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Effects of Nitrogen Sources and C/N Ratios on the Lipid-Producing Potential of *Chlorella* sp. HQ

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Microalgae are being researched for their potential as attractive biofuel feedstock, particularly for their lipid production. For maximizing biofuel production, it is necessary to explore the effects of environmental factors on algal lipid-producing potential. In this study, the effects of nitrogen (N) sources (NO_2-N, NO_3-N, urea-N, NH_4-N, and N-deficiency) and carbon-to-nitrogen ratios (C/N = 0, 1.0, 3.0, and 5.0) on algal lipid-producing potential of *Chlorella* sp. HQ were investigated. The results showed that for *Chlorella* growth and lipid accumulation potential, NO_2-N was the best amongst the nitrogen sources, and NO_3-N and urea-N also contributed to algal growth and lipid accumulation potential, but NH_4-N and N-deficiency instead caused inhibitory effects. Moreover, the results indicated that algal lipid-producing potential was related to C/N ratios. With NO_2-N treatment and carbon addition (C/N = 1.0, 3.0, and 5.0), total lipid yield was enhanced by 12.96–20.37%, but triacylglycerol (TAG) yields decreased by 25.52–94.31%. As for NO_3-N treatment, carbon addition led to a 17.82–57.43%/25.86–82.67% reduction of total lipid/TAG yields. When NH_4-N was used as the nitrogen source, total lipid/TAG yields were increased by 46.67–113.33%/28.99–74.76% with carbon addition. The total lipid/TAG yields of urea-N treatment varied with C/N ratios. Overall, the highest TAG yield (TAG yield: 38.75 ± 5.21 mg/l; TAG content: 44.16 ± 4.35%) was achieved under NO_2-N treatment without carbon addition (C/N = 0), the condition that had merit for biofuel production.

Keywords: *Chlorella* sp. HQ, lipid accumulation, triacylglycerols, nitrogen sources, C/N ratios

Introduction

Facing traditional fuel depletion and environmental pollution, the exploration of efficient and environmental-friendly fuel substitutes has aroused worldwide concern. Microalgal biodiesel is considered a promising substitute for fossil fuel because of algal rapid growth, and non-toxic and biodegradable characteristics of lipids, and potential value for wastewater purification [5]. Although microalgae have many advantages, they are not yet commercially viable for biodiesel production. Scientists are eager to break through serious bottlenecks to enlarge the cultivation scale, enhance the lipid accumulation potential, and conquer the high cost of biofuel from microalgae.

For narrowing the bottlenecks above, an effective dual-purpose strategy is proposed. It is beneficial to cultivate microalgae for feedstock production and wastewater treatment synchronously. Many dual-purpose algae with high nutrient removal and lipid-producing potential were used for the above purpose. Our previous reports show an isolated freshwater microalga, *Chlorella* sp. HQ, had a great capacity for nutrient uptake and lipid accumulation [32]. In the Ge and Champagne [9] study, *Chlorella vulgaris* could remove the nutrients and obtain a high lipid productivity efficiently when growing in centrate wastewater. Additionally, *Scenedesmus obliquus* was also found to achieve high nutrient removal and lipid accumulation potential simultaneously [22].

To achieve improvement in nutrient uptake and lipid yield simultaneously, many studies were conducted to investigate the effects of environmental factors on microalgae, such as wastewater types, microorganisms, contaminants, and nutrients [4, 27, 29]. Meanwhile, aiming at the optimization of algal biofuel production, many research studies have focused on screening for best culture conditions (temperature, pH, light wavelength, nutrients, micronutrients, CO_2, *etc*) [10, 23, 24]. Both nitrogen (N) and carbon (C) are considered as essential factors for microalgal lipid-producing potential [10, 18, 23].

As known, the composition of nitrogen source is complicated in wastewater. In general, ammonium-N, nitrate-N, urea-N, and nitrite-N are the primary nitrogen sources utilized by microalgae [21, 22], and organic nitrogen is able to be degraded in wastewater [8]. In addition, the extent and rate of algal lipid accumulation were dependent on carbon sources, and carbon was simultaneously involved in nitrogen assimilation. In particular, the carbon concentration might also influence the algal growth and lipid accumulation like nitrogen [7, 11]. Unexpectedly, nitrogen starvation (including N-depletion and N-deficiency) is considered a low-cost and easily controlled approach to enhance lipid-producing potential among many strategies, including light limitation, nitrogen or phosphorus starvation, iron and salinity addition, *etc*. [1, 10, 19]. Most studies focus on N-depletion, and the effects of N-deficiency on algal lipid-producing potential have seldom been reported.

Many research studies have focused on screening optimal nitrogen sources or C/N ratios for algal lipid-producing potential. However, it is also important to find out the best nitrogen source and C/N ratios for certain algae (*e.g.*, *Chlorella* sp. HQ with high nutrient removal and lipid-producing potential). Moreover, we first investigated the combined effects of nitrogen sources and C/N ratios on algal lipid-producing potential, particularly. The aim of this work was to study the independent/dependent effects of nitrogen sources (ammonium-N, nitrate-N, urea-N, nitrite-N, and N-deficiency) and C/N ratios (C/N = 0, 1.0, 3.0, and 5.0) on the lipid-producing potential of *Chlorella* sp. HQ. It will provide in-depth understanding and practical data for further application of this coupling technique.

Materials and Methods

Microalgae Species and Culture Medium

An oleaginous green alga, *Chlorella* sp. HQ (collection no. GCMCC7601 in China General Microbiological Culture Collection Center), preserved in SE medium [32] was used in this study. The mBG11 medium with 1.5 mg P/l and 15 mg N/l (simulating the typical concentration of phosphorus and nitrogen in secondary effluent) was used as the growth medium in Experimental set-up (including Part one. Effects of nitrogen sources on lipid-producing potential of *Chlorella* sp. HQ; and Part two. Effects of C/N ratios on lipid-producing potential of *Chlorella* sp. HQ). In all experiments, $K_2HPO_4{\cdot}3H_2O$ (1.5 mg P/l) and $NaHCO_3$ were used as the phosphorus and carbon sources, respectively. With the exception of nitrogen and carbon, the composition of other elements was the same as with mBG11 medium [32].

Experimental Set-Up

Before inoculation, the alga was domesticated in a P-N-free mBG11 medium for 5 days, and then a certain volume (1.40 ml) of exponentially growing *Chlorella* sp. HQ cells were inoculated in 180 ml of autoclaved mBG11 medium (121°C, 30 min) in 250 ml Erlenmeyer flasks with initial cells density of 2.5×10^5 cells/ml.

Part one: Effects of nitrogen sources on lipid-producing potential of *Chlorella* sp. HQ. *Chlorella* sp. HQ was inoculated in cultures with five N sources without external carbon, including ammonium chloride (NH_4Cl, 15 mg N/l), sodium nitrate ($NaNO_3$, 15 mg N/l), urea (H_2NCONH_2, 15 mg N/l), sodium nitrite ($NaNO_2$, 15 mg N/l), or no added N source (0 mg N/l).

Part two: Effects of C/N ratios on lipid-producing potential of *Chlorella* sp. HQ. *Chlorella* sp. HQ was inoculated in 20 kinds of artificial wastewaters (see Table S1) containing five kinds of nitrogen sources (*e.g.*, N-deficiency, $NaNO_3$, NH_4Cl, H_2NCONH_2, and $NaNO_2$) with various C/N ratios (0, 1.0, 3.0, and 5.0). C/N = 0 means no carbon addition ($NaHCO_3$), which was defined as the control group in this study.

All the experiments were conducted in an artificial climate chamber (HPG-280H, HDL, China) under controlled laboratory conditions (temperature 25°C, light intensity of 60 μmol photons m^{-2} s^{-1} and a light/dark cycle of 14 h/10 h). Additionally, all experiments were carried out at least in quadruplicates and the Erlenmeyer flasks were hand shaken two or three times per day.

Analytical Methods

Algal growth assay. The microalgal density (*N*, cells/ml) was determined by counting the number of cells with a hemocytometer under an optical microscope (XSZ-HS3; Chongqing Optical & Electrical Instrument Company, China). The morphological characteristics of the cells were observed and photographed simultaneously.

The algae growth was modeled by the Logistic Model [2], where *N* is the algal density at time t (d), K (cells/ml) stands for the carrying capacity and is the maximum algal density reached in the culture, R_{max} is the maximum sustainable yield (cells ml^{-1} d^{-1}), r (d^{-1}) is the specific growth rate, and a is a constant. The values of K, R_{max}, r, and a can be obtained by logistic fit with the data series of N and t, and K, R_{max}, and r are used for the comparison of algal growth.

Biomass and pH evaluation. After cultivation for 45 days, the

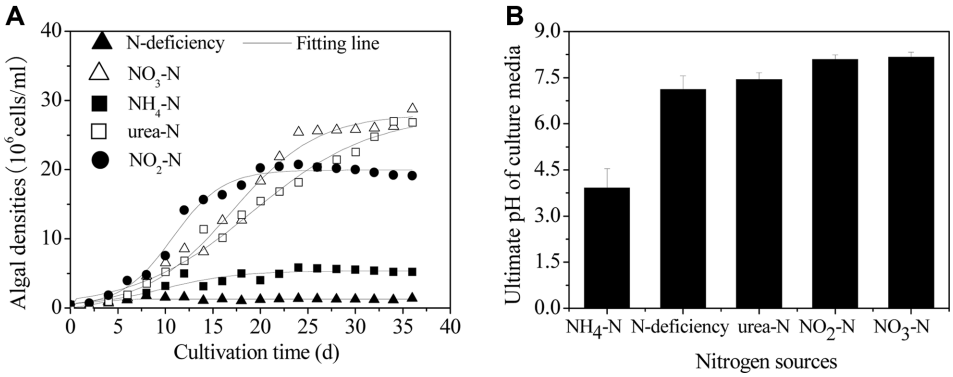

Fig. 1. Algal cells densities and logistic model fitting line of *Chlorella* sp. HQ and ultimate pH values (45 d) in culture media with five test nitrogen sources (N-deficiency, NO_3-N, NH_4-N, urea-N, and NO_2-N).
(**A**) Algal cells densities and logistic model fitting line. (**B**) Ultimate pH values (45 d).

pH values in the culture were tested with a pH meter (PB-10; Sartorius, Germany). Additionally, the biomass was determined as follows: quadruplicate samples (40 ml) were filtered through pre-weighed 0.45 μm membranes. Then the membranes with algal cells were dried in an oven (MOV-112F; Sanyo, Japan) at 110°C for 24 h and the dry weight of algal biomass was determined gravimetrically with a precision electronic balance (AW220, Shimadzu, Japan)

Algal total lipids and triacylglycerol determination. After 45 days of cultivation, quadruplicates of 40 ml cultures transferred from the flasks were concentrated to a volume of about 0.8 ml by a high-speed freezing centrifuge (CR22G; Hitachi, Japan) for 10 min at 12,000 rpm and 4°C. The total lipids were extracted by adding 2 ml of chloroform, 2 ml of methanol, and 1 ml of distilled water. Then the liquids were mixed. After centrifugation at 4,000 rpm for 10 min, the mixture was separated into three layers. The chloroform layer was transferred into a pre-weighed glass tube, the chloroform was evaporated to obtain the dry lipid in a nitrogen evaporator (DC-12; Anpel, China), and then the total lipids were quantified gravimetrically. After the determination of total lipid yields, the dried lipids were dissolved in 0.4 ml of isopropyl alcohol and mixed thoroughly. Then triacylglycerol (TAG) yields were estimated by enzymatic colorimetric method with a TAGs Assay Kit (Beijing BHKT Clinical Reagent Co., Ltd., No. 2400076, China) according to the manufacturer's instructions. Briefly, 1,000 μl of TAG enzymatic reagent was added to the above mixture (10 μl) and incubated at 37°C for 10 min. Subsequently, the chromogenic substrate was monitored at the wavelength of 500 nm. TAGs were used as the standard for calibration [32]. Additionally, total lipid contents (total lipids yield/biomass ×100%) and TAG contents (TAG yield/total lipids yield ×100%) were calculated.

Statistical Analysis

Unless otherwise noted, all the data were expressed as mean values ± standard error (SE) of four independent experiments. All the mean ± SE data were statistically analyzed by one-way or two-way analysis of variance (ANOVA). The least significant difference (LSD) test was used to make multiple comparisons among treatment means from significant ANOVA tests (level of significance: $p < 0.05$). All statistics were performed using SPSS ver. 18.0.

Results and Discussion

Effects of Nitrogen Sources on Lipid-Producing Potential of *Chlorella* sp. HQ

Effects of nitrogen sources on growth potential of *Chlorella* sp. HQ. In order to study the effects of nitrogen sources on the lipid-producing potential of *Chlorella* sp. HQ, the algal growth and lipid accumulation potential were studied. In this section, algal growth potential was analyzed (Fig. 1). Moreover, the logistical model was applied to describe the relationship between algal growth potential and cell density. The values of logistical parameters (the maximum sustainable yield, R_{max}; maximal cell density, K; specific growth rates, r) were obtained through calculation, as presented in Table 1 (referred to the control groups, C/N = 0).

During the first 20 days, the cell density after NO_2-N treatment increased rapidly and was higher than that of the other four nitrogen sources (Fig. 1A). The highest R_{max} ($1.87 \pm 0.00 \times 10^6$ cells ml^{-1} d^{-1}) was achieved with NO_2-N treatment, which was 1.21-fold, 1.67-fold, 5.67-fold, and 6.45-fold more than that of NO_3-N, urea-N, NH_4-N, and N-deficiency, respectively. Hence, NO_2-N was considered to be the best nitrogen source for growth of *Chlorella* sp. HQ. In general, different microalgae species respond to nitrogen sources differently. For instance, the growth rate of *Botryococcus braunii* KMITL was significantly higher in NO_3-N (KNO_3) treatment compared with other N treatments

Table 1. Logistic parameters of *Chlorella* sp. HQ with different nitrogen sources combined with four C/N ratios.

Nitrogen sources	[a]C/N ratios	r/ (d^{-1})	K/ (10^6cells/ml)	R_{max}/ 10^6cells ml^{-1} d^{-1}	Adj. R-Square
[c]N-deficiency	[b]0	0.89 ± 0.39	1.30 ± 0.05	0.29 ± 0.01	0.75
	1	0.00 ± 0.00	1.17 ± 0.11	0.00 ± 0.00	0.20
	3	0.08 ± 0.34	1.00 ± 0.04	0.02 ± 0.00	0.71
	5	0.63 ± 0.26	0.86 ± 0.03	0.14 ± 0.00	0.69
[d]NO_3-N	0	0.22 ± 0.02	28.09 ± 0.86	1.55 ± 0.00	0.99
	1	0.29 ± 0.06	30.84 ± 1.47	2.23 ± 0.02	0.95
	3	0.33 ± 0.05	29.16 ± 0.90	2.41 ± 0.01	0.97
	5	0.30 ± 0.04	27.32 ± 0.96	2.05 ± 0.01	0.97
[d]NH_4-N	0	0.26 ± 0.06	5.14 ± 0.23	0.33 ± 0.00	0.91
	1	0.26 ± 0.05	26.99 ± 1.17	1.76 ± 0.02	0.95
	3	0.28 ± 0.05	22.56 ± 0.89	1.58 ± 0.01	0.96
	5	0.37 ± 0.04	18.77 ± 0.40	1.74 ± 0.00	0.98
[d]urea-N	0	0.16 ± 0.01	27.96 ± 1.28	1.12 ± 0.00	0.99
	1	0.33 ± 0.04	26.83 ± 0.65	2.21 ± 0.01	0.98
	3	0.37 ± 0.03	24.38 ± 0.38	2.26 ± 0.00	0.99
	5	0.29 ± 0.04	22.95 ±0.64	1.66 ± 0.01	0.98
[d]NO_2-N	0	0.38 ± 0.03	19.72 ± 0.30	1.87 ± 0.00	0.99
	1	0.38 ± 0.05	24.83 ± 0.47	2.36 ± 0.01	0.98
	3	0.28 ± 0.04	33.61 ± 1.13	2.35 ± 0.01	0.96
	5	0.26 ± 0.03	27.16 ± 0.66	1.77 ± 0.01	0.98

[a]C/N ratios mean carbon concentration to nitrogen concentration (the corresponding carbon concentration of 1.0, 3.0, and 5.0 was 15, 45 and 75 mg C/l, respectively; $NaHCO_3$ was used as the carbon source).

[b]C/N = 0 means no dissolved inorganic carbon addition, which was considered as the control groups.

[c]N-deficiency means no nitrogen addition.

[d]The nitrogen concentration is 15 mg N/l.

[21]. Urea-N (H_2NCONH_2) is generally preferred by *Nannochloropsis salina* for algal growth potential (cell density) with respect to other nitrogen sources [2]. *Ellipsoidion* sp. cultured in medium with NH_4-N (NH_4Cl) as the nitrogen source provided a significantly higher growth rate than cells grown in medium with the other nitrogen sources [30]. That is likely becauese the microalgae used in the studies come from different genera and species, and their regulatory genes involved in algal metabolism might be different. In addition, the actual cultivation condition is various, and thus, the combined effects of other environmental factors such as temperature, pH, culture media, *etc*. might also induce various preferable N-sources per algal species [10].

After 20 days of cultivation, *Chlorella* sp. HQ with NO_2-N treatment reached the stationary phase. For the NO_3-N treatment, the critical day for the transition from exponential-phase to stationary-phase was 24 days. As for urea-N treatments, the cell density showed a steady increase in growth up to day 36 (Fig. 1A). In other words, the transition day for stationary-phase of *Chlorella* sp. HQ with NO_2-N treatment occurred faster than with NO_3-N or urea-N treatments. Concomitantly, the color of cultures with NO_2-N treatment varied from green to yellow when cells grew to stationary-phase (21–36 days) (Fig. S1) and was the result from the reduction in chlorophyll [34]. This may be due to the intracellular conversion of NO_2-N by nitrite reductase and ferredoxin, which might consume a great amount of cellular nicotinamide adenine dinucleotide hydrogen (NADH), and the decrease in NADH might lead to the reduction of chlorophyll [20]. Moreover, the reduction of chlorophyll might result in lessening of photosynthesis, which suggested the reason for the cell growth stopping after 20 days of cultivation. In addition, *Chlorella* sp. HQ with NO_3-N and urea-N treatments reached the stationary phase successively, probably as a

consequence of nitrogen depletion in the culture media successively (Fig. S2). In other words, it might be that the lower utilization rate of urea could result in the nitrogen starvation of algal cells.

Meanwhile, NO_3-N and urea-N treatments also contributed to the cell growth of *Chlorella* sp. HQ, but NH_4-N did not (Fig. 1A). NO_3-N and urea-N treatments showed increase in R_{max} by 369.70% and 239.39% with respect to NH_4-N treatment, respectively (Table 1). In addition, a phenomenon observed after cultivation was that the pH value of NO_3-N or urea-N maintained between 7.44 ± 0.23 and 8.17 ± 0.16, whereas that of NH_4-N treatment decreased to 3.92 (Fig. 1B). In other words, the algal culture medium turned acidic when NH_4-N was used as the N source. A similar phenomenon was reported when the assimilation of ammonium ions caused a decrease in pH value to 4.0 with cultivation of *C. sorokiniana*, which led to an inhibitory effect on algal growth potential [20]. That is likely due to NH_4^+ releasing H^+ into the culture medium during assimilation of ammonium ions in the algal cultivation process [28] (In this study, the NH_4-N concentration in culture medium of *Chlorella* sp. HQ with NH_4-N added gradually decreased. See Fig. S2). As known, acidic culture might disrupt cellular respiration through depletion of tricarboxylic acid cycle intermediates; as a result, the algal cells grew slower. Meanwhile, the color of cultures changed from green to yellowish-white under NH_4-N treatment (Fig. S1), attributed to the breakdown of chlorophyll [34]. Moreover, the breakdown of chlorophyll might further inhibit the cell growth owing to photosynthesis lessening. Consequently, NH_4-N treatment resulted in an inhibitory effect on algal growth potential.

In order to obtain the maximum lipid-producing potential for biofuel production, nitrogen starvation (including N-deficiency and N-depletion) has been proposed as a good strategy. In our study, the total nitrogen starvation (*i.e.*, N-deficiency) was evaluated. Under N-deficiency condition, the cell density of *Chlorella* sp. HQ increased approximately 7-fold during the first 8 days (Fig. 1A). That was attributed to chlorophyll in algal cells degrading for nutrient recycling to support algal growth under N-deficiency condition [34], and concomitantly, the color of culture broths changed from normally green into yellowish-white (Fig. S1). However, the chlorophyll degradation for algal growth during the early days was considered as cell self-destruction, and in effect, the cell density dropped sharply after 8 days (Fig. 1A). As a result, the final cell density of *Chlorella* sp. HQ with N-deficiency treatment was significantly lower than that of the other nitrogen sources (Fig. 1A). The R_{max} of N-deficiency treatment showed 6.45-fold, 5.34-fold, 3.86-fold, or 1.14-fold decrease relative to that of NO_2-N, NO_3-N, urea-N, or NH_4-N treatments, respectively (Table 1). Similarly, the data of Zhu *et al*. [34] showed that N-deficiency was not an efficient approach for the growth of *Chlorella zofingiensis* compared with N-added condition. As known, nitrogen is an essential component in many macromolecules like RNA, DNA, chlorophyll, and protein. Consequently, N-deficiency will impair photosynthesis and protein synthesis, which could impede cell division and contribute to a decrease in cell density [6, 26]. Taken together, N-deficiency treatment was not as efficient as N-addition for algal growth potential.

Effects of nitrogen sources on lipid accumulation potential of *Chlorella* sp. HQ. As described previously in details, the growth of *Chlorella* sp. HQ varies under different nitrogen treatment conditions. Next, the effects of nitrogen sources on the lipid accumulation potential of *Chlorella* sp. HQ was studied, including the algal biomass, total lipids yield, and TAG yields (Fig. 2A), and the total lipids content per biomass (%, dry weight) and TAG content per total lipids yield (%) as well (Fig. 2B).

For biofuel production, TAGs have priority over other non-TAG lipids (*e.g.*, diacylglycerols, DAG; monoacylglycerols, MAG; glycolipids, GL; phospholipids, PL) [16, 31] owing to their higher fatty acid contents and advantage of converting into biodiesel directly. Moreover, TAGs have higher calorific value than structural lipids as storage lipids. Hence, high TAG-producing potential has been proposed as a crucial factor for optimization of microalgal biofuel production. In *Chlorella* sp. HQ, the maximum TAG yield (38.75 ± 5.21 mg/l) was obtained under NO_2-N cultivation, which was significantly higher than that of NO_3-N ($p < 0.001$), urea-N ($p < 0.001$), or NH_4-N ($p < 0.001$) treatments, ranging between 5.86 ± 2.86 and 19.99 ± 4.56 mg/l (Fig. 2A) (one-way ANOVA, LSD multiple comparisons test; Table S2). Moreover, the TAG content of 44.16 ± 4.35% with NO_2-N treatment was higher than that of other N treatments (*i.e.*, NO_3-N, urea-N, or NH_4-N) (Fig. 2B). Through comprehensive analysis, NO_2-N treatment had priority over the other nitrogen sources for lipid accumulation potential in *Chlorella* sp. HQ.

Previous reports have documented that the removal of nitrite during the wastewater treatment process was quite difficult but important, because nitrite can be toxic to the algal environment (aquatic life). For instance, the total lipid content of *C. vulgaris* was dropped to 13.04 ± 1% with nitrite-N (NO_2^-) addition [19]. In this study, *Chlorella* sp. HQ showed a high nitrite removal potential (also seen from Fig. S2) and a good tolerance to nitrite, and achieved a

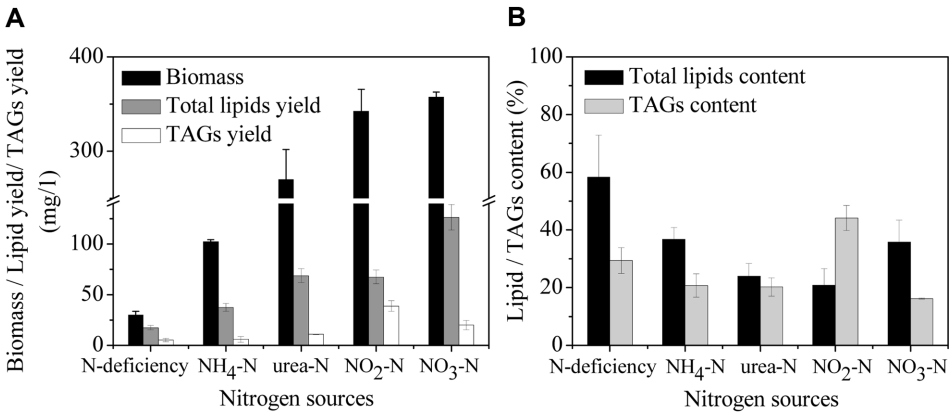

Fig. 2. Biomass/total lipids/TAG yield and total lipid content per algal biomass (%, dry weight) and TAG content per total lipid yield (%) at 45 days of *Chlorella* sp. HQ in five test nitrogen sources (N-deficiency, NO_3-N, NH_4-N, urea-N, and NO_2-N). (**A**) Biomass/total lipids/TAG yield. (**B**) Total lipid content per algal biomass.

higher TAG accumulation (Figs. 2A and 2B). Thus, *Chlorella* sp. HQ would be a preferred candidate for biofuel production by using the nitrite accumulated in wastewater. That is likely due to lipid biosynthetic pathways preferred to the formation of storage compounds (such as TAGs) instead of structural compounds under adverse conditions [14]. The site for lipid synthesis was found to be in chloroplasts, and GL, as its main component, played as an intermediate for TAG synthesis [17]. Therefore, TAG biosynthesis might result in chlorophyll breakdown. Meanwhile, the color of culture broths changed from green to yellow (as discussed above). These results suggested that NO_2-N treatment may force the lipid biosynthetic pathways towards TAGs in *Chlorella* sp. HQ.

However, the optimal nitrogen sources for lipid accumulation potential was algal species-specific, so it is important to control the nitrogen source correctly for microalgae in order to obtain the highest lipid-producing potential. Lin and Lin [13] found that NO_3-N ($NaNO_3$) was the best nitrogen source for *S. rubescens*, contributing to increases in total lipid content up to 35%. In *Chlorella* sp. HQ, the highest algal biomass (357.50 ± 5.30 mg/l) was achieved by adding NO_3-N, which showed a slight advantage over NO_2-N (342.50 ± 22.98 mg/l) ($p > 0.05$) and represented 1.32-fold and 3.49-fold more than that of urea-N (270.00 ± 31.82 mg/l) ($p < 0.001$) and NH_4-N (102.50 ± 1.77 mg/l) ($p < 0.001$), respectively (one-way ANOVA, LSD multiple comparisons test, Table S2). In addition, the total lipid yields of *Chlorella* sp. HQ with NO_3-N treatment (126.25 ± 12.63 mg/l) resulted in 83.64%, 87.03%, and 236.67% increases compared with urea-N (68.75 ± 6.88 mg/l) ($p < 0.001$), NO_2-N (67.50 ± 6.75 mg/l) ($p < 0.001$), and NH_4-N (37.50 ± 3.75 mg/l) ($p < 0.001$) treatment, respectively (Fig. 2A) (one-way ANOVA, LSD multiple comparisons test, Table S2). Moreover, the total lipid content of 35.82 ± 7.65% with NO_3-N treatment was higher than that of the other nitrogen treatments (*i.e.*, urea-N, NO_2-N and NH_4-N) (Fig. 2B). Unfortunately, the TAG yield ($p < 0.001$) and TAG content ($p < 0.001$) were significantly lower than that of NO_2-N (Figs. 2A and 2B) (one-way ANOVA, LSD multiple comparisons test, Table S2). Hence, NO_3-N could be not considered as the best nitrogen source for lipid accumulation potential of *Chlorella* sp. HQ. On the other hand, if new technology could realize the conversion from non-TAG lipids to TAGs, NO_3-N might be better used for algal lipid accumulation potential.

In the study of Ramanna *et al*. [20], urea-N (H_2NCONH_2) was the best nitrogen source for *C. sorokiniana,* where the highest relative fluorescence per cell (rf/cell) lipid peaked at 48.31%. In our study, the total lipid yield of urea-N treatment (68.75 ± 6.88 mg/l) was only lower than that of NO_3-N ($p < 0.001$) but higher than that of NO_2-N ($p < 0.001$) and NH_4-N ($p < 0.001$) treatments (one-way ANOVA, LSD multiple comparisons test, Table S2). That is likely due to intracellular conversion of urea-N, forming ammonium and CO_2 by urease enzymes (or urea amido hydrolase pathway). In solution, the additional CO_2 dissociated to HCO_3^- or CO_3^{2-}, providing excess carbon flux towards lipid accumulation and promoting algal growth [20]. However, the TAG yield ($p < 0.001$) of *Chlorella* sp. HQ with urea-N treatment was lower than that of NO_2-N treatment (one-way ANOVA, LSD multiple comparisons test, Table S2). Consequently, the urea-N treatment was not the best nitrogen source for lipid accumulation potential of *Chlorella*

sp. HQ.

Xu *et al*. [30] reported that NH_4-N (NH_4Cl) appeared favorable for total lipid accumulation (33.3 ± 0.39%) of *Ellipsoidion* sp. The biomass of *Chlorella* sp. HQ with NH_4-N treatment decreased by 71.33% and 70.07% as compared with NO_3-N treatment and NO_2-N treatment, respectively. The total lipid yield (37.50 ± 3.75 mg/l) of *Chlorella* sp. HQ with NH_4-N treatment was 29.70% and 55.55% of NO_3-N (126.25 ± 12.63 mg/l) ($p < 0.001$) and NO_2-N (67.50 ± 6.75 mg/l) ($p < 0.001$), separately (one-way ANOVA, LSD multiple comparisons test, Table S2). The NH_4-N treatment resulted in TAG yields of 5.86 ± 2.86 mg/l, which were 70.69% and 84.88% lower than that of NO_2-N treatment ($p < 0.005$) and NO_3-N treatment ($p < 0.001$), respectively (Fig. 2A) (one-way ANOVA, LSD multiple comparisons test, Table S2). These results demonstrated the lipid accumulation potential of *Chlorella* sp. HQ with NH_4-N added to be lower than that of NO_2-N and NO_3-N treatments. That is likely due to NH_4-N addition being unbeneficial for cell growth (as discussed above), and lipids were used up as a form of storage energy for algal growth under high concentration of ammonium-N, because of cell growth declined [20].

In the current study, the highest total lipid content (58.33 ± 14.48%) was found in N-deficiency treatment, which was about 2.80-fold higher than NO_2-N treatment ($p < 0.001$), 2.42-fold higher than urea-N treatment ($p < 0.001$), 1.63-fold higher than NO_3-N treatment ($p < 0.01$) and 1.58-fold higher than NH_4-N treatment ($p < 0.01$), respectively (Fig. 2B) (one-way ANOVA, LSD multiple comparisons test, Table S2). Similar phenomena were found on *S. obliquus* and *C. pyrenoidosa*, of which higher total lipid contents (47.7% and 54.49%) were achieved under N-deficiency compared with N-added conditions [26]. The phenomenon may be explained by two reasons. Primarily, nutrient stress (*e.g.*, nitrogen deficiency) channels metabolic flux to lipid biosynthesis when photobiosynthesis is active [6] because lipids are found to be the preferred storage compounds in helping cell survival under adverse conditions (such as nutrition starvation) and owing to highly reduced molecular states. Secondly, it might be the result of gene expression, which might decline cell division and promote the accumulation of lipids under N-deficiency. The idea is similar to Liu and Benning [15], who reported that *NRR1* is a key regulatory gene involved in lipid accumulation potential in *Chlamydomonas* cells under nitrogen-starvation conditions.

An inherent disadvantage of N-deficiency is, however, that it could lead to a dramatic decrease in biomass, total lipid yield, and TAG yield in *Chlorella* sp. HQ. The decrease ranged 70.73–91.61% for biomass, 53.33–86.14% for total lipid yield and 8.87–86.21% for TAG yields in comparison with the other four nitrogen sources (Fig. 2A). As described above, it is likely due to the inhibitory effect on cell growth. Consequently, N-deficiency treatment was regarded as an inefficient strategy for algal lipid accumulation potential compared with N-added treatments.

Significant effects of nitrogen sources on the total lipid yield ($p < 0.001$), TAG yield ($p < 0.001$), total lipid content ($p < 0.005$), and TAG content ($p < 0.001$) were also observed by using statistical analysis (one-way ANOVA, Table S2). In summary, NO_2-N was the best nitrogen source for algal lipid-producing potential; both NO_3-N and urea-N also contributed to algal lipid-producing potential; and NH_4-N and N-deficiency treatments are an inefficient strategy to enhance lipid-producing potential of *Chlorella* sp. HQ, attributed to substantial loss in biomass, total lipid yield, and TAG yield.

Effects of C/N Ratios on Lipid-Producing Potential of *Chlorella* sp. HQ

Effects of C/N ratios on growth potential of *Chlorella* sp. HQ. To provide in-depth understanding and practical data for its further application, the effects of C/N ratios on algal growth potential of *Chlorella* sp. HQ under different nitrogen sources (NO_2-N, NO_3-N, NH_4-N, urea-N, or N deficiency) should be explored. Fig. 3 shows the cell density of *Chlorella* sp. HQ under NO_2-N, NO_3-N, NH_4-N, urea-N, or N-deficiency treatments with four C/N ratios (C/N = 0, 1.0, 3.0, and 5.0). A logistical model [2] was fitted as shown with the values of r, K, and R_{max} (Table 1). (The logistic model fitting line is in Fig. S3.)

As known, dissolved inorganic carbon (DIC) might impact the metabolism of algae and be converted into organic carbon [14]. In algal growth potential studies, cell densities, r, K, and R_{max} are considered together. In this study, carbon addition (C/N = 1.0 and 3.0) enhanced algal growth potential of *Chlorella* sp. HQ with NH_4-N, NO_2-N, urea-N, or NO_3-N treatments compared with no carbon addition (C/N = 0) treatments, but was found to be limited by high C/N ratios (C/N = 5.0) (Table 1 and Fig. 3). That is likely due to the lack of DIC (HCO_3^-) resulting in photosynthesis lessening [18], which in turn resulted in a decrease in algal growth potential. By contrast, carbon addition ($NaHCO_3$) could be effective in keeping the DIC concentration high [11]. However, the high concentration of $NaHCO_3$ might cause salt osmotic and toxic ionic stress generated by Na^+, which in turn results in reduced chlorophyll contents and photosynthetic rate [7, 11].

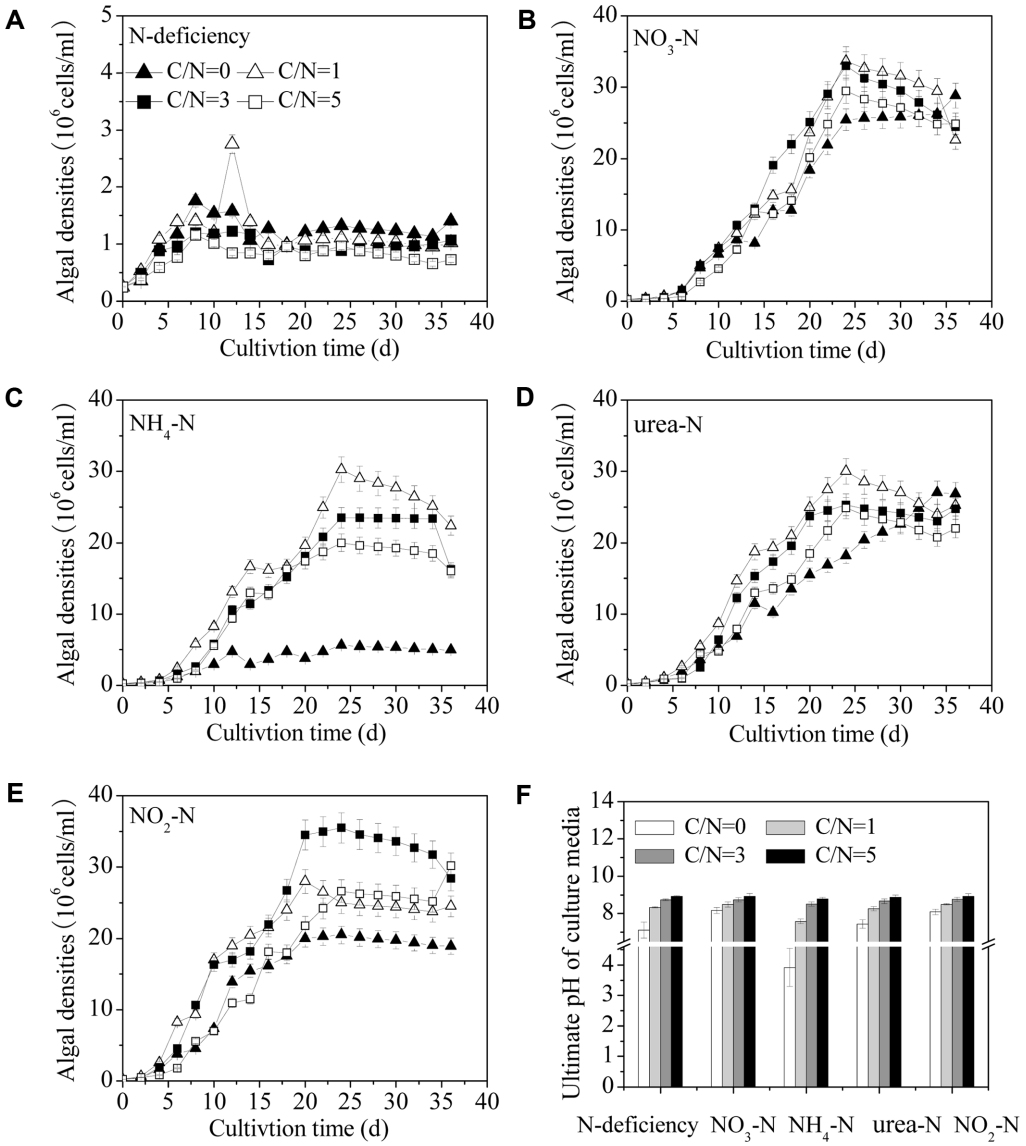

Fig. 3. Growth curves of *Chlorella* sp. HQ and ultimate pH values (45 d) in culture media with five test nitrogen sources (**A**, N-deficiency; **B**, NO_3-N; **C**, NH_4-N; **D**, urea-N; **E**, NO_2-N) under various C/N ratios (0, 1.0, 3.0, and 5.0).
(**A**–**E**) Growth curves of *Chlorella* sp. HQ. (**F**) Ultimate pH values (45 d). C/N = 0 means no carbon addition ($NaHCO_3$), which is considered as the control group.

In addition, the effect of C/N ratios on algal growth potential was nitrogen-dependent; cell densities of NH_4-N or NO_2-N treatments was influenced by C/N ratios more significantly than that of urea-N or NO_3-N treatments (Figs. 3B–3E). In particular, the effect of C/N ratios on algal growth potential of *Chlorella* sp. HQ in NH_4-N treatment was more obvious. For instance, C/N = 0 with NH_4-N treatment resulted in R_{max} of only 0.33 ± 0.00 ×10^6 cells ml^{-1} d^{-1}, which was 79–81% lower than C/N = 1.0, 3.0, and 5.0. Concomitantly, the ultimate pH of NH_4-N under C/N = 0 was only 3.92, whereas the ultimate pH under 1.0, 3.0, and 5.0 stabilized between 8.17 ± 0.16 and 8.92 ± 0.16 (Fig. 3F). As reported by our previous study, favorable pH for the growth of *Chlorella* sp. HQ was 7–9 [33]. These results indicated that NH_4-N treatments might decrease the algal growth potential of *Chlorella* sp. HQ, attributing to the low pH derived from no carbon addition (C/N = 0). By contrast, carbon addition ($NaHCO_3$) as an excellent buffer could be effective in keeping the DIC concentration high and, as a result, the pH was favorable for algal growth

potential [11]. Hence, to control the phenomenon caused by low pH from no carbon addition under NH_4-N addition condition, some measurements like carbon addition could be taken to adjust the condition of wastewater pretreatment.

The optimal C/N ratios for algal growth potential of *Chlorella* sp. HQ with NH_4-N, NO_2-N, urea-N, or NO_3-N treatments were found to be 1 and 3 (15 and 45 mg C/l). With C/N = 1.0–3.0, *Chlorella* sp. HQ cultivated in the four nitrogen sources achieved high K and r of (22.56–33.61) × 10^6 cells/ml and 0.26–0.38 d^{-1}, respectively. The R_{max} also maintained a relatively high level, ranging between 1.58 × 10^6 and 2.41 × 10^6 cells ml^{-1} d^{-1}. However, the best C/N ratio for algal growth potential was algal species-specific. For example, the appropriate control of C/N was 1:25 (570 mg C/l $NaHCO_3$, $NaNO_3$) to reach the highest growth rate of 0.103 ± 0.021 d^{-1} in *Nannochloropsis oculata* CS179 [14]. The growth rate of *Chlorella* sp. peaked at (6.91 ± 2.30) × 10^{-2} d^{-1} under C/N = 0.7 (86.4 mg C/l $NaHCO_3$, $NaNO_3$) [18]. El-Sheekh *et al*. [7] observed that the optimal C/N ratio (carbon concentration) for algal growth potential (OD_{680}) of *S. obliquus* was 0.06 (70 mg C/l $NaHCO_3$, KNO_3). The inconsistent conclusions might be caused by the different microalgae used in the studies.

As for N-deficiency, cell density under C/N = 1.0, 3.0, and 5.0 exhibited a decrease as compared with C/N = 0, but was found to be insignificant (Fig. 3A). With respect to C/N ratios (0, 1, 3, and 5), the cell density of N-deficiency treatment was significantly lower than that of nitrogen addition condition (NO_2-N, NO_3-N, NH_4-N, or urea-N) (Figs. 3A–3E). These results demonstrated that the effects of nitrogen sources on algal growth potential are more important than C/N ratios.

Effects of C/N ratios on lipid accumulation potential of *Chlorella* sp. HQ. The effects of C/N ratios on algal growth potential of *Chlorella* sp. HQ under different nitrogen sources are described above. The effects of C/N = 0, 1.0, 3.0, and 5.0 on the algal lipid accumulation potential were analyzed based on the biomass, total lipid yield and TAG yield (Fig. 4) and the total lipid and TAG contents (Fig. 5).

With NO_2-N treatment, carbon addition (C/N = 1.0, 3.0, and 5.0) enhanced the total lipid yields by 12.96–20.37% when compared with no carbon addition (C/N = 0) (Fig. 4). However, carbon addition decreased the TAG yields by 25.52–94.31% (Fig. 4). In particular, C/N = 3.0 led to a 94.31% decrease in TAG yield (2.20 ± 5.20 mg/l) ($p < 0.001$) and an over 16.67% increase in total lipid yield (78.75 ± 7.88 mg/l) ($p > 0.05$) when compared with C/N = 0 (TAG yield: 38.75 ± 5.25 mg/l; total lipid yield: 67.50 ± 6.75 mg/l) (one-way ANOVA, LSD multiple comparisons test, Table S3).

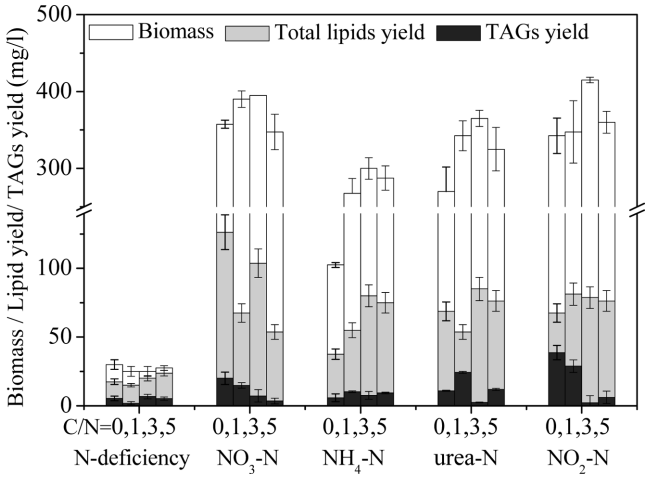

Fig. 4. Biomass, total lipid, and TAG yields at 45 days of *Chlorella* sp. HQ in five test nitrogen sources (*i.e.,* N-deficiency, NO_3-N, NH_4-N, urea-N, and NO_2-N) under different C/N ratios (0, 1.0, 3.0, and 5.0).
C/N = 0 means no carbon addition ($NaHCO_3$), which is considered as the control group.

As a result, the TAG content also dropped to the lowest 4.30 ± 4.35% (Fig. 5B). In addition, the color of the culture broths changed to yellow under low carbon addition (C/N = 0 and 1.0) and to green under high carbon addition (C/N = 3.0 and 5.0) after 36 days of cultivation (data not shown). The phenomena suggested that with high carbon additions, chlorophyll contents were higher than that with low carbon addition conditions. As reported previously, high chlorophyll content contributed to enhanced photosynthesis, which might suggest that TAG biosynthesis from GL (as discussed above) might decrease, and biosynthetic pathways towards photosynthesis might be involved with the process. Consequently, the carbon addition conditions led to a reduction in TAGs of *Chlorella* sp. HQ for NO_2-N treatments. In other words, *Chlorella* sp. HQ had high lipid-producing potential without carbon addition when NO_2-N was used as the nitrogen source (C/N = 0–5). Interestingly, the TAG contents increased more at the point of C/N = 5 compared with C/N = 3, which is likely because a high concentration of C/N ratio caused salt osmotic and toxic ionic stress for algal growth and biomass production (Fig. 4). As a result, TAGs begin to accumulate in microalgal cells under the conditions of physical or chemical stress[10].

On the other hand, these results also revealed that carbon addition promoted non-TAG lipid accumulation but decreased TAG accumulation in *Chlorella* sp. HQ when NO_2-N was used as the nitrogen source. This means that

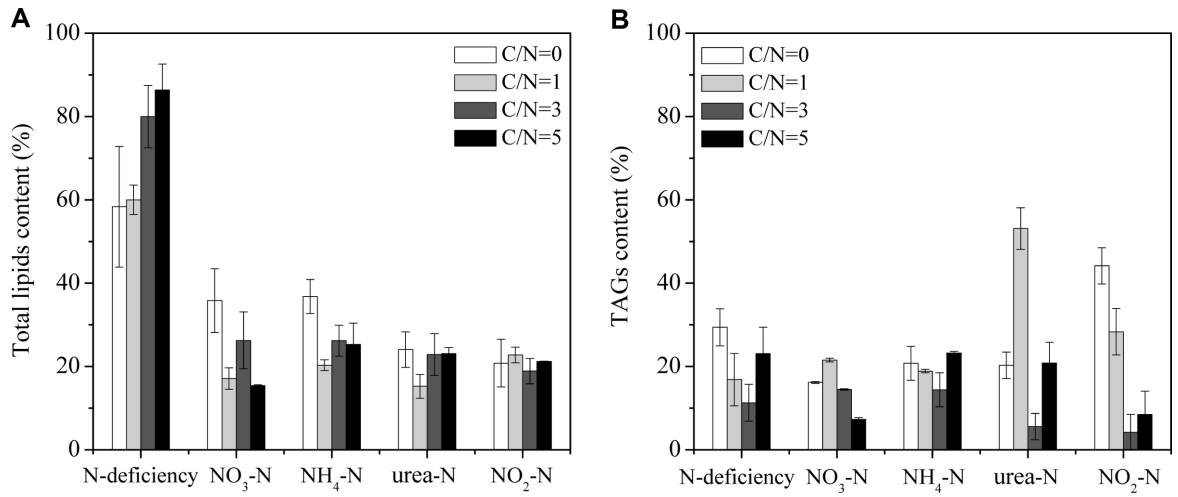

Fig. 5. Total lipid content per algal biomass (%, dry weight) and TAG content per total lipid yield (%) at 45 days of *Chlorella* sp. HQ in five test nitrogen sources (N-deficiency, NO_3-N, NH_4Cl, H_2NCONH_2, and NO_2-N) combined with three C/N ratios (0,1.0, 3.0, and 5.0).

(**A**) Total lipid content per algal biomass (%, dry weight). (**B**) TAG content per total lipid yield (%). C/N = 0 means no carbon addition ($NaHCO_3$), which is considered as the control group.

the effects of C/N ratios on algal lipid accumulation potential were dependent on lipid types. Similarly, carbon addition promoted the saturated fatty acids productivity but decreased the fatty acid methyl esters productivity of *Nannochloropsis oculata* CS 179 (when nitrate was used as the nitrogen source) [14]. For *Chlorella* sp. HQ, when NO_3-N was used as the nitrogen source, carbon addition (C/N = 1.0, 3.0, and 5.0) decreased the total lipid and TAG yields by 17.82–57.43% ($p < 0.001$, $p < 0.05$, and $p < 0.001$) and 25.86–82.67% ($p > 0.05$, $p < 0.05$, and $p < 0.001$), respectively, as compared with no carbon addition (C/N = 0) (Fig. 4) (one-way ANOVA, LSD multiple comparisons test, Table S3). Its total lipid and TAG contents with carbon addition were also lower than those without carbon addition, with the exception of TAG content under C/N = 1.0 (Figs. 5A and 5B). The TAG content (21.54 ± 0.44%) ($p < 0.001$) under C/N = 1.0 was increased by 33.21% in comparison with no carbon addition (Fig. 5B) (one-way ANOVA, LSD multiple comparisons test, Table S3), which is likely due to the decrease in total lipid yield being more significant than the decrease in TAG yield under C/N = 1.0 (46.53% decrease in total lipids yield and over 25.86% decrease in TAG yield as compared with C/N = 0). Consequently, the final lipid accumulation potential with carbon addition was still lower than that without carbon addition of *Chlorella* sp. HQ in NO_3-N treatments. Similarly, carbon addition ($NaHCO_3$, KNO_3) induced reduction in esterified fatty acids of *S. obliquus* [7]. As known, algal lipid accumulation usually occurred under adverse conditions. The algal growth potential and lipid accumulation in cells are inversely related. Both NO_3-N and carbon addition were considered as favorable conditions for algal growth potential, which might relieve the stress of *Chlorella* sp. HQ during the formation and accumulation of lipids.

Interestingly, *Chlorella* sp. HQ with urea-N treatment obtained the highest total lipid yield (85.00 ± 8.50 mg/l) but the lowest TAG yield (2.52 ± 0.24 mg/l) under middle C/N ratio (C/N = 3.0), and the lowest total lipid yield (53.75 ± 5.37 mg/l) but the highest TAG yield (24.34 ± 0.79 mg/l) under low C/N ratio (C/N = 1.0). Concurrent with NO_2-N treatments, the highest total lipid yield of 78.75 ± 7.88 mg/l (only 3.08% lower than C/N = 1.0) and the lowest TAG yield of 2.20 ± 5.21 mg/l existed under middle C/N ratio (C/N = 3.0), whereas having the lowest total lipid yield of 67.50 ± 6.75 mg/l but the highest TAG yield of 38.75 ± 5.21 mg/l was achieved under low C/N ratio (C/N = 0). In other words, there were inconsistent yields between non-TAG lipids and TAGs. According to the reports on *C. reinhardtii*, algae could convert non-TAG lipids (DAG, MAG, GL, PL) to TAGs by using enzymes (phospholipid:diacylglycerol acyltransferase) as biocatalysts [31]. During TAG synthesis, monogalactosyldiacylglycerol, as an intermediate, was hydrolyzed to release C18:1 (fatty acids) as well [12]. Considering both aspects, the inconsistent yields between non-TAG lipid yields and TAG yields hint that C/N ratios influence the conversion direction between

non-TAG lipids and TAGs, and the existing competitive pathways could be responsible for their synthesis and transformation. Previous studies have reported that non-TAG lipids included DAG, MAG, GL, PL, *etc*. [12, 31]. The accurate quantity of non-TAG lipids were studied in later work and further investigation should be done to clarify how the conversion happens and what molecules are involved in it.

For the NH_4-N treatment, although carbon addition decreased total lipid/TAG contents in cells compared with no carbon addition for *Chlorella* sp. HQ (Figs. 5A and 5B), carbon addition (C/N = 1.0, 3.0, and 5.0) increased the total lipid/TAG yields by 46.67–113.33%/28.99–74.76% compared with no carbon addition (Fig. 4). In particular, the total lipid yield under C/N = 3.0 ($p < 0.001$) and TAG yield under C/N = 1.0 ($p < 0.05$) of NH_4-N treatment reached values of 80.00 ± 8.00 mg/l and 10.64 ± 0.61 mg/l, respectively, while being 113.33% and 74.78% higher than no carbon addition, respectively (one-way ANOVA, LSD multiple comparisons test, Table S3). Consequently, the final lipid accumulation potential was enhanced with carbon addition. Similarly, the biomass and lipid productivity of *Tetraselmis suecica* and *Chlorella* sp. were enhanced with addition of sodium bicarbonate [18]. As described above, carbon addition could increase the pH value up to a favorable range for algal growth. As a result, a favorable pH value might relieve the inhibitory effect on algal growth. Meanwhile, the ultimate color of culture broths changed from yellowish-white (without carbon addition) to green (with carbon addition) (data no shown). The phenomena might also hint that photosynthesis of *Chlorella* sp. HQ with carbon addition was more effective than that without carbon addition. Therefore, the algal biomass with carbon addition was increased (Fig. 4). Consequently, carbon addition promoted the final lipid accumulation potential. Notably, the total lipid/TAG contents in cells of NH_4-N treatments without carbon addition were higher than that of carbon addition. In other words, microalgae favored lipid accumulation in cells under no carbon addition condition. It is likely due to biosynthesis pathways changing towards lipid biosynthesis rather than photosynthesis under adverse condition for algal growth (*i.e.*, no carbon addition condition). These results suggested that favorable conditions for algal growth could enhance lipid-producing potential, attributing to the enhancement in biomass; the adverse condition for algal growth potential might also improve lipid-producing potential by forcing the algal biosynthesis towards lipid accumulation in cells.

Based on the above, it is necessary to control nitrogen sources and C/N ratios correctly in order to obtain the optimal combination for algal growth and lipid accumulation potential. For algal biofuel production, no carbon addition (C/N = 0) was regarded as the best C/N ratio of *Chlorella* sp. HQ with NO_3-N or NO_2-N treatment, and C/N = 1.0 was considered as the optimal C/N ratio for NH_4-N or urea-N treatment (Figs. 4 and 5). Specifically, the highest TAG production (TAG yield: 38.75 ± 5.21 mg/l; TAG content: 44.16 ± 4.35%) was achieved under NO_2-N treatment without carbon addition (C/N = 0), which has the advantage of being converted into biodiesel directly in biofuel production. However, the optimal value of C/N ratio for lipid accumulation potential varied in microalgae. In *S. obliquus*, Shen *et al*. [22] reported that low C/N ratio of 1.6 (80 mg TOC/l glucose) was deemed the optimal C/N ratio for lipid-producing potential (total lipids content) under non-stress condition. The optimal C/N ratio was considered be 7, and under that the maximum total lipid content was more than 40% in *C. sorokiniana* [3]. Considering both aspects, it is necessary to find the optimal C/N ratio for a special microalga to accumulate lipids.

As for N-deficiency treatments, the required C/N ratios to achieve the highest total lipid yield and TAG yield were 5.0 and 3.0, respectively, which were higher than that of NH_4-N or urea-N, NO_3-N or NO_2-N treatment (Figs. 4 and 5). This is because continuous carbon influx appeared after exogenous nitrogen depletion [25]. These results indicated that the carbon addition was more important for lipid accumulation potential under no nitrogen addition condition. Carbon addition (C/N = 1.0, 3.0, and 5.0) decreased biomass but improved total lipid/TAG yields of N-deficiency as compared with no carbon addition (C/N = 0); and particularly, C/N = 5.0 increased the total lipid content of *Chlorella* sp. HQ to reach the maximum of 86.36 ± 6.26% ($p < 0.01$) (one-way ANOVA, LSD multiple comparisons test, Table S3). However, all the biomasses of N-deficiency under C/N = 0, 1.0, 3.0, and 5.0 were obviously lower than that of NO_3-N, urea-N, NO_2-N, or NH_4-N (Figs. 4 and 5).

Taken together, lipid accumulation potential (total lipid yield, TAG yield, total lipid content, and TAG content) were processed significantly in response to not only nitrogen sources (all $p < 0.001$) but also C/N ratios (all $p < 0.001$), which were further studied by using statistical analysis (two-way ANOVA analysis, Table S4). Two-way ANOVA analysis also presented significant interaction between nitrogen sources and C/N ratios (*i.e.*, C/N×N) on the total lipid yield, TAG yield, total lipid content, and TAG content of *Chlorella* sp. HQ (all $p < 0.001$). In other words, the best combination of nitrogen source and C/N

ratio might not be the combination of the best nitrogen source under one C/N ratio and the best C/N ratio under one nitrogen source, attributing to the interaction. Moreover, the optimal combination of nitrogen sources and C/N ratios for algal biofuel production was algal species-specific. For biofuel production, much needs to be done to find out the optimal culture condition for the microalgae with high lipid-producing potential. In this study, the highest TAG yield was achieved under NO_2-N treatment without carbon addition (C/N = 0), the condition of which had merit for biofuel production.

In conclusion, NO_2-N was demonstrated to be the best nitrogen source for lipid-producing potential of *Chlorella* sp. HQ. Meanwhile, NO_3-N and urea-N also contributed to algal lipid-producing potential, but not NH_4-N and N-deficiency because of the proposed inhibitory effect. Moreover, algal lipid-producing potential was related to C/N ratios (C/N = 0, 1.0, 3.0, and 5.0). With NO_2-N or NO_3-N treatment, carbon addition (C/N = 1.0, 3.0, and 5.0) enhanced algal growth but decreased lipid accumulation. As for urea-N or NH_4-N treatments, carbon addition could promote algal growth and lipid accumulation.

In summary, NO_2-N treatment without carbon addition (C/N = 0), producing the highest TAG yield (TAG yield: 38.75 ± 5.21 mg/l; TAG content: 44.16 ± 4.35%), was the best condition that had merit for biofuel production.

Acknowledgments

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