Bioresource Technology 292 (2019) 121887

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Characteristics of external carbon uptake by microalgae growth and associated effects on algal biomass composition

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ARTICLE INFO

Keywords:
Microalgae
Organic carbon
Growth characteristics
Elemental composition

ABSTRACT

Water eutrophication may be affected not only by nutrients but also the coexisting organic carbon. In order to reveal the effect of external carbon on algal growth, an experimental study was conducted using *Chlorella vulgaris* as the representative microalgae to investigate their growth under varied N and P levels with/without added glucose at TOC = 18 mg/L. The TOC consumption by microalgae growth depended much on N and P concentrations and N/P ratio especially when P was sufficient. This ultimately increased the specific growth rate and resulted in higher N and P accumulations but lower carbon fixation in algal biomass in contrast to non-TOC addition. The biomass dry weight became much lower with TOC addition, along with an apparent change of algal composition shown by the much lower chlorophyll contents in the microalgae cells, which might associate the extent of two carbon fixation pathways – anabolism vs catabolism.

1. Introduction

Discharge of untreated domestic sewage, non-standardized industrial and agricultural wastewater deteriorates water quality and enhances nutrient loads in lakes or rivers causing eutrophication (Hansen et al., 2017; Lürling et al., 2016). Microalgae are the base of the trophic chain and have significant effects on stability of the aquatic ecosystem. Severe algal blooms are a primary eutrophication symptom, resulting in high turbidity and anoxic conditions that cause fish kills and disturb the balance of ecological structure and function (Bhagowati and Ahamad, 2018). Green algae is one of the most important functional groups that plays a central role in trophic transfers in aquatic ecosystems, particularly in inland lakes (Cao et al., 2016). *Chlorella vulgaris* is green eukaryotic microalgae that can be cultivated in autotropic, heterotrophic and mixotrophic cultures. The composition and structure of *C. vulgaris* is assumed to reflect nutrient stress through cell assimilation in lake environments (Safi et al., 2014). Although the idea of limiting nitrogen (N) and phosphorus (P) contents to prevent algae blooms dates back almost half a century, there is still continuous interest in coupled cycling of C, N and P using typical microalgae species as indicators to understand the cause and mechanism underlying eutrophication (Dupas et al., 2015; Jarvie et al., 2018; Li et al., 2018).

Carbon sources, including carbon dioxide (CO_2) and organic carbon uptake are the main energy conversion and utilization processes of microalgae. Carbon consumption and fixation in biomass are directly affected by the supply of nutrients and control microalgal production (Gao et al., 2019; Li et al., 2018). The most common growth mode for microalgae is autotrophic cultivation using CO_2 and light, while the heterotrophic mode only utilizes organic compounds without light illumination (Gao et al., 2019; Safi et al., 2014). The mixotrophic mode uses both light and external organic compounds to provide more energy and intermediates for cellular growth and metabolism (Li et al., 2018). Adequate light intensity contributes to the generation of photo-assimilated compounds through CO_2 assimilation and conversion into chemical energy for cell growth (Sacristán de Alva et al., 2018). Furthermore, microalgae have a much higher growth potential with organic carbon supply (Li et al., 2014a). It has also been illustrated that nutrient limitations significantly affect the intrinsic physiological properties and principal component proportions of microalgae (Markou and Georgakakis, 2011). Microalgae accumulate N and P in biomass for chlorophyll, protein and rRNA synthesis with sufficient nutrient contents, while the nitrogenous compounds are consumed and slow down protein synthesis as well as cell division rates under nutrient limited conditions (Pancha et al., 2014). Microalgae adjust N and P contents of

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https://doi.org/10.1016/j.biortech.2019.121887
Received 7 June 2019; Received in revised form 23 July 2019; Accepted 24 July 2019
Available online 25 July 2019
0960-8524/

their inner biomass depending on the nutrient supply in the medium (Beuckels et al., 2015). Some recent studies have mainly focused on growth, nutrient uptake, carbohydrate content, lipid accumulation, fatty acid methyl ester (FAMEs) and biomass production of microalgae for bio-products utilization with carbon supply levels of several $g{\cdot}L^{-1}$ and total N and P concentrations of 25–250 $mg{\cdot}L^{-1}$ and 6–17 $mg{\cdot}L^{-1}$, respectively (Gao et al., 2019; Gupta et al., 2016; Li et al., 2018; Nzayisenga et al., 2018; Sacristán de Alva et al., 2018; Song and Pei, 2018), while others have paid more attention to microalgae-based wastewater treatment (Beuckels et al., 2015; Luo et al., 2016). However, the nutrient range detected in the aquatic environment is far lower than the above mentioned data (Huang et al., 2018a; Li et al., 2019; Wang et al., 2019). Yao et al. (2018) investigated 38 lakes representing different nutrient concentrations and different eutrophication levels in China, and the average N and P contents were 1.73 ± 0.423 and 0.35 ± 0.033 $mg{\cdot}L^{-1}$, with maximum N and P contents of 9.17 ± 0.406 and 1.68 ± 0.147 $mg{\cdot}L^{-1}$, respectively. As C, N and P contents vary in the aquatic environment, linking flexible N and P loads to assess microalgal growth with/without an external organic carbon source and identifying the effects on microalgal growth and biomass characteristics is very necessary. Therefore, a more stringent, reliable and meaningful investigation is needed (Cao et al., 2016; Poikane et al., 2019).

Based on the above discussion, *C. vulgaris*, which can grow under both autotrophic and heterotrophic culture conditions, was selected for further investigation. This study aimed to determine to what extent *C. vulgaris* can be influenced by the carbon source at aquatic environment levels of N and P, and to explore the effect of carbon source availability on *C. vulgaris* growth characteristics and the associated effects on biomass composition. The total organic carbon (TOC) consumption rate, carbon fixation ability, specific growth rates, growth model parameters, biomass production, photosynthetic pigments and elemental composition of the microalgae with and without glucose addition at multiple combinations of N and P were quantified. The results will provide a new perspective for further investigations of microalgal growth in the aquatic environment.

2. Material and methods

2.1. Microalgae cultivation

Wild *C. vulgaris* (FACHB-24) was obtained from the Freshwater Algae Culture Collection at the Institute of Hydrobiology, FACHB-collection, Wuhan, China. The *C. vulgaris* varieties were initially preserved on agar jelly to avoid bacterial contamination, which was inoculated and activated in BG11 medium under photoautotrophic conditions during two cultivation periods. The 500 mL stoke cultures were maintained in 1000 mL sterilized Erlenmeyer flasks. The initial inoculation density was 2×10^5 $cells{\cdot}mL^{-1}$. The microalgae were placed in an incubator at 25 °C with continuous illumination of 45–50 μmol photon $m^{-2}{\cdot}s^{-1}$ light intensity using a white fluorescent light (12/12 h light/dark periods). The pH of the medium was adjusted to 7.1 using 1 M NaOH or HCl with a WTW pH3110 SET1 pH meter (Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany). All chemicals used in this study were of analytical grade. All glasswares were autoclaved at 121 °C for 30 min before use, and the inoculum was added under a sterile hood to ensure that the cultures were of a single variety. In order to evaluate how the carbon source and various nutrients supplied affected growth and carbon assimilation of *C. vulgaris*, the cultivation medium was based on autoclaved BG11 medium with varying concentrations of N and P for *C. vulgaris* growth testing with or without additional organic carbon. A concentrated glucose solution was added to the BG-11 medium after sterilization using a 0.22 μm filter to provide the organic carbon source. According to the Class V ranking of the environmental water quality standard (GB3838-2002), the glucose concentration in the medium was kept at a maximum chemical oxygen demand limit of 40 $mg{\cdot}L^{-1}$ (TOC = 18 $mg{\cdot}L^{-1}$). For both cultivation groups with or without glucose addition, a total of 25 treatments prepared with different concentrations of N (1, 2, 3, 6 and 8 $mg{\cdot}L^{-1}$, as $NaNO_3$) and P (0.1, 0.2, 0.4, 0.6 and 1 $mg{\cdot}L^{-1}$, as K_2HPO_4) in all possible combinations are referred to as the Class V ranking of GB3838-2002. The design of the experiment was replicated three times and the results were taken as the average of triplicate cultivations (mean ± standard deviation [SD], n = 3).

2.2. Microalgae growth measurements

2.2.1. Growth rates

Microalgal cell density was determined daily using a laboratory-scale algal density meter (Cellometer® auto T4, Nexcelom Bioscience, Lawrence, MA, USA) based on accurate cell counting of the collected samples each with a small volume of 20 μL. The specific growth inhibitory rate (μ) was calculated by the following equation:

$$\mu = \frac{\ln N_1 - \ln N_0}{t_1 - t_0} \quad (1)$$

where N_1 is the cell density of the test group at t_1, and N_0 is the cell density of the control group at t_0.

2.2.2. Photosynthetic pigments

Total chlorophyll and carotenoid contents were measured using the method described by Lichtenthaler and Buschmann (2001) with modifications (Huang et al., 2018b). Absorbance of the samples at 664, 649 and 470 nm was determined with a visible spectrophotometer (TU-1901, Beijing Purkinje General Instruments Co., Ltd., Beijing, China), and the concentrations of chlorophyll *a* (c_a), chlorophyll *b* (c_b) and the carotenoids (c_{x+c} = xanthophylls + carotenes) were calculated using the following equations:

$$c_a(\mu g{\cdot}mL^{-1}) = 13.36OD_{664} - 5.91OD_{649} \quad (2)$$

$$c_b(\mu g{\cdot}mL^{-1}) = 27.43OD_{649} - 8.12OD_{664} \quad (3)$$

$$c_{x+c}(\mu g{\cdot}mL^{-1}) = (1000OD_{470} - 2.13c_a - 97.64c_b)/209 \quad (4)$$

2.2.3. Carbon, nitrogen and phosphorus measurements

A sample of the microalgal culture was centrifuged at 5000 rpm for 10 min, and the supernatant was filtered through a 0.45 μm membrane to determine TOC, nitrate-N (NO_3^-) and phosphorus-P (PO_4^{3-}). TOC was measured with a total organic carbon analyzer (TOC-VCPN, Shimadzu, Tokyo, Japan). Two to three replicates were measured for each sample, giving a relative standard deviation of < 2%. N and P contents were determined based on Chinese state standard testing methods (Monitoring Methods for Water and Wastewater, 2002).

The substrate consumption rate (Ri) was calculated using Eq. (5):

$$\mathrm{Ri} = \frac{S_0 - S_t}{t_t - t_0} \quad (5)$$

where S_0 is the initial substrate concentration ($mg{\cdot}L^{-1}$) as TOC, TN or TP at t_0, and S_t is the corresponding substrate concentration ($mg{\cdot}L^{-1}$) at t_t.

2.2.4. Biomass dry weight and elemental composition analyses

The microalgal biomass was harvested by centrifugation (10 min, 8000 rpm) for the elemental composition analysis at the end of cultivation (day 9). The pellet was freeze-dried and stored at −40 °C in a lyophilizer for further experiments (Boyikang, Model FD-1-50, Beijing, China). Total carbon and nitrogen contents in the dry microalgal biomass (1–2 mg) were quantified in tin capsules and were placed in an elemental analyzer (Vario PYRO cube, Elementar Co., Hanau, Germany) with an auto sampler at the end of the experiment. C and N in the samples were converted to CO_2 and NO_x gas via combustion at 1020

°C (Liu et al., 2016). Helium was used as the carrier gas in the system to bring the combustion gases to the gas chromatography separation and thermal conductivity detector. Sulfanilamide was used as the calibration standard. Elemental P was pretreated by diluting 1 mg dry microalgal biomass in Milli-Q water and subjecting it to a Hach digestion instrument (DBR200, Hach, Loveland, CO, USA). The P concentration was measured based on Chinese state standard testing methods (Malachite green-phosphomolybdic acid spectrophotometry). The detection range was 0.001–0.3 $mg{\cdot}L^{-1}$.

Microalgal biomass dry weight was detected by filtering 20 mL samples in solution through a cellulose acetate membrane filter (0.45 μm). The membrane was dried at 105 °C for 3 h and subsequently cooled to room temperature before weighing (Li et al., 2014b). The dry cell weight of the microalgae was obtained by subtracting the dry weight of the membrane blank from the loaded filter. Triplicate measurements were made for each sample during the experiment.

At the end of the cultivation period, the volume of the remaining microalgae and the weight of the freeze-dried microalgal biomass pellet were measured. The biomass carbon contents in dry cells were determined with the elemental analyzer. Fixed carbon was calculated using Eq. (6):

$$\text{Fixed carbon } (\text{mg } C{\cdot}L^{-1}) = C \times DW/V - C_i \quad (6)$$

where C is the total carbon concentration (% of dry weight) in the final cultivated dry microalgal biomass, and DW (mg) and V (L) are the dry weight and volume of the microalgal culture at the end of the cultivation period. C_i is the initial carbon concentration (mg $C{\cdot}L^{-1}$) in the inoculated cell biomass.

2.3. Kinetic modeling

Most kinetic models describing microalgal growth are expressed as a function of a single nutrient. These models are usually classified into Monod and Droop models, depending on whether the nutrient supply is in the cultivation medium or in the microalgal biomass, respectively (Eze et al., 2018).

2.3.1. Monod model

The Monod model assumes that growth rate is controlled by an external nutrient concentration. Models in this group are always widely applied for convenient data acquisition. To describe the microalgal growth rate in the presence of low nutrient concentrations and further explore the difference between the growth of *C. vulgaris* with/without added glucose, the Monod model (Eq. (7)) was used to describe the relationship between the maximum specific growth rate $\mu_{max,\ N(P)}$ and initial N and/or P concentration.

$$\mu = \frac{\mu_{max,\ N(P)}}{1+\frac{K_S}{S_{N(P)}}} \quad (7)$$

where μ_{max} and μ are the maximum specific growth rate and the specific growth rate of microalgae (day^{-1}); $S_{N(P)}$ is the substrate concentration ($mg{\cdot}L^{-1}$) of N and P; and Ks is the saturation constant for the substrate.

2.3.2. Droop model

The Droop model proposes the concept of cell quota (the amount of intercellular nutrients per cell), which is based on the assumption that the growth rate of *C. vulgaris* depends on the internal nutrient concentration in the cell. The Droop equation has been applied to describe algal growth in natural ecosystems with N or P as a limiting nutrient (Eq. (8)).

$$\mu = \mu_{max,N(P)}\cdot\left(\frac{Q_{N(P)} - Q_{min,\ N(P)}}{Q_{N(P)}}\right) \quad (8)$$

where μ_{max} and μ are the maximum specific growth rate and the specific growth rate of microalgae (day^{-1}), respectively; $Q_{N(P)}$ and $Q_{min,\ N(P)}$ are the cell quota of internal nitrogen and phosphorus for growth and the minimum N(P) cell quota (mg N(P)·g^{-1} DW).

Because of the technical difficulty measuring the nutrient quota, as well as a lack of clear interpretation of the quota, the Droop model is limited and rarely used. This study proposed a new direction to measure cell quota and investigated the parameters of *C. vulgaris* growth with/without glucose addition. The cell quota can be divided into N cell quota and P cell quota, which can be estimated from the obtained data. $Q_{N,(P)}$ is the N(P) content in the biomass that can be obtained by the elemental N(P) composition using the method described in Section 2.2.4.

2.4. Data analysis

All experimental measurements in this study were performed in triplicate. IBM SPSS Statistics 20.0 software (IBM Co., Armonk, NY, USA) was used for the statistical analysis. Specifically, the results of three independent experiments in each culture were analyzed, and these values were expressed as mean ± standard deviation. The differences between treatments were tested by one-way analysis of variance. This method was used to determine the differences in the specific growth rates, biomass dry weight, chl-*a*, chl-*b* and carotenoid contents and the elemental composition under supply of various nutrients in the *C. vulgaris* groups without/with added glucose. A p-value < 0.05 was considered significant. First optimization (1stopt) was used to fit the parameters of the growth kinetic models under different nutrient concentrations.

3. Results and discussion

3.1. TOC consumption and carbon fixation

The changes in TOC contents and specific TOC consumption rate of glucose-added *C. vulgaris* at various N-P content combinations are shown in Fig. 1. TOC content decreased rapidly at all N-P levels during the cultivation period due to their fast assimilation by *C. vulgaris* cultivated with glucose addition. As shown in Fig. 1, the TOC consumption rate was less affected by N-P contents when $P \leq 0.2\ mg{\cdot}L^{-1}$. Interestingly, when initial P was $\geq 0.4\ mg{\cdot}L^{-1}$, the TOC consumption rate decreased significantly with the increase in N content. The N/P ratio also affected the TOC consumption rate in this study, particularly when $P \geq 0.4\ mg{\cdot}L^{-1}$, indicating that TOC consumption was influenced by the nutrient supply in the medium. This was attributed to the finding that under the N-P limited conditions, assimilation of carbon is

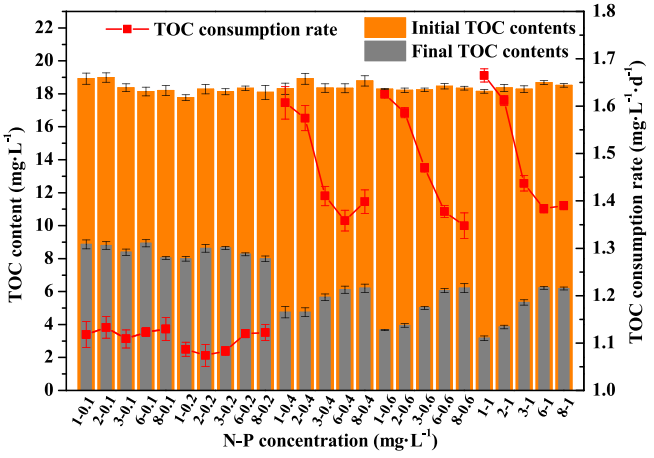

Fig. 1. The changes in total organic carbon (TOC) contents and the TOC consumption rate of glucose-added *Chlorella vulgaris* at various N-P content combinations. Each data point represents the mean of three replicates, and the error bars represent the standard deviation.

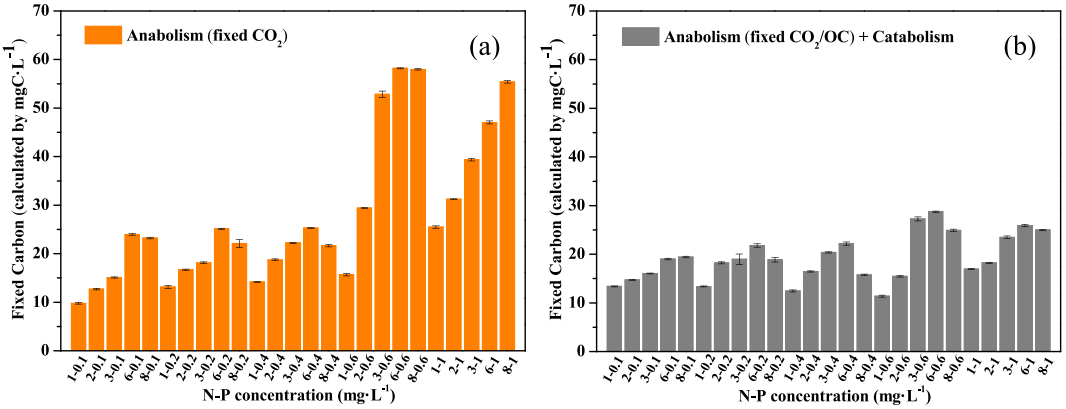

Fig. 2. Microalgal carbon fixation supplemented with various initial N and P concentrations (a) without and (b) with glucose addition. Each data point represents the mean of three replicates, and the error bars represent the standard deviation.

accelerated to synthesize essential components and energy by *C. vulgaris* (Li et al., 2018). Moreover, when the initial TOC/TN ratio is relatively low, *Chlorella* sp. metabolism is less affected by organic matter in the wastewater (Gao et al., 2019). The TOC consumption rate for $P \leq 0.2\ mg{\cdot}L^{-1}$ was always lower than the value for $P \geq 0.4\ mg{\cdot}L^{-1}$ (Fig. 1). This result indicates that phosphorus is important for ATP production and the energy cycle in algal cells (Procházková et al., 2014; Beuckels et al., 2015). A sufficient P supply helps produce energy from mitochondria and then supports transport of organic compounds between the cell compartment and TOC assimilation (Zhai et al., 2017). However, no significant difference ($P > 0.05$) in the TOC consumption rate was observed when $P \leq 0.2\ mg{\cdot}L^{-1}$ at various N contents from 1 to $8\ mg{\cdot}L^{-1}$, indicating that uptake of TOC is closely related to P content. As carbon source uptake is the main mechanism for energy conversion and utilization by microalgae, the consumption rate of carbon would be directly affected by nutrient supply, and further control microalgal production (Gao et al., 2019; Li et al., 2018).

Fixed carbon was calculated based on the biomass carbon data acquired with the elemental analyzer. Fig. 2 shows the fixed CO_2 in the cultivation group without organic carbon addition and fixed carbon (CO_2 + OC) in *C. vulgaris* with glucose addition. Feeding the biomass with CO_2 and proper illumination caused biomass C (%) for the non-TOC added *C. vulgaris* to increase in the cells by photosynthesis. It has been demonstrated that autotrophic microalgae cause a net carbon increase after balancing the consumption of CO_2 for photosynthesis and CO_2 emissions from respiration (Nordlander et al., 2017). In addition to anabolism of CO_2 in autotrophic mode by the TOC-added type of *C. vulgaris*, the increase in total carbon was calculated using the absorption of organic carbon and catabolism by respiration. The fixed carbon source (calculated by mg C·L^{-1}) at various N-P contents without glucose addition was always similar ($N \leq 3\ mg{\cdot}L^{-1}$ and $P \leq 0.2\ mg{\cdot}L^{-1}$) or higher (average of 1.53 times) than the glucose-supplied samples. Maximum C fixation for both cultivation modes without and with glucose were 58.19 mg C·L^{-1} and 28.74 mg C·L^{-1}, respectively. This result shows that microalgae supplied with organic carbon caused a net carbon decrease by catabolism compared with the non-glucose-added type.

3.2. Microalgal growth characteristics with and without external carbon source

3.2.1. Specific growth rate

Fig. 3 shows the specific growth rate of *C. vulgaris* during cultivation with/without glucose addition under various initial N and P concentrations. The glucose-added type of *C. vulgaris* exhibited significantly ($P < 0.05$) more rapid growth at all combinations of N-P contents. Specific growth rate can be accelerated as high as 0.178 d^{-1}

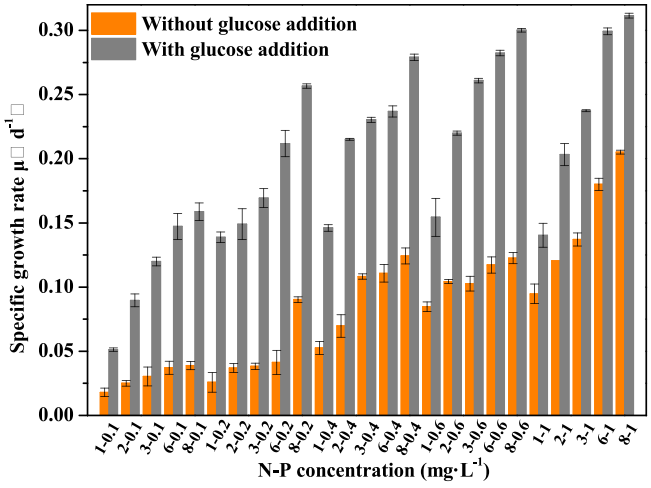

Fig. 3. Specific growth rate of *Chlorella vulgaris* under various N and P levels with/without glucose addition. Each data point represents the mean of three replicates, and the error bars represent the standard deviation.

during the cultivation phase, while the average growth rate of the glucose-added type was 1.37 times higher than that of the non-glucose-added type. Previous studies illustrated that microalgae cultivated with added glucose enhance the acetyl CoA/malonyl CoA pool as a basis for increasing metabolism (Lin and Wu, 2015). The presence of organic carbon altered photosynthesis and metabolism in *C. vulgaris*. Gupta et al. (2016) reported that *C. vulgaris* exclusively transports glucose into microalgal cells, which is further broken down in mitochondria via oxidative phosphorylation to generate ATP for cell division. During the cultivation period, chemical energy (organic carbon) and light energy are simultaneously assimilated to produce ATP and NAD(P)H, so that algal growth is reinforced with a reduced dependency on light utilization (Song and Pei, 2018). These observations suggest that *C. vulgaris* utilizes not only the energy from light in the non-glucose-added mode but also the organic carbon source available for cell anabolism to obtain a remarkably higher growth rate. Moreover, Lin and Wu (2015) reported that the specific growth rate of mixotrophic *C. vulgaris* is significantly higher than the sum of those from photoautotrophic and heterotrophic growth, illustrating independent growth mechanisms. Fig. 3 also shows that the specific growth rates of both types of *C. vulgaris* supplied with/without glucose were significantly ($P < 0.05$) affected by the initial N and P concentrations. These values increased with an increase in initial N (1–8 mg·L^{-1}) or P (0.1–1.0 mg·L^{-1}) concentration

Table 1
Monod parameters of *Chlorella vulgaris* growth at different N and P concentrations with/without glucose addition.

Parameters		$\mu = \mu_{max}\frac{S_N^*}{K_N + S_N}$	$\mu = \mu_{max}\frac{S_P^*}{K_P + S_P}$	$\mu = \mu_{max}\frac{S_N}{K_N + S_N}\frac{S_P^*}{K_P + S_P}$
μmax 10^6 cells·(mL d)$^{-1}$	Without glucose	1.23	1.904	2.99
	With glucose	3.01	2.03	4.13
K_N (K_P) mg·L^{-1}	Without glucose	1.89	0.705	1.575 (0.677)
	With glucose	1.87	0.105	1.964 (0.109)

*Calculated by applying S_N and S_P as the initial N (1–8 mg·L^{-1}) and P (0.1–1.0 mg·L^{-1}) concentrations in the mode with glucose addition (R^2 = 0.9306) and without glucose addition (R^2 = 0.9919).

3.2.2. Growth kinetics

Monod model

The Monod growth parameters of *C. vulgaris* with/without glucose addition in growth medium with different initial nutrient concentrations are shown in Table 1 ($P < 0.01$) through "1stOpt 15PRO". Reasonably, the maximum algal density increased with an increase in the initial N or P concentration in both cultivation types. There was a clear difference between the two growth conditions despite starting with the same inoculation concentrations of nutrients depending on whether external glucose was supplied. The highest specific growth rate values achieved in the glucose-added cultures resulted in a 5.38-fold increase compared to the values obtained in the non-glucose added cultures. The higher μmax of the cultivation mode with added organic carbon resulted in a faster growth rate and a greater increase in cell number during the cultivation period according to the calculated Monod equation.

Droop model

The amount of intercellular N and P per cell can be measured by the elemental composition data based on the method described in Section 2.2.4. This result was expected because the Droop model accurately represents the capability of algae to consume nutrients at rates that exceed immediate requirements for growth. The Droop growth parameters of *C. vulgaris* with/without glucose addition in the growth medium at various intercellular N and P contents are shown in Table 2 ($P < 0.05$). The minimum N cell quota values were 16.67 and 16.62 mg N·(g biomass)$^{-1}$, while the minimum P cell quota values were 2.81 and 6.40 pg P·cell^{-1} for *C. vulgaris* cultivated with/without glucose addition, respectively. This is because the organic carbon supply controls the phosphate uptake rate, which is closely correlated with P quota. Microalgae store surplus P (in the form of P quota) inside of cells, and cellular P significantly decreases during cultivation under a nutrient limited condition (Ou et al., 2014). Addition of carbon source mostly results in a decrease in the P quota when using biomass P as a nutrient source (Klanjšček et al., 2016). The great variation in the minimum P cell quota indicates that the supplied P concentrations (0.1–1.0 mg·L^{-1}) restricted microalgal growth with carbon source addition. The maximum N cell quota of 200 mg N· (g biomass)$^{-1}$ and a higher minimum N cell quota of 14 mg N·(g biomass)$^{-1}$ have been reported to show the great potential of microalgae for exploitation (Adesanya et al., 2014; Scott et al., 2010). In recent modelling studies, the P cell quota in phytoplankton cells was reported to be from 0.083 to 17.6 pg P·cell^{-1} (Klanjšček et al., 2016; Lee et al., 2015; Ou et al., 2014). The values of $Q_{min,\ N\ (P)}$ obtained from this study fall within the range for the N and P cell quota reported in previous studies. The μ_{max} value obtained by the Droop model revealed significantly enhanced growth under the fed-batch cultivation supplied with glucose as a carbon source.

Both models showed that under the same cultivation levels of N (1–8 mg·L^{-1}) and P (0.1–1.0 mg·L^{-1}) supply at any possible combination, the specific growth rate of *C. vulgaris* cultivated with glucose addition was always higher than the non-glucose added type. The glucose-added type of *C. vulgaris* had a stronger ability to absorb and transform nutrients. Adding glucose saves energy output in the form of inorganic CO_2 fixation so that it can be more effectively used in carbon assimilation for cell reproduction (Adesanya et al., 2014), produce sufficient energy by catabolizing TOC via respiration, and convert illumination into chemical energy, which strengthens nutrient uptake and transport; thus, leading to better growth of mixotrophic mode than the autotrophic mode (Li et al., 2018). Similarly, Abreu et al. (2012) reported that all doses of organic carbon used in algae cultivation result in faster growth rates. The data obtained in the present study could provide a reference for fitting a *C. vulgaris* growth model under lower C, N and P conditions.

3.3. Microalgal biomass characteristics with and without external carbon source

3.3.1. Biomass dry weight

Fig. 4 shows the final dry weight (DW) of *C. vulgaris* at the end of cultivation with and without glucose addition under various N and P levels. The final values varied from 28.52 to 107.07 mg·L^{-1} for the non-glucose-added *C. vulgaris* and from 26.52 to 66.58 mg·L^{-1} for the glucose-added type. Interestingly enough, the maximum biomass weight in the culture without glucose addition was 1.79 times higher than that of the cultivation with glucose addition. The final dry weight of the microalgal biomass was about 2.25–60.15% higher for the non-glucose supplied *C. vulgaris* than for the glucose-supplied type when compared pairwise for each nutrient combination. This is inconsistent with most previous findings that mixotrophic cultivation of *C. vulgaris* enhances biomass production up to 4.8 times compared with autotrophic

Table 2
Droop parameters of *Chlorella vulgaris* growth at various intercellular N and P concentrations with/without glucose addition.

Parameters		$\mu = \mu_{max,N}\cdot(\frac{Q_N - Q_{min,N}}{Q_N})^*$	$\mu = \mu_{max,P}\cdot(\frac{Q_P - Q_{min,P}}{Q_P})^*$
$\mu_{max,\ N(P)}$ 10^6 cells·(mL d)$^{-1}$	Without glucose	1.423	1.124
	With glucose	3.261	1.962
Q_{min} mg·g^{-1}	Without glucose	16.62	0.67 (6.40 pg P·cell^{-1})
	With glucose	16.67	0.30 (2.81 pg P·cell^{-1})

*Calculated by applying Q_N and Q_P as the cell quota (mg N (P)·g^{-1} DW) of internal n and p concentrations with carbon source addition and (R^2 = 0.9479) without carbon source addition (R^2 = 0.9946).

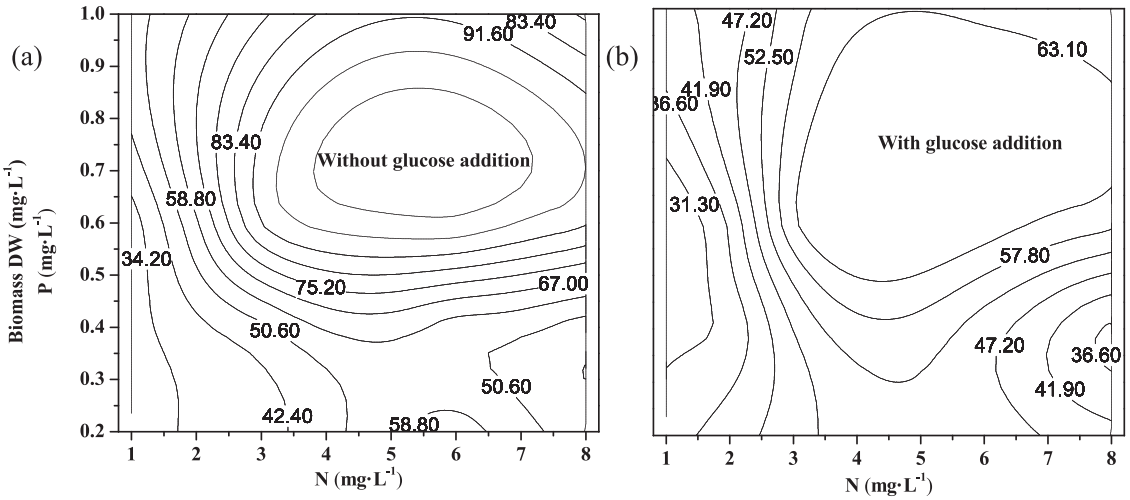

Fig. 4. Biomass dry weight ($mg{\cdot}L^{-1}$) of *Chlorella vulgaris* at the end of cultivation (a) without and (b) with glucose addition under various N and P levels.

conditions (Heredia-Arroyo et al., 2011; Liang et al., 2009; Zhan et al., 2016). However, a similar phenomenon was observed by Gao et al. (2019) when isolating a *Chlorella* sp. strain. This may be attributed to the finding that the glucose content for microalgal cultivation in this study was relatively low (TOC = 18 $mg{\cdot}L^{-1}$). Under an insufficient supply of exogenous organic carbon during the logarithmic growth phase, cultivated *C. vulgaris* would utilize intracellular substances, such as carbon, to lose weight and maintain growth conditions by heterotrophic metabolism (Wang et al., 2016; Gao et al., 2019). It was also demonstrated that heterotrophic growth was the dominant growth mode when microalgae grew in the mixotrophic cultures in the presence of glucose. Once the glucose was consumed, microalgae switch from heterotrophic to photoautotrophic metabolism by slowing down the increase in algal dry weight (Deng et al., 2018). These results also confirm the fact that an increased maximum growth rate does not necessarily correlate with increased biomass production under the same conditions (Paranjape et al., 2016). The lower dry weight production suggested that lower contents of glucose (TOC = 18 $mg{\cdot}L^{-1}$) might have been assimilated and converted to energy directly leading to cell growth but not converted into storage compounds that increase biomass dry weight. Biomass production was also affected by N and P supply. The final dry weight of *C. vulgaris* increased with the N supply at lower N content in the medium and was reduced by about 6.82% and 19.64% at higher nitrogen content for *C. vulgaris* cultivated with/without glucose addition. The biomass yields of both types at higher N contents were also significantly ($P < 0.05$) affected by the P concentration. The dry cell weight increased with P supplied at lower levels in the medium and was reduced by about 7.55% and 24.5% at higher P content for *C. vulgaris* cultivated with/without glucose addition (Fig. 4a, b). Similarly, Beuckels et al. (2015) reported that there is a link between N and P supply and biomass yield to show a marginal influence of nutrient supply.

3.3.2. Photosynthetic pigments

The microalgal pigments decreased greatly after fed-batch cultivation with glucose addition compared with the non-glucose-added mode and different nutrient levels (Fig. 5). Chl-*a*, Chl-*b* and carotenoid contents in *C. vulgaris* increased gradually at the beginning of the cultivation period and stabilized or leveled off after 6–9 days in the non-glucose-added mode and after 3–9 days in the glucose-added mode. Chlorophyll has been considered an intracellular nitrogen reservoir for cell growth under nitrogen starvation, as it is rich in nitrogen (Deng et al., 2018). The reduction of pigments during cultivation could be explained by a nitrogen limitation. Cultures with/without external carbon source addition consume the nitrogenous pigments to maintain the intracellular nitrogen quota for normal metabolic functions (Jiao et al., 2017). At the end of the cultivation period, the average decrease in chl-*a*, chl-*b* and carotenoid contents at all nutrient supply combinations of the glucose-added type were 36.88, 33.41 and 25.97% compared with the non-glucose-supplied type, respectively. Mondal et al. (2017) reported that chlorophyll contents decrease under heterotrophic period in the absence of light during mixotrophic cultivation, which changes the color of the algal cells from dark green to yellowish because chlorophyll production is mainly stimulated by light. The metabolism of photosynthetic pigments can be affected by the consumption of external organic carbon sources through lowering photosynthetic yield and altering respiration (Song and Pei, 2018). The "glucose-bleaching effect" has also been proposed to explain the decline in pigment composition when glucose serves as the carbon source, as the photosynthetic rate decreased with the assimilation of external carbon for cell growth, and the microalgae downregulate chlorophyll synthesis to conserve energy (Gupta et al., 2016). Chl-*a*, chl-*b* and carotenoid contents in both types of *C. vulgaris* all increased significantly ($P < 0.05$) with the elevated N supply at fixed P contents. Therefore, the gradual decrease of pigments in the glucose-added mode could also be explained by advanced N stress. This result is in keeping with the finding that glucose induces and activates the hexose/H^+ symport system to facilitate uptake of nutrients during N deficiency (Deng et al., 2018).

3.3.3. Elemental composition

N is an important macro element contributing to biomass production (1–10% of total mass) and energy transportation, and it is essential for many functional components of algae, including structural proteins and enzymes, nucleic acids and chlorophyll (Kube et al., 2018). P is also essential for the biosynthesis of phospholipids, proteins and nucleic acids, and is critical to the energy cycle of algal cells (Procházková et al., 2014). The elemental composition of N and P increased considerably after fed-batch cultivation with glucose addition compared with the non-glucose-added mode (Fig. 6a, b). The *C. vulgaris* cultivated with glucose addition contained more biomass N (4.76 ± 1.80%) and biomass P (0.71 ± 0.44%) than the non-glucose-supplied *C. vulgaris* with 4.66 ± 1.32% for N and 0.52 ± 0.24% for P. This is because the faster growing rate resulted in a more rapid uptake of nutrients and extra energy from assimilation of organic carbon, which could be used to synthesize amino acids proteins, phospholipids, nucleic acids and other essential N and P-containing components, as observed in this study and that of Sacristán de Alva et al. (2018). Under both trophic modes, the elemental N levels in the biomass were reasonably high when the supply of N in the medium was high and low when the nutrient supply was low. The elemental P contents showed a downward

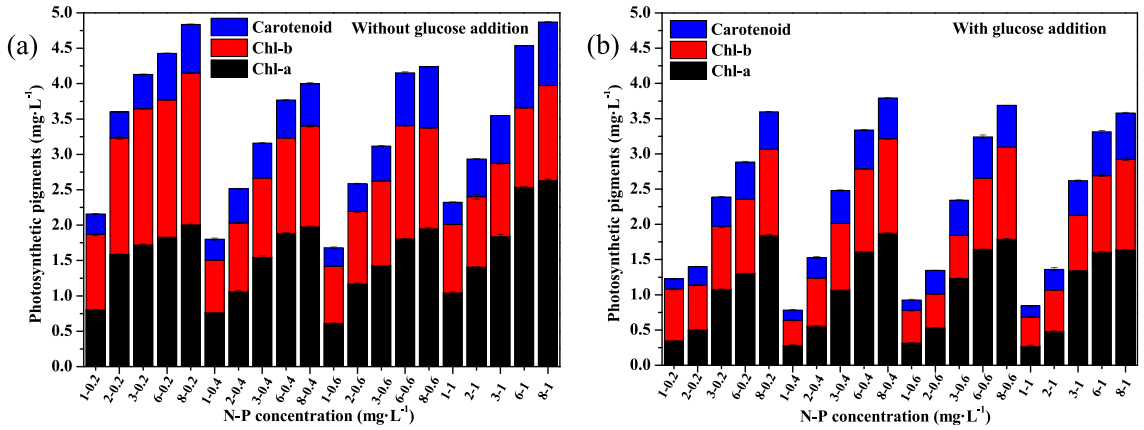

Fig. 5. Composition of chlorophyll contents (chl-*a*, chl-*b* and carotenoid) in *Chlorella vulgaris* cells on day 9 (a) without and (b) with glucose addition under various nutrient levels. Each data point represents the mean of three replicates, and the error bars represent the standard deviation.

trend with the increase of the N supply. This observation can be explained by the function of N and P in cellular synthesis. The increase of N accelerated the synthesis of amino acids, which integrate into proteins and form sugars, lipids and starch (Xin et al., 2010), while elemental P is utilized to reduce ribosomes for enhanced cell division and to form ATP for the energy cycle (Beuckels et al., 2015). This finding should be taken into account when exploring to what extent the C, N or P supply affect the microalgal elemental composition in the aquatic environment.

The nutrient conversion efficiency of N and P during cultivation mode with/without glucose addition was calculated to determine if the consumption of nutrients from the medium matched the accumulation of nutrients in the microalgal biomass. Table 3 shows the average nutrient conversion efficiency of both glucose and non-glucose added *C. vulgaris* at fixed nitrogen or phosphorus contents. On average, conversion efficiency was 57.79 ± 4.17% for N and 45.56 ± 5.92% for P in *C. vulgaris* cultivated without an added carbon source and 73.16 ± 10.90% for N and 77.68 ± 7.42% for P in *C. vulgaris* cultivated with glucose addition. The nutrient conversion ability of glucose-added *C. vulgaris* was always higher than that of the non-glucose-supplied type to show the influence of the carbon source on nutrient transformation and accumulation capacity.

Table 3
The N and P conversion efficiency of *Chlorella vulgaris* with/without glucose addition at various nutrient levels.

Conversion efficiency	Nutrient contents ($mg{\cdot}L^{-1}$)	Without glucose	With glucose
Nitrogen (%)	N1 = 1	56.64 ± 5.33	69.85 ± 11.31
	N2 = 2	51.41 ± 13.99	55.52 ± 6.59
	N3 = 3	59.48 ± 10.51	82.13 ± 5.34
	N4 = 6	62.67 ± 7.07	78.42 ± 9.46
	N5 = 8	58.73 ± 10.89	79.89 ± 6.12
Phosphorus (%)	P1 = 0.1	37.17 ± 6.03	86.32 ± 5.92
	P2 = 0.2	43.07 ± 9.66	74.47 ± 9.21
	P3 = 0.4	52.61 ± 1.23	82.23 ± 11.00
	P4 = 0.6	45.69 ± 2.65	78.39 ± 3.31
	P5 = 1.0	49.26 ± 7.15	66.99 ± 11.28

The data are expressed as mean ± standard deviation of three replicate flasks.

4. Conclusions

Carbon fixation is an important process associated with photosynthesis. Although the actions of nutrients have been clarified by numerous studies, little was known on the effect of organic carbon on algal growth. The present study revealed that comparing with autotrophic photosynthesis where CO_2 fixation provided the sole carbon source, the utilization of dissolved organic carbon resulted in significant change in the growth rate, biomass content, and elemental composition of the microalgae cells. These preliminary findings may direct future studies on the effect of carbon source type and concentration on algal growth, which may assist eutrophication control for aquatic environment improvement.

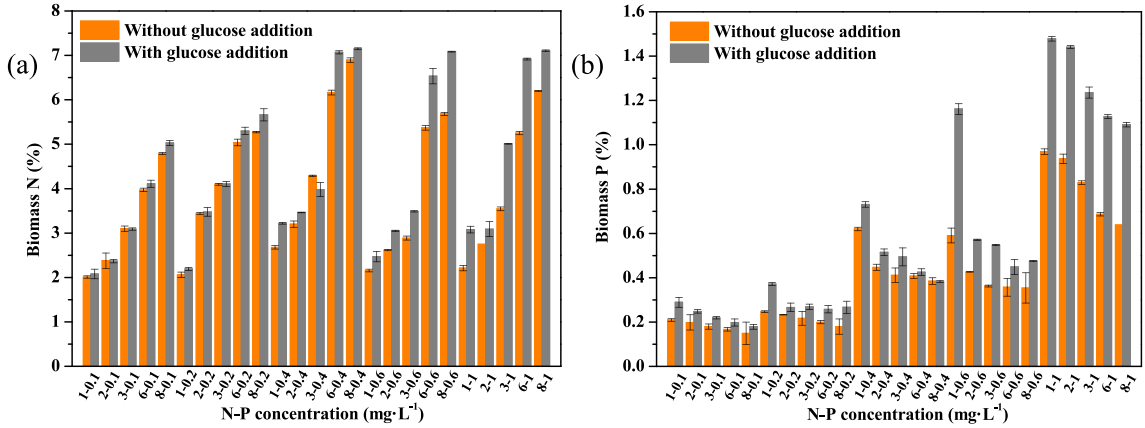

Fig. 6. Biomass N and P concentrations (% of dry weight) of *Chlorella vulgaris* at the end of cultivation (a) without and (b) with glucose addition under various N and P levels. Each data point represents the mean of three replicates, and the error bars represent the standard deviation.

Acknowledgements

This work was supported by the National Natural Science Foundation of China No. 51778522, the Scientific Research Program Funded by Shaanxi Provincial Education Department (grant number 18JS056), the Science Foundation for Fostering Talents of Xi'an University of Architecture and Technology (RC1721) and the Program for Innovative Research Team in Shaanxi Province (No. 2013KCT-13). E-supplementary data of this work can be found in online version of the paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2019.121887.

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