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The Utilization of Two Commercial Indonesian Fertilizers as Microalgal Growth Medium for *Chlorella pyrenoidosa* and *Botryococcus braunii* Cultivation

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Abstract

In the current trend of the upstream stage of microalgal bioprocessing studies, many works mainly focused on optimizing microalgal cultivation parameters for obtaining a high yield of either biomass or a particular metabolite. Meanwhile, efforts to alternatively provide affordable growth medium are considered still limited to be performed. This study aims to provide an alternative microalgal growth medium from commercial fertilizers and supplements provided by a particular Indonesian company. Our proposed microalgal growth medium was developed by considering the chemical contents of the products with the essential components of an established Guillard f/2 microalgal growth medium. By employing the developed growth medium in Chlorella pyrenoidosa and Botryococcus braunii cultivation, similar growth profiles were shown in both green microalgae with optimum time for biomass harvesting encouraged within two weeks of cultivation. Regarding biomass productivity, 0.19 g and 0.20 of dried biomass were averagely obtained from 1 L cultures of Chlorella pyrenoidosa and Botryococcus braunii, respectively. This finding implies that two Indonesian fertilizer products could be utilized as the main ingredient of moderate performance of green microalgal growth medium.

Keywords: *Alternative Growth Medium; Guillard f/2; Microalgal Biomass; Microalgal Bioprocessing; Microalgal Growth Medium*

1 Introduction

Indonesia has an enormous area of seawater bodies enabling promising various types of tropical marine microorganisms to grow well beneath those seawater bodies. Current studies regarding the potential of marine natural resources in Indonesia are still limited. One example of the Indonesian marine resources that should be thoroughly studied is microalgae [1]. Microalgae have been widely recognized as a prospective marine bioresource for various applications, e.g., food, bioenergy, pharmaceutical, and cosmetic industries. Several microalgae have been shown an antimicrobial, antifungal, antivirus [2], antioxidant, and antibacterial compounds [3]. Although the microalga provides a broad range of applications, several challenges are still faced, especially in the affordability of their growth medium.

Microalgal growth mediums could be defined as an artificial environment to provide optimal nutrition, light, and physicochemical conditions for microalgal growth. This growth medium has a role in microalgae growth, either cultivated in laboratory or industrial conditions. Technically, the growth medium contains nutrients for microalgae to grow well, consisting of macronutrients and micronutrients. Macronutrients include C, H, N, P, K, S, Mg, and Ca, while micronutrients contain Cu, Fe, Co, Mn, Zn, Mo, Vn, and Si [4]. Those nutritional contents would affect the optimal growth of microalga and the biomass yield as well. One of the common growth mediums used in microalgae cultivation is the Guillard f/2 medium [5]. The Guillard f/2 medium is notably a mixture of various inorganic salts and biochemical components are most of those components should be imported from

countries outside Indonesia. This case limits the utilization of Guillard f/2 medium for routine use of microalgal cultivation, especially on a large scale, since the import scheme would increase the cost of operation for microalgal cultivation. Therefore, an alternative growth media for microalgal cultivation needs to be established to provide a relatively cheaper and more affordable option for cultivation.

Meanwhile, Indonesian companies focusing on agrochemicals regularly provide many commercial fertilizers. Some fertilizers are designed with particular levels of macronutrient contents that could significantly affect crop yields, such as nitrate, phosphate, urea, Potassium, or a specific combination of those compounds or elements. Meanwhile, some fertilizers provide micronutrients required in relatively low concentration but slightly essential for plants, for example, several metal ions that can act as cofactors for many enzymes. NPK Mutiara 16-16-16® and Meroke Fitoflex® are two examples of fertilizers provided by a particular Indonesian company for agrochemicals that contain macronutrients and micronutrients, respectively. Technically, those nutrient profiles are slightly similar with several components of common microalgal growth medium so they could be used as alternative sources of microalgal nutrients.

In this study, we develop an alternative microalgal growth medium through the formulation of NPK Mutiara 16-16-16® and Meroke Fitoflex®. The growth medium receipt was mainly developed by some substitution of several macro- and micronutrient components referred to existing common microalgal growth mediums' components, i.e., Guillard f/2 components, subsequently. Afterwards, the developed growth mediums were used for the cultivation of two green microalga isolates then their growth and biomass profiles could be analyzed.

2 Method

The materials used in this study consist of *Botryococcus braunii* and *Chlorella pyrenoidosa* isolates, NPK Mutiara 16-16-16® fertilizer, Meroke Fitoflex® fertilizer, coarse salt, distilled water, vitamin B_1, vitamin B_{12}, and biotin (vitamin IPI). The equipment employed in this work includes an analytical balance, Erlenmeyer flask, beaker, volumetric flask, Mohr pipette, measuring glass, salinity measuring device, glass storage bottles, stirring rod, autoclave, dropper pipette, aerator, oven, glass funnel, Whatman No. 41 filter paper, Buchner funnel, vacuum pump, 15 L carboy, and UV-vis spectrophotometer.

The growth media were developed by adapting the Guillard f/2 medium which consists of separated solutions with each different composition, i.e., solutions A, C, and D. Note that solution B of Guillard f/2 medium was not adapted for this study since its solution is a silicate-based component which is essential for another group of microalgae, i.e., diatom group. In this work, Solution A and C of Guillard f/2 were substituted by 10 g of NPK Mutiara 16-16-16® fertilizer (10 g/100 mL water), and Meroke Fitoflex® fertilizer (25 g/100 mL water), respectively. Meanwhile, solution D of Guillard medium was substituted by a mixture of 0.1 mL of vitamin B_{12}, 1 mL of biotin, and 0.4 g of vitamin B_1 dissolved in 1 L of distilled water. The A and B solutions were stored at room temperature, while the C solution was stored in the refrigerator with a temperature range of 4±2°C.

To prepare a ready-to-use growth medium, a small amount of those solutions was mixed with saline water. Each 1 mL of solutions A and C was acquired and added, subsequently, into 1 L of artificial saline water (28 ppt of salinity). Then, the medium was autoclaved for 15 minutes at 121°C. After cooling down at room temperature for 12 hours, 1 mL of solution D is added into a sterilized medium.

Microalgae activation was carried out by adding 5 mL of starter microalgae isolate (*Botryococcus braunii* or *Chlorella pyrenoidosa*) into 100 mL of the growth medium. Those 100 mL scales of microalgal culture were cultivated without additional aeration at room temperature and under direct sunlight exposure during the dry season. After activation had been successful, 30 mL of each activated microalgae was acquired and added into 15 L of growth media, subsequently, for cultivation with additional aeration. The cultivation design was the microalgal batch type the nutrient is only added on day 0 and its biomass is fully harvested without any addition of nutrients during the cultivation steps. The microalgae growth curve was determined by measuring the optical density (absorbance) of the microalgae isolate culture using a UV-Vis spectrophotometer every day at a wavelength of 450 nm. The microalgae growth curve is constructed based on

the optical density data measured every day for 25 days.

Harvesting for microalgal biomass was conducted during the exponential growth phase by using a Büchner funnel lined with Whatman No. 41 filter paper and a vacuum pump. After filtration, the biomass was dried in an oven at 105°C for 3 hours and the net biomass weight was determined, subsequently.

3 Result and Discussion

The alternative microalgal growth medium was principally developed by substituting several Guillard f/2 components with components within the fertilizers. Table 1 displays a comparison of components and their levels within the common Guillard f/2 and studied fertilizers.

Table 1. Comparison between Guillard f/2 Medium and Fertilizer Components

Component	Guillard f/2 Medium (g/L)	Alternative Fertilizer Medium	
		NPK (%)	Meroke (%)
Solution A: Phosphate and Nitrate			
$NaNO_3$	84.15	N (12%)	-
$NaH_2PO4.H_2O$	6.00	P_2O_5 (12%)	-
$FeCl_3.6H_2O$	2.90	-	Fe (2,5%)
$Na_2EDTA.2H_2O$	10.00	-	-
Solution C: trace metal			
$CuSO_4.5H_2O$	1.96	$CuSO_4$*	Cu (2%)
$ZnSO_3.7H_2O$	4.40	$ZnSO_4$*	Zn (5%)
$(NH_4)_2Mo_7O_{24}.4H_2O$	1.26	-	Mo (0,1%)
$MnCl_2.4H_2O$	36.00	-	Mn (7%)
$CoCl_2.6H_2O$	2.0	-	-
Solution D: Vitamin			
Vitamin B	0.4	See remarks**	
Vitamin B_{12}	0.002 mg	See remarks**	
Biotin	0.1 mg	See remarks**	
The component that does not exist within Guillard f/2			
-	-	K_2O (17%)	-
-	-	MgO (2%)	-
-	-	Borate*	Boron (2%)

Remarks: *Borate, $CuSO_4$, $ZnSO_4$ (0,07%); **vitamin solutions were prepared with the same amount of Guillard f/2 components

Table 1 shows that both fertilizers contain most of the common nutrients that are commonly contained within the Guillard f/2 medium. However, particular macro- and micronutrients of Guillard f/2 were unavailable in both fertilizers, i.e., $Na_2EDTA.2H_2O$ and $CoCl_2.6H2O$, respectively. It was probably sodium content that could be slightly compensated by saline water artificially made from coarse salts. On the other hand, both fertilizers also provide components unavailable within Guillard f/2 medium, i.e., K_2O, MgO, Borate, and Boron.

Macronutrients required in microalgal growth medium include carbon (C), hydrogen (H), calcium (Ca), magnesium (Mg), sulfur (S), nitrogen (N), phosphorus (P), and potassium (K). The f/2 medium contains two essential nutrient elements crucial for microalgae development, which are phosphate and nitrate originating from NaH_2PO_4 and $NaNO_3$ compounds, respectively. While, nitrate, phosphate, and potassium contents are abundantly present in NPK Mutiara 16-16-16® fertilizer. Nitrate serves a crucial role in microalgae metabolism, including the formation of proteins, nucleic acids (DNA and RNA), and microalgal bio pigments, while phosphate is essential for reproduction and energy transfer processes like ATP production [7]. Both of these elements are highly important for microalgae growth, and therefore, they are required in optimal amounts [8]. Regarding metabolism, Potassium may enable an optimal performance of osmotic regulatory and enzymatic activities.

Micronutrients required in microalgae growth media include micronutrient elements such as iron (Fe), copper (Cu), manganese (Mn), zinc (Zn), cobalt (Co), molybdenum (Mo), boron (Bo), vanadium (Vn) and silicon (Si). Fe, Mn, Zn, Cu, Co, and Mo are mainly contained in the Guillard f/2. Those micronutrients were predominantly found in Meroke Fitoflex® fertilizers as well. Cobalt is an element that solely does not exist within both fertilizers. Whereas Co has an essential role related to vitamin B_{12} function (although there is also vitamin B_{12} added as solution D).

Activation of both microalga isolates was successfully conducted through small-scale non-aerated cultivation. There were visual color changes in microalgal cultures indicating that the cell density was steadily increased during the cultivation. Figure 1 depicts the appearance of both microalgal cultures in the initial (0 day), middle (14 days), and last days (25 days) of cultivation.

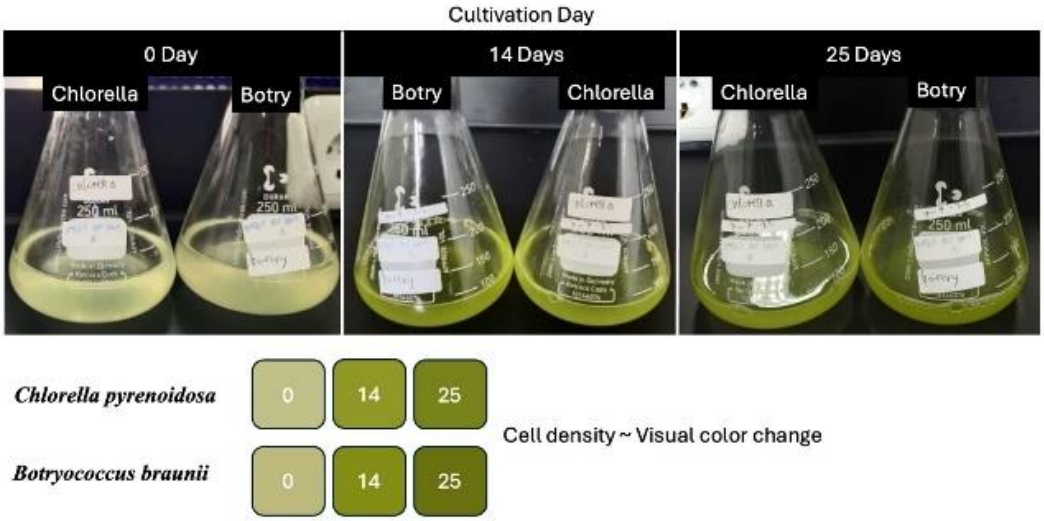

Figure 1. The visual appearance of a 100 mL microalgae culture from 0 to 25 days and representative color changes

The growth rate of microalgae could be measured using a UV-Vis spectrophotometer, which is based on the increase in culture absorbance, as demonstrated from previous work [9]. This related work also showed that the absorbance on the growth curve represents the number of cells in the culture, i.e., the higher the absorbance, the greater the number of cells in the growth medium [9]. The growth curve was then generated based on those absorbance data to determine the harvesting time for microalgae cultures. The growth curves of *Botryococcus braunii* and *Chlorella pyrenoidosa* microalgae are shown in Figure 2.

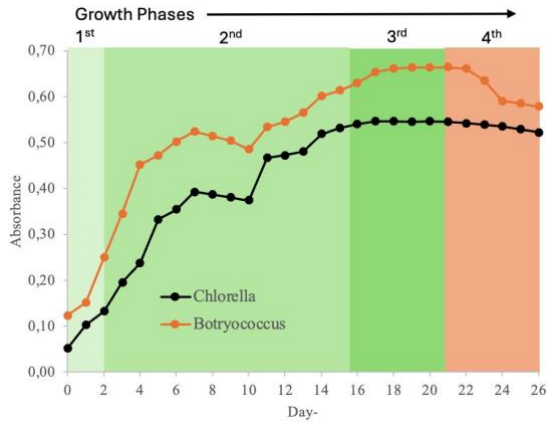

Figure 2. Growth curve of *Chlorella pyrenoidosa* and *Botryococcus braunii* microalgaes in 25 days

Based on the 25 days-growth curves of *Chlorella pyrenoidosa* and *Botryococcus braunii*, it can be observed that microalgae growth undergoes five growth phases, i.e. the adaptation phase (lag phase), the logarithmic phase (log phase), the declining growth phase, the stationary phase, and the death phase [10]. The first phase was the adaptation phase (lag phase) that occurs from day 0−2. In this phase, microalgae may undergo various changes in structure, metabolism, and cellular responses as part of the transition from their previous environment to the new one. Typically, this adaptation phase is considered an initially sensitive period that could lead to a decrease in microalgae growth or productivity over a certain period. However, microalgae would generally adapt and enter the exponential growth phase, indicating a dramatic increase in their growth.

The second phase of this cultivation was classified as the logarithmic phase, occurring from day 2−16. In this phase, the microalgal cells were exponentially increased. Thus, it was established as the exponential growth phase for both cultures. The logarithmic phase is crucial in microalgae cultivation because it is where biomass production and growth reach their highest levels. However, it is important to carefully control and monitor environmental conditions during this phase to ensure optimal growth and to prevent issues such as tight nutrient competition and the accumulation of potentially toxic byproducts. Most microalgal cells usually initiate to multiply themselves in the exponential phase for 3-7 days [11]. Interestingly, the exponential phase in this study was relatively longer, i.e., around 14 days.

The third phase is the decline phase which both cultures occurred from day 17−21. There was an indication of a decrease in the microalgae growth rate. This condition occurs after the microalgae reach their maximum biomass level combined with the limited amount of the nutrient

level within the cultures resulting in slower growth rates.

The fourth phase of this cultivation scheme was started on day 22, indicated by the dramatic decrease in both cultures. We defined this phase as a stationary phase instead of a death phase since the visual observation showed that most microalgal cells were still in relatively fresh condition and the indication of deterioration of cultures was shown after 40 days of cultivation.

The fifth phase, or the final phase of microalgae growth, is the death phase. This phase begins on day 26. During this phase, there is a decline in the number of microalgae cells due to the death of some cells. The death phase is not shown in the growth curve because the microalgae were fully harvested, and the cell count was not measured.

Scaling-up of both microalga isolates was also successfully performed. An aerated cultivation was chosen for this larger cultivation since air bubbles could also homogenize microalgal cells in the entire culture medium so that most cells could obtain nutrients and gases, equally. Figure 3 displays both 15 L - scaled microalgal cultures in the day established for the harvesting step.

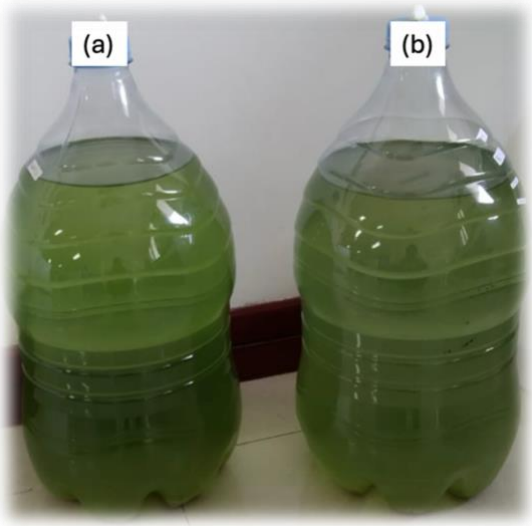

Figure 3. The appearance of ready-harvested microalgae cultures with 15 L of volume: a) *Chlorella pyrenoidosa* and b) *Botryococcus braunii cultures*

Chlorella pyrenoidosa and *Botryococcus braunii* biomass were harvested in the logarithmic or exponential phase [12]. Biomass was harvested through vacuum filtration by using Whatman No. 41 filter paper. Afterward, the wet biomass was dried in an oven at 105°C for 3 hours. Figure 4 displays the appearances of the wet and dry biomass of microalga.

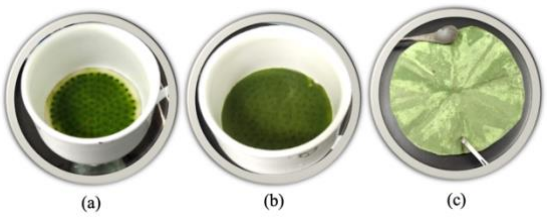

Figure 4. Visual apperance of a) the wet biomass of *Chlorella pyrenoidosa*, b) *Botryococcus braunii*, and c) the dry biomass of *Chlorella pyrenoidosa*

Those dry biomass were carefully measured in order to figure out the productivity of both microalgal cultures. Table 2 provides the biomass yield of both cultures.

Table 2. Microalgae dry biomass

Microalgae isolates	Weight (g)	
	Biomass (in 15 L culture)	Biomass (per L culture)
Chlorella pyrenoidosa	2.97	0.19
Botryococcus braunii	2.98	0.20

According to Table 2, both microalga isolates have relatively the same biomass productivity after being cultivated by using the developed medium. Although both biomass productivities are still slightly lower than the dry biomass of *Botrycoccus brauni* and *Chlorella* sp. cultivated in Chu growth medium (0.8 – 7.0 g/L) and Bold Basal growth medium (1.4 – 6.8 g/L), respectively [13], as well as *Botrycoccus brauni* cultivated in the modified Chu 13 medium (0.382 – 0.628 g/L) [14], the displayed biomass yields remain promising to be further scaled up, especially for manufacturing scale. For instance, 1 L of this fertilizer-based microalgal growth medium, made from several pouches of NPK Mutiara 16-16-16® and Meroke Fitoflex® fertilizers, could produce 190 g of valuable microalgal biomass. The microalgal biomass from affordable and simple ingredients of growth medium is interesting from an industrial perspective. Furthermore, selecting simpler raw materials rather than employing serial chemical products for producing microalgal growth medium could indirectly diminish the utilization of animal-origin material to fulfill some consumers' preferences [15].

4 Conclusion

The Indonesian' fertilizer-based microalgal growth medium developed in this study demonstrated proper cultivation profiles of *Chlorella pyrenoidosa* and *Botryococcus braunii*. Moreover, the consistent finding regarding biomass yields indicates that this growth medium would be versatile enough for different green microalgae.

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