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Heuristic Optimization of Culture Conditions for Stimulating Hyper-Accumulation of Biomass and Lipid in *Golenkinia* SDEC-16

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Abstract: Overproduction of biomass and hyper-accumulation of lipids endow microalgae with promising characteristics to realize the cost-effective potential of advanced bioenergy. This study sought to heuristically optimize the culture conditions on a rarely reported *Golenkinia* sp. The results indicate that *Golenkinia* SDEC-16 can withstand the strong light intensity and grow in a modified BG11 medium. The optimal culture conditions for the favorable tradeoff between biomass and lipid accumulation were suggested as follows, 25,000 lux of light intensity, 9 mM of initial nitrogen concentration, and 20 mM of initial sodium chloride concentration. Under these conditions, the biomass concentration and productivity reached 6.65 g/L and 545 mg/L/d, and the synchronous lipid content and productivity reached 54.38% and 296.39 mg/L/d. Hypersalinity significantly promoted lipid contents at the cost of biomass and resulted in an increase of cell size but loss of spines of *Golenkinia* SDEC-16. The results shed new light on optimizing biomass and lipid productivity.

Keywords: *Golenkinia* SDEC-16; Biomass concentration; High lipid content; Culture conditions; Carotenoids

1. Introduction

Oleaginous microalgae for biomass and lipid production has attracted immense attention in recent years, owing to their rapid growth rates, high lipid contents, valuable bio-products, and eco-friendly effects [1–3]. The widely used strains for biomass and lipid studies are green microalgae and cyanobacteria including, *Chlorella* spp, *Scenedesmus* spp, *Spirulina* spp., and *Chlamydomonas* spp. (Supplementary Figure S1). From the perspective of microalgal output, the lipid content of 30% is quite common in most of the species. In addition, strains with higher lipid content tend to grow slower than low lipid content strains [4].

Recently, we discovered *Golenkinia* SDEC-16 with a large and spiny cell and easy self-sediment for harvest [5], exhibiting great potential in both biomass yield and lipid accumulation. However, the biomass concentration (2.09 g/L) and lipid content (30.43%) of *Golenkinia* SDEC-16 [5] are still lower than the reported values (e.g., 4.72 g/L of biomass concentration and 71.45% of lipid content in *Eustigmatos* cf. *polyphem*) [6]. Thus, maximizing the production of both biomass and lipid remains a contemporary challenge [7]. Therefore, the motivation of this study was to find a strategy to maximize both biomass yield and lipid accumulation.

Additionally, studies around the *Golenkinia* sp. under different culture conditions are rare in comparison to the widely used strains. To our knowledge, the topics are concentrated on synthesis of chlorophyll [8], nutrition utilization under heterotrophic or autotrophic [9–11], physiology and ultrastructure [12,13], phylogenetic analysis [14–16], feedstock for target organic matters synthesis [17], hydrolysis of cells for carbohydrate extraction [18,19], biomass for bioenergy and wastewater treatment [5,20], etc. So, it is necessary to test the effects of culture conditions on *Golenkinia* SDEC-16.

Mounting evidence over the past decade shows that biomass and lipids are mostly influenced by light, nutrient utilization, and salinity (see Supplementary Figure S2). Generally, microalga proliferates rapidly as the increase in light intensity but stops growing when the light intensity is beyond light saturation [1]. In addition, the light intensity can change bio-composition and affect the production of starch or lipid. For example, higher productivity of lipid and starch was attained under high light intensity ($600 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) [21].

Nitrogen is a crucial nutrient to support microalgal growth but constrains lipid synthesis at a high concentration [4], thus balancing nitrogen uptake is vital in its commercial and sustainability success [22]. For instance, *Eustigmatos* cf. *polyphem* exhibited a hyper-accumulation of lipid (72.01% of dry weight) when supplied with 1 mM initial nitrogen [6]. When nitrogen is deficient, microalgal cells remobilize the carbon stream to enrich more lipids and starch [23]. Unfortunately, lipid hyper-accumulation by nitrogen deficiency is at the cost of microalgae growth, so the strategy failed to maximize the overall lipid productivity [24]. For instance, the biomass concentration of *Eustigmatos* cf. *polyphem* was only 4.72 g/L at 1 mM initial nitrogen concentration, which is inferior to 7.80 g/L at 18 mM nitrogen [6].

Salinity plays a positive role in lipid synthesis [25]. As an example, an increment of initial NaCl concentration from 0.5 to 1.0 M in *Dunaliella* resulted in higher intracellular lipid content from 60% to 67% [26]. However, microalgae would loss biomass if the salinity remained at extremely high levels [27]. Importantly, the adaptability to change culture conditions is dependent on specific species. Therefore, it is necessary to determine appropriate initial nitrogen and NaCl concentrations to maintain the balance between the biomass concentration and lipid content.

The purpose of this study was to propose an effective strategy in achieving high biomass and lipid productivity in *Golenkinia* SDEC-16. We carried out heuristic optimizations of light intensities, initial nitrogen, and NaCl concentrations, and the results show that biomass and lipid content in *Golenkinia* SDEC-16 are among the highest among green microalgae. The insights gained in this study open a new avenue in optimizing culture conditions of *Golenkinia* SDEC-16 for renewable energy.

2. Method and Materials

2.1. Experimental Design

2.1.1. Microalgal Strains

The microalga strain used in this study was *Golenkinia* SDEC-16 (GeneBank accession number: KT180320) isolated from a local lake in Jinan, Shandong Province, China. Previous cultivations of *Golenkinia* SDEC-16 were performed in the photo-bioreactors with BG11 liquid medium at $25 \pm 2 \text{ }^\circ\text{C}$ to attain healthy cells for further study.

2.1.2. Light Intensity

The following experiments were conducted in the Erlenmeyer flasks containing 500 mL BG11 medium with aeration of 1.0 L/min under a photoperiod (24: 0 h of light: dark period) at $25 \pm 2 \text{ }^\circ\text{C}$ for 12 days, and all of the experiments were carried out in triplicate. The light was provided by a row of LEDs that were horizontally fixed on the shelves on one side. Four of the Erlenmeyer flasks received continuous light intensities (10,000, 15,000, 20,000, and 25,000 lux), measured by a photometer by changing the positions toward the light source, which was considered to be the light intensity value used in the experiments.

2.1.3. Initial Nitrogen Concentration

In this study, the basal medium was BG11, which contained sodium nitrate as the nitrogen source. Two initial NaNO_3 concentrations (1 mM and 9 mM) were exposed to the 10,000 lux and 25,000 lux, respectively. The experiments with aeration of 1.0 L/min were carried out in the Erlenmeyer flasks containing 500 mL modified BG11 medium, under a photoperiod (24: 0 h of light: dark period) at 25 ± 2 °C for twelve days, and all of the experiments are carried out in triplicate.

2.1.4. Initial Sodium Chloride Concentration

In this study, the basal medium was optimized BG11 (initial nitrogen concentration of 9 mM). The initial sodium chloride concentrations were 20, 160, 320, and 640 mM with aeration of 1.0 L/min upon exposure to the 25,000 lux light intensity. The experiments were carried out in Erlenmeyer flasks containing 500 mL modified BG11 medium with NaCl, under a photoperiod (24: 0 h of light: dark period) at 25 ± 2 °C for 12 days, and all of the experiments were carried out in triplicate.

2.2. Microalgal Analyses

2.2.1. Biomass Measurements

The microalgal growth was determined by weighing the constantly dry biomass. A 5 mL volume of microalgal solution was filtered by a pre-dried and pre-weighed 0.45 μm cellulose nitrate membrane to remove the supernatant. The filtered microalgae were transferred to a clean and weighed aluminum foil dish, which was then dried to become constant mass in a thermostatically controlled oven at 60 °C. The biomass productivity, P_b (mg L/day) was calculated according to Equation (1),

$$P_b = \frac{X_m - X_0}{(\Delta t)V} \quad (1)$$

where X_m and X_0 are the respective concentrations of biomass (g/L) at the end and beginning of a batch run, V is the volume of one sample, and Δt is the duration of the run.

2.2.2. Lipid Content

The lipid content was measured by solvent extraction and gravimetry [28]. The harvested microalgae were freeze-dried and then ground to an approximately uniform powder for lipid extraction. About 0.1 g of dry biomass powder was mixed with 10 mL of chloroform-methanol solution (2:1, v/v) in a centrifuge tube. The mixture was treated by ultrasonication (Ultrasonic Cell Crusher, SCIENTZ-IIID, China) for 10 min and subsequently centrifuged at 2000 g for 10 min at 4 °C. The supernatant was moved to a clean and dry 60 mL separatory funnel. The entire extraction process was repeated twice. After the extraction, NaCl solution (0.9%) of one fifth the volume of the supernatant was added into the separatory funnel. The mixture was shaken well for 1 min and then kept still for 15 min to stratify. The volume of the lower-phase solution was measured, and 5 mL of the lower-phase solution was then transferred to a clean 10 mL glass tube. The solvent in the 5 mL sample then evaporated under a nitrogen stream. The tube containing lipid was dried in an oven at 60 °C to attain a constant weight. The lipid content (LC) was expressed as a fraction of the dry weight and calculated based on the formula

$$LC = (m_2 - m_1) \times V / (5 \times m_0) \quad (2)$$

where m_0 (g) is the weight of algae powder, m_1 (g) and m_2 (g) are the weights of the empty glass tube and the tube containing dried lipid, respectively, and V (mL) is the volume of the lower phase.

Lipid productivity (LP , g/L/d) was determined according to the following formula:

$$LP = P_b \times LC / \Delta t \quad (3)$$

where LC (%) is the corresponding lipid content, and Δt (days) is the total algal cultivation time.

2.2.3. Microalgal Cell Size Measurement

Lengths of cells' radiuses and outer spines at the end of the experimental groups are recorded by an inverted fluorescence microscope (Ti-s, Nikon, Japan). Statistical analyses of the algal cells were computed with NIS-Elements D 4.20.00 software (Nikon, Japan).

2.2.4. Pigments Measurements

Pigments contents measurement procedures were as follows. First, a 2 mL culture was centrifuged at 9000 g for 10 min to discard the supernatant. The pellet was mixed with methanol and broken via grinding. Then, the mixture was incubated in darkness for 24 h at 45 °C. Subsequently, the solution was centrifuged at 9000 g for 10 min. The absorbance of the supernatant was measured at wavelengths of 470 nm, 652.4 nm, 665.2 nm, and 750 nm on a UV-visible spectrophotometer (ZDS-10, Shanghai Cany Precision Instrument, China). Concentration was calculated according to following the formulas [29]:

$$\text{Chlorophyll a } (\mu\text{g/mL}) = 16.72A_{665.2} - 9.16A_{652.4} \quad (4)$$

$$\text{Chlorophyll b } (\mu\text{g/mL}) = 34.09A_{652.4} - 15.28A_{665.2} \quad (5)$$

$$\text{Carotenoids } (\mu\text{g/mL}) = (1000A_{470} - 1.63chl_a - 104.9chl_b)/221 \quad (6)$$

Absorbances at 470, 652.4, and 665.2 nm were corrected for turbidity by subtracting absorbance at 750 nm.

2.3. Nutrients Measurements

Filtered samples were used to test total organic carbon concentration by TOC analyzer (TOC-L CPN CN200, Shimadzu, Japan). The total nitrogen (TN), ammonium, and total phosphorus (TP) were determined according to the Chinese state standard testing methods [30].

2.4. Experiment of Water Replenishment

The effects of water loss on microalgal biomass production were evaluated under the optimized culture conditions. The settings were as follows: the control group was cultivated without any additional operations, and the other one was added distilled water to ensure identical to the initial volume every day. The biomass concentrations and real production were recorded.

3. Results

3.1. Effects of Culture Conditions on Biomass Concentration

Figure 1 shows the characteristics of the biomass concentrations and lipid contents in *Golenkinia* SDEC-16 under light intensities, initial nitrogen, and NaCl concentrations. The maximal biomass concentration was 5.27 g/L under the light intensity of 25,000 lux, followed by 5.18, 4.98, and 4.88 g/L under 20,000, 15,000, and 10,000 lux, respectively.

Reduced initial nitrogen concentration significantly affected biomass yield. At a 1 mM initial nitrogen concentration, the biomass concentrations decreased to 3.5 g/L and 3.8 g/L under light intensities of 10,000 lux and 25,000 lux, respectively, which were lower than those in the BG11 medium under the same light intensities. When the initial nitrogen concentration was 9 mM, the biomass concentrations were 4.45 g/L and 5.28 g/L, respectively, upon exposure to 10,000 lux and 25,000 lux, which were nearly identical to those in BG11 upon exposure to the same light intensities. The results illustrated that the over-reduction of initial nitrogen concentration exerted an adverse effect on microalgal growth.

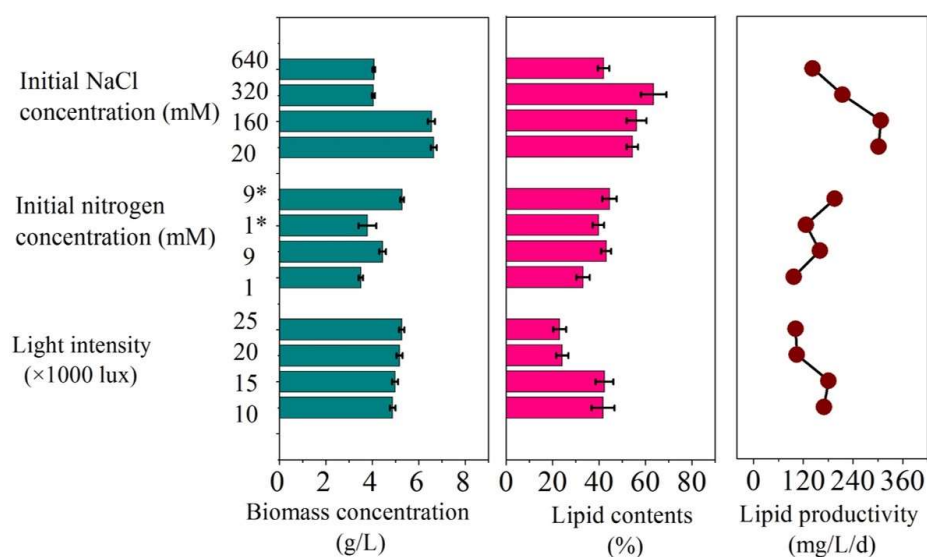


Figure 1. Biomass concentrations, lipid contents, and lipid productivities of *Golenkinia* SDEC-16 were exposed to different culture conditions. In the initial nitrogen concentration group, 1 and 9 represent 1 mM and 9 mM initial nitrogen concentrations under the 10,000 lux; 1* and 9* represent 1 mM and 9 mM initial nitrogen concentrations under the 25,000 lux.

The biomass concentration decreased in the medium with the increase in initial NaCl concentration, and a maximum yield was obtained (6.65 g/L) at a level of 20 mM NaCl. This biomass yield (6.65 g/L) was the highest in comparison to other experimental groups, which was about 1.3 times as much as the maximal biomass yield (5.28 g/L) in the medium without NaCl.

3.2. Effects of Culture Conditions on Lipid Accumulation

As shown in Figure 1, a significant change in lipid contents was observed under different light intensities. Under 10,000 lux and 15,000 lux, the lipid contents were 41.77% and 42.34%, which were appreciably higher than those under 20,000 (24.19%) and 25,000 (23.07%) lux. Given the conflict between biomass concentration and lipid accumulation, a strategy of reduced initial nitrogen concentration was introduced, and lipid synthesis was enhanced. Specifically, under 10,000 lux, the lipid content reduced to 33.17%, which is lower than that in the BG11 medium, when the initial nitrogen concentration was 1 mM. However, the lipid content (43.16% of dry biomass) at the 9 mM initial nitrogen concentration was similar to that in BG11. In contrast, under 25,000 lux, the lipid contents reached 39.82% and 44.59% of dry biomass at 1 mM and 9 mM of the initial nitrogen concentrations, respectively, which were 1.65 and 1.93 times higher than those obtained in BG11 upon exposure to the same light intensities. Hence, the 9 mM of initial nitrogen concentration can compensate for the low lipid content and exert no effects on biomass concentration under the 25,000 lux.

To further enhance lipid accumulation, NaCl was introduced. An increase in initial NaCl concentrations from 20 mM to 320 mM resulted in the enhancement of lipid content up to 63.51%, but lipid content decreased at 640 mM NaCl. Thus, the optimized NaCl concentration is 320 mM.

Lipid productivity can reflect both biomass concentration and lipid accumulation, and it is an essential indicator for biofuel production. It is worth noting that higher lipid productivities (166.01 and 176.93 mg/L/d) were attained under low light intensities (10,000 and 15,000 lux), which illustrated that lipid production needs to consider both biomass concentrations and lipid contents. Regarding the nitrogen group, the highest lipid productivity, 189.91 mg/L/d, was attained at a 9 mM initial nitrogen concentration under 25,000 lux light intensity. Similar to the case of lipid content, introduced NaCl facilitates lipid productivity, and the highest value was 296.39 mg/L/d in the medium supplemented with NaCl at 20 mM, which was approximately 1.5 and 1.4 times higher than that at 0 and 320 mM of initial NaCl concentrations. Taken together, the optimal culture conditions for

synchronous accumulation of biomass and lipid are as follows, 25,000 lux, 9 mM initial nitrogen concentration, and 20 mM initial NaCl concentration.

3.3. Effects of Culture Conditions on Microalgal Morphology

Cell size plays a pivotal role in maintenance and culture productivity [31]. The holotype of *Golenkinia* sp presents that the cells possessed 5–10 μm of radius, and cells are irregularly arranged numerous spines [32]. As depicted in Figure 2, cell radiuses and spine lengths of *Golenkinia* SDEC-16 were substantially affected by the ambient change. The average radiuses in the light group were 7.61, 8.00, 8.47, and 8.13 μm , corresponding to the 10,000, 15,000, 20,000, and 25,000 lux. Regarding initial nitrogen concentrations, upon exposure to the 10,000 lux and 25,000 lux light intensity, the average radiuses were 8.01 μm and 7.93 μm at 9 mM, and were 8.95 μm and 8.70 μm at 1 mM, respectively. The cell radius was enlarged as the salinity was increased, while reduced at higher concentration, with the highest values (14.34 μm and 18.50 μm for average and maximum, respectively) occurring at 320 mM initial NaCl concentration.

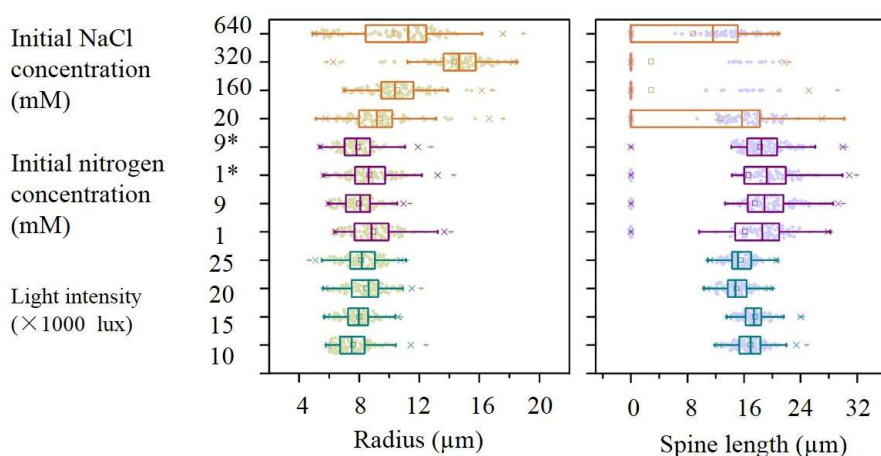


Figure 2. Cell radiuses and spine lengths of *Golenkinia* SDEC-16 were exposed to different culture conditions. In the initial nitrogen concentration group, 1 and 9 represent 1 mM and 9 mM initial nitrogen concentrations under the 10,000 lux; 1* and 9* represent 1 mM and 9 mM initial nitrogen concentrations under the 25,000 lux.

In terms of spines, the spine lengths of *Golenkinia* SDEC-16 at reduced initial nitrogen concentrations were all longer than those in the light intensity groups. Conversely, the increasing salinity is the main factor shortening the spine lengths. Cultivations at 160 mM and 320 mM initial NaCl make the most of algal cells discard their spines, resulting in zero of the median value. There was a proportion of cells without spines, although the higher median values were 15.72 μm and 11.59 μm at 20 mM and 640 mM initial sodium chloride concentrations.

3.4. Effects of Culture Conditions on Pigments Production

Green microalgae are rich in pigments, especially chlorophyll, which is a valuable byproduct for improving human health. Changes in chlorophyll were also measured in the present work to reveal the effect of change in culture conditions (Figure 3). The chlorophyll-a content was strongly affected by light intensities. Initially, the chlorophyll-a increased upon exposure to 10,000 lux compared with 80,000 lux, with the highest value (17.83 mg/g), and then decreased gradually as light intensified. The chlorophyll-a contents decreased with decreasing initial nitrogen concentration in comparison to the BG11. Besides, the increase in salinity retarded chlorophyll-a accumulation.

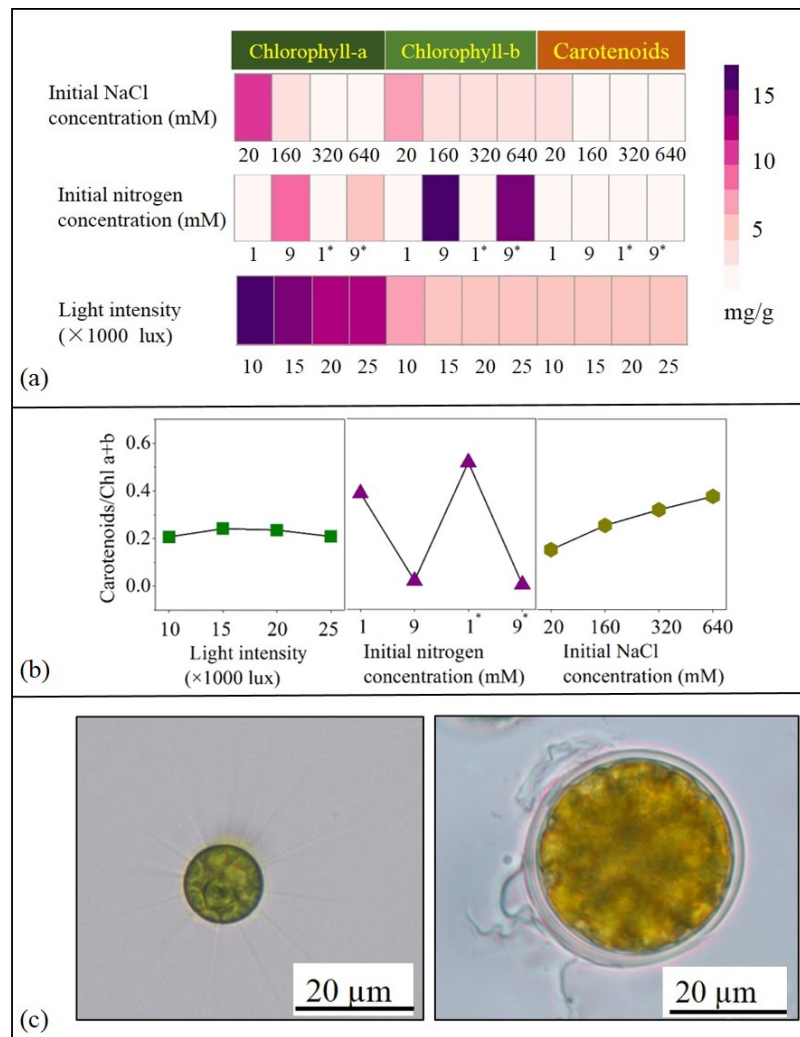


Figure 3. Chlorophyll contents of *Golenkinia* SDEC-16 were exposed to different culture conditions: (a) chlorophyll-a, chlorophyll-b, and carotenoids contents; (b) the ratio between carotenoids and chlorophyll a and b; (c) photos display *Golenkinia* SDEC-16 in BG11 and enlarged cells under salinity stress conditions. In the initial nitrogen concentration group, 1 and 9 represent 1 mM and 9 mM initial nitrogen concentrations under the 10,000 lux; 1* and 9* represent 1 mM and 9 mM initial nitrogen concentrations under the 25,000 lux.

The chlorophyll-b was weakly affected by changes of light intensities, and salinity, except for initial nitrogen concentrations. The contents for chlorophyll-b were up to 17.13 mg/g and 13.75 mg/g at 9 mM initial nitrogen concentration under the 10,000 lux and 25,000 light intensities, respectively. The lower values of chlorophyll-b were 1.33 mg/g and 0.91 mg/g in media, which were provided by 1 mM initial nitrogen concentration under the same light intensities. High values of carotenoids were attained under light intensities of 10,000 lux and 15,000 lux, corresponding to 4.96 mg/g and 4.97 mg/g. The nitrogen deficiency and the hypersalinity played a negative role in carotenoids accumulation.

3.5. Effects of Culture Conditions on Nutrient Utilisations

Nutrients are central to the metabolisms of microalgae. Figure 4 quantitatively depicts the nutrient variation in algae. The total organic carbon (TOC) concentrations were low at begin, owing to autotrophic mode, and then increased in all of the treatment groups in the end since natural complexes, such as exopolysaccharide and glycoproteins, exerted from microalgae or dead cells [33,34]. The peaks of TOC concentrations were 896.07 mg/L under the light intensity of 20,000 lux and 531.21 mg/L at

9 mM initial nitrogen concentration. A downtrend was observed with an increase in initial NaCl concentrations, and the highest value for TOC concentration was 338.86 mg/L in the media with initial NaCl at 20 mM.

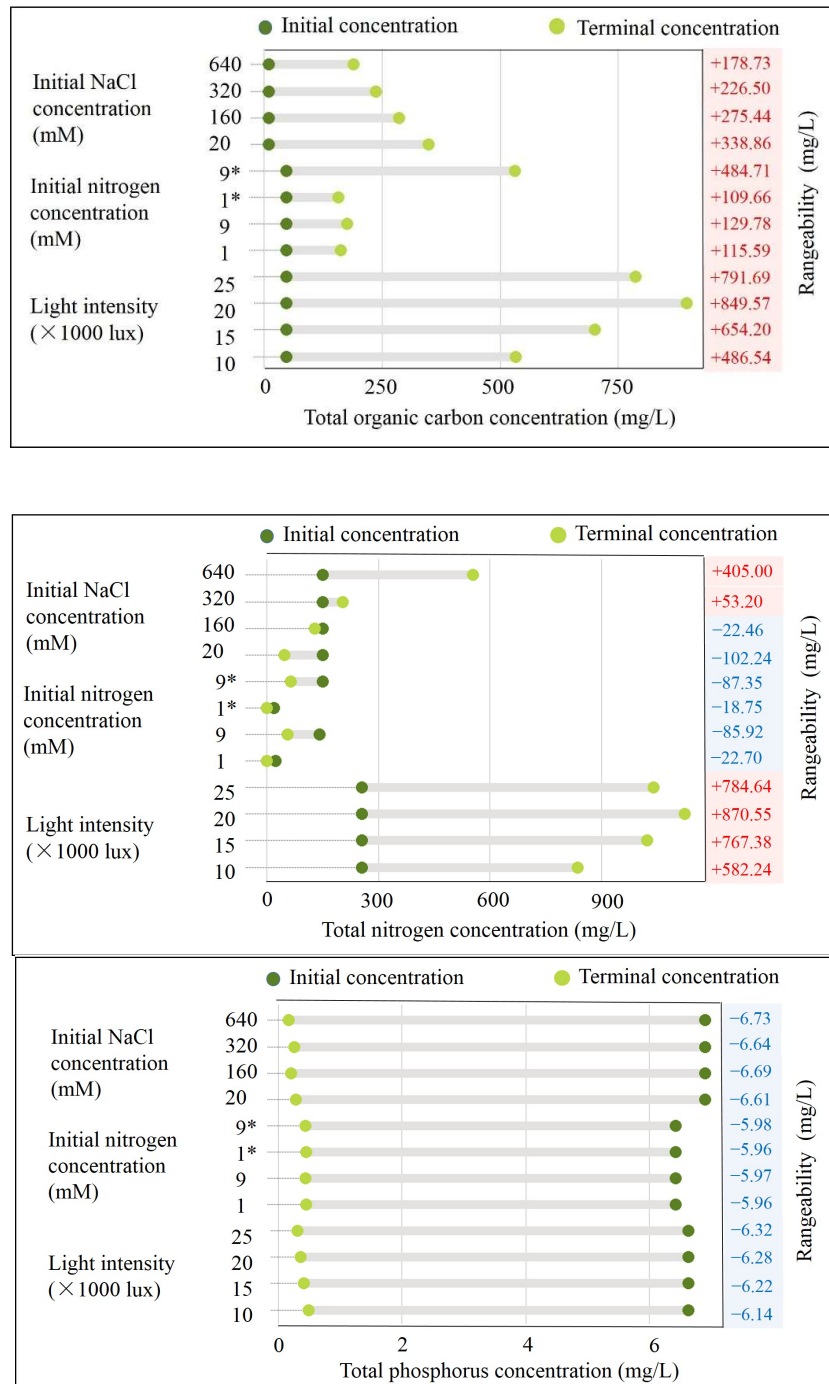


Figure 4. The residue concentrations of total organic carbon, total nitrogen, and total phosphorus were exposed to different culture conditions. In the initial nitrogen concentration group, 1 and 9 represent 1 mM and 9 mM initial nitrogen concentrations under the 10,000 lux, and 1* and 9* represent 1 mM and 9 mM initial nitrogen concentrations under the 25,000 lux.

The maximal residue of total nitrogen concentrations was 1039.01 mg/L under 20,000 lux, followed by 1124.93, 1021.76, 836.62 mg/L under 20,000, 15,000, and 10,000 lux. The residue total nitrogen

concentration decreased when the initial nitrogen concentration reduced. The residue total nitrogen concentrations were 56.14 mg/L and 63.33 mg/L at 9 mM initial nitrogen concentrations under light intensities of 10,000 lux and 25,000 lux, respectively. Under the same light intensities, the total nitrogen was utterly taken up at 1 mM of initial nitrogen concentration. The residue total nitrogen concentration increased gradually with higher initial NaCl concentrations, the lowest residue of concentration of which was 47.51 mg/L in a media that supplemented with 20 mM initial NaCl. Phosphorus is also vital for microalgal metabolism. Phosphorus participates into generating ATP through phosphorylation along with adenosine diphosphate (ADP) [4]. In the present work, total phosphorus was consumed entirely in all the experimental groups.

3.6. The Relationship between Microalgal Indicators

Correlations among microalgal indicators of *Golenkinia* SDEC-16 are depicted in Figure 5. The biomass concentrations exhibited no statistically significant associations with other indicators. Lipid content significantly shows a positive correlation with the cells' radius and significantly negative relationships with spine length and the residues of TOC concentration and total nitrogen concentration. Chlorophyll-a shows a negative association with radiuses and significantly positive correlations with carotenoids, the residue of TOC, and the total nitrogen. Carotenoids contents also exhibited a positive relationship with the residue of TOC and nitrogen concentration. A statistically significant negative correlation existed between radius and spine, and a positive correlation existed between the residue of TOC and nitrogen concentrations.

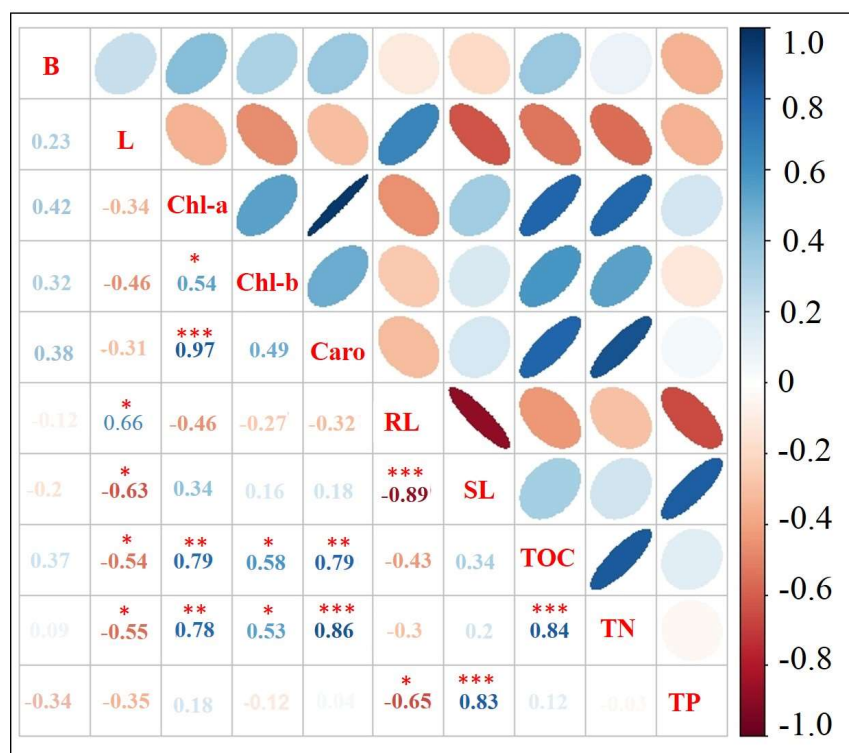


Figure 5. Correlation between microalgal indicators. Biomass (B) represents the final microalgal biomass concentration. Lipid (L) represents the final microalgal lipid contents. Chlorophyll-a (Chl-a) represents chlorophyll-a contents. Chlorophyll-b (Chl-b) represents chlorophyll-b contents. Caotenoid (Caro) represents carotenoid contents. Radius lengths (RL) represents the radius of cells. Spine lengths (SL) represents the microalgal spine lengths. Total organic carbon (TOC) represents the final total organic carbon concentration. Total phosphorus (TP) represents the final total phosphorus concentration. Total nitrogen (TN) represents the final total nitrogen concentration.

4. Discussion

Changes in light intensity remarkably affected the biomass concentration and lipid content of *Golenkinia* SDEC-16. It is well acknowledged that microalgae grew rapidly with an increase in light intensity, but it stopped growing when exposed to strong light intensity. The excess light causes photooxidation to PSII components, which results in photoinhibition. Photoinhibition damaged essential proteins required for the electron transfer in photosynthesis, which resulted in lowering biomass productivity [35]. Nevertheless, the densest biomass concentration was attained under the 25,000 lux at the end of the culture. It might be ascribed to light counteraction by microalgal self-shading against damage by intense light when the cell density turned dense.

Lipid content is enhanced by increasing light intensity through activating critical enzymes in the synthesis process of fatty acid [36]. Previous work showed lipid content for *Golenkinia* SDEC-16 was 37.8% in BG11 under the 5,000 lux on day eight [5]. In the present work, the lipid content was promoted under the 10,000 lux and 15,000 lux but sharply dropped under the 20,000 lux and 25,000 lux, respectively, stating that there is a peak of light intensity that can benefit lipid synthesis. Similarly, *Chlorella vulgaris* was found to obtain the maximal lipid content under the peak ($560 \mu\text{mol m}^{-2}\text{s}^{-1}$) of given light intensities [37]. Given that distinct behaviors of biomass concentration and lipid accumulation under 10,000 lux and 25,000 lux, strategies that reduce initial nitrogen concentrations were subsequently evaluated to make compensation to low lipid content.

Nitrogen is one of the essential nutrients for microalgae, and limitation or redundancy of nitrogen supply can affect the biomass concentration and change the biochemical composition. Microalgae utilize nitrate by nitrate enzymes and transferred nitrate as ammonium to participate in metabolism [4]. According to Figures 1 and 4, the results indicate that the 1 mM initial nitrogen concentration is too insufficient to support the *Golenkinia* SDEC-16 growth and the 9 mM initial nitrogen concentration can play an identical role to the BG11 in the growth of *Golenkinia* SDEC-16. Additionally, the 9 mM initial nitrogen could be greatly consumed in comparison to the BG11, illustrating that the 9 mM is the economical level in the application of microalgae cultivation.

As for lipid synthesis, the most commonly known is that nitrogen limitation effectively increased the lipid accumulation [38]. Generally, nitrogen limitation imposes changes upon the microalgae in three aspects. First, the cellular content of the thylakoid membrane is decreased. Second, acyl hydrolase is activated, and the phospholipid hydrolysis is stimulated. Third, the intracellular content of fatty acid acyl-CoA may be enhanced via those changes and be converted to triglyceride via activated diacylglycerol induced by nitrogen limitation. Therefore, nitrogen limitation could facilitate both lipid and TAG synthesis in microalgal cells [4]. The lipid content of green algae under the nitrogen deprivation could be multiplied up to two-to-three-fold [4]. Interestingly, both increases and decreases in the lipid contents have been reported in some microalgae, depending on the species [4,6]. For instance, the lipid contents of five *Eustigmatophytes* species significantly increased as the initial nitrogen concentration decreased from 18 mM to 1 mM, except for *Vacuoliviride* sp., which exhibited an opposite trend [6]. In the present work, the results indicate that the reduction of initial nitrogen concentration under 25,000 lux can facilitate the lipid synthesis but exert little effects on lipid synthesis under 10,000 lux.

For the sake of further enhancement of lipid content, supplementation with NaCl was evaluated. Salinity stress affected microalgae in three types—(1) ionic stress broke the balance of ion homeostasis, (2) high salt-reduced osmotic potential and water uptake, and (3) generation of reactive oxygen species (ROS) caused oxidative stress [39]. ROS exerted adverse effects on cells and made microalgae responded by facilitating antioxidative enzymes. Moreover, salinity stress changed membrane composition by regulating ion transporters [40]. That is the reason that microalga cannot survive in a high salinity environment.

In accordance, media with hypersalinity retarded the biomass yield in the present work. Surprisingly, low initial salinity facilitated the biomass accumulation in comparison to previous experimental groups. We speculated that *Golenkinia* SDEC-16 took up more nutrients, as evidenced

by the higher reduction of nitrogen concentration (102.24 mg/L) at 20 mM initial NaCl concentration. The lipid content increased gradually with salinity, while the content decreased with the initial 640 mM NaCl. The results indicate the 640 mM NaCl gave rise to severe impairs to *Golenkinia* SDEC-16 with the observations of low biomass concentration and pigment contents. The lipid content sharply decreased, primarily because the hypersalinity could not support the microalgal metabolism by the destruction of the thylakoid membranes and the reduction in photosynthetic activity and electron transport efficiency [27]. Therefore, we suggested culture conditions aiming at the maximal output are as follows, light intensity (25,000 lux), initial nitrogen concentration (9 mM), and initial NaCl concentration (20 mM).

In terms of pigments, algae have developed specialized mechanisms to regulate more carotenoids with the help of a non-photochemical process to dissipate excess light energy [35]. Carotenoids can quench triplet excited states of chlorophylls through non-photochemical quenching and help chlorophylls received energy [41]. It is reported that up-regulations of the transcripts of carotenogenic genes, like *PDS*, *BKT*, and *CHYb*, upon exposure to intense light irradiations [41]. Salt stress is another accepted driving force to enhance carotenoids accumulation [32,41] by up-regulation of carotenogenic genes *BKT* [41].

In the present study, the highest carotenoids were attained under the 10,000 lux and 15,000 lux, although the orange-reddish of the cells occurred upon exposure to salinity stress. It is explained by the high ratios of carotenoids and chlorophyll a+b at high salinity (Figure 3b,c). Moreover, hypersalinity decreased chlorophyll a, chlorophyll b, and carotenoids via damages of photosystem II reaction centre [42]. The highest value of carotenoids in this work is higher than that in *Golenkinia* sp. FAUBA-3 (2.1 mg/g) [32]. Compared with published studies, contents for carotenoid were reported to range from 0.3 mg/g to 6.6 mg/g among 22 separate microalgal species [42]. Carotenoid content in *Golenkinia* SDEC-16 is in a high middle position compared with reported strains [42] but far below the value reported for a *Scenedesmus* sp. (34.2 mg/g of total carotenoid) [43]. Further studies will be undertaken to enhance carotenoid productivity through optimization of other culturing conditions. Therefore, we suggested culturing conditions aiming at the carotenoids output are as follows: light intensity (10,000 lux) and BG11 media.

Regarding associations among the microalgal indicators in Figure 5, no statistically significant correlations with biomass concentration illustrated that the description of biomass concentration varying should be limited to culturing conditions. For instance, biomass concentration decreased with an increase in lipid contents under the condition of growth from 20 mM to 320 mM of initial NaCl, which was consistent with the trend of conflict between biomass and lipid accumulation. However, both biomass concentration and lipid content in the media with 20 mM initial sodium chloride increased in comparison to mediums without salt under the same light intensities, which is against the view of the conflict between biomass and lipid accumulation.

The morphology of a longer cell radius with a shorter spine and the hyper-accumulation of lipid content concurrently occurred upon exposure to the salt stress. The extended cell size caused by hypersalinity gave rise to difficulties for the outer cell wall to envelop the whole cell (see Supplementary Figure S4). A positive relationship between the high TOC and nitrogen concentration indicates that cells were broke so that parts of the natural complexes in cells were discarded into the medium (see Supplementary Figure S4). The abundant nutrient might create a mixotrophic mode, improving efficiency in photosynthesis, which explained the positive association between pigments and nutrients.

Aside from the promotion of microalgal growth under optimal culturing conditions, water loss is a crucial factor to lead to hyper biomass concentration. The present work compared experimental groups with or without water replenishment. As depicted in Supplementary Figure S3, the final biomass concentration without water replenishments is far beyond group without water replenishment, although the final biomass production is similar, indicating high biomass concentrations benefited from water loss. The promotion of biomass and lipid productivity was achieved without water

replenishment, and the residue culture medium can reduce cost, owing to less volume liquid required for harvesting.

Retrospective to the present work as seen in Table 1, both the biomass concentration and lipid content in *Golenkinia* SDEC-16 are clearly enhanced by the optimized culture conditions in comparison to the same genus in the published work. The results indicate the effective efforts in improving the *Golenkinia* SDEC-16 production. Regarding the microalgae under the autotrophic mode, the maximal biomass concentration (6.65 g/L) in *Golenkinia* SDEC-16 under the optimized culture conditions caught up with some top outputs, such as *Chlorella vulgaris* FACHB-31 (5.57 g/L) [37], *Eutigmator cf. polyphem* (4.72 g/L) [6]. In addition, the lipid content (54.38%) in *Golenkinia* SDEC-16 under the optimal culture condition located in a high position in the reported lipid contents compared with the widely used strains, such as *Chlorella* sp., *Scenedesmus* sp., illustrating the promising potential for renewable energy.

Table 1. Comparison of the biomass concentrations and lipid contents in *Golenkinia* SDEC-16 with published results.

Species	Main Strategy	Biomass Concentration (g/L)	Lipid Content (%)	Reference
<i>Golenkinia</i> SDEC-16	High light intensity	4.88–5.27	23.07–42.34	This study
<i>Golenkinia</i> SDEC-16	High light intensity; Decrease in nitrogen	3.5–5.28	33.17–44.59	This study
<i>Golenkinia</i> SDEC-16	High light intensity; Decrease in nitrogen; Salinity stress	4.04–6.65	41.98–63.51	This study
<i>Golenkinia</i> SDEC-16	No stress	2.05	30.43	[5]
<i>Golenkinia</i> sp. FAUBA-3	Salinity stress	1.02	37.2	[32]
<i>Eutigmator cf.</i> <i>polyphem</i>	Nitrogen deficiency	4.72	72.01	[6]
<i>Chlorella vulgaris</i>	High light intensity	2.8	53.57	[21]
<i>Chlorella kessleri</i> NIES-2159	High light intensity	5.6	21.43	[21]
<i>Scenedesmus</i> <i>obliquus</i> XJ002	Salinity stress	0.22	32.26	[27]
<i>Chlorella vulgaris</i> FACHB-31	High light intensity	5.57	41.66	[37]
<i>Chlorella</i> sp	No stress	1.53	24.97	[42]
<i>Scenedesmus bijugus</i>	No stress	1.57	24.24	[42]

5. Conclusions

For the sake of hyper-accumulation of biomass and lipid, the best culturing parameters were 25,000 lux of light intensity, 9 mM of initial nitrogen concentration, and 20 mM of initial NaCl concentration. Under these conditions, the final biomass concentration reached 6.65 g/L, and the biomass productivity was 545 mg/L/d. At the same time, the lipid content was as much as 54.38% of biomass and lipid productivity achieved 296.39 mg/L/d. The highest carotenoid value was 4.96 mg/g under the 10,000 lux, which can bring additional values.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1996-1073/13/4/964/s1>, Figure S1: The distribution of published articles related to used strains and topics, Figure S2: The distribution of published articles related to culture conditions and research objectives, Figure S3: The biomass concentration and production of *Golenkinia* SDEC-16 under the conditions of water replenishment or not, Figure S4: Photos of *Golenkinia* SDEC-16 under the salty stress.

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