

Growth Rate of Microalgae *Nannochloropsis oculata* at Different Culture Scales

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Abstract

Nannochloropsis oculata is a microalgae that acts as a natural food source in fisheries. The need for *N. oculata* can be met through progressive culture starting from laboratory scale culture to provide inoculant stock to mass culture using concrete tubs. The culture of *N. oculata* begins with the preparation and sterilization of culture media and equipment, fertilizer production, microalgae culture starting from the laboratory scale, intermediate scale, and mass scale, observing the density of microalgae and water quality. The lag phase of *N. oculata* occurs on the first and second days, the exponential growth phase lasts for four days, followed by a stationary phase that occurs on the seventh day. Peak population density of *N. oculata* on all culture scales occurred on day 6 with culture density in glass containers 14.58×10^6 cells/mL, carboy culture 11.6×10^6 cells/mL, intermediate culture 5.1×10^6 cells/mL, and mass culture 9.85×10^6 cells/mL. The growth rate of microalgae *N. oculata* from the beginning of growth to day 6 was highest on the intermediate scale. The size of the container and the different composition of fertilizer at each culture scale caused differences in the peak population density and growth rate of *N. oculata*. Production of *N. oculata* can be carried out in bulk while still paying attention to every stage of culture preparation and microalgae culture activities as a standard operating procedure for microalgae culture to prevent contamination.

Keywords: progressive culture, microalgae, *Nannochloropsis oculata*

INTRODUCTION

Microalgae are a natural food source for the growth of aquaculture organisms, so their existence is vital in fish farming activities. The selection factors for good natural food for larvae are high nutrition, size according to mouth opening, ease to culture in bulk, easy to digest, and non-toxic (Ismi *et al.*, 2012). One of the microalgae that can meet these needs is *Nannochloropsis oculata*. The protein content in *N. oculata* was $34.11 \pm 0.7\%$, carbohydrates $19.81 \pm 2.02\%$, and lipids $12.51 \pm 1.01\%$ (Benerjee *et al.*, 2011). In addition, *N. oculata* also contains vitamin B12 and *Eicosapentaenoic Acid*

(EPA) by 34% and total content of Omega 3 Highly Unsaturated Fatty Acids (HUFAs) of 42.7%. Vitamin B12 contained in *N. oculata* can increase the population of rotifers and EPA is very important for nutrition for juvenile and marine fish larvae (Mukhlis *et al.*, 2017). According to Fakhri and Arifin (2016), *Nannochloropsis* has high unsaturated fatty acids, high protein content, and a fast growth rate. Therefore, this microalgae is very suitable to be used as natural food for fish larvae. *Nannochloropsis sp* is widely cultivated as natural food because it has a role in the hatchery of fish, shrimp, crabs, and other marine

biotas (Endrawati and Riniatsih, 2013). In addition, it can also be used as feed for primary consumers such as rotifers, and artemia. According to Sari and Manan (2012), to meet the availability of sustainable feed, in sufficient quantities, and on time, laboratory-scale microalgae culture is needed to obtain pure cultures to mass scale. Therefore, scientific information is needed on the cell density and growth rate of *N. oculata* through progressive culture using culture containers of different volume sizes.

RESEARCH METHODS

Material

The materials used for the culture of *N. oculata* were sea water, Walne Pro Analysis (PA) fertilizer for laboratory scale consisting of NaNO₃ (100 gr), NaH₂PO₄ (20 gr), Na²EDTA (45 gr), H₃BO₃ (33.6 gr), MnCl₂ (0.36 g), FeC₁₃ (1.3 g), and Aquades (1 L). Walne grade Technical Growth (TG) fertilizer was used for the culture of *N. oculata* on an intermediate scale, consisting of KNO₃ (1 kg), NaH₂PO₄ (100 gr), Na²EDTA (10 gr), FeC₁₃ (13 gr), and freshwater (10 L). The fertilizers used for mass-scale culture consisted of Urea (210 g), Za (140 g), TSP (70 g), FeC₁₃ (3.5 g), EDTA (3.5 g), and seawater (7 L). Additional materials used for the laboratory scale are cobalamin (B1) (100 gr) and thiamin (B12) (5 gr).

Method

Sterilization of Equipment and Culture Media

Sterilization of culture

equipment such as Petri dishes, test tubes, and Erlenmeyer with *stericell* at 100°C for 15 minutes. The jars and carboys were washed with a detergent solution and then rinsed with fresh water. The aeration equipment was immersed in 200 ppm chlorine solution for 24 hours to reach the inside of the hose, then washed with a detergent solution, rinsed with fresh water, and dried. Sterilization of culture tanks (concrete and fiber tanks) with a detergent solution, rinsed with seawater, and then dried until ready to be filled with seawater.

The sterilization of seawater as a culture medium depends on the volume of seawater used as a culture medium. For pure culture media in Erlenmeyer, the seawater used was sterilized by autoclave at 12°C for 15 minutes. Sterilization of seawater for intermediate and bulk scale culture using a filter bag. The media was given chlorine as much as 10 ppm and left for 8 hours then neutralized with 5 ppm Na-Thiosulfate. The media neutrality test uses a chlorine test. Neutral media shows a clear color, while yellow indicates that the media is not yet neutral (Sari and Manan, 2012).

Nannochloropsis Oculata Culture

N. oculata culture activities as natural food source at Balai Perikanan Budidaya Air Payau (BPBAP), Situbondo done progressively. The first step is to perform unaltered culture to obtain pure microalgae stocks. The results of the isolation of plankton in the media were cultured in a 10 mL test tube to multiply cells, then developed again with a larger volume using a 50-

100 mL Erlenmeyer. According to Creswell (2010), stock culture serves as an inoculant for larve volume of phytoplankton production that can be harvested as natural feed. Erlenmeyer culture results are used as an inoculant or pure culture starter in a 3-5L glass container. pure culture in a glass container on a small scale aims to adapt microalgae to new environments such as the use of aeration.

Inoculant density from the culture in Erlenmeyer for culture in glass containers was $\pm 9 \times 10^6$ cells/mL, the glass container is covered with plastic and aerated. Carboy scale culture used a plastic container with a volume of 10-12 L with an inoculant density of $\pm 9 \times 10^6$ cells/mL from a small-scale culture in a glass container. The cultures were given Walne fertilizer and vitamins at a dose of 1 mg/L each, then the carboy was covered and aerated. Laboratory scale culture equipped with 40 Watt TL lamp.

The intermediate scale culture used a concrete tank with a volume of 400 L and a fiber bath of 500 L. The results of the Carboy scale culture which reached an exponential phase were used as inoculants with a seed density of $\pm 9 \times 10^6$ cells/mL. The culture was fed with Walne-grade TG fertilizer at a dose of 1 mg/L. According to Creswell (2010), to maintain high-quality cultures, inoculant transfer must be carried out during the exponential growth phase, with an inoculant of at least 10 – 20 percent from the total volume or initial concentration of about 10^5 cell/ml, to accelerate population growth. Mass scale culture for big

volume production is using 10 tons concrete tub. The inoculant used originated from an intermediate scale culture, because it is not sufficient then it is taken from another concrete tub. The inoculant density given was $\pm 9 \times 10^6$ cells/mL. The fertilizer used was 7 L of agricultural fertilizer containing TSP, Urea, Za, FeCl_3 , and EDTA. To prepare mass scale fertilizer, first TSP is dissolved so it does not clump during mixing. Next Urea, Za, FeCl_3 , and EDTA mixed into 7 L of seawater and stirred out until dissolved. Fertilizer is only given once at start of culture (Hermanto *et al.*, 2011).

***Nannochloropsis oculata* Growth Rate and Density**

N. oculata density was conducted every day at the same time using *haemocytometer*. Small block methods is used for counting microalgae density (Ruliaty *et al.*, 2019) with 100x microscope magnification. *N. oculata* growth rate was calculated using the equation according to Campaña-Torres *et al.* (2012):

$$K = (\ln B_n / B_o) / (t_n - t_o),$$

Where:

K = growth rate

B_o = initial density of microalgae

B_n = the density at any time $t_n - t_o$ is the culture period.

Water Quality

External conditions that can affect the growth of *N. oculata* are the quality of the culture media water. Water quality parameters observed at the beginning of the culture included: pH, temperature, light intensity, and

salinity.

DISCUSSION AND RESULTS

The lag phase of *N. oculata* culture occurs on the first and second days. The density of laboratory-scale *N. oculata* in a glass container on the first day was $3,6 \times 10^6$ cells/mL, whereas the culture inside of carboy plastic was $4,6 \times 10^6$ cells/mL. The lag phase of intermediate and mass scale *N. oculata* culture also occurred on the first and second days with the population density on the first day being $0,38 \times 10^6$ cells/mL and $2,85 \times 10^6$ cells/mL, respectively. When the microalgae are put into the culture container, the microalgae cells adapt to their environment. According to Prayitno (2016), microalgae cells are physiologically prepared to carry out cell division by producing enzymes and other metabolic compounds needed for cell division.

In this phase, the number of dividing cells is still small so there is not much increase in population, therefore it is called the lag phase. The lag phase ranges from 2-3 days, depending on the amount of inoculum used (initial cell density), algae species, irradiation, and temperature (Creswell, 2010). According to Ru'yatin et al. (2015), parameters that can affect adaptation time are the type, age of microalgae cells, inoculum size, and growing media conditions. If the media lacks nutrients, the adaptation phase will take longer because the cells must produce enzymes that are suitable for the type of nutrients present.

The exponential growth phase that occurred on the 3rd to 6th day of

culture in jars was higher than that of the carboy scale culture (Figure 1). This is influenced by the differences in the use of containers and differences in the incoming light intensity. Wahyuni et al. (2018) stated that the intensity of light entering the glass jar scale culture was higher and the temperature could be maintained well, otherwise light was difficult to penetrate the plastic containers used for the carboy scale culture.

The intensity of light can also affect the growth of microalgae cells because it is used as a photosynthesis process while temperature affects the metabolic processes of microalgae. The exponential phase of intermediate and mass scale *N. oculata* culture occurred from day 3 to day 6. According to Creswell (2010), after the adaptation phase, algal cells divide rapidly, and the population increases logarithmically. The exponential growth phase can last for four days or more. The increase in microalgae growth in the exponential phase indicates that microalgae perform cell division by utilizing nutrients in the media so that the growth rate and population density increase many times in this phase (Purwitasari et al., 2012).

Peak population density of *N. oculata* on all culture scales occurred on day 6 with culture density in glass containers $14,58 \times 10^6$ cells/mL, carboy culture $11,6 \times 10^6$ cells/mL, intermediate culture $5,1 \times 10^6$ cells/mL, and mass culture $9,85 \times 10^6$ cells/mL (Figure 1). The growth rate of microalgae *N. oculata* from the beginning of growth to day 6 was highest in the intermediate scale culture (Table 1). Chiu et al.

(2008) proved that the culture of *N. oculata* in a 0.8 L container and given CO₂ 0.03-2% reached its peak density on days 6-8.

The difference in population density (Figure 1) and the growth rate of *N. oculata* (Table 1) at each culture scale of *N. oculata* was due to the size of the container and the composition of the fertilizer used at each culture scale. Laboratory scale culture using Walne grade PA fertilizer. PA-grade fertilizer has a complete and appropriate nutrient composition and concentration to meet the nutritional needs of microalgae so it is very good for microalgae growth (Yanuaris et al., 2012). In the laboratory, scale culture is also needed for vitamins. The vitamins used are cobalamin (B1) (100 g) and thiamin (B12) (5 g). Vitamin B12 serves to build essential proteins to promote growth. Vitamin B1 functions in the catabolism process by breaking down complex compounds into simpler forms, to produce energy in the cell division process (Widiastoety et al., 2009). Intermediate scale culture using Walne fertilizer grade technical growth. The composition of Walne grade TG fertilizer consists of KNO₃, NaH₂PO₄, Na²EDTA, and FeCl.

The composition of the fertilizer media has a very important benefit for the growth of *N. oculata* cells. NaNO₃ and KNO₃ are sources of nitrogen which are the most important elements for microalgae cell growth. The high concentration of N in NaNO₃ and KNO₃ affects metabolic activity which can take place in the optimum time because nutrient needs are met, so cell division

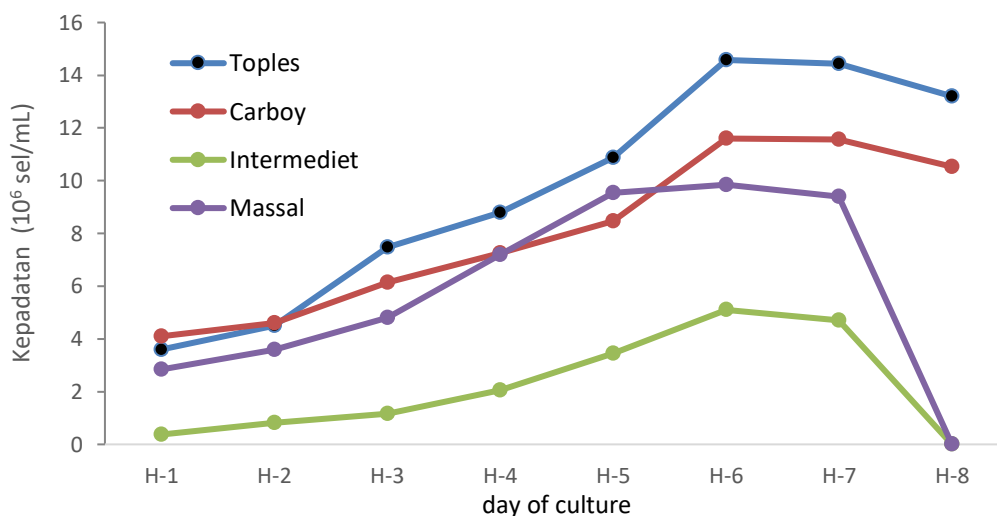
continues until the exponential phase. NaH₂PO₄ is a source of phosphorus that can increase the number of cells (Suminto, 2009). The element Mn functions in cell division and the process of photosynthesis. Boric acid (H₃BO₃) functions in regulating pH or as a buffer component (BPBAP, 2017). The need for fertilizer on a mass-scale culture can cost a lot if you use Walne fertilizer, so agricultural fertilizers are used for microalgae as a source of nitrogen and phosphate for growth because they are relatively cheap (Lam and Lee, 2012). Urea and Za are sources of nutrients because they contain high nitrogen elements. Nitrogen is an important nutrient for microalgae and plays a role in increasing microalgae growth. TSP is a macronutrient that functions as a source of phosphorus for cell metabolic processes in the growth and development of microalgae (Mukhlis et al., 2017).

On day seventh, *N. oculata* culture entered the stationary phase until the death phase. According to Creswell (2010), the exponential growth phase is followed by a stationary phase, when cell division decreases and there is no longer an increase in cell density. Decreased growth due to changes in nutrient concentration, self-shading (high cell density reduces the amount of light available to algal cells), and changes in the culture medium, such as increased pH, a buildup of metabolic waste products or substances called autoinhibitory secreted by some species (mostly diatoms). Endrawati and Riniatsih (2013) also argue that the growth of *N. oculata* is highly

dependent on the availability of nutrients, light intensity, carbon dioxide, pH, temperature, and salinity.

According to Benerjee et al. (2011), *N. oculata* grows in a temperature range between 25 - 30°C, and pH 7 - 9. Creswell (2010) argues that most microalgae strains grow best at temperatures ranging from 16 - 27°C

with an optimal temperature of around 24°C, while the pH ranges from 7.5 - 8.2 for marine species. Microalgae culture in an enclosed space must take into account the size of the room, the heat source, and the volume and temperature of the air pumped into the culture vessel.



Picture 1. *Nannochloropsis oculata* growth graph

Table 1. *Nannochloropsis oculata* growth rate from early growth to exponential phase

Culture Scale	Initial density (sel/mL)	Day-6 density (sel/mL)	Growth rate (K)
Jar	3,6 x 10 ⁶	14,58 x 10 ⁶	0,28
Carboy	4,1 x 10 ⁶	11,6 x 10 ⁶	0,21
Intermediate	0,38 x 10 ⁶	5,1 x 10 ⁶	0,52
Mass	2,85 x 10 ⁶	9,85 x 10 ⁶	0,25

The laboratory scale culture temperature of *N. oculata* was 25°C, the intermediate scale was 28°C and the mass scale was 30°C. This temperature is still optimal for indoor and outdoor cultural activities. The pH value in culture containers of all scales is 9. According to Creswell (2010), some nutrients will precipitate, algae growth will be inhibited, and microalgae

cultures can collapse at a pH of around 10. Persoone et al. (1980) argue that pH can be maintained by providing aeration. In addition, physical mixing takes place so that nutrients and cells are evenly distributed, reduces self-shading and/or photoinhibition (decreased photosynthesis due to excess light), and avoids thermal stratification in outdoor systems. In the intermediate

scale culture and mass scale, the light source comes from sunlight, while the laboratory scale culture uses a light source that is given through a 40 Watt TL lamp with a light intensity of 5000 lux. According to Prayitno (2016), light is the main component needed by microalgae for the formation of biomass and the carbon fixation process. The optimal light intensity for the growth of *N. oculata* is 5000 lux (Wahidin et al., 2013), while Kurniastuti and Isnansetyo (1995) argue that the required light illumination ranges from 100 - 10,000 lux.

Salinity affects the osmotic pressure and osmoregulation of microalgae. The salinity range for the growth of *N. oculata* is 25 - 45 ppt (Gu et al., 2012). The salinity of the laboratory scale culture is 37 ppt, while on the intermediate and bulk scales it is 36 ppt. The salinity is in the range of 25 - 45 ppt so that *N. oculata* can grow well on culture media. Rotifer contamination in mass-scale culture causes a decrease in microalgae density. According to Hoff and Snell (2008), contamination is a threat to microalgae culture.

Chemical or biological contaminants can come from one or more sources. Chemical contaminants are residual chlorine from the sterilization process, while biological contaminants may include: 1) excessive bacteria visible from cloudy water, 2) protozoa or rotifers, 3) other microalgae resulting in competition (changes in color or scale adhering to the walls of the culture container). and 4) macroalgae (green or brown strands

attached to the walls of the culture container).

CONCLUSION

The need for *N. oculata* as natural feed can be met through progressive culture starting from laboratory scale culture for inoculant stock to mass culture using concrete tanks. The difference in the size of the container and the composition of the fertilizer used in each culture scale caused differences in the peak population density of *N. oculata*. At all culture scales, the peak population density of *N. oculata* occurred on day 6 with culture density in glass containers 14.58×10^6 cells/mL, carboy culture 11.6×10^6 cells/mL, intermediate culture 5.1×10^6 cells/mL, and culture intermediates 5.1×10^6 cells/mL. bulk 9.85×10^6 cells/mL. The growth rate of microalgae *N. oculata* from the beginning of growth to day 6 was highest in the intermediate scale culture.

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