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Heterotrophy of filamentous oleaginous microalgae *Tribonema minus* for potential production of lipid and palmitoleic acid

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HIGHLIGHTS

- Heterotrophic ability of microalgae *Tribonema minus* was identified for the first time.
- Glucose and urea were the optimal sources for heterotrophic fermentation of *T. minus*.
- Highest biomass and lipid productivity of 30.8 g L^{-1} and 730 mg L^{-1} d^{-1} were obtained.
- The percentage of palmitoleic acid in fatty acid profile reached up to 50.1%.
- Effects of nutrient, pH and temperature on *T. minus* heterotrophy were investigated.

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ABSTRACT

Heterotrophic fermentation and high valuable co-product producing are thought to be effective ways to improve the economic viability and feasibility of commercial production of microalgae biofuels. This work reported the heterotrophic cultivation of *Tribonema minus* for lipid and palmitoleic acid (a novel functional fatty acid) production. Firstly, the heterotrophic ability of *T. minus* was identified for the first time with significant promotion in biomass and lipid productivity, and glucose and urea were then selected as the optimal carbon and nitrogen sources. Moreover, nutrient concentrations and culture conditions were optimized. Highest biomass and lipid productivity of 30.8 g L^{-1} and 730 mg L^{-1} d^{-1} were obtained respectively by adding 80 g L^{-1} glucose at once. In addition, 2 g L^{-1} urea, 0.8 g L^{-1} K_2HPO_4, 24 mg L^{-1} ammonium ferric citrate, initial pH of 6, and temperature of 27 °C were determined as the appropriate conditions for heterotrophic growth and lipid production.

1. Introduction

The recent decade witnessed a bloomed interest in oleaginous microalgae as a promising sustainable source for biodiesel production associated with positive impacts on environmental mitigation. However, microalgae biofuel technology remains in its infancy. One of the most critical problems is the inefficiency and high cost of the production of microalgae biomass and lipid. Microalgae can grow with CO_2 as the carbon source and sunlight as the energy supply. However, traditional photoautotrophic cultivation of microalgae is highly dependent on the climatic and seasonal conditions, and the long cultivation period, especially low biomass and lipid productivities damage the economic viability and the potential of commercial production (Wijffels and Barbosa, 2010). In comparison to photoautotrophic cultivation, heterotrophic fermentation allows microalgal cells to grow very fast, accumulate dense biomass and lipid oil within less time and the scale-up is much easier. Thus, it offers a potential pathway to produce oil for diesel production in large scale (Griffiths and Harrison, 2009; Rodolfi et al., 2009). Accumulating studies indicate that various species of microalgae can be cultured heterotrophically, however, there are only a few *Chlorella* sp. strains such as *C. protothecoides*, *C. pyrenoidosa* and *C. vulgaris*, and *Crypthecodinium* sp. strains such as *C. cohnii* demonstrated efficient fermentation capacity using organic carbon sources, especially glucose (de Swaaf et al., 2003; Xiong et al., 2008; Wei et al., 2009; Han et al., 2012; Xiao et al., 2015).

It has been suggested that microalgal strains producing valuable co-products, such as feed, pigments, chemicals or pharmaceuticals, would further improve the economic viability of microalgal biofuel production (Pienkos and Darzins, 2009; Brennan and Owende, 2010; Singh and Gu, 2010). To date, omega-3 fatty acid, eicosapentanoic acid, decosahexaenoic acid and chlorophyll have been shown to be potentially valuable co-products of microalgal

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biodiesel production (Spolaore et al., 2006; Becker, 2007; Harun et al., 2010). Palmitoleic acid (16:1), also called cis-9-Hexadecenoic acid, which is belonged to omega-7 fatty acid, has been paid increasing interest as one of novel functional fatty acid. Palmitoleic acid is an important contributor to human health and sustainable industry, and it received increasing attention in recent years (Wu et al., 2012). Immense amounts of studies released recent years indicate that palmitoleic acid shows application values in increasing insulin sensitivity, increasing cell membrane fluidity, reducing inflammation, protecting cardiovascular system, and inhibiting oncogenesis (Stefan et al., 2010; Maedler et al., 2003; Mozaffarian et al., 2010; Welters et al., 2006). Currently, soft gels containing purified palmitoleic acid have been put into market as healthcare products (http://omega-7.org/). Moreover, palmitoleic acid is thought to be better to provide low temperature property than other saturated fatty acid when it was converted into biodiesel because of its carbon chain length and unsaturated double bond number (Knothe, 2010; Cao et al., 2014). Besides, palmitoleic acid could be used to produce 1-octene which is highly consumed in the expanding production of linear low density polyethylene (Rybak et al., 2008; Nguyen et al., 2010).

Up to date, this fatty acid, with a variety of functions, is mainly obtained from sea buckthorn (*Hippophae rhamnoides* L.), which contains about 43% palmitoleic acid in its total lipid but confronts the limitation of commercialization due to its very low lipid content in buckthron pulp, low yield and poor agronomic characteristics (Wu et al., 2012; Yang and Kallio, 2001). Oils from large scale planted oil crops, such as Sunflower and Jatropha, contain less than 2% palmitoleic acid (Salas et al., 2004; Okullo et al., 2012). A number of unicellular oleaginous microalgae have been identified as sustainable sources for fatty acids production, but most of them only contains 8%–25% palmitoleic acid in fatty acid profiles (de Swaaf et al., 2003; Xiong et al., 2008; Wei et al., 2009; Han et al., 2012; Lang et al., 2011; Klok et al., 2014).

Fortunately, in our previous work, a filamentous oleaginous microalgae specie, *Tribonema minus*, with unbranched filaments composed of a single row of elongated and cylindrical cells, was identified as an alternative candidate for the production of lipid and palmitoleic acid due to its high lipid content (over 50%) in biomass and dominated component of palmitoleic acid (about 50%) in fatty acid profile (Wang et al., 2013; Guo et al., 2014; Wang et al., 2016a,b). In addition, the constructed autotrophic cultivation system has successfully demonstrated that *T. minus* has other advantages in easy harvest and resistance to the predation of grazers due to its filamentous feature (Wang et al., 2013). However, as mentioned before, on the views of feasibility of mass production of palmitoleic acid enriched lipid oil and economic viability, heterotrophic fermentation of *T. minus* with organic carbon source would be more encouraging.

In this study, the distinctive heterotrophic ability of *T. minus* was reported at first. As follows, the influences of different nutrients including the organic carbon sources, nitrogen sources and other trace elements, initial pH value and cultivation temperature on biomass density and lipid content were investigated, and finally an optimal heterotrophic condition for *T. minus* was obtained. Results have demonstrated great commercialized potential for producing both lipid oil and palmitoleic acid component by heterotrophic fermentation with filamentous microalgae *T. minus*.

2. Materials and methods

2.1. Microalgae strain

Freshwater filamentous microalgae *T. minus* was purchased from SAG culture collection, University of Gottingen, and maintained in BG11 medium. Inoculum of microalgal cells was prepared in 250 mL flask with 100 mL BG11 medium under illumination intensity of 50 μmol photons m^{-2} s^{-1}, the orbital shaking rate was 180 rpm and temperature was maintained at 27 ± 1 °C.

2.2. Experimental methods

All the cultures were carried out in 250 mL flask with 100 mL modified BG11 medium (see Table 1). The photoautotrophy of *T. minus* was carried out as control with the illumination intensity of 50 μmol photons m^{-2} s^{-1}. The heterotrophy test was carried out in silver paper covered flask to shield the irradiance at 27 ± 1 °C, and the heterotrophic medium was the modified BG11 medium with glucose of 10 g L^{-1}.

Considering the importance of carbon source and nitrogen on fermentation, four organic carbon sources including glucose, sucrose, glycerol and sodium acetate and four popular used nitrogen sources including $NaNO_3$, yeast extract, NH_4Cl and urea were added into modified BG11 medium respectively to screen the optimal organic carbon source and nitrogen source. Though peptone is an appropriate nitrogen source (Wang et al., 2017), however here it was not taken into account due to its high price. The concentration of organic carbon source was 10 g L^{-1}, and the concentration of the four nitrogen sources was $NaNO_3$ 1.5 g L^{-1}, yeast extract 2 g L^{-1}, NH_4Cl 0.89 g L^{-1} and urea 0.5 g L^{-1}, respectively, which indicated the same nitrogen level of 0.017 mol L^{-1}.

After determined glucose and urea as the optimal organic carbon source and nitrogen source (see Section 3.2), the influence of glucose concentration from 5 g L^{-1} to 80 g L^{-1} in modified BG11 medium (in which 2 g L^{-1} urea was used to take place $NaNO_3$) on the heterotrophy of *T. minus* was investigated. Different concentrations of urea from 0 g L^{-1} to 2 g L^{-1} and other nutritional conditions such as K_2HPO_4 (from 0 g L^{-1} to 0.8 g L^{-1}) and ammonium ferric citrate (from 0 mg L^{-1} to 48 mg L^{-1}) were also designed to investigate the performance of heterotrophic culture under the same glucose concentration of 20 g L^{-1} in the medium.

Moreover, the influences of initial pH value (4.0, 6.0, 7.9, 9.0 and 10.0) of medium and temperature (17, 22, 27 and 32 °C) on fermentation were also investigated.

2.3. Analytical methods

For biomass analyses, 5 mL (for autotrophy) or 2 mL (for heterotrophy) broth (V) were taken from the flasks everyday. The sample was filtered to a pre-weighted 0.45 μm GF/C filter membrane (Whatman, DW_0) and washed for three times by distilled water. Then the membrane was oven dried at 105 °C overnight and weighted (DW_1). The dry weight (DW) was calculated as $(DW_1 - DW_0)/V$ (Wang et al., 2013).

Total lipid content was determined by gravimetric analysis according to Chen et al. (2012) with some modifications. Approximately 50 mg of dried microalgal cells were extracted with methanol-chloroform (2:1, v/v) after grinding and then separated into chloroform and aqueous methanol layers by adding chloro-

Table 1
Composition of modified BG11 medium.

Modified BG11 medium			
$NaNO_3$	1500 mg L^{-1}	$ZnSO_4·7H_2O$	0.222 mg L^{-1}
K_2HPO_4	400 mg L^{-1}	$CuSO_4·5H_2O$	0.079 mg L^{-1}
$MgSO_4·7H_2O$	375 mg L^{-1}	$MnCl_2·4H_2O$	1.81 mg L^{-1}
$CaCl_2·2H_2O$	180 mg L^{-1}	$Na_2MoO_4·2H_2O$	0.39 mg L^{-1}
Na_2CO_3	100 mg L^{-1}	$Co(NO_3)_4·6H_2O$	0.0494 mg L^{-1}
Citric acid	30 mg L^{-1}	H_3BO_3	2.86 mg L^{-1}
Ammonium ferric citrate	24 mg L^{-1}	Na_2EDTA	5 mg L^{-1}

form and 1% NaCl solution to make a final volume ratio of chloroform: methanol: 1% NaCl of 1:1:0.9. The chloroform layer was evaporated to dryness by nitrogen-blowing, and the lipid content was then calculated gravimetrically.

Fatty acid profiles were determined post-conversion to fatty acid methyl esters (FAMEs) and analyzed on a Varian 450GC (Varian Inc., USA). Lipid, together with nonadecanoic acid (C19:0) added as an internal standard, was converted to FAMEs with sulfuric acid/methanol (1:50, w/v) and incubated at 85 °C for 2.5 h. Nitrogen was used as carrier gas during FAMEs analysis on 450GC, and the injector temperature was set at 280 °C with an injection volume of 2 μL under split mode of 10:1. The individual FAMEs were identified by chromatographic comparison with authentic standards (Sigma) and calculated by the way reported by Chen et al. (2012).

For glucose concentration analyses, samples taken from the flasks were filtered by 0.45 μm filter membrane, and the filtrate was collected to detect the glucose concentration by using a biosensor (SBA-40D, China). Glucose-biomass conversion efficiency, namely the cell growth yield on glucose, was calculated as $(B_1 - B_0)/(G_0 - G_1)$, where B_0 and G_0 were the initial biomass and glucose concentration, and B_1 and G_1 were the final biomass and glucose concentration, respectively.

2.4. Statistical analysis

All the experiments were performed in parallel triplicates and repeated twice for validation, and data was denoted as mean value ± SE (standard error). One-way analysis of variance (ANOVA) was used to evaluate the difference of biomass and lipid content among the treatments at confidence level of 0.05.

3. Results and discussions

3.1. Identification of heterotrophic ability of T. minus

The comparison of *T. minus* biomass in photoautotrophy and heterotrophy with 10 g L^{-1} glucose was presented in Fig. 1A. It showed that *T. minus* grow very fast by heterotrophy, and the biomass density reached 6.14 g L^{-1}at day 4, while the biomass density was only 2 g L^{-1} at day 5 and 2.5 g L^{-1} at day 6 via photoautotrophy. It demonstrated that *T. minus* could use glucose as carbon substrate for fast heterotrophic growth. After 4 days fermentation, the glucose concentration in medium was decreased from 10 g L^{-1} to 0 g L^{-1}, corresponding to the carbon/biomass conversion rate of 0.56 g g^{-1}. The changes of lipid content by autotrophy and heterotrophy were plotted in Fig. 1B. With the prolong of the cultivation, the lipid content of biomass by autotrophy gradually increased from 25% in inoculum to about 37.9% at day 6, while the lipid content of biomass by heterotrophy decreased to 10% at day 2 and then gradually increased to only about 18.8%, much lower than that by autotrophy. Low lipid content was also observed in *C. protothecoides* when heterotrophic cultivation was employed (Wei et al., 2009; Li et al., 2013, 2015). A possible reason for this phenomenon might be that the major products of biosynthesis are structural substances e.g., polysaccharides and proteins for microalgae cell construction, rather than the energy storage chemicals such as lipids in the rapid growth phase of heterotrophy with glucose. In spite of this, the lipid productivity was improved to about 200 mg L^{-1} d^{-1} by heterotrophy compared with 158 mg L^{-1} d^{-1} by phototrophy. It should be noted that there was still a lot of room to improve the biomass growth rate and lipid content if an optimized heterotrophic condition such as controlled C/N ratio of medium (Wei et al., 2009) or further lipid induction strategy was adopted.

Fatty acid profiles of *T. minus* of the both autotrophy and heterotrophy at day 6 were determined and displayed in Table 2. In contrast to the difference on biomass density and lipid content, scarcely differences were observed in fatty acid profiles between phototrophic and heterotrophic cultures. There is insignificant difference of palmitoleic acid content for both the biomass by autotrophy and heterotrophy and palmitoleic acid is the dominated fatty acid, constituting up to 50%. While oleic acid, which was usually in majority in most green algae (Griffiths et al., 2011), was only 3.0%–3.4% in *T. minus*. The second main composition of the lipid of the two cultures of *T. minus* was palmitic acid (16:0), taking a proportion of 28.0%–28.4%, and as for the rest components, proportions were slight and inappreciable. In conclusion, analysis results revealed that heterotrophic growth of *T. minus* with glucose kept the excellent performance of producing palmitoleic acid, as the phototrophic growth exhibited.

In recent years, heterotrophic fermentation of microalgae has been paid much attention in order to solve the problem of fast production of biomass. However all the reported microalgae species which could be efficiently fermented with glucose are limited to only a few unicellular microalgae species, of which *Chlorella* sp. are the most used (Xiong et al., 2008; Han et al., 2012; Li et al., 2015; Perez-Garcia et al., 2011). This reported *T. minus* is the first one of filamentous microalgae specie. And due to the functional usage of palmitoleic acid as healthcare product, the identification of heterotrophic growth ability of filamentous oleaginous *T. minus* is very likely to lead a profitable bio-refinery technology for both rare omega-7 fatty acid and biodiesel production.

3.2. Screening of organic carbon source and nitrogen source

Different organic carbon sources and nitrogenous substances were used to evaluate the optimum carbon and nitrogen sources for heterotrophic growth of *T. minus* (Fig. 2). From the results shown in Fig. 2A, glycerol and sodium acetate had a slight but of no worth promotion on *T. minus* growth, and sucrose could barely offer any biomass accumulation, as well as xylose, galactose, mannose, L-rhamnose, maltose and starch investigated in this study but not shown here. Zhang et al. (2016) reported that sodium acetate could be utilized as organic carbon source for *Haematococcus pluvialis* heterotrophy. Sucrose is another optional organic carbon substrate for microalgae heterotrophy, but it needs to be hydrolyzed prior to use (Wang et al., 2016c). Comparably, glucose is the most commonly used organic carbon source for heterotrophic fermentation of microalgae because of its excellent performance, cheap and easy to get compared to any other substrates (Perez-Garcia et al., 2011). The efficient use of glucose by *T. minus* for heterotrophy provides the feasibility of fermentation in industrial scale.

Nitrates were usually employed as nitrogen source for microalgae phototrophy while other nitrogenous substances such as yeast extracts and urea were preferable in heterotrophic culture of microalgae (Xiong et al., 2008; Li et al., 2015). The heterotrophic growth of *T. minus* with four types of nitrogen sources at the same nitrogen level in medium was presented in Fig. 2B. There was a significant influence of the nitrogen source on *T. minus* heterotrophic growth while different nitrogen sources were employed. Except ammonium chloride, *T. minus* had a well growth with the three other nitrogen sources. Especially for the urea, the biomass density was observed about 7.1 g L^{-1} in only 2 days cultivation, which was much higher than that of sodium nitrate and yeast extract ($p < 0.05$). There were little differences among the lipid contents of *T. minus* with various nitrogen sources except ammonium chloride (Fig. 2C). With ammonium chloride as nitrogen source, the lipid content increased to about 34.2% of dry weight after 6 days fermentation, which was higher than that of sodium nitrate

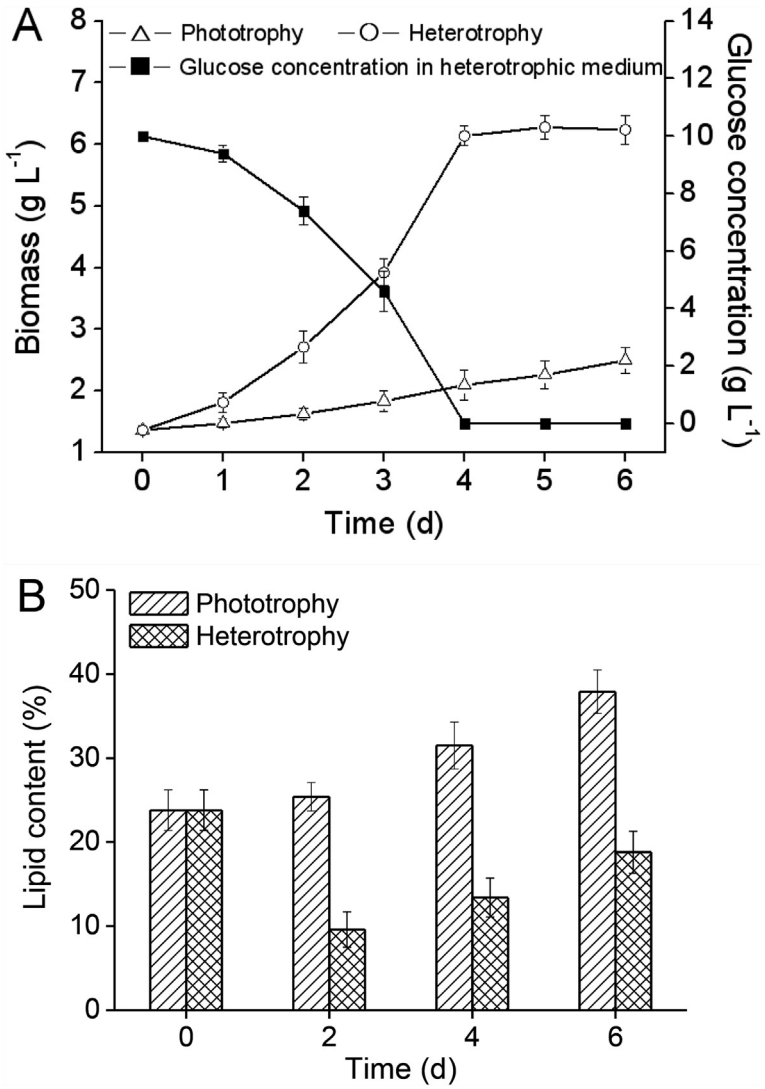

Fig. 1. Growth (A) and lipid accumulation (B) of *T. minus* under phototrophic and heterotrophic culture conditions.

Table 2
Fatty acid profiles of *T. minus* under photoautotrophic and heterotrophic culture conditions.

Fatty acid composition	Photoautotrophy	Heterotrophy
14:0	6.9	8.7
16:0	28.4	28.0
16:1	50.7	50.1
18:0	1.0	1.7
18:1	3.0	3.4
18:2	1.0	0.3
20:4	3.0	2.4
20:5	3.0	2.8
Others	3.0	2.6

(18.8% of DW), yeast extract (25.5% of DW) and urea (20.4% of DW). However as shown in Fig. 2B, its biomass density was much lower than the other nitrogen sources, so its lipid productivity was very poor. The reason may be attributed to the serious inhibition or stress of ammonium nitrogen by ammonium chloride on algal cell. The lipid productivity reached the highest of 255 mg L^{-1} d^{-1} when urea was used as nitrogen source, indicating that urea was the most appropriate nitrogen source for heterotrophic culture of *T. minus*.

3.3. Influence of medium nutrient composition on T. minus heterotrophy

The influences of medium nutrient composition including glucose, urea, K_2HPO_4 and ammonium ferric citrate on heterotrophic growth of *T. minus* were investigated and the results were shown in Fig. 3. With the increase of glucose concentration from 5 g L^{-1} to 80 g L^{-1}, the final biomass density at day 6 were 4.9 g L^{-1}, 9.9 g L^{-1}, 19.9 g L^{-1}, 25.6 g L^{-1} and 30.8 g L^{-1} respectively (Fig. 3A). The changes of residual glucose concentration in medium were shown in Fig. 3B. With extension of fermentation, glucose was consumed for the construction of *T. minus* cells. Comparing Fig. 3A and B, when the initial glucose concentration was 5, 10, 20 and 40 g L^{-1}, all the glucose was exhausted at day 2, day 3, day 4 and day 5, respectively, at which the biomass density reached the highest. However, with initial concentration of 80 g L^{-1} glucose, the highest biomass density of 30.8 g L^{-1} was reached at day 5 while 37 g L^{-1} glucose was residual. Glucose-biomass conversion efficiency, namely the cell growth yield on glucose, is one of important technical indicators for fermentation. For the five initial glucose concentration gradients, the glucose-biomass conversion efficiency was calculated as 0.66, 0.73, 0.87, 0.58 and 0.62 g biomass g^{-1} glucose, respectively. Shi et al.

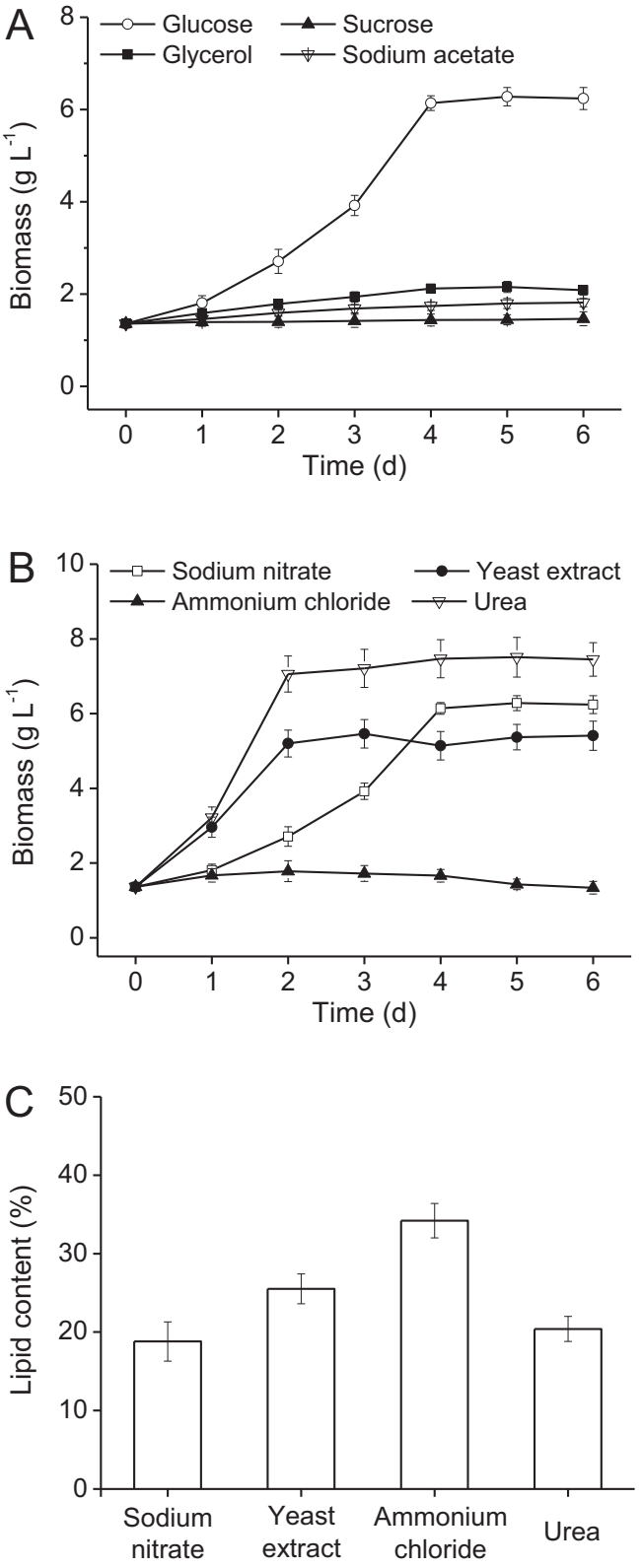

Fig. 2. Heterotrophic growth of *T. minus* with different carbon sources (A) and nitrogen sources (B), and lipid accumulation (C) of *T. minus* with different nitrogen sources.

(1999) reported that, in heterotrophic culture of *C. protothecoides* CS-41, the maximum biomass increased from 4.9 g L^{-1} to 31.2 g L^{-1} with an increase in initial glucose concentration from 10 g L^{-1} to 80 g L^{-1}, which was similar to this work. However, the glucose-biomass conversion efficiency of *C. protothecoides* was only 0.39–0.46 g g^{-1}, which was much lower than that of *T. minus*. Another research released by Jiang and Chen (2000) revealed that *C. cohnii* could also grow well heterotrophically over a range of glucose concentrations (5–40 g L^{-1}) and obtained a growth yield on glucose of 0.6 g g^{-1} at initial glucose concentration of 20 g L^{-1} in medium. Anyway, appropriate lower initial glucose concentration, such as 20 g L^{-1} would be better on the view of substrate utilization efficiency.

The biomass production was significantly improved when the concentration of urea was increased from 0 to 0.25 g L^{-1} (Fig. 3C) ($p < 0.05$). By further addition of urea, the fermentation was dramatically facilitated, for instance, the culture time to reach the highest biomass density was shortened to only 3 days when the urea concentration was 2 g L^{-1}, which was half of that when the urea concentration was 0.5 g L^{-1}. As for K_2HPO_4 and ammonium ferric citrate, the analogous effect on heterotrophic growth of *T. minus* was observed and shown in Fig. 3D and E, respectively. Both of the growth and final biomass density were enhanced by increasing K_2HPO_4 and ammonium ferric citrate concentrations, but it should be noted that excess nutrients may induce no more facilitation and cause the nutrients costly for heterotrophic growth of *T. minus*.

The lipid contents of heterotrophic *T. minus* under different nutrients concentrations were determined and presented in Fig. 3F, which revealed diverse effects among different nutrients and different concentrations on lipid content. High glucose concentrations were conducive to the accumulation of lipid, while the lower groups leaded to a significant reduction of lipid content, which could be speculated as the result of carbon insufficiency after rapid growth. On the contrary, the deprivation of nitrogen resulted in a promotion of lipid accumulation. Such phenomenon has been widely validated by most reports of microalgae by both autotrophy and heterotrophy. For example, under the nitrogen starvation, the productivity of fatty acids of heterotrophic *C. vulgaris* was determined as three times greater than that under nitrogen sufficient conditions (Shen et al., 2016). However, for this *T. minus*, in our previous work of *T. minus* autotrophy, nitrogen starvation resulted in lower lipid content (Guo et al., 2014). The opposite effect of nitrogen starvation on lipid accumulation between phototrophic and heterotrophic cultivation methods could be a complicated process, and yet it needs to be further studied intensively. Except the free K_2HPO_4 experiment, the lipid content was observed a little increase when the concentration of K_2HPO_4 in medium increased from 0.05 g $L^{-1}$to 0.8 g L^{-1}. The concentration of ammonium ferric citrate seems have insignificant influence on the lipid content.

Overall, the lipid content of heterotrophic *T. minus* under various nutrients conditions was only in the range from 11% to 31%, which was much lower than that of phototrophic culture, 50–60% (Wang et al., 2013), but considering the biomass density, the highest lipid productivity obtained under the condition of 80 g L^{-1} glucose was 730 mg L^{-1} d^{-1}, which is almost 5 folds compared with autotrophy, indicating heterotrophy could enormously improve the efficiency of lipid production. If further optimization such as C/N controlling and fed-batch were adopted, the potential of heterotrophy for lipid production is worth looking forward to.

3.4. Effect of pH and temperature influence on T. minus heterotrophy

Effects of initial pH value of medium on heterotrophic growth of *T. minus* were evaluated and shown in Fig. 4A. It shown that mild pH environments ranged from 6 to 9 was adaptive for heterotrophy of *T. minus*, but at weakly alkaline, there was a little longer of the lag phase ($p < 0.05$).While the final algal density and lipid content at pH 6.0 and 7.9 approached the same level after three days fermentation, it indicated that the medium close to neutral had

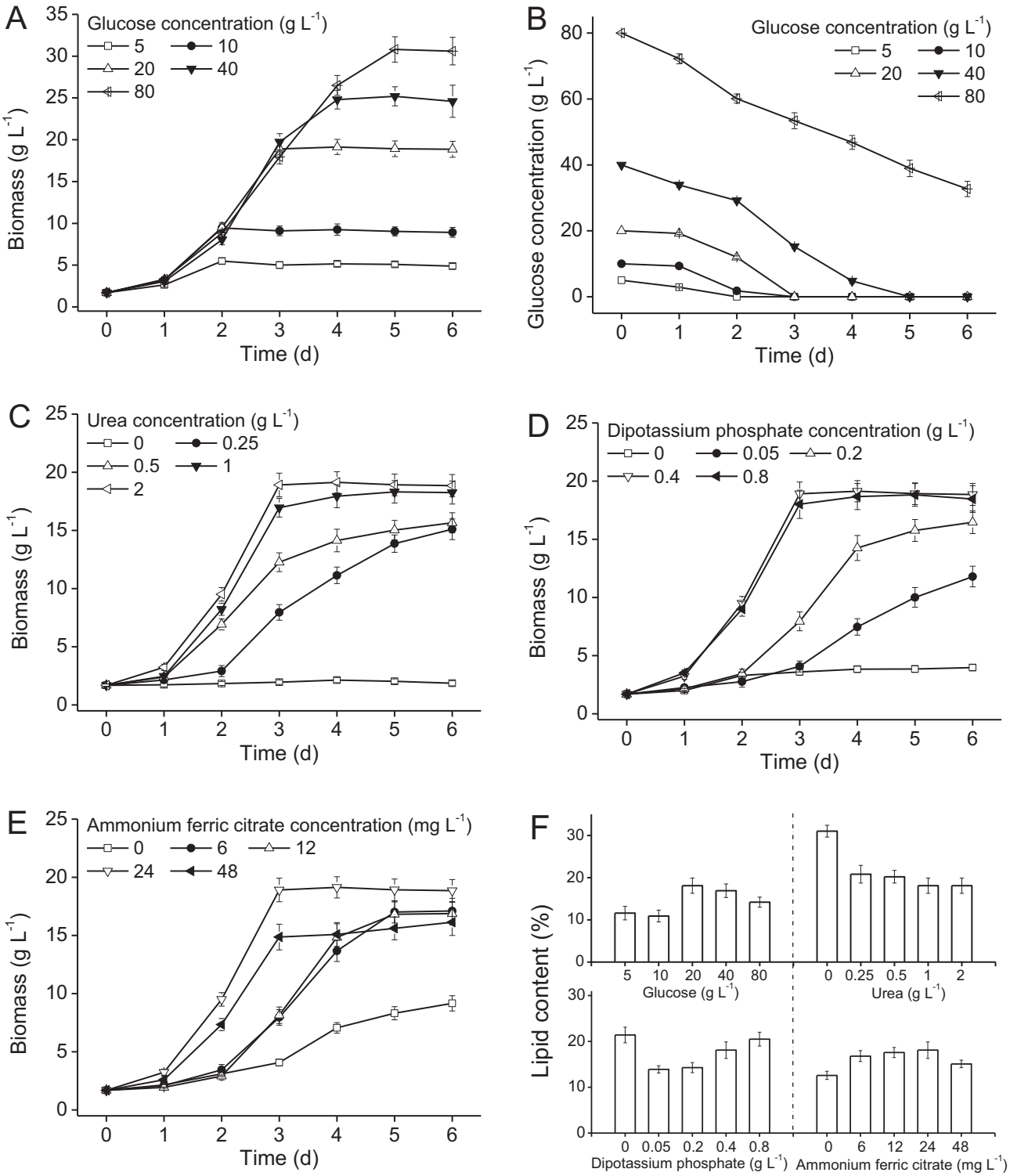

Fig. 3. Effects of different nutrient concentrations on growth and lipid accumulation of *T. minus*: effects of different glucose (A), urea (C), dipotassium phosphate (D) and ammonium ferric citrate (E) concentrations on growth; time courses for glucose concentration (B) in medium under different initial glucose concentration conditions; effects of different nutrient concentrations on lipid accumulation (F).

insignificant influence on fermentation. However the heterotrophic growth was seriously inhibited when the initial pH value was too high to 10.0 or too low to 4.0 ($p < 0.05$). Results from Shi et al. (2006), Jiang and Chen (2000) also suggested that the pH values of 6.0 and 7.2, a closely neutral condition of medium, were optimal for heterotrophic culture of *C. protothecoides* and *C. cohnii* respectively in order to obtain the highest biomass. A slight rise of lipid content when initial pH of medium was increased from 7.9 to 10.0 is reasonably contributed to the alkaline stress.

The effect of temperature on *T .minus* heterotrophic growth was shown in Fig. 4B., the growth of *T. minus* was notably expedited when the temperature was raised from 17 °C to 27 °C, and the culture time to reach the highest biomass was shortened by 2 days (Fig. 4B) ($p < 0.05$). Shi et al. (2006) investigated the heterotrophic

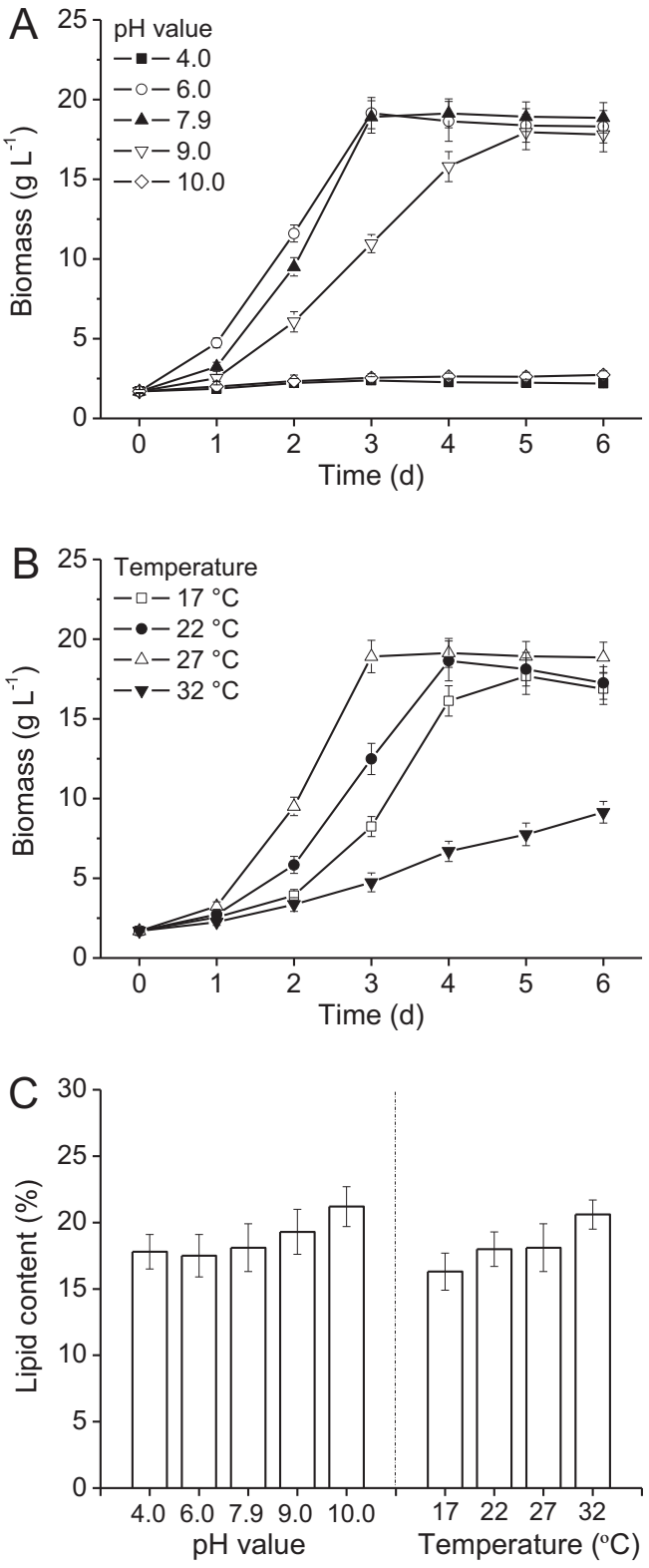

Fig. 4. Effects of different culture conditions on growth and lipid accumulation of *T. minus*: effects of initial pH value (A) and temperature (B) on growth; effects of different culture conditions on lipid accumulation (C).

growth of *Chlorella* and suggested 28 °C around was the most appropriate temperature. There was, whereas, a dramatic reduction of growth rate when the fermentation temperature was raised to 32 °C. This temperature region has a little difference to that by autotrophy. Wang et al. (2016b) reported that *T. minus* showed an increasing promotion in biomass by the raising of temperature from 5 °C to 35 °C. A possible reason for this was that the activity of enzymes, especially those related to glucose metabolism, was restrained by the high temperature in heterotrophic conditions.

Inconspicuous differences were observed in lipid content between different conditions of pH value and temperature (Fig. 4C), it indicated that both high pH value and high temperature were slightly beneficial to the lipid accumulation of heterotrophic *T. minus*, but resulted in a reduction in lipid productivity due to their inhibition on growth. It seemed that in the long run, two-step fermentation strategy, that is, appropriate low pH value to 6.0 and temperature to 27 °C for biomass fast growth in first step and then a little higher pH value to 10.0 and temperature to 32 °C for the promotion of lipid accumulation in the second step, may be an effective option of *T. minus* heterotrophy for lipid and palmitoleic acid production.

4. Conclusions

This work first reported the ability of heterotrophy of *T. minus* with glucose. Glucose and urea were the most appropriate carbon and nitrogen source to boost the fermentation. Highest biomass and lipid productivity of 30.8 g L^{-1} and 730 mg L^{-1} d^{-1} were obtained by adding 80 g L^{-1} glucose at once while the highest glucose-biomass conversion efficiency of 0.87 g g^{-1} was obtained at initial glucose concentration of 20 g L^{-1}. Culture conditions for heterotrophy of *T. minus* were optimized. Results provided a promising way of heterotrophic fermentation with *T. minus* for biomass and lipid, especially palmitoleic acid production.

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