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INFLUENCE OF ESSENTIAL NUTRIENT COMPOSITION OF CULTURE MEDIA OF *CHLORELLA VULGARIS* ON CHLOROPHYLL PRODUCTION

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ABSTRACT

This study focused on the concentration of nutrients (nitrogen and phosphorus) and the substitution of Carbon source from Glucose for $NaHCO_3$ affected microalgal growth and chlorophyll a. Microalgal strain of *Chlorella vulgaris* SAG 211-19 were grown in media that increased or decreased by half of $NaNO_3$ and KH_2PO_4 and replaced of $NaHCO_3$ with glucose compared to control samples cultured in Bold's Basal Medium (BBM). The results showed that chlorophyll a concentrations varied with fluctuations in essential nutrients such as nitrogen, phosphorous, and carbon. Specifically, as reducing the concentration of sodium nitrate salt in the exponential phase by half, the chlorophyll a of synthetic microalgae only reached 7.7 mg/L while the control sample was at 12.45 mg/L. Meanwhile, with reducing or increasing KH_2PO_4 by half in BBM, chlorophyll synthesis was abundant with values recorded for 13.5 or 14.5 mg/L, respectively. Chlorophyll a that was extracted and measured at the end of culture showed the same trend as during the exponential phase, but there was a slight increase in its concentration when the final biomass was harvested. Finally, the chlorophyll a of the microalgal biomass obtained was 11.4 mg/L; 11.7 mg/L; 14.6 mg/L; and 15.75 mg/L, respectively, in culture media where the concentration of $NaNO_3$ or KH_2PO_4 is increased by one and a half times or reduced by half.

Keywords: chlorophyll, essential nutrients, microalgal cultivation, microalgal harvesting.

INTRODUCTION

Microalgae are recognized as an important source of valuable functional products, including proteins, lipids, polysaccharides, minerals, vitamins, pigments and polyunsaturated fatty acids (PUFA), which are commercially value and beneficial for health. Among these compounds, natural pigments are particularly promising for exploitation. Besides their role as coloring agents, these microalgal pigments are evaluated for health benefits such as antioxidant, anti-cancer, anti-inflammatory properties, as well as their effectiveness as artificial colorants (Guiry, 2012; Schoefs, 2002; Sun *et al.*, 2023). Three types of pigments are commonly found in microalgae: phycobiliproteins, carotenoids, and chlorophylls.

On the other hand, chlorophyll is utilized as a colorant because of its capacity to selectively absorb light at specific wavelengths, making green easily identifiable. Fluctuations in market demand and regislations have necessitated the use of natural colorants in food products instead of artificial ones. Nowadays, coloring is crucial for both consumers and manufacturers, as many foods lose their natural color due to chemical processes during processing and production. Consumers seek products with their original appearance intact, while manufacturers strive for uniformity across all products. Chlorophyll in plants is associated with chloroplasts, where it forms complexes not only with phospholipids, polypeptides, and tocopherols but also benefits from protection by a hydrophobic membrane (Humphrey, 2004; Spears, 1988; Timberlake & Henry, 1986). However, during processing, chlorophyll molecules are exposed to weak acids, oxygen, or light, accelerating their oxidation and leading to the formation of numerous degradation products. This phenomenon is exemplified by the existence of two types of chlorophyll: chlorophyll a and chlorophyll b. Meanwhile, scientists have identified chlorophyll a and b two typical pigments crucially involved in photosynthesis. The first pigment, chlorophyll, is the primary in photosynthesis, storing light energy and releasing high-energy electrons to two photosystems. The latter, chlorophyll b, is a secondary pigment that transfers trapped energy to the chlorophyll a. Therefore, the fundamental difference between chlorophyll a and b lies their roles in photosynthesis. Chlorophyll a is found in all photosynthetic organisms on earth, imparting them their green color, while chlorophyll b contributes to their yellow-green color *(Halim et al.,* 2010; Silva *et al.*, 2020). In this study, chlorophyll a was chosen as the primary compound for investigation. We examined whether altering the microalgae's culture environment affects the production of this natural pigment.

MATERIALS AND METHODS

Strain and growth conditions

In line with previous research, *Chlorella vulgaris* SAG 211-19 (*C. vulgaris* SAG 211-19) was initially cultivated in 400ml of BBM medium with the following ingredients: 189.7 mg/L of N in 1151.98 mg/L $NaNO_3$; 140.00 mg/L $MgSO_4$. $7H_2O$, 22.8 mg/L of P in 100.00 mg/L KH_2PO_4; 120 mg/L of C in 840.00 mg/L $NaHCO_3$, 25.00 mg/L $CaCl_2.2H_2O$, and 0.5 mL of micro-mineral solution. The culture conditions included LEDs light with an intensity of 150 µmoL/m^2/s for 12 hours followed by darkness for the remaining period. For easy of further manipulations, a correlation between microalgal growth's absorbance at 680 nm and its chlorophyll-a content was determined using the method described by Nguyen *et al.,* 2014.

The culture media of *C.vulgaris* were manipulated by decreasing and augmenting the nitrogen composition of $NaNO_3$ by half (M1 and M2), adjusting the phosphorous compound KH_2PO4 (M3 and M4), and substituting $NaHCO_3$ with 450 mg/L of glucose (M5).

Experimental setup

A starter medium was made by adding an initial concentration of 5% v/v microalgae with a cell density (OD680 nm) of 0.8 into the BBM medium to grow the biomass in a 250 mL Erlenmeyer flask, and this culture medium was also used as a control sample as performing experimental series from M1 to

M5. The growth of *C. vulgaris* was noted daily by absorbance for final chlorophyll a assessment before harvest. Specifically, the growth rate of each sample is extracted to evaluate the life cycle of this strain in different environments in order to be able to use linear regression between cell density and chloropyll a with OD < 1.0. Visually, since *C. vulgaris* was at the phase of death, the chlorophylla a was recorded.

Methods

Linear regression related to cell density and chlorophyll a

Quantification of the pigments was conducted by measuring the absorbance spectrum following their extraction with methanol.

The culture volume V1 is centrifuged (Minispin, Eppendorf) at 13,400 rpm for 5 minutes. The medium is aspirated using a Pasteur pipette, followed by the addition of volume V2 of methanol for pigment extraction. The suspension is then kept in darkness for one hour in an oven at 44 °C.

At the end of the incubation, the samples are centrifuged for 5 minutes at 13,400 rpm to remove cellular debris. The absorbances of the supernatant at the following wavelengths (652, 665 and 750 nm) are then measured using a spectrophotometer (Labmed, USA). The concentrations of chlorophyll a, expressed in mg/L, is deduced from the absorbances measured by the following relationships (Ritchie, 2006):

$$C_{Chl_A} = [-8{,}092\,.\,(OD_{652} - OD_{750}) + 16{,}5169\,.\,(OD_{682} - OD_{750})]\frac{V_2}{V_1\,.\,l}$$

Analysis

The extraction of chlorophyll a from microalgal cultures was performed as illustrated above. Statistical analyses were conducted using one-way ANOVA followed by Tukey's post hoc test for comparisons. Data with $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Linear regression related to cell density and chlorophyll a

The linear regression relating cell density to absorbance at 680 nm and chlorophyll a concentration was constructed by the following equation: y = 0.106x, where y represents absorbance at 680 nm (OD680), x is chlorophyll a (mg/L), and R^2 = 0.9962 (Figure 1). Using this graph, the sampling days during the culture period can be simplified by measuring the OD680 and then substituting the value into the linear equation to determine chlorophyll a concentration in just a few seconds. However, this graph is accurate only when the biomass concentration of microalgae does not exceed 1.0 g/L.

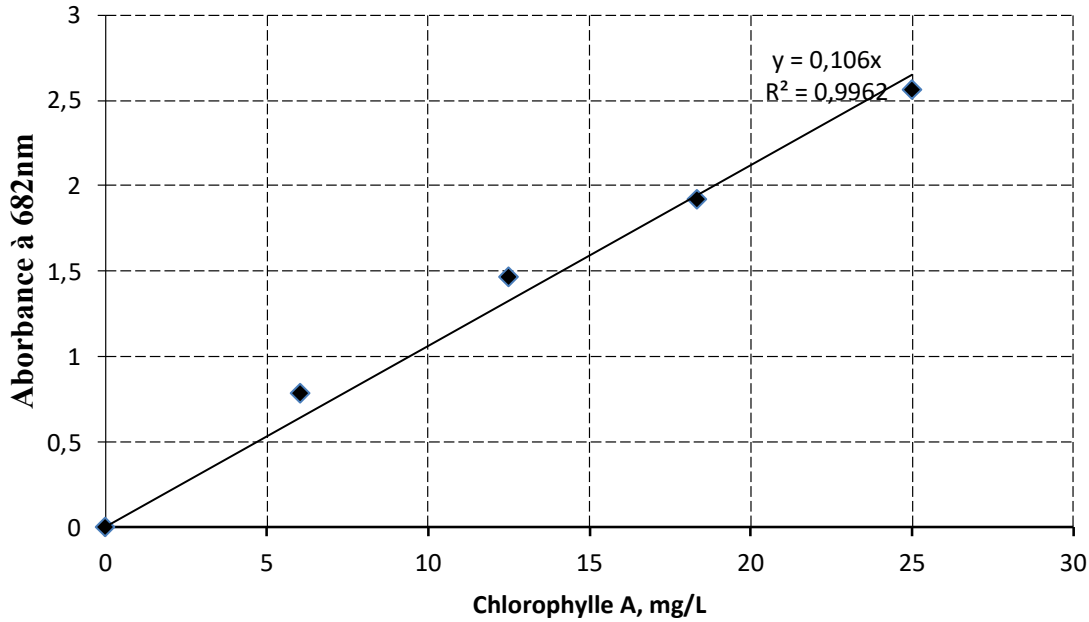

Figure 1. The correlation of cell density and chlorophyll a.

Determination of chlorophyll a in the fluctuation of essential nutrient composition of microalgal culture media

Because chlorophyll a shows a green color, it is visually evident that changes in a medium, such as increasing or decreasing $NaNO_3$ or KH_2PO_4 by half, and replacing $NaHCO_3$ with glucose, result in noticeable differences in green color. In Figure 2, although the decrease or increase in $NaNO_3$ concentration of essential nutrients for microalgal growth also affects the chlorophyll a, the green color of microalgae becomes pales compared to the control sample when the $NaNO_3$ concentration is reduced by half. In particular, the green color of chlorophyll a is replaced by the yellow-green one of chlorophyll b when this salt concentration is increased by half. On the contrary, changes in phosphorus concentration, whether increasing or decreasing, make the green color of microalgae darker than the control sample. Similarly, replacing $NaHCO_3$ by glucose also makes the chlorophyll of the microalgae darker green than the control sample. These initial results have created the basis for changes in nutrien composition of culture medium to the ability of chlorophyll synthesis. However, to get a more convincing answer than the observational results, the data in Figure 3 was drawn.

In Figure 3, changing the essential nutrients in the culture medium such as N, P and C influenced on the chlorophyll a production. Previous studies have clarified that Nitrogen is an essential macronutrient for microalgal growth, which contributes to the synthesis of proteins, lipids and carbohydrates. For instance, reseatch by Yodsuwan *et al*. (2017) published that *Phaeodactylum tricornutum*, a marine diatom, had high lipid accumulation during photoautotrophic nitrogen-deficient cultivation. In the study, it was demonstrated that the total nitrogen

(TN) – total phosphorous (TP) have a close relationship with chlorophyll a in the predicted regenerative lake layers, which was linear, calculated using the trophic ecoregions based on the synthesis of Omernik Level III ecoregions. On the other hand, some studies suggested that low N and P ratios gave microalgae a competitive advantage and that abundant N availability limited phytoplankton growth. In fact, the tendency of eutrophic lakes to have low N and P ratios is thought to be a plausible reason why cyanobacteria tend to outgrow the phytoplankton in eutrophic lakes. More specifically, the findings suggested that cyanobacteria are outgrown in lakes with low N and P ratios in general and that decreasing nitrogen input as a phytoplankton control measure would be a waste of effort and was almost unsuccessful because cyanobacteria can easily substitute for the missing nitrogen through nitrogen fixation. Therefore, scientists recommended that water managers should focus on reducing P to increase the N/P ratio in water resources. (Downing & McCauley, 1992; Smith, 1983; Yodsuwan *et al*., 2017; Yuan & Pollard, 2014). Meanwhile, another ratio that also interests scientists is the ratio of carbon and chlorophyll a. The ratio of phytoplankton carbon to chlorophyll a (Cph:Chl a) is quite different in many environmental conditions such as coastal and oceanic, even though the optical layer in both environments is the same. Therefore, this high ratio found in the ocean may be due to Cph variation, as insignificant differences in illumination with similar photolayers would not effect the Chl a content (Crespo *et al*., 2011). In this study, the results regarding the change of C source in the microalgal culture medium are also consistent with their findings.

Figure 2. Changes in chlorophyll a in culture media are based on visual observations.

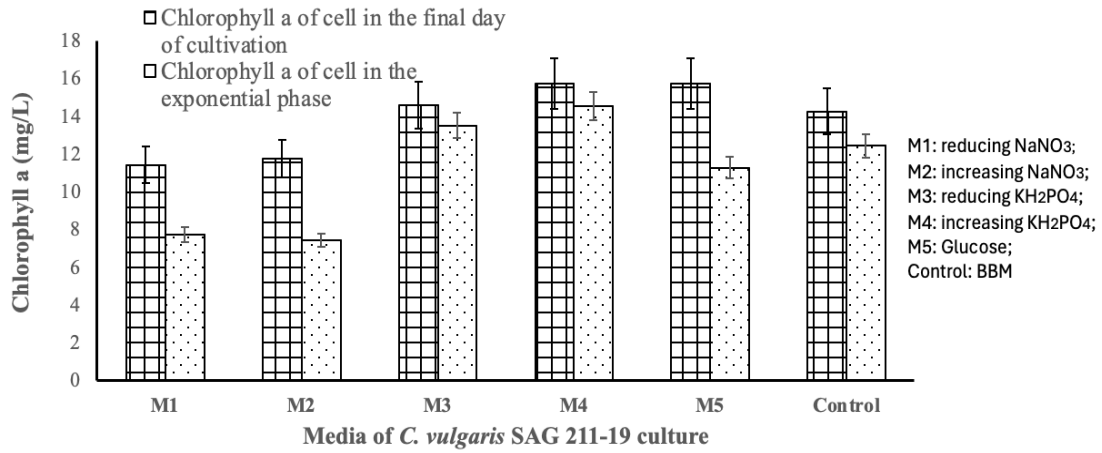

Figure 3. Chlorophyll a of *C. vulgaris* – a function of essential nutrient concentration.

Furthermore, this study provided a different perspective on the relationship between main nutritional components, such as N and P starvation or excess, as well as carbon sources, and chlorophyll a in the growth phase of *C. vulgaris*. As shown in Figure 3, the chlorophyll a obtained from the extraction of *C. vulgaris* cells in the exponential phase exhibited a higher ratio of Chlorophyll and growth rate. This can be explained by the fact that, during the process of cell division, stress in culture conditions can increase the growth rate, leading to an increase in the chlorophyll synthesis. Essential nutrients like phosphorous are crucial for the growth and cell division of *C. vulga*ris, and their requirements vary widely among different microalgal species. Phosphorus starvation is an effective environmental stress to induce lipid accumulation. *Scenedesmus* sp. accumulated more lipids as phosphorus decreased. In this study, when the phosphorus concentration was reduced or increased, the growth rate of the microalgae increased, leading to a rise in chlorophyll a in the cells (Xin *et al*., 2010; Yaakob *et al.*, 2021).

CONCLUSION

This study investigated the influence of essential nutrients in the culture media of *C.vulgaris* on chlorophyll synthesis. The results indicated that chlorophyll a in the microalgae decreased significantly when the $NaNO_3$ concentration in the culture medium was either reduced or increased. However, when the KH_2PO_4 concentration was reduce or increase chlorophyll a levels increased compared to the control sample.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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