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Effect of Using Different Culture Media on the Growth of Phytoplankton *Tetraselmis chuii*

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Abstract. This study was conducted to determine the effect of different culture media on the growth of *Tetraselmis chuii*. This study used the experimental method. The treatments used in this study were P1 (GRIM Standard), P2 (GRIM+Clewat-32), P3 (Jasfa Yaeyama), and P4 (GRIM KNO_3 Clewat-32). The experimental procedure consisted of a pre-research process to observe and calculate the population density and cell size observation of *Tetraselmis chuii*. The results showed that different culture media affected the population density of *Tetraselmis chuii*. Optimum growth was obtained in the culture medium GRIM + KNO_3 Clewat-32, reaching 30.3× 10^6 cells/ml. One-way ANOVA test with a significance level of 95% showed that the difference in media had a significant effect on the growth of *Tetraselmis chuii* with a value of $F = 1.09$, $P < 0.05$. The results of the Duncan test showed that cell density in media A (Grim Standard) was significantly different from media treatments C (Jasfa Yaeyama) and D (GRIM KNO_3 Clewat-32). Still, treatment A was not significantly different from treatment B (GRIM Clewat-32), nor was treatment B significantly different from treatments C and D. The cell size of *Tetraselmis chuii* at the time of the study in all treatments obtained an average value of 6.84 - 11.55 µ. The water quality required for *T. chuii* is suitable for culture media.

Keywords *Tetraselmis chuii*, growth, media culture, cells, density

I. INTRODUCTION

North Bali has several types of main potential in fish resources, consisting of grouper, snapper, tuna, and other types of reef fish. In addition to fish, other catches are small squid [1]. Reference [21] added that the potential of waters in North Bali, precisely in Buleleng Regency, besides having the potential for capture fisheries, also has potential in aquaculture. In aquaculture, cultivated commodities include milkfish, grouper, ornamental fish and reef fish, pearls, and seaweed. The North Bali water area is one of Indonesia's largest milkfish, grouper, and sea bass aquaculture centers. The success of fish hatcheries is highly dependent on natural fish larval feed availability. The availability of raw food plays an essential role in fish farming, especially in the fry stage, and is the main requirement that must be provided because it can improve the survival of fish larvae.

Microalgae have an essential role as natural food for zooplankton and fish larvae because they contain complete carbohydrates, proteins, fats, minerals, and amino acids. Microalgae that are good and often used as natural food for fish larvae and zooplankton such as rotifers, one of which is *Nannochlropsis* sp. This plankton is easy to cultivate, and the population is relatively high. However, its availability is often problematic because this microalgae is too sensitive to environmental changes, such as lack of sunlight during the rainy season. An obstacle often encountered with *Nannochloropsis* sp. is mass culture. The reduced cell density of *Nannochloropsis* sp. can affect cultivation activities because the quality of *Nannochloropsis* sp. used for initial larval feed and rotifer culture greatly affects the success of hatcheries [20]. One type of natural food that can be used as a substitute for *Nannochloropsis sp*. is *Tetraselmis chuii*.

T.chuii is a type of phytoplankton from the Clorophyceae class that can be used as a natural food for fish and non-fish larvae and used in rearingseawater fish larvae with a *green water* system. *Tetraselmis* sp. has been widely used in marine fish hatcheries such as shrimp larvae, ornamental fish, and sea cucumber larvae [14].

According to reference [15], *Tetraselmis chuii* is one type of natural food that can fulfill aquaculture feed needs. The advantages of this plankton include being available in nature, having a size that matches the mouth opening of fish larvae, and having movements that can stimulate aquatic organisms to prey on it. Another advantage is that it can be used as a biopharmaceutical, biofuel, and bioethanol and as an alternative material for biodiesel. *T.chuii* contains 48.42% protein, 12.10% carbohydrate, and 9.70% fat. *T.chuii* extract has antioxidant activity ranging from 2.55-31.29 mg/ml and total chlorophyll ranging from 3.65-19.20 mg/g [7].

Tetraselmis chuii has many benefits and uses, so its availability is needed in large quantities. *Continuous* provision of T. *chuii as a* natural food is very difficultif only collecting it from *nature*, so it is necessary to produce *T. chuii*, which can be increased by culturing on a production scale. *T. chuii* was chosen to becultured and fed as a natural food because it is an alternative to non-diatomic *Nannochloropsis* sp., which is fed as fish food, especially in large-scale production of milkfish and grouper fry, due to the difficulty of developing mass or semi-mass culture.

Related to this problem, a study was conducted using modified media GRIM Standard, GRIM + Clewat-32, Jasfa Yaeyama, and GRIM KNO_3 Clewat-32 on the growth of *Tetraselmis chuii* as fish larvae feed. The use of technical fertilizer in the culture medium is expected to increase the density of *Tetraselmis chair phytoplankton because this fertilizer is an inorganic fertilizer that has a* complete macronutrient and micronutrient content compared to organic fertilizers.

II. METHODS

A. Research Time and Location

The research was carried out on November 22, 2022 - January 16, 2023, with culture in the treatment carried out within 36 days at the Biotechnology Laboratory of the National Research and Innovation Agency (BRIN), CWS Gondol, Bali. For more details, the following map illustrates the location of the intended research site.

B. Experiment Procedure

The type of research conducted is experimental research by making a set of experiments to see whether or not there is an effect of different culture media on the growth rate of *Tetraselmis chuii*. This study used a research design, namely; 1) *Pre-test post-test control group design* as treatment design; 2) Completely Randomized Design (CRD) for placing experimental units with four treatments with three replicatesin each treatment. The treatments are; 1) treatment A with GRIM media standard technical fertilizer 0.175 ml/L; 2) treatment B with GRIM media + Clewat-32 technical fertilizer 0.164 ml/L; 3) treatment C with Jasfa Yaeyama media technical fertilizer 0.129 ml/L; 4) treatment D with GRIM-KNO_3 media + Clewat-32 technical fertilizer 0.164 ml/L.

The tools used in this study include an Erlenmeyer volume of 2000 ml, as many as 12 pieces, an aeration hose, *beaker glass*, measuring pipette, measuring cup, TL lamp and watt lamp, air conditioner, culture rack, room thermometer, microscope, drop pipette, micropipette, hemocytometer, hand counter,refractometer, Baruno water quality meter, gas stove, drying rack, container tub, sterilization jar, bucket, cover glass, aluminum foil, small Erlenmeyer, microtube, WinROOF manual, Eppendorf tube, 0.01g scale, NIKON microscope along with WinROOF application, funnel, sponge, sterilization container, jar, and *sand filter*. The materials used in this study include *Tetraselmis chuii* seeds, GRIM Standard media, GRIM + Clewat-32 media, Jasfa Yaeyama media, GRIM KNO_3Clewat-32 media, $FeCl_3$ fertilizer, urea fertilizer, Clewat-32, EDTA, TSP, NPK/Ammonium sulfate, KNO3, KW21 fertilizer sodium thiosulfate 2.5 grams, sterile seawater, chlorine, alcohol, *tissue*, HCl solution, and liquid laundry soap.

The experimental procedure was carried out through several stages of activity, namely; 1) sterilization of tools; 2) making fertilizer; 3) expansion of pure culture; 4) preparation of culture water; 5) preparation of culture media; 6) seedling selection; 7) planting inoculants. Sterilization of tools is done by preparing all the tools that will be used, then sterilizing by washing using soap and running water and giving HCl to kill inoculants or other unwanted microorganisms such as microbes that are still left in the tool. After being sterilized by washing,the tools are arranged on a storage rack and then sprayed using alcohol as a disinfectant and left to dry. Related to the fertilizer manufacture, the fertilizer made is KW21 type fertilizer for pure culture with the dosage used in the Erlenmeyer as much as 1 ml/1000 ml of inoculant. The fertilizer to be cultured in the treatment was prepared. The modified fertilizer for the4 treatments was weighed first according to the dosageused, previously calculated using the formula. After weighing, the fertilizer was put into a container and stored. Regarding the pure inoculant, the pure inoculant was obtained from the Biotechnology Laboratory of the National Research and Innovation Agency (BRIN), CWS Gondol, Bali. This study was conducted for 36 days. The algae density at the beginning of the inoculation experiment was about 300,000 cells/ml. The culture containers used in this study were 12 erlenmeyers of 2,000 ml volume. The water sanitation system used is a high-pressure *sand filter of* 2 units. The initial stock of pure inoculant was cultured in 500 ml erlenmeyer for 10 days. The inoculant was cultured using

a 3,000 ml volume erlenmeyer for seven days. The population density during pure culture was 1,300,000 cells/ml. The pure culture was carried out using boiled seawater and KW21 fertilizerat 1 ml/1000 ml of inoculant. Related to the preparation of culture water, culture water uses seawater that has been filtered with a *sand filter* and accommodated in a 20 L plastic container, after whichit is given 5 ml of chlorine and allowed to stand first to be sterilized for 24 hours until homogeneous. The culture process begins with neutralizing water in the culture medium. After 24 hours, it is given thiosulfate as much as 2.5 grams, left for 3 hours and ready for culture. Related to the preparation of culture media, the preparation was carried out using erlenmeyer with a capacity of 2000 ml filled with sterile water with aeration given to each culture container (erlenmeyer). Each culture container was given technical fertilizer according to the treatment. The fertilizer waited until it was well mixed. The seedling selection was done by first turning off the aeration, then precipitating the inoculant, and selecting inoculants that floated solid green with high density. The selected inoculants were then planted byfirst calculating the initial density with the formula according to reference [9]

$$V1 = N2 \times V2 / N1 \times 100\%$$

Description:
V1 = seedling volume for initial translation (ml)
V2 = desired volume of culture medium (ml)
N1 = seedling/stock density (cells/ml)
N2 = desired seedling density (cells/ml)

Inoculants were put into the prepared culture medium. *Tetraselmis chuii* inoculant was put into the culture medium with a ratio of seawater and inoculantof 770:230 ml.

C. Data Collection Method

Data were collected using observation, counting and measurement methods. Observations were made of the state of *Tetraselmis chuii* placed on a *haemocytometer* using a microscope with a magnification of 100 times, 200 times, and 400 times.Aspects observed were the shape and color of *Tetraselmis chuii*, while calculations were made on the density of *Tetraselmis chuii*, measurements were made on the cell size of *Tetraselmis chuii.* Density was calculated using a *hand counter*, while cell size was measured using the Win ROOF application.

D. Data Analysis Technique

Observational data from the results of this studyis the cell density of *Tetraselmis chuii* which is calculated using a *haemocytometer*. The density data is then presented as a table containing the results of the calculation of daily cell density of *Tetraselmis chuii* and in the form of graphs analyzed using the *Microsoft Excel for Windows* program. The data were then processed using SPSS version 26 to determine the values in the normality test, homogeneity test, *one-way* ANOVA test, and Duncan'sfurther test.

Fig. 1. Location Map of the Research Site

III. RESULTS AND DISCUSSION

A. Population Density of *Tetraselmis chuii*

The media in each treatment at the beginning of the culture, namely on day 0 and day 1, is light green and clear, which indicates that the plankton will growwell. The color of *Tetraselmis chui* phytoplankton a few days later changed color to dark green and dense.This indicates that the plankton grew and experienced an increase in cell density. This statement is in accordance with the opinion of reference [19] that the growth of plankton during cultivation or culture can be seen visually marked by a change in color from initially clear to light green and changes again to green/dark green. This color change occurs accompanied by a decrease in transparency. This indicates an increase in cells whichwill directly affect the density level. The image at the beginning of the culture, namely on day -0 and day five can be seen in Figure 2 and Figure 3 as follows:

Fig. 2. Treatment at the Beginning of Culture (Day 0)

Fig. 3. Treatment After Several Days of Culture (Day 5)

Cell density was calculated using a *haemocytometer* under a microscope with 100x magnification every 24 hours, the data was entered into *Microsoft Excell for Windows and* then analyzed in the form of graphs which can be seen below:

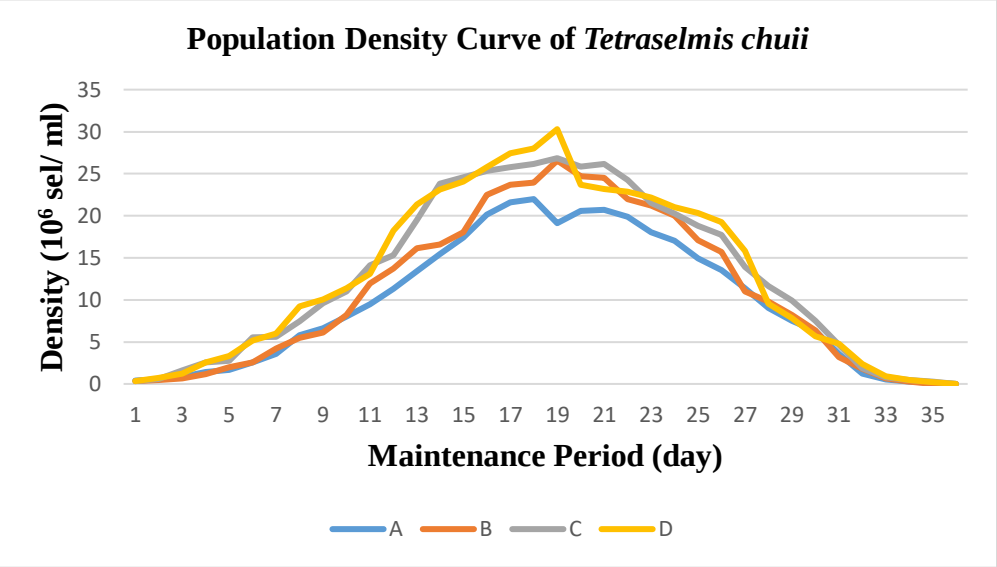

Fig. 4. Population density curve of *Tetraselmis chuii*

The density given to each treatment of *Tetraselmis chuii* culture is the same, namely 0.3×10^6 cells/ml. The treatment using GRIM Standard reached the exponential phase on day 18, which is as much as 21.983333×10^6 cells/ml. GRIM + Clewat-32 reached the exponential phase on day 19 at 26.566666×10^6 cells/ml. Jasfa Yaeyama treatment experienced an exponential phase on day 19 reaching 26.85×10^6 cells/ml. GRIM+KNO_3 Clewat-32 experienced an exponential phase on day 19, reaching 30.3×10^6 cells/ml. The inoculant experienced a stationary phase until the death phase on day 36. The highest population density successively occurred in treatments D, C, B, and A. Cell density in different treatments and replicates can experience different amounts of population density. This can occur due to several factors. According to reference [6] microalgae experience an increase in cell density in the exponential phase which occurs because there is sufficient space to move in the growth process. The availability of nutrients and environmental factors also affect cell population density, such as sufficient temperature and light in the culture medium. All treatments experienced a decrease in growth rate after reaching the peak phase. This was thought to be due to the reduction of nutrients in the culture medium which caused a decrease in the cell population. Reference [13] states that a decrease in the number of cells occurs because the organism cannot maintain it self because after experiencing peak growth, the inoculant will experience a decrease due to lack of nutrients and light penetration and sufficient light intensity due to "*self-shading*" which means that the inoculant is protected from its own shadow which causes limited light to be obtained.

The highest cell population density of *Tetraselmis chuii was* obtained in the treatment using GRIM+KNO_3 Clewat-32 media which reached 30.3×10^6 cells/ml. According to reference [3] providing the right KNO_3 can make phytoplankton experience optimum growth and affect growth patterns, biomass, and crude protein content in phytoplankton. The density of *Tetraselmis chuii* in the microscope 400× magnification can be seen in the following Figure 5:

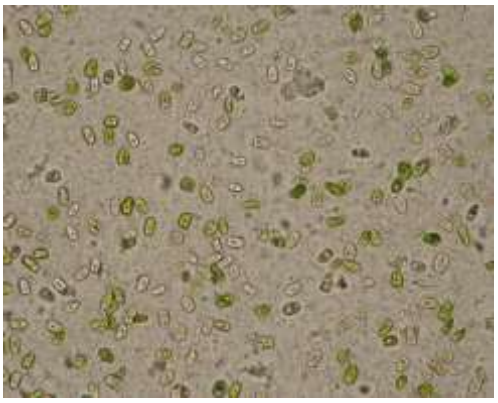

Fig. 5. *Tetraselmis chuii* under the microscope (400x magnification)

T. chuii cells experienced a phase of decreasing density starting on day 19 in the treatment using GRIM Standard treatment (A). The other treatments also

experienced a decreasing phase starting on day 20, and all treatments experienced the death phase on day 36. The death phase (declination) can be characterized by a change in color to light green or clear, which was originally dark green, foam appearing on the culture medium's surface, and *Tetraselmis chuii* cells sticking or settling on the walls of the Erlenmeyer. This is in accordance with the statement of [9] that morphologically in the deathphase, many algae cells die rather than divide. This phase is characterized by the changing color of the culture water, froth on the surface of the culture media, and clumps of algae cells that settle to the bottom of the culture container. The death phase in microalgae occurs due to the reduction or decrease of nutrients inthe culture medium and the ability of old cells to metabolize, resulting in a very rapid decrease in the number of cells compared to their growth.

Population growth or cell density of *T.chuii* was analyzed using SPSS *software* version 26. The analysis obtained the results of normally distributed and homogeneous data. The next test was conducted *one-way* ANOVA with a significance level of 95%. The test results showed that cell density using different fertilizers significantly affected on the growth of *T. chuii* with a value of $F = 1.09$, $P < 0.05$. It was continued with the Duncan test to determine the real difference between treatments. The results of Duncan's test showed that cell density in media A (Grim Standard) was significantly different from media treatments C (Jasfa Yaeyama) and D (GRIM KNO_3 Clewat-32), but treatment A was not significantly different from treatment B (GRIM Clewat-32), nor was treatment B significantly different from treatments C and D. The results of Duncan's test showed that cell density in media A (Grim Standard) was significantly different from treatment B (GRIM Clewat-32).

B. Cell Size of *Tetraselmis chuii*

The cell size of *Tetraselmis chuii* can be seen in the following table:

TABLE 1.
CELL SIZE OF *Tetraselmis chuii*

Parameters	Medium Treatment			
	A	B	C	D
Cell *length* (µ)	11,22 ± 1,16	11,15 ± 2,68	11,60 ± 2,42	12,25 ± 2,88
Cell width (µ)	5,66 ± 0,84	6,69 ± 1,94	7,33 ± 1,64	7,67 ± 2,60

The cell size of *T. chuii* observed under a microscope obtained the largest cell size in treatment D with a length of 12.25 ± 2.88 µ and a width of 7.67 ± 2.60 µ, while the smallest cell size was obtained in treatment A with a length of 11.22 ± 1.16 µ and a width of 5.66 ± 0.84 µ.

The cell size of *Tetraselmis chuii* at the time of the study in all treatments obtained an average value of 6.84 - 11.55 µ. This is in accordance with the statement of [12] the cell size of *Tetraselmis chuii* ranges from 7-12 µ.

C. Water Quality

Water quality is an important medium to support phytoplankton life. Based on the results of water quality measurements in the study, the value of water quality parameters showed tolerance limits according to the maintenance conditions. Water quality influences on the growth of phytoplankton that is maintained. Water quality measurements for temperature, pH, DO, salinity, nitrate, and phosphate measured at the beginning of rearing on the first day and the second week on the eighth day. This was done to determine the feasibility and influence of changes in water quality parameters in the lag/adaptation phase and when the population experienced an exponential phase during the culture of *Tetraselmis chuii*.

TABLE 2.
WATER QUALITY RANGE OF *Tetraselmis chuii* CULTURES DURING THE STUDY

Parameters	Parameters			
	A	B	C	D
Temperature (°C)	18,95±2,05	20,3±0,28	20,55±0,35	20,25±0,77
pH	7,94±0,21	8,13±0,16	8,1±0,07	8,15±0,07
Salinity (ppt)	35,5±0,70	35,5±0,70	35,5±0,70	35,5±0,70
DO (mg)	7,09±0,01	6,73±0,44	6,69±0,30	6,72±0,43
Phosphate (ppm)	6	3,75	2,50	1,25
Nitrate (ppm)	100	100	100	50

The average pH in this study was around 8.08. Nurmalitasri et al. [12] stated that the optimal pH level for Tetraselmis chuii growth ranges from 8 to 9.5. The study's average dissolved oxygen (DO) level was 6.81 mg/L. This isin accordance with Citra [1], which states that the optimal dissolved oxygen levels for the growth of*Tetraselmis chuii* in marine waters are around 11 mg/lat 0°C and 7 mg/l at 25°C. Dissolved oxygen levels innatural waters are usually less than 10 mg/l.

The average temperature obtained during maintenance is 20.01°C. These results are to Martosudarmo and Wulani [9] statement that Tetraselmis chuii can grow with a tolerance temperature between 15-36°C, and the optimal temperature ranges from 23-25°C. Reference [2] added that the optimum temperature range forphytoplankton growth in waters is 20-30°C.

Salinity during maintenance obtained an average value of 35.5 ppt. This is in accordance with [16] opinion that the optimum salinity of *Tetraselmis chuii* is 25-35 ppt. Reference [18] added that *Tetraselmis chuii* has a very high tolerance that ranges from 20-36 ppt because *Tetraselmis chuii* is a single-celled algaethat is very tolerant of large salinity changes.

The phosphate value during the study in treatments A, B, C, and D consecutively amounted to 6 ppm, 3.75 ppm, 2.50 ppm, and 1.25 ppm. The value of phosphate in maintenance exceeds the specified threshold. According to reference [2], the phosphate content in water is 0.005-0.02 mg/l. Total phosphate levels exposed in natural waters rarely exceed 1mg/l. Orthophosphate, based on its level in the water, can be divided into 3: oligotrophic with a phosphate range of 0.003 - 0.001 mg/l, mesotrophic with a phosphate range of 0.001 - 0.02 mg/l, and eutrophic with a phosphate range of 0.031 - 0.1 mg/l. Phosphate is non-toxic to humans, animals, and fish. The presenceof phosphate in water is significant, especially in the formation of proteins and metabolism for organisms. However, it is feared that high phosphate can cause eutrophication in the form of algae *blooming*, which is terrible for aquaculture. The source ofphosphate in waters comes from livestock waste, human waste, especially detergents, agriculture, especially the use of inorganic fertilizers such as TSP(*Triple Super Phosphate*), industrial waste, and natural processes in the environment [5]. Nitrateand Phosphate content will affect the growth of algae and aquatic plants. According to reference [5], phosphate is one of the main elements for protein formation and organism cell metabolism. Several external factors influence the availability of phosphate in waters by algae. The absorptionof phosphate by algae is strongly influenced by light, especially in limited conditions (CO_2), then the use ofphosphate by algae will decrease in a dark room.Optimal algae growth will occur when phosphate concentration is high and nitrate as a nitrogen source. Phosphate is also strongly influenced by pH, pH plays a role in changing the speed ofphosphate absorption by changing enzyme activity, namely the permeable properties of cell membranes orthe degree of phosphate ionization.

The nitrate value during the study in treatments A, B, and C was 100 ppm, and treatment D was 50 ppm. The nitrate value exceeded the threshold value. According to reference [4], the optimum nitrate content for phytoplankton growth is between 0.9 and 3.5 mg/l. The high value of nitrate content is thought to be due to the input or increase in nutrients from the rest of the fertilizer that settles under the erlenmeyer along with the reduced volume of inoculants so that the color of the water is getting greener and denser cell density. According to reference [22], the increase in nutrients causes the growth of algae and aquatic plants in excess, which is also called eutrophication. According to reference [17], nitrate functions as a controller of primary productivity of waters in the euphotic zone and can be a fertilizer for aquatic plants.

IV. CONCLUSION

The results showed that different culture media significantly affected differences in *Tetraselmis chuii* growth. Treatment D by using GRIM KNO_3 Clewat-32 media gave the best growth with density indicators reaching 30.3×10^6 cells/ml and cell size with a length of 12.25±2.88 µ and width of 7.67±2.60 µ.

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