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Chlorellaceae Feedstock Selection under Balanced Nutrient Limitation

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Abstract: Microalgae are an attractive source of biomass for fossil fuel alternatives and renewable energy sources. Regardless of their potential, the development of microalgal biofuels has been limited due to the associated economic and environmental costs. We followed and compared the biomass properties of six *Chlorellaceae* strains with a specific interest in lipid-based biofuels. The strains were cultivated under balanced nutrient limitation inducing a gradual limitation of nutrients that triggered reserve accumulation. The final biomass of each strain was characterized by its elemental and biochemical composition. Due to its high lipid content and overall composition, *Chlorella vulgaris* NIES 227 was identified as an ideal feedstock for biofuels with the best energy-content biomass. Its fatty acid profile also showed superior qualities for biodiesel production. Balanced nutrient limitation promoted not only the accumulation of storage compounds in all strains, but also resulted in a low content of heteroatom precursors and ashes for biofuel applications.

Keywords: biofuel; microalgal biomass; *Chlorella*; lipids

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1. Introduction

The wide diversity of microalgae and their intrinsic qualities make them an attractive source of biomass for fossil fuel alternatives and other energy sources [1]. These microorganisms are capable of converting solar energy to biomass through photosynthesis, capturing CO_2, and storing this carbon in energy-rich compounds [2]. Regardless of the potential of microalgae as an alternative fuel source, microalgal biofuels' race against fossil fuels has been constrained by the economic and environmental costs of their production [3]. In order to compete with fossil fuels, it is essential to consider and optimize every step of the process, from the strain selection and production of the biomass to its conversion into biofuel and further upgrading. Various strategies have been considered to improve the economic value and environmental footprint of biofuel production. Microalgae cultivation in wastewater, for example, has been recognized as a win-win scenario for water treatment and nutrient recovery [4]. Process effluents, in particular, such as the wastewater recovered after biomass harvesting or after biomass conversion into biocrude, are proposed as a step towards resource sustainability [5].

When it comes to the biomass production stage, a lot of attention has been given on the yield of compounds of interest, such as lipids, carbohydrates, pigments, or other metabolites. The biosynthesis of some of these compounds serves as a survival mechanism for microalgae under stress conditions, such as nutrient deficiencies [6]. At the same time, these compounds serve as energy carriers during biomass conversion into biofuels. These biochemical characteristics of the feedstock, in combination with the process reacting conditions, determine the properties and yield of the biofuel [7,8]. Consequently, the

biomass has a major impact further down the process, as it may vary according to the metabolic capacities, cultivation techniques, and abiotic factors, like light and nutrient supply [9]. An effective and conscious use of nutrients defines both the signaled metabolic pathways and the amount of produced biomass. Nitrogen, for example, is a key element that enables cellular division when in sufficient supply, and triggers carbon accumulation, when deficient in supply, as starch or lipids [10].

Microalgal biomass can then be transformed into energy products, such as the following: biodiesel through transesterification of the lipids; bio-oil via thermochemical conversions like hydrothermal liquefaction (HTL) and pyrolysis; bio-ethanol, hydrogen and methane production by biological pathways [11]. Particularly, HTL has been recognized as an efficient method for microalgal biomass conversion into a bio-crude without the need for complete water removal, which is a high energy-intensive step that also increases the production cost [12]. During this process, the biomass is exposed to high temperatures and high pressure, during which the major biomolecules are broken down into simpler compounds and undergo further chemical reactions. Finally, the energy is recovered in the form of a biocrude, for its subsequent upgrading and biofuel production, in addition to some by-products: a gaseous phase, an aqueous phase, and a solid phase [13]. The main energy-carrier molecules during the HTL process are lipids, followed by proteins, and lastly carbohydrates [12]. Another route for lipid-based biofuels is the production of biodiesel, where lipids are directly extracted from the biomass for further transesterification [14]. Similarly, carbohydrate-rich biomass can also be transformed into bioplastics or bioethanol [15].

To work toward environmentally friendly energy sources, it is important to select strains where not just the yield and productivity of the compound of interest are considered, but also the implications on the fed biomass along the process. Having this in mind, it was the aim of our study to identify a suitable strain for lipid-based biofuels among six strains. Although it was out of the scope of this study, it was still important to take into account a possible inclusion of process effluents, which often contain high loads of organic compounds, ammonium, and some heavy metals that could inhibit microalgal growth [16]. For this, we considered the need to focus on strains that would be able to thrive in harsh conditions. *Chlorellaceae*, divided into two sister clades, *Chlorella* and *Parachlorella*, covers a wide variety of attributes that are attractive for the production of biomass for biofuel purposes [17]. This microalgae family is well known for its fast growth rate, and resistance to adverse growth conditions [18]. Additionally, different studies have shown the potential of *Chlorella* sp. to produce biomass with a high content of carbohydrates or lipids when grown under stress conditions, like nutrient limitation [19–21].

For this study, we followed a one-stage strategy with balanced nutrient limitation, where nutrients were provided in sufficient conditions to induce biomass accumulation while recovering all macronutrients from the medium. Once the microalgae consumed the nutrients from the media, the six strains were studied under nutrient-depleted conditions to potentially produce biomass with high lipid content. The synergistic effect of the microalgae metabolism, together with its efficient use of nutrients, were the main aspects chosen to select a strain with essential characteristics for lipid-based biofuels. Moreover, the close relationship between the energy content of the biomass and the different components of the microalgae was explored.

2. Materials and Methods

2.1. Microalgae Strains

The strains *Chlorella vulgaris* CCALA 256 and CCALA 269 and *Parachlorella kessleri* CCALA 251 and 253 were obtained from the Culture Collection of Autotrophic Organisms (CCALA), Czech Republic. The strains *C. vulgaris* NIES 227 and *C. sorokiniana* NIES 2173 were obtained from the National Institute for Environmental Studies (NIES), Japan. These are further referred to as the following: CCALA 256, CCALA 269, CCALA 251, CCALA 253, NIES 227, and NIES 2173, respectively.

2.2. Cultivation Media

The stock cultures were grown with Fresh Water Medium (FWM) in 125 mL flasks. The FWM 1/2 was composed of: 729.5 mg/L $NaNO_3$, 51 mg/L NH_4Cl, 116.5 mg/L KH_2PO_4, 71.5 mg/L $MgSO_4 \cdot 7H_2O$, 0.75 mg/L $CaCl_2 \cdot 2H_2O$, and 0.5 mL/L of Hutner's trace element solution [22]. The initial pH was set to 7.0 without buffer addition and no manual adjustment during cultivation. The culture pH oscillated between the CO_2 exchange and the nutrient consumption, mainly staying around 7.5–8.0. Cultivation conditions were kept constant at 25 °C in an automated incubator ZWYC 290A (LABWIT Scientific, Melbourne, Australia). The light intensity was set to 100 µmol/m^2/s, with a photoperiod of 20 h light and 4 h darkness. Agitation was kept at 130 RPM and the air was enriched with 2% CO_2.

2.3. Experimental Design: Cultivation of High-Lipid Content Microalgae under Nutrient Limitation

To assess the biomass characteristics of the microalgae, the six strains were cultivated for a period of 17 days in three biological replicates. Balanced nutrient limitation was the strategy used to trigger storage compounds. Here, we used modified FWM, medium, originally designed to provide enough macronutrients (N, P, and S) for *Chlorophyceae*-type strains to produce 4 g/L of biomass without nutrient deficiencies [22]. The calculations were made based on elemental analyses of the biomass of *Parachlorella kessleri* NIES 2152 with the following elemental composition: 50.1% C, 7.21% H, 6.68% N, 1.33% P, 0.465% S, 0.848% K, 0.428% Mg and 0.00446% Ca. Analyses were performed by SOCOR (France).

For this study, the nutrient content of the medium was reduced by 50% (FWM 1/2) to induce a natural depletion of the nutrients (particularly N, S and P), while aiming for a complete macronutrient consumption. This approach supported a one-stage starvation, where nutrient deficiency was reached as the nutrients were depleted from the medium. Once the nutrients were completely consumed by the microalgae, the cultures were left growing under nutrient starvation to determine their compound accumulation capacities. Cultivation conditions were kept the same as for the stock cultures with the exception of light intensity, which was increased to 230 µmol/m^2/s.

2.4. Growth Parameters

Microalgae growth was monitored by the determination of the number and size of the cells, optical density, and dry weight. The optical density of the culture was measured at 880 nm using a UV-Vis Epoch2 (BioTek Instruments, Winooski, VT, USA). Cellular density and volume were determined using a particle sizer and counter (Multisizer 4 Counter, Beckman Coulter, Brea, CA, USA). Depending on the optical density of the sample, an aliquot between 10 uL and 100 uL was diluted in 10 to 20 mL of filtered isotonic water (Isoton®® II Diluent, Beckman Coulter, Brea, CA, USA). Dry weight was determined with 2–10 mL of the culture. Cells were centrifuged for 10 min at 4500 rpm (4410× *g*) in an Allegra X15R (Beckman Coulters, Brea, CA, USA), equipped with a SX4750A Swinging Bucket Rotor. The supernatant was collected to determine nutrient consumption by ion chromatography, while the pellet was washed with distilled water and centrifuged again. The pellet was recovered in pre-weighed aluminum plates and dried at 105 °C for at least 24 h to determine the dry weight (DW) of the sample.

2.5. Intracellular Nutrient Content

Nutrient consumption was followed daily by ion chromatography (940 Professional IC Vario, Metrohm, Herisau, Switzerland). A Metrosep C4 was used for cations (ammonium, magnesium, potassium, and sodium) with an elution solution made of nitric and dipicolinic acids at 34 mM. A Metrosep A column was used for anions (acetate, chloride, nitrate, phosphate, and sulfate) with an elution solution made of sodium carbonate at 72 mM. The N content of the biomass was estimated by the consumption of N sources in the media, and it was further verified in the final biomass by elemental analysis. Consumption was considered as the difference between the initial and remaining N concentration in the

media. The amount of N that was taken up by cells was expressed as intracellular N-quotas in terms of biomass and (mg-N/g) and number of cells (pg-N cell/L).

2.6. Biomass Characterization

On day 4, day 11, and day 17, samples of 50 mL were collected, centrifuged at 4410× *g* in an Allegra X15R (Beckman Coulters, Brea, CA, USA), washed with distilled water, and centrifuged again for 10 min. The frozen pellets were lyophilized and stored at −20 °C until further analyses. The lipid content was quantified using an adapted Folch method from Axelsson and Gentili [23]. Briefly, the lipids of biomass samples of 10–20 mg were extracted with the addition of 2.5 mL of methanol, followed by the addition of 5 mL of chloroform. Samples were incubated for 10 min in an ultrasonic bath and homogenized with a vortex. When the samples were well suspended, 1 mL of a 0.73% NaCl solution was added to the mix and the sample was centrifuged at 1500 rpm (1470× *g*) for 10 min. Then, the top layer was removed, and the hydrophobic phase was manually recovered with a Pasteur pipette. The leftover biomass and top layer were suspended with 2 mL of Chloroform for a second lipid recovery and the hydrophobic phase was recovered again after centrifugation. The carbohydrate content of the final biomass (day 17) was measured according to a modified Dubois protocol [24]. The protein content was calculated based on the N-to-protein conversion factor of 5.04, according to Templeton and Laurens [25]. The lyophilized biomass was also analyzed for its CHONS composition, using a FLASH 2000 (Thermo Fisher Scientific, Walthman, MA, USA). Approximately 1 mg of microalgae was weighed and injected in the equipment. The C, H, N, and S analysis was performed via the analysis of the CO_2, H_2O, N_2, and SO_2 gases formed during the combustion of the samples at 900 °C, respectively. On the other hand, the oxygen analysis was performed by quantifying CO issued from the pyrolysis (inert atmosphere) at 1000 °C. The formed gases were analyzed with a TCD detector. The higher heating value (HHV) was estimated, based on the final elemental composition according to the following equation [26], where C, H, O, N, S and A are the mass percentages of carbon, hydrogen, oxygen, nitrogen, sulfur, and ash content, respectively:

$$\text{HHV (MJ/kg)} = 0.3491\,\text{C} + 1.1783\,\text{H} + 0.1005\,\text{S} - 0.10340\,\text{O} - 0.0151\,\text{N} - 0.0211\,\text{A}$$

2.7. Fatty Acid Profile

To characterize the final fatty acid profile in the microalgae (day 17), samples of 5–20 mg of lyophilized biomass were subjected to transmethylation to obtain fatty acid methyl esters (FAMEs). The samples were incubated for 1 h at 85 °C with 3 mL of the transesterification agent (1.25M hydrogen chloride in methanol, Ref: 17935, Supelco) and 0.2 mL of the 15:0-Me internal standard (prepared at 3 mg/mL in anhydrous hexane). After cooling down the samples at room temperature, 3 mL of hexane were added to recover the lipid fraction, followed by 1 mL of distilled water. Finally, the hexane was recovered after centrifugation at 1500 rpm for 5 min.

The total FAMEs and fatty acid (FA) profile were determined using gas chromatography–flame ionization detection (GC–FID) system. The FAME samples were injected with a volume of 0.5 or 1 µL in split mode by a Shimadzu AOC-20i-s autosampler into a GC2010 Pro gas chromatograph (Shimadzu, Kyoto, Japan). The chromatograph was equipped with a 30 m × 0.32 mm ID FAMEWAX capillary column (Restek, Bellefonte, PA, USA), filled with a 0.25 µm Crossbond polyethylene glycol stationary phase (polar phase). The column temperature was increased from 150 °C to 240 °C at a rate of 5 °C/min. Methyl pentadecanoate (C15:0, reference 91446-5G, Sigma Aldrich, St. Louis, MO, USA) was used as an internal standard for the semi-quantitative determination of individual and total FAs. The GC-FID data acquisition was carried out using LabSolutions software (Shimadzu, Kyoto, Japan). The compound identification was performed by the retention indices method and confirmed by two sets of standards: a mix of six FAMEs (Ref: 07631-1AMP, Supelco), and a mix of 37 FAMEs (Ref: CRM47885, Supelco, St. Louis, MO, USA). With these

standards, 95% of the fatty acid profile was validated. To further validate the identification of some specific FAMEs (C16:2 and C16:3), samples were sent to an external laboratory to confirm compounds by means of the GC-mass spectroscopy (MS) detection system.

2.8. Biodiesel Calculations

The resulting FA profiles were studied and the qualities for its utilization as biodiesel were estimated. according to Arguelles et al. [27], while considering the 95% of the FA profile that was cross-validated.

3. Results and Discussion

3.1. Growth and Biomass Accumulation under Nutrient Deficient Conditions

During the cultivation, all strains maintained a constant biomass accumulation, regardless of the reduced nutrient content of the medium (Figure 1A). Nitrogen was depleted from the mediums as they reached a biomass concentration of 2 g/L. This was correspondent to the medium composition (FWM 1/2) designed to produce this amount of biomass from a *Chlorophyceae*-type strain with nutrient deficiencies [22]. *Chlorella sorokiniana* NIES 2173 had the highest final biomass concentration and overall biomass productivity, corresponding to 5.63 g/L and 0.33 g/L/d, followed by *C. vulgaris* CCALA 256 and *C. vulgaris* NIES 227, as seen on Table 1. *C. vulgaris* CCALA 269 had the lowest overall performance, with a final biomass concentration of 3.91 g/L and overall biomass productivity of 0.23 g/L/d.

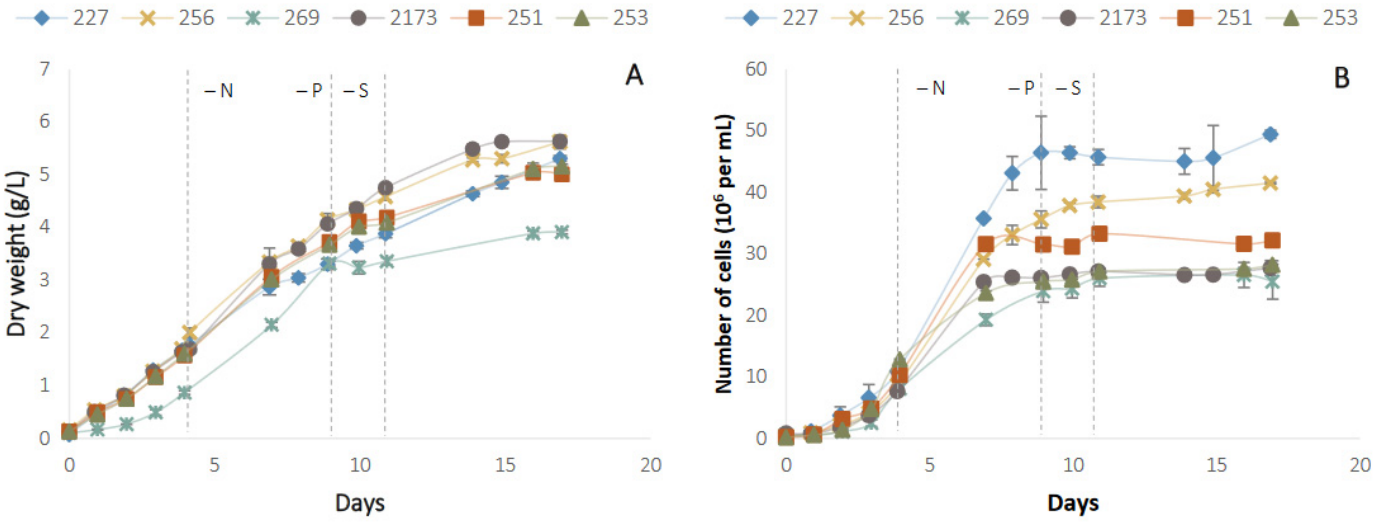

Figure 1. Growth comparison based on (**A**) Dry weight and (**B**) Number of cells of six *Chlorellaceae* spp. strains: *C. vulgaris* NIES 227 and CCALA 256 & 269, *C. sorokiniana* NIES 2173, *P. kessleri* (CCALA 251 & 253). Dashed line denotes the onset of N (-N), P (-P) and S (-S) starvation (except for CCALA 269, for which N depletion occurred on day 7).

Table 1. Microalgae growth, biomass, and lipid values comparison of six *Chlorellaceae* spp. strains: *C. vulgaris* NIES 227 and CCALA 256 & 269, *C. sorokiniana* NIES 2173, *P. kessleri* (CCALA 251 & 253).

Microalgae Strain	Growth Rate (μ/d)	Biomass			Lipids		Carbohydrates	
		(g/L)	Overall P (g/L/d)	Max P (g/L/d)	(%)	Max P (mg/L/d)	(%)	Max P (mg/L/d)
C. vulgaris NIES 227	1.67 ± 0.1	5.31 ± 0.1	0.31 ± 0.00	0.48 ± 0.01	54 ± 2%	212 ± 3	14 ± 1%	92 ± 04
C. vulgaris CCALA 256	1.55 ± 0.2	5.62 ± 0.1	0.32 ± 0.01	0.58 ± 0.08	40 ± 1%	156 ± 4	38 ± 5%	220 ± 24
C. vulgaris CCALA 269	1.20 ± 0.1	3.91 ± 0.0	0.23 ± 0.00	0.58 ± 0.03	27 ± 1%	91 ± 3	27 ± 3%	131 ± 16
C. sorokiniana NIES 2173	0.90 ± 0.1	5.63 ± 0.1	0.33 ± 0.01	0.70 ± 0.01	30 ± 4%	149 ± 1	37 ± 6%	244 ± 33
P. kessleri CCALA 251	1.60 ± 0.1	5.00 ± 0.0	0.29 ± 0.00	0.50 ± 0.00	35 ± 1%	124 ± 3	48 ± 1%	236 ± 19
P. kessleri CCALA 253	1.25 ± 0.1	5.16 ± 0.1	0.30 ± 0.00	0.47 ± 0.02	29 ± 1%	108 ± 2	53 ± 1%	199 ± 30

In contrast to the produced biomass, nutrient limitation strongly affected the cell cycle of the microalgae. Cell division ceased soon after all macronutrients were depleted from the mediums on day 9 (Figure 1B). Once cell division stopped, the cells continued to increase in volume, suggesting the accumulation of storage compounds, which was reflected by the continuous increase in biomass and cellular volume (Figure A1). Interestingly, even if N was depleted from the medium on day 4 for most of the strains (except for CCALA 269, for which N depletion occurred on day 7), cell division did not stop immediately, and a stationary phase was observed from day 9 for all microalgae. After day 10, all strains were lacking not only N, but also P and S (Figure A2). It is unknown how the lack of P and S could have affected each of the tested strains regarding the specific growth and compound accumulation. However, how N-starvation is the main trigger for the accumulation of storage compounds, like lipids and carbohydrates, has been well studied [6]. In some strains, like in *C. vulgaris* CCALA 924, sulfur deprivation resulted in a higher starch accumulation than in N-deprived conditions [28]. Sakarika et al. also observed higher intracellular lipid content under S-limitation and a higher maximum biomass and lipid productivity under P-limitation [29].

Nitrogen is a vital nutrient for the biosynthesis of proteins necessary for cell growth and metabolic requirements [30]. However, N-depletion from the mediums did not reflect the real metabolic status of the cells, since more impactful stress was produced as the cells continued to consume the internal N-reserves. Remarkably, when comparing the intracellular N-quotas between the onset of extracellular N-depletion (day 4) and after almost two weeks of intracellular N-starvation (day 17), a reduction of almost four times was observed among the strains (Table 2), indicating the consumption of intracellular N after N was completely depleted from the mediums. Based on the intracellular N quota on day four, we estimated that protein content was drastically reduced in all strains, from an initial protein content of 40–45% to reach values as low as 12–16% on day 17 (Figure 2). Microalgae can assimilate high amounts of N for the buildup of protein and nucleic acids, consisting of up to 40–60% of their dry weight [25]. Under N-limiting conditions, microalgae degrade non-essential proteins in order to support basic cellular requirements [31]. It was observed that microalgal cells continued to divide, by using their own N reserves, even after extracellular N-depletion. Cell division continued until reaching a limiting intracellular N content during the second week of cultivation.

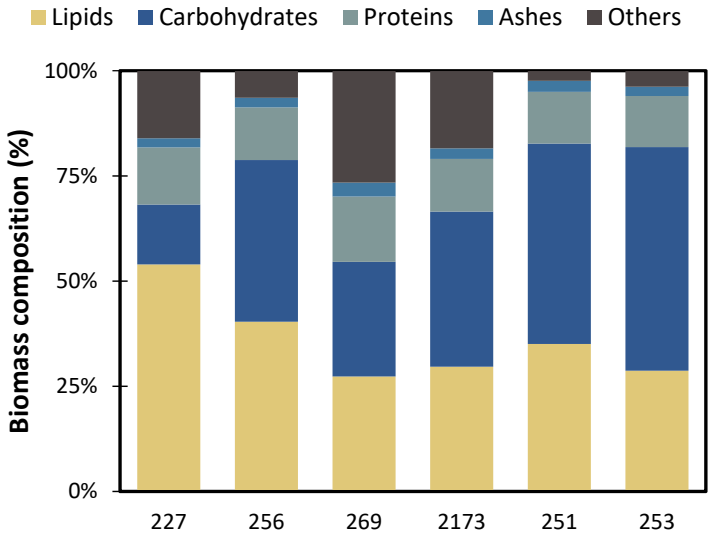

Figure 2. Final biomass (day 17) composition of six *Chlorellaceae* spp.: *C. vulgaris* NIES 227, CCALA 256 & 269, *C. sorokiniana* NIES 2173 and *P. kessleri* CCALA 251 & 253.

Table 2. Intracellular N content dynamics per (A) number of cells and (B) per biomass of six *Chlorellaceae* spp. strains: *C. vulgaris* NIES 227 and CCALA 256 & 269, *C. sorokiniana* NIES 2173, *P. kessleri* (CCALA 251 & 253).

		Intracellular N Quota					
	Day	**NIES 227**	**CCALA 256**	**CCALA 269**	**NIES 2173**	**CCALA 251**	**CCALA 253**
(A) pg of N per cell	4	1.29 ± 0.05	1.62 ± 0.03	1.88 ± 0.22	2.01 ± 0.18	1.41 ± 0.03	1.15 ± 0.01
	17	0.28 ± 0.02	0.33 ± 0.01	0.62 ± 0.08	0.51 ± 0.01	0.45 ± 0.01	0.52 ± 0.01
(B) mg of N per g of biomass	4	85.4 ± 1.9	88.1 ± 3.7	183.8 ± 8.9	89.9 ± 2.8	98.6 ± 2.9	98.7 ± 1.7
	17	26.0 ± 0.3	24.9 ± 0.6	40.0 ± 0.6	24.7 ± 0.4	29.2 ± 0.1	29.1 ± 0.3

3.2. Accumulation of Storage Compounds under Nutrient Deficient Conditions

After almost two weeks of N-starvation, the prolonged metabolic stress triggered the accumulation of storage compounds, such as lipids and carbohydrates, at different levels, depending on the strain (Figure 2). Each strain presented a different biochemical and lipid profile, even among the same species of *Chlorellaceae*. showing potential and diversity. In general, all strains had a higher lipid content compared to the 10–20% found in nutrient sufficient conditions (data not shown). Notably, different lipid accumulation patterns could be observed among the microalgae (Figure 3). NIES 227 had the earliest trigger for lipid accumulation and a continuous lipid increase since the onset of N-starvation. On the opposite side, for the strains CCALA 251 and 253, lipid biosynthesis was triggered after almost two weeks of N-starvation. The highest lipid contents were observed in NIES 227 and CCALA 256 with 54% and 40%, respectively (Table 1). Although NIES 227 had only moderate biomass productivity, this strain significantly surpassed the lipid productivities of all the tested strains with a value of 212 mg/L/day, compared to CCALA 256 and NIES 273 with 156 and 149 mg/L/day. The reduced lipid content observed in some strains coincided with a preference for carbohydrate storage. Four of the strains had a carbohydrate content of at least 35%. The highest carbohydrate content was found in both *P. kessleri* strains, CCALA 251 and 253 with 48% and 53%, respectively. NIES 227 had the lowest carbohydrate content with 14%. During nutrient limitation, total carbohydrates can reach up to 55% of the biomass dry weight [28].

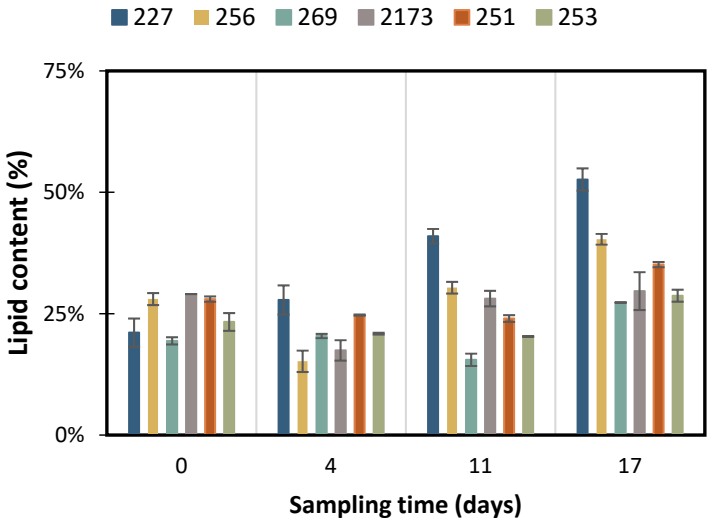

Figure 3. Lipid accumulation (as biomass %) under nutrient limitation over time: day 0, 4, 11 and 17 for six *Chlorellaceae* spp. strains: *C. vulgaris* NIES 227 and CCALA 256 & 269, *C. sorokiniana* NIES 2173, *P. kessleri* (CCALA 251 & 253).

The observed differences between the strains could be explained by the different rates of carbohydrate synthesis and degradation inherent to each strain. Nitrogen stress has been widely studied for its effect on both lipid and starch accumulation and the degree and extent to which this stress may have different responses among microalgae, and remarkably even within the same species [32]. The specific preference of each strain towards starch or lipid storage under nitrogen stress depends on each microalgae's metabolic capacities and energy requirements for the biosynthesis of these molecules [33]. Nordin et al. identified, by proteomic studies, that *Chlorella vulgaris* (UPSI-JRM01) responded to the absence of N by producing both storage compounds for different purposes [10]; where the storage of starch corresponded to an immediate response to N starvation, and a subsequent carbohydrate degradation and lipid biosynthesis took place for long-term energy storage. The transition from starch storage to lipid biosynthesis might differ from one strain to the other, explaining the differences in lipid and carbohydrate contents that we observed.

Furthermore, a different growth rate was observed among the strains, resulting in a dissimilar number of cells for a similar amount of biomass. Cellular division of NIES 227 resulted in twice the number of cells compared to NIES 2173 for the same amount of nutrients. When we compared the intracellular N content, or the protein content of the biomass, minor differences were observed. However, when comparing the intracellular N quotas, we observed that each strain had different intracellular N reserves. Interestingly, the strains with a lower intracellular N-quota (<0.5 pg-N/cell), such as NIES 227 (0.28 pg-N/cell) and CCALA 256 (0.33 pg-N/cell), were the strains with a higher lipid content. Markou et al. compared several levels of N-limitation to induce diverse N-content in the biomass of *Chlorella vulgaris* (SAG 211-11b). It was found that a lower N content (29 mg-N/g) promoted a higher lipid content in the biomass, 48%, compared to a higher N content (41 mg/N g) with 28% of lipids [34]. In our study, it could be observed that the biomass N content could diverge from the intracellular N quotas, depending on the cellular division of the strains. A similar N content in the strains CCALA 256 (24.9 mg-N/g) and NIES 2173 (24.7 mg-N/g) resulted in different intracellular quotas of 0.33 pg-N/cell and 0.51 pg-N/cell respectively, and distinctive biomass compositions.

3.3. Impact of the Biomass Composition as a Biofuel Feedstock

Although the biomass properties change during, and after, any conversion, it is essential to highlight how high-lipid biomass and a specific lipid profile can serve as a promising feedstock for the production of bio-crude (via HTL) or biodiesel (via transesterification). As important as the biochemical composition is, the elemental composition has become one of the most effective determinations to estimate energy generation of any biomass [35]. For our study, the nutrient deficient conditions resulted in final biomasses with high C content and decreased N content. Typically, C content in microalgae varies between 45–55% of dry biomass [30], values within 52% to 62% were found among the studied strains. The high-lipid biomass of NIES 227 presented the highest C and H (62.2% and 9.3%) content and the lowest O content (27%). In contrast, the high-carbohydrate biomass of CCALA 253 presented the lowest C and H content (52% and 7.6%, respectively) and high O content of (39.3%) (Table 3).

The results showed the influence of the C content present among the different macromolecules. For instance, carbon in the biomass decreased when carbohydrates were the predominant storage compound, since the carbohydrate backbone has a lower C content (44%) compared to lipids and proteins, 76% and 53%, respectively [31]. A positive correlation (R^2 = 0.80, *p*-value: <0.05) was determined between the lipid content of the strains and HHV. The opposite trend was observed with the sugar content (R^2 = 0.77, *p*-value: <0.05). The highest HHV was found in NIES 227 (29.8 MJ/kg), in comparison to CCALA 253 (22.9 MJ/kg), which had the highest sugar content. Correspondingly, C and O have a contrasting effect on the potential HHV of the biomass, while C has a positive correlation and O has the opposite effect [35].

Table 3. Microalgae CHONS elemental composition and calculated higher heating value (HHV) six *Chlorellaceae* spp. strains: *C. vulgaris* NIES 227 and CCALA 256 & 269, *C. sorokiniana* NIES 2173, *P. kessleri* (CCALA 251 & 253).

Microalgae Strain	C (wt. %)	H (wt. %)	O (wt. %)	N (wt. %)	S (wt. %)	HHV (MJ/kg)
C. vulgaris NIES 227	62.2 ± 0.1	9.3 ± 0.1	27.0 ± 2.2	2.6 ± 0.0	0.101 ± 0.01	29.8
C. vulgaris CCALA 256	56.3 ± 2.6	8.2 ± 0.3	35.1 ± 0.6	2.4 ± 0.1	0.097 ± 0.03	25.7
C. vulgaris CCALA 269	55.7 ± 2.1	8.1 ± 0.5	33.4 ± 0.3	3.0 ± 0.1	0.099 ± 0.01	25.4
C. sorokiniana NIES 2173	53.5 ± 0.3	7.9 ± 0.1	40.6 ± 0.8	2.4 ± 0.0	0.102 ± 0.01	23.7
P. kessleri CCALA 251	54.7 ± 0.4	7.9 ± 0.1	35.3 ± 1.2	2.4 ± 0.1	0.104 ± 0.01	24.7
P. kessleri CCALA 253	52.0 ± 0.2	7.6 ± 0.1	39.3 ± 1.5	2.4 ± 0.0	0.100 ± 0.01	22.9

Microalgae are considered high-N content biomass, mostly varying within 5–9% and reaching values as high as 12%, in comparison to 1–5% found in other biofuel feedstocks and 1%, or even less, in lignocellulosic biomass [36,37]. In this study, 2–3% of N was measured in the final biomass of all strains, which potentially could decrease the N content in the biofuel and consequent emissions. Sulfur, a less mentioned component of microalgae, which varies around 0.15% to 1.6% [38], can also have repercussions during the biomass conversion and further SOx emissions during the biofuel combustion. Remarkably, this element was also on the lower end in all the strains, with a commonly shared value of 0.10 ± 0.01%.

Mineral and trace element levels also vary among microalgae (2–20%) depending on the specific metabolic requirements and the availability in the mediums [38,39]. This ash fraction of the feedstock could later on interfere during the biomass conversion to biocrude and its further upgrading, and it could cause damage to the combustion system [40]. Remarkably, a low ash content of 2.2–3.3% was found in the final biomass of the strains (Figure 2). A comparison between conversion of a low-ash and a high-ash microalgal biomass showed the preference for a lower ash content biomass, as the high-ash biomass reduced the conversion efficiency [41].

3.4. Fatty Acid Profile for Biodiesel Applications

An alternative road for biofuel production is the extraction and transesterification of lipids to produce biodiesel. In parallel to lipid productivity, it is also important to assess the fatty acid (FA) profile of the microalgae. In our study, 65–80% of the total FA profile corresponded to C18 chains in all strains, followed by C16 chains (18–33%) (Figure 4). Major differences were observed in the saturation level of the different FA profiles. NIES 227 and CCALA 269 presented 55% and 36% of the total FAs as mono-unsaturated FAs (MUFAs), predominantly in the form of oleic acid (C18:1), with 49% and 32%, respectively. For the other strains, about 60% of the FAs were in the form of PUFAs, which are not ideal for biodiesel, but which are of great nutraceutical interest [14]. Notably, the other four strains presented a high content of linoleic acid (C18:2), known for its health properties, with values ranging from 30% to 41%.

To obtain a biodiesel with balanced properties, certain generalizations can be made, but it is the entire composition that dictates the qualities of the biodiesel. For this, the different FA profiles were studied and the qualities for its utilization as biodiesel were estimated, according to Arguelles et al. [27]. Based on these properties, NIES 227 presented not only an outstanding lipid content, but also a high-quality FA profile for its direct use as biodiesel. Interestingly, this strain was the only one covering certain regulations stated by the European standard for biodiesel [42], such as a linoleic acid (C18:3) content lower than 12% (9%), a cetane number (51) which should be at least 51 and an IV of less than 120 (Table 4).

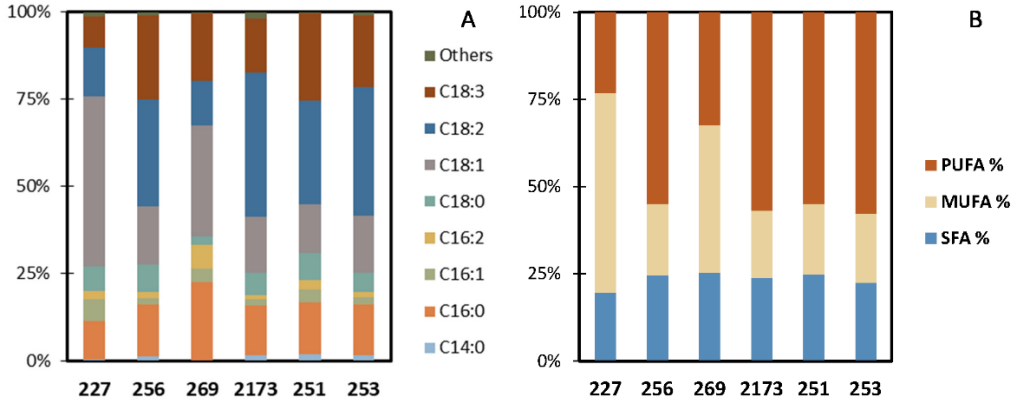

Figure 4. (**A**) FAME profile of six *Chlorellaceae* spp: *C. vulgaris* NIES 227, CCALA 256 & 269, *C. sorokiniana* NIES 2173 and *P. kessleri* CCALA 251 & 253. (**B**) Saturation distribution: Saturated fatty acids (SFA), Monounsaturated fatty acids (MUFA), Poly-unsaturated fatty acids (PUFA).

Table 4. Biomass properties for biofuel applications.

Microalgae Strain	CN (min)	HHV (MJ/kg)	C18:3 (wt. %)	IV (g I_2/ 100 g of fat)	SV (mg/ KOH g)	OS (h)	CFPP (°C)
C. vulgaris NIES 227	51.6 ± 0.2	46.5 ± 0.1	9%	99.9 ± 0.6	196.7 ± 0.2	7.7 ± 0.1	−1.5 ± 0.1
C. vulgaris CCALA *256*	42.5 ± 0.0	45.3 ± 0.0	24%	138.9 ± 0.0	198.7 ± 0.1	4.7 ± 0.1	0.9 ± 0.0
C. vulgaris CCALA 269	50.4 ± 0.1	46.3 ± 0.2	19%	108.8 ± 0.4	191.1 ± 0.2	6.2 ± 0.1	−5.5 ± 0.7
C. sorokiniana NIES 2173	43.7 ± 0.7	45.4 ± 0.4	15%	133.8 ± 1.57	198.8 ± 2.8	4.7 ± 0.1	−2.3 ± 1.7
P. kessleri CCALA 251	42.6 ± 0.2	45.4 ± 0.1	25%	138.9 ± 0.6	198.4 ± 0.2	4.7 ± 0.1	0.5 ± 0.1
P. kessleri CCALA 253	41.8 ± 0.1	45.2 ± 0.0	21%	140.6 ± 0.3	201.1 ± 0.3	4.6 ± 0.1	3.1 ± 0.0
European Standard EN 14214 [42]	≥51	-	<12%	≤120	-	≥8	≤5/≤−20

Cetane number (CN), iodine value (IV), saponification value (SV), higher heating value (HHV), oxidation stability (OS), degree of saturation (DU), cold filter plugging point (CFPP).

Certain fatty acids (FAs) could have contrasting effects on different properties of the biodiesel. Poly-unsaturated FAs (PUFAs), for example, give better properties for low temperatures, but they decrease the oxidation stability of the biodiesel [43]. Stansell et al. reported that a feedstock ideally should have a high content of MUFA, and highlighted the importance of the composition of the saturated FA (SFA) [43]. In general, a balance between a low unsaturation level of the FA and a higher proportion of longer FA chains (C16–C18) is desirable [27].

3.5. Chlorellaceae as a Source of Biomass for Biofuel Production

Multiple studies and reviews have discussed the ideal characteristics of microalgal strains that are essential for closing the gap in microalgae applications, particularly for the production of low-value products like biofuels. The following ideal characteristics have been identified rapid growth rate, high lipid productivity, tolerance to a wide range of culture conditions and ease of harvesting [44]. Additional criteria are frequently overlooked, which, in fact, could potentially increase the cost and complexity of the downstream processing and that are critical to the production of environmentally-friendly biofuels. In contrast to high-value products from microalgae, for the commercialization of microalgal biofuels it is essential to optimize every step of the process. Depending on the biomass conversion pathway, the overall biomass composition has an impact at different levels. The biomass influences not only the biofuel yield and properties, but its environmental impact as well. This is the case regarding heteroatom precursors (cyclic N-, O- and S-containing molecules) present in the biomass, such as proteins and carbohydrates. These molecules can affect the quality of the biofuel, reduce the energy content of the biomass, cause corrosion and contribute to NOx and SOx emissions during the combustion of

the biofuel [45]. Therefore, a low content of these molecules is highly desirable in any biofuel feedstock.

Chlorella, one of the most studied microalgae, cover a wide variety of characteristics that are attractive for different applications. During our study, the rich potential of the genus, and its distinctive profiles, even within strains from the same species, *Chlorella vulgaris*, was highlighted. From a small study group of strains, *Chlorella vulgaris* NIES 227 stood out as an ideal candidate for lipid-based biofuels, such as biodiesel and bio-oil production. At the same time, *Chlorella sorokiniana* NIES 2173 presented a strong biomass production, while able to produce both storage compounds at interesting levels. Although CCALA 256 did not have the highest lipid productivity in our study, Přibyl et al. (2012) described this strain as having the highest lipid productivity among other strains at much higher light intensity (500 $\mu mol/m^2/s$) [19]. CCALA 269 (CCAP 211-11b) has been equally reported, in numerous studies, to be an interesting candidate for lipid-based applications [29]. Despite having a good set of characteristics for biodiesel, under the studied conditions, an ideal lipid and biomass productivity were not achieved for this strain. This strain had an overall limited growth, possibly indicating that favorable conditions were not met for this specific strain. Moreover, the two *P. kessleri* strains, CCALA 251 and 253, remain promising candidates for sugar-based applications, due to their high carbohydrate content and productivity.

However, the higher content of carbohydrates found in *Chlorellaceae*, compared to oleaginous strains, could limit its application in lipid-based conversion pathways, like HTL [46]. *Chlorella vulgaris* NIES 227 was previously noted for its high lipid content (88%) under heterotrophic conditions, with 12 g/L of glucose, in combination with N-starvation [21]. Although this outstanding value was not achieved in our study, autotrophic conditions led to in an important amount of lipids compared to the other strains. The overall composition of the strain resulted in the highest-quality biomass for HTL, as it did not only present a high amount of lipids, but also a reduced carbohydrate content and protein content.

Finally, the use of an equilibrated medium at a macronutrient level (N, S, P), resulted in low heteroatom content in the biomass and advantageous properties as biofuel feedstocks. In addition to the accumulation of storage compounds, an optimized use of nutrients is strategic to produce sustainable energy sources. For this, it is critical to find a balance between nutrient limitation and an efficient use of nutrients. In our study, we applied a one-stage strategy to gradually trigger natural nutrient deficiency, with complete nutrient consumption. The results of this study show the potential in using a tailored medium to steer microalgal metabolism. Several advantages, like its simplicity and total nutrient consumption, could potentially be taken to a larger scale. Further research is necessary to study biomass and compound accumulation production beyond laboratory-scale experiments.

4. Conclusions

The synergy of a balanced medium and strain-specific metabolism resulted in different biochemical and lipid profiles. The observed metabolic diversity, even among the same *Chlorella* species, showed the potential of this microalgal family for different applications. *C. vulgaris* NIES 227 was identified as an ideal lipid-based feedstock, due to its superior lipid productivity and reduced content of heteroatom precursors. In parallel, *Parachlorella* strains presented the potential for sugar-based applications. A balanced nutrient limitation is proposed to produce high-lipid or high-sugar biomass. This one-stage strategy supported biomass accumulation with advantageous properties as biofuel feedstocks, while fully recovering the macronutrients from the media.

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

Appendix A

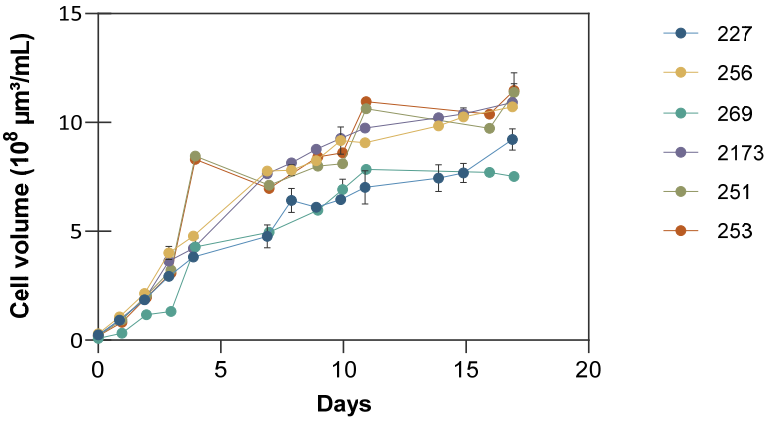

Figure A1. Cellular volume increase during compound accumulation of *C. vulgaris* NIES 227, CCALA 256 & 269, *C. sorokiniana* NIES 2173 and *P. kessleri* CCALA 251 & 253.

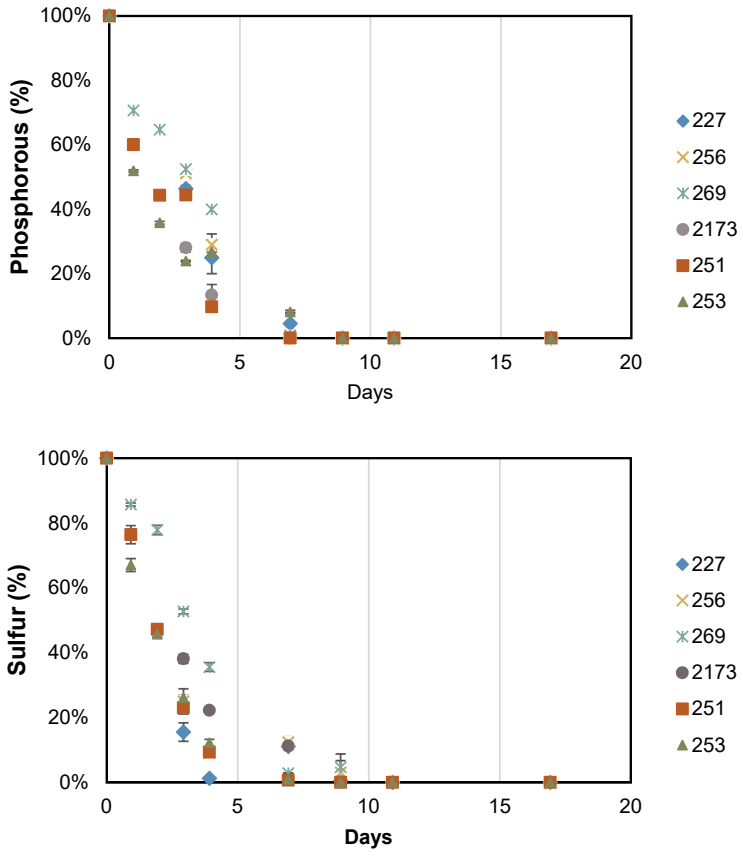

Figure A2. *Cont.*

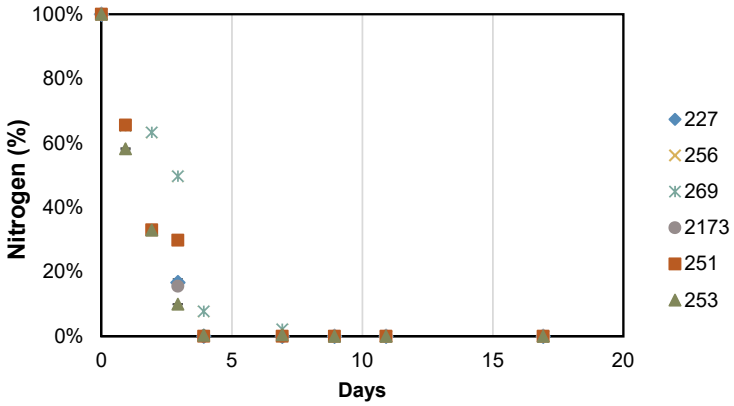

Figure A2. Nutrient availability (%) in culture media in relation to the initial content of: Phosphorous, Sulfur, and Nitrogen. *C. vulgaris* NIES 227, CCALA 256 & 269, *C. sorokiniana* NIES 2173 and *P. kessleri* CCALA 251 & 253.

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