmus* sp., *Chlorella vulgaris*, and *Scenedesmus obliquus*) and grown at laboratory scale (0.5 and 2 L) with drainage wastewater taken from hydroponic tomato crops. Measures of OD_{750} and OD_{680} revealed *Desmodesmus* sp. as the strain with the best performance, achieving 1.4 and 1.8, respectively, in the 0.5 L assay and 1.08 and 1.3 in the 2 L assay. These results were confirmed in another assay that compared *Desmodesmus* sp. growth in drainage wastewater and in control media. Then, *Desmodesmus* sp. was tested at industrial scale. Results show the columnar PBR reached 1.8 $g \cdot L^{-1}$ compared to 1.1 $g \cdot L^{-1}$ achieved with raceways. Finally, the resulting biomass was tested in a biocatalysis process for biostimulant production, suggesting an innovative system to use microalgae biomass cultured in wastewater as a valuable product such as biostimulant.

Keywords: microalgae; wastewater; *Desmodesmus*; circular economy; biostimulant

Citation: Álvarez-Gil, M.; Blanco-Vieites, M.; Suárez-Montes, D.; Casado-Bañares, V.; Delgado-Ramallo, J.F.; Rodríguez, E. Revolutionizing Agriculture: Leveraging Hydroponic Greenhouse Wastewater for Sustainable Microalgae-Based Biostimulant Production. *Sustainability* **2023**, *15*, 14398. https://doi.org/10.3390/su151914398

Academic Editors: Teodor Rusu, Franco Capocasa and Nesibe Ebru Kafkas

Received: 31 August 2023
Revised: 15 September 2023
Accepted: 28 September 2023
Published: 30 September 2023

1. Introduction

Overwhelming growth in the population, which is projected to reach 8.9 billion people by 2050, is forcing farmers to increase agricultural land and production [1]. In recent years, farming techniques have focused on intensive systems to ensure the supply of groceries to feed this constantly growing population, leading to an increase in the addition of excessive amounts of nutrients and biopesticides [2], both of which have a significant environmental impact. Pesticides have contaminated almost all parts of our environment and their residues are found in soil, air, and different aquatic bodies [3]. Regarding chemical fertilizers, their greatest environmental impact is due to greenhouse gas (GHG) emissions during their production. Nevertheless, contamination by environmental runoff of nutrients, not absorbed by crops, can also cause serious eutrophication problems [4].

In recent decades there has been a change to a more sustainable model in agriculture practices, mainly focused on organic agriculture [5]. In 2021, 76.4 million hectares were under organic agricultural management worldwide, increasing 1.7% compared to the

previous year [6]. Scientific results support the increase in yields and productivity due to the use of biofertilizers and biostimulants. For example, research results show that the use of certain biostimulants and organic fertilizers for strawberry cultivation increases efficiency, improves yields, and allows soil enhancement [7]. However, yields from organic agriculture do not reach the levels of conventional agriculture because organic sources of nutrients are not sufficient to increase the crop yield. Therefore, one of the crucial challenges still faced by sustainable agriculture is meeting the global demand for safe food while preserving the environment and mitigating climate change, coupled with reducing the residue of chemicals and pesticides on food [4,8].

On this basis, researchers are called to develop innovative tools to increase the quality and quantity of produced crops while reducing the carbon footprint and harmful consequences.

Hydroponic systems offer a sustainable tool for industrialized crop production, especially due to the recirculation of nutrient solutions, which reduces nutrient addition. Moreover, this technology can offer a growth rate up to 50% higher than that of traditional crops [9]. Hydroponic systems are based on a substrate consisting mainly of a nutrient-rich water solution, with technical benefits including being cost effective and disease free, having a higher yield, and being more sustainable, with fewer requirements for space and labor [10]. The optimization of water consumption has also been studied, resulting in the agrological technology with higher water-saving potential [2]. Despite the success of these systems, concerns about some environmental disadvantages are rising, mainly based on the negative effect of the nutrient-rich drainage solution in water bodies [11].

Accordingly, drainage is currently treated as wastewater, losing the potential as a source of valuable compounds. The contents of nutrients can be within a range of about 200–300 $mg \cdot L^{-1}$ for nitrogen and 40–100 $mg \cdot L^{-1}$ phosphorus [12,13].

Microalgae are photosynthetic microorganisms that use inorganic compounds and solar energy to synthesize organic molecules needed for their survival [14]. Their capacity to adapt to different physical-chemical conditions (e.g., pH, temperature, CO_2) is due to their efficient use of photosynthesis [15,16]. They convert water and carbon dioxide into carbon compounds using light as a source of energy and other nutrients such as nitrogen and phosphorus [17], which can be provided as inorganic salts or can be taken from wastewater [18].

Microalgae biomasses have biostimulant and biofertilizer properties. They also increase nutrient absorption and tolerance to abiotic stress, achieving better yield and improved quality in several crops [19]. Furthermore, microalgal extracts have also been shown to boost protection against bacterial and fungal diseases, which might help in the reduction in pesticide application [20].

Biostimulants are considered to be environmentally friendly, mainly because of their reduced environmental impact and high crop production. Interest in them is currently increasing and they are becoming more attractive to growers [18].

Production of microalgae coupled to wastewater treatment has been widely reported as a reliable process. Microalgae can be applied to different wastewater targets, including for largely different effluents such as sewage, centrate, and manure [21–23]. However, growing microalgae using a nutrient-rich solution from hydroponic systems is a novel approach. Moreover, the conversion of these drainage wastes into recoverable resources to produce high-value products (e.g., biostimulants), fits into the circular bioeconomy approach [11]. Within the circular economy paradigm, microalgae can use nutrients from a wide range of wastewaters, while producing biomass and value-added compounds such as pigments, antioxidants, and proteins [20].

Different studies have already been performed, from laboratory to pilot scale. Moreover, different species and different hydroponic crops have been tested. Accordingly, the potential of microalgae as a solution for the elimination of nutrient-rich effluents has been demonstrated.

A systematic review of the cultivation of *Arthrospira* sp. in alternative culture media based on wastewater effluent was carried out, and demonstrated a supplement with a synthetic medium for the use of obtained biomass for commercial purposes [24].

Regarding the cultivation of *Chlorella*, it was found that the composition of minerals reaches higher concentrations when it is grown in the most concentrated hydroponic wastewater from lettuce cultivation, thereby showing the use of this residue in a sustainable way for obtaining high-valued biomass [25].

More recent studies about the treatment of drainage solution from hydroponic tomato greenhouse included not only *Chlorella vulgaris* but also an indigenous microalgal community. A significant reduction in nitrogen of between 34.7 and 73.7 mg/L, and in phosphorus, of between 15.4 and 15.9 mg/L, was observed, although the large reduction in nutrients was achieved through growth of the indigenous microalgal community. Nevertheless, biomass composition analysis of the algae showed inferior content compared to results obtained with conventional growth media [12].

A trend of using consortia or indigenous communities instead of monospecific cultures was observed in other studies. A comparison between four microalgae strains (*Chlorella vulgaris*, *Parachlorella kessleri*, *Scenedesmus obliquus*, *Scenedesmus quadricauda*) and an indigenous consortium of photosynthetic microorganisms was made in terms of bioremediation capacity of wastewater from hydroponic tomato cultures. The best results were shown by *Scenedesmus obliquus* and the consortia, which in less than seven days obtained the highest reduction in nitrate and phosphate concentrations (average removal of 98.2 and 87.1%, respectively) [26].

Other experiments using wastewater from hydroponic lettuce cultivation to grow a microbial consortium and axenic culture (*Paracercomonas saepenatans*) achieved nearly 100% removal of nitrate and between 41 and 100% removal of phosphate. It was also revealed that among the most important strains of the consortium were *Vorticella* and *Scenedesmus* [27].

Scenedesmus is a strain that has been used to achieve very good results in the reuse of nutrients from hydroponic wastewater or drainage. It was found to be capable of achieving removal efficiencies from cucumber hydroponic drainage close to 35% for nitrates and 98% for phosphates (where this efficiency was maximized at higher pH) while growing in PBR conditions [28]. These removal efficiencies could be increased up to 100% of N and P using a pilot-scale indoor photobioreactor equipped with LEDs [29].

Despite the good results obtained in these previous studies, such as nutrient reduction through microalgae cultivation with wastewaters, it is necessary to continue investigating to overcome certain technological challenges. Among these investigations, the study of new species and the optimization of culture protocols that achieve maximum nutrient absorption and higher quality biomass production is crucial. On the same basis, it is necessary to develop strategies for the recovery of produced biomass, as well as its industrial and/or commercial applications [29].

Therefore, these challenges are addressed in this study. First, the growth rate of different microalgae strains was evaluated when they were cultivated within enriched nutrient wastewater from hydroponic tomato crops. The main objective was to select a microalgae strain capable of growing using wastewater drainage as a culture medium, whose biomass could be used as a source of biostimulant production. On the same basis, the growth of the selected strain was evaluated within different culture systems by measuring the biomass production yield. Afterwards, a biocatalysis process based on enzymatic degradation was developed, leading to biostimulation production. Enzymatic degradation possesses several advantages, especially when compared to acid hydrolysis, including the absence of secondary inhibitory by-products, fewer corrosion issues, and low utility consumption [30]. This innovative approach enhances the circularity and sustainability of agriculture practices, since wastewater from hydroponic crops is used to grow microalgae, while using this biomass to develop a novel biostimulant that can be further applied on crops.

2. Materials and Methods

2.1. Microalgae Strains

The initial stages of the investigation were focused on making a selection of microalgae species capable of growing in wastewater, whose biomass can be used for the production of biostimulants [31,32]. *Desmodesmus* sp. has been proven to have a clear positive impact on plant development, as suggested in some previous studies [31]. Other selected strains were *Chlorella vulgaris*, which is one of the most cultured strains worldwide and has also been previously studied for its biostimulant effect [33], and a *Tetradesmus* sp. strain (hereinafter referred to as *Scenedesmus*, its former name), which is also a well-known species for bioremediation of different kinds of organic wastewater [34].

The *Desmodesmus* strain was the property of STAM SLR (Italian Engineering company) and a sample was shipped to Neoalgae for preliminary tests. *Chlorella vulgaris* was provided by the Culture Collection of Algae and Protozoa (CCAP 211/109), and *Tetradesmus obliquus CE.402* (formerly known as *Scenedesmus obliquus*) was provided by the strain repository of Neoalgae Micro Seaweed Products (Gijon, Spain), and was isolated from an urban waste landfill in Asturias, Spain [35].

2.2. Culture Media

The used cultured medium was BG-11 [23], which is a widely used culture medium for Chlorophyte strains such as the ones selected, as suggested by several banks of algal strains [36]. The chemical composition of this culture medium is shown in Tables 1 and 2.

Table 1. BG-11 culture medium composition.

Chemical Compound	Concentration in Stock Solutions ($g \cdot L^{-1}$)	Addition per 1 L of Culture
$NaNO_3$		1.5 g
$K_2HPO_4 \cdot 3H_2O$	40.00	1 mL
$MgSO_4 \cdot 7H_2O$	75.0	1 mL
$CaCl_2 \cdot 2H_2O$	36.0	1 mL
Citric acid	6.0	1 mL
Ammonium ferric citrate	6.0	1 mL
$MgNa_2EDTA \cdot H_2O$	1.0	1 mL
Na_2CO_3	20	1 mL
Trace metals solution *		1 mL

* Preparation of the trace metals solution (Table 2).

Table 2. Composition of the trace metals solution.

Chemical Compound	Concentration ($g \cdot L^{-1}$)
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H2O$	0.22
$Na_2MoO_4 \cdot 2H_2O$	0.39
$CuSO_4 \cdot 5H_2O$	0.08
$Co(NO_3)_2 \cdot 6H_2O$	0.05

All reagents used were of analytical grade and purchased from Labbox Labware (Barcelona, Spain), with exception of $NaNO_3$ and $MgSO_4$, which were purchased from Vadequímica (Barcelona, Spain).

For the experimental set-up, which focused on microalgae cultivation with wastewater, hydroponic drainage obtained from tomato crops of the University of Thessaly (UTH) in Volos was used [37]. The drainage wastewater was not exposed to any chemical or physical treatments in order to preserve its original characteristics. This decision was taken in contemplation of the final objective of this technology, which is based on the culture of microalgae within hydroponic wastewater in a simple and efficient system.

UTH cultivates different crops in an experimental polyethylene covered greenhouse (ground area of 160 m^2), located at the University of Thessaly near Volos (latitude 39°44′, longitude 22°79′, altitude 85 m) on the coastal area of eastern Greece. The selected wastewater was analyzed by UTH following the standard analytic laboratory protocols. Extraction was performed using the Kjeldahl nitrogen method (TKN) based on the Kjeldahl (1883) protocol [38]. Nutrient elements were determined using ICP (ICP-OES, SPECTRO Analytical Instruments GmbH, Kleve, Germany).

According to the results shown in Table 3, the drainage wastewater from tomato crops showed higher nutrient content, and thus selected for this investigation.

Table 3. Nutrient composition of the used wastewater from different crops in University of Thessaly (UTH) in Volos.

Compound	Hydroponics Cucumber Drainage (mmol L^{-1})	Hydroponics Tomato Drainage (mmol L^{-1})	Fish Tanks (mmol L^{-1})	Aquaponics Tomato Drainage (mmol L^{-1})
NO_3^-	12.50	12.95	2.19	1.14
NH_4^+	0.50	0.57	0.06	0.02
PO_4^{3-}	0.90	1.93	0.19	0.07
K	3.83	8.17	0.71	0.31
Ca	6.98	6.60	1.04	1.20
Na	1.83	3.18	1.78	2.55
Mg	2.34	3.10	0.50	0.50

2.3. Laboratory Scale Culturing

At laboratory scale, it was decided to develop three experiments at different volumes. First, *Chlorella*, *Scenedesmus*, and *Desmodesmus* strains were inoculated in 0.5 L of wastewater and supplemented with BG-11 culture medium (see Figure 1). On this basis, experimental and control cultures were supplemented with 0.5 mL of each of the stock solutions specified in Table 1.

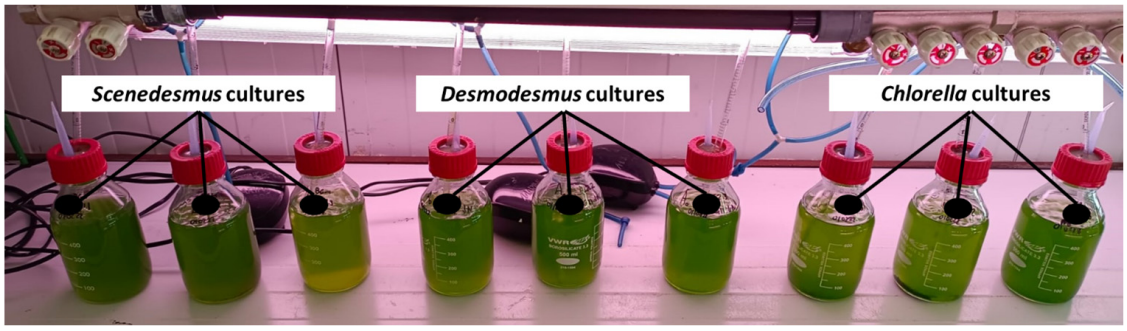

Figure 1. A 0.5 L experimental assay with three strains of Chlorophyte microalgae.

Then, these cultures were used as inoculums for the next experiment in 2 L bottles, in which it was expected to consolidate the previous outcomes. Before inoculation, all of the cultures used as inoculums were diluted to a similar starting OD750 (with a value of 0.2), in order to enhance the homogeneity at the beginning of the experiment.

Finally, the 2 L assay was repeated with 2 different groups of the same strain: the experimental group (cultured in presence of 100% wastewater) and the control group (absence of wastewater) (Figure 2). Thus, the *Desmodesmus* inoculum was taken from the first 2 L experiment, following the hypothesis that, when pre-cultured in hydroponics drainage, this strain could become better acclimated to it and develop mechanisms that enhance its growth rate and survival.

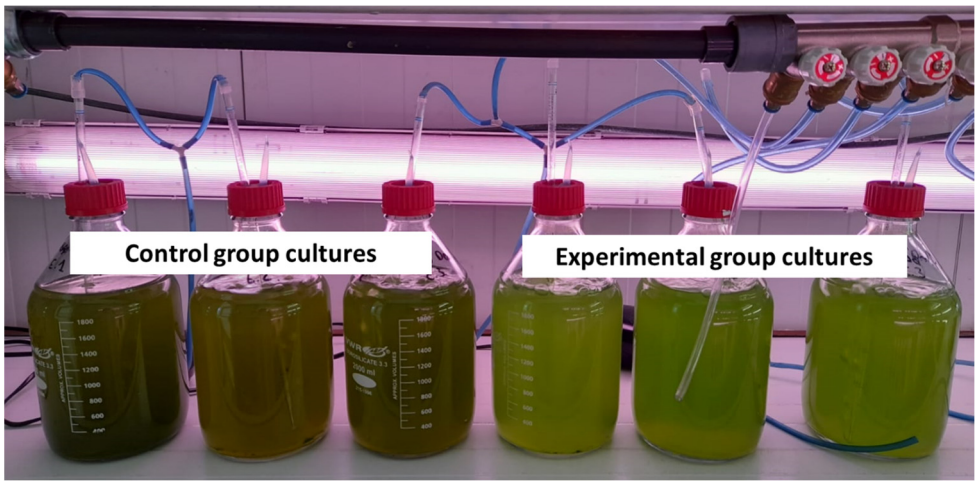

Figure 2. Two-liter trials with experimental and control groups of *Desmodesmus* sp.

In the three experiments, the cultures were maintained under constant controlled conditions in order to minimize the influence of different parameters on culture growth and wastewater tolerance. Accordingly, environmental settings were kept constant, with a photoperiod of 16 h of light and 8 h of darkness. Light was provided through lamps with a continuous photon flux of 80–100 $\mu mol \cdot s^{-1} \cdot m^{-2}$. Moreover, temperature was also maintained at 25 °C. Furthermore, cultures were agitated through the addition of aeration [22]. This was done using air pumps with a flow rate of 100 $L \cdot h^{-1}$. All experimental cultures were carried out in triplicate, including a control group that was not exposed to stressors such as the presence of hydroponic drainage wastewater. Once the results obtained were evaluated, the optimum strain was selected for further steps in pilot-scale culturing.

2.4. Pilot-Scale Culturing

Once the stationary phase of cellular density was reached, 2 L cultures were used as inoculums for pre-industrial 10 L plastic bottles (Nalgene, United States). These bottles were kept in the Neoalgae greenhouse facility in Gijón, Spain (43.52326797371128, −5.701862389558017). The change from a controlled-conditions chamber to a climate-dependent greenhouse can be a source of stress for microalgae, along with the scaling-up process [39]. The variances in light intensity, as well as temperature, can be translated into growth rate declines, and reductions in microalgae development and even their collapse. Accordingly, such a source of stress is widely recognized as a critical point in industrial-scale cultivation of microalgae. On this basis, it was decided to make several renewals of the 10 L cultures (a total of 5 renewals at intervals of 10 days) in order to achieve a steady growth curve. It was decided to do so in contemplation of achieving a *Desmodesmus* strain that was fully acclimatized to the selected culture conditions. On this basis, cultures were kept in these bottle-like systems until reaching the late exponential growth phase, and were then used as inoculums for vertical photobioreactors (PBRs; Aqualgae, Spain) (Figure 3, left). PBRs are industrial production systems that consist of closed vertical glass columns that are 3 m high and 30 cm wide, in which air can be added through their base. These systems have a maximum volume of 100 L. After evaluating the growth of the selected strain, PBR cultures were used as inoculums for open culturing systems named raceways (Fibrastur, Spain) (Figure 3, right), since there are the most widely used tool for industrial-scale microalgae biomass production [40]. Thus, raceway ponds were chosen as culturing facilities. These open-type production systems for microalgae cultivation consist of an oval open pond with a paddle wheel that continuously homogenizes the culture in order to facilitate gas interchange and culture agitation. These systems are 12 m long and 30 cm deep and have a total volume of 4500 L.

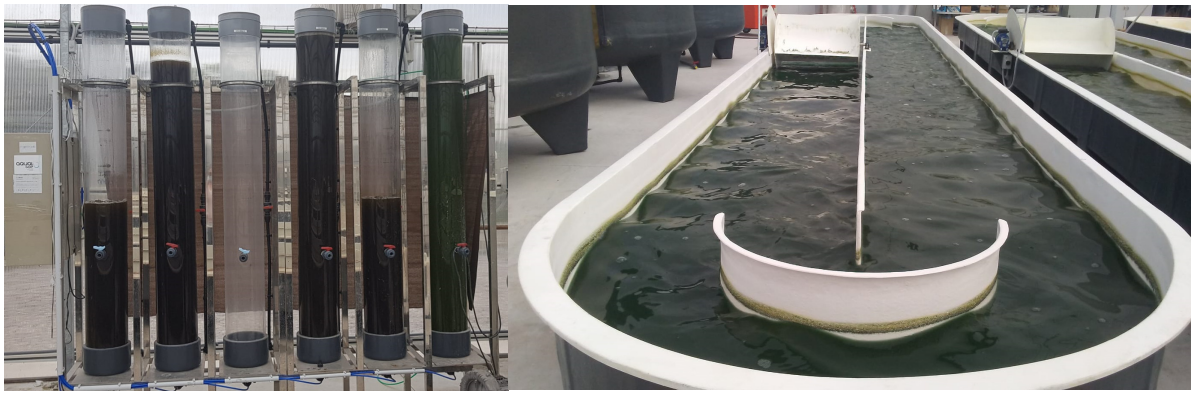

Figure 3. Pilot-scale microalgae cultivation systems at NEOALGAE: vertical closed PBRs (**left**), open raceways (**right**).

2.5. Growth Parameters

For microalgae growth analysis, it was decided to measure optical density at 750 nm (OD_{750}) in order to evaluate the evolution of the culture opacity, by sampling every 48 h [41]. Moreover, chlorophyll concentration was also analyzed via optical density measurements at an absorbance of 680 nm (OD_{680}) [42] in order to evaluate if the opacity observed was related to a variation in the concentration of living cells. Sampling was conducted under sterile conditions and the samples were agitated before measurements to avoid errors due to cellular precipitation. The optical densities were measured using a spectrophotometer (BioChrome Libra S11) by taking 1 mL of each sample per replicate. Sampling was conducted in sterile conditions, and the samples were agitated with a vortex for 5 s before the optical density was measured. Background fluorescence was determined using a blank sample obtained by mixing the culture medium and wastewater following the experimental percentages.

Dry weight was obtained gravimetrically. Firstly, the initial weight was obtained by drying (24 h) GF/C glass microfiber filters (Whatman, Cambridge, UK) inside Petri plates. Around 10 mL of culture was filtrated and washed two times with distilled water to eliminate the salt excess. Then, filters were dried again for 36 h at 60 °C and the final biomass was obtained by calculating the difference between the final weight (filter and cell biomass) and initial weight (only filter) [43].

In addition, microscopy analysis was carried out using an optical microscope (Bioblue BB.1153-PLi) in order to evaluate the culture's health, and especially to check for the possibility of grazers or other unwanted microorganisms that could damage the experimental cultures. Moreover, microscopy analysis also helped to evaluate microalgal stress since the selected microalgae form aggregates when exposed to undesirable conditions. Sampling was conducted by micro-pipetting (200 µL) in sterile conditions.

2.6. Optimization of a Biocatalysis Process

The biocatalysis process consisted of enzymatic hydrolysis without previous harvesting steps, which strongly facilitates the operating conditions while reducing the installation costs [44]. It was decided to use a process based on the sedimentation of the culture.

The biocatalysis process was based on the hydrolytic activity of non-GMO enzymes called EnzMix complex (Neoalgae Micro Seaweeds Products, Asturias, Spain). The experimental design was based on the addition, at room temperature, of different concentrations of the EnzMix complex to several samples of *Desmodesmus* culture with the objective of obtaining the minimum volume of enzymatic complex required to maximize the quantity of broken cellular walls. Moreover, the experimental design was divided into 2 different groups: group A (where microalgae culture was pre-concentrated to $\frac{1}{4}$ of the initial volume) and group B (no preconcentration of microalgae culture). Therefore, 250 mL samples were taken for each of the replicates of the experimental groups. Those for group

A were pre-concentrated through gravity precipitation of the culture used for the group B assay. The final experimental design is presented in Table 4, which shows the % and µL of EnzMix added to each sample of each group. Culture samples were exposed to each of the enzymatic complexes and were examined after agitation for 24 h, along with a control sample that was agitated for the same time but without the addition of enzymes. After 24 h, samples were exposed to an analysis of the chlorophylls present (OD680) under the assumption that broken cell walls produce the degradation of the photosynthetic systems while degrading the chlorophyll. Each test was performed in triplicate and the results are shown as averages with variance in the outcomes obtained. Group A consisted of a pre-concentration through gravity precipitation of the culture used for the group B assay, specifically at 1/4 of the initial volume.

Table 4. Experimental design for biocatalysis optimization of pre-concentrated and non-concentrated biomass. The samples are named as follows: EM means EnzMix, A or B letter means experimental group, C means control, and number represents the % of EnzMix.

Experimental Group	Sample Number	EnzMix Complex (%)	EnzMix Complex (µL)
A	EM-AC	Control	0
	EM-A1	1%	270
	EM-A4	4%	1080
	EM-A8	8%	2160
	EM-A10	10%	2700
	EM-A12	12%	3240
B	EM-BC	Control	0
	EM-B1	1%	100
	EM-B4	4%	400
	EM-B8	8%	800
	EM-B10	10%	1000
	EM-B12	12%	1200

3. Results

3.1. Strain Selection at Laboratory Scale Culturing

Figure 4a shows the results in terms of opacity measures (OD_{750}) for the studied microalgae species (*Scenedesmus*, *Chlorella*, and *Desmodesmus*) cultured with 0.5 L of wastewater. The first measure of the inoculum on day 0 displayed similar values among the cultures evaluated (0.5). However, during the first two days, *Scenedesmus* and *Chlorella* did not show changes in this value, while values for *Desmodesmus* increased to 0.6. For the rest of the experiment, there was a growing trend for the three curves; however, *Scenedesmus* showed another drop on day 8 to 1.0, which was less than the values achieved by *Desmodesmus* and *Chlorella*. *Desmodesmus* did not present any observable drop, showing higher values during all experiments and finally achieving the maximum value of 1.4.

Regarding chlorophyll concentration analysis (OD_{680}) (Figure 4b), measurement results showed similar trends to opacity results. The experiments started with the same value of 0.5 for the three inoculums. Then, *Desmodesmus* was the culture that reached the highest values of OD_{680}, achieving the highest value of 1.8 on day 10 and not presenting any drop during all the experiments. *Scenedesmus* and *Chlorella* showed the lowest values and, during the first two days, showed an initial drop, evidencing no growth during these first moments.

These experimental cultures were used as inoculums for 2 L bottles in the following experimental assay; Figure 5 shows the results in terms of measures of OD_{750} (opacity evaluation) and OD_{680} (chlorophyll content evaluation).

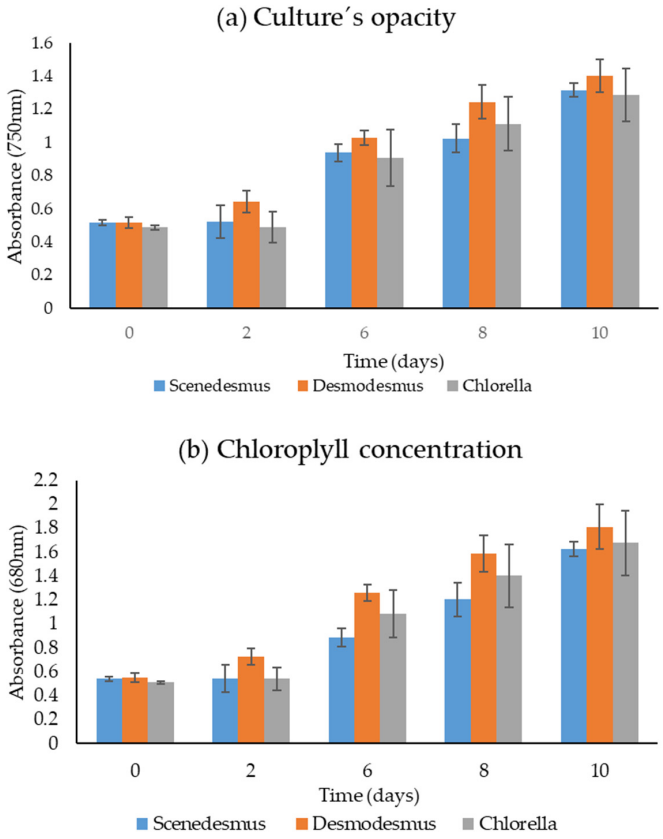

Figure 4. Measurements in 0.5 L experiments with wastewater: (**a**) absorbance at OD_{750} for opacity evaluation, (**b**) absorbance measurements at OD_{680} for chlorophyll content evaluation.

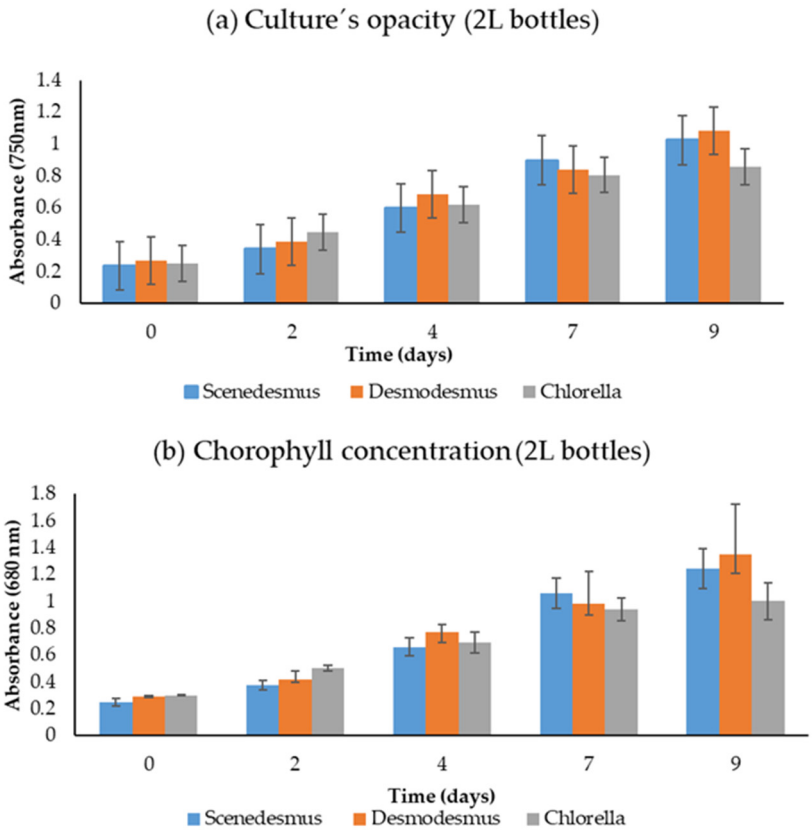

Figure 5. Measurements in 2 L experiments with wastewater: (**a**) absorbance at OD_{750} for opacity evaluation, (**b**) absorbance measurements at OD_{680} for chlorophyll content evaluation.

Accordingly, initial measurements of the inoculum at time 0 of experimentation displayed the same values of absorbances at 750 nm and 680 nm of 0.2 and 0.3, showing that the three experimental groups presented similar opacity and chlorophyll content, respectively.

Regarding culture opacity, contrary to what was observed during the 0.5 L assays, no initial drops were observed during the first two days of the 2 L trials. Nevertheless, *Scenedesmus* cultures showed a slightly reduced growth rate during the first two days, especially when compared to *Desmodesmus* cultures (0.34 and 0.38 in each case on day 2). These species presented a constant growing rate that lasted until day 12 of the experimental design, where it seemed to achieve a stationary phase with maximum values of 1.02 and 1.08. On the contrary, *Chlorella* cultures presented a higher growth rate at the beginning of the experiment, achieving 0.44 on day 2. Nonetheless, this intense growth rate was not maintained during the whole experiment. During the subsequent measurements, the observed opacity in *Chlorella* cultures showed a reduction in terms of growth rate, which was less than that of the rest of the strains, achieving a maximum of 0.8 on day 12. Regarding chlorophyll concentration, trends were similar in all the cases. On the second day, *Chlorella* reached the highest value of 0.5, while *Scenedesmus* and *Desmodesmus* stayed at 0.3 and 0.4, respectively. However, the growth for *Chlorella* was less than that of the other two, reaching the lowest value on day 12 (1.0)

Figure 6 shows the results for the *Desmodesmus* culture growth in different media, i.e., control culture growth in the absence of wastewater and experimental group growth with 100% wastewater.

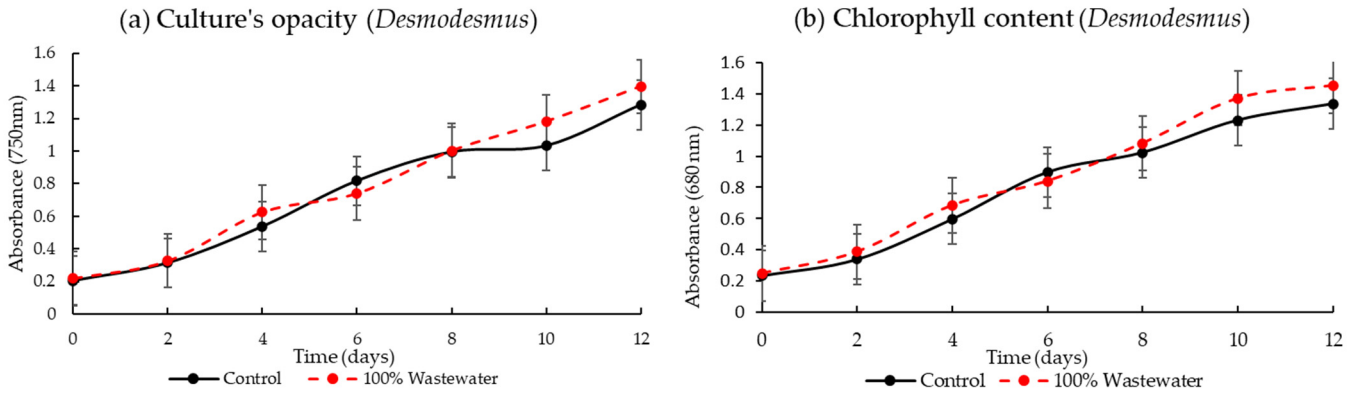

Figure 6. Measurements in 2 L experiments with *Desmodesmus* sp. growth with wastewater and the control (without wastewater): (**a**) absorbance at OD_{750} for opacity evaluation, (**b**) absorbance measurements at OD_{680} for chlorophyll content evaluation.

At inoculation (day 0), both control and experimental cultures showed significantly close starting values (0.2) in terms of opacity (OD_{750}) and chlorophyll concentration (OD_{680}). Both groups developed closely during the early stages of the assay; nevertheless, the experimental group presented a slight peak of 0.6 in opacity measurements on day 4 of experimentation, which increased in the following days (0.7 on day 6, 1.0 on day 8, and reaching the maximum value of 1.4 on day 12). Although control groups reached a higher value on day 6 (0.8), the rest of the values were always below those of the experimental group, which also showed an exponential growth phase from day 6 to 12. Control group cultures showed a fluctuation by the end of the experimental assay (days 8 to 12), showing a smaller exponential growth phase (only for days 10 to 12).

On the other hand, chlorophyll concentration analysis showed very similar tendencies to the culture opacity measures, which is visible in Figure 6. Nevertheless, control group cultures presented a curve with a more linear trend than the opacity studies. Accordingly, the exponential section of the observed curve lasted longer (from day 3 until the end of the experimental assay). This phase led to a stationary phase from day 8. Regarding experimental group cultures, by the middle period of the experimentation (day 6), the experimental group showed a clear stationary phase, which led to the obtained data being

below the yield of the control group. Furthermore, the experimental group presented a second exponential phase on day 8, reaching final maximum values higher than those observed in control group cultures.

3.2. Selection of Best Microalgae Cultivation System at Industrial Scale

After finishing the 2 L assays, *Desmodesmus* cultures were scaled up to 10 L bottles in order to increase the inoculum volume. The selected cultures survived the switch in conditions and grew with optimum results. After the acclimation assay, 10 L *Desmodesmus* sp. bottles were used as inoculums for three vertical column-type photobioreactors (PBRs), for a final volume of 100 L (Figure 3, left).

After inoculation, the cultures' opacity (OD_{750}) and chlorophyll concentration (OD_{680}) were evaluated; results are shown in Figure 7. Cultures presented a starting stationary phase that lasted from the beginning of experimentation to the 2nd day. Nevertheless, this period led to an exponential growth phase that ended on day 9 with a second stationary phase. Thus, 9 days was considered to be the maximum growth period for this assay.

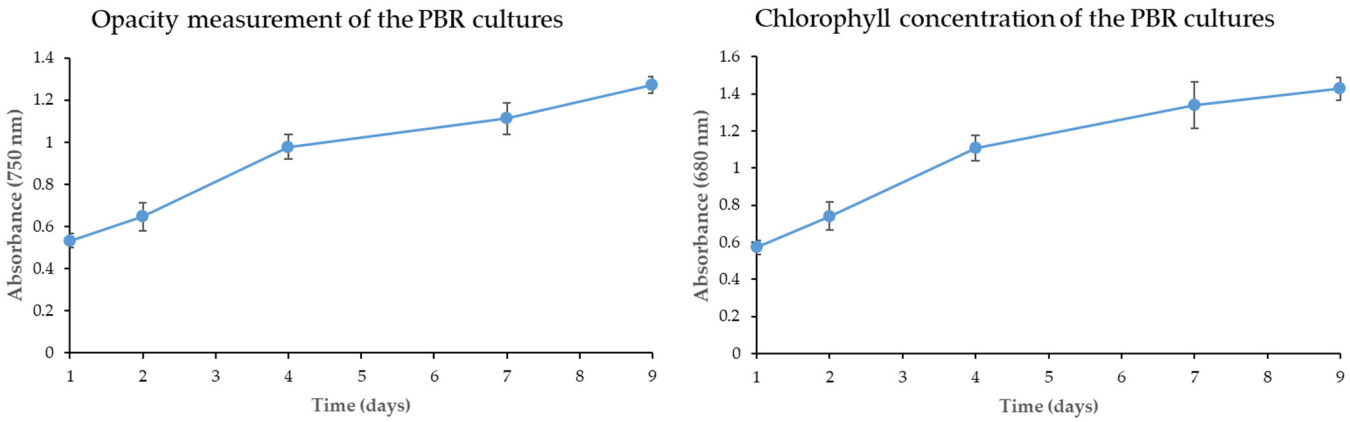

Figure 7. OD_{750} and OD_{680} measurements during closed-type PBR culturing.

It was also decided to evaluate the biomass generation via the study of the progress of the dry weight per liter ($g \cdot L^{-1}$) (see Figure 8).

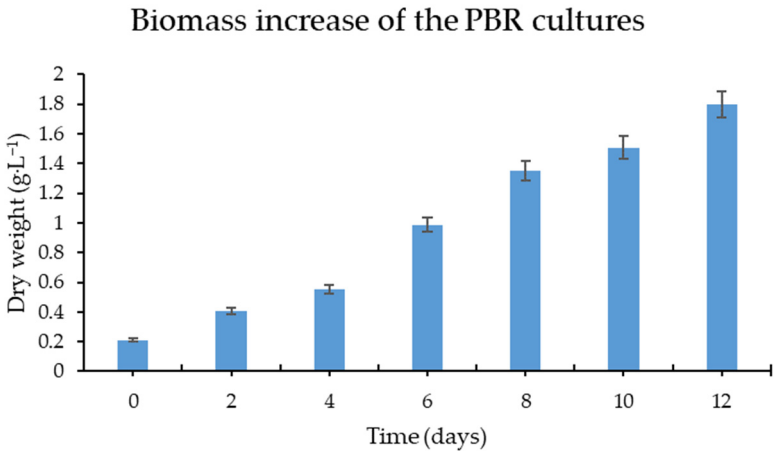

Figure 8. Dry weight biomass measurements during closed-type PBR culturing.

Dry weight biomass analysis showed that the absorbance increase that was shown in the previous absorbance analysis during the early stages of the assay (days 1 to 4) does not experience significant growth in terms of biomass synthesis (below 0.5 $g \cdot L^{-1}$). Moreover, by the 6th day of experimentation, the dry weight analysis showed that the exponential growth phase seen during previous measurements was related to a significant augmentation of

the microalgal biomass that was present in the sample, reaching up to 1.8 g·L^{-1} on day 9. Despite the fact that maximum opacity measurements were seen by day 9, dry weight analysis showed increases until day 12. On this basis, significant growth in the present biomass was observed until the end of the experimental assay.

As shown in Figure 3, right, the *Desmodesmus* cultures grown in PBRs were used as inoculums for the raceway cultivation system.

As seen during the PBR assay, microalgal growth presented a continuously growing trend, which did not show any drop in terms of opacity or chlorophyll concentration. As shown in the OD_{750} analysis in Figure 9, the growth was constant from day 0 to day 12, reaching 1.08. During days 6 to 8, the measures showed a small decreasing tendency, but recovered by day 10.

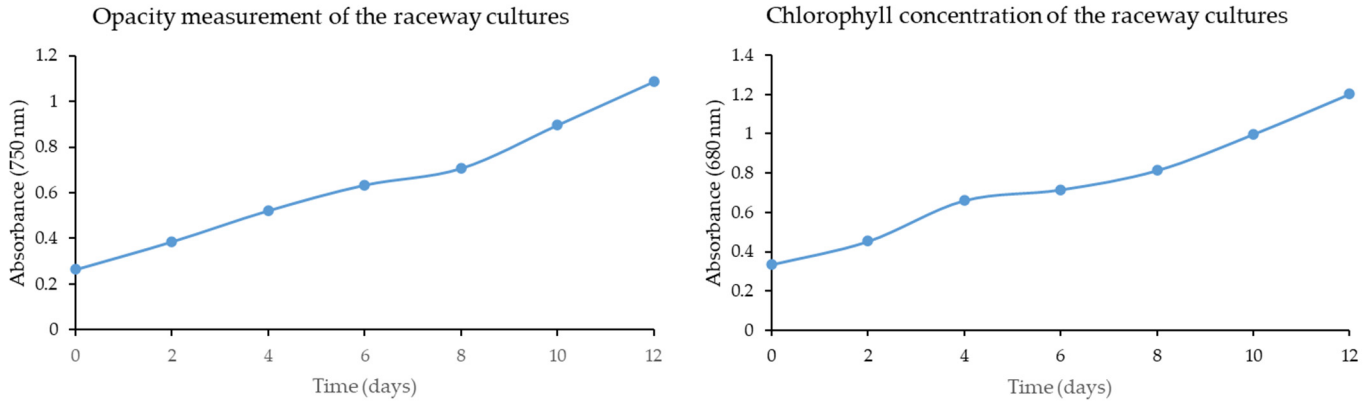

Figure 9. Absorbance measurements of the raceway cultures of *Desmodesmus* sp.

On the other hand, the chlorophyll concentration analysis also showed a starting exponential growth phase during the first 4 days, then a stationary phase, and finally from day 8 to day 12, a second exponential growth phase.

In order to evaluate the biomass generation potential of this strain and compare it among the culture systems (closed-type vs. open-type), it was decided to conduct a biomass generation study along with the absorbance measurements; results are visible in Figure 10.

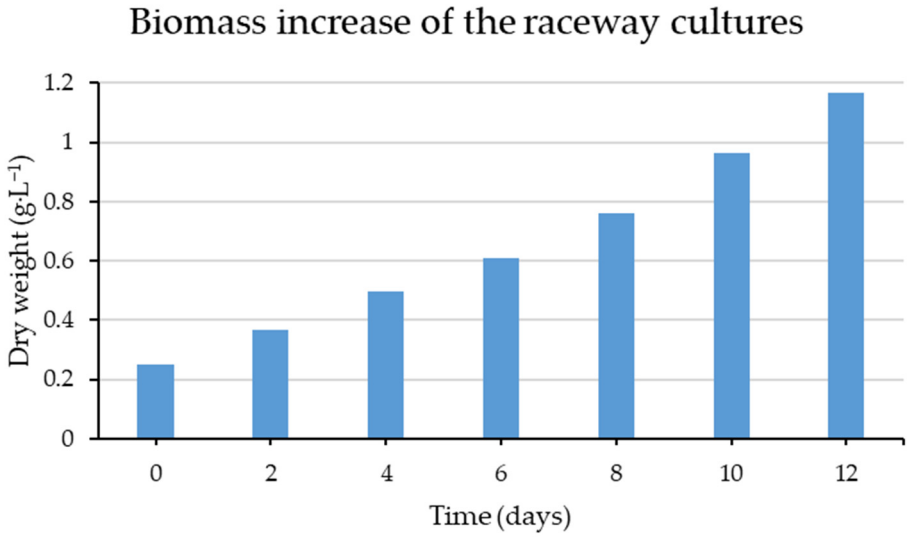

Figure 10. Dry weight biomass measurements of the raceway cultures of *Desmodesmus* sp.

The obtained results showed that this strain presented a continuous tendency that maintained a relatively steady rate during the whole experimentation, achieving 1.1 g·L^{-1}

on day 12. Furthermore, this strain showed an exponential growth phase from day 8, in terms of dry weight synthesis, which matches with the results of the opacity measurements.

3.3. Development of the Biocatalysis Process

As observed in Figure 11, the control group of the pre-concentrated assay showed a maximum OD_{680} of 1.742, which did not vary significantly with the addition of 1% and 4% of the EnzMix complex. Nevertheless, the addition of 8% of the EnzMix complex produced a clear decrease in the absorbance measurements taken, from 1.742 to 1.565. This decrease was even more significant when 10% of the EnzMix complex was applied, with the value falling to 1.393. At this point, the reduction in the concentration of chlorophylls was considered to be significant. Nevertheless, the addition of 12% of the EnzMix complex produced a slight increase in the values measured.

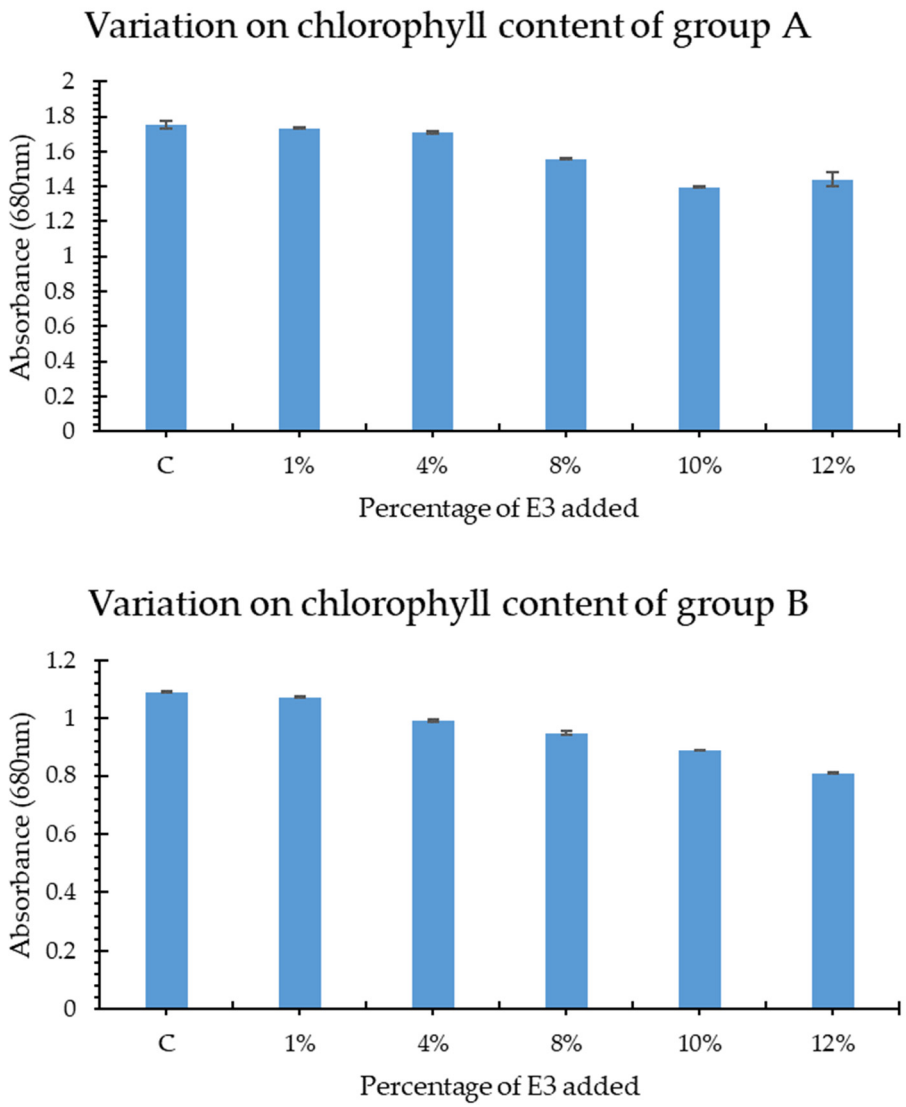

Figure 11. Variation in the chlorophyll content of the pre-concentrated culture (group A) and non-concentrated culture (group B) when hydrolyzed with EnzMix complex for 24 h.

Regarding the group B trials, the results obtained show that the addition of 1% of the EnzMix complex does not modify the chlorophyll concentration, as observed during the OD_{680} measurements. Moreover, the 4% group showed a visible decrease in the measurements taken, which relates to a better relation between substrate and enzyme if compared to the 1% group. On the same basis, the 8 and 10% groups each showed

a greater decrease in the percentage of chlorophyll present in the sample, which aligns with what was stated about the optimum relation between the quantity of substrate and enzymatic molecules. Similarly, the 12% group was consistent with the correlation between the increasing percentage of enzyme and the reduction in the absorbance measured at 680 nm.

Furthermore, these results were also supported by the visual study of the samples (see Figure 12).

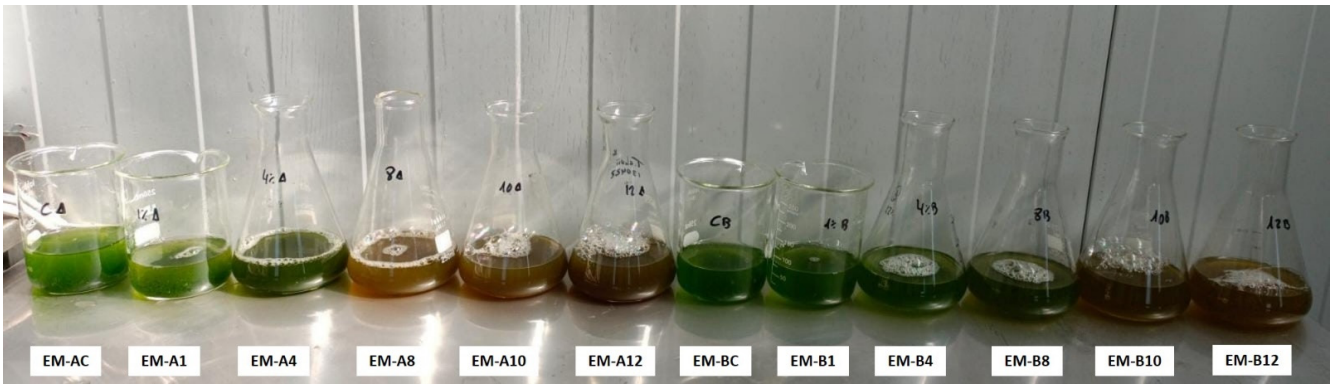

Figure 12. Example of the visual degradation of the samples used for the study of chlorophyll content of the pre-concentrated culture (group A) and non-concentrated culture (group B) when hydrolyzed with EnzMix complex for 24 h.

Colorimetric study of the results revealed that the darkening of the sample became more intense as the percentage of EnzMix complex was increased. The variance among the control group and the experimental assays was visually evident, which correlates with the efficient activity of the enzymatic complex chosen. Moreover, the influence of EnzMix on cellular integrity was more significant between the 4% and the 8% groups, which might be related to the increased percentage of enzymes. Moreover, the 8% group still presented a light-green color mixed with deep brown. This light-green color completely disappeared with the 10% EnzMix complex. On the same basis, it was decided to evaluate the cellular structure under optical microscopy in order to evaluate the degradation of the external walls (see Figure 13).

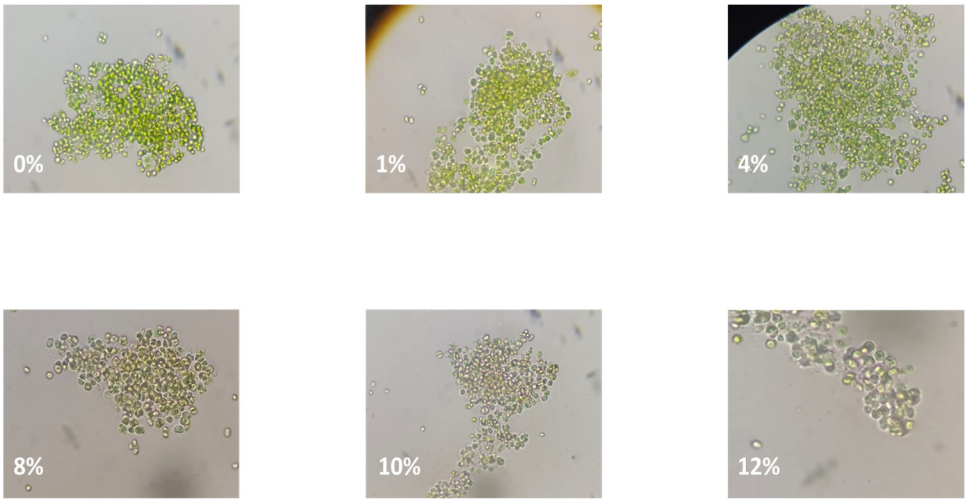

Figure 13. Microscopic view of the group A samples after 24 h of hydrolytic process.

As can be seen in Figures 12 and 13, control groups maintained a bright-green color after 24 h of agitation in the absence of the studied enzymatic complex. Moreover, the

same color was kept with the 1% and 4% EnzMix complexes, which is related to what was observed in Figure 12, mainly because the color presented in the used flasks is a magnification of what is observed at a microscopic level. On the contrary, the group that was exposed to the 8% EnzMix complex for 24 h presented a notable loss in the green color of the sample observed. This change is also related to the brown-like color observed in the visual analysis of the sample measured. Nevertheless, the observed cells preserved a slight green color; moreover, no broken cell walls were seen during the analysis of this experimental group. Furthermore, the group that was treated with a 10% EnzMix complex presented an absence of color and the structure of most of the cells was heavily damaged, which relates to the totally brown color observed during visual analysis of the flasks. On the same basis, the 12% group presented the same visual results, with no colorimetric change when compared to the 10% assays. Moreover, the study of the cellular walls showed no observable difference between the integrity of the observed samples of the 10% and 12% groups.

4. Discussion

4.1. Strain Selection in Laboratory-Scale Culturing

At a 0.5 L volume, the three studied microalgal species (*Chlorella vulgaris*, *Scenedesmus*, and *Desmodesmus* sp.) tolerated the presence of hydroponic drainage wastewater. It was observed that there was no decrease in terms of cellular population during the experimental culturing process. On this basis, drainage wastewater at 100% concentration did not present any hazardous effect on any of the selected strains. According to Figure 4, the three species showed similar trends in OD_{750} and OD_{680} measurements. Thus, these findings support the obtained results. *Scenedesmus* (which showed the lowest growth results) and *Chlorella* cultures presented diverse rising and falling stages, indicating possible metabolic stress. Both species showed no growth during the two first days of the experimentation, which may mean that time is needed to acclimate to the wastewater. *Desmodesmus* sp. cultures did not present any observable drop in the culture opacity measurements, which means that the presence of 100% drainage wastewater did not represent a source of stress for this strain. Indeed, *Desmodesmus* sp. cultures presented a continuously rising trend that was constant during the whole experimental assay. On the same basis, the experimental cultures belonging to this strain presented an opacity and chlorophyll content that were higher than those of the rest of the experimental cultures.

At a volume of 2 L, cultures behaved differently from what was observed in 0.5 L. Up to day 4, *Scenedesmus* presented more limited growth, but, from that point, showed the fastest evolution, reaching nearly the highest values of absorbance at 750 nm and 680 nm. However, *Chlorella* showed the opposite evolution, showing the highest values up to day 4 and then dropping, before reaching a stationary phase.

These results support the findings from previous works regarding cultivation of *C. vulgaris* and *S. obliquus* with agricultural drainage water (ADW) from maize fields [45]. These previous findings suggested that there were no relevant inhibitory compounds for microalgae growth, but rather that microalgae nutrients were taken up from ADW. Global productivity ($g \cdot L^{-1} \cdot$day-1) of *C. vulgaris* and *S. obliquus* increased from 0.145 to 1.96 and from 0.263 to 0.385, respectively, when the nutrient media were supplemented with ADW, but decreased to 0.071 and 0.062 when cultivated with 100% ADW. Previously, another study [12] found that *C. vulgaris* growth over nine days with drainage solution from a commercial greenhouse achieved a similar biomass dry weight ($mg \cdot L^{-1}$) compared to the control (267 and 296, respectively), which also supports the results from our investigation.

Finally, *Desmodesmus* sp. presented the highest culture opacity by the mid-term of the assay, but this then fell to be below the results yielded by *Scenedesmus*. Nevertheless, this peak was brief and *Desmodesmus* sp. ended the assay as the strain with the highest growth rate, and was shown to be the strain that presented the best acclimation to hydroponic drainage wastewater.

After the analysis of the obtained results during these first two experiments, all of the used microalgal species yielded similar results in terms of acclimation to the presence of 100% wastewater, as well as the growth rate under controlled conditions. Nevertheless, the selected *Desmodesmus* strain used during the experimental design showed slightly superior performance when compared to *Chlorella* and *Scenedesmus* strains in terms of culture opacity, chlorophyll concentration, and maximum growth rate.

A previous study [46] compared, at laboratory scale, the growth of five species of *Scenedesmus* and two species of *Desmodesmus* in potato wastewater over 7 days. Despite that fact that cellular density of the species increased significantly during the experimental assay, one of the species, *Scenedesmus* sp. (HXY5), yielded the best growth results (2.64 $g \cdot L^{-1}$).

The results yielded by *Desmodesmus* during our initial study were confirmed in the assay of 2 L, with two *Desmodesmus* groups (one control without wastewater and the other with 100% wastewater). With the exception of the measurements obtained on day 6, in which values of experimental groups decreased by 0.05 in terms of absorbance at 750 nm and 680 nm, the values were constantly higher than those of the control groups. According to the data obtained, we could not assess that the presence of wastewater significantly disturbs the development of *Desmodesmus* sp. cultures. Indeed, higher growth was shown when cultured in 100% wastewater (achieving a maximum of 1.37 in OD_{680} on day 10, while the control showed 1.37, 1.45, and 1.33 on day 12). Previous studies [47] carried out experiments to grow *Desmodesmus maximus* in the presence of different wastewaters (swine, municipal, dairy, and poultry wastewaters), achieving values of OD_{680} on day 10 between 0.8 and 1.1. Accordingly, our study improves on the growth performance of *Desmodesmus* culturing in the presence of wastewater achieved in the presented previous works.

4.2. Selection of the Best Microalgae Cultivation System at Industrial Scale

According to the results obtained for biomass productivity ($g \cdot L^{-1}$) of *Desmodesmus* cultures in PBR and raceway systems, Figure 8 shows that values are higher in PBRs, reaching 1.7 $g \cdot L^{-1}$ on day 12, than in the case of raceways (see Figure 10), which reached a maximum of 1.1 $g \cdot L^{-1}$ on day 12. Previous studies [48] enhanced the growth of two *Desmodesmus* species by up to 3.5 and 4.7 gL^{-1} after 14 days of growth using an optimal light stress (400 µmol m^{-2} s^{-1}). On this basis, we can suggest that further works can enhance the biomass production of *Desmodesmus* sp. under the described conditions in our study in order to improve this type of circular economy system.

Regarding results of *Desmodesmus* in PBRs, dry weight biomass analysis showed that, although the absorbance increased at early stages of the assay (days 1 to 4), cultures did not experience significant growth in terms of biomass synthesis. This could be explained by the natural development of microalgae by binary fission [49], which intensively affects the optical density measurements but does not produce a notable increase in the total dry weight of the sample analyzed. This effect is not shown in the raceway system where, although biomass values are lower, the tendency is more constant. Compared to the outcomes shown in PBRs, raceway cultures did not present an initial steady phase in terms of biomass generation. However, both values (OD_{750} and OD_{680}) showed clearly that this species grows with optimum results in closed-type photobioreactors such as the PBRs used in this study.

Previous works achieved similar values of biomass growth of 2 $g \cdot L^{-1}$ in half the time (6 days), growing *Desmodesmus* sp. in a bubble column photobioreactor but at a smaller scale (2.5 L) and under controlled temperature and photoperiod conditions [50]. The same culture in nitrogen-deficient conditions could increase the growth to 9 $g \cdot L^{-1}$. Other studies [51] also achieved higher biomass but in heterotrophic conditions, ranging from 2.82 $g \cdot L^{-1}$ to 3.53 $g \cdot L^{-1}$ when two-stage cultivation strategies were applied.

4.3. Optimization of a Biocatalysis Process

The results obtained indicate that 10% (regarding biomass present in the sample) is the optimum concentration of the EnzMix complex for efficient cell wall disruption, while

keeping the addition of enzymatic complexes at the minimum possible. The control group did not vary significantly with the addition of 1% and 4% of the target compound. This result might be explained by the reduced relation among the substrate (microalgae cells) and the enzymatic molecules, so there were no changes in the data obtained. However, the decrease in the absorbance measurements when a higher percentage of EnzMix was added (8% to 10%) is related to the efficient degradation of the present cellular walls. Nevertheless, the addition of 12% EnzMix produced a slight increase in the values measured, which relates to the turbidity caused by the presence of the enzymatic complex. Due to the novelty of this compound (EnzMix), we could not find any previous studies that tested this technology in microalgae disruption. On this basis, it is suggested that further studies need to develop the suggested system for biostimulant synthesis.

Previous studies examined biostimulant activity resulting from biomass suspension and aqueous extract of *Desmodesmus* [31], from *Desmodesmus* cells (culture centrifuged and washed twice with sterile distilled water) [32], and from *Desmodesmus* sp. extracts prepared with cells lysed in distilled water [52]. To the best of our knowledge, this is the first report using a biocatalysis process with *Desmodesmus* sp., which is suggested as a novel and sustainable way to produce biostimulants at an industrial scale.

The results obtained suggest an innovative and sustainable system for the reuse of hydroponic drainage wastewater in value-added products (such as biostimulants) through the cultivation of microalgae and within the framework of the circular economy paradigm. Among the microalgae species studied in this work, *Desmodesmus* sp. was found to have the best performance growing in drainage wastewater, despite being a new species in this type of application and not being studied at industrial scale. Nevertheless, further studies remain necessary to assess the biostimulant effect of *Desmodesmus* sp. biomass hydrolyzed with the EnzMix complex.

Author Contributions: M.Á.-G.: Conception and design of study, Acquisition of data, Analysis and/or interpretation of data, Writing—original draft, Writing—review and editing. M.B.-V.: Conception and design of study, Analysis and/or interpretation of data, Writing—review and editing. V.C.-B.: Conception and design of study, Analysis and/or interpretation of data, Writing—review and editing. D.S.-M.: Conception and design of study, Writing—review and editing. J.F.D.-R.: Conception and design of study, Writing—review and editing. E.R.: Conception and design of study, Writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This study was carried out within the European Union's Horizon 2020 research and innovation programme under the Green Deal grant agreement No. 101037128, project PestNu. This manuscript only reflects the authors' views and opinions and neither the European Union nor the European Commission can be considered responsible for them.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher on reasonable request.

Acknowledgments: The authors are thankful to the following partners of the PESTNU project: STAM SRL for sending Desmodesmus strains and UTH-University of Thessaly (Nikolaos Katsoulas) for sending wastewater samples of their tomato hydroponic crops and sharing information about wastewater analytic results.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Tahat, M.M.; Alananbeh, K.M.; Othman, Y.A.; Leskovar, D.I. Soil Health and Sustainable Agriculture. *Sustainability* **2020**, *12*, 4859. [CrossRef]
2. Preite, L.; Solari, F.; Vignali, G. Technologies to Optimize the Water Consumption in Agriculture: A Systematic Review. *Sustainability* **2023**, *15*, 5975. [CrossRef]
3. Aktar, W.; Sengupta, D.; Chowdhury, A. Impact of Pesticides Use in Agriculture: Their Benefits and Hazards. *Interdiscip. Toxicol.* **2009**, *2*, 1–12. [CrossRef] [PubMed]
4. Waqas, M.; Hawkesford, M.J.; Geilfus, C. Feeding the world sustainably: Efficient nitrogen use. *Trends Plant Sci.* **2023**, *28*, 505–508. [CrossRef]
5. Quintero-Angel, M.; González-Acevedo, A. Tendencies and Challenges for the Assessment of Agricultural Sustainability. *Agric. Ecosyst. Environ.* **2018**, *254*, 273–281. [CrossRef]
6. Willer, H.; Schlatter, B.; Czech Organic. *The World of Organic Agriculture. Statistics and Emerrging Trends 2023*; IFOAM—Organics International: Bonn, Germany, 2023. [CrossRef]
7. Kilic, N. Synergistic Effect of Organic and Biofertilizers on Strawberry Cultivation. *Sustainability* **2023**, *15*, 8206. [CrossRef]
8. Timsina, J. Can Organic Sources of Nutrients Increase Crop Yields to Meet Global Food Demand? *Agronomy* **2018**, *8*, 214. [CrossRef]
9. Huo, S.; Liu, J.; Addy, M.; Chen, P.; Necas, D.; Cheng, P.; Li, K.; Chai, H.; Liu, Y.; Ruan, R. The Influence of Microalgae on Vegetable Production and Nutrient Removal in Greenhouse Hydroponics. *J. Clean. Prod.* **2020**, *243*, 118563. [CrossRef]
10. Jan, S.; Rashid, Z.; Ahngar, T.A.; Iqbal, S.; Naikoo, M.A.; Majeed, S.; Bhat, T.A.; Gul, R.; Nazir, I. Hydroponics—A Review. *Int. J. Curr. Microbiol. Appl. Sci.* **2020**, *9*, 1779–1787. [CrossRef]
11. Ajeng, A.A.; Rosli, N.S.M.; Abdullah, R.; Yaacob, J.S.; Qi, N.C.; Loke, S.P. Resource Recovery from Hydroponic Wastewaters Using Microalgae-Based Biorefineries: A Circular Bioeconomy Perspective. *J. Biotechnol.* **2022**, *360*, 11–22. [CrossRef]
12. Hultberg, M.; Carlsson, A.S.; Gustafsson, S. Treatment of Drainage Solution from Hydroponic Greenhouse Production with Microalgae. *Bioresour. Technol.* **2013**, *136*, 401–406. [CrossRef] [PubMed]
13. Supraja, K.V.; Behera, B.; Balasubramanian, P. Performance Evaluation of Hydroponic System for Co-Cultivation of Microalgae and Tomato Plant. *J. Clean. Prod.* **2020**, *272*, 122823. [CrossRef]
14. Barbosa, M.J.; Janssen, M.; Südfeld, C.; D'Adamo, S.; Wijffels, R.H. Hypes, Hopes, and the Way Forward for Microalgal Biotechnology. *Trends Biotechnol.* **2023**, *41*, 452–471. [CrossRef] [PubMed]
15. Bello, A.S.; Saadaoui, I.; Ben-Hamadou, R. "Beyond the Source of Bioenergy": Microalgae in Modern Agriculture as a Biostimulant, Biofertilizer, and Anti-Abiotic Stress. *Agronomy* **2021**, *11*, 1610. [CrossRef]
16. Blanken, W.; Postma, P.R.; De Winter, L.; Wijffels, R.H.; Janssen, M. Predicting Microalgae Growth. *Algal Res.* **2016**, *14*, 28–38. [CrossRef]
17. Gonçalves, A.L.; Pires, J.C.M.; Simões, M. A Review on the Use of Microalgal Consortia for Wastewater Treatment. *Algal Res.* **2017**, *24*, 403–415. [CrossRef]
18. Brennan, L.; Owende, P. Biofuels from Microalgae—A Review of Technologies for Production, Processing, and Extractions of Biofuels and Co-Products. *Renew. Sustain. Energy Rev.* **2010**, *14*, 557–577. [CrossRef]
19. Ronga, D.; Biazzi, E.; Parati, K.; Carminati, D.; Carminati, E.; Tava, A. Microalgal Biostimulants and Biofertilisers in Crop Productions. *Agronomy* **2019**, *9*, 192. [CrossRef]
20. Costa, J.A.V.; Freitas, B.C.B.; Cruz, C.G.; Silveira, J.; Morais, M.G. Potential of Microalgae as Biopesticides to Contribute to Sustainable Agriculture and Environmental Development. *J. Environ. Sci. Health B* **2019**, *54*, 366–375. [CrossRef]
21. Acién Fernández, F.G.; Gómez-Serrano, C.; Fernández-Sevilla, J.M. Recovery of Nutrients From Wastewaters Using Microalgae. *Front. Sustain. Food Syst.* **2018**, *2*, 59. [CrossRef]
22. Blanco-Vieites, M.; Casado, V.; Battez, A.H.; Rodríguez, E. Culturing Arthrospira Maxima in Mining Wastewater: Pilot-Scale Culturing and Biomass Valorisation into C-Phycocyanin and Crude Lipid Extract. *Environ. Technol. Innov.* **2023**, *29*, 102978. [CrossRef]
23. Blanco-Vieites, M.; Suárez-Montes, D.; Delgado, F.; Álvarez-Gil, M.; Battez, A.H.; Rodríguez, E. Removal of Heavy Metals and Hydrocarbons by Microalgae from Wastewater in the Steel Industry. *Algal Res.* **2022**, *64*, 102700. [CrossRef]
24. Rodrigues, Y.V.S.; Almeida, E.D.S.; Vieira, E.D. Characterization of Arthrospira sp (Spirulina) Biomass Growth in Hydroponic Waste Solution: A Review. *J. Bioeng. Technol. Appl. Health* **2020**, *3*, 354–358. [CrossRef]
25. Bertoldi, F.C.; Sant'Anna, E.; Oliveira, J.L.B. Teor de clorofila e perfil de sais minerais de Chlorella vulgaris cultivada em solução hidropônica residual. *Ciênc. Rural* **2008**, *38*, 54–58. [CrossRef]
26. Delrue, F.; Ribeiro De Jesus Cerqueira, M.; Compadre, A.; Alvarez, P.; Fleury, G.; Escoffier, C.; Sassi, J.-F. Hydroponic Farm Wastewater Treatment Using an Indigenous Consortium. *Processes* **2021**, *9*, 519. [CrossRef]
27. Lee, J.Y.; Rahman, A.; Behrens, J.; Brennan, C.; Ham, B.; Kim, H.S.; Nho, C.W.; Yun, S.-T.; Azam, H.; Kwon, M.J. Nutrient Removal from Hydroponic Wastewater by a Microbial Consortium and a Culture of Paracercomonas Saepenatans. *N. Biotechnol.* **2018**, *41*, 15–24. [CrossRef]
28. Salazar, J.; Valev, D.; Näkkilä, J.; Tyystjärvi, E.; Sirin, S.; Allahverdiyeva, Y. Nutrient Removal from Hydroponic Effluent by Nordic Microalgae: From Screening to a Greenhouse Photobioreactor Operation. *Algal Res.* **2021**, *55*, 102247. [CrossRef]

29. Salazar, J.; Santana-Sánchez, A.; Näkkilä, J.; Sirin, S.; Allahverdiyeva, Y. Complete N and P Removal from Hydroponic Greenhouse Wastewater by Tetradesmus Obliquus: A Strategy for Algal Bioremediation and Cultivation in Nordic Countries. *Algal Res.* **2023**, *70*, 102988. [CrossRef]
30. Colla, G.; Nardi, S.; Cardarelli, M.; Ertani, A.; Lucini, L.; Canaguier, R.; Rouphael, Y. Protein Hydrolysates as Biostimulants in Horticulture. *Sci. Hortic.* **2015**, *196*, 28–38. [CrossRef]
31. Mazepa, E.; Malburg, B.V.; Mógor, G.; de Oliveira, A.C.; Amatussi, J.O.; Corrêa, D.O.; Lemos, J.S.; Ducatti, D.R.B.; Duarte, M.E.R.; Mógor, Á.F.; et al. Plant Growth Biostimulant Activity of the Green Microalga Desmodesmus Subspicatus. *Algal Res.* **2021**, *59*, 102434. [CrossRef]
32. Lopes, G.B.; Goelzer, A.; Reichel, T.; De Resende, M.L.V.; Duarte, W.F. Potential of Desmodesmus Abundans as Biofertilizer in Common Bean (*Phaseolus vulgaris* L.). *Biocatal. Agric. Biotechnol.* **2023**, *49*, 102657. [CrossRef]
33. de Lima, J.F.; de Queiroz, A.J.M.; de Figueirêdo, R.M.F.; de Souza, W.R.; Debonsi, H.M.; dos Santos, V.F.; Dantas, A.M.N.; Araújo, R.H.C.R. Utilization of Chlorella Sp. as Biostimulant in the Germination of Melon Seeds (*Cucumis melo* L.). *J. Agric. Stud.* **2020**, *8*, 750–773. [CrossRef]
34. Oliveira, A.C.; Barata, A.; Batista, A.P.; Gouveia, L. Scenedesmus Obliquus in Poultry Wastewater Bioremediation. *Environ. Technol.* **2019**, *40*, 3735–3744. [CrossRef] [PubMed]
35. Suarez-Montes, D.; Borrell, Y.J.; Gonzalez, J.M.; Rico, J.M. Isolation and Identification of Microalgal Strains with Potential as Carotenoids Producers from a Municipal Solid Waste Landfill. *Sci. Total Environ.* **2022**, *802*, 149755. [CrossRef]
36. Allen, M.M. Simple Conditions For Growth of Unicellular Blue-Green Algae On Plates. *J. Phycol.* **1968**, *4*, 1–4. [CrossRef]
37. Elvanidi, A.; Benitez Reascos, C.; Gourzoulidou, E.; Kunze, A.; Max, J.; Katsoulas, N. Implementation of the Circular Economy Concept in Greenhouse Hydroponics for Ultimate Use of Water and Nutrients. *Horticulturae* **2020**, *6*, 83. [CrossRef]
38. Kjeldahl, J. Neue Methode zur Bestimmung des Stickstoffs in organischen Körpern. *Fresenius Z. Anal. Chem.* **1883**, *22*, 366–382. [CrossRef]
39. Borowitzka, M.A.; Vonshak, A. Scaling up Microalgal Cultures to Commercial Scale. *Eur. J. Phycol.* **2017**, *52*, 407–418. [CrossRef]
40. Sarker, N.K.; Kaparaju, P. A Critical Review on the Status and Progress of Microalgae Cultivation in Outdoor Photobioreactors Conducted over 35 Years (1986–2021). *Energies* **2023**, *16*, 3105. [CrossRef]
41. Griffiths, M.J.; Garcin, C.; Van Hille, R.P.; Harrison, S.T.L. Interference by Pigment in the Estimation of Microalgal Biomass Concentration by Optical Density. *J. Microbiol. Methods* **2011**, *85*, 119–123. [CrossRef]
42. Michael, A.; Kyewalyanga, M.S.; Lugomela, C.V. Biomass and Nutritive Value of Spirulina (Arthrospira Fusiformis) Cultivated in a Cost-Effective Medium. *Ann. Microbiol.* **2019**, *69*, 1387–1395. [CrossRef]
43. Andersen, R.A. (Ed.) *Algal Culturing Techniques*; Elsevier/Academic Press: Burlington, MA, USA, 2005; ISBN 978-0-12-088426-1.
44. Okoro, V.; Azimov, U.; Munoz, J.; Hernandez, H.H.; Phan, A.N. Microalgae Cultivation and Harvesting: Growth Performance and Use of Flocculants—A Review. *Renew. Sustain. Energy Rev.* **2019**, *115*, 109364. [CrossRef]
45. Alvarenga, P.; Martins, M.; Ribeiro, H.; Mota, M.; Guerra, I.; Cardoso, H.; Silva, J.L. Evaluation of the Fertilizer Potential of Chlorella Vulgaris and Scenedesmus Obliquus Grown in Agricultural Drainage Water from Maize Fields. *Sci. Total Environ.* **2023**, *861*, 160670. [CrossRef] [PubMed]
46. Yuan, S.; Ye, S.; Yang, S.; Luo, G. Purification of Potato Wastewater and Production of Byproducts Using Microalgae Scenedesmus and Desmodesmus. *J. Water Process Eng.* **2021**, *43*, 102237. [CrossRef]
47. Das, V.; Dunford, N.; Deka, D. Waste Utilization and Biodiesel Production from Desmodesmus Maximus Grown in Swine Wastewater Using Waste Eggshells as a Catalyst. *Aquac. Eng.* **2022**, *99*, 102293. [CrossRef]
48. Mehariya, S.; Plöhn, M.; Leon-Vaz, A.; Patel, A.; Funk, C. Improving the Content of High Value Compounds in Nordic Desmodesmus Microalgal Strains. *Bioresour. Technol.* **2022**, *359*, 127445. [CrossRef] [PubMed]
49. Zachleder, V.; Bišová, K.; Vítová, M. The Cell Cycle of Microalgae. In *The Physiology of Microalgae*; Borowitzka, M.A., Beardall, J., Raven, J.A., Eds.; Springer International Publishing: Cham, Switzerland, 2016; pp. 3–46. ISBN 978-3-319-24943-8.
50. Pozzobon, V.; Levasseur, W.; Guerin, C.; Gaveau-Vaillant, N.; Pointcheval, M.; Perré, P. *Desmodesmus* Sp. Pigment and FAME Profiles under Different Illuminations and Nitrogen Status. *Bioresour. Technol. Rep.* **2020**, *10*, 100409. [CrossRef]
51. Li, Z.; Peng, S.; Li, Q.; Wei, S.; Zhang, Q.; An, X.; Li, H. Exploration of Two-Stage Cultivation Strategy Using Nitrogen Limited and Phosphorus Sufficient to Simultaneously Improve the Biomass and Lipid Productivity in Desmodesmus Intermedius Z8. *Fuel* **2023**, *338*, 127306. [CrossRef]
52. Lamb, T.I.; Berghahn, E.; Pita, F.M.; De Oliveira Neves, L.; Dos Reis Blasi, É.A.; Hofstetter, J.S.; Dammann, M.; Da Silva, L.C.O.; Buffon, G.; Dullius, A.; et al. Isolation and Selection of Microalgae Capable of Stimulating Rice Plant Development and Seed Production. *Algal Res.* **2023**, *74*, 103203. [CrossRef]