Effect of Temperature on Biological Macromolecules of Three Microalgae and Application of FT-IR for Evaluating Microalgal Lipid Characterization

Ting Zhao, Xiaotian Han,* and Huidi Cao

ABSTRACT: This study had shown the growth of Phaeodactylum tricornutum, Chlorella vulgaris, and Nannochloropsis sp. under different temperatures and their structure and relative content of polysaccharide, protein, and lipid. Lipid was more suitable to accumulate under the condition of low temperature; however, polysaccharide and protein were not; they had a similar change trend but different amounts. The correlation between the relative content of the lipid and the total lipid and fatty acid in a single microalga cell was also analyzed. The results showed that the relative content of the lipid detected by Fourier transform infrared (FTIR) spectroscopy and the total lipid and the unsaturated fatty acids (UFAs) obtained by a gravimetric method in a single microalga cell had a good linear relationship ($R^2 \approx 0.8$) while the correlation of saturated fatty acids is poor ($R^2 < 0.5$). These studies had demonstrated that temperature was a key factor for phytoplankton that can influence their growth and biological macromolecule content. Moreover, FTIR spectroscopy was proved to be a meaningful technology for selecting the microalgae rich in total lipid and UFAs.

1. INTRODUCTION

Microalgae, the basic organism in the tropical level of aquatic systems, especially in the marine ecological environment, are acknowledged as a source of several biomacromolecules. Microalgal bioactive compounds with a great diversity of ecological functions, such as polysaccharide, protein, and lipid, have many excellent and valuable properties and functions. These compounds make the algae be exploited economically, can meet the need of nutrient and health benefit, and are utilized agro-industrially as human functional food supplements and the energy source of population. In addition, the growth rate of microalgae is so fast that it can provide a guarantee for its industrialization.

Proteins from microalgae can be made into commodities with the function of nutrient value and therapeutic, especially for the formulating of food applications, such as fortified foods, nutrition shakes, and sports drinks. The biological function of microalgal polysaccharide had been ever evaluated and found to have the effect of anti-asthmatic, immune stimulation and antiviral, antioxidant activities, and anti-inflammatory activities. Lipids in microalgae were of high value, and it had been studied as a promising feedstock of biodiesel production and other high value-added productions such as essential fatty acids and essential nutrients for humans.

The microalgal culture conditions such as nutrient, light, pH, and radiation that might affect its growth, biomass, and the composition and content of bioactive compounds had been investigated in many previous studies. The biochemical synthesis, photosynthesis activity, metabolic ability, enzyme activity, absorption, and utilization efficiency of nutrients were affected by temperature apparently. As a crucial stress factors, lack of studies were about the combined effects of various temperatures at a fixed pH. In order to bridge this knowledge gap, the factor of temperature was considered in this work to explore the effect whether it could affect the status of phytoplankton. In this study, three microalgal strains (Bacillariophyceae: Phaeodactylum tricornutum, Chlorophyceae: Chlorella vulgaris, and Eustigmatophyceae: Nannochloropsis sp.) were applied as the test species. The absolute lipid contents were determined by a classical gravimetric method and the relative lipid contents were detected with Fourier transform infrared (FTIR) spectroscopy; meanwhile, the correlation analysis between the relative lipid content and...
Figure 1. The fitting results of growth curve used the logistic growth model (the points were the experimental data, and lines were the fitting curve). The value of the error bar is standard deviation. For all data sets, each point represents the mean (±SE) of three replicate culture flasks.

| Table 1. Maximum Biomass \( B_i \) (10^6 cells/L) and Maximum Growth Rate \( \mu_{\text{max}} \) (10^6 cells/L/h) of Three Marine Microalgae |
|--------------|---------------|---------------|---------------|
|              | \( B_i \)     | \( \mu_{\text{max}} \) | \( R^2 \)     |
| \( P. \text{tricornutum} \) | \( C. \text{vulgaris} \) | \( N. \text{nannochloropsis} \) |
| 15 °C        | 11.81         | 8.38          | 40.23         |
| 20 °C        | 7.43          | 12.16         | 31.43         |
| 25 °C        | 12.23         | 3.41          | 44.30         |
|               | 0.81          | 0.91          | 3.46          |
|               | 0.997         | 0.995         | 0.957         |
|               | 0.970         | 0.999         | 0.955         |

2. RESULTS AND DISCUSSION

2.1. Effects of Temperature on the Growth of Three Microalgae. The logistic growth model was used to simulate the growth process of \( P. \text{tricornutum} \), \( C. \text{vulgaris} \), and \( N. \text{nannochloropsis} \) at different temperatures (Figure 1). The correlation coefficients \( R^2 \) was 0.93–0.99, indicating that the logistic growth model could describe the growth curve of these three microalgae (Table 1), the maximum biomass \( B_i \) (cell/L) and maximal growth rate \( \mu_{\text{max}} \) (cell/L/h) were estimated by MATLAB software (Table 1). According to the fitting results in Figure 1 and Table 1, the growth of microalgae varies with temperature, and the biomass and growth rate of \( P. \text{tricornutum} \) reached highest at 25 °C, which was 12.23 \times 10^6 cells/L and 2.14 \times 10^6 cells/L/h, respectively. The growth rate changes of \( P. \text{tricornutum} \) at 15 and 20 °C were consistent with the results of Liang and Zang. \( C. \text{vulgaris} \) had the maximum biomass and highest growth rate at 20 °C, 12.16 \times 10^6 cells/L and 1.17 \times 10^6 cells/L/h, respectively. Zhang proposed that \( C. \text{vulgaris} \) can adapt to a wide range of temperatures, the optimal growth temperature ranged from 20 to 35 °C, while the growth rate was down to 0.39 cells/L/h at 25 °C in this experiment; the difference may be due to the experimental condition of high light intensity and input of CO2 gas in this work. The biomass and growth rate of \( N. \text{nannochloropsis} \) sp. were higher than that of the two microalgae at these three different temperatures, and the biomass and growth rate at 25 °C were 3 times as high as those at 15 and 20 °C, almost up to 44.3 \times 10^6 cells/L and 10.75 \times 10^6 cells/L/h; this was consistent with the results of a wide range of temperature adaptabilities of 0–30 °C of \( N. \text{nannochloropsis} \) sp. found by Jiang.

2.2. Macromolecular Structure in Marine Microalgae. The distinct absorption bands of the three microalgae cells were shown over the wavelength range 4000–6000 cm\(^{-1}\) (Table 2). The bands at 1200–950 cm\(^{-1}\) characterized the C–O–C stretching vibration absorption of polysaccharide, and its absorption strength could be used to quantify the total carbohydrate content. The infrared wavelength around 1630 and 1540 cm\(^{-1}\) was dominated by the protein amide I (mainly C=O stretching) and amide II (mainly N–H bending) vibrational bands, respectively, and the band around 1450–1430 cm\(^{-1}\) could represent the CH\(_2\) and CH\(_3\) bending vibration from protein. Lipid had characteristic absorption bands around 1740 cm\(^{-1}\), representing the ester C=C stretching vibration, around 3000–2800 cm\(^{-1}\) could represent the C–H symmetric vibration, the band at 1740 cm\(^{-1}\) primarily was from lipids, and the other region (3000–2800 cm\(^{-1}\)) represented saturated CH. The vibration frequency attributed to different functional groups, and the bands were assigned to specific molecular groups and were generally classified as polysaccharide, protein, and lipid. The classified peak area results represented the relative content of the three biological macromolecules.

2.3. Relative Contents of Polysaccharide, Protein, and Lipid in a Single Microalgal Cell under Different
Temperatures. The peak profile of these three microalgae was similar, while the peak intensity was different, which indicated that the content of each substance in microalgae was different. The polysaccharide, protein, and lipid contents of marine microalgae were closely related to the growth conditions. The relative absorption peak areas of biological macromolecules under different temperatures were dissimilar. The contents of the three bioactive substances were quantified by the peak area during their wavelength range.

The integral peak areas of the polysaccharide, protein, and lipid in a single microalgae cell are shown in Figure 2A. The content change of polysaccharide and protein was basically similar. The relative absorption peak area of polysaccharide and protein in *P. tricornutum* was the highest at 20 °C on the 9th day with a maximum of 6.02 (% P < 0.05) and 2.78 (% P < 0.05), respectively (Figure 2D,E). For *C. vulgaris*, the relative absorption peak area of polysaccharide and protein at 25 °C was far greater than that at 15 and 20 °C, and the maximum was on the 6th day and the 12th day, 28.32 (% P < 0.01) and 7.82 (% P < 0.01), respectively (Figure 2F,G). The variation of lipid was different from that of polysaccharide and protein, and the relative absorption peak area of lipid on the 6th day and the 9th day at the low temperature (15 °C) was the largest in *P. tricornutum*. The results of Renaud showed that the lipid content in microalgae would increase under low temperature to maintain the mobility of the cell membrane, ensuring the normal life of the cell. However, on the 12th day, the lipid contents of these three algae were the highest at 25 °C. The three biological macromolecules in *C. vulgaris* occupied an absolute advantage at a temperature of 25 °C, which indicated that *C. vulgaris* was rich in nutrients and was an excellent source providing abundant polysaccharide, protein, and lipid.

### 2.4. Total Lipid and Fatty Acid of the Single Microalgae Cell under Different Temperatures

The total lipid contents of *P. tricornutum* were significantly high at 15 °C on the 6th and 9th day, 11.56 and 17.18% (% P < 0.01), respectively. The maximum total lipid content appeared at 25 °C on the 12th day, 13.94% (% P < 0.05). The high contents of unsaturated fatty acids (UFAs) were 6.24% (% P < 0.01).
5.37% ($P < 0.05$), which occurred on the 9th day at 15°C and 12th day at 20°C. The contents of saturated fatty acids (SFAs) in P. tricornutum were high at 20°C on the 9th and 12th day, which were 16.02% ($P < 0.05$) and 15.84%, respectively. The contents of total lipid, UFA, and SFA in Nannochloropsis sp. were lower than those in P. tricornutum and C. vulgaris, and the maximum content of total lipid in Nannochloropsis sp. was 3.69%, which occurred at 15°C on the 6th day. The maximum content of UFAs and SFAs were 2.71% ($P < 0.05$) and 6.30% ($P < 0.05$) at 25°C on the 12th day, which were almost 2 times higher than those in the other two temperatures. The tendency of total lipid, UFAs, and SFAs of C. vulgaris were the most at 25°C ($P < 0.01$) and far superior to those of the other two temperatures and other two microalgae.

These tendencies were probably due to the difference of energy supply, energy storage, and photosynthetic activity at different temperatures. The difference of algal strains, culture condition, and culture mode that researchers conducted were relevant to the differences of results. Temperature had the distinct effects on the composition and unsaturated degree of fatty acid of phytoplankton. The change of fatty acid was characterized with the decrease of the percentage of polyunsaturated fatty acids (PUFAs), while SFAs and monounsaturated fatty acids rose as the temperature increases. The optimum temperature for fatty acid synthesis varied from species, and extreme temperature might lead to synthetic limitations and cause an irreversible damage to the involved enzyme. Low temperature could promote the synthesis of PUFAs in Nannochloropsis sp., while high temperature could not. Indeed, a number of studies had shown that the PUFA content would increase with the decreasing ambient temperature. UFAs increased the mobility of the cell membrane lipids, which was the physiological need of the organism itself to maintain its normal physiological function. Changes in fluidity would in turn affect the activity of the entire membrane protein, such as translocators and ion channels, among others. Temperature might affect the activity of various chain elongase, desaturase, and the transcriptional activity of desaturase gene in the process of UFA synthesis. At the same time, the change of temperature can effectively promote the transformation between fatty acids. In this work, extremely high/low temperatures were not conducted and appropriate culture temperature would be beneficial to lipid production (Figure 3).

**2.5. Correlation Analysis of Lipid and Fatty Acid Content.** The correlation between the relative absorption peak area of the lipid and total lipid and fatty acid content was analyzed to confirm whether the FTIR detection method could display the actual contents extracted by a gravimetric method. The exponential growth phase was generally on the 9th day for these three microalgae, and during this period, the biological macromolecule in the microalgae cell was more complicated...
than that in growth adaptation period and exponential stability period; the correlation coefficient \( R^2 \) was less than 0.3; therefore, the correlation on the 6th and 12th day was analyzed.

Relatively good linear relationships were observed on the 6th day (\( R^2 = 0.841 \)) and 12th day (\( R^2 = 0.817 \)) between the relative absorption peak area of the lipid and the total lipid content in single microalgae cells (Figure 4A,D). Similarly, the UFAs also had a relatively good linear relationship (Figure 4C,F) on the 6th day (\( R^2 = 0.701 \)) and 12th day (\( R^2 = 0.781 \)), while the correlation of SFAs was poor, and the correlation coefficient was less than 0.5 (Figure 4B,E). FTIR, different from the method of Nile Red staining, could detect the lipid content in microalgal cells\(^{23,38} \) and was a meaningful tool for selecting the oil-rich microalgae. In this work, the results of correlation showed that FTIR could not only be used to indicate the total lipid content in a single microalgae cell but also indicated the content of UFAs per cell. Currently, FTIR was used to determine total lipid and UFAs in microalgae; these two kinds of components were of interest in the field of microalgae, but more in-depth investigations were needed.

3. CONCLUSIONS

This study had shown the growth of \( P. \) tricornutum, \( C. \) vulgaris, and \( Nannochloropsis \) sp. and their structure and relative contents of polysaccharide, protein, and lipid under different temperatures. The correlation between the relative content of the lipid and the total lipid and fatty acid in a single microalgae cell was also analyzed. These studies had shown that temperature was an indispensable factor for phytoplankton to influencing their growth status. FTIR could identify the structure in microalgae effectively and might be an efficient and rapid tool for appraising the total lipid content and UFA content in a single microalgae cell.

4. MATERIALS AND METHODS

4.1. Materials and Algae Cultivation. \( P. \) tricornutum, \( C. \) vulgaris, and \( Nannochloropsis \) sp. used in this work were provided by the Key laboratory of Marine Ecology and Environmental Science, Institute of Oceanology, Chinese Academy of Sciences. The culture medium was prepared with filtered and autoclaved natural seawater. The algal inoculum of 400 mL was transferred into the culture vessels (5 L conical flasks containing 2 L of culture medium) and cultured with a f/2 medium.\(^{39} \) The cultures then acclimatized under an average irradiance of 150 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) with a light/dark regime of 12/12 h, and the experiments were conducted at 15, 20, and 25 °C with three replicates. The flasks were placed in the light incubator (GXZ-280C-CO2-LED) with 10,000 ppm CO2 introduced (maintained a stable pH) for 12 days. The algal cell was fixed with Lugol solution, and the cell density was measured by calculating with a hemocytometer using an optical microscope (Olympus, CKX53) on days 0, 3, 6, 9, and 12.

4.2. Experimental Design. 4.2.1. Pretreatment of Microalgae Cells. Microalgae suspension (200 mL) of 0, 3, 6, 9, and 12 days were centrifuged (1400 \( g \), 10 min, 4 °C) to obtain the algae cell. The collected cell was washed twice and
centrifuged again, then lyophilized, and ground into algae powder with liquid nitrogen.

4.2.2. Extraction of Total Lipid. The extraction of the total lipid was conducted according to Bligh and Dyer\textsuperscript{40} and Dos Santos.\textsuperscript{31} About 10 mg (m) of algae powder was applied to extract lipid using 3 mL of CH\textsubscript{2}OH–CHCl\textsubscript{3} (v/v = 2:1) mixed solution. The extract liquid was sonicated for 5 min and centrifuged for 15 min, then the organic phase was poured into a separatory funnel, and 2.5 mL of CHCl\textsubscript{3} and 3 mL of 1% NaCl were added, mixed, and stratified. All the lower layer liquid was placed in an empty stoppered tube (m\textsubscript{1}) and then nitrogen was blown to a constant weight (m\textsubscript{2}). The gravimetric method was used to calculate the total lipid content with the following equation

\[ w = \frac{(m_2 - m_1)}{m} \times 100\% \]

where m is the weight of algal powder, m\textsubscript{1} is the wet weight of the emptied stoppered tube, and m\textsubscript{2} is the total weight of the stoppered tube and extracted lipid.

4.2.3. Synthesis and Determination of Fatty Acid Methyl Ester. The synthesis of fatty acid methyl ester (FAME) was carried out according to Browse\textsuperscript{42} and Garces and Mancha.\textsuperscript{43} The total lipid in the tube was saponified with 2.0 mL of 0.04 M KOH–CH\textsubscript{2}OH solution in a water bath of 75 °C for 60 min, shaken per 10 min, and then cooled to room temperature. HCl–CH\textsubscript{2}OH (2.0 mL, v/v = 1:9) was then added and homogenized for 20 min at 60 °C in a water bath and shaken every 10 min for methyl esterification. The saturated NaCl solution and hexane were added to the cooled methyl esterified solution and mixed homogeneously. The hexane layer was dehydrated with anhydrous sodium sulfate and chromatographed.

Gas chromatography–mass spectrometry (GC–MS) (Agilent 7890A GC-5975C MSD) was employed to conduct chromatography. The HP-SMS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) (J&W Scientific) was used to separate. The initial temperature was 50 °C, which was held for 1 min, raised to 190 °C at 20 °C/min, then raised to 240 °C at 4 °C/min, then ramped up to 280 °C at 10 °C/min, and finally held at 280 °C for 2 min. The inlet temperature was 280 °C. The injection volume was 1 μL with the splitless mode. The constant carrier gas flow rate was at 1.0 mL/min. In the full-scan mode, the transfer line temperature was 280 °C, the ion source temperature was 230 °C, and the quadrupole analyzer temperature was 150 °C. The FAMEs were identified by comparing their relative retention time with a standard FAME mixture.

4.2.4. FTIR Spectroscopy. Microalgal powder (1 mg) and 100 mg of KBr were ground gently in an agate bowl under the infrared light, then pressed to a thin tablet, and measured. Spectra were acquired with a Nicolet iS 10 FTIR spectrometer (Thermo Scientific, USA) fitted with a DTGS–KBr detector. The absorbance spectra were collected between 4000 and 600 cm\textsuperscript{-1} at a spectral resolution of 6 cm\textsuperscript{-1} with 16 scans. The data interval is 1.929 cm\textsuperscript{-1}, the beam splitter is KBr, and the window is diamond. Omnic 8.0 software was used to deal with the spectra measured by FTIR.

4.3. Statistical Analysis. One-way variance analysis and Duncan multiple comparisons were performed to analyze the effects of growth, the macromolecular content, and the total lipid and fatty acid level by SPSS. Origin 8.6 (OriginLab, USA) was used to perform graphical work. The results were displayed as the form of average values ± standard deviation in this work.

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**Notes**

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