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Full Length Research Paper

The characteristics of biomass production, lipid accumulation and chlorophyll biosynthesis of *Chlorella vulgaris* under mixotrophic cultivation

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The main objective of this study was to investigate the behaviors of *Chlorella vulgaris* for biomass production, lipid accumulation and chlorophyll biosynthesis under mixotrophic cultivation. The obtained results show that mixotrophism might be a competitive pattern for the culture of *C. vulgaris* on a large scale based on the achieved maximum biomass and volumetric productivities of lipid and chlorophyll. Glucose was the optimal carbon source for mixotrophic cultivation of *C. vulgaris* and the effects of glucose content on the alga growth under mixotrophic conditions were considerable because lower glucose content (1 g/l) promoted the production of biomass and photosynthetic pigments; higher glucose contents (≥ 5 g/l) increased the biomass and lipid accumulation but inhibited the chlorophyll biosynthesis. The microalga could not grow well without pH control when ammonium and organic nitrogen were the sole nitrogen sources in the mixotrophic cultures because of the remarkable drop in pH value, while the critical urea concentration was observed at 0.50 g/l. It was concluded that mixotrophic cultivation of *C. vulgaris* is a feasible approach for lipid accumulation and chlorophyll biosynthesis that are dependent on the enhancement of biomass content and volumetric productivity.

Key words: *Chlorella vulgaris*, mixotrophic cultivation, biomass production, lipid accumulation, chlorophyll biosynthesis.

INTRODUCTION

As a promising source for the production of biodiesel and natural pigment, microalgae have drawn more and more attention of researcher because they possess high growth rate and provide lipids fraction for biofuel production; rapidly increases the biomass production and the productivity of lipid and other cellular composition and decreases the cost of biodiesel production become essential (Song et al., 2008). The photoautotrophic mechanism in microalgae cells can convert atmospheric CO_2 into biomass, protein and lipid, as well as other biologically active substances; one of them is chlorophyll (Chisti, 2007; Spolaore et al., 2006). Chlorophyll provides a chelating agent activity which can be used in ointment, food, treatment for pharmaceutical benefits especially liver recovery and ulcer treatment (Humphrey, 2004).

Many algal organisms are capable of using either metabolism process (autotrophic or heterotrophic) for growth, meaning that they are able to photosynthesize as well as ingest prey or organic materials (Zhang et al., 1999). The ability of mixotrophism to process organic substrates means that cell growth is not strictly dependent on photosynthesis. Therefore, light energy is not an absolutely limiting factor for growth and light or organic carbon substrates can support the alga growth, hence, there is less biomass loss during the dark phase (Andrade and Costa, 2007). Chojnacka and Noworyta (2004) compared *Spirulina* sp. growth in photoautotrophic, heterotrophic and mixotrophic cultures. They found that mixotrophic cultures reduced photoinhibition and

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improved growth rates over both autotrophic and heterotrophic cultures. These features infer that mixotrophism can be an ideal nutritional mode for the microalgae biofuels production and functional pigments biosynthesis.

In this paper, the characteristics of biomass production, lipid accumulation and chlorophyll biosynthesis of *Chlorella vulgaris* under mixotrophic cultivation were investigated. First, we revealed the effects of nutritional modes on the cell growth, lipid production and chlorophyll accumulation. Subsequently, the influence of carbon sources, glucose content, nitrogen sources and urea content on the above behaviors of *C. vulgaris* were examined.

MATERIALS AND METHODS

Microalgae and growth conditions

C. vulgaris was purchased from the Culture Collection of Algae, Institute of Hydrobiology, Chinese Academy of Sciences, and was grown on modified soil extract medium (SEM) which consisted of (per litre): 0.25 g $NaNO_3$; 0.175 g KH_2PO_4; 0.075 g K_2HPO_4; 0.075 g $MgSO_4·7H_2O$; 0.025 g NaCl; 0.025 g $CaCl_2·2H_2O$; 5 mg $FeCl_3$; 0.287 mg $ZnSO_4·7H_2O$; 0.169 mg $MnSO_4·H_2O$; 0.061 mg H_3BO_3; 2.5 µg $CuSO_4·5H_2O$; and 1.24 µg $(Na)_6Mo_7O_{24}·7H_2O$. The pH was adjusted to 7.2 prior to autoclaving at 120℃ for 20 min. All cultures were maintained at 25℃ in 250 ml flasks containing 100 ml culture under illumination at 2500 lux with 12 h light, 12 h dark (except heterotrophic group) and shaken at 120 rpm on an orbital shaker.

Cultures were harvested on day six.

Experimental design

In the experiment of nutritional modes, autotrophic group was cultured in SEM; 10 g/l glucose was added in mixotrophic and heterotrophic group, respectively. The heterotrophic group was cultured in dark condition. In the groups of carbon sources, 1 g/l different carbon sources including sodium bicarbonate, sodium acetate, glucose, sucrose and glycerol were added in SEM, respectively, and the control was cultured in SEM. For the tests of glucose content, different content of glucose (1 to 20 g/l) was supplied in the medium and cultured under illuminated condition. In the trials of nitrogen sources, the nitrogen in SEM was replaced with potassium nitrate, urea, ammonium sulfate, ammonium nitrate, peptone and beef extract at the content of 0.5 g/l, respectively, and 10 g/l glucose was supplemented in each culture. For urea content, the nitrogen in SEM was replaced with urea and the content ranging from 0 to 1.0 g/l, as well as 10 g/l glucose was added in each culture. During the cultivation, the pH values in the medium were measured with Orion 868 pH meter.

Determination of biomass content and productivity

Algal growth curves and biomass concentrations were determined by measuring the absorbance at 660 nm and dry cell weight, respectively. Cells were centrifuged at 5000 rpm for 10 min, rinsed twice with distilled water and dried at 70℃ for 24 h to give the dry cell weight (g/l).

At the end of each run, specific growth rate (μ, day^{-1}) of *C. vulgaris* at the exponential phase was calculated according to the equation $\mu = (\ln X_t - \ln X_0)/(t_x - t_0)$, the biomass content (g/l) was recorded and the productivity (P, g/l/day) was calculated from the equation $P = (X_t - X_0)/(t_x - t_0)$, where X_t and X_0 are the dry cell weight concentration (g/l) at time t_x and t_0, respectively (Andrade and Costa, 2007).

Lipid extraction and determination

Cells were harvested by centrifugation, washed with distilled water, and then dried by a freeze dryer. The dry biomass was homogenised in mortar and extracted with *n*-hexane for 30 min and centrifuged. The extraction process was repeated three times and supernatant was transferred to pre-weighed glass vial and evaporated on rotary evaporator, the alga lipid was recovered and dried at 70℃ completely. The weight of glass vial containing oil was measured gravimetrically and the lipid concentration was expressed as dry weight percentage (Dayananda et al., 2005; Miao and Wu, 2006). Meanwhile, the productivity of lipid (P, mg/l/day) was calculated.

Chlorophyll extraction and determination

10 ml algal cultures were centrifuged at 5000 × g for 10 min, rinsed twice with distilled water and the pellet was extracted with 10 ml 90% (v/v) ethanol two times until the algal faded in 4℃ refrigerator, followed by centrifugation at 5000 × g for 10 min and the supernatant was used for chlorophyll determination. The contents of Chl a, Chl b and total Chl a+b in the algal cells were determined by UV-VIS spectrometer (Lichtenthaler, 1987) and the productivity of chlorophyll (P, mg/l/day) was calculated.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) from three independent parallel experiments. The analysis of variance was performed by ANOVA and significant differences among the means of samples were analyzed by Tukey's test with a 95% confidence level.

RESULTS AND DISCUSSION

Effect of different nutritional modes on biomass production, lipid accumulation and chlorophyll biosynthesis of *C. vulgaris*

For biomass production of microalgae, many cultivation modes, such as open pond, raceway and heterotrophic fermenter, have been established (Borowitzka, 1999; Miao and Wu, 2006). Mixotrophic growth offers a possibility of greatly increasing microalgal cell concentration and volumetric productivity in batch systems (Chojnacka and Noworyta, 2004). Cultivation modes affected the growth rates and cellular compositions of *C. vulgaris* (Figure 1a). Compared with the control photoautotrophic group, the cultures under mixotrophic and heterotrophic grew more quickly, and reached stationary phase ahead of the control. Especially, the mixotrophic group displayed its obvious predominance in the stationary phase.

As shown in Figure 1b, the pH values in

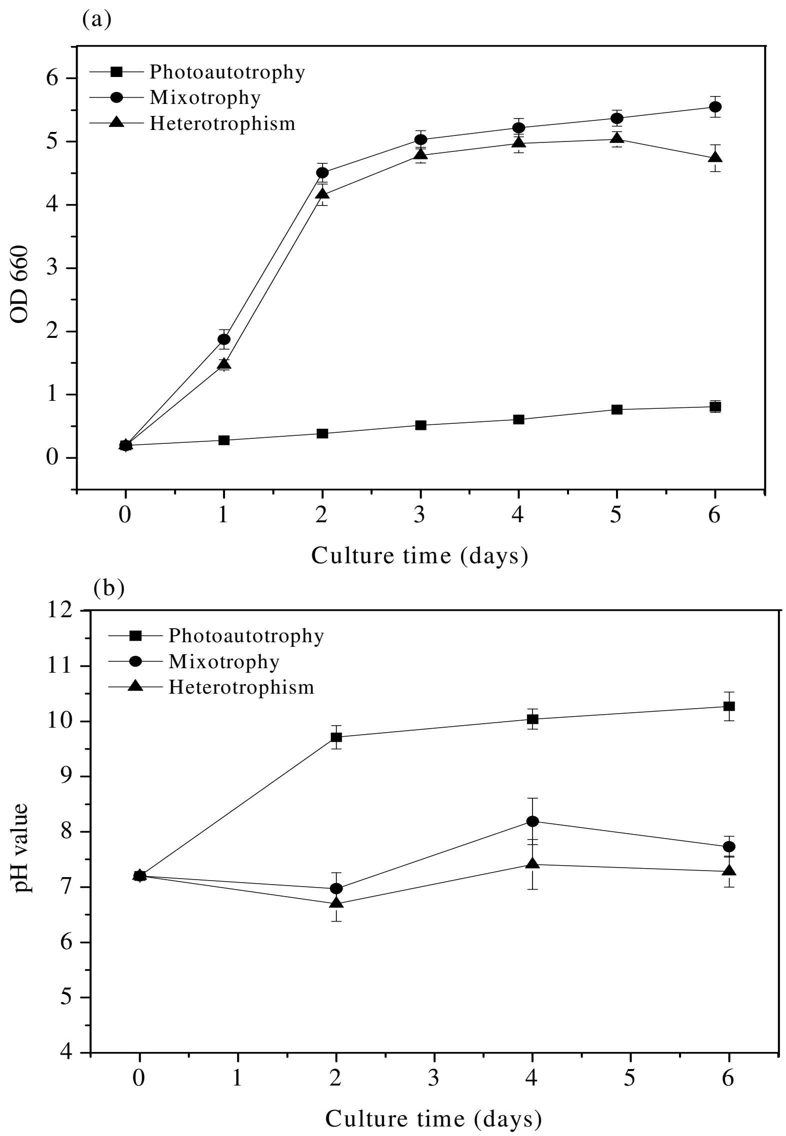

Figure 1. Effect of different nutritional modes on the growth of *C. vulgaris* (A) and pH value (B) in the culture medium.

photoautotrophic group increased with the culture time and exceeded 10 at the end of cultivation. The pH values in mixotrophic and heterotrophic groups fluctuated around 7. The differences resulted from the assimilation and release of carbon dioxide during photosynthesis and respiration, respectively. Moreover, organic acids metabolism during respiration can also decrease the pH values in cultures (Yu et al., 2000).

The algal cells specific growth rates, biomass concentrations and biochemical compositions were significantly influenced by the nutritional modes (Table 1). The biomass contents of mixotrophic and heterotrophic cultures showed a 7.31 and 6.24-fold increase over that in the photoautotrophic, respectively. The maximum specific growth rate (1.08 day^{-1}), biomass productivity (0.35 g/l/day), lipid content (12.64%) and lipid productivity (44.68 mg/l/day) were obtained under the mixotrophic cultivation, which was higher than the photoautotrophic and heterotrophic groups. Although, the chlorophyll content in the algal cells cultured under photoautotrophy gave the highest value, maximum chlorophyll productivity was also achieved in the group of mixotrophy because of its maximum biomass content.

It was found that in the growth of *Spirulina* sp. there are three metabolic possibilities of culture: autotrophic, heterotrophic and mixotrophic. In mixotrophic growth there are two distinctive processes within the cell; photosynthesis and aerobic respiration. The former is influenced by light intensity and the latter is related to the organic substrate concentration (glucose) (Chojnacka and Noworyta, 2004). The ATP formed from the photochemical reactions should accelerate the anabolism

Table 1. The biomass production, lipid accumulation and chlorophyll biosynthesis of *C. vulgaris* under different nutritional modes.

Nutritional mode	Photoautotrophy	Mixotrophism	Heterotrophism
Biomass content (g/l)	0.29 ± 0.03[a]	2.12 ± 0.10[c]	1.81 ± 0.08[b]
Specific growth rate (μ, day^{-1})	0.32 ± 0.02[a]	1.08 ± 0.07[b]	1.07 ± 0.06[b]
Biomass productivity (g/l/day)	0.05 ± 0.004[a]	0.35 ± 0.001 [c]	0.30 ± 0.014[b]
Lipid content (mass %)	6.72 ± 0.69[a]	12.64 ± 1.32[b]	11.27 ± 0.91[b]
Lipid productivity (mg/l/day)	3.31 ± 0.62[a]	44.68 ± 4.98[c]	34.00 ± 4.22[b]
Chlorophyll content (mg/g)	27.99 ± 1.4 [c]	6.32 ± 0.30[b]	3.31 ± 0.17[a]
Chlorophyll productivity (mg/l/day)	1.38 ± 0.18[b]	2.23 ± 0.12[c]	1.00 ± 0.04[a]

Values are mean ± S.D., *N* = 3; mean values in the same line with different letters in the superscript are significantly different ($p < 0.05$).

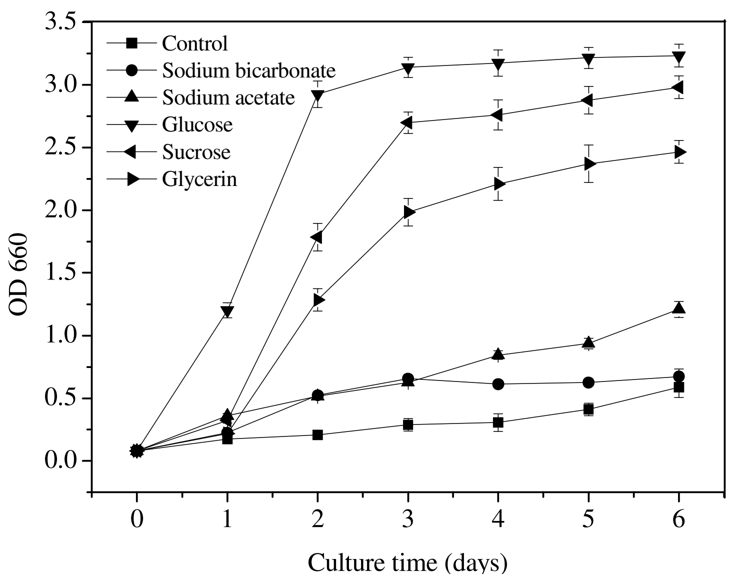

Figure 2. Effect of different carbon sources on the growth of *C. vulgaris.*

from glucose in the mixotrophic culture of *Euglena gracilis* and this should be a reason for increased growth in the culture (Yamane et al., 2001). Moreover, good production of chlorophyll (39.4 mg/l) and carotenoids (13.8 mg/l) were attained in the mixotrophic culture of *E. gracilis*, giving the highest fermenter productivity with respect to biomass as well as chlorophyll and carotenoids.

Jiménez et al. (2009) reported that *C. protothecoides* can grow under photoautotrophic, mixotrophic and heterotrophic conditions. The highest biomass production and lipid accumulation was obtained under heterotrophy; the total lipid content in cells reached a value around 40%, overcoming the data obtained in photoautotrophic mode (eight folds). Similar results were observed in our work. In mixotrophic and heterotrophic culture, the lipid content was much higher than that in the autotrophic culture (1.74 to 1.88 times), whereas, the cellular chlorophyll content was much lower than that in the autotrophic culture. The mixotrophic cultures experienced an increase in lipids and photosynthetic pigments productivities that are dependent on the increase in biomass content.

Effect of carbon sources on biomass production, lipid accumulation and chlorophyll biosynthesis of *C. vulgaris*

The obtained results show that *C. vulgaris* assimilated and grew in the presence of inorganic and organic substrates, that is, sodium bicarbonate, sodium acetate, glucose, sucrose and glycerol, in the light (mixotrophic growth) (Figure 2). Compared with the autotrophic control, the growth rates of the cultures supplied different carbon sources enhanced markedly. Particularly, the effects of organic substrates on the growth rates of *C. vulgaris* were higher than the inorganic carbon sources. The cultures added glucose, sucrose and glycerol at 1 g/l content, respectively, reached their stationary phases at three days. The growth curve indicated that glucose is the optimal organic carbon source for mixotrophic cultivation of *C. vulgaris*.

As shown in Table 2, the effects of carbon sources on biomass production, lipid accumulation and chlorophyll biosynthesis of *C. vulgaris* were significant. After six days

Table 2. Effect of carbon sources on biomass production, lipid accumulation and chlorophyll biosynthesis of *C. vulgaris.*

Carbon sources (1 g/l)	Control	Sodium bicarbonate	Sodium acetate	Glucose	Sucrose	Glycerin
Biomass content (g/l)	0.21 ± 0.03^a	0.24 ± 0.02^a	0.45 ± 0.02^b	1.23 ± 0.02^e	1.13 ± 0.02^d	0.93 ± 0.04^c
Specific growth rate (μ, day^{-1})	0.43 ± 0.02^a	0.70 ± 0.03^b	0.69 ± 0.02^b	1.22 ± 0.03^d	1.17 ± 0.02^d	1.07 ± 0.06^c
Biomass productivity (g/l/day)	0.04 ± 0.005^a	0.04 ± 0.003^a	0.07 ± 0.004^b	0.20 ± 0.003^e	0.19 ± 0.003^d	0.16 ± 0.07^c
Lipid content (mass %)	7.51 ± 0.31^a	7.79 ± 0.31^a	8.13 ± 0.22^a	8.45 ± 0.81^a	7.94 ± 0.35^a	7.26 ± 0.81^a
Lipid productivity (mg/l/day)	2.62 ± 0.28^a	3.15 ± 0.32^a	6.08 ± 0.41^b	17.30 ± 1.89^d	14.96 ± 0.78^d	11.28 ± 1.12^c
Chlorophyll content (mg/g)	10.63 ± 0.54^a	21.77 ± 1.36^c	22.71 ± 0.36^c	16.85 ± 0.05^b	10.47 ± 0.92^a	8.54 ± 0.48^a
Chlorophyll productivity (mg/l/day)	0.37 ± 0.05^a	0.88 ± 0.09^b	1.70 ± 0.11^d	3.45 ± 0.09^e	1.97 ± 0.21^d	1.33 ± 0.06 ^{c}

Values are mean ± S.D., *N* = 3; mean values in the same line with different letters in the superscript are significantly different ($p < 0.05$).

cultivation, the SEM with 1 g/l glucose obtained the maximum biomass concentration of 1.23 g/l, specific growth rate of 1.22 day^{-1} and biomass productivity of 0.20 g/l/day, which was higher than those of the control group of 5.86, 2.84 and 6.67-fold, respectively. Biomass productivities of the trials supplied glucose, sucrose and glycerol was enhanced notably due to their high cell density.

For lipid contents, ranging from 7.51 to 8.45% (dry weight), no statistically significant differences between control mean and others were observed. The volumetric lipid productivities of the cultures, however, which was supplied organic substrates, that is, glucose, sucrose and glycerol was enhanced notably due to their high cell density. The highest lipid productivity of 17.30 mg/l/day was achieved in the culture with 1 g/l glucose in SEM, which exceeded the control 6.60-fold.

The chlorophyll contents of the alga cells varied with the different substrates. The effects of sodium bicarbonate and acetate on the biomass production of the algal cells were weaker than the organic substrates. However, the chlorophyll biosynthesis was promoted by the inorganic carbon sources. The maximum chlorophyll content of 22.71 mg/g was achieved in the culture supplied sodium acetate, which was higher than the control 2.14-fold. The information from Tables 1 and 2 suggest that the concentration of glucose in the medium influenced the photosynthesis and pigment biosynthesis.

The ability of obligate photoautrophy microalgae to grow mixotrophycally (or photoheterotrophically) is a phenomenon which appears to exist in a number of genera and species distributed throughout the major taxonomic divisions (Ukeles and Rose, 1976). Bouarab et al. (2004) reported that *Micractinium pusillum* Fresenius grew in the presence of organic substrates, that is, glucose and acetate, under mixotrophic condition as well as in the heterotrophic growth. The growth was much more important in the light than in the dark and more in the presence of glucose than of acetate. Ukeles and Rose (1976) and Hayward (1968) studied the effect of a wide range of externally supplied carbon compounds on the growth of *P. tricornutum* Böhlin in mixotrophic conditions. In their studies, glycerol, sodium acetate and sodium lactate, among others, were tested at same concentration (0.01 M). Ukeles and Rose (1976) reported growth stimulatory effect for the three substrates, whereas Hayward (1968) observed this behaviour only for glycerol.

In this work, we found that the ability of *C. vulgaris* to utilized different carbon sources is diversiform. At low concentration (1 g/l), the addition of organic carbon sources in SEM under mixotrophic conditions shortened the growth cycle and promoted the harvesting biomass content remarkably, however, the lipid accumulation had no striking effect. Whereas, the inorganic substrates can stimulate the pigments synthesis through enhance photosynthesis in consideration of the chlorophyll content at dry weight level. The above results suggest that mixotrophic cultivation of *C. vulgaris* supplied organic carbon source and illumination was a desired approach for high density culture of microalgae in view of the volumetric productivities of biomass, lipid and chlorophyll.

Effect of glucose content on biomass production, lipid accumulation and chlorophyll biosynthesis of *C. vulgaris*

Glucose plays a vital role in promoting cell growth of *C. vulgaris* in mixotrophic culture. Cells grew poorly under photoautotrophic conditions in which no glucose was supplied, whereas, supplementation of glucose in SEM, ranging from 1 to 20 g/l, led to a significant improvement of the algal cell growth (Figure 3). The growth curve also indicated that, however, high glucose level (>5 g/l) might prolong lag phase slightly, the cell growth entered into the log phase quickly after those momentary inhibition, and

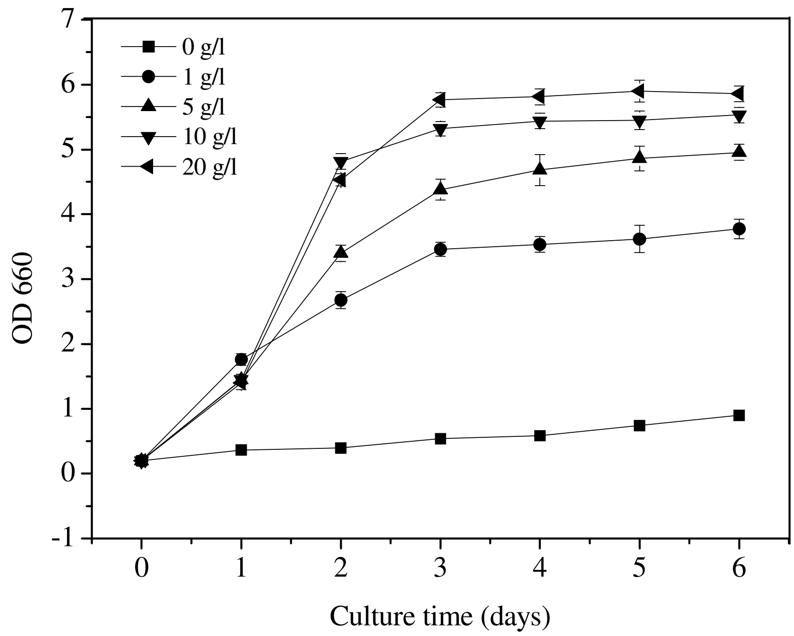

Figure 3. Effect of glucose content on the growth of *C. vulgaris.*

Table 3. Effect of glucose content on biomass production, lipid accumulation and chlorophyll biosynthesis of *C. vulgaris.*

Glucose content (g/l)	0	1	5	10	20
Biomass content (g/l)	0.33 ± 0.02^{a}	1.44 ± 0.10^{b}	1.89 ± 0.13^{c}	2.11 ± 0.07^{c}	2.24 ± 0.26^{c}
Specific growth rate (μ, day^{-1})	0.33 ± 0.03^{a}	0.95 ± 0.07^{b}	1.03 ± 0.09^{b}	1.09 ± 0.06^{b}	1.12 ± 0.17^{b}
Biomass productivity (g/l/day)	0.05 ± 0.03^{a}	0.24 ± 0.017^{b}	0.32 ± 0.02 ^{c}	0.35 ± 0.01^{c}	0.37 ± 0.04^{c}
Lipid content (mass %)	7.57 ± 0.45^{a}	8.12 ± 0.42^{a}	9.25 ± 0.79^{a}	12.79 ± 0.88^{b}	17.74 ± 0.43^{c}
Lipid productivity (mg/l/day)	4.16 ± 0.43^{a}	19.44 ± 1.35^{b}	29.22 ± 3.9 ^{b}	45.02 ± 1.83^{c}	66.25 ± 9.41^{d}
Chlorophyll content (mg/g)	28.56 ± 1.93^{b}	29.20 ± 0.98^{b}	6.06 ± 0.81^{a}	5.37 ± 0.54^{a}	4.10 ± 0.43^{a}
Chlorophyll productivity (mg/l/day)	1.57 ± 0.19^{a}	7.01 ± 0.74^{b}	1.92 ± 0.93^{a}	1.89 ± 0.18^{a}	1.53 ± 0.32^{a}

Values are mean ± S.D., *N* = 3; mean values in the same line with different letters in the superscript are significantly different ($p < 0.05$).

achieved stationary phase at three days culture except for the photoautotrophic control. Similar results were found in another green microalga, *Chlorella protothecoides*, in which better growth was observed with increasing glucose concentration from 10 to 80 g/l, but a further increase in glucose concentration (up to 100 g/l) resulted in decreases in both the specific growth rate and cell growth yield (Shi et al., 1999), which might probably be due to substrate inhibition (Chen and Johns, 1996).

As shown in Table 3, supplementation of glucose in SEM led to a significant improvement in biomass concentration, for specific growth rate and biomass productivity of *C. vulgaris*. The maximum values of 2.24 g/l, 1.12 day^{-1} and 0.37 g/l/day were obtained at the glucose concentration of 20 g/l in SEM, which were higher than that of the photoautotrophic control 6.79, 3.39 and 7.40-fold, respectively. High glucose concentrations in the medium contributed to lipid accumulation in *C. vulgaris*, the maximum lipid content and productivity achieved at the glucose content of 20 g/l with 17.74% and 66.25 mg/l/day, respectively. Botham and Ratledge (1979) argued that the glucose conversion into lipids was triggered, when nitrogen was exhausted, due to the high-energy charge (ratio of ATP: AMP) present, which might be the reason for enhancement of lipid production under high glucose concentration. Similar results also reported that the growth and lipid productivity of *C. vulgaris* were much enhanced by increasing the concentration of inorganic carbon source (CO_2) (Widjaja et al., 2009).

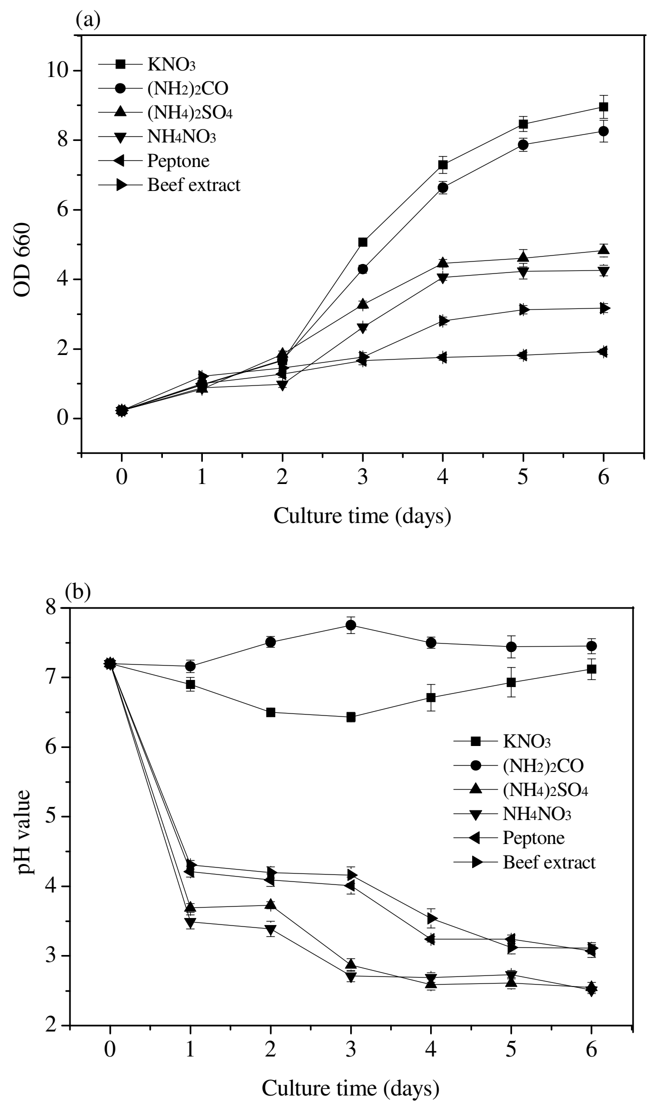

Figure 4. Effect of nitrogen sources on the growth of *C. vulgaris* (A) and pH value (B) in culture medium.

However, the effects of glucose content on the chlorophyll biosynthesis in *C. vulgaris* were interesting. Compared with the autotrophic culture, addition of low concentration glucose (1 g/l) in SEM accelerated the photopigment biosynthesis of the algal cell. But, higher glucose content (> 5 g/l) inhibited the chlorophyll production. The lowest chlorophyll content of 4.10 mg/g was obtained in the culture supplied 20 g/l glucose, which was lower that than of the control value of 28.56 mg/g notably. The low concentration of glucose stimulated the cells growth under mixotrophic condition. During the culture beginning, the algal cells might switch to photoautotrophic mode and synthesize photosynthetic pigments after consumption of glucose in the medium, which could be also revealed from the maximum chlorophyll productivity obtained at 1 g/l glucose in SEM. Heterotrophic respiration might be the primary metabolic pattern in *C. vulgaris* cells at high glucose content.

Effect of nitrogen sources on biomass production, lipid accumulation and chlorophyll biosynthesis of *C. vulgaris* under mixotrophic cultivation

A wide variety of nitrogen sources, such as potassium nitrate, urea, ammonium sulfate, ammonium nitrate, peptone and beef extract, were used as nitrogen sources for mixotrophic growing of *C. vulgaris*. Figure 4 shows the effects of different nitrogen sources on the growth of *C. vulgaris* and the pH values in culture medium under mixotrophic cultivation. The results from Figure 4 and Table 4 implicate that the cultures supplemented with potassium nitrate and urea displayed satisfactory growth states, for instance, extension of the logarithmic growth

Table 4. Effect of nitrogen sources on biomass production, lipid accumulation and chlorophyll biosynthesis of *C. vulgaris* under mixotrophic cultivation.

Nitrogen sources (0.5 g/l)	KNO_3	$(NH_2)_2CO$	$(NH_4)_2SO_4$	NH_4NO_3	Peptone	Beef extract
Biomass content (g/l)	3.43 ± 0.15^d	3.16 ± 0.08^d	1.84 ± 0.10^c	1.62 ± 0.18^{bc}	0.72 ± 0.03^a	1.20 ± 0.26^b
Specific growth rate (μ, day^{-1})	0.87 ± 0.04^c	0.84 ± 0.05^c	0.74 ± 0.08^{bc}	0.72 ± 0.09^{bc}	0.51 ± 0.07^a	0.63 ± 0.02^{ab}
Biomass productivity (g/l/day)	0.57 ± 0.02^d	0.53 ± 0.01^d	0.31 ± 0.016^c	0.27 ± 0.029^{bc}	0.12 ± 0.004^a	0.20 ± 0.044^b
Lipid content (mass %)	8.23 ± 0.35^b	8.18 ± 0.96^b	6.55 ± 0.66^{ab}	5.46 ± 0.53^a	11.33 ± 1.00^c	14.89 ± 0.38^d
Lipid productivity (mg/l/day)	47.10 ± 3.55^d	43.13 ± 5.35^d	20.09 ± 3.03^b	14.75 ± 2.99^{ab}	13.62 ± 1.70^{ab}	29.87 ± 7.31^{bc}
Chlorophyll content (mg/g)	8.27 ± 0.72^c	22.93 ± 1.77^d	5.67 ± 0.51^b	5.23 ± 0.20^b	1.69 ± 0.16^a	2.34 ± 0.22^a
Chlorophyll productivity (mg/l/day)	4.73 ± 0.36^c	12.09 ± 0.90^d	1.74 ± 0.22^b	1.41 ± 0.21^b	0.20 ± 0.03^a	0.47 ± 0.15^{ab}

Values are mean ± S.D., *N*=3; mean values in the same line with different letters in the superscript are significantly different ($p<0.05$).

phase and enhancement of biomass content and productivity. The growth of the tests done with ammonium sulfate and ammonium nitrate as the sole nitrogen was feeble because of the severe drop in culture pH to below pH 4. Without the control of the pH values in flask cultures, the dropping of pH in the cultures with ammonium sulfate and ammonium nitrate was greater than peptone and beef extract as nitrogen source. After six days cultivation, the pH values in cultures supplemented with ammonium sulfate, ammonium nitrate, peptone and beef extract dropped to 2.55, 2.51, 3.07 and 3.11, respectively. Whereas, the pH values in the cultures with potassium nitrate and urea fluctuated around 7.2. The reasons of the pH values drop in the mixotrophic cultures might attribute to the increase of releasing H^+ with the utilization of ammonium ion and the metabolism of organic acids during aerobic respiration by the alga (Shi et al., 2000; Yu et al., 2000).

In consideration of specific growth rate, biomass content and productivity, potassium nitrate or urea is the suitable nitrogen source for mixotrophic cultivation of *C. vulgaris.* The culture with potassium nitrate achieved the maximum specific growth rate (0.87 day^{-1}), biomass content (3.43 g/l), biomass productivity (0.57 g/l/day) and lipid productivity (47.10 mg/l/day), meanwhile, the culture with urea gained the maximum chlorophyll content (22.93 mg/g) and productivity (12.09 mg/l/day). The organic nitrogen sources, such as peptone and beef extract, were bad for biomass production and chlorophyll biosynthesis in *C. vulgaris* cells, however, the level of lipid content obtained was 11.33 and 14.89%, respectively.

It was found that *C. vulgaris* preferentially absorbed ammonium and higher algal yields were obtained when nitrate was replaced with ammonium in the autotrophic culture. This preference resulted from less energy expenditure on the absorption of ammonium by algae than needed for the uptake of nitrate (Syreth and Morris, 1963). The use of ammonium as nitrogen source for *Ellipsoidion* sp. also resulted in higher growth rate and lipid content than that of using urea and nitrate under photoautotrophic conditions (Xu et al., 2001). While, *N. oleoabundans* with nitrate grew faster and accumulated higher lipid than that with urea, the cell grew poorly in medium with ammonium as the nitrogen source (Li et al., 2008). In the report of eicosapentaenoic acid (EPA) production by the diatom *Nitzschia laevis* in heterotrophic cultures, nitrate and urea were found to be the preferred nitrogen sources for both cell growth and EPA content, tryptone and yeast extract were respectively added to the medium and both of them were found to enhance EPA production compared with the control (Wen and Chen, 2001). The above results imply that the abilities of different microalgae to utilize nitrogen sources varied with the species and trophic modes.

However, among the organic nitrogen sources, urea gained important generally in large-scale algal cultivation, because the cost of urea is lower than others. With respect to dry cell weight, lipid productivity, total chlorophyll yields, as well as cost, urea is the best nitrogen source for mixotrophic culture of *C. vulgaris* in our study.

Effect of urea content on biomass production, lipid accumulation and chlorophyll biosynthesis of *C. vulgaris* under mixotrophic cultivation

Nitrogen is known to have a strong influence on the growth and metabolism of lipids and fatty acids in various microalgae. Many studies have focused on the effect of nitrogen concentration and starvation on the growth and lipid content in algae grown in autotrophic and

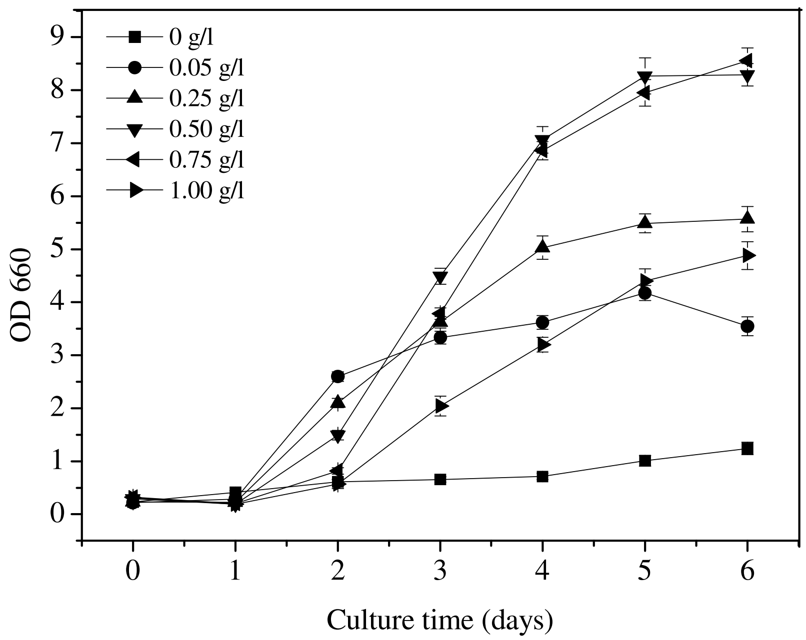

Figure 5. Effect of urea content on the growth of *C. vulgaris*.

Table 5. Effect of urea content on biomass production, lipid accumulation and chlorophyll biosynthesis of *C. vulgaris* under mixotrophic cultivation.

Urea content (g/l)	0	0.05	0.25	0.50	0.75	1.00
Biomass content (g/l)	0.46 ± 0.05^{a}	1.35 ± 0.20^{b}	2.13 ± 0.12^{c}	3.18 ± 0.55^{d}	3.28 ± 0.30^{d}	1.86 ± 0.05^{bc}
Specific growth rate (μ, day^{-1})	0.30 ± 0.05^{a}	0.59 ± 0.04^{b}	0.63 ± 0.04^{b}	0.67 ± 0.04^{b}	0.64 ± 0.03^{bc}	0.53 ± 0.03^{bd}
Biomass productivity (g/l/day)	0.08 ± 0.00^{a}	0.22 ± 0.033^{b}	0.35 ± 0.02^{c}	0.53 ± 0.092^{d}	0.55 ± 0.049^{d}	0.31 ± 0.009^{bc}
Lipid content (mass %)	13.66 ± 0.60^{d}	9.75 ± 0.40^{c}	7.98 ± 0.43^{b}	6.10 ± 0.76^{a}	5.48 ± 0.35^{a}	5.05 ± 0.18^{a}
Lipid productivity (mg/l/day)	10.48 ± 1.58^{a}	21.92 ± 4.06^{b}	28.30 ± 2.64^{b}	32.28 ± 9.08^{b}	29.95 ± 4.56^{b}	15.67 ± 0.97^{a}
Chlorophyll content (mg/g)	5.86 ± 0.49^{a}	12.03 ± 0.54^{b}	16.02 ± 1.08^{c}	22.74 ± 0.84^{d}	25.98 ± 0.16^{e}	24.05 ± 0.84^{de}
Chlorophyll productivity (mg/l/day)	0.45 ± 0.09^{a}	2.70 ± 0.51^{ab}	5.68 ± 0.46^{b}	12.04 ± 2.54^{c}	14.20 ± 1.22^{c}	7.46 ± 0.40^{b}

Values are mean ± S.D., *N*=3; mean values in the same line with different letters in the superscript are significantly different ($p<0.05$).

heterotrophic bioreactors (Illman et al., 2000; Li et al., 2008; Xu et al., 2001; Wen and Chen, 2001), but not much on the mixotrophic cultivation systems. In this study, the effects of urea contents on the mixotrophic growth of *C. vulgaris* and chemical components were examined, and the results are summarized in Figure 5 and Table 5. Urea concentrations of 0.05, 0.25, 0.50, 0.75, and 1.00 g/l were used as the initial nitrogen source to investigate the effects on the alga growth and cellular composition by batch mode operation. In our experimental results, the alga grew poorly in nitrogen free medium with urea as the sole nitrogen source. The alga had obvious growth predominance in its early cultivation at low content of urea (< 0.50 g/l), yet the higher urea content (0.75, 1.00 g/l) prolonged the lag phase of *C. vulgaris*. Fortunately, the growth of cultures supplied the higher urea content had more advantages in the extension of exponential phase than that in the lower nitrogen at the later growing stage.

The results from Table 5 indicate that the growth and biochemical composition of the alga varied with the level of urea concentration in flask cultures. After six days cultivation, higher initial urea concentrations of the nutrient medium led to an increase in biomass concentration, and the highest biomass content of 3.28 g/l was obtained by cultivation with an initial urea feed of 0.75 g/l. Additionally, the specific growth rate of the algal cell increased with the urea content in culture medium until 0.50 g/l. The highest specific growth rate of 0.67 day^{-1} was obtained with the urea concentration of 0.50 g/l, which was higher than that of the control test 2.23-flod. The growth rate displayed a decreasing situation when the urea concentration exceeded 0.50 g/l. However, no appreciable inhibitory effect on the heterotrophic growth of *Chlorella protothecoides* was observed over a nitrogen concentration range of 0.85 to 1.70 g/l (Shi et al., 2000).

The results also suggest that the cells had high lipid content (13.66%) but low biomass concentration (0.46 g/l) with nitrogen starvation. Larger amounts of urea improved cell growth but decreased total lipid content. The critical urea concentration was observed at 0.50 g/l because the cells had both a high specific growth rate (0.67 day^{-1}) and high total lipid productivity (32.28 mg/l/day), compared with those cultivated with urea free and at 1.00 g/l. The above results were consistent with some other reports; for example, the total lipid content of *Neochloris oleoabundans* and *Chlorella* sp. increase by a factor of two at low nitrogen concentrations (Illman et al., 2000; Li et al., 2008), and the growth rate and lipid accumulation of microalgae were strongly related to nitrogen concentration (Hsieh and Wu, 2009). According to literature reports, nitrogen limitation may increase the intracellular content of fatty acid acyl-CoA and activate diacylglycerol acyltransferase, which converts fatty acid acyl-CoA to triglyceride (Sukenik and Livne, 1991). That may be the cause of low urea concentration and the rise of the total lipid content.

In addition, the chlorophyll biosynthesis increased with the promotion of urea content in cultures. The maximum chlorophyll content (25.98 mg/g) and productivity (14.20 mg/l/day) were obtained at 0.75 g/l urea in the culture. Previous work indicated that the high contents of chlorophylls and primary carotenoids at 1.1 g/l nitrate might be a factor suppressing the biosynthesis of the secondary carotenoids and astaxanthin in *Chlorella zofingiensis* (Ip et al., 2004). Boussiba and Vonshak (1991) reported that nitrogen (nitrate) was essential for astaxanthin accumulation in *Haematococcus pluvialis*; they suggested that nitrogen was required for continuous synthesis of protein responsible for supporting the pigment formation. Consequently, an optimized supply of urea is considered to be a mixotrophic cultivation strategy for microalgal biomass production, lipid accumulation and chlorophyll biosynthesis.

Conclusion

In summary, mixotrophic cultivation of *C. vulgaris* is a feasible approach for lipid accumulation and chlorophyll biosynthesis that are dependent on the increase in biomass content and volumetric productivity. Glucose is the best carbon source for mixotrophic cultivation of *C. vulgaris* and the effects of glucose content on the alga growth under mixotrophic conditions are considerable because lower glucose content (1 g/l) promotes the production of biomass and photosynthetic pigments, while, higher glucose contents (≥ 5g/l) increase the biomass and lipid accumulation but inhibit the chlorophyll biosynthesis, which may be caused by the conversion of photoautotrophic mode into heterotrophic respiration under the conditions of sufficient glucose in mixotrophic culture medium.

The behaviors of *C. vulgaris* digests nitrogen source under mixotrophic cultivation are different from photosynthetic mode. The microalga could not grow well without pH control when ammonium and organic nitrogen were the sole nitrogen sources in the mixotrophic cultures because of the remarkable drop in pH value. Urea is a suitable nitrogen source for mixotrophic cultivation of *C. vulgaris* for the sake of lipid production and photosynthetic pigments accumulation in consideration of the alga growth behaviors and the costs of nutrient.

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