Original Research Article

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

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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*. 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 43,2 mmolC/L and 2,94 mmolN/L 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 days-1 in treatment supplemented with glucose at ratio C:N=18:1.

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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].

Thus, 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

Chlorella vulgaris algae strain is preserved in the Algae Technology room, Faculty of Biology-Environment, University of Education - University of Da Nang. The strain is kept 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.

The experiment on the effect of some organic carbon sources on the growth of *C. vulgaris* algae in mixed nutrient cultivation, three organic carbon sources including glucose, sodium acetate, and sucrose were used at a carbon concentration of 0.236g/L. A control formula using BBM medium without carbon supplementation was used as a control. Each formula was repeated 3 times. The experiment lasted for 3 days, and the algae density was monitored daily.

For the experiment on the effect of C:N ratio on the growth of *C. vulgaris* algae, ratios of 1:1, 6:1, 12:1, 18:1, and 24:1 were studied by changing the carbon and nitrogen concentrations compared to the control medium (C=19.7 mmol/L, N=2.94 mmol/L). The specific concentrations in each formula are shown in Table 1. Each formula was repeated 3 times, and the experiment lasted for 3 days. Algae density was monitored daily.

Table 1. Carbon and nitrogen concentrations in each formula

Rate C/N	**1:1**	**6:1**	**12:1**	**18:1**	**24:1**
Rate C/N (N fixed)	2.94:2.94	19.70:2.94	35.28:2.94	52.92:2.94	70.56:2.94
Rate C/N (C fixed)	19.70:19.70	19.70:2.94	19.70:1.64	19.70:1.01	19.70:0.82

The growth of *C. vulgaris* algae was evaluated by monitoring the cell density during the cultivation process. The cell density was checked daily. Algae cells were observed under a 4X objective lens, photographed on a Neubauer counting chamber, and counted using ImageJ software. The growth rate was calculated using the formula

$$\mu = \frac{\left(ln(N1) - ln(N2)\right)}{t}$$

where: N1, N2 are the cell densities at two different time points and t is the time interval between two measurements.

The collected data were analyzed using descriptive statistical analysis. The statistical significance of differences between the groups under investigation was assessed by analysis of variance (ANOVA). All data processing steps were carried out using the R software [11].

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 0.52±0.02 day^{-1}, significantly higher than other treatments (*p-values*< 0.05)

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(Fig. 1). Specifically, the growth rate was 1.2 times higher than that in the control treatment (autotrophic culture - 0.43±0.01 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±0.01 day^{-1} and 0.45±0.02 day^{-1}, respectively) were not significantly different from the control treatment (*p-values*> 0.05).

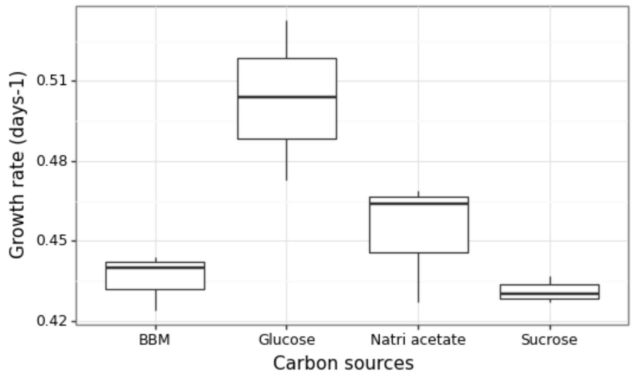

Fig. 1. The growth rate of C. vulgaris in different carbon sources supplemented media

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 107×10^6cells/mL. 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.

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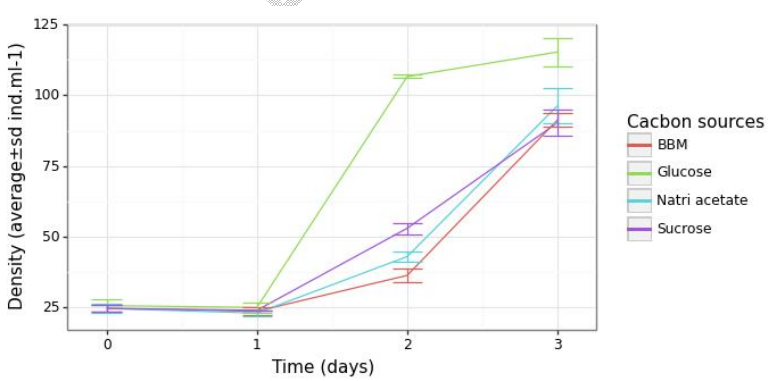

Fig. 2. Growth curve of C. vulgaris in different carbon sources supplemented media

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 [12], [13]. 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 [14].

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 [15]. 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 [16]. When sucrose was supplemented with a type of yeast found in the culture medium of infected algae, the density reached 151.2×10^6 cells/mL in just two days because the yeast was able to hydrolyze sucrose and accumulate monosaccharides [16]. Compared to other carbon sources, glucose requires less complex metabolic exchange processes to provide energy for algal development [17].

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 *Chlamydomonas reinhardtii*, *Haematococcuspluvialis*, *Chlorella sorokiniana*, *Chlorella sp.*, *Nannochloropsissp*… [18], [19]. 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 mmolC/L and 2.94 mmolN/L. 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±0.02 day^{-1}).

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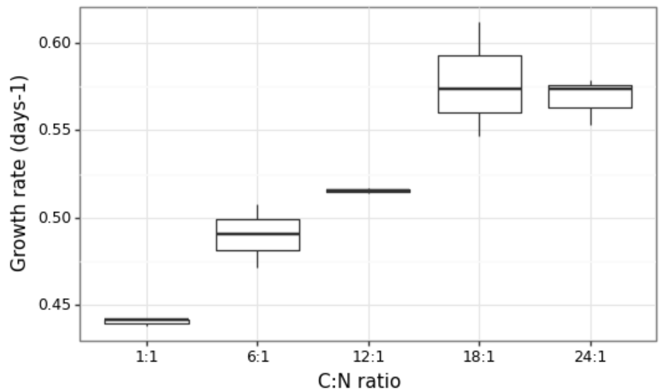

Fig. 3. The growth rate of *C. vulgaris* at different C:N ratios (with a fixed nitrogen concentration of 2.94 mmol/L)

Conversely, when the carbon concentration was fixed at 19.698 mmol/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 0.35 to 0.4 day^{-1}. These values were significantly lower than those of the control medium (0.49±0.02 day^{-1}) (*p-values*< 0.05).

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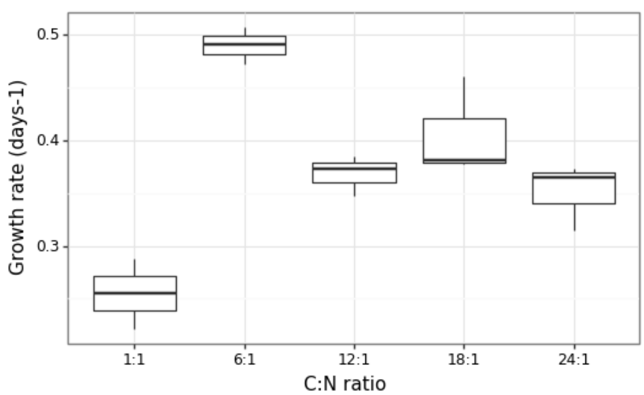

Fig. 4. The growth rate of *C. vulgaris* at different C:N ratios (with a fixed carbon concentration of 19.698 mmol/L)

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 [19], who reported that the optimal glucose concentration for growing *C. vulgaris* was 1% (10 g/L) [19]. 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 [18], [19]. Ward et al. suggested that a high Carbon:Nitrogen 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 [20]. 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. In a nutrient medium containing 43.2 mmolC/L (from glucose) and 2.94 mmolN/L, corresponding to a C:N ratio of 18:1, the algal growth rate was the highest at 0.58±0.03 day^{-1}.

Comment [TEP9]: Briefly explain the effect of the C:N ratio and make suggestions for improvements in future research

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