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The effects of temperature on the growth, lipid accumulation and nutrient removal characteristics of *Chlorella* sp. HQ

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ABSTRACT

As a promising feedstock for biodiesel production, the oleaginous microalgae have attracted the attention worldwide. The effects of temperature on the growth, lipid accumulation and nutrient removal characteristics of a freshwater microalga *Chlorella* sp. HQ were evaluated. The results show that it could grow in a wide range of temperature between 12 and 25˚C, but hardly grow at extremely high temperature of 38˚C. After 15 d of batch cultivation, the algal biomass was in the range of 68.8 ± 15.9–176.7 ± 13.8 mg L^{-1} and the peak was obtained at 18˚C. The lipid content per algal biomass decreased linearly from 41.8 to 6.9% as the temperature rose from 12 to 25˚C. The lipid yields at 12 and 18˚C were almost the same (around 23.8 mg L^{-1}), and at 25˚C, the lipid yield was remarkably lower (8.8 ± 1.8 mg L^{-1}). The highest TAGs content (10.2 ± 1.1%) was obtained at 25˚C, and the TAGs yields were ranging from 0.53 ± 0.15 to 0.89 ± 0.13 mg L^{-1}. In the range of 18–25˚C, water nitrogen (N) (up to 94.9 ± 0.3%) and phosphorus (P) (up to 99.0 ± 1.7%) were removed efficiently. Through comprehensive analysis, the optimal temperature for *Chlorella* sp. HQ to produce biodiesel and purify wastewater is suggested in the range of 18 and 25˚C.

Keywords: *Chlorella* sp.; Temperature influence; Growth rate; Lipid accumulation; Triacylglycerol; Nutrient removal

1. Introduction

Rapid development of the human society has caused huge depletion of fossil fuels, intensifying energy crisis in the twenty-first century. Additionally, the over combustion of fossil fuels aggravates the global warming due to the release of large amounts of greenhouse gases. Hence, exploiting for sustainable, renewable, and clean energy has become a hot issue. Promisingly, biodiesel is a high quality biofuel, which is comparable with fossil diesel and even better in terms of emissions and performance [1]. Hence, it has aroused significant attention worldwide in recent years.

As an alternative feedstock for biodiesel production, microalgae are superior in several aspects over traditional feedstocks, such as microalgae have shorter generation time and higher photosynthesis efficiency for the biomass production, occupy less arable land, can fix huge amounts of CO_2, and the residual algal biomass can be used as fertilizer or to produce methane after oil extraction, etc. [1]. As a neutral lipid in microalgae, triacylglycerols (TAGs) serve as a

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storage form of carbon and energy, being considered as an ideal source for biodiesel [2]. Therefore, quantitative evaluation of cells growth status and TAGs content is essential for efficient and scalable biofuel production in bioprocess engineering.

In addition to producing biodiesel, microalgae can be used to purify wastewater under autotrophic or mixotrophic conditions. Hence, a concept combing biodiesel production with wastewater treatment based on microalgae emerged, which was considered as an effective approach to improve the economic and environmental feasibility, ascribing to the significant decrease in large amounts of freshwater sources and nutrients as well as the chance of eutrophication. Currently, this combined concept has aroused great attention worldwide, i.e., Álvarez-Díaz et al. studied the characteristics of microalga *Scenedesmus obliquus* in two cultivation stages: the first stage was in batch culture in real wastewater and the second stage was maintaining the stationary phase with different culture conditions to improve the lipid content [3]. Abou-Shanab et al. evaluated the lipid accumulation and nutrient removal characteristics of six microalgal species in piggery wastewater and found that *Chlamydononas mexicana* was one of the most promising candidates for synchronous high-efficient biodiesel production and nutrient removal [4]. Gomez et al. reported that the microalgal biomass productivity and lipid content per biomass of *Muriellopsis* sp. could reach up to 0.5 g L^{-1} d^{-1} and 33%, respectively, in secondary-treated wastewater [5].

Moreover, several researches show the growth rate, lipid content, and nutrient removal efficiency changed with cultivation conditions, such as illumination, temperature, nutrient concentration (nitrogen (N), phosphorus (P), iron), etc. As reported, a sharp increase in total fatty acids was observed with high light at exponential phase and lower light caused increases in the relative abundance of unsaturated fatty acids [6]. The prolonged illumination time was beneficial to remove NH_4-N and PO_4^{3-} [7]. Han et al. found that 30℃ was the optimal daytime temperature for *Chlorella pyrenoidosa* to achieve high biomass and lipid, and elevating the daytime temperature could lessen the loss of night biomass and induce the lipid accumulation [8]. In response to nutrient limitation, the lipid accumulation of *Nannochloropsis oculata* increased up to three or fourfold, in comparison with sufficient conditions [9].

This study attempts to investigate the changes in properties of growth, lipid accumulation, and nutrient removal of an oleaginous freshwater microalga *Chlorella* sp. HQ isolated in our earlier study under varied cultivation temperature and provide an optimal cultivation temperature for this alga to synchronously produce biodiesel and purify wastewater.

2. Materials and methods

2.1. Microalgae and culture medium

Chlorella sp. HQ (No. GCMCC7601) was collected in China General Microbiological Culture Collection Center. It was preserved in modified BG11 (mBG11) medium composed of 15 mg L^{-1} N and 1.5 mg L^{-1} P simulating secondary effluent of municipal wastewater treatment plants, 37.5 mg L^{-1} $MgSO_4{\cdot}7H_2O$, 18 mg L^{-1} $CaCl_2{\cdot}2H_2O$, 3 mg L^{-1} citric acid, 3 mg L^{-1} ferric ammonium citrate, 0.5 mg L^{-1} EDTA, 10 mg L^{-1} Na_2CO_3, 1 mg L^{-1} A_5 + Co. The $NaNO_3$ and $K_2HPO_4{\cdot}3H_2O$ were added as N and P sources, respectively. The A_5 + Co solution contained 2.86 g L^{-1} H_3BO_3, 1.81 g L^{-1} $MnCl_2{\cdot}H_2O$, 222 mg L^{-1} $ZnSO_4{\cdot}7H_2O$, 79 mg L^{-1} $CuSO_4{\cdot}5H_2O$, 390 mg L^{-1} $Na_2MoO_4{\cdot}2H_2O$, 49 mg L^{-1} $Co(NO_3)_2{\cdot}6H_2O$.

2.2. Experimental setup

The alga was cultivated in 200 mL autoclaved culture medium (in 500-mL conical flasks) with initial inoculation density of 2×10^5 cells mL^{-1} in an artificial climate chamber (HPG-280H, HDL, China). To determine the effects of temperature, the illumination and cyclic illumination period were set at 60 μmol photons·(m^2 s)$^{-1}$ and 14 (light):10 (dark), respectively. The temperature was set as follows: 12, 18, 25 and 38℃ by adjusting the parameters of the climate chamber. The experimental groups were shaken 2–3 times per day, and all the tests were repeated three times.

The algal density was counted every day and the dry weight of algal biomass, lipid content per biomass and TAGs content per lipid were determined after 15 d of cultivation, the concentrations of N and P from cultures were analyzed simultaneously.

2.3. Algal density and biomass determination

The algal density was determined by counting cell numbers (cells mL^{-1}) using a hemocytometer under an optical microscope (XSZ-HS3, COIC, China). A 40-mL sample was filtered through pre-weighed 0.45-μm membranes, on which the algal cells were dried in an oven (MOV-112F, SANYO, Japan) at 110℃ to constant weight and then was determined gravimetrically by a precision electronic balance (AW220, SHIMADZU, Japan).

2.4. Lipid and TAGs determination

The algal cells were harvested by centrifugation at 12,000 rpm min^{-1} (high-speed freezing centrifuge,

HITACHI CR22G, Japan) for 10 min under the temperature of 4℃. And then, the total lipid content was extracted with 5 mL solution composed of chloroform/methanol/distilled water (2/2/1, v/v/v), and then, the extracted lipid was separated into three layers after centrifugation at 4,000 rpm for 10 min. The bottom layer (chloroform layer) was collected to concentrate lipid and the methanol layer including water was concomitantly removed. Afterward, the chloroform in mixed extracts was blown away using a nitrogen evaporator (DC-12, ANPEL, China) to obtain the total algal lipid, and then quantified gravimetrically [10]. After the determination of total lipid, 0.4 mL isopropanol was added into the dry algal lipid and mixed well, then the TAGs were tested by enzymatic colorimetric method using commercial kit from Beijing BHKT Clinical Reagent Co. Ltd. No. 2400076 [11].

2.5. Water quality analysis

The algal culture was filtered through 0.45-μm membranes, and then, the filtered supernatant was transferred into another clean tube. Subsequently, a sample of 8 mL was used for the determination of N concentration by using a total organic carbon analyzer (TOC-VCPH, SHIMADZU, Japan). A sample of 1 mL was digested for 30 min at 130℃ by adding into 3 mL distilled water and 1 mL potassium persulfate (5%) in the digestion apparatus (DRB 200, HACH, America). Finally, the P concentration was determined by the ammonium molybdate spectrophotometric method by using a spectrophotometer (DR 5000, HACH, America) at the wavelength of 700 nm [12].

3. Results and discussion

3.1. Growth property of *Chlorella* sp. HQ at different cultivation temperature

After 15 d of cultivation, the growth curves of *Chlorella* sp. HQ obtained at different temperature are shown in Fig. 1. It is obvious to observe that the cells densities at different temperature varied in a significant difference. During algal growth process, the cell densities at temperature of 12, 18, and 25℃ were extremely higher than 38℃. The algal density cultivated at 38℃ varied in a significantly small range of (1.3 ± 0.14)–(2.1 ± 0.24) × 10^5 cells mL^{-1}, which can be hardly observed the increase tendency. However, the alga grew well at other temperatures. At 12℃, the algal cells turned into the stationary phase since the thirteenth day and the maximal cells density was (1.1 ± 0.14) × 10^7 cells mL^{-1}, which is slightly lower than 25℃ ((1.9 ± 0.34) × 10^7 cells mL^{-1}). The peak of

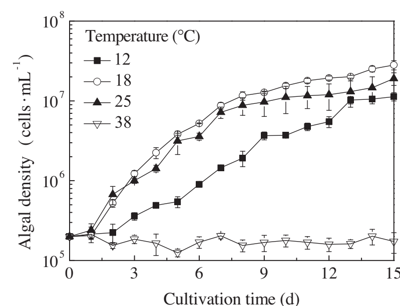

Fig. 1. Growth curves of *Chlorella* sp. HQ in culture medium at different cultivation temperature.

algal density was obtained at 18℃, reaching up to (2.8 ± 0.37) × 10^7 cells mL^{-1}.

Generally, the effects of cultivation temperature on the growth of microalgae may be species-dependent. As reported, the maximum densities of a freshwater microalga *Scenedesmum* sp. LX1 isolated from tap water after 15 d of cultivation under different temperature of 10, 20, 25, and 30℃ had no significant difference [13]. And the growth rate of *Nannochloropsis* sp. had no response to temperature, while that of *Isochrysis galbana* increased linearly with temperature, with small differences [14]. It was found that the algal densities of 3 benthic diatom species (*Cocconeis sublittoralis*, *Achnanthes longipes*, and *Navicula* cf. *jeffreyi*) declined after the cultivation of 18 weeks coinciding with a drop in temperature from 20.4 to 16.2℃ [15]. Moreover, at extremely low (15℃) and high (40℃) temperatures, the chlorophyll of *Trachydiscus minutus* (Bourr.) H. Ettl decreased and the cells growth was inhibited [16]. In this study, *Chlorella* sp. HQ had significant responses to different cultivation temperature and could grow well at the cultivation temperature between 12 and 25℃. The result indicates that *Chlorella* sp. HQ was a promising microalgae species which could adapt to a wide range of cultivation temperature from 12 to 25℃, but could not endure high temperature of 38℃.

3.2. Lipid accumulation property of *Chlorella* sp. HQ at different cultivation temperature

After 15 d of cultivation, the algal biomass (mg L^{-1}, dry weight), lipid content per algal biomass (%) and total lipid yield (mg L^{-1}, dry weight) of *Chlorella* sp. HQ were obtained, as shown in Fig. 2. The algal biomass concentrations at different cultivation temperature were in significant differences. At 38℃, *Chlorella* sp. HQ could hardly accumulate any algal biomass. Among other experimental groups, algal biomass concentrations were obtained in the

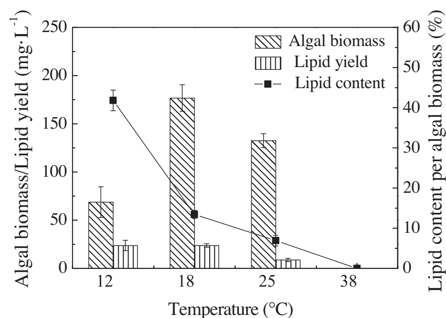

Fig. 2. Algal biomass (mg L^{-1}, dry weight), lipid content per algal biomass (%), and lipid yield (mg L^{-1}, dry weight) of *Chlorella* sp. HQ at different cultivation temperature.

range of (68.8 ± 15.9)–(176.7 ± 13.8) mg L^{-1}. The peak of biomass was obtained at the temperature of 18℃, the minimal value occurred at 12℃. At the normal temperature of 25℃ for algal cultivation, the biomass maintained at a relatively high level, reaching 132.5 ± 7.1 mg L^{-1}. Li et al. reported that the microalgal biomass of *Scenedesmus* sp. LX1 were almost the same at the temperature of 20, 25, and 30℃, which were significantly higher than that at low temperature of 10℃ [13]. In this study, in order to produce as high microalgal biomass level as possible, the optimal cultivation temperature for *Chlorella* sp. HQ should be controlled at 18℃.

Notably, the lipid content per algal biomass decreased sharply and linearly as the cultivation temperature rose. At normal cultivation temperature of 25℃, the lipid content per algal biomass was 6.9 ± 1.3%. At higher temperature of 38℃, the lipid content per algal biomass was 0% because there were no algal cells in this case. At lower temperature of 18℃, the lipid content per algal biomass reached to 13.4 ± 1.0%, nearly 2 times as that at 25℃. When the cultivation temperature was 12℃, the lipid content per algal biomass achieved the maximal value of 41.8 ± 2.6%, significantly higher than the other experimental results.

In general, at various taxonomic levels, some interspecific differences were as great as intraspecific differences with response to cultivation temperature, i.e., *Chaetoceros* cf. *wighamii* achieved the highest lipid content at 25℃ comparing with the ones at temperature of 20 and 30℃ [17]; the lipid content of *N. oculata* was practically doubled (from 7.9 to 14.9%) with an increase in temperature from 20 to 25℃, while an increase from 25 to 30℃ caused a significant decrease in lipid content of *Chlorella vulgaris* from 14.7 to 5.9% [18]. The influence of cultivation temperature on lipid content per biomass of *Chlorella* sp. HQ was different from either of the results above. In this study, low cultivation temperature of 12℃ could induce the lipid accumulation of *Chlorella* sp. HQ to a certain degree and result in significantly higher lipid content per algal biomass. This result coincides with the conclusion that at lower temperature of 10 and 20℃, the lipid contents per biomass of *Scenedesmus* sp. LX1 were significantly higher than the ones at temperature of 25 and 30℃ reported by Li et al. [13].

Due to the combined effects of algal biomass concentrations and lipid content per algal biomass, the lipid yields were almost the same at the temperature of 12 and 18℃, reaching around 23.8 mg L^{-1}. At 25℃, the lipid yield was 8.8 ± 1.8 mg L^{-1}, which was remarkably lower in comparison with the results at 12 and 18℃ due to the relatively lower lipid content in this case. The lipid yield at high temperature of 38℃ was also 0 mg L^{-1}. Li et al. found that the peak of lipid productivity of *Scenedesmus* sp. LX1 was obtained at the temperature of 20℃ [13]. For *Chlorella* sp. HQ, the optimal cultivation temperature to produce lipid was suggested to be in the range of 12–18℃.

After 15 d of cultivation, the TAGs content per lipid (%) and total TAGs yield (mg L^{-1}, dry weight) of *Chlorella* sp. HQ under different cultivation temperature were obtained, as shown in Fig. 3. It is clear that both the TAGs content per lipid and total TAGs yield varied in great differences, and the similar changing trend was observed. Same with the phenomenon in biomass and lipid accumulation, the alga had no TAGs content per lipid and TAGs yield at temperature of 38℃. The TAGs content per lipid decreased from 3.9 ± 0.2 to 1.9 ± 0.0% with cultivation temperature from 12 to 18℃, whereas it increased sharply to 10.2 ± 1.1% when the temperature rose to 25℃. The TAGs yields were almost the same at temperature of 12 and 25℃, reaching 0.89 ± 0.13 mg L^{-1} which was significantly higher than the one (0.53 ± 0.15 mg L^{-1}) at 18℃.

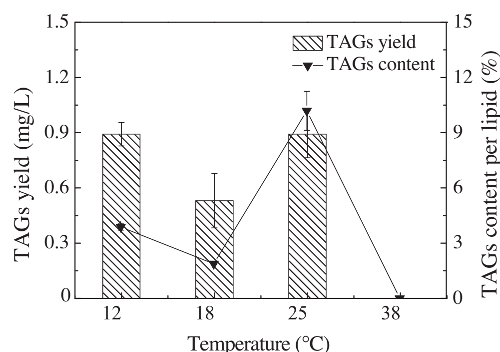

Fig. 3. TAGs content per lipid (%) and TAGs yield (mg L^{-1}, dry weight) of *Chlorella* sp. HQ at different cultivation temperature.

Li et al. reported that the TAGs content per lipid of *Scenedesmus* sp. LX1 had a positive correlation with the cultivation temperature from 10 to 30℃ [13]. Additionally, Chen et al. found that the TAGs content per lipid decreased with the cultivation temperature decreasing [19]. Besides, many other approaches have been certified to enhance the TAGs content, i.e., exposing algae cells with trace amount (1.0–2.0 mg L^{-1}) of EMA in comparison with the one without EMA treatment, the TAGs content per lipid and TAGs productivity of *Scenedesmus* sp. LX1 were increased by 79 and 40%, respectively [11]; the TAGs of four microalgal species (*Pavlova viridis*, *Chaetoceros* sp., *Phaeodactylum tricornutum,* and *N. oculata*) increased gradually under P-limitation [20]; the TAGs level of *Chlamydomonas reinhardtii* increased rapidly in response to nutrient limitation, especially sulfur starvation [21].

3.3. Nutrient removal property of Chlorella sp. HQ at different cultivation temperature

After 15 d of cultivation, the N and P removal efficiencies of *Chlorella* sp. HQ were obtained, as presented in Fig. 4. At the temperature of 12℃, the N was removed by 47.0 ± 6.9%, which was significantly lower than the ones at temperature of 18 (94.9 ± 0.25%) and 25℃ (84.5 ± 0.65%). This phenomenon may be caused by the relatively less algal cells at 12℃ in comparison with those at 18 and 25℃. The P removal efficiency had negative correlation with cultivation temperature between 12 and 25℃, decreasing from 99.0 ± 1.7 to 93.8 ± 8.9%. When the cultivation temperature rose to 38℃, the concentration of N and P in culture medium increased by 34.5 ± 0.41 and 48.4 ± 1.97%, respectively.

Generally, the nutrient removal depending on microalgae can be affected by many factors, i.e., Ip et al. found that too high concentration of ammonia nitrogen (>50 mg L^{-1}) inhibited the algal growth and further influenced the nutrient removal [22]; Li et al. reported that increasing in the pH value was beneficial for microalgae to remove $NH_3{\cdot}H_2O$ and PO_4^{3-}, whereas the algal growth would be affected with too high value of pH [23]; both the limited or excessive light intensity had inhibitory effect on the algal growth and the nutrient removal [8], etc. However, the relationship between cultivation temperature and nutrient removal was rarely investigated. Powell et al. reported that elevated temperature increased the rate of intracellular polyphosphate accumulation, while resulted in no-utilization of the stored acid-insoluble polyphosphate because the biomass was not starved of phosphate [24]. Notably, in this study, the concentrations of N and P in the culture medium at cultivation temperature of 38℃ did not decrease, on the contrast, increased to a great extent. It may be explained as that the algal cells at 38℃ turned to death gradually with the cultivation time increase and released the intracellular nutrients, which resulted in the significant increase of N and P concentrations. The results indicate that the cultivation temperature for microalgae to purify wastewater should be taken into consideration deliberately. The optimal cultivation temperature for *Chlorella* sp. HQ is suggested to be controlled between 12 and 25℃.

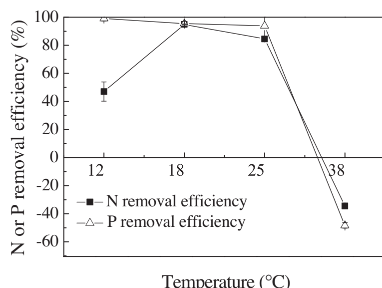

Fig. 4. The N and P removal efficiencies of *Chlorella* sp. HQ at different cultivation temperature.

4. Conclusions

A freshwater microalga *Chlorella* sp. HQ could grow in a wide range of temperature between 12 and 25℃, but hardly grow at extremely high temperature of 38℃. The maximal algal biomass was obtained at temperature of 18℃, reaching 176.7 ± 13.8 mg L^{-1}. The lipid content per algal biomass decreased linearly from 41.8 ± 2.6 to 6.9 ± 1.3% as the cultivation temperature rose from 12 to 25℃, while the lipid yields were almost the same at the temperature of 12 and 18℃ (around 23.8 mg L^{-1}) and at 25℃ the lipid yield was remarkably lower (8.8 ± 1.8 mg L^{-1}). The nitrogen (N) (up to 94.9 ± 0.2%) and phosphorus (P) (up to 99.0 ± 1.7%) were removed efficiently with cultivation temperature in the range of 18–25℃. As a result, the optimal cultivation temperature for *Chlorella* sp. HQ to produce biodiesel and purify wastewater is suggested to be in the range of 18–25℃.

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