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Modifications of major pigment content of *Chlorella vulgaris* cultured in different media

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Abstract. Currently, to avoid the harmful effects of artificial dyes, scientists strongly encourage the use of natural pigments derived from organisms such as microalgae. Although microalgae are known to outperform all other microorganisms in terms of pigment production, their natural occurrence is insufficient for the increasing commercial demand. Therefore, further enhancing the pigment production in the algae cells is of tremendous significance. Some environmental factors known to regulate natural pigment synthesis are changes in pH, temperature, light, salinity and nutrients of the environment. This study indicates that further manipulation of major nutrient parameters, such as nitrogen (N), phosphorus (P), and carbon (C), in the culture medium can be the easiest way to intervene among other physical parameters with the aim of cultivating microalgae at an industrial scale and commercial production. It also emphasises that if the N content is doubled compared with the standard Bold's Basal Medium (BBM), the Chlorophyll *a* content obtained will be higher, reaching (37 ± 0.08) mg/L, while doubling the P content cannot increase chlorophyll production but increases carotenoid synthesis with the highest content compared with the remaining media. Similarly, replacing the C source of glucose with $NaHCO_3$ also changes the synthesis of pigments, and the concentrations of chlorophyll *a*, *b* and carotenoids are all higher than in BBM. Therefore, the results of this study might indicate that if the N/P ratio is doubled compared with its ratio in the BBM medium, the ability to synthesise chlorophyll and carotenoids will increase. This will be the basis for cultivating microalgae in other environments besides the traditional BBM medium to collect chlorophyll or pigments.

Keywords: chlorophyll, carotenoids, microorganism, microalgae culture media, pigment

1 Introduction

The food industry is one of the most important sectors in the world with a high demand for food in the global market. Most food products contain dyes, ranging from natural to synthetic pigments. Plant pigments are chemicals responsible for the colourful appearance and visual appeal of fruits and vegetables. Their consumption has been associated with a reduced risk of various diseases in humans [1]. Artificial pigments are the most commonly used in the cosmetic, food, and pharmaceutical industries because of their commendations related to colour ranges, costs, antioxidant capacity, and solubility. Meanwhile, natural dyes have become more attractive worldwide because of their therapeutic and medical effects and the high toxicity of synthetic colourants. However, natural pigments have numerous shortcomings, such as a lack of stability and low bioavailability and reproduction [2]. Therefore, microalgae are currently considered a promising solution to these drawbacks and the production of valuable compounds because of their farming advantages compared with other plants [3]. For example, valuable pigments derived from microalgae and cyanobacteria, such as astaxanthin, β-carotene, phycocyanin, phycoerythrin, and fucoxanthin, have been

exploited and applied in human life in recent years [4, 5]. Although microalgae are known to outperform other organisms in terms of pigment, their natural production still struggles to meet the growing commercial demand. Therefore, efforts to enhance their production yield are necessary regarding research on changing environmental factors in microalgae cultivation and molecular biological aspects. Some environmental factors affecting and regulating natural pigment synthesis are fluctuations in pH, temperature, light, salinity and nutrients of the microalgal culture media [6].

In this study, we study the changes in the nutrient composition of the microalgae culture media to demonstrate their influence on the synthesis of natural pigments. Changes in major nutrients, such as nitrogen, phosphorus, and carbon, have also been studied in previous research. These composition changes have also been shown to influence the growth of microalgae cells.

2 Materials and methods

2.1 Strain and growth conditions

Microalgae strain of *Chlorella vulgaris* SAG 211-19 was cultivated in the laboratory of The University of Danang – University of Technology and Education (16°04'36.9"N 108°12'49.0"E). The medium chosen for the starter culture was Bold's Basal Medium (BBM) [7] with ingredients in mg/L as follows (Table 1):

Table 1. Ion composition of microalgal culture medium with 0.5 mL/L of Hutner's trace elements solution [8]

Ion	Concentration (mg/L)	Type of salt
Ca^{2+}	6.8	$CaCl_2{\cdot}2H_2O$
Mg^{2+}	13.8	$MgSO_4$
Na^{+}	545.5	$NaHCO_3$
K^{+}	28.7	KH_2PO_4
Cl^{-}	12.7	$CaCl_2{\cdot}2H_2O$
NO_3^{-}	840.4	$NaNO_3$
HCO_3^{-}	610	$NaHCO_3$
SO_4^{2-}	54.6	$MgSO_4$
PO_4^{3-}	69.8	KH_2PO_4

* Na^{+} is calculated as the sum of Na^{+} composition in $NaNO_3$ and $NaHCO_3$.

2.2 Experiment design

In this study, the pigments were monitored for changes in concentration according to the variation in the main nutritional components of the culture medium, namely nitrogen (N), phosphorus (P), and carbon (C). Experimental series were established by changing these major ions compared with nitrogen and phosphorus components in Table 1, and the values were raised or reduced by half; component C was replaced with glucose while other components were kept the same. Specifically, the M1 and M2 media had the NO_3^- and PO_4^{3-} components reduced by half to 420.2 mg/L and 35 mg/L, respectively. Besides, the M3 and M4 media had the NO_3^- and PO_4^{3-} components increased by half to 1260.6 mg/L and 104.8 mg/L, respectively. Finally, M5 had 300 mg/L of glucose instead of $NaHCO_3$. After the culture media were prepared, 1% (v/v) of the

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starter culture was added to 250 mL of these media in 500 mL Erlenmeyer flasks. The microalgal growth in the media (M1–M5) and BBM as a control was recorded by measuring the absorption intensity at a wavelength of 682 nm. During microalgal growth monitoring, the suspension in each culture media was withdrawn to record the concentration of pigments every two days. Other culture conditions, such as light intensity of 150 μmol/m²/s, pH of 7.5 and temperature of 25 °C, were set up in the same way as the cultivation of *C. vulgaris*, as described by Nguyen et al. [8].

The microalgae cultivation stage involves a number of essential parameters, including the concentration of essential nutrients, pH, light intensity, and temperature, which require rigid monitoring as they are crucial to achieving efficient biomass productivity and the production of chemical compounds in microalgae cultivation. In this study, essential nutrients chosen to investigate the production of pigments were the N, P, and C resources (Table 2).

Table 2. Composition of nutrients in different culture media (mg/L)

Ion	M1	M2	M3	M4	M5	Control
Ca^{2+}	6.8	6.8	6.8	6.8	6.8	6.8
Mg^{2+}	13.8	13.8	13.8	13.8	13.8	13.8
Na^{+*}	389.62	545.5	701.48	545.5	315.5	545.5
K^{+}	28.7	14.35	28.7	43.05	28.7	28.7
Cl^{-}	12.7	12.7	12.7	12.7	12.7	12.7
NO_3^{-}	420.2	840.4	1260.6	840.4	840.4	840.4
HCO_3^{-}	610	610	610	610	0**	610
SO_4^{2-}	54.6	54.6	54.6	54.6	54.6	54.6
PO_4^{3-}	69.8	35.0	69.8	104.8	69.8	69.8

*Na^{+} is calculated as the sum of Na^{+} composition in $NaNO_3$ and $NaHCO_3$.

**C in $NaHCO_3$ was subtituted with C in 300 mg/L glucose

2.3 Analytical methods

Pigment concentration was recorded by using the spectrophotometric method. Before measuring the optical density (OD) at the wavelengths from 480 to 750 nm, pigments were extracted from microalgae with methanol. According to Richie's method [9], 1 mL of microalgal suspensions in different culture media was collected and transferred into an Eppendorf with 2 mL of methanol. The suspension was placed in an oven at 40 °C for an hour. Then, centrifugation for 7 min at 13400 rpm (Minispin, Eppendorf) was performed to collect the suspension for the next analysis. After measuring the absorbances of the suspension at 480, 652, 665 and 750 nm on a spectrometer (Lambda 2S, Perkin Elmer), the chlorophyll *a*, *b* (Chl *a*, Chl *b*) and carotenoid values were calculated by using the following equations as in [9]

$$[\text{Chl } a]\mu\text{g/mL} = -8.0962 \times \text{OD}_{652} + 16.5169 \times \text{OD}_{665} \quad (1)$$

$$[\text{Chl } b]\mu\text{g/mL} = 27.4405 \times \text{OD}_{652} - 12.1688 \times \text{OD}_{665} \quad (2)$$

$$[\text{Carotenoids}]\mu\text{g/mL} = 4 \times OD_{480} \quad (3)$$

The optical density measured at 480, 652 and 665 nm was corrected from the turbidity by subtracting the optical density measured at 750 nm. During growth monitoring, the concentration of pigments was also determined every two days.

The measurements were performed in three replicates. Significant differences between multiple groups were evaluated by using a one-way analysis of variance (ANOVA) followed by Tukey's test, where $p < 0.05$ was considered significant.

3 Results and discussions

3.1 Chlorophyll

Chlorophylls are the most common pigments present in nature. They are found mainly in photosynthetic organisms such as cyanobacteria, algae, and plants [10]. The amount of chlorophyll in these organisms is an essential indicator, in particular Chl *a*, which is the primary pigment in most known oxygenic photoautotrophic organisms. This work surveyed the impacts of some fluctuations of essential nutrients of microalgal culture media on the amount of Chl *a*.

The graph in Figure 1 displays that the values of Chl *a* and Chl *b* changed significantly according to the composition of essential nutrients in the standard culture medium (control sample), as illustrated in Table 2. During 22-day microalgal cultivation, the growth and chlorophyll production showed a close relationship, specifically with the beginning of the growth phase; the microalgae cells accelerate their division to raise the number of cells, leading to an increase in cell density from day 10 to day 14. This also augments the concentration of Chl *a* in each cell. However, this increase depends on the composition of the culture medium. For example, the concentration of chlorophyll *a* obtained in the M5 medium has the highest value at 37.17 µg/L on day 12 of culture when compared with the highest value in the control sample at 10.82 µg/L. The same results were obtained in the M3 medium, when increasing the nitrogen concentration in the medium by one and a half times compared with the control medium also increased Chl *a* concentration three times. Meanwhile, decreasing the nitrogen concentration or increasing the phosphate concentration did not result in a significant change in this chlorophyll value, as compared with the BBM. On the other hand, the concentration of Chl *b* exhibited the opposite result to that of Chl *a* during the microalgal culture period. That is, towards the end of the culture period, the concentration of Chl *a* gradually decreased with the growth of *C. vulgaris*, but that of Chl *b* gradually increased and also had a maximum value in media M5 and M3 [11]. The end of the culture period is the stage when *C. vulgaris* was in the death phase. At this time, the cells stopped dividing to synthesize other organic compounds, leading to the gradual settling of the cells down because of gravity to form bioflocs. Therefore, the cell density of microalgae recorded during this period was very low. According to Pruvost et al., the effects of nitrate starvation in the culture medium on the growth of *Neochloris oleoabundans* decreased the cell density, leading to a decline of the chlorophyll content [12]. Although the best growth of *C. vulgaris* was achieved in the CHU medium compared with the BG 11 medium with NH_4NO_3 and BBM with NH_4NO_3, as nitrogen source, the change of the N source in the culture medium of this strain indicated that Chl *a* and Chl *b* increased 2-fold in the BG11 medium [13]. With the composition of N in the Conway medium, Johnson (KNO_3 of 100 mg/L), and in the F2 medium ($NaNO_3$ of 75 mg/L), the Chl *a* of cells extracted from the biomass of *Dunaliella salina* obtained from the first medium was the highest [14]. In numerous studies, there is a lack of data that detailed the influence of the changes in the essential nutrients of the culture media on the concentration of chlorophyll. By contrast, the concentration of chlorophyll *a* and *b* in this study was controlled by the essential nutrients of the culture medium. More specifically, Chl *a* reached

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the highest value in the environment where $NaHCO_3$, as a carbon source, is replaced with glucose. Meanwhile, Chl *b* increased significantly during the death phase of *C. vulgaris* when the cell density in the suspension was very low in most of the five media.

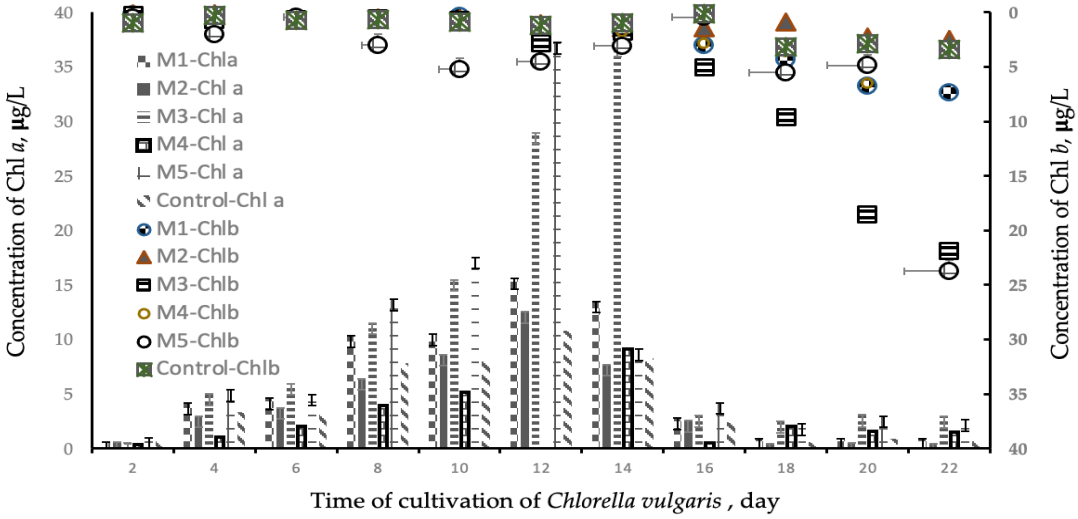

Fig. 1. Response of chlorophyll content to variations of essential nutrients in different culture media of *C. vulgaris* cultivation

3.2 Carotenoid

Carotenoids are a type of organic pigment found naturally in plants and other photosynthetic organisms, especially algae. More than 600 types of carotenoids have been identified to date, belonging to two groups: xanthophylls and carotenes. In this study, the content of carotenoids was recorded when varying the essential nutrient composition of the culture media of *C. vulgaris*. These variation has an influence on the production of carotenoids following a specific configuration. The nutrients affect the carotenoid production in a specific profile. Specifically, during the growth of *C. vulgaris*, the content of carotenoids increases gradually until entering the death phase. The highest content reached a value of 7.9 µg/L in the M3 medium when the nitrogen content increased one and a half times (Figure 2).

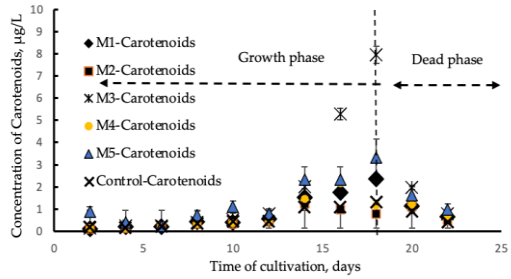

Fig. 2. Time course profile in fluctuations of essential nutrients in culture media of *C. vulgaris*

Compared with the maximum concentration of carotenoids extracted from biomass of *C. vulgaris* SAG 211-19 in BBM (control sample) at 1.32 µg/L, the variations of the composition of N, P and C increased the concentration of carotenoids, except for the samples in M2. As discussed by Wa Iba et al. [15], the carotenoid production was affected by essential nutrients of the microalgae culture media. Particularly, the nutrient ratio of C/N/P was a major regulator in carotenoid biosynthesis. Comparing several culture media of *C. vulgaris*,

they found that organic media of fermented hyacinths with a concentration of 1.0% produced the maximal growth of this strain with the highest concentration of carotenoid at 0.545 μg/mL [15]. Another study also analyzed the composition and production of major pigments (chlorophyll and total carotenoids) in *C. vulgaris* cultures under different molar ratios of N to P (0.44–576) and indicated that the carotenoid production could be enhanced with increasing P concentration under N-deficiency conditions and accumulated efficiently when the N/P molar ratio was less than 10.44. On a per-cell basis, the carotenoid production was lower despite the increased total carotenoid production. It is possible that increased glucose concentrations may result in an osmotic effect, in which osmotic pressure increases when glucose concentrations increase, reducing the availability of water for microorganism growth. Or, it increases probably because of the inhibition of primary metabolisms, such as protein synthesis, while cell growth may be reduced because of the inhibition of this metabolism; high C/N/P ratios may have a negative effect on this process, promoting the observed phenotypic changes [16].

4 Conclusion

In this study, the composition and production of chlorophyll and total carotenoids in *Chlorella vulgaris* cultures were analyzed with different N, P, and C concentrations in the medium. The production of these pigments, especially Chl *a* in the cultures, increased with increasing P concentration under reduced N/P ratio compared with that achieved in BBM. Specifically, the obtained Chl *a* content reached 37 mg/L if the P content was doubled compared with that in the BBM medium, suggesting that reducing the N/P ratio could be a promising culture strategy to increase Chl *a* as well as carotenoid productivity in this microalga. Meanwhile, the most significant increase in the carotenoid content achieved at 7.9 μg/L in the microalgae culture medium when the nitrogen content increased one and a half times compared with the BBM medium. Furthermore, the colour change of the cultures was correlated with the relative chlorophyll and total carotenoid contents. Therefore, the accumulation of Chl *b* due to the substitution in the C source could explain the yellow colour of the cultures because of the remarkable change in Chl *b* concentration obtained in the M5 medium. This study could be instructive for enhancing and monitoring chlorophyll and carotenoid productivity in microalgae.

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