Effects of different nitrogen concentrations and light intensities on lipid accumulation and growth of
Chlamydomonas reinhardtii

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Abstract. This study sets different nitrogen concentration gradients to explore the impact of nitrogen concentration on biomass and lipid accumulation of Chlamydomonas reinhardtii under different light intensities. The results showed that the conditions for the accumulation of lipid in the algae were 30μE·m⁻²·s⁻¹ light intensity and 100 mg·mL⁻¹ nitrogen concentration with the lipid content of C. reinhardtii was 39.79% and chlorophyll content was 17.08 mg·mL⁻¹. This study provides an experimental basis for the regulation of lipid production of C. reinhardtii by using nitrogen-deficient culture and provides a foundation for further utilization of genetic engineering methods to regulate the lipid metabolism of C. reinhardtii.

1. Introduction
The consumption of new energy has become an urgent task because of traditional chemical energy will emit sulfur and nitrogen pollutants during the production process [1]. CO₂ is converted into organic matter by photosynthesis then released into the atmosphere again by combustion in the production process of bioenergy, therefore it is a new type of low-emission, low-pollution clean energy [2]. Algae can convert CO₂ and water into oxygen, carbohydrates and lipids through photosynthesis [3]. Under stress conditions, such as excessive illumination or nutritional deficiencies, some algae could accumulate large amounts of lipids, like triacylglycerol. Therefore, microalgae were considered to be the ideal material for biological energy source, because of the fast growth and high lipid content. Algae that have been found to produce high lipid include Chrysophytes, Haptophytes, Dinophytes, Xanthophyceae, Rhodophytes, and their average lipid content was 27.1% of dry weight when they were growing in normal environment, but it rose to 44.6% under environmental pressure [4,5]. Chlamydomonas reinhardtii is a unicellular green alga and its genome has been completely sequenced, fast growth, low cost of cultivation, and could produce lipid under nitrogen-deficient conditions, therefore it is considered to be a model species for lipid production [6]. Like other microalgae, the growth of C. reinhardtii is inhibited in nitrogen-deficient conditions, and its biomass and lipid accumulation are negatively correlated with the degree of nitrogen deficiency [6-9], which leads to lower lipid production efficiency and accumulation than its theoretical value. In this study, biomass and lipid yields of C. reinhardtii were detected under different nitrogen concentrations and light intensities in order to determine the optimal nitrogen content and light intensity for lipid accumulation and growth of C. reinhardtii.
2. Materials and methods

2.1. Experimental materials and cultivation methods

*C. reinhardtii* cc849 was a type of cell deficient strain. The algae grew in TAP (Tris-acetate-phosphate) medium (pH=7.0) photoheterotrophy under 0~200 μE▪m⁻²▪s⁻¹ at 25±1 °C. Nitrogen elements in TAP medium was replaced with chloride salts (TAP-N) [10, 11].

2.2. Nitrogen deficiency treatment

When the algal cells grew to saturation period, the cells were collected by centrifuging at 4500g, 25°C for 5 min. The algal sediment was washed gently using TAP-N medium three times in order to get rid of nitrogen thoroughly then 400 mL TAP-N medium and TAP medium (in proportion) was added in flask in varying concentrations of nitrogen, 100, 200, 300 mg∙mL⁻¹. The sample in flask was shaken gently so that algae and medium mixed well. The flasks were placed under light intensities of 30, 60, 100, 200 μE▪m⁻²▪s⁻¹. The sample cultured in pure TAP and TAP-N medium were used as the controls.

2.3. Determination of algal growth

The absorbance value at 750nm was measured to represent the cell density. Chlorophyll was extracted with 95% ethanol, and the steps were as follows: 1mL algae was centrifuged at 8000rpm for 2min, then the supernatant was removed and 1ml 95% ethanol was added to resuspend the algae. Finally, the supernatant was centrifuged at 8000rpm for 2min, and the supernatant was absorbed to detect the absorbance value at 665nm and 649nm.

\[
\text{Chlorophyll content (mg/L) } = \text{OD}_{665} \times 6.01 + \text{OD}_{649} \times 20.04 \quad [11]
\]

\[
\text{Chl=Chla+Chlb}=6.10\times A_{665} + 20.04\times A_{649} \quad (1)
\]

Measurements were performed for at least three independent experiments.

2.4. Extraction and detection of lipid content

The method of Bligh et al. (1959) was used to detect the lipid content in *C. reinhardtii* and measurements were performed for at least three independent experiments [12]. 400 mL of algal cells was collected by centrifuging at 7500 g for 10 min and then washed three times with fresh TAP-N medium. Then put the solid sample into a dry weighing bottle and dry it in an oven at 80 °C for 24 h until the quality no longer decreases. 0.2 g of dry cells (denoted as W₀) was transferred into a centrifuge tube, and then 5 mL of a mixture of chloroform and methanol (1:1 by volume) was added to the centrifuge tube. The cells in the centrifuge tube were shaken on a shaker for 30 minutes and then centrifuged at 8000 g for 10 min, and the supernatant was taken. All the supernatants (W₁) were collected and transferred to a dry rotary evaporator with a known weight and evaporated to dryness (W₂). The above steps were repeated until the extracted supernatant became colorless.

\[
C_i = \frac{(W_2 - W_1)}{W_0}\times 100\%.
\]

2.5. Data analysis

The t-test was used to analyze the significant difference between the lipid yield of the experimental group and the control group. The significance level was set to p <0.05. The results of the significant difference are marked with * in the figure. All data processing processes were completed in SPSS19.0. Meanwhile, the two-factor analysis of variance was used to explore the effects of light intensity, nitrogen and the interaction of light and nitrogen on lipid content.

3. Results

3.1. The growth of *C. reinhardtii* in different nitrogen concentrations

The growth of *C. reinhardtii* in different nitrogen concentrations and light intensities is shown in Figure 1.
The OD_{750} of algae increased gradually with the increase of culture time, which reached 0.08, 1.61, 1.62, and 2.97 on the 7th day with 30 μE·m⁻²·s⁻¹ light intensity for 0, 100, 200, 300, and 375 mg · L⁻¹ (TAP medium) concentration of nitrogen in the medium, respectively (Figure 1 a). Similar with the OD_{750}, chlorophyll contents of algae increased gradually with the increase of culture time and reached 0.04, 7.08, 17.87, 26.01, and 27.84 mg · mL⁻¹ on day 7 with 30 μE·m⁻²·s⁻¹ light intensity for 0, 100, 200, 300, and 375 mg · L⁻¹ (TAP medium) concentration of nitrogen in the medium (Figure 1 b). Compared to C. reinhardtii in TAP medium, the growth of C. reinhardtii in nitrogen-deficient conditions was inhibited, especially in TAP-N culture, the growth of C. reinhardtii was significantly inhibited. The OD_{750} and chlorophyll content of C. reinhardtii was reduced by 97.3% and 99.8%, respectively in TAP-N culture compared with TAP culture. The OD_{750} of C. reinhardtii in 100, 200, and 300 mg·L⁻¹ nitrogen concentration of culture were 45.8%, 45.8%, and 45.5%, respectively lower than those in TAP culture. The chlorophyll contents in nitrogen deficient culture decreased were more significant, which decreased by 74.6%, 35.8%, and 6.6% compared with in TAP culture.

Similar with 30 μE·m⁻²·s⁻¹, as shown in Figure 1 (c, d, e, f), the OD_{750} of algae increased gradually with the increase of culture time under 60 μE·m⁻²·s⁻¹ and 100 μE·m⁻²·s⁻¹ light intensity. The maximum values were reached 2.96 and 3.01 (Figure 1 c, e) and the maximum chlorophyll concentrations were 29.26 mg · mL⁻¹ and 28.88 mg · mL⁻¹ on day 7 in TAP-N culture as shown in Figure 1 (d, f). The results showed that the growth was affected after nitrogen deficiency treatment on 60 μE·m⁻²·s⁻¹ or 100 μE·m⁻²·s⁻¹ light intensity.

It’s worth noting that the value of OD_{750} in TAP-N reduced by 92.9-97.0% and the chlorophyll contents were reduced by 98.8-99.4% compared with those of C. reinhardtii in TAP culture.

3.2. Lipid yields of C. reinhardtii in different nitrogen concentrations

The former results indicated that the growth of C. reinhardtii reached saturation on day 2 under different nitrogen concentrations and light intensity conditions. Therefore lipid contents of C. reinhardtii were detected under different nitrogen concentrations and light intensities on day 2 and results are shown in Figure 2 and Table 1. The results showed that nitrogen deficiency could promote the accumulation of lipids in C. reinhardtii and the lower the nitrogen concentration, the higher the total lipid content in C. reinhardtii, the maximum lipid content of C. reinhardtii was obtained in TAP-N culture. In 30 μE·m⁻²·s⁻¹ light intensity, the total lipid content (mass fraction) of C. reinhardtii cultured in TAP-N medium was 48.6%, which was 3.2 folds than that of TAP culture, and the total lipid content in other nitrogen concentration cultures were 2.7, 2.4 and 1.7 folds than the control group (TAP culture). Interestingly, in 60μE · m² · s⁻¹ light intensity condition, the total lipid content of C. reinhardtii also decreased gradually with the increase of nitrogen concentration, especially in the TAP-N culture, the maximum lipid content was 42.2%, i.e., 3.2 folds higher than the control group (in TAP culture). Total lipid contents in other nitrogen concentration cultures were 2.5, 1.8 and 1.4 folds of the control group. Similarly, under 100 μE·m²·s⁻¹ light intensity condition, the total lipid content of C. reinhardtii also decreased with the increase of nitrogen concentration, and the maximum lipid accumulation was 39.2% in the TAP-N culture, i.e., 3.3 folds of the control group. The total lipid content under other nitrogen concentration conditions was 3.0, 2.9 and 1.9 times of the control group. The t-test was used to analyze the significant difference. The t-test result showed that the total lipid content of C. reinhardtii cultured in the TAP-N culture was significantly improved compared to those in TAP culture.
Figure 1. The growth of *C. reinhardtii* in different nitrogen concentration cultures, OD750nm (a, c, e), chlorophyll content (b, d, f).
Figure 2. Lipid content of *C. reinhardtii* in different nitrogen concentration cultures. * indicates significant difference between the experimental and control group.

Table 1. Lipid contents of *C. reinhardtii* under different nitrogen concentrations and light intensities on 2nd day.

| Nitrogen concentration (mg∙mL⁻¹) | 30μE∙m⁻²∙s⁻¹ | 60μE∙m⁻²∙s⁻¹ | 100μE∙m⁻²∙s⁻¹ |
|----------------------------------|--------------|---------------|----------------|
| Lipid content (%)                | Biomass (Chl) (mg∙mL⁻¹) | Lipid content (%) | Biomass (Chl) (mg∙mL⁻¹) | Lipid content (%) | Biomass (Chl) (mg∙mL⁻¹) |
| 0                                | 48.59        | 0.34          | 42.23          | 0.40          | 39.20          | 0.35          |
| 100                              | 39.79        | 17.08         | 31.97          | 18.24         | 35.96          | 16.09         |
| 200                              | 35.44        | 17.87         | 23.00          | 20.83         | 34.63          | 19.40         |
| 300                              | 25.35        | 26.01         | 18.07          | 30.51         | 22.53          | 26.15         |
| 375                              | 15.00        | 27.84         | 13.00          | 29.78         | 12.00          | 28.88         |

In order to explore the effects of light and biomass on lipid production in this research, a two-factor analysis of variance for the three sets of data was conducted and the results are shown in Table 2. The results show that the interaction of light intensity and nitrogen concentration on lipid production of *C. reinhardtii* was significant. This result shows that the interaction of two factors should be considered at the same time when investigating the accumulation of lipid in *C. reinhardtii*.

Table 2. Two-factor analysis of variance for light intensity and nitrogen concentration on lipid production of *C. reinhardtii*.

| Factors                          | df | F         | P-value |
|----------------------------------|----|-----------|---------|
| Nitrogen concentration           | 4  | 414.03    | <0.001  |
| Light intensity                  | 2  | 67.70     | <0.001  |
| Nitrogen concentration × Light intensity | 8  | 9.47      | <0.001  |

**R²=0.98, P-value<0.001**

4. Discussions
The lipid content of microalgae is relatively low under natural conditions, while the production of lipid and carbohydrates could increase with the condition of insufficient nitrogen [12]. Therefore, research on microalgae lipid production was based on nitrogen deficiency with the expense of biomass. Choosing a proper nitrogen concentration to increase biomass and lipid production of *C. reinhardtii* in an equilibrium state was the key point in this research. Lipid accumulation in *C. reinhardtii* is directly
related to its biomass [13] and other factors such as light intensity and pH of culture [14] and the biomass of C. reinhardtii is also related to light intensity. Cells of C. reinhardtii were cultures under five different nitrogen concentrations and three different light intensity conditions to explore the appropriate conditions for regulating lipid production and biomass of C. reinhardtii. The results showed that the growth of C. reinhardtii was inhibited under nitrogen-deficient conditions compared to that of TAP culture. Since nitrogen is the main component of chlorophyll, therefore, the chlorophyll synthesis of C. reinhardtii is greatly affected after nitrogen deficiency, which could be further verified by detecting the activity of the photosystem II of C. reinhardtii. Kim et al. (2006) published that high light intensity could damage cells of C. reinhardtii, and the low photosynthesis is also not conducive to its biomass accumulation [14]. Therefore it is necessary to find a suitable light intensity for biomass accumulation of C. reinhardtii. A two-factor analysis of variance on light regulation and nitrogen concentration was conducted in this research. The results showed that both factors have an important role in lipid accumulation. Therefore, the optimal light intensity for biomass and lipid accumulation of C. reinhardtii was 30 μEꞏm⁻²ꞏs⁻¹ in this study. At the same time, Hu and Wang et al. (2008, 2016) proposed that nitrogen deficiency in the culture would promote the lipid accumulation of C. reinhardtii [15, 16]. Similarly, lipid content has been increased most significantly in the absence of nitrogen, 100 mg∙mL⁻¹ was chosen as the optimal nitrogen concentration considering the biomass factor. In this study, 100 mg∙mL⁻¹ and 30 μEꞏm⁻²ꞏs⁻¹ were chosen as the most suitable conditions for lipid production, meanwhile the lipid content of C. reinhardtii was 39.79% (Table 1). Because the initial cell concentration of C. reinhardtii was too low (OD₇₅₀ was only 0.34) in this study, which resulted in a low biomass of C. reinhardtii. At the same time, the gradient of nitrogen concentration in this study was too large. Therefore, the next experiment could improve the initial cell concentration of C. reinhardtii and reduce the nitrogen gradient (such as 10, 20, 50, 70 and 100 mg∙mL⁻¹) in order to explore the effect of nitrogen on lipid accumulation of C. reinhardtii.

5. Conclusions
This study explored the suitable conditions for lipid production and biomass of C. reinhardtii grown under different nitrogen concentrations and light intensities. The results showed that nitrogen deficiency could promote the accumulation of lipid and different nitrogen concentrations have different effect of lipid accumulation. In this study, the optimal nitrogen concentration of C. reinhardtii was 100 mg∙mL⁻¹ in 30 μEꞏm⁻²ꞏs⁻¹ light intensity. This study will provide a theoretical basis for further studies on increasing lipid accumulation in C. reinhardtii by controlling the culture conditions and lay an experimental foundation for metabolic regulation and gene regulation of lipid production in C. reinhardtii based on nitrogen-deficient culture.

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