ISSN 1330-9862
(FTB-2897)

*original scientific paper*

# Effect of Glycerol and Glucose on the Enhancement of Biomass, Lipid and Soluble Carbohydrate Production by *Chlorella vulgaris* in Mixotrophic Culture

*Wei-Bao Kong[1,2]*, Hong Yang[2], Yun-Tao Cao[2], Hao Song[1], Shao-Feng Hua[1] and Chun-Gu Xia[1]**

[1]State Key Laboratory for Oxo Synthesis and Selective Oxidation, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou, 730000, PR China

[2]College of Life Science, Northwest Normal University, Lanzhou, 730070, PR China

Received: July 20, 2011
Accepted: March 14, 2012

**Summary**

Biodiesel-derived glycerol is a promising substrate for mixotrophic cultivation of oleaginous microalgae, which can also reduce the cost of microalgal biodiesel. The objective of this study is to investigate the potential of using glycerol and glucose as a complex carbon substrate to produce microalgal biomass and biochemical components, such as photosynthetic pigments, lipids, soluble carbohydrates and proteins by *Chlorella vulgaris*. The results show that *C. vulgaris* can utilize glycerol as a sole carbon substrate, but its effect is inferior to that of the mixture of glycerol and glucose. The effect of glycerol and glucose could enhance the algal cell growth rate, biomass content and volumetric productivity, and overcome the lower biomass production on glycerol as the sole organic carbon source in mixotrophic culture medium. The utilization of complex organic carbon substrate can stimulate the biosynthesis of lipids and soluble carbohydrates as the raw materials for biodiesel and bioethanol production, and reduce the anabolism of photosynthetic pigments and proteins. This study provides a promising niche for reducing the overall cost of biodiesel and bioethanol production from microalgae as it investigates the by-products of algal biodiesel production and algal cell hydrolysis as possible raw materials (lipids and carbohydrates) and organic carbon substrates (soluble carbohydrates and glycerol) for mixotrophic cultivation of microalgae.

*Key words*: *Chlorella vulgaris*, glycerol, glucose, biomass production, biochemical components, mixotrophic cultivation

## Introduction

As a promising source for the production of biodiesel and active ingredients such as pigments, proteins and unsaturated fatty acids, microalgae are drawing more and more attention of researchers because of their high growth rate and lipids required for biofuel production. It is essential to increase biomass production and the productivity of lipids and other cellular compounds rapidly and to decrease the cost of biodiesel production (*1*). The price of algal biofuel ultimately depends on the substrate cost, lipid yield, and the quality of the products formed by the downstream processing (*2*). The cost of carbon source represents 50 % of the cost of the medium for algal cultivation (*3*). With the aim of commercializing biodiesel production from algae, a substantial effort has been devoted to the development of improved algal strains and more efficient cultivation process. Recent studies have found that the biomass and lipid content of algae can be increased by changing cultivation condi-

*Corresponding author; Phone: ++86 931 496 8089; Fax: ++86 931 496 8129; E-mail: kwbao@163.com, cgxia@lzb.ac.cn

tions such as $CO_2$ fixation rate, temperature, salinity and nutrient concentration (*4–6*). Particularly, the effects of nitrogen sources and their concentrations on lipid accumulation by microalgae have been examined widely (*7*).

The ability of growth transition from photoautotrophic to mixotrophic mode in microalgae is a phenomenon which appears to exist in a number of genera and species distributed throughout the major taxonomic divisions (*8*). Many algal organisms are capable of using either metabolic process, autotrophic or heterotrophic, for growth, meaning that they are able to photosynthesize as well as utilize organic materials (*9*). The ability of mixotrophs to process organic substrates means that the cell growth is not strictly dependent on photosynthesis. Therefore, light energy is not an absolutely limiting factor for growth, but both light and organic carbon substrates can support the algal growth; hence, there is less biomass loss during the dark phase (*10*). Hayward (*11*) studied the effect of a range of externally supplied carbon compounds on the growth of *Phaeodactylum tricornutum* in the dark and in the light. *P. tricornutum* is able to respire glucose, mannitol and lactate without the sufficient energy for growth. Bouarab *et al.* (*12*) also reported that *Micractinium pusillum* grew in the presence of organic substrates, *i.e.* glucose and acetate, under mixotrophic conditions as well as under heterotrophic conditions. The growth of *M. pusillum* was much more eugonic in the light than in the dark and more in the presence of glucose than of acetate. It can be concluded from the above that mixotrophism is an ideal nutritional mode for high-density cultivation of microalgae for the production of biofuels and functional components.

During the biodiesel manufacturing process, one of the major by-products is crude glycerol (*13*). With the rapid growth of biodiesel production, the market is flooded with crude glycerol. As a result, biodiesel producers must seek new uses for this waste stream. Recently, a process using crude glycerol as a substrate for the fermentation of the microalga *Schizochytrium limacinum* has been developed (*14*). The oleaginous *S. limacinum* is capable of producing significant amounts of total lipids and docosahexaenoic acid (DHA, C 22:6 *n*-3), especially when grown on a variety of carbon sources such as glucose, glycerol or fructose (*15*). The above findings suggest that biodiesel-derived glycerol is a potential substrate for mixotrophic cultivation of oleaginous microalgae, with a further purpose of utilization of glycerol and reducing the production cost of microalgal biodiesel.

However, there are few reports on the effects of carbon sources, especially on the biomass production and algal cell components under mixotrophic cultivation (*10, 12*). In this paper, the effects of glycerol and glucose on the enhancement of biomass, lipid and soluble carbohydrate production by *C. vulgaris* under mixotrophic conditions are investigated.

## Materials and Methods

### *Microalgal growth conditions*

*Chlorella vulgaris* was purchased from the Culture Collection of Algae, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, PR China, and was grown on modified soil extract medium (SEM) which consisted of (per liter): $NaNO_3$ 0.25 g, $KH_2PO_4$ 0.175 g, $K_2HPO_4$ 0.075 g, $MgSO_4{\cdot}7H_2O$ 0.075 g, NaCl 0.025 g, $CaCl_2{\cdot}2H_2O$ 0.025 g, $FeCl_3$ 5 mg, $ZnSO_4{\cdot}7H_2O$ 0.287 mg, $MnSO_4{\cdot}H_2O$ 0.169 mg, $H_3BO_3$ 0.061 mg, $CuSO_4{\cdot}5H_2O$ 2.5 μg and $Na_6Mo_7O_{24}{\cdot}7H_2O$ 1.24 μg. The pH was adjusted to 7.2 prior to autoclaving at 120 °C for 20 min. In the tests, different concentrations of glycerol (1, 5 and 10 g/L) and 2 g/L of glucose were supplied to the medium and cultured under illumination. The autotrophic control group was cultivated in SEM with illumination. All cultures were maintained at (30±1) °C in 250-mL flasks containing 100 mL of culture under illumination at 2500 lux with 12 h light/12 h dark periods and shaken at 120 rpm on an orbital shaker. Cultures were harvested on day 4 (96 h).

### *Determination of biomass concentration and productivity of Chlorella vulgaris*

Algal growth curves and biomass concentrations were determined by measuring the absorbance at 660 nm and dry cell mass, respectively. Cells were centrifuged at 2000×*g* for 10 min, rinsed twice with distilled water and dried at 70 °C for 24 h to give the dry cell mass (g/L).

The specific growth rate ($\mu$/day$^{-1}$) of *C. vulgaris* at the exponential phase was calculated according to the following equation:

$$\mu=(\ln X_2-\ln X_1)/(t_2-t_1) \qquad /1/$$

where $X_2$ and $X_1$ are the dry cell mass concentration (g/L) at time $t_2$ and $t_1$, respectively.

The biomass concentration (g/L) was recorded and the productivity [$r_P$/(mg/(L·day))] was calculated from the equation:

$$r_P=(X_2-X_1)/(t_2-t_1) \qquad /2/$$

where $X_2$ and $X_1$ are the dry cell mass concentration (g/L) at time $t_2$ and $t_1$, respectively (*10*).

### *Extraction and determination of photosynthetic pigments*

A volume of 4 mL of algal cultures was centrifuged at 2000×*g* for 10 min and rinsed twice with distilled water. The pellet was extracted twice with 8 mL of 80 % (by volume) ethanol, followed by centrifugation at 2000×*g* for 10 min. The contents of chlorophyll a, chlorophyll b, total chlorophyll (a+b) and carotenoids in the supernatant were determined by UV-VIS spectroscopy (*16*).

### *Extraction and determination of lipids*

Cells were harvested by centrifugation, washed twice with distilled water, and then dried in a freeze dryer. The dry biomass (100 mg) was homogenized in a mortar, extracted with *n*-hexane (20 mL) for 30 min and centrifuged. The extraction process was repeated three times, the supernatant was transferred to a preweighed glass vial and evaporated on a rotary evaporator. The algal lipids were recovered after solvent evaporation and dried completely at 70 °C. The mass of the glass vial containing oil was measured gravimetrically and the lipid content expressed as dry mass percentage (*17,18*). Meanwhile, the lipid productivity [$r_P$/(mg/(L·day))] was calculated (*10*).

### *Extraction and determination of soluble carbohydrates*

Cells were harvested by centrifugation, washed twice with distilled water, and then dried in a freeze dryer. The dry sample was homogenized in a mortar, extracted with boiled water for 1 h and centrifuged at 2000×*g* for 10 min. The extraction process was repeated twice and the soluble carbohydrate content in the mixed supernatant was estimated. Anthrone-sulphuric acid method was adopted to determine the content of soluble carbohydrates (*19*). Briefly, 0.5 mL of the sample were mixed with 0.5 mL of distilled water and added to 5.0 mL of anthrone agent. Homogeneous mixture was incubated in a boiling water bath for 10 min. After chromogenic reaction and cooling, the absorbance was measured at 620 nm with a spectrophotometer. Glucose was used as a carbohydrate standard. The soluble carbohydrate content was expressed as dry mass percentage.

### *Extraction and determination of soluble proteins*

*Chlorella vulgaris* cells were homogenized and extracted in 10 mL of 90 % ethanol for 2 h, and the homogenate was centrifuged at 2000×*g* for 10 min. The obtained pellet was mixed with 5 mL of 10 % trichloroacetic acid (by mass per volume) and recentrifuged. The pellet was repeatedly washed with ethanol (95 %) for complete removal of trichloroacetic acid, dissolved in 0.1 M NaOH and extracted twice for 1 h at 60 °C in a water bath, maintaining the volume at 50 mL. Soluble protein content was quantified using Coomassie Brilliant Blue with bovine serum albumin as a protein standard (*20*) and expressed as dry mass percentage.

### *Statistical analysis*

The data are presented as mean values of at least four replicates per treatment±standard deviation (S.D.). Each experiment was conducted in duplicate. The differences between mean values were calculated using Tukey's test at the 0.05 level by Origin software (v. 7.5, OriginLab, Northampton, MA, USA).

## Results and Discussion

### *Growth curves, kinetics and biomass production of Chlorella vulgaris*

Results presented in Fig. 1 and Table 1 demonstrate the effects of glycerol and glucose on the growth curves, kinetics and biomass production of *C. vulgaris* under mixotrophic cultivation (96 h). With the cultivation of *C. vulgaris* under 30 °C and 2500 lux, all of the cultures had an obvious growth except for the control group. The samples supplied with organic carbon sources (glycerol and glucose) were superior in their growth compared to the autotrophic control. Also, the mixtures of glycerol and glucose performed better than the sole glycerol. After the lag phase (about 12 h), the algal cells entered their logarithmic growth phase. After 84 h, the cell growth was in stationary phase. At an early stage, the growth of algal cells was inhibited by high concentrations of glycerol (10 g/L). Fortunately, this trend disappeared during the exponential phase. In comparison with autotrophic control, the specific growth rates of *C. vulgaris* were promoted by glycerol and glucose in the medium under illumination and the maximum values of 0.99 $day^{-1}$ were obtained in the samples supplied with 5 g/L of glycerol and 2 g/L of glucose or 10 g/L of glycerol and 2 g/L of glucose.

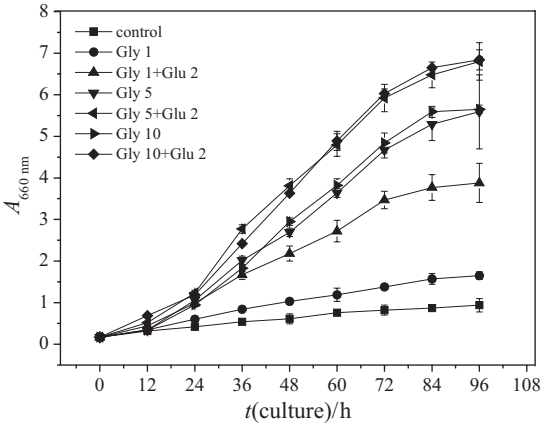


**Fig. 1.** Effect of glycerol (Gly) and glucose (Glu) mass concentrations (g/L) on the growth of *Chlorella vulgaris*. Values are mean± S.D., *N*=4

After 96 h of cultivation, the maximum biomass content of 2.62 g/L was obtained in the culture with 10 g/L of glycerol and 2 g/L of glucose added, which is 7.71 times higher than in the control group; however, there was no significant difference ($p<0.05$) from the culture medium supplied with 5 g/L of glycerol and 2 g/L of glucose (2.60 g/L). Meanwhile, the results of biomass productivity of *C. vulgaris* were similar; it increased with the supplementation of glycerol and glucose, as well as with the glycerol concentration. The maximum biomass productivity was 654.17 mg/(L·day) in the culture with 10 g/L of glycerol and 2 g/L of glucose, which was significantly different ($p<0.05$) from the control sample (85.42 mg/(L·day)), but not from the sample supplied with 5 g/L of glycerol and 2 g/L of glucose (650.00 mg/(L·day)).

Table 1. Effect of glycerol (Gly) and glucose (Glu) mass concentrations on the biomass content, specific growth rate ($\mu$) and productivity ($r_P$) of *Chlorella vulgaris*

| Parameter | $\gamma$/(g/L) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | Gly 1 | Gly 1+Glu 2 | Gly 5 | Gly 5+Glu 2 | Gly 10 | Gly 10+Glu 2 |
| $\gamma$(biomass)/(g/L) | $(0.34\pm0.06)^a$ | $(0.62\pm0.03)^a$ | $(1.48\pm0.19)^b$ | $(2.13\pm0.34)^c$ | $(2.60\pm0.18)^d$ | $(2.16\pm0.04)^{cd}$ | $(2.62\pm0.10)^d$ |
| $\mu$/$day^{-1}$ | $(0.48\pm0.05)^a$ | $(0.63\pm0.01)^b$ | $(0.84\pm0.03)^c$ | $(0.94\pm0.04)^d$ | $(0.99\pm0.02)^d$ | $(0.94\pm0.02)^d$ | $(0.99\pm0.01)^d$ |
| $r_P$/(mg/(L·day)) | $(85.42\pm15.73)^a$ | $(154.17\pm7.22)^a$ | $(368.75\pm47.19)^b$ | $(533.33\pm83.93)^c$ | $(650.00\pm43.75)^d$ | $(539.58\pm9.55)^{cd}$ | $(654.17\pm25.26)^d$ |

Values are mean±S.D., *N*=4; mean values in the same line with different letters in the superscript are significantly different ($p<0.05$)

For biomass production of microalgae, many cultivation methods, such as open pond, raceway and heterotrophic fermentation, have been tested (*12,21*). Some studies have focused on the more economical methods of making biodiesel from microalgae. One of them is to decrease substrate costs by using alternative carbon sources such as corn powder hydrolysate (*22*) or sweet sorghum juice (*23*). Alternatively, other studies have sought to find marketable uses for the by-products of algal biodiesel production, such as glycerol (*24*).

It has been found that there are three metabolic possibilities of cultivation in *Spirulina* sp.: autotrophic, heterotrophic and mixotrophic growth. In the mixotrophic growth there are two distinctive processes, photosynthesis and aerobic respiration. The former is influenced by light intensity, and the latter is related to the organic substrate (glucose) concentration (*25*). The high cell density of mixotrophic cultures demonstrates that the growth-stimulating effects of light and $CO_2$ in mixotrophic cultures were as strong as the effects of glucose (*24*). An early report showed that mixotrophic growth offered a possibility to increase greatly the microalgal cell concentration and volumetric productivity in a batch system (*26*). It was established that the adenosine triphosphate formed in the photochemical reactions accelerated the glucose anabolism in the mixotrophic culture of *Euglena gracilis*. This is the reason why the growth in the mixotrophic culture increased (*26*). The results of our study suggest that *C. vulgaris* has a potential to be an excellent biofuel producer because the growth rate of the strains can be stimulated by organic materials.

Recent work (*25*) has shown that *Chlorella prototheco-ides* was slightly inhibited by glycerol, and could still grow even when the salinity of the culture medium reached 35 g/L. *C. vulgaris*, however, had much lower tolerance of glycerol and its growth was inhibited when the glycerol concentration reached 15 g/L. In our study, slight inhibition by glycerol was observed during initial cultivation when its concentration reached 10 g/L. However, the growth dynamics parameters (specific growth rate, biomass content and productivity) of the algae were not significantly different when 5 or 10 g/L of glycerol were added to the medium. Furthermore, this inhibition decreased when the mixture of glycerol and glucose was added. The results showed that using glycerol and glucose as complex carbon sources for mixotrophic cultivation of *C. vulgaris* is a feasible way to solve the problem of low algal cell density when glycerol is used as the sole carbon source and to stimulate further utilization of glycerol by *C. vulgaris.*

### *The content and productivity of photosynthetic pigments*

Chlorophyll is one of the cellular compounds on the basis of which microalgal biomass in the culture is estimated and it can be used to measure cell growth. As shown in Table 2, the effect of glycerol and glucose on the photosynthetic pigment content and productivity of mixotrophic *C. vulgaris* was significant. After 96 h of cultivation, the maximum mass fractions, based on the dry cell mass, of chlorophyll a, chlorophyll b, total chlorophyll and carotenoids of 23.53, 12.55, 36.08 and 4.88 mg/g, respectively, were obtained in the autotrophic control culture. All the above values were higher than when 1, 5 or 10 g/L of glycerol, or 2 g/L of glucose ($p<0.05$) were added. However, with the increase of glycerol concentration (from 1 to 10 g/L) and the supplementation of 2 g/L of glucose to mixotrophic cultures, the photosynthetic pigment content decreased significantly. When 1 g/L of glycerol, or 1 g/L of glycerol and 2 g/L of glucose were added to the medium, the decrease was more significant compared to the autotrophic control sample ($p<0.05$). The effect of glycerol concentrations of 5 and 10 g/L on the photosynthetic pigment content was not significant ($p<0.05$).

At the end of culture period (96 h), the maximum photosynthetic pigment productivity was obtained when 1 g/L of glycerol and 2 g/L of glucose were added to the culture medium, and the values of total chlorophyll and carotenoid productivity were 5.74 and 0.75 mg/(L·day), which was 1.87 and 1.83 times higher, respectively, than in the autotrophic control. However, the differences in chlorophyll and carotenoid productivity between the samples supplied with 5 and 10 g/L of glycerol were not significant ($p<0.05$). According to a previous report, the utilization of an external organic carbon source may affect the photoautotrophic growth processes, such as photosynthesis and respiration (*27*). Glucose inhibited the photosynthetic $CO_2$ fixation tenfold and modified the pigmentary system. Light inhibited glucose uptake and assimilation,

Table 2. Effect of glycerol (Gly) and glucose (Glu) mass concentrations on the photosynthetic pigment content ($w$) and productivity ($r_P$) of *Chlorella vulgaris*

| Parameter | $\gamma$/(g/L) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | Gly 1 | Gly 1+Glu 2 | Gly 5 | Gly 5+Glu 2 | Gly 10 | Gly 10+Glu 2 |
| $w$(chlorophyll a)/(mg/g) | $(23.53\pm1.32)^{a}$ | $(17.62\pm1.30)^{b}$ | $(11.78\pm0.48)^{c}$ | $(6.51\pm0.10)^{d}$ | $(5.64\pm0.27)^{de}$ | $(5.94\pm0.26)^{de}$ | $(4.46\pm0.31)^{e}$ |
| $r_P$(chlorophyll a)/(mg/(L·day)) | $(2.00\pm0.11)^{a}$ | $(2.73\pm0.20)^{b}$ | $(4.36\pm0.18)^{c}$ | $(3.47\pm0.05)^{d}$ | $(3.67\pm0.18)^{d}$ | $(3.21\pm0.14)^{de}$ | $(2.92\pm0.20)^{be}$ |
| $w$(chlorophyll b)/(mg/g) | $(12.55\pm0.76)^{a}$ | $(8.30\pm0.45)^{b}$ | $(3.72\pm0.35)^{c}$ | $(1.49\pm0.06)^{d}$ | $(1.25\pm0.10)^{d}$ | $(1.60\pm0.13)^{d}$ | $(1.28\pm0.14)^{d}$ |
| $r_P$(chlorophyll b)(mg/(L·day)) | $(1.07\pm0.06)^{a}$ | $(1.29\pm0.07)^{b}$ | $(1.38\pm0.13)^{b}$ | $(0.79\pm0.03)^{c}$ | $(0.81\pm0.07)^{c}$ | $(0.86\pm0.07)^{c}$ | $(0.84\pm0.09)^{c}$ |
| $w$(total chlorophyll)/(mg/g) | $(36.08\pm1.21)^{a}$ | $(25.93\pm1.72)^{b}$ | $(15.50\pm0.83)^{c}$ | $(8.00\pm0.16)^{d}$ | $(6.89\pm0.38)^{de}$ | $(7.55\pm0.39)^{de}$ | $(5.74\pm0.45)^{e}$ |
| $r_P$(total chlorophyll)/(mg/(L·day)) | $(3.07\pm0.10)^{a}$ | $(4.02\pm0.27)^{b}$ | $(5.74\pm0.31)^{c}$ | $(4.26\pm0.08)^{b}$ | $(4.48\pm0.24)^{b}$ | $(4.07\pm0.21)^{b}$ | $(3.76\pm0.29)^{bd}$ |
| $w$(carotenoids)/(mg/g) | $(4.88\pm0.44)^{a}$ | $(4.23\pm0.06)^{b}$ | $(2.03\pm0.15)^{c}$ | $(1.16\pm0.02)^{d}$ | $(1.03\pm0.05)^{d}$ | $(1.14\pm0.05)^{d}$ | $(0.79\pm0.01)^{d}$ |
| $r_P$(carotenoids)/(mg/(L·day)) | $(0.41\pm0.04)^{a}$ | $(0.66\pm0.01)^{b}$ | $(0.75\pm0.05)^{c}$ | $(0.62\pm0.01)^{b}$ | $(0.67\pm0.03)^{b}$ | $(0.62\pm0.03)^{b}$ | $(0.52\pm0.01)^{d}$ |

Values are mean±S.D., $N$=4; mean values in the same line with different letters in the superscript are significantly different ($p<0.05$)

but under mixotrophic conditions maximal utilization of glucose was obtained. However, in *C. vulgaris* UAM 101, both light and dark respiration rates were enhanced by the addition of glucose, although the net photosynthetic rate was not influenced (*28*). The study of *Spirulina platensis* showed that both the photosynthetic and respiration rates were unchanged after the addition of glucose (*29*). Organic carbon assimilation under mixotrophic conditions induces changes in both respiratory and photosynthetic metabolism in cyanobacteria (*30*). Marquez *et al.* (*29*) suggested that the photosynthetic activity and the organic carbon-dependent respiratory activity operated separately in *S. platensis* cells, because the total growth yield of *S. platensis* in mixotrophic cultures equals the yield of photoautotrophic growth in the light together with the heterotrophic growth in the dark. However, Nieva and Fernández Valiente (*31*) observed a decreased rate of $CO_2$ fixation under mixotrophic growth, which suggested that some aspects of $CO_2$ metabolism may be modulated by organic compounds. This may be the reason for the inhibition of photosynthetic pigment synthesis under mixotrophic growth. Yamane *et al.* (*26*) indicated that a good production of chlorophyll (39.4 mg/L) and carotenoids (13.8 mg/L) was obtained in the mixotrophic culture of *E. gracilis*, due to the highest fermenter productivity with respect to biomass as well as chlorophyll and carotenoids. Our results showed that the mixotrophic cultures experience an increase in photosynthetic pigment productivity that was dependent on the increase of microalgal biomass content.

### *Lipid content and productivity*

As summarized in Fig. 2, the effect of glycerol and glucose on the lipid content and productivity of *Chlorella vulgaris* under mixotrophic conditions was significant. The lowest lipid content, based on the dry cell mass, of 7.48 % was obtained in the autotrophic control culture, and the highest value of 10.64 % was obtained when 10 g/L of glycerol and 2 g/L of glucose were supplied to the SEM. With the increase of glycerol concentration, the lipid content increased slightly. The SEM samples supplied with 10 g/L of glycerol (and 2 g/L of glucose) were

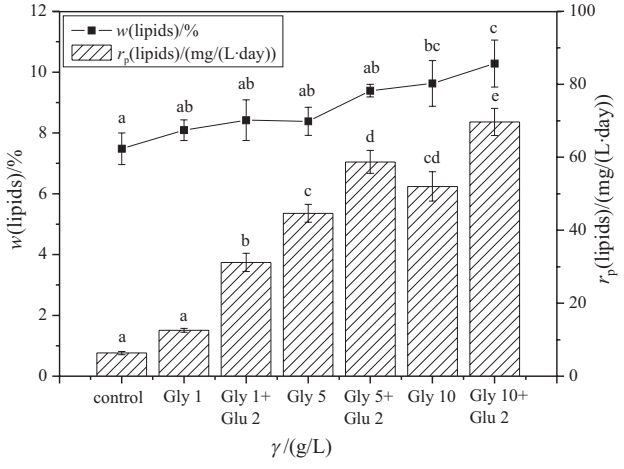


**Fig. 2.** Effect of glycerol (Gly) and glucose (Glu) mass concentrations on the lipid content ($w$) and productivity ($r_P$) of *Chlorella vulgaris.* Values are mean±S.D., $N$=4; the significance of differences is denoted by different letters ($p<0.05$)

significantly different compared to the control ($p<0.05$). However, the addition of glucose did not promote lipid accumulation ($p<0.05$). The differences in lipid volumetric productivity caused by the effects of glycerol and glucose addition were more expressed than in the lipid content. Lipid volumetric productivity of *C. vulgaris* was stimulated by glycerol and glucose since the organic carbon sources promoted the algal biomass content notably. The maximum lipid productivity (69.68 mg/(L·day)) was achieved when 10 g/L of glycerol and 2 g/L of glucose were added, which was 10.96 times higher than the control. In the mixotrophic samples, the lipid productivity was higher when complex carbon sources were used than when different concentrations of glycerol were added as the sole organic carbon source in SEM.

Recent work has revealed that *C. vulgaris* could grow under autotrophic, mixotrophic and heterotrophic conditions, and the mixotrophic cultivation especially could produce more cell biomass than the autotrophic or heterotrophic cultures individually or combined (*25*). Furthermore, the substrate concentration significantly influenced the final cell yield of the mixotrophic cultivations, while the cell lipid content remained relatively constant. The sensitivity analysis results showed that the initial glycerol concentration was the most significant factor for algal growth and lipid production (*32*). The new study has demonstrated simultaneous high growth rates of and lipid yields by *Chlorella protothecoides* heterotrophically grown on mixtures of glycerol and glucose (*33*). In that work, the growth of and lipid production by *C. protothecoides* using glycerol as the carbon source was evaluated to demonstrate the utility of recycling the glycerol created during biodiesel production. Glycerol was examined as both the sole carbon source and in combination with glucose. Algal cultures grown only on glycerol in shake flasks showed a specific growth rate of 0.1 $h^{-1}$ and final lipid yield of 0.31 g/g of substrate. The values were similar to those observed on pure glucose, 0.096 $h^{-1}$ and 0.24 g of lipid per g of substrate, respectively. When the medium contained a mixture of glycerol and glucose, simultaneous uptake of the two substrates was observed. Due to the difference in the rates of lipid storage, lipid productivity was 0.077 g/$h^{-1}$ during growth on glycerol, while the growth on glucose had a productivity of 0.096 g/$h^{-1}$. During growth on the 9:1 mixture of glycerol and glucose, lipid productivity was 0.098 g/$h^{-1}$. Another report indicated that in batch mode, the biomass and lipid concentration in *C. protothecoides* cultivated in a crude glycerol medium were 23.5 and 14.6 g/L, respectively, in a 6-day cultivation (*34*). In the fed-batch mode, the biomass and lipid concentration improved to 45.2 and 24.6 g/L, respectively, after 8.2 days of cultivation. This work demonstrates the feasibility of crude biodiesel glycerol as an alternative carbon substrate to glucose for microalgal cultivation; and a cost reduction of carbon substrate feed in microalgal lipid production may be expected. Similar results were observed in our work. In mixotrophic cultures, the lipid contents were 1.08–1.42 times higher than in the autotrophic culture. However, the mixotrophic cultures supplied with glycerol and/or glucose experience an increase in lipid productivity that depends on the increase in biomass content.

### *Soluble carbohydrate and protein content and productivity*

The effects of glycerol and glucose on the soluble carbohydrate content and productivity of *C. vulgaris* under mixotrophic cultivation can be seen in Fig. 3. The lowest soluble carbohydrate content and productivity of 6.04 % and 5.13 mg/(L·day), respectively, were obtained in the control sample. However, the maximum values of 8.74 % and 57.26 mg/(L·day) were achieved when 10 g/L of glycerol and 2 g/L of glucose were added to the SEM.

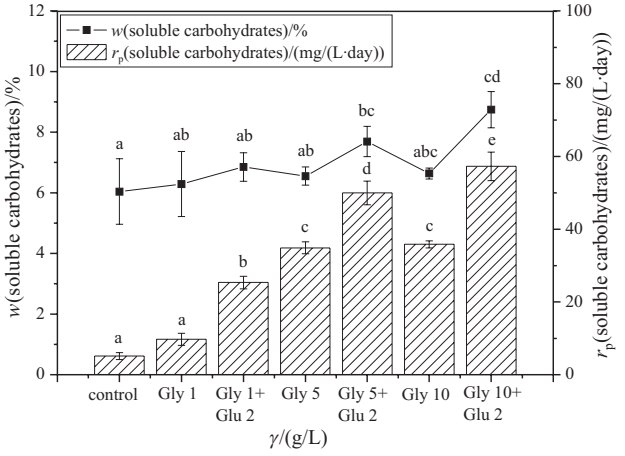


**Fig. 3.** Effect of glycerol (Gly) and glucose (Glu) mass concentrations on the soluble carbohydrate content ($w$) and productivity ($r_P$) of *Chlorella vulgaris*. Values are mean±S.D., $N$=4; the significance of differences is denoted by different letters ($p<0.05$)

In comparison with autotrophic control sample, the cultures supplied with glycerol and glucose had higher soluble carbohydrate content and productivity. At the level of 1 g/L of glycerol, the differences of soluble carbohydrate content were not significant compared to the control, but they became significant with the increase of glycerol concentration (5 and 10 g/L). Moreover, each culture supplied with glycerol and glucose mixture had higher carbohydrate content than the one supplied only with glycerol. The differences in soluble carbohydrate productivity among the experimental groups were significant and enhanced by the glycerol concentration and glucose feed.

Fig. 4 shows the effects of the supplementation of glycerol and glucose on the soluble protein content and productivity of *C. vulgaris*. The effects of mixotrophic (illumination and organic carbon stimulation) conditions on the algal soluble protein anabolism were different from the one on lipid and soluble carbohydrates, but similar to the effect on photosynthetic pigments biosynthesis. The minimum soluble protein content (1.58 %) and productivity (10.37 mg/(L·day)) were obtained when 10 g/L of glycerol and 2 g/L of glucose were added to the SEM, and the maximum values of 18.13 % and 15.41 mg/(L·day), respectively, were achieved in the autotrophic control group.

Carbohydrates are found as the intermediary reserves in some algae, due to the fact that they are required when

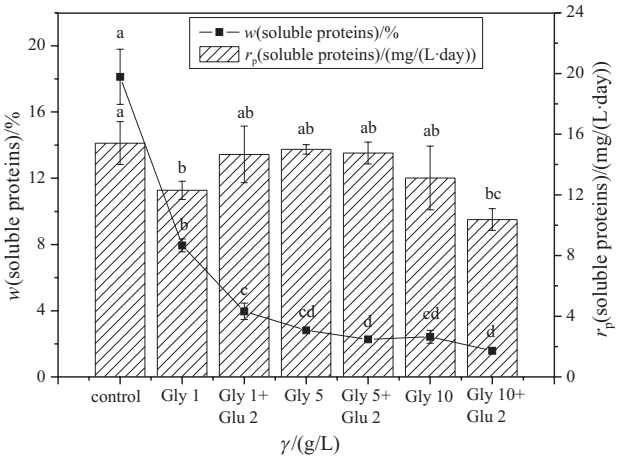


**Fig. 4.** Effect of glycerol (Gly) and glucose (Glu) mass concentrations on the soluble protein content ($w$) and productivity ($r_P$) of *Chlorella vulgaris*. Values are mean±S.D., $N$=4; the significance of differences is denoted by different letters ($p<0.05$)

the nitrogen becomes limited in the lipid synthesis. In the present study, when protein content in *C. vulgaris* decreased, both lipid and carbohydrate content increased (Figs. 2–4). These changes in the constituents are in agreement with reports of other authors who mentioned that there was a reduction in the protein content in mixotrophic *C. vulgaris* UAM 101 cells, which was compensated by an increase in lipid and carbohydrate content (*28*). Ogbonna and Tanaka (*35*) reported that during the night, decreases were observed in the biomass concentration and carbohydrate content of *C. pyrenoidosa* cells while their protein content increased. These changes implied that in the absence of light, intracellularly stored carbohydrates were metabolized as an energy source for cell maintenance and protein synthesis. Similar results were obtained in our study. When 2 g/L of glucose and different concentrations of glycerol were added to the SEM, the higher the glycerol concentration was, the higher the soluble carbohydrate content but lower the protein content in *C. vulgaris* were obtained based on the dry cell mass. The mixotrophic conditions and supplementation of organic carbon sources promoted the biomass, lipid and carbohydrate production, while reduced the pigment and protein biosynthesis, which implied that the mixotrophic conditions changed the metabolic pathways of nitrogen and carbon.

Previous work reported that nitrogen (nitrate) was essential for astaxanthin accumulation in *Haematococcus pluvialis* (*36*). The authors suggested that nitrogen was required for continuous synthesis of the protein responsible for supporting the pigment formation. Another study concluded that higher chlorophyll and protein content was found in *C. vulgaris* cultures with higher ammonium concentrations (*37*), and the algal growth was accompanied by a decrease in nitrogen content in the medium, indicating that nitrogen removal was due to the algal uptake and assimilation. Our results suggested that the supplementation of organic carbon source and energy (light and glucose) might convert the algal cell metabolic pathway. These results suggested that changes in the cellular biochemical composition were influenced by the trophic conditions and nutrient concentration in the medium.

## Conclusions

The results in this paper showed that *Chlorella vulgaris* can utilize glycerol as a sole carbon substrate, but that its effect was inferior to that of the mixture of glycerol and glucose for the production of biomass and biochemical components. The effect of glycerol and glucose as a mixed substrate could enhance the *C. vulgaris* growth, biomass content and volumetric productivity, and overcome the lower biomass yield caused by glycerol as the sole organic carbon source. Besides, the utilization of mixed organic carbon sources could stimulate the accumulation of lipids and soluble carbohydrates as the raw materials for biodiesel and bioethanol production, respectively, while reduce the anabolism of photosynthetic pigments and proteins. This provides a feasible way to reduce the cost of bioenergy production from microalgae by reutilization of glycerol and hydrolysis of algal cell carbohydrate.

*Acknowledgements*

Financial support was provided by National Science Found for Distinguished Young Scholars of China (Grant No. 20625308) and Research Fund for Young Teachers of Northwest Normal University (Grant No. NWNU-LKQN--10-30).

## References

1. D. Song, J. Fu, D. Shi, Exploitation of oil-bearing microalgae for biodiesel, *Chin. J. Biotechol. 24* (2008) 341–348 (in Chinese).
2. J.S. Yang, J.X. Huang, J.R. Ni, Mathematical modeling of batch fermentation of *Zoogloea* sp. GY3 used for synthesizing polyhydroxyalkanoates, *J. Chem. Technol. Biotechnol. 81* (2006) 789–793.
3. Y. Cheng, Y. Lu, C. Gao, Q. Wu, Algae-based biodiesel production and optimization using sugar cane as the feedstock, *Energy Fuels, 23* (2009) 4166–4173.
4. S.Y. Chiu, C.Y. Kao, M.T. Tsai, S.C. Ong, C.H. Chen, C.S. Lin, Lipid accumulation and $CO_2$ utilization of *Nannochloropsis oculata* in response to $CO_2$ aeration, *Bioresour. Technol. 100* (2009) 833–838.
5. Z.Y. Liu, G.C. Wang, B.C. Zhou, Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*, *Bioresour. Technol. 99* (2008) 4717–4722.
6. A.R. Rao, C. Dayananda, R. Sarada, T.R. Shamala, G.A. Ravishankar, Effect of salinity on growth of green alga *Botryococcus braunii* and its constituents, *Bioresour. Technol. 98* (2007) 560–564.
7. A. Converti, A.A. Casazza, E.Y. Ortiz, P. Perego, M. Del Borghi, Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production, *Chem. Eng. Process. 48* (2009) 1146–1151.
8. R. Ukeles, W.E. Rose, Observations on organic carbon utilization by photosynthetic marine microalgae, *Marine Biol. 37* (1976) 11–28.
9. X.W. Zhang, Y.M. Zhang, F. Chen, Application of mathematical models to the determination optimal glucose concentration and light intensity for mixotrophic culture of *Spirulina platensis*, *Process Biochem. 34* (1999) 477–481.
10. M.R. Andrade, J.A.V. Costa, Mixotrophic cultivation of microalga *Spirulina platensis* using molasses as organic substrate, *Aquaculture, 264* (2007) 130–134.
11. J. Hayward, Studies on the growth of *Phaeodactylum tricornutum*. II. The effect of organic substances on growth, *Physiol. Plant. 21* (1968) 100–108.
12. L. Bouarab, A. Dauta, M. Loudiki, Heterotrophic and mixotrophic growth of *Micractinium pusillum* Fresenius in the presence of acetate and glucose: Effect of light and acetate gradient concentration, *Water Res. 38* (2004) 2706–2712.
13. D.T. Johnson, K.A. Taconi, The glycerin glut: Options for the value-added conversion of crude glycerol resulting from biodiesel production, *Environ. Prog. 26* (2007) 338–348.
14. Z. Chi, D. Pyle, Z. Wen, C. Frear, S. Chen, A laboratory study of producing docosahexaenoic acid from biodiesel--waste glycerol by microalgal fermentation, *Process Biochem. 42* (2007) 1537–1545.
15. T. Yokochi, D. Honda, T. Higashihara, T. Nakahara, Optimization of docosahexaenoic acid production by *Schizochytrium limacinum* SR21, *Appl. Microbiol. Biotechnol. 49* (1998) 72–76.
16. H.K. Lichtenthaler, Chlorophylls and carotenoids: Pigments of photosynthetic biomembrane, *Methods Enzymol. 148* (1987) 350–382.
17. C. Dayananda, R. Sarada, S. Bhattacharya, G.A. Ravishankar, Effect of media and culture conditions on growth and hydrocarbon production by *Botryococcus braunii*, *Process Biochem. 40* (2005) 3125–3131.
18. X.L. Miao, Q.Y. Wu, Biodiesel production from heterotrophic microalgal oil, *Bioresour. Technol. 97* (2006) 841–846.
19. E.W. Yemm, A.J. Willis, The estimation of carbohydrates in plant extracts by anthrone, *Biochem. J. 57* (1954) 508–514.
20. M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72* (1976) 248–254.
21. M.A. Borowitzka, Commercial production of microalgae: ponds, tanks, tubes and fermenters, *J. Biotechnol. 70* (1999) 313–321.
22. A. Wei, X. Zhang, D. Wei, G. Chen, Q. Wu, S.T Yang, Effects of cassava starch hydrolysate on cell growth and lipid accumulation of the heterotrophic microalgae *Chlorella protothecoides*, *J. Ind. Microbiol. Biotechnol. 36* (2009) 1383–1389.
23. C. Gao, Y. Zhai, Y. Ding, Q. Wu, Application of sweet sorghum for biodiesel production by heterotrophic microalga *Chlorella protothecoides*, *Applied Energy, 87* (2010) 756–761.
24. T. Heredia-Arroyo, W. Wei, R. Ruan, B. Hu, Mixotrophic cultivation of *Chlorella vulgaris* and its potential application for the oil accumulation from non-sugar materials, *Biomass Bioenergy, 5* (2011) 2–10.
25. K. Chojnacka, A. Noworyta, Evaluation of *Spirulina* sp. growth in photoautotrophic, heterotrophic and mixotrophic cultures, *Enzyme Microbial Technol. 34* (2004) 461–465.
26. Y. Yamane, T. Utsunomiya, M. Watanabe, K. Sasaki, Biomass production in mixotrophic culture of *Euglena gracilis* under acidic condition and its growth energetics, *Biotechnol. Lett. 23* (2001) 1223–1228.
27. J. Lalucat, J. Imperial, R. Pares, Utilization of light for the assimilation of organic matter in *Chlorella* sp. VJ79, *Biotechnol. Bioeng. 26* (1984) 677–681.
28. M.L. Orús, E. Marco, F. Martínez, Suitability of *Chlorella vulgaris* UAM 101 for heterotrophic biomass production, *Bioresour. Technol. 38* (1991) 179–184.
29. F.J. Marquez, K. Sasaki, T. Kakizono, N. Nishio, S. Nagai, Growth characteristics of *Spirulina platensis* in mixotrophic and heterotrophic conditions, *J. Ferment. Bioeng. 76* (1993) 408–410.
30. A. Vonshak, S.M. Cheung, F. Chen, Mixotrophic growth modifies the response of *Spirulina (Arthrospira) platensis* (cyanobacteria) cells to light, *J. Phycol. 36* (2000) 675–679.

*31.* M. Nieva, E. Fernández Valiente, Inorganic carbon transport and fixation in cells of *Anabaena variabilis* adapted to mixotrophic conditions, *Plant Cell Physiol. 37* (1996) 1–7.

*32.* J. Yang, E. Rasa, P. Tantayotai, K.M. Scow, H. Yuan, K.R. Hristova, Mathematical model of *Chlorella minutissima* UTEX 2341 growth and lipid production under photoheterotrophic fermentation conditions, *Bioresour. Technol. 102* (2011) 3077–3082.

*33.* J. O'Grady, J.A. Morgan, Heterotrophic growth and lipid production of *Chlorella protothecoides* on glycerol, *Bioprocess Biosyst. Eng. 34* (2011) 121–125.

*34.* Y.H. Chen, T.H. Walker, Biomass and lipid production of heterotrophic microalgae *Chlorella protothecoides* by using biodiesel-derived crude glycerol, *Biotechnol. Lett. 33* (2011) 1973–1983.

*35.* J.C. Ogbonna, H. Tanaka, Night biomass loss and changes in biochemical composition of cells during light/dark cyclic culture of *Chlorella pyrenoidosa*, *J. Ferment. Bioeng. 82* (1996) 558–564.

*36.* S. Boussiba, A. Vonshak, Astaxanthin accumulation in the green alga *Haematococcus pluvialis*, *Plant Cell Physiol. 32* (1991) 1077–1082.

*37.* N.F.Y. Tam, Y.S. Wong, Effect of ammonia concentrations on growth of *Chlorella vulgaris* and nitrogen removal from media, *Bioresour. Technol. 57* (1996) 45–50.