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Maximizing Nitrogen Removal and Lipid Production by Microalgae under Mixotrophic Growth Using Response Surface Methodology: Towards Enhanced Biodiesel Production

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Abstract: The present study aimed to optimize synthetic wastewater composition as a mixotrophic medium for enhanced growth and lipid accumulation coupled with high nitrogen removal by the green microalga *Chlorella* sp. Individual effects of the three main independent variables (nitrate concentration, seawater ratio, and glycerol supplementation) were tested initially, then response surface methodology (RSM) was subsequently performed to explore the optimum combined conditions. The highest lipid productivity of 37.60 mg/L day was recorded at 25% seawater. Glycerol supplementation enhanced both lipid content and biomass production, which resulted in the highest recorded lipid productivity of 42.61 mg/L day at 4 g/L glycerol. Central composite design followed by numerical optimization was further applied which suggested $NaNO_3$ concentration at 101.5 mg/L, seawater ration of 23.8%, and glycerol supplementation of 0.25 g/L as the optimum conditions for dual maximum lipid productivity and nitrogen removal of 46.9 mg/L day and 98.0%, respectively. Under the optimized conditions, dry weight and lipid content increased by 31.9% and 20.3%, respectively, over the control, which resulted in increase in lipid productivity by 71.5%. In addition, optimization process resulted in pronounced changes in fatty acid proportions where saturated fatty acids increased by 7.4% in the optimized culture with simultaneous reduction of polyunsaturated fatty acids. The estimated biodiesel characteristics calculated from the fatty acid methyl ester (FAMEs) profile showed agreement with the international standards, while optimized cultures showed an 8.5% lower degree of unsaturation, which resulted in higher cetane numbers and lower iodine values. This study provides economical approach for optimization and efficient nutrient recycling through cultivation of *Chlorella* sp. for further enhanced biodiesel production.

Keywords: optimization; biofuel; factorial design; *Chlorella* sp.; lipid productivity

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1. Introduction

Nowadays, exploring renewable energy resources is becoming an urgent issue, not only due to continued reduction of fossil fuel reserves but also for environmental and economic concerns. Solar energy, tidal energy, hydro energy, wind energy, and biomass energy are the most popular renewable energy sources. Among the different renewable energy resources, biofuels are receiving increasing consideration, where the first biofuel-flight took place in 2018, followed by more than 150,000 flights using biofuel blends [1]. However, the current biofuel share represents <0.1% of total aviation fuel usage [2]. From energy and environmental aspects, biodiesel has many advantages, including renewable clean characteristics, biodegradability, and comparable performance to fossil diesel [3]. From the total biodiesel production cost, feedstock represents 70–80% [4], where vegetable oils are the main biodiesel feedstock currently used. In addition to the elevated biodiesel production cost, using edible oil raises the *food-versus-fuel* debate due to competition with human food, possibly leading to soaring food prices [5,6]. Therefore, using non-edible

feedstocks could reduce the biodiesel production cost and enhance its commercialization to replace fossil diesel.

Microalgae have been discussed as a promising biomass feedstock for different kinds of biofuels [7], including the transesterification of algal lipids into biodiesel [8,9]. Despite the numerous advantages of microalgae compared to terrestrial plants, several issues still need to be addressed in order to achieve sustainable microalgal-based biofuels. The costs of cultivation of microalgae and further downstream processing represent the main challenge needed to be overcome. For cultivation, nutrient cost plays a considerable factor in the elevated production cost of microalgal biomass [10]. In addition, high lipid content is the main limiting factor for acceptable biomass characteristics. Therefore, achieving enhanced microalgal growth coupled with high lipid content is a crucial step towards enhanced volumetric biodiesel yield in a certain period of time (known as productivity) [11]. In addition, replacing costly synthetic growth media with waste streams through integrated routes can achieve economically-feasible microalgal biomass production systems [12]. Thus, microalgae cultivation on wastewater for biodiesel production has been discussed to reduce the overall cost with simultaneous wastewater treatment [13]. In case of microalgae, cellular metabolism can be controlled by changing the cultivation conditions, providing a simple tool to enhance certain desired metabolites in the biomass [14]. Among different growth conditions, salinity optimization was reported as a promising approach for enhanced microalgal growth and lipid accumulation [15]. Recycling of biodiesel byproducts, mainly waste glycerol, could be used to achieve environmentally and economically sustainable system for microalgae-based biofuel production [16]. It is estimated that every 100 kg of the produced biodiesel results in 10 kg of waste glycerol [17]. Microalgae with ability to grow mixotrophically or heterotrophically can utilize glycerol as an exogenous carbon source for enhanced growth and lipid production [18]. The microalgal stress response owing to nitrogen starvation is another cost-effective mechanism to enhance lipid accumulation in microalgal cells [19]. However, nitrogen deficiency inhibits cellular growth with accumulation of oil in starved cells, which results in lower biomass/lipid productivity [20]. Thus, tuning nitrogen concentration of the growth medium to achieve the maximum lipid yield is of great importance.

Many studies evaluated the effect of the aforementioned factors individually on microalgae for enhanced biomass and lipid recovery, lacking the possible impact of their interaction. For optimization, response surface methodology (RSM) has been recommended to develop a multivariate method at the minimum experimental runs for reduced analysis cost at a shorter experimental time [21–23]. Studying the effect of combined factors including nitrogen concentration, salinity stress, and glycerol supplementation on growth and lipid production coupled with wastewater treatment was not previously evaluated. Therefore, the present study optimized the composition of mixotrophic growth medium using synthetic wastewater for enhanced microalgal growth, lipid accumulation, and fatty acid methyl ester (FAMEs) quality coupled with high nitrogen removal by the chlorophyte *Chlorella* sp. The three main independent variables were tested and studied individually, then central composite design (CCD-RSM) was applied to achieve the optimum conditions for enhanced lipid production and high nitrogen removal. In addition, optimum conditions obtained from CCD-RSM model were experimentally tested and validated. Moreover, simulated biodiesel characteristics at the optimized conditions were evaluated in FAMEs comparing to the typical synthetic wastewater medium. This study provides economical approach for optimization and efficient nutrient recycling through microalgae cultivation for enhanced biodiesel production.

2. Materials and Methods

2.1. Microalga and Growth Conditions

Chlorella sp. (GenBank accession number MW817150.1) was isolated from Yingming lake, Chengdu University campus (Figure S1, Supplementary data). It was cultivated in 700 mL of synthetic wastewater at initial optical density (OD_{680}) of 0.04. Wastewater was

composed of (mg/L); NH_4Cl 230, $MgSO_4 \cdot 7H_2O$ 400, K_2HPO_4 200, $NaNO_3$ 100, $NaCOOCH_3$ 100, Na_2HPO_4 100, $CaCl_2 \cdot 2H_2O$ 100, and KH_2PO_4 50 at pH 7 [4]. The three independent factors of nitrogen concentration, salinity stress, and glycerol supplementation were tested under different levels through three separate individual experiments to assess their effect on cellular growth and lipid production. Concentrations of sodium nitrate [100 mg/L (control), 75 mg/L (−25%), 50 mg/L (−50%), 25 mg/L (−75%), and 125 mg/L (+25%)]; seawater ratio [0% (control), 25%, 50%, 75%, and 100%]; and glycerol supplementation [0 g/L (control), 2, 4, 6, and 8 g/L)] were initially studied. Microalgal cultures were incubated for 20 days at 25 °C and 100 µmol photons/$m^2 \cdot s$, and aerated with 2% CO_2-enriched air. After defining the optimum value for each factor from individual experiments, the optimum mixture value for maximum lipid productivity and nitrogen removal was determined by applying randomized central composite design (CCD) using the control and the defined optimum value as lower and upper limits using Design-Expert (Stat-Ease Inc., Minneapolis, MN, USA). Finally, numerical optimization was carried out to determine the optimum values for maximum lipid productivity and nitrogen removal. The suggested conditions were selected and applied experimentally, then lipid production and fatty acid profile were experimentally evaluated at the optimized conditions compared to the control.

2.2. Growth Measurement

Cellular growth was measured every other day by monitoring the optical density at 680 nm (OD_{680}). To calculate biomass productivity (Equation (1)), algal cellular dry weight (CDW) was measured by filtration of 10 mL of the culture through 0.45 µm pore size pre-weighted filter, then dried at 105 °C until of constant weight.

$$\text{Biomass productivity (g/L day)} = (CDW_L - CDW_E)/(T_L - T_E) \quad (1)$$

where CDW_L and CDW_E represent the CDW (g L^{-1}) at late and early exponential phases (T_L and T_E), respectively.

2.3. Total Lipid Content

Lipid extraction was performed using chloroform/methanol mixture according to the method of Folch et al. [24]. Briefly, 10 mL or 20 mL of algal culture (at late and early exponential phases, respectively) were centrifuged for 10 min at 3000× *g*, then 10 mL of 2:1 (*v/v*) chloroform/methanol was added to the cell pellet and acidified using 30 µL of 1 M HCl. Mixture was incubated at room temperature for 2 h, then 1 mL of 0.9% NaCl was added and vigorously vortexed. The mixture was centrifuged for 2 min at 200× *g* to separate the 2 phases, then the lower organic phase was siphoned into a pre-weighted glass vial, dried at 80 °C for 10 min, and weighted again to determine the weight of lipids. Lipid productivity was calculated using the following equation;

$$\text{Lipid productivity (mg/L day)} = (LC_L - LC_E)/(T_L - T_E) \quad (2)$$

where LC_L and LC_E refer to lipid production (mg L^{-1}) at late and early exponential phases (T_L and T_E), respectively.

2.4. Fatty Acid Profile

The transesterified fatty acids were analyzed according to the modified method of Christie [25], as previously described [26]. Briefly, 10 mL of culture was centrifuged at 3000× *g* for 10 min, then cell pellet was heat-treated for 3 min in a boiling water bath to deactivate lipases. Before extraction, 40 µg of glycerin-trinonadecanoate was added and lipids were extracted as described in the previous section. After solvent evaporation using a stream of argon, 333 µL of 1:1 methanol:toluene and 167 µL of sodium methoxide (0.5 M) was added and vigorously mixed. Mixtures were incubated at room temperature for 20 min, then 0.5 mL of NaCl (1 M) and 50 µL of HCl (37%) were added. Hexane (1.5 mL) was added to extract FAMEs. After evaporation of hexane by a stream of argon, 40 µL

acetonitrile was added into FAMEs and analyzed by GC-FID (GC-2200, Yunbo instruments, China) equipped with 30 m DB-FFAP column (Agilent, Santa Clara, CA, USA) using argon as a carrier gas. Detector and injector temperature was kept constant at 240 °C. The oven temperature started at 100 °C for 3 min, increased to 140 °C (20 °C/min), then increased to 260 °C (4 °C/min), where it was maintained for 5 min.

2.5. Estimated Biodiesel Characteristics

Many predictive models have been suggested to evaluate the main biodiesel characteristics based on fatty acid composition [27,28]. In the present work, the main biodiesel properties, including specific gravity (SG), cloud point (CP), iodine value (IV), cetane number (CN), kinematic viscosity (KV), and higher heating value (HHV), were calculated according to previous studies [27,29] using Equations (3)–(9);

$$ADU = \sum N \times M_f \quad (3)$$

$$KV = -0.6313\ ADU + 5.2065 \quad (4)$$

$$SG = 0.0055\ ADU + 0.8726 \quad (5)$$

$$CP = -13.356\ ADU + 19.994 \quad (6)$$

$$CN = -6.6684\ ADU + 62.876 \quad (7)$$

$$IV = 74.373\ ADU + 12.71 \quad (8)$$

$$HHV = 1.7601\ ADU + 38.534 \quad (9)$$

where ADU represents the unsaturation degree, N refers to the number of carbon double bonds, and M_f represents mass fraction of each fatty acid constituent.

2.6. Statistical Analysis

All experiments were conducted in triplicates and results are shown as the mean ± standard deviation (SD). Statistical analyses were performed using SPSS, and degree of significance was determined using one-way analysis of variance followed by least significant difference test at probability level (p) ≤ 0.05.

3. Results and Discussion

3.1. Effect of Sodium Nitrate

Nitrogen is one of the most essential macronutrients for microalgal growth as it is incorporated in lipids, proteins, and carbohydrate synthesis as well as the formation of nucleic acids [30,31]. The effect of different sodium nitrate concentrations on growth pattern of *Chlorella* sp. was evaluated, which showed the end of exponential growth phase at the 16th day of incubation (Figure 1). Results confirmed pronounced effect of nitrogen concentration on the microalgal growth. Reduction of $NaNO_3$ showed reduction of cellular density while, increasing of $NaNO_3$ by 25% enhanced the growth over the control. The same results were confirmed by biomass, where 25%, 50%, and 75% reduction of $NaNO_3$ showed significant reduction in the cellular dry weight by 13.7%, 28.5%, and 50.6%, respectively, compared to the control (Table 1). However, increasing of $NaNO_3$ by 25% resulted in significant increase in the dry weight and biomass productivity by 14.5% and 12.3%, respectively, over the control. Inhibition of photosynthetic efficiency is a typical response of microalgae for nitrogen limitation due to the strong relationship between pigment synthesis and nitrogen metabolism [32–34], where a 1.5–2.5-fold reduction in five proteins of N-starved diatom *Thalassiosira pseudonana* is involved in the synthesis of chlorophyll [35].

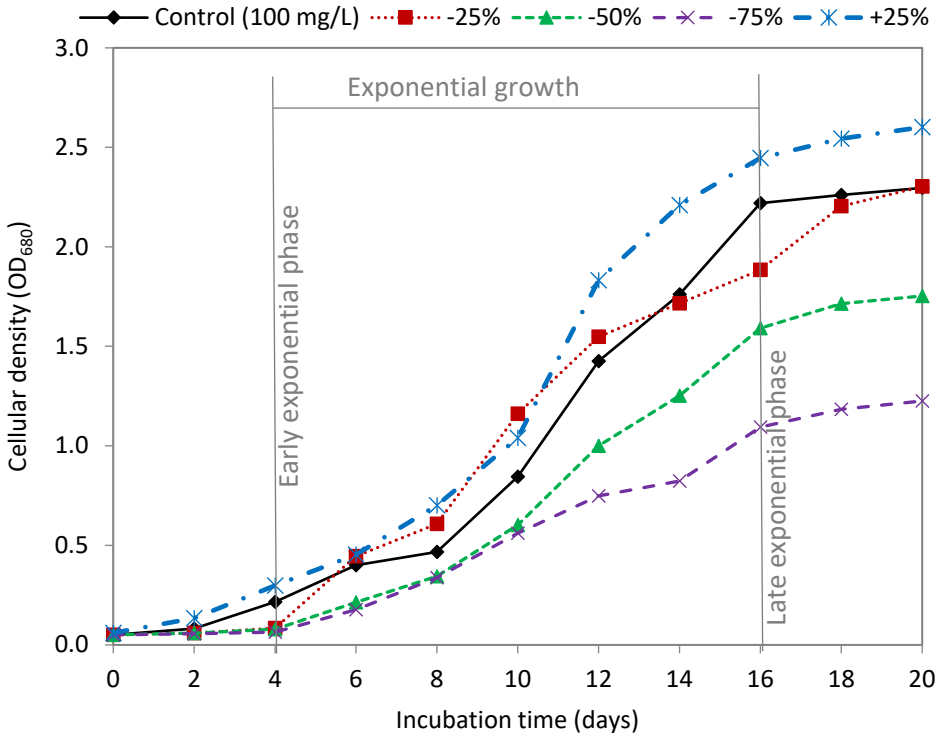

Figure 1. Effect of different concentrations of sodium nitrate on growth of *Chlorella* sp. cultivated for 20 days in synthetic wastewater.

Table 1. Effect of different concentrations of sodium nitrate on biomass and lipids of *Chlorella* sp. cultivated in synthetic wastewater and harvested at the late exponential phase.

Sodium Nitrate	Dry Weight (g/L)	Biomass Productivity (g/L Day)	Lipid Content (mg/g DW)	Lipid Productivity (mg/L Day)
Control (100 mg/L)	2.49 ± 0.079 ^{a}	0.187 ± 0.007 ^{a}	160.06 ± 4.27 ^{a}	27.27 ± 0.728 ^{a}
−25%	2.15 ± 0.071 ^{b}	0.171 ± 0.006 ^{b}	175.72 ± 6.39 ^{b}	25.36 ± 0.978 ^{ab}
−50%	1.78 ± 0.011 ^{c}	0.141 ± 0.001 ^{c}	204.81 ± 8.99 ^{c}	24.57 ± 0.013 ^{b}
−75%	1.23 ± 0.013 ^{d}	0.096 ± 0.001 ^{d}	258.23 ± 3.61 ^{d}	22.26 ± 0.319 ^{c}
+25%	2.85 ± 0.054 ^{e}	0.210 ± 0.004 ^{e}	158.23 ± 7.55 ^{a}	35.76 ± 1.965 ^{d}

Values in the same column with the same letter showed insignificant differences, while those with different letters showed significant differences (at $p \leq 0.05$).

Concerning lipid content, the results in Table 1 show that severe reduction in $NaNO_3$ by 75% led to significant increase in lipid content by 61.3%. However, increase in $NaNO_3$ by 25% showed insignificant impact on lipid content. It was confirmed that microalgal biomass production is inversely related to lipid content in most cases and, and therefore, lipid productivity was suggested as a more efficient parameter than lipid content for organism selection or conditions optimization for biodiesel production [36]. Many studies confirmed that microalgae tend to accumulate lipids under nitrogen deficient conditions, where nitrogen starvation was reported as one of the most effective strategies to enhance lipid storage in microalgae [37–39]. It is reported that nitrogen starvation stimulates the response of microalgae through degradation of nitrogen-containing intracellular macromolecules and reservation of carbon compounds, such as polysaccharides and fats [40,41]. However, the reduction of cellular growth under nitrogen starvation remains the main challenge for enhanced lipid productivity. In the present study, lipid productivity was significantly reduced by reduction of $NaNO_3$ showing the minimum recorded value of 22.26 mg/L day (which was 18.4% lower than the control). However, high concentrations of $NaNO_3$ (+25%) significantly increased lipid productivity by 31.1% over the control (Table 1). Thus, these

findings identified $NaNO_3$ at 125 mg/L as the optimum concentration for enhanced lipid productivity of *Chlorella* sp.

3.2. Effect of Seawater

The effect of different seawater ratios on the growth pattern of *Chlorella* sp. was monitored as changes in the cellular density (Figure 2). Increasing of seawater ratio by 25% and 50% showed higher growth than the control, while further increase in seawater ratio resulted in sharp reduction in cellular density. Therefore, the increasing in the seawater ratio by 25% significantly stimulated biomass productivity to the maximum recorded values of 0.237 g/L day, representing 27.4% above the control (Table 2). However, further increase in the seawater ratio significantly reduced the biomass productivity, showing the minimum value of 0.079 g/L day at 100% seawater ratio. These results are in agreement with El-Sheekh et al. [11], who recorded the enhancement of growth of *Scenedesmus obliquus* using 25% and 50% seawater ratios. Several studies confirmed the significant impact of salinity on freshwater microalgae due to the importance of NaCl in chlorophyll function, activity of photosystem II, oxygen evolution, and photochemistry [42].

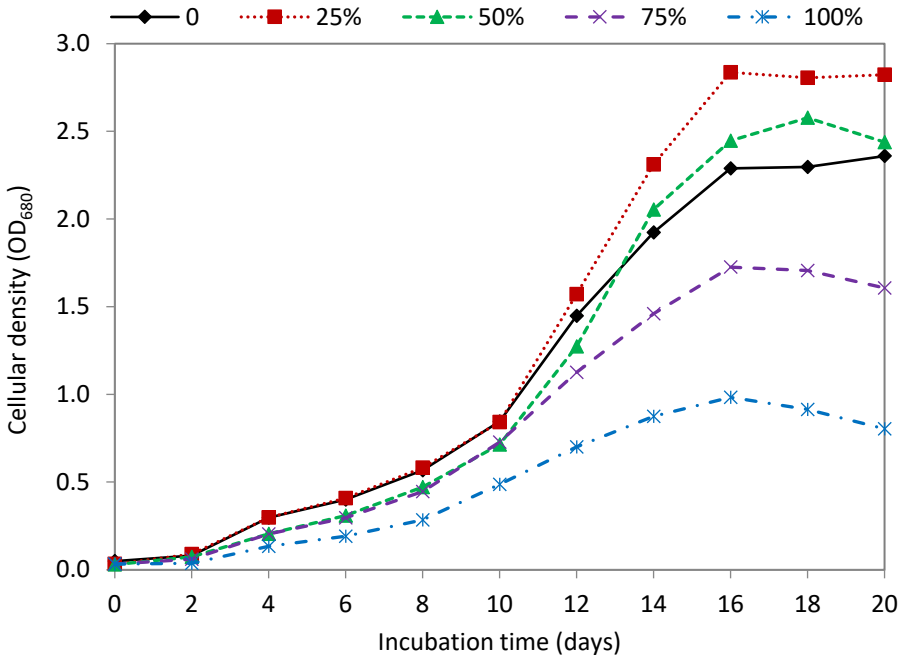

Figure 2. Effect of different seawater ratios on growth of *Chlorella* sp. cultivated for 20 days in synthetic wastewater.

Table 2. Effect of different seawater ratios on biomass and lipids of *Chlorella* sp. cultivated in synthetic wastewater and harvested at the late exponential phase.

Seawater Ratio (%, *v*/*v*)	Dry Weight (g/L)	Biomass Productivity (g/L Day)	Lipid Content (mg/g DW)	Lipid Productivity (mg/L Day)
Control (0)	2.56 ± 0.011 ^{a}	0.186 ± 0.005 ^{a}	157.81 ± 8.78 ^{a}	27.46 ± 0.893 ^{a}
25%	3.18 ± 0.039 ^{b}	0.237 ± 0.003 ^{b}	164.90 ± 5.06 ^{a}	37.60 ± 0.459 ^{b}
50%	2.74 ± 0.121 ^{c}	0.209 ± 0.009 ^{c}	132.27 ± 5.04 ^{b}	27.05 ± 0.504 ^{a}
75%	1.93 ± 0.020 ^{d}	0.142 ± 0.003 ^{d}	123.17 ± 7.09 ^{b}	16.80 ± 0.720 ^{c}
100%	1.10 ± 0.043 ^{e}	0.079 ± 0.003 ^{e}	111.24 ± 3.23 ^{c}	8.38 ± 0.664 ^{d}

Values in the same column with the same letter showed insignificant differences, while those with different letters showed significant differences (at $p \leq 0.05$).

Interestingly, lipid content showed insignificant changes using 25% seawater compared to the control (Table 2). Further increase in the seawater ratio led to significant reduction of lipid content and lipid productivity, reaching the lowest values at 100% seawater (29.5% and 69.5%, respectively, lower than the control). Growth and lipid reduction at high salinity might be attributed to the inhibition of photosynthesis by accumulation of reactive oxygen species and reduction of chlorophyll synthesis due to toxic ionic stress [43]. As a result of increased growth at 25% seawater ratio with insignificant change in lipid content, the maximum lipid productivity of 37.60 mg/L day was recorded, which was 36.9% higher than the control. Thus, using 25% seawater was identified as the optimum ratio to enhance lipid productivity of *Chlorella* sp.

3.3. Effect of Glycerol

Glycerol supplementation was suggested to stimulate the microbial growth and lipid accumulation as an organic carbon source and one of the precursors of lipid biosynthesis [16]. In the present study, initial increase in glycerol up to 4 g/L enhanced the microalgal growth, which showed reduction by further increase in glycerol supplementation (Figure 3). The highest significant dry weight and biomass productivity were recorded at 2 g/L of glycerol (2.74 g/L and 0.195 g/L day, respectively) (Table 3). The negative impact of high concentrations of glycerol on growth might be attributed to increasing of C/N ratio in the growth medium [44]. Moreover, glycerol is osmotically-active compound which influences the medium osmotic potential [45], leading to osmotic stress which influences the growth and enhances lipid accumulation [46]. In that context, lipid accumulation showed continuous increase up to 204.15 mg/g dw at 6 g/L glycerol, which was 24.9% higher than the control (Table 3). Thus, glycerol enhanced both lipid content and growth, with the highest recorded lipid productivity of 42.61 mg/L day at 4 g/L glycerol (Table 3), which was identified as the optimum concentration in the present study.

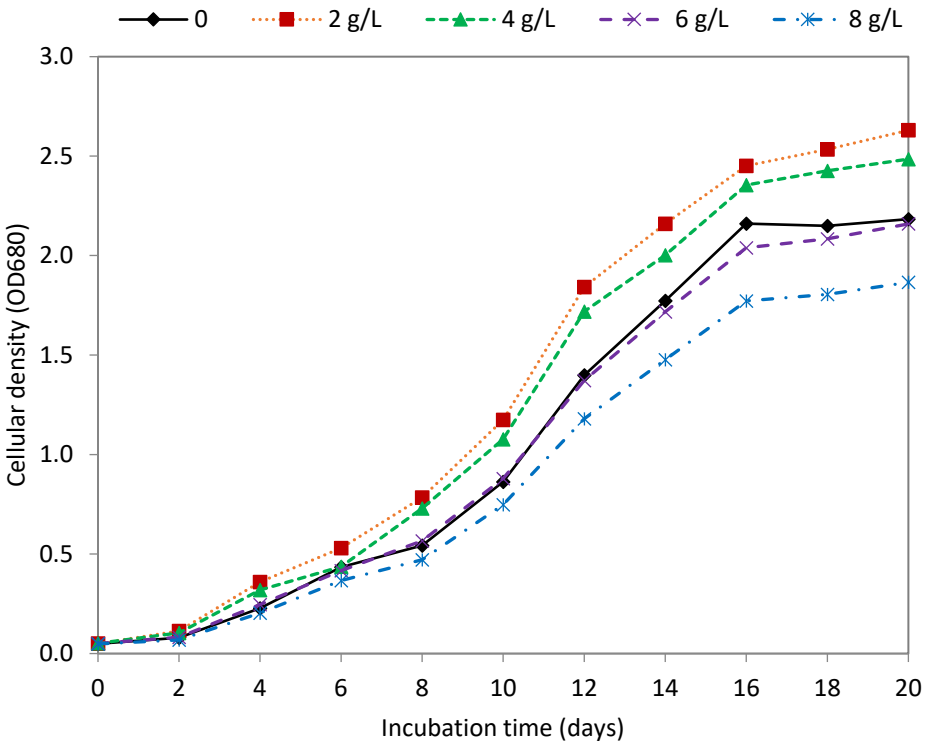

Figure 3. Effect of different concentrations of glycerol on growth of *Chlorella* sp. cultivated for 20 days in synthetic wastewater.

Table 3. Effect of different concentrations of glycerol on biomass and lipids of *Chlorella* sp. cultivated in synthetic wastewater and harvested at the late exponential phase.

Glycerol (g L^{-1})	Dry Weight (g/L)	Biomass Productivity (g/L Day)	Lipid Content (mg/g DW)	Lipid Productivity (mg/L Day)
Control (0)	2.42 ± 0.053 ^{a}	0.180 ± 0.003 ^{ab}	163.48 ± 5.02 ^{a}	27.17 ± 0.388 ^{a}
2	2.74 ± 0.060 ^{b}	0.195 ± 0.007 ^{c}	181.69 ± 8.09 ^{b}	33.29 ± 0.759 ^{b}
4	2.64 ± 0.225 ^{ab}	0.190 ± 0.013 ^{ac}	203.63 ± 5.33 ^{c}	42.61 ± 0.898 ^{c}
6	2.28 ± 0.129 ^{c}	0.167 ± 0.012 ^{b}	204.15 ± 3.50 ^{c}	37.13 ± 0.624 ^{d}
8	1.98 ± 0.174 ^{d}	0.146 ± 0.014 ^{d}	188.32 ± 3.27 ^{b}	29.29 ± 0.861 ^{e}

Values in the same column with the same letter showed insignificant differences, while those with different letters showed significant differences (at $p \leq 0.05$).

3.4. Interaction Optimization

In order to identify the optimum concentration of the three studied parameters for both lipid productivity and wastewater treatment, CCD was used by applying the lower and higher values as control and optimum values of each individual parameter. A total of 15 runs were designed (Table S1, supplementary data) using −1 and +1 values as 100 mg/L and 125 mg/L ($NaNO_3$), 0% and 25% (Seawater), and 0 g/L and 4 g/L (glycerol). Application of 125 mg/L $NaNO_3$, 0 seawater ratio, and 4 g/L glycerol showed the highest lipid productivity of 46.74 mg/L day (Figure 4). These results confirm that increasing the nitrogen concentration could balance the C/N ratio of the medium and alleviate the negative impact of the high glycerol concentration. However, the lowest lipid productivity of 27.3 mg/L day was recorded using 100 mg/L $NaNO_3$, 0 seawater ratio, and 0 g/L glycerol (control), which was significant with all other runs (Figure 4). On the other hand, the highest nitrogen removal of 97.45% was recorded at 112.5 mg/L $NaNO_3$, 25% seawater ratio, and 2 g/L glycerol, while the lowest value of 82.45% was recorded at 100 mg/L $NaNO_3$, 25% seawater ratio, and 4 g/L glycerol (Figure 4). Thus, the pattern of lipid productivity and nitrogen removal were quite different and further optimization was conducted to obtain the maximum lipid productivity with highest nitrogen removal.

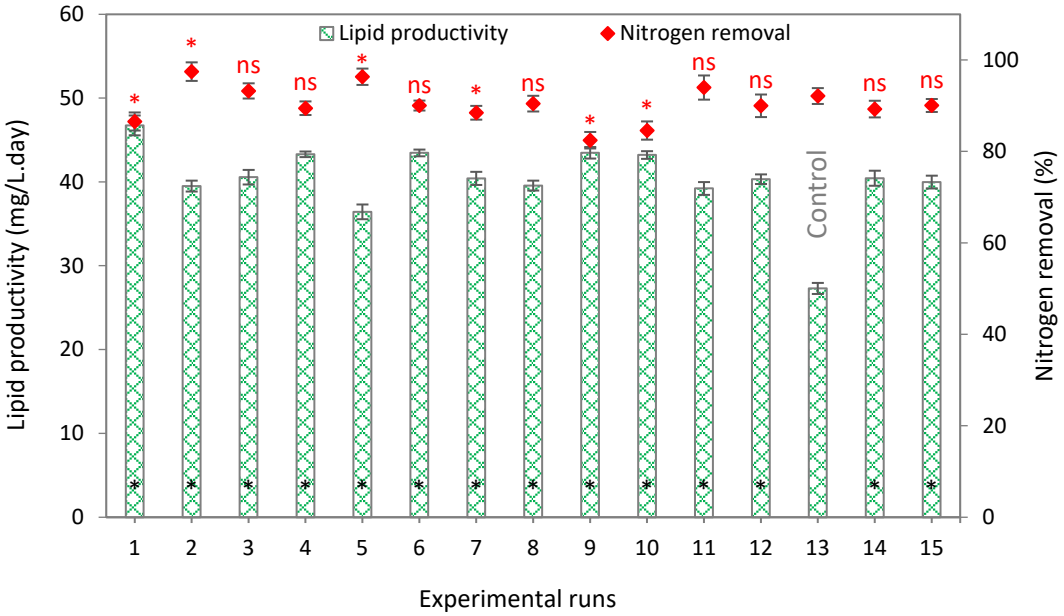

Figure 4. Lipid productivity and nitrogen removal by *Chlorella* sp. cultivated at different sodium nitrate concentrations, seawater ratios, and glycerol supplementation using randomized central composite design as shown in Table S1. * and ns represent significant and non-significant differences, respectively, with respect to the control (Run #13) at $p \leq 0.05$.

For lipid productivity, combination of seawater ratio with $NaNO_3$ or glycerol showed the highest values at the highest applied concentration individually, while combination resulted in reduction of lipid productivity (Figure 5). In addition, combination of glycerol with $NaNO_3$ showed the highest lipid productivity at the highest glycerol concentration with lowest $NaNO_3$. Overall, results of the developed model showed high agreement between the predicted values and experimental values (Figure 5D) with high significance ($p < 0.0266$) of the entire model as shown in the ANOVA test (Table S2). In addition, a high correlation (R^2) of 0.9553 was recorded for output responses, which confirms that the experimental results are strongly correlated to the applied model. Overall, the current findings confirm that using RSM to develop the regression model is a very accurate tool which precisely reflects the impact of the three studied variables on lipid productivity. Nitrogen removal is the main parameter to monitor the wastewater treatment in wastewater treatment plants (WWTPs) [47], and therefore, it is the focus of the present study. The highest nitrogen removal was recorded at the highest seawater ratio with lowest glycerol (Figure 6). It can be attributed to increase in autotrophic growth at high seawater ratio, while addition of glycerol could reduce the nitrogen removal rate. In the current model, experimental values were comparable to the predicted values (Figure 6D), with very high significance ($p < 0.0001$), as shown in the ANOVA test (Table S3). In addition, the applied model showed a high correlation (R^2) of 0.9975, confirming its accuracy with the experimental results.

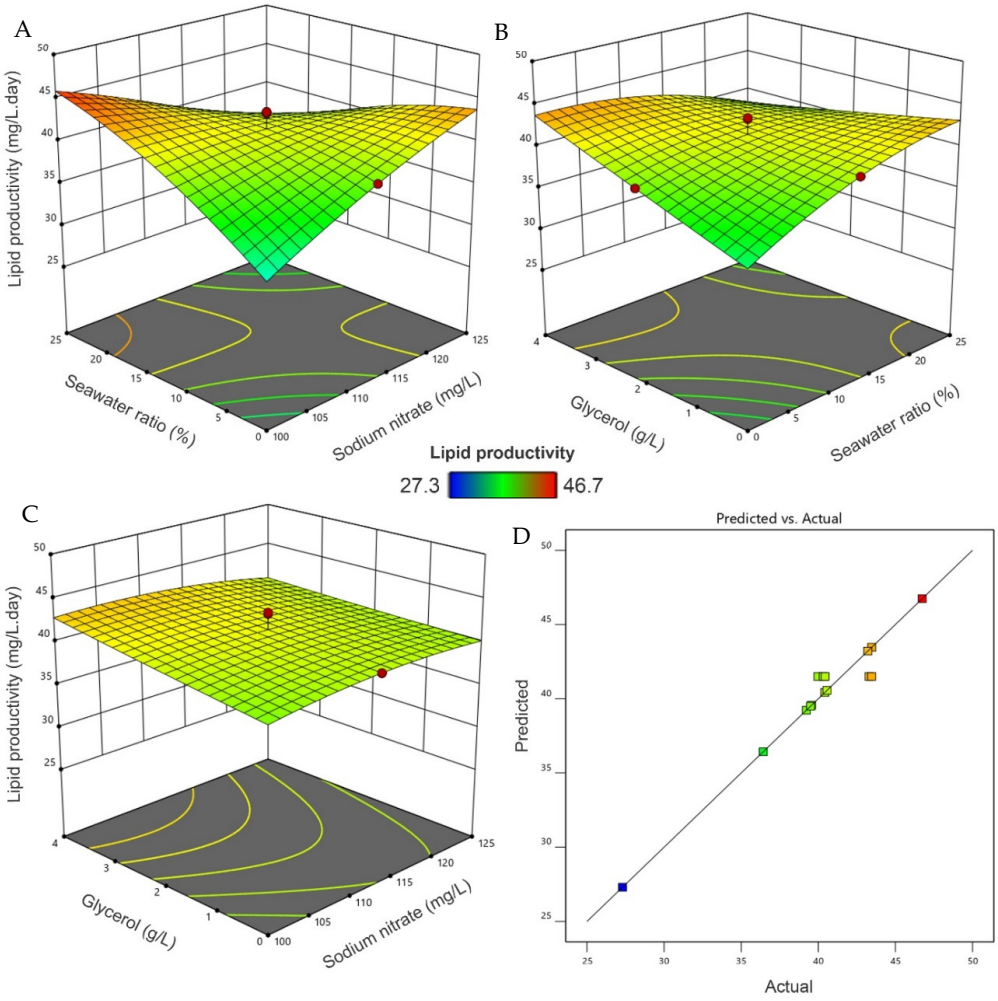

Figure 5. Central composite design showing the lipid productivity responses to different studied conditions of sodium nitrate, seawater, and glycerol (**A**–**C**) with the fitting graph of actual and predicted values (**D**).

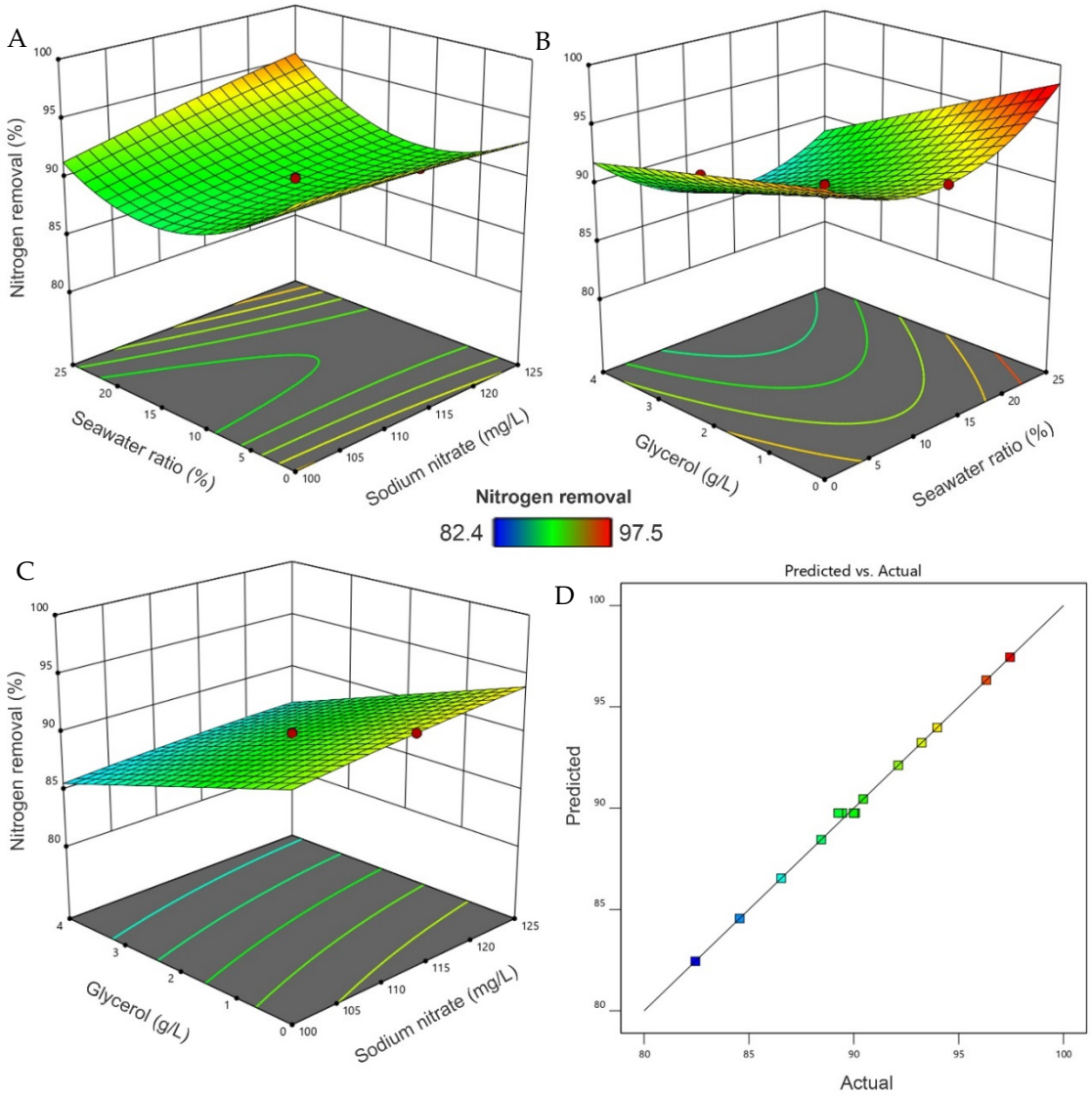

Figure 6. Central composite design showing the nitrogen removal responses to different studied conditions of sodium nitrate, seawater, and glycerol (**A**–**C**) with the fitting graph of actual and predicted values (**D**).

This work aimed to optimize $NaNO_3$, seawater, and glycerol as the three main parameters for enhanced microalgal growth, lipid productivity, and nitrogen removal; therefore, numerical optimization was further applied on the RSM results. It is an efficient tool for optimization of a combination of multi-factors [22,48]. Numerical optimization methods have been used in various applications with the advantage of that it can be applied to a wide range of problems. Recently, RSM optimization coupled with numerical methods has been suggested to give rise to an adaptive, novel, and heuristic approach [49]. In that regard, RSM methodology using numerical optimization showed effective results in predicting the optimal conditions for different studied factors [23]. The optimization was further proceeded by selecting the maximum lipid productivity and nitrogen removal while allowing the $NaNO_3$, seawater, and glycerol values within the study range (Figure S2, Supplementary data). The model suggested an $NaNO_3$ concentration of 101.5 mg/L, seawater ration of 23.8%, and glycerol supplementation of 0.25 g/L as the optimum conditions for maximum lipid productivity and nitrogen removal of 46.9 mg/L day and 98.0%, respectively. Therefore, these conditions were applied and further evaluation of biodiesel was compared with the control. For large-scale application, controlling the nitrogen content in

natural wastewater can be achieved by mixing local wastewater with other wastes/water based on the nitrogen content. For instance, wastewater with high nitrogen can be mixed with seawater or low-nitrogen industrial wastewater to reduce the total nitrogen to the desired level. However, wastewater with low nitrogen content can be supplemented with nitrogen-rich wastes such as anaerobic digestate, or nitrates can be added directly to the culture. Glycerol can be supplemented from the biodiesel production process, where it is a byproduct of transesterification, providing a closed-loop zero-waste approach.

3.5. Lipid Productivity and Estimated Biodiesel Characteristics

Under optimized conditions, dry weight and lipid content were enhanced by 31.9% and 20.3%, respectively, over the control (Table 4). Therefore, lipid productivity increased by 71.5% under the optimized conditions. Although lipid content increased by 20.3% by optimization, FAMEs recovery increased by 23.0% (Table 4), confirming the increase in lipids in the form of storage triglycerides [36]. Previous studies confirmed the potential of microalgae to grow and accumulate lipids in different kinds of wastewater with high potential of nitrogen removal (Table 5). For instance, total nitrogen removal efficiency of 82.7% was recorded by *C. zofingiensis* grown in piggery effluent [35]. Compared to the other studies shown in Table 5, lipid productivity and/or nitrogen removal efficiency in the present study are higher than that reported in other studies [50–56]. Thus, these findings provide basics for further enhancement of wastewater treatment and lipid production by microalgae.

Table 4. Biomass, lipid production, and fatty acid profile of *Chlorella* sp. grown under optimized conditions in comparison with the control.

Parameters	Control	Optimized
Dry weight (g/L)	2.48 ± 0.06	3.27 ± 0.06 *
Lipid content (mg/g dw)	161.62 ± 3.66	194.41 ± 9.01 *
Lipid productivity (mg/L day)	27.40 ± 1.55	46.98 ± 3.38 *
Total FAMEs (mg/g dw)	147.2 ± 3.25	181.0 ± 8.53 *
C14:0	1.12	1.47
C16:0	20.18	25.84
C16:1*n*7	10.96	9.45
C16:1*n*9	3.88	2.36
C16:3*n*4	4.1	6.14
C18:0	16.47	18.18
C18:1*n*9	15.96	17.05
C18:1*n*7	3.45	5.96
C18:2*n*6	9.85	6.04
C18:3*n*3	6.47	5.42
C18:4*n*3	2.07	1.13
C20:0	5.49	0.96
SFAs	43.26 ± 1.05	46.45 ± 1.06 *
MUFAs	34.25 ± 0.95	34.82 ± 0.86 ^{ns}
PUFAs	22.49 ± 0.85	18.73 ± 0.74 *

^{ns} showed non-significant difference with the corresponding control; * showed significant difference with the corresponding control (at $p \leq 0.05$).

C16:0, C18:0, and C18:1*n*9 were the dominant fatty acids in both cultures (Table 4). However, optimization resulted in pronounced changes in fatty acid proportions where saturated fatty acids increased by 7.4% in the optimized culture in favor of polyunsaturated fatty acids. Unsaturation degree is a significant parameter for adaptation of microalgae to the environmental conditions. In the optimized conditions, the addition of seawater showed the highest impact comparing to glycerol and sodium nitrate (Figure S2). In that context, Xu and Beardall [57] reported that salinity stress results in increased saturated fatty acids in *Dunaliella salina*, with simultaneous reduction in polyunsaturated fatty acids. One of the mechanisms to keep the membrane fluid and prevent its destruction at elevated salinity is to change the fatty acid profile [58]. Renaud and Parry [59] evaluated the

growth of *Isochrysis* sp. at elevated NaCl and recorded a significant reduction in the polyunsaturated fatty acids C18:5 and C22:6. The lower unsaturation degree of lipids in response to high salinity was also reported for the algae *Dunaliella* sp., *Nannochloropsis* sp., and *N. frustulum* [57,60]. Thus, fatty acid profile is significantly influenced by the growth conditions, which in turn affects the biodiesel characteristics and quality [61,62].

Table 5. Comparison of biomass productivity (P_{mass}), lipid productivity (P_{lipid}), and nitrogen removal efficiency (RE) in the present study under optimized conditions with previous studies.

Wastewater	Microalgae	P_{mass} (g/L Day)	P_{lipid} (mg/L Day)	RE (%)	Ref.
Municipal wastewater	*Chlorella sorokiniana*	0.073	16.20	74.2 ^{NO3} 83.3 ^{NH4}	[50]
Urban wastewater	*Chlorella vulgaris*	0.190	14.31	87.9 ^{NO3}	[51]
Cattle manure leachate	*Coelastrum* sp.	0.171	11.08	72.3 ^{NO3}	[52]
Municipal wastewater	*Scenedesmus* sp. LX1	0.007	8.00	98.5 ^{NO3}	[53]
Domestic effluents	*Botryococcus braunii*	0.065	15.80	62.0 ^{NO3}	[54]
Carpet mill	*Chlorella saccharophila*	0.023	4.20	-	[56]
Municipal wastewater	*Chlorella pyrenoidosa*	0.229	48.90	59.4 ^{NH4}	[55]
Optimized synthetic wastewater	*Chlorella* sp.	0.277	46.98	98.0 ^{TN}	This study

^{NO3}, ^{NH4}, and ^{TN} represent removal efficiency of NO_3–N, NH_3–N, and total nitrogen, respectively.

The main biodiesel characteristics were mathematically estimated based on the fatty acid profile, where all parameters showed agreement with the recommended international standards, while optimized cultures showed an 8.5% lower degree of unsaturation (Table 6). A lower degree of unsaturation of the biodiesel enhances its oxidative stability. Moreover, a higher degree of unsaturation results in high undesirable emissions and lower hydrocarbons (HCs) [63]. Iodine value is another parameter that is affected by the unsaturation degree, which refers to the iodine amount required to achieve complete saturation in 100 g of oil. According to European biodiesel standards [64], iodine values up to 120 g I_2/100 g oil are recommended for the best engine performance. In addition, the cetane number is another parameter to determine the ignition quality of the biodiesel [65]. The estimated cetane number of the optimized culture was higher than that of the control, with a lower iodine value. Therefore, the present study provides a tool for optimized conditions that result in triple effect of enhanced wastewater treatment, biodiesel yield, and biodiesel quality.

Table 6. The main estimated biodiesel characteristics of the *Chlorella* sp. grown under optimized conditions in comparison with the control and calculated based on fatty acid profile.

Parameters	Control	Optimized	US (ASTM D6751-08)	Europe (EN 14214)
ADU	0.94	0.86	-	-
Kinematic viscosity ($mm^2\ s^{-1}$)	4.61	4.66	1.9–6.0	3.5–5.0
Specific gravity	0.88	0.88	0.85–0.9	-
Cloud point	7.45	8.49	-	-
Cetane number	56.61	57.13	Min. 47	51–120
Iodine value (g I_2/100 g oil)	82.58	76.75	-	Max. 120
HHV (MJ kg^{-1})	40.19	40.05	-	-

ADU, average degree of unsaturation; HHV, higher heating value.

4. Conclusions

The present study aimed to enhance the growth and lipid accumulation coupled with high nitrogen removal by the green microalga *Chlorella* sp. in mixotrophic growth medium under different nitrate, salinity, and glycerol conditions. Studying the impact of the three parameters individually enhanced lipid productivity up to 42.61 mg/L day at 4 g/L glycerol (56.3% higher than the control). The CCD model suggested the application of 125 mg/L $NaNO_3$, 0 seawater ratio, and 4 g/L glycerol for the maximum lipid productivity of 46.74 mg/L day. However, the highest nitrogen removal of 97.45% was suggested at

112.5 mg/L $NaNO_3$, 25% seawater ratio, and 2 g/L glycerol. Numerical optimization was further applied, which suggested $NaNO_3$ concentration of 101.5 mg/L, seawater ration of 23.8%, and glycerol supplementation of 0.25 g/L as the optimum conditions for dual maximum lipid productivity and nitrogen removal of 46.9 mg/L day and 98.0%, respectively. Under the optimized conditions, saturated fatty acids increased by 7.4%, which resulted in higher cetane numbers and lower iodine values for the produced biodiesel. Further studies are needed to evaluate the impact of the optimized conditions using municipal and industrial wastewater in large-scale production systems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8120682/s1, Figure S1: The phylogenetic tree of the isolate CDU-W11 showing high similarity of 95.77% with *Chlorella* sp. isolated from Cheshme Sabz lake, Iran (accession number MN738559.1); Figure S2. Numerical optimization ramp for input factors and responses of lipid productivity and nitrogen removal at the maximum with desirability of 1. Table S1: The experimental runs of the optimization experiment using randomized central composite design; Table S2: ANOVA analysis for central composite design of lipid productivity responses at different studied conditions of sodium nitrate, seawater, and glycerol; Table S3: ANOVA analysis for central composite design of nitrogen removal responses at different studied conditions of sodium nitrate, seawater, and glycerol.

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