Original Research Article

Effects of some nutritional factors on the growth of *Chlorella vulgaris* in a mixotrophic cultivation

ABSTRACT

The microalgae *Chlorella vulgaris* is not only known as a source of lipid compounds for the biofuel industry but also as a potential source of biomass production for aquaculture. This study investigated the influence of some nutritional factors (organic carbon source and C:N ratio) on the growth rate of *Chlorella vulgaris*. Glucose, sodium acetate, and sucrose were used to check the effect of the carbon sources. Meanwhile, five different ratios of C:N (1:1, 6:1, 12:1, 18:1, and 24:1) were tested. Results showed that glucose was the most suitable source of organic carbon for the biomass development of the *Chlorella vulgaris*; the ratio C:N=18:1 equivalent to 52.92mmol/Lcarbon and 2.94 mmol/L nitrogen is the most appropriate ratio to promote biomass increase. The maximum growth rate of *C. vulgaris* recorded in this study was 0.58±0.03 day^{-1} in treatment supplemented with glucose at ratio C:N=18:1.

Keywords: Chlorella vulgaris; mixotrophic; organic carbon; C:N ratio

1. INTRODUCTION

Microalgae not only play an essential role in natural ecosystems but also have been extensively applied in various fields of production and life. They are regarded as a renewable source of feed and an important natural food source for aquaculture. In particular, microalgae are exploited for use as functional food and pharmaceuticals for humans [1]–[4].

Microalgae can be grown under different conditions, including autotrophic, heterotrophic, and mixotrophic growth. In autotrophic growth, most microalgae perform photosynthesis for development (photoautotrophic), and thus, light is necessary. As the microalgae density increases, the demand for light also increases [5]. Natural light is difficult to provide stably to meet this demand (due to significant fluctuations depending on weather and difficulty to adjust to the optimal range for microalgae), leading to the use of artificial light sources in production. However, this has led to the problem of energy costs for large-scale models [6]. Additionally, the ability to increase biomass productivity is also significantly limited by the "self-shading" phenomenon, which hinders the ability of microalgae cells in the culture to receive light.

Mixotrophic growth overcomes these limitations because when an organic carbon source is added to the culture medium, microalgae can use both energy sources to develop. Thus, mixotrophic growth is not only capable of increasing biomass productivity but also promotes a much higher accumulation of other compounds than autotrophic culture [7].

In mixotrophic cultivation, finding appropriate organic carbon sources to ensure high biomass yield, stability, and at the same time meet production cost requirements is essential. Glucose, glycerol, and acetate have been demonstrated to be effective in enhancing the

growth rate of some microalgae species [4,8,9]. However, the effectiveness level depends on the strain and specific cultivation conditions. In addition, adding organic carbon can enhance or inhibit microalgae growth depending on nitrogen concentration. Therefore, the mixotrophic growth process requires strict control of the C:N ratio to optimize the growth and accumulation of target compounds [10]. For instance, the microalgae *Tetraselmischuii* was reported to obtain a high density and biomass at C:N ratios of 16 and 12 while it synthesized more polysaccharide at a C:N ratio of 24 [11].In the experiments of Silaban et al. for a Louisiana native coculture of microalgae (*Chlorella vulgaris*) and cyanobacteria (*Leptolyngbya sp.*), the highest mean biomass productivity was obtained at a C:N ratio of 15:1 in the medium cultures with sodium acetate [12K].

This study was conducted to identify suitable carbon sources for *Chlorella vulgaris* and investigate the optimal C:N ratio for the growth of this microalgal species. The findings of this study can be applied to enhance the biomass production of *C. vulgaris* at a larger scale.

2. MATERIAL AND METHODS

Microalgae strain

Chlorella vulgaris algae strain is preserved in the Algae Technology Laboratory, Faculty of Biology and Environmental Science, The University of Science and Education, Da Nang University. The strain is cultivated in the BBM (Bold-Basal Medium) environment at a temperature of 25 □, light intensity of 2500 lux, and a light-dark cycle of 18:6.

Experiments on the effects of different organic carbon sources

To evaluate the effects of organic carbon sources on the growth of *C. vulgaris* in mixed nutrient cultivation, three organic carbon sources including glucose, sodium acetate, and sucrose were used at a carbon concentration of 0.236 g/L (Table 1). Besides, a BBM medium without carbon supplementation was used as a control sample. The initial microalgae cell density was 20 x 10^6 cells/mL and the cultural environmental condition (temperature: 25 ℃, light density: 2500 lux, light-dark cycle: 18:6) was similar for all formulas. Each formula was taken in triplication and lasted for 3 days. The algae density and growth rate were monitored daily.

Table 1. Formulas for investigation the effects of organic carbon sources on Chlorella vulgaris' growth

Formula	Cultural medium (C: 0.236 g/L)	Number of samples
Control	BBM	3
1	BBM + glucose	3
2	BBM + sodium acetate	3
3	BBM + sucrose	3

Experiments on the effects of different C:N ratio

To investigate the effects of the C:N ratio on the growth of *C. vulgaris* in mixotrophic cultivation, the experiment was arranged with 12 formulas with five C:N ratios of 1:1, 6:1, 12:1, 18:1, and 24:1 as shown in Table 2. Specifically, the experiments were conducted for 3 days in 250 mL flasks containing microalgae with an initial cell density and the cultural environmental condition like those in the experiments of different organic carbon sources described above. The first experimental group investigated the effect of the C:N ratio with a fixed nitrogen source content of 2.94 mmol/L and increased the carbon content (from 2.94 mmol/L to 70.56 mmol/L) to obtain the respective C:N ratios. Meanwhile, in the second

experiment group, the carbon concentration was fixed at 19.70 mmol/L while the nitrogen concentration was reduced from 19.70 mmol/L to 0.82 mmol/L. All the experiments were also conducted in parallel with a control sample of carbon and nitrogen concentrations of 19.7 mmol/L and 2.94 mmol/L, respectively. Each formula was repeated 3 times, and the experiment lasted for 3 days. The density and growth rate of the microalgae in each formula was assessed every day throughout the culture period.

Table 2. Carbon and nitrogen concentrations in each formula

C:N ratio	1:1	6:1	12:1	18:1	24:1
Concentration of C: N (mmol/L) (N fixed)	2.94:2.94	19.70:2.94	35.28:2.94	52.92:2.94	70.56:2.94
Concentration of C: N (mmol/L) (C fixed)	19.70:19.70	19.70:2.94	19.70:1.64	19.70:1.01	19.70:0.82

Microalgae density and growth rate identification

Algae cells were observed under a 4X objective lens of a Hund (H6000) microscope, photographed on a Neubauer counting chamber (0.1 mm height), and counted using ImageJ software.

The growth rate μ (d^{-1}) of the microalgae was calculated using the formula:

$$\mu = \frac{(ln(N_1) - ln(N_2))}{t}$$

where: N_1, N_2 are the cell densities at two different time points (cells/mL) and t is the time interval between two measurements.

Data analysis

The collected data were analyzed using descriptive statistical analysis. The results were expressed as mean ± standard deviation (SD). The statistical significance of differences between the groups under investigation was assessed by analysis of variance (ANOVA) with p-values of less than 0.05 was regarded as significant. All data processing steps were carried out using the R software [13].

3. RESULTS AND DISCUSSION

3.1 Influence of organic carbon sources on the growth of *Chlorella vulgaris* in mixed culture

The results showed that *C. vulgaris* grew best in glucose-supplemented medium with a growth rate of 0.50±0.04 day^{-1}, significantly higher than other treatments (*p-values*< 0.05) (Figure 1, Table 3). Specifically, the growth rate was 1.2 times higher than that in the control treatment (autotrophic culture 0.44±0.008 day^{-1}). In contrast, the addition of sodium acetate and sucrose to the culture medium did not stimulate the growth and development of *C. vulgaris* in mixed culture, as their growth rates (0.46±0.02 day^{-1} and 0.43±0.008 day^{-1}, respectively) were not significantly different from the control treatment (*p-values*> 0.05).

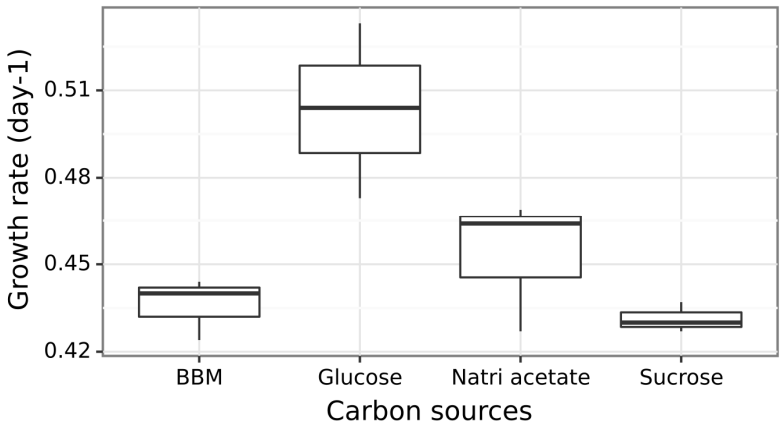

Fig. 1. The growth rate of *C. vulgaris* in different carbon sources supplemented media

Table 3. The growth rate of *C. vulgaris* in different carbon sources

Media	BBM (control)	BBM + Glucose	BBM + Natri acetate	BBM + Sucrose
Growth rate (day^{-1})	0.44±0.008	0.50±0.04	0.46±0.02	0.43±0.008

Observing the growth curve (Figure 2), it can be seen that the algae density did not change much after 24 hours of the experiment. The difference in growth became significant after 2 days when the density of the glucose-supplemented treatment spiked to 107×10^6 cells/mL (Figure 2). After that, the density continued to increase slightly to 115×10^6 cells/mL at the end of the experiment. The cell density in the sodium acetate and sucrose-supplemented media did not show significant differences from the control treatment, with an average density of about 90×10^6 cells/mL.

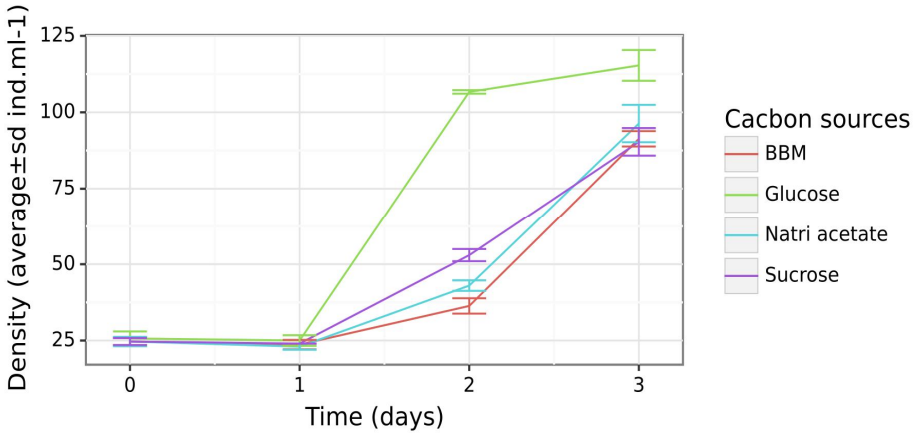

Fig. 2. Growth curve of C. vulgaris in different carbon sources supplemented media

Carbon is one of the essential elements for cell development, so the concentration and source of carbon used in the nutrient medium have a significant effect on biomass and lipid accumulation in algae. As the use of CO_2 and organic carbon sources occurs simultaneously in mixed culture conditions, the supply of CO_2 and organic compounds need to be optimized to achieve the best productivity.

In this study, *C. vulgaris* showed the best growth performance in a medium supplemented with glucose due to the stimulating effects of light and CO_2 on growth and an increase in the rate of glucose metabolism under nutrient-rich conditions [14], [15]. These findings are consistent with the study by Yun et al. in 2021, which also identified glucose as the best organic carbon source for enhancing biomass productivity and the concentration of valuable bio-compounds in algae [16].

One reason why adding carbon from sources such as sodium acetate and sucrose did not promote growth compared to standard nutrient conditions (BBM) is that cells lack the enzymes to co-metabolize the two carbon sources [17]. Tian et al. suggested that disaccharides such as sucrose are difficult to use in cultivation and nutrition because there is no extracellular sucrase to hydrolyze sucrose and accumulate monosaccharides [18]. When sucrose was supplemented with a type of yeast found in the culture medium of infected algae, the density reached $151.2x10^6$ cells/mL in just two days because the yeast was able to hydrolyze sucrose and accumulate monosaccharides [18]. Compared to other carbon sources, glucose requires less complex metabolic exchange processes to provide energy for algal development [19].

Acetate is one of the most commonly used organic carbon sources for mixed cultivation. Numerous studies have shown that supplementing with sodium acetate can promote both growth and lipid accumulation in many algae species such as *Chlamydomonasreinhardtii*, *Haematococcuspluvialis*, *Chlorella sorokiniana*, *Chlorella sp.*, *Nannochloropsissp*… [20], [21]. However, it should be noted that acetate may not always promote growth rate and can be toxic to many species at high concentrations. In this study, when sodium acetate was added at a concentration of 0.236 g/L, the growth rate of the algae increased, but not significantly compared to the control group (*p-values*> 0.05).

3.2. The influence of the C:N ratio on the growth of *Chlorella vulgaris* in mixotrophic cultivation

The research results showed that the C:N ratio had a significant impact on the growth of *C. vulgaris* algae in mixed nutrient cultivation (Figure 3). When the nitrogen concentration was fixed at 2.94 mmol/L and the carbon concentration was gradually increased to the desired C:N ratios, the growth rate tended to increase proportionally with the carbon concentration in the cultivation medium. *C. vulgaris* algae exhibited the best growth after 3 days of cultivation in a medium with a C:N ratio of 18:1, corresponding to 52.92 mmol/L carbon and 2.94 mmol/L nitrogen. The growth rate recorded in this medium was 0.58 ± 0.03 day^{-1}, which was 1.2 times higher than that of the control medium with a C:N ratio of 6:1 (0.49 ± 0.018 day^{-1}) (Table 4).

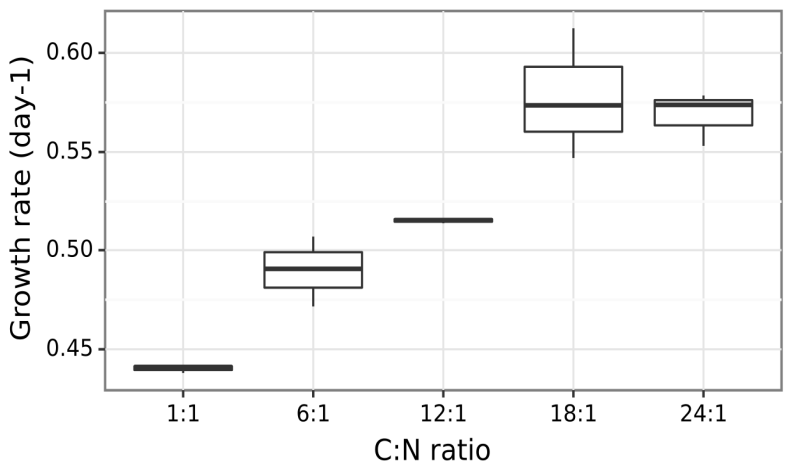

Fig. 3. The growth rate of *C. vulgaris* at different C:N ratios (with a fixed nitrogen concentration of 2.94 mmol/L)

Table 4. The growth rate of *C. vulgaris* at different C:N ratios

C:N ratio	1:1	6:1	12:1	18:1	24:1
Concentration of C: N (mmol/L)(N fixed)	2.94:2.94	19.70:2.94	35.28:2.94	52.92:2.94	70.56:2.94
Growth rate (day^{-1})	0.44±0.002	0.49±0.018	0.52±0.001	0.58±0.03	0.57±0.01
Concentration of C: N (mmol/L)(C fixed)	19.7:19.7	19.70:2.94	19.70:1.64	19.70:1.01	19.70:0.82
Growth rate (day^{-1})	0.255±0.03	0.49±0.018	0.37±0.019	0.41±0.047	0.35±0.032

Conversely, when the carbon concentration was fixed at 19.70mmol/L and the nitrogen concentration was varied, the results were not optimistic (Figure 4). Increasing or decreasing the nitrogen concentration compared to the control medium (C:N= 6:1) both resulted in lower growth rates. In the medium with a C:N ratio of 1:1, the growth rate after 3 days was only 0.255±0.033 day^{-1}, while in higher C:N ratios (12:1, 18:1, 24:1), the growth rate was about 0.37 to 0.41 and 0.35 day^{-1}(Table 4). These values were significantly lower than those of the control medium (0.49±0.018 day^{-1}) (*p-values*< 0.05).

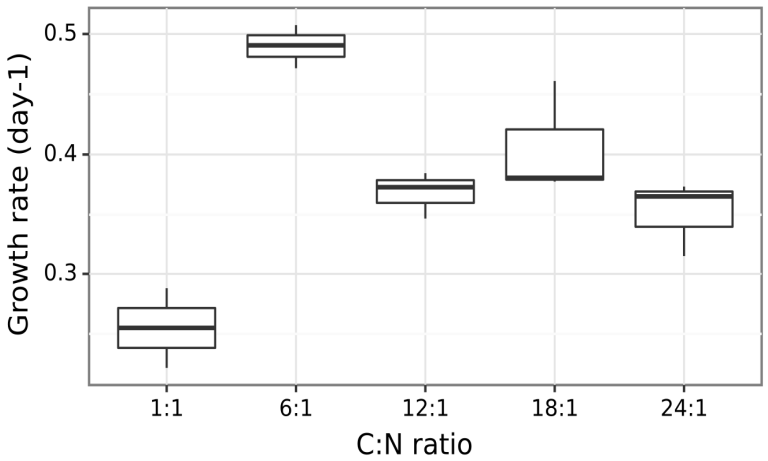

Fig. 4. The growth rate of *C. vulgaris* at different C:N ratios (with a fixed carbon concentration of 19.698 mmol/L)

The comparison of the results of two experiments on the influence of C:N ratio showed that *C. vulgaris* algae grew best in the experiment with C:N ratio of 18, corresponding to around 10.5 g/L glucose. This result is similar to the study by Liang et al. in 2009 [21], who reported that the optimal glucose concentration for growing *C. vulgaris* was 1% (10 g/L) [21]. In the experiment with increased carbon concentration, the growth rate of algae increased proportionally to the amount of carbon supplied to the nutrient environment, except for the experiment with fixed C:N ratio of 24:1. Excessive glucose concentration inhibited the growth of *C. vulgaris* algae [20], [21]. Ward et al. suggested that a high C:N ratio typically stimulates lipid accumulation in algae, but nitrogen deficiency compared to carbon is also a limiting factor for the overall growth of the algae community [22]. The results from the experiment with fixed carbon concentration and decreased nitrogen concentration in this study also support this conclusion.

4. CONCLUSION

This study demonstrates that mixotrophic cultivation is an effective method to enhance the growth rate of *C. vulgaris*. Among the carbon sources investigated, glucose was found to be the most favorable in promoting growth rate at 0.52±0.02 day^{-1}. Additionally, the C:N ratio was also considered to optimize biomass productivity. The growth rate of C. vulgaris was positively correlated with carbon concentration in an experimental range of C:N ratio of from 1:1 to 18:1. At a higher C:Nratio, the microalgal growth rate began to decrease. Our results showed that the algae reached the highest growth rate (0.58±0.03 day^{-1})in a nutrient medium containing 52.92mmol/L carbon (from glucose) and 2.94 mmol/L nitrogen, corresponding to a C:N ratio of 18:1. Aside from biomass productivity, C. vulgaris is also a good source of is a good source of protein and lipid. Therefore, it is suggested that future studies should be implemented to identify the suitable C:N ratio for achieving the highest protein and lipid accumulation.

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