International Journal of Natural Science Studies and Development (IJOSS)
Volume 02 Issue 01 2025, pp 114-123
E-ISSN: 2615-1383
DOI: https://doi.org/10.55299/ijoss.v2i1.18

Growth of *Nannochloropsis oculata* Cultured using Tofu Liquid Waste Fertilizer

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Abstract

Growth of Nannochloropsis oculata is affected by nutrients in its culture media. Tofu liquid waste which is processed into fertilizer is expected to be an alternative nutrient that is more economical for microalgae culture activities. The purpose of this study was to determine the effect of tofu liquid waste fertilizer with different concentrations on the growth of Nannochloropsis oculata. This study used an experimental method with Completely Randomized Design (CRD). The treatment was different concentrations namely P1 (1 ml L^{-1}), P2 (3 ml L^{-1}), P3 (5 ml L^{-1}), P4 (7 ml L^{-1}) and P0 (Walne nutrient 1 ml L^{-1}) each treatment were repeated four times. The main parameters in this study were cell density, cell growth rate and cell doubling time. Data obtained were analyzed using Analysis of Variance (ANOVA) and continued with Duncan's Multiple Range Test (DMRT). Results showed that the treatment of tofu liquid waste fertilizer with different concentrations had a significant different (p<0,05) on the growth of Nannochloropsis oculata. Concentration of 5 ml L^{-1} (P3) was the best concentration for the growth of Nannochloropsis oculata with peak cell density 1,335±13.54x10^4 cells ml^{-1}, cell growth rate 0.900±0.001 cells ml^{-1} day^{-1} and cell doubling time 18.493±0.026 hours.

Keywords: *Tofu Liquid Waste Fertilizer, Growth, Nannochloropsis oculata*

1. INTRODUCTION

Nannochloropsis oculata is a microalgae whose growth is closely related to the nutrient content in culture media. Walne nutrient is a standard nutrient to support the growth of these microalgae. Walne nutrient has a disadvantage due to its less economical price because it's composed of a variety of synthetic chemical formulations.

Tofu liquid waste has the potential to be used as fertilizer in *Nannochloropsis oculata* culture because it's contains macronutrients and micronutrients that can be utilized by microalgae. Dianursanti et al. (2014) and Widayat et al. (2017) showed that tofu liquid waste can be used for green microalgae cultures but the cell density was still low. This was allegedly caused by the use of tofu liquid waste directly. The results of the preliminary test showed that tofu liquid waste has organic matter of 2,157.18 mg L^{-1} and N:P ratio of 0.79:1. Tofu liquid waste should be treated first by reducing organic matter and adjusting the ratio of nitrogen: phosphorus (N:P) according to growth conditions.

History:
Received : December 22, 2024
Revised : January 24, 2025
Accepted : January 29, 2025
Published : January 30, 2025

Publisher: Inovasi Pratama Int. Press

Effective microorganisms (EM) are suitable microorganism cultures to degrade these organic matter. The test results showed that N:P ratio of tofu liquid waste after fermentation of 0.88:1. The N:P ratio in tofu liquid waste after fermentation with EM also needs to be increased by adding nitrogen. The standard N:P ratio commonly used in *Nannochloropsis oculata* cultures is 17:1, which refers to Walne nutrient. The purpose of this study was to determine the effect of tofu liquid waste fertilizer with different concentrations on the growth of *Nannochloropsis oculata* and to know the concentration that can provide the best growth.

2. METHOD

The materials used in this study were culture rack, aerator, tubular light (TL) set, lux meter, T branch, culture tube, aeration hose, cleaning brush, glass drop pipette, haemocytometer, glass cover, binocular microscope, sprayer, hand counter, optilab camera, glass jar, erlenmeyer, autoclave, aeration, thermometer, refractometer, pH meter, measuring cup, analytic balance, measuring pipettes, bulbs, storage bottles, jerry cans, *Nannochloropsis oculata* from BPBAP Situbondo, tofu liquid waste, plastic wraps, Effective Microorganism (EM), molasses, aquades, 70 % alcohol, liquid chlorine, cotton, tissue, sodium thiosulfate, Walne nutrient, sea water, paper label and fresh water.

This research used an experimental method with Completely Randomized Design (CRD). The treatment used were tofu liquid waste fertilizer with different concentrations namely P1 (1 ml L^{-1}), P2 (3 ml L^{-1}), P3 (5 ml L^{-1}), P4 (7 ml L^{-1}) and P0 (Walne nutrient 1 ml L^{-1}) each treatment were repeated four times. The main parameters in this study were cell density, cell growth rate and cell doubling time. Data obtained were analyzed using Analysis of Variance (ANOVA) and continued with Duncan's Multiple Range Test (DMRT). The nutrients content of each treatments are presented in Table 1.

Table 1. Nutrient Content in Each Treatment

Treatment	Nitrogen (N) (mg L^{-1})	Phosphorus (P) (mg L^{-1})	N:P Ratio	Iron (Fe) (µg L^{-1})	Zinc (Zn) (µg L^{-1})	Manganese (Mn) (µg L^{-1})
P0	1.510	0.087	17:1	0.312	0.010	0.100
P1	10.999	0.647	17:1	1.604	0.321	0.402
P2	32.997	1.941	17:1	4.812	0.963	1.206
P3	54.995	3.235	17:1	8.020	1.605	2.010
P4	76.993	4.529	17:1	11.228	2.247	2.814

Preparation for Tofu Liquid Waste Fertilizer Stock

Effective microorganism (EM) was activated by mixing EM, molasses and distilled water in a ratio of 1:1:20 in a glass jar. The mixture was stirred evenly and left for 12 hours under anaerobic conditions. Fermentation is done by mixing EM which has been active with tofu liquid waste using a ratio of 1:20 in a glass jar and then left for 7 days under anaerobic conditions. Tofu liquid waste that has been through the fermentation process was filtered again to eliminating the lumps formed during the fermentation process and then carried out the autoclave sterilization process at a temperature of 121 ^{o}C and a pressure of 1 atm for 15 minutes. Tofu liquid waste before and after fermentation examined on the content of nitrogen (N), phosphorus (P) and organic matter. Results can be seen in Table 2.

Table 2. Tofu Liquid Waste Examination Result

Tofu Liquid Waste	N-Total (mg L^{-1})	P-Total (mg L^{-1})	N:P Ratio	Organic Matter (mg L^{-1})
Pre Fermentation	330	414	0.79:1	2,157.18
Post Fermentation	572	647	0.88:1	873.42

Results showed that the N:P ratio of tofu liquid waste after fermentation was still not in accordance with the standard, namely 17:1 so that it was necessary to add nitrogen from urea fertilizer. Urea fertilizer contain nitrogen of 45.17 %. The calculation results showed to increase the N:P ratio from 0.88:1 to 17:1, it was necessary to add 23.0839 grams of urea fertilizer in 1 liter of fermented tofu liquid waste.

Preparation for Walne Nutrient Stock

Walne nutrient in this study was obtained from BPBAP Situbondo. Walne nutrient contains N:P ratio of 17:1 with a concentration of 1 ml L^{-1} of culture media. Each 1 liter Walne nutrient contains 100 grams of $NaNO_3$, $NaH_2PO_4.2H_2O$ 20 grams, Na_2EDTA 45 grams, H_3BO_3 33.6 grams, $FeCl_3.6H_2O$ 1.5 grams, $MnCl_2.4H_2O$ 0.36 grams, $Na_2SiO_3.9H_2O$ 40 grams, trace solution 1 ml metal and 1 ml vitamin solution (Endar et al., 2012).

Sterilization of Culture Equipment and Media

The glassware sterilized by being wrapped using opaque paper then autoclave at 121 ^oC and pressure of 1 atm for 15 minutes. Large glass equipment and aeration sterilized using a chlorine solution of 150 mg L^{-1} for 24 hours then rinsed with fresh water until the smell of chlorine was lost and dried. Sterilization of culture media was carried out using chlorine 60 mg L^{-1} for 24 hours and neutralized with sodium thiosulfate 30 mg L^{-1} with aeration for 24 hours.

Nannochloropsis oculata Culture

The culture in this study used a volume of 500 ml. Preparation was carried out by mixing sterile sea water with salinity of 30 ppt then adding fertilizer according to the treatment and inoculum with an initial cell density of 10^4 cells ml^{-1} then cultured for 14 days. The expected culture environment was temperature of 32-36 ºC, pH 7-9, salinity 30 ppt, light intensity 5.000 lux. The photoperiod used in this study was 24 hours bright.

Cell Density Calculation (N)

Cell density of *Nannochloropsis oculata* was calculated using the Tulashie and Salifu (2017) method every 24 hours for 14 days from the beginning of culture. The formula for calculating cell density as follows:

$$\text{Cell Density (N) (cells ml}^{-1}) = \frac{Na+Nb+Nc+Nd+Ne}{5 \times 4 \times 10^{-6}} \quad (1)$$

Note :

N_a, N_b, N_c, N_d, N_e : Cell density in the square a, b, c, d, e (cells)
konstanta 5 : Total square used to count
4×10^{-6} : Square a, b, c, d, e volume (ml)

Cell Growth Rate Calculation (μ)

Cell growth rate was calculated when it reached the peak of the exponential phase. Calculation of cell growth rate refers to the method of Paes et al. (2016) as follows:

$$\text{Cell Growth Rate } (\mu)\ (\text{cells ml}^{-1}\ \text{day}^{-1}) = \frac{\ln N_t - \ln N_0}{\Delta t} \quad (2)$$

Note
N_t : Cell density at exponential phase peak (sel ml^{-1})
N_0 : Cell density at the beginning (sel ml^{-1})
Δt : Different in cell density calculation time (day)

Cell Doubling Time Calculation (t_d)

Cell doubling time was calculated when it reached the peak of the exponential phase. Calculation of cell doubling time refers to the method of Schweitzer et al. (2014) as follows:

$$\text{Cell Doubling Time } (t_d)\ (\text{hour}) = \frac{\ln 2}{\mu} = \frac{0{,}693}{\mu} \quad (3)$$

Note
t_d : Cell doubling time (hour)
μ : Cell growth rate (sel ml^{-1} $hour^{-1}$)

3. RESULTS AND DISCUSSION

Results

The results of Analysis of Variance (ANOVA) showed that the treatment of tofu liquid waste fertilizer with different concentrations had a significant different (p<0.05) on cell density (Figure 1), cell growth rate and cell doubling time of *Nannochloropsis oculata*. Results of Duncan's Multiple Range Test (DMRT) at the cell population peak (day 8) showed that there were significant different (p<0.05) between treatments (Table 3). Results of Duncan's Multiple Range Test (DMRT) on growth rate and cell doubling time time data (Table 4) showed that there were significant differences (p<0.05) between treatments. Result of environment condition can be seen in Table 5.

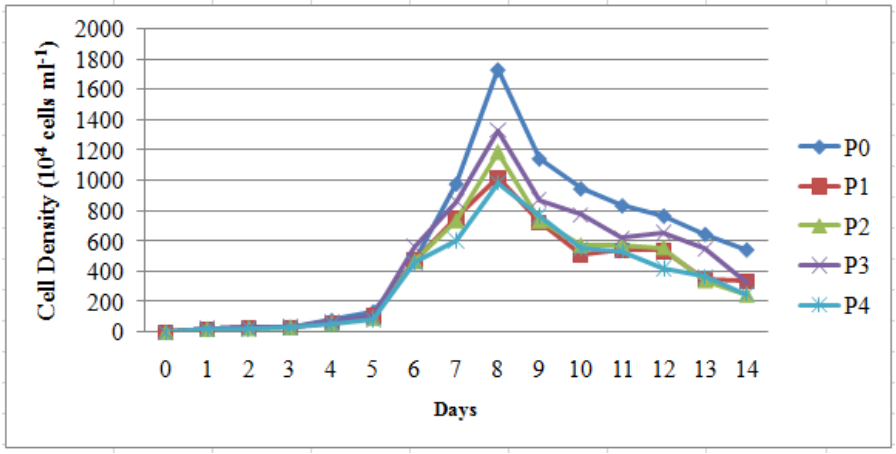

Figure 1. Growth Curve of *Nannochloropsis oculata*

Table 3. *Nannochloropsis oculata* Average Cell Density

Culture Period (Day)	Cell Density (Average±SDx10^4 cells ml^{-1})				
	P0	P1	P2	P3	P4
0	1±0	1±0	1±0	1±0	1±0
1	21.25±2.5^{ab}	20±0^{b}	23.75±2.5^{a}	23.75±2.5^{a}	20±0^{b}
2	32±0.81^{a}	27.25±0.5^{b}	26.5±0.57^{b}	27±0.81^{b}	25±1.15^{c}
3	35.25±1.25^{a}	32.5±0.57^{c}	33±1.15^{bc}	34±0.81^{ab}	30.5±0.57^{d}
4	78.75±0.95^{a}	57.25±1.5^{d}	60.75±0.95^{c}	71.5±0.57^{b}	55.25±1.5^{e}
5	135.5±3.69^{a}	105.25±2.06^{b}	96.75±3.86^{c}	105.25±1.5^{b}	82.25±2.06^{d}
6	473.5±4.04^{b}	474.5±24.44^{b}	466.25±30.41^{b}	565.5±7.85^{a}	459±7.52^{b}
7	980.75±12.99^{a}	752.5±15.02^{c}	735.25±8.30^{c}	864.25±9.17^{b}	605±23.80^{d}
8	**1,736.25±17.01^{a}**	**1,020±4.08^{d}**	**1,186.25±7.5^{c}**	**1,335±13.54^{b}**	**985±10.80^{e}**
9	1,147.5±10.4^{a}	725±4.08^{d}	732.5±10.4^{d}	876.25±4.78^{b}	766.25±4.78^{c}
10	950±12.9^{a}	512.25±11.05^{d}	573.75±26.57^{c}	778.25±18.67^{b}	550.75±26.87^{c}
11	837.5±32.01^{a}	542.5±6.45^{d}	573.75±2.5^{c}	622.5±6.45^{b}	536.25±20.96^{d}
12	770±9.12^{a}	536.25±20.15^{c}	557.5±15.0^{c}	658.75±21.36^{b}	421.25±43.08^{d}
13	648.75±33.26^{a}	350±34.88^{c}	342.5±13.22^{c}	552.5±23.27^{b}	366.25±35.44^{c}
14	545.75±31.76^{a}	334.5±18.73^{b}	248.25±33.04^{c}	334.5±17.15^{b}	251.5±29.03^{c}

Note : P0 (Walne nutrient 1 ml L^{-1}), P1 (tofu liquid waste fertilizer 1 ml L^{-1}), P2 (tofu liquid waste fertilizer 3 ml L^{-1}), P3 (tofu liquid waste fertilizer 5 ml L^{-1}), P4 (tofu liquid waste fertilizer 7 ml L^{-1}) and SD = Standard Deviation, different superscripts on the same line show significant different (p<0.05).

Table 4. *Nannochloropsis oculata* Average Cell Growth Rate and Cell Doubling Time

Treatment	Cell Growth Rate (Average±SD cells ml^{-1} day^{-1})	Cell Doubling Time (Average±SD hour)
P0	0.932±0.001^{a}	17.841±0.023^{a}
P1	0.866±0.001^{d}	19.211±0.011^{d}
P2	0.883±0.001^{c}	18.801±0.017^{c}
P3	0.900±0.001^{b}	18.493±0.026^{b}
P4	0.862±0.001^{e}	19.308±0.031^{e}

Note : P0 (Walne nutrient 1 ml L^{-1}), P1 (tofu liquid waste fertilizer 1 ml L^{-1}), P2 (tofu liquid waste fertilizer 3 ml L^{-1}), P3 (tofu liquid waste fertilizer 5 ml L^{-1}), P4 (tofu liquid waste fertilizer 7 ml L^{-1}) and SD = Standard Deviation, different superscripts on the same row show significant different (p<0.05).

Table 5. Environment Condition of *Nannochloropsis oculata* Culture

Parameter	Results					Normal Range
	P0	P1	P2	P3	P4	
Culture Media Temperature (°C)	27.7-35.3	27.6-34.9	27.7-35	27.7-35	27.7-34.7	^{a}25-36
Culture Chamber Temperature (°C)	28-32	28-32	28-32	28-32	28-32	^{b}25-30
Culture Media pH	7.6-8.6	7.6-8.7	7.6-8.8	7.6-8.7	7.6-8.7	^{a}7-9.4
Culture Media Salinity (ppt)	30-38.5	30-38.5	30-38.5	30-38.8	30-38.3	^{c}25-45
Culture Chamber Light Intensity (lux)	5,230-5,882	5,210-5,922	5,407-5,960	5,270-5,960	5,277-5,960	^{d}3,000-9,000

Note : ^{a}Banerjee et al., 2011; ^{b}Sun et al., 2018; ^{c}Gu et al., 2012; ^{d}Budiono et al., 2018 and P0 (Walne nutrient 1 ml L^{-1}), P1 (tofu liquid waste fertilizer 1 ml L^{-1}), P2 (tofu liquid waste fertilizer 3 ml L^{-1}), P3 (tofu liquid waste fertilizer 5 ml L^{-1}), P4 (tofu liquid waste fertilizer 7 ml L^{-1}).

Discussion

Growth of *Nannochloropsis oculata*

Results on growth parameters were cell density (Table 3), cell growth rate and cell doubling time (Table 4) showed that treatment P3 provides the highest cell density, cell growth rate and the shortest cell doubling time compared to treatments P1, P2 and P4 but treatment P3 has lower cell density, cell growth rate and longer cell doubling time compared to P0 (Walne nutrient). It was assumed that nutrients contained in tofu liquid waste fertilizer were suitable to supported the growth of *Nannochloropsis oculata*, but the nutrients contained in Walne nutrient were still more optimal to support growth compared to nutrients in tofu liquid waste fertilizer.

The nitrogen:phosphorus (N:P) ratio, nutrient concentration and nutrient sources are factors that influence microalgae growth (Ashour and El-Wahab, 2017). The N:P ratio in all treatments was 17:1. The difference in growth that occurs was thought to be due to different in the concentration of nutrients and sources of nitrogen and phosphorus from the two nutrients.

In the treatment of tofu liquid waste fertilizer, treatment P3 has the best growth when compared with treatments P1, P2 and P4. It was assumed that nutrient concentrations in treatment P3 can be absorbed properly by cell and were at the appropriate concentration to support growth compared to treatments P1 and P2. In treatment P4, it was assumed that nutrient concentrations in culture media exceed cell requirements and there was an imbalance in concentration between culture media and cell cytoplasm, causing some cells become lysis and the growth result was lower than treatment P3.

In treatment P0 (Walne nutrient) has lower nutrient concentration than treatment P1 but has better growth than treatment P3. This was allegedly due to differences in sources of nitrogen and phosphorus on Walne nutrient with tofu liquid waste fertilizer. Endar et al. (2012) stated that Walne nutrient contained nitrate (NO_3^-) as nitrogen source from $NaNO_3$ and orthophosphate (PO_4^{2-}) as phosphorus source from $NaH_2PO_4.2H_2O$. Tofu liquid waste fertilizer contained ammonium (NH_4^+), nitrate (NO_3^-) and amino acid ($NH_4COO\text{-}R'$) as nitrogen source from proteins breakdown by lactic acid bacteria (de Mesquita et al., 2017) and orthophosphate (HPO_4^{2-} dan $H_2PO_4^-$) as phosphorus source from dissolution of organic phosphorus by Actinomycetes (Alori et al., 2017).

Hii et al. (2011) stated that *Nannochloropsis* cultured using fertilizer contain nitrate (NO_3^-) and ammonium (NH_4^+) had lower growth compared to those cultured using fertilizer contain nitrate (NO_3^-) as a single nitrogen source. This was due to different in ion types and molecular weight. Ammonium (NH_4^+) will reduce the effectiveness of nitrate (NO_3^-) absorption so that the growth result was lower (Sanz-Luque et al., 2015). *Nannochloropsis* is a genus of microalgae which is more likely to use nitrate (NO_3^-) as nitrogen source compared to ammonium (NH_4^+) and amino acids ($NH_4COO\text{-}R'$) (Hii et al., 2011).

Mayhead et al. (2018) explains that microalgae are easier to use orthophosphate with the form of PO_4^{2-} than HPO_4^{2-} and $H_2PO_4^-$. PO_4^{2-} is more effective to used as a source of phosphorus in photophosphorylation reactions to forming adenosine triphosphate (ATP) with the support of ATP synthase enzymes than HPO_4^{2-} and $H_2PO_4^-$ because it has smaller molecular size (Masojidek et al., 2013). The more effective formation of ATP will accelerate the formation of genetic material, namely DNA for the cell division process.

The results showed that cell growth rate had a positive correlation with cell density but had a negative correlation with cell doubling time. Short cell doubling times produce high growth rates and cell densities. Treatment P3 had the best results compared to treatments P1, P2 and P4 but treatment P0 (nutrient walne) had better results than treatment P3. The correlation between doubling time, growth rate and cell density is affected by the metabolism of *Nannochloropsis oculata*. Nutrients contained in tofu liquid waste fertilizer become one of the factors that influence metabolic activity.

Nitrogen (N), phosphorus (P), iron (Fe), zinc (Zn) and manganese (Mn) in tofu liquid waste fertilizers are nutrients that play an important role in the cell division process which then affects doubling time, growth rate and cell density. Bisova and Zachleder (2014) explain that nitrogen (N) and phosphorus (P) play a role in the preparation of DNA, which is nucleic acid that very instrumental in the process of mitosis when binary fission occurs by parent cells which then produces daughter cells. Nitrogen (N) and phosphorus (P) in the right concentration and source will cause the process of DNA replication in the process of cell division to be faster. Flynn and Raven (2017) explain the rate of growth of microalgae cells is also influenced by the activity of the enzyme ribulose-1,5-biphosphate carboxylase/oxydase (RuBisCO) which serves to accelerate the binding of CO_2 for use in photosynthesis which will produce ATP for energy in the cell division process . The effectiveness of the RuBisCO enzyme works depends on the micronutrient concentration of iron (Fe), zinc (Zn) and manganese (Mn) (Dou et al., 2013). In treatment P3, it is suspected that the activity of RuBisCO enzyme runs more optimally so that cell generation time was shorter and had higher growth rate and cell density compared to treatments P1, P2 and P4. In the treatment P0 (Walne nutrient) was thought to have a concentration and a better source of nutrients to support the work of the RuBisCO enzyme so that it had a shorter cell doubling time and a higher growth rate and cell density than the treatment P3.

Nannochloropsis oculata in this study had growth curve consisting of adaptation, exponential and death phases. The adaptation phase in treatments P0, P1, P2, P3 and P4 starts on day 1 until day 5. The long-term adaptation process occurs because inoculated *Nannochloropsis oculata* adapts to the conditions of the new culture media. Adaptation carried out to temperature, pH, salinity of culture media and light intensity of the culture chamber. The exponential phase occurs in treatments P0, P1, P2, P3 and P4 starting from day 5 until day 8. The exponential phase that occurs on day 5 until day 8 was caused by the nutrients present in the culture media that can be optimally utilized on the day 5 to day 8. On the day 8 *Nannochloropsis oculata* in treatments P0, P1, P2, P3, P4 reached peak of cell populations. The death phase in treatments P0, P1, P2, P3 and P4 occurred from day 9 until day 14. This was occurs because *Nannochloropsis oculata* snatched space in the culture media and the nutrient content in culture media decreases significantly so the cell death rate occurs was higher than the cell reproduction rate.

Environment Condition of *Nannochloropsis oculata* Culture

The temperature of media and culture chamber during the study showed significant increase from 27.6 ^{o}C until 35.3 ^{o}C for culture media and 28.0 ^{o}C until 32.0 ^{o}C for culture chamber. Normal range of temperatures for culture media are 25.0-36.0 ^{o}C (Banerjee et al., 2011) and for culture chamber are 25.0-30.0 ^{o}C (Sun et al., 2018). The increase in temperature was thought to be a factor that causes the adaptation period to last a long time, starting on day 1 until day 5. This was presumably because the air circulation was

running poorly so that the heat from the radiation of the tubular lamp (TL) was trapped in the culture chamber. The temperature of the culture media measured during the study was still in the normal range but the measured temperature of culture chamber was slightly higher than the upper limit of the normal range.

The pH of the culture media in this study ranged from 7.6 until 8.8. The normal range of culture media pH is 7.0-9.4 (Banerjee et al., 2011). The increase of pH caused by photosynthesis carried out by microalgae. Carbon dioxide in water forms a bicarbonate acid compound (H_2CO_3). This compound then decomposes $H_2CO_3 \rightarrow H^+ + HCO_3^-$. *Nannochloropsis oculata* utilized carbonic acid (HCO_3^-) for photosynthesis by absorbing CO_2 and releasing OH^-. Cultures that used 24 hour bright photoperiods cause high CO_2 requirements so that OH^- were always formed so increase pH in culture media.

The salinity of the culture media in this study ranged from 30.0 to 38.8 ppt. The normal range of culture media salinity is 25.0-45.0 ppt (Gu et al., 2012). The increase in salinity caused by the evaporation process of culture media due to heat from tubular lamp (TL) was used as a light source. Evaporation results higher dissolved mineral concentrations so that salinity increase.

The light intensity of the culture chamber in this study ranged from 5,210-5,960 lux. The normal range of light intensity of culture chamber is 3,000-9,000 lux (Budiono et al., 2018). Light intensity in the study has been able to support the growth of *Nannochloropsis oculata*.

4. CONCLUSIONS AND SUGGESTIONS

Tofu liquid waste fertilizer with different concentrations affects the growth of *Nannochloropsis oculata* and the concentration that can provide the best growth found in the treatment P3, namely the tofu liquid waste fertilizer 5 ml L^{-1}.

5. ACKNOWLEDGE

We as authors would like to express our sincere gratitude to all individuals and institutions that supported this research. Special thanks to the Faculty of Fisheries and Marine Airlangga University for providing the facilities and resources necessary for conducting this study.

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