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The Growth of Microalgae Chlorococcum sp. Isolated from Ampenan Estuary of Lombok Island in Walne's Medium

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Abstract. Microalgae, also known as plankton, are one of the aquatic organisms which conduct photosynthesis similar to higher plants. Microalgae can live in any aquatic environment and are relatively easy to culture. There have been many studies that have shown the commercial applications of microalgae which have been continuously driving the development of microalgae-related research for decades. As for this research, the aim was to determine the growth phase of microalgae *Chlorococcum* sp isolated from Ampenan Beach estuary of Lombok Island. In this study, isolate from solid medium obtained from previous research were transferred to liquid medium enriched with Walne's nutrition. The conditions of the culture were: room temperature (25 °C), light intensity of 2000-3000 lux, photo period of light:dark (24:0) hours, pH range 7-8, 24 hours of aeration, and culture under sterile environment. Based on every day observation of cell numbers, it is known that *Chlorococcum* sp began to enter the death phase on the fifth day so that the observations were only carried out for seven consecutive days. The initial cell number was 187,500 cells/ml and the optimum cell numbers (on the fourth day) was 331,250 cells/ml. In conclusion, the optimum growth of microalgae occurred on the fourth day from the first day of culture and it began to enter its death phase on the fifth day.

INTRODUCTION

Microalgae are photosynthetic microorganisms living in watery environment, whether in saline water or in fresh water. Common people call these organisms as plankton. So, in the aquatic ecosystem, microalgae play an important role as the primary producer of oxygen and also as the lowest food chain in the water [1]. As for human, microalgae can give a various benefit for the viability of the human race. Due to its uniqueness, microalgae can't be classified using one method only. There are several ways offered to classify them. The commonly used methods to classify microalgae are based on the size, pigment composition, storage profile, diversity of ultrastructural features, etc [2]. But we can also distinguish them in a very simple way. For example, microalgae can be classified based on their cell. Based on the color of the cells, microalgae can be classify into seven classes, those are: green algae (Chlorophyta), red algae (Rhodophyta), yellow-green algae (Xanthophyta), brown algae (Paeophyta), golden-brown algae and diatoms (Chrysophyta), fire algae (Pyrrophyta), and eugnoloids (Euglenophyta) [3]. The differences in cell color in various types of microalgae are influenced by the dominant photosynthetic pigments that vary in each alga. For example, the green algae is green because of the dominant pigment in their cell is chlorophyll. While the red microalgae showing the red color is because of the abundance of phycoerythrin and pycocyanin pigment in their cells. As for the brown and golden microalgae, the colors of their cell are caused by the dominancy of fucoxanthin pigment [4].

Research in microalgae have been one of the interesting topics for decades because their various applications potency for commercial uses. Some or whole parts of microalgae can be used for human food and supplements, animal feed, biofertilizer, pharmaceutical products, cosmetics, feedstock, and bioenergy such as biodiesel, bioethanol, biohydrogen, solar cells, etc [5-11]. Indonesia, as a maritime country, certainly has a quite high diversity of microalgae. In addition, as a tropical country, sunlight, which is needed by microalgae to photosynthesize, is abundant throughout the year. Thus, Indonesia is a high-potential country to develop microalgae-related research. And this is also applicable in Lombok Island.

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So far, there are only a few research that have been reported for microalgae in Lombok Island. In 2015, a research conducted by Cokrowati in Mapak Beach of West Lombok, successfully identified 18 microalgae [12]. In 2016, Subagio conducted research in Cemara Beach of East Lombok and successfully identified 35 species of microalgae [13]. In 2017, a study conducted by Astuti, *et al.* in Pelangan Estuary of West Lombok, successfully identified 85 species of microalgae [14]. And in 2018, Astuti, *et al.* carried out another study in Ampenan Estuary of West Lombok, which successfully identified 48 species of microalgae. Even though there are already so many species of microalgae discovered in few areas in Lombok Island, unfortunately, only few of them have been isolated. The microalgae from Ampenan Estuary that have been successfully isolated in the laboratory are as many as four species, namely *Chloridella sp, Chlorococcum humicola, Chlorococcum sp, and Chroococcus sp* [15].

Further exploration cannot be done if the microalgae have not been isolated and cultured in the laboratory. So, in this research, one of the isolated microalgae, *Chlorococcum sp,* was cultured to multiply for further study. The aim of this research was to determine the growth phase of *Chlorococcum sp* microalgae isolated from Ampenan Estuary of Lombok Island. The determination of growth phase of microalgae is important to find out the best time to harvest the microalgae culture and to seek out the optimum growth for specific metabolite production.

MATERIALS AND METHODS

Method

This study was carried out at the Biology Laboratory of Mataram University. *Chlorococcum* sp was the microalgae species used in this research and was collected by [14]. The initial cell number in Walne's culture media was 187,500 cells/ml. All the activities were conducted in sterile environment.

Microalgae Identification

The identification of microalgae was done before and after the culture process. The observation and identification were repeated 10 times in each sample. Microalgae were identified as described by Davis (1955) [16].

Culture Conditions

Microalgae were cultured using standard procedure as describe by APHA/AWWA/WPCH (1989) and Anderson (2005) [17-18]. Cultured medium used included Walne's solutions as nutrient sources. Details of the culture conditions are shown in **Table 1. Table 2** and **3** show the composition of specific solid media for isolation of *Chlorococcum* sp to separate it from another species. Isolates were then transferred to liquid medium (to culture) enriched with Walne's solutions.

TABLE 1. Detail of culture conditions

Photo period (light:dark) hour(s)	Light (lux)	Temp (^{o}C)	pH	Airing
24:0	2000-3000	25	7-8	Mild

TABLE 2. Specific solid media for isolations of Chlorococcum sp

A solution compound	Quantity
$NaNO_3$	20 gr/L
$NaH_2PO_4.2H_2O$	40 gr/L
$FeCl_3.6H_2O$	2.6 gr/L
H_3BO_3	67.2 gr/L
$MnCl_2.4H_2$	0.72 gr/L
EDTA titriplek III	90 gr/L
Aquadest	1000 ml
B solution	**Quantity**
$ZnCl_2$	2.1 gr/L
$CoCl_2.5H_2O$	2 gr/L
$(NH_4)_6.Mo_7O_{24}.4H_2O$	0.9 gr/L
$CuSO_4.5H_2O$	2 gr/L
Aquadest	100 ml

TABLE 3. Walne culture media

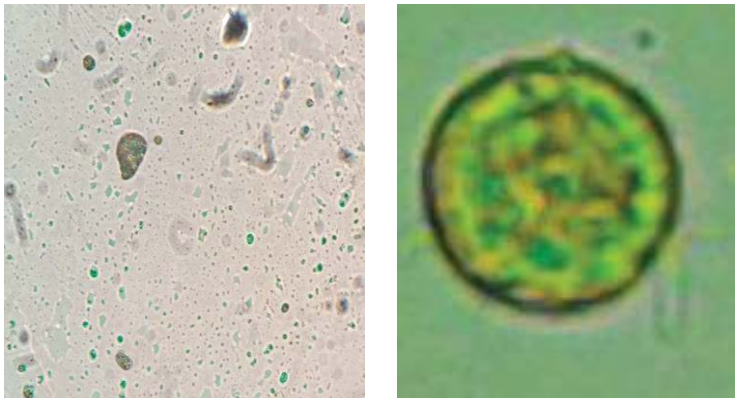
A solution (nutrient)	Quantity
$NaH_2PO_4.2H_2O$	20 gr/L
$NaNO_3$	100 gr/L
Na_2EDTA	5 gr/L
Na_2SiO3	40 gr/L
$MnCl_2.H_2O$	0.36 gr/L
$FeCl_3$	1.3 gr/L
H_3BO_3	10 gr/L
Aquadest	1000 ml
B solution (trace metal)	**Quantity**
$ZnCl_2$	21 gr/L
$CoCl_2.6H_2O$	2 gr/L
$(NH_4)_8.Mo_7O_{24}.4H_2O$	0.9 gr/L
$CuSO_4.7H_2O$	20 gr/L
$FeCl_3.6H_2O$	3.15 gr/L
Aquadest	100 ml
C solution (vitamin)	**Quantity**
B12	0.1 gr/L
Thiamine	20 gr/L
Biotin	0.1 gr/L

Morphological Observation

The microscopic observations were done before and after culture to ensure the target species were growing well. The observations were made at different stages of the life cycle, the sizes of the cells, either cells in the exponential phase or on cells in late stationary phase of growth. The total cells in culture media were counted once every day using a haemacytometer and a counter under the microscope with a magnification of 40x [18] until the cell number (cell density) was decrease gradually. The observations were repeated by a minimum of three times. The determination of total cell number was using the following protocol: 1) Take the average cell count from each of the sets of 16 corner squares of the counting chamber, 2) Multiply by 10,000 (10^4). The unit of the count is cells/ml [19].

RESULTS AND DISCUSSION

Microalgae were observed under the microscope with a magnification of 40x (see Figure 1). From the observation results, it can be concluded that *Chlorococcum sp* tend to grow in solitary or without forming a colony and appear as a green spot. Thus, *Chlorococcum sp* can be classified as a green alga, which means that it has the abundance of chlorophyll pigment. Upon taking a closer look, this microalgae is rounded in shape and dominantly show the colors of green and yellow in its cell. The green color in microalgae is produced by chlorophyll pigment, while the yellow color can be produced by carotenoid compound pigments [20]. So, it can be expected that Chlorococcum sp is high in chlorophyll and carotenoid pigments.



(a) (b)

FIGURE 1. Chlorococcum sp cell under microscope: (a) grow solitaire in the culture, (b) rounded in shape and showing green and yellow colors

The previous daily counted cell numbers are shown in **Table 4**. From the table, it can be seen that the highest cell number was at day 4 (331,250 cells/ml), which is almost doubled the number of the initial cells (187,500 cells/ml). Then, it can be concluded that the peak growth of Chlorococcum sp was at day 4. From this data, the growth curve of Chlorococcum sp (see Figure 2) were made using the Excel program.

TABLE 4. Daily cell counting

Day(s)	0	1	2	3	4	5	6	7
Cells number count (cells/ml)	187,500	111,250	120,000	185,000	331,250	31,250	30,000	21,250

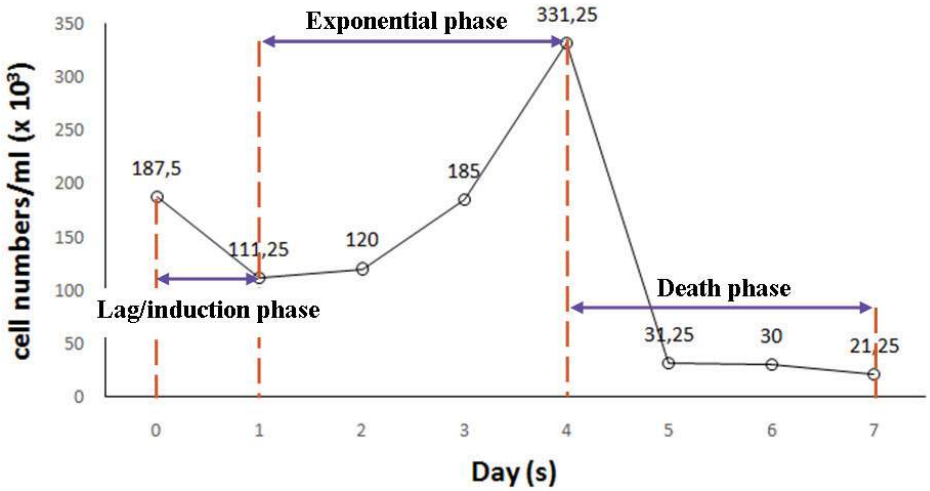


FIGURE 2. Growth curve of *Chlorococcum sp*

Based on the growth curve, it can be seen that *Chlorococcum sp* went through three phases of growth during cultivation. These are the lag/induction phase, the exponential phase, and the death phase [21-22]. The lag/induction phase appeared between days 0 to 1. In this phase, microalgae were through the adaptation phase in the new medium. That is why the cell numbers were declining. The drastic reduction of *Chlorococcum sp* cells can be understood because the algae were just being transferred from two different types of growth media (from solid to liquid) and two different types of nutrition media (from Conway to Walne) making it hard to adapt in the new environment. The second phase is exponential phase. This phase appeared between day 1 to day 4. At this phase, microalgae were drastically increasing in numbers according to a logarithm function. At this phase, growth rate is maximum [21-22]. This is because the cells have adapted to the new environment that makes it start to grow steadily. The last phase is the death phase, which appeared since day 5. During this phase, water quality worsens and nutrients are depleted that makes cells unable to sustain its growth. Thus, the cell density decreases geometrically because the cells die faster than growing more [21-22]. Normally, microalgae cell will go through 5 phases during it growth namely, induction phase, exponential phase, phase of declining growth, stationary phase, and death phase [21-22]. But unfortunately, the phase of declining growth and the stationary phase were not able to be observed in this research, probably due to its relatively short life cycle. Whereas other algae on average live for a week, the microalga *Chlorococcum sp* in this research only lives for 4 days long. To make the two other phases appear in the growth curve, it can be suggested for another researcher to count the cell density less than a day or in count of hours to get a more accurate cell growth curve. In addition, another study can be conducted to seek out the optimum culture conditions to get the maximum biomass or specific secondary metabolites productions. For example, using a different medium of nutrition, using various concentrations of saline water (20-24 g/l for optimum growth) [22], etc.

CONCLUSION

Based from the growth curve, the life cycle of microalga *Chlorococcum* sp was 4 days long. Where, the highest growth occurred on the fourth day from the first day of culture and it began to enter its death phase on the fifth day. For further study, the optimum culture conditions for *Chlorococcum* sp growth must be sought out to get the best growth for maximum biomass and metabolite production.

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