

Effect of inorganic fertilizer on the growth of freshwater *Chlorella* sp.

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Abstract. One of the alternative nutrient sources in the culture of *Chlorella* sp. is inorganic fertilizer. The aim of this study was to determine the effect of inorganic fertilizer composition in the culture medium on the growth of *Chlorella* sp. This research was conducted by using the 2.5 L transparent containers containing 2 L freshwater. The culture was carried out in laboratory facilitated with an air conditioner, fluorescent light and continuous aeration for 14 days of culture. The treatments consisted of fertilizer composition of Urea:ZA:TSP i.e. P1 (1:1:0,50), P2 (2:2:0,70), P3 (3:3:1), P4 (4:4:1,25) and P0 (Walne medium) as a positive control. Each treatment was performed in triplicates and arranged based on the Completely Randomized Design. *Chlorella* sp. was inoculated with the initial density of 30×10^4 cells·mL⁻¹. The results of this study showed that the inorganic fertilizer composition of 3:3:1 provided the highest growth rate of 25.9 %/day, and the highest population density reached 2.348×10^4 cells·mL⁻¹. The doubling time was not affected by the inorganic fertilizer treatments.

1. Introduction

Plankton is natural feed for fish larvae, either zooplankton or phytoplankton. Plankton should be high nutrient, not harmful to fish, not pollute the environment, suitable with the size of fish's mouth and has a high tolerance to environmental change [1]. One of the phytoplankton used as natural feed is *Chlorella* sp.

The factors affecting the growth of *Chlorella* in the cultivation include light intensity, dissolved oxygen, temperature and nutrients. Indoor culture generally use 80 watt fluorescent bulb, with the light intensity of 5,000 lux–10,000 lux [2]. Good dissolved oxygen content for the growth of *Chlorella* sp. is > 2 mg L⁻¹ [3]. A common temperature for the growth of *Chlorella* sp. ranges from 26 to 33 °C [4]. The nutrients needed for *Chlorella* consist of macro and micro nutrients. Macro nutrients are C, H, N, P, K, S, Mg and Ca, meanwhile micro nutrients consist of Fe, Cu, Mn, Zn, Co, Mo, Bo, Vn and Si [3]. Among these nutrients N and P are often limiting factors for the growth of microalgae. In addition to macro and micro nutrients, algae also need vitamins in supporting its growth. The vitamins needed by algae consist of cobalamin (vitamin B₁₂), thiamin (vitamin B₁) and biotin.

The nutrient required by *Chlorella* in the cultivation is obtained from culture medium. Water as a growing medium of *Chlorella* sp. only has limited mineral content. Thus, the addition of minerals from outside is absolutely necessary. The additional nutrient on laboratory scale cultivation is usually in the form of Walne medium while on a mass scale is in the form of inorganic fertilizers. The inorganic fertilizer commonly used is urea, TSP and ZA. Urea is an artificial fertilizer containing primary nutrient which is nitrogen with the chemical formula CO (NH₂)₂. Urea contains 46 % element of nitrogen [5].



Ammonium sulfate (ZA) has the chemical formula $(\text{NH}_4)_2\text{SO}_4$. ZA in a pure state contains 21 % element of nitrogen (N) and 23 % nutrient of sulphur (S). Phosphorus (P) in the fertilizer is indicated in P_2O_5 . TSP (Triple Super Phosphate) contains phosphorus of 44 % P_2O_5 . The dose of inorganic fertilizer given to the cultivation media will affect the growth of *Chlorella* population, hence this study aim was to determine the effect of the different doses of inorganic fertilizer on the growth of *Chlorella* sp.

2. Materials and methods

The research was conducted from February to May 2016 in the Laboratory of Aquaculture Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada. This research was conducted by the experimental method using Completely Randomized Design with four treatments and one control, each treatment consisted of three replications. The factors observed were the effect of inorganic fertilizers with ratios of urea: ZA: TSP as follows P1= 1:1:0.05; P2 = 2:2:0.70; P3 = 3:3:1; P4 = 4:4:1.25.

Chlorella sp. was obtained from a pure culture developed by the Technology Development Center of Marine and Fisheries Yogyakarta. The culture medium at the control treatment used in the study was Walne medium (Table 1). Cultures of *Chlorella* sp. was conducted in a jar containing 2 L fresh water media for 2 weeks. Observation was performed on the growth of *Chlorella* sp. population and water quality. The water quality consist of temperature, pH, carbon dioxide-free, nitrate, phosphate and ammonia.

The research was carried out by maintaining a pure culture of *Chlorella* sp. in the jar. Water culture medium was freshwater which came from groundwater. The culture medium in the jar was enriched with inorganic fertilizers and performed the initial stocking density of *Chlorella* sp. at 30×10^4 cells·mL⁻¹ which was determined by using the formula [6]:

$$V1 \times N1 = V2 \times N2$$

Description:

V1 = the volume of the desired seed in the stocking

V2 = the volume of media culture

N1 = stocking density

N2 = the desired initial stocking density

The density of *Chlorella* sp. was observed daily by haemocytometer periodically at 10:00 am. The density observation was started on the second day until the death phase. The density of *Chlorella* sp. was enumerated by following [7].

$$\text{Cell density (cells mL}^{-1}\text{)} = \frac{\text{The cell total in 4 block (=4)}}{\text{The sum of block (=4)}} \times 10^4$$

The growth of *Chlorella* sp. was determined based on the specific growth rate which was the change in the number of cell mass per unit of time. The specific growth rate was determined by following [8]:

$$K = \frac{\ln N_t - \ln N_0}{t} \times 100 \%$$

Description:

K = Specific growth rate (cells/day)

Nt = the density of *Chlorella* sp. in the peak population

No = the density of *Chlorella* sp. in the initial population

t = the time required to reach the maximum population

Table 1. Composition of Walne Medium.

	Content	In 1000 mL H ₂ O
1	NaNO ₃	100.00 mg
	Na ₂ EDTA	45.00 mg
	H ₃ BO ₃	33.60 mg
	NaH ₂ PO ₄ . 2 H ₂ O	20.00 mg
	FeCl ₃ . 6 H ₂ O	1.30 mg
	MnCl ₂ . 4 H ₂ O	0.36 mg
2	Vitamin	
	B ₁	0.100 mg
	B ₁₂	0.005 mg
3	Micro Metallic Solution	
	ZnCl ₂	0.021 mg
	CoCl ₂ . 6 H ₂ O	0.020 mg
	(NH ₄) ₆ . Mo ₇ O ₂₄ . 4 H ₂ O	0.009 mg
	CuSO ₄ . 5 H ₂ O	0.020 mg

Source: [6]

The doubling time was calculated by using the formula [9]:

$$k = \frac{t \log 4}{\log b - \log a}$$

Description:

k = the doubling time (day)

t = the time to reach maximum population (day)

a = the initial density of *Chlorella* sp. ($\times 10^4$ cells·mL⁻¹)

b = the maximum density *Chlorella* sp. ($\times 10^4$ cells·mL⁻¹)

Water quality including water temperature, pH, light intensity, content of ammonia, phosphate, nitrate and free carbon dioxide were observed. The temperature measurement was carried out everyday. The measurements of free carbon dioxide, water pH, contents of ammonia, phosphate, nitrate, and the light intensity were performed at the beginning, middle and end of the culture.

3. Results and Discussion

3.1. The growth rate of *Chlorella* sp.

Cell density, population peak, doubling time, specific growth rate and growth rate are parameters that commonly used to estimate the growth of cells in the culture medium [10]. The cell density was measured every day during the culture until the cell growth reached the death phase.

Daily population density of *Chlorella* sp. in all treatments increased from the first day. The treatment with the composition of fertilizers 3:3:1 resulted the greatest growth rate which was 25.9 %/day. Based on the Dunnet analysis of the growth rate of *Chlorella* sp., it was found that a composition of inorganic fertilizer 3:3:1 resulted the similar growth rate with the control treatment.

The differences of population growth rate of *Chlorella* sp. in all treatments was resulted from the difference dosage of fertilizer [11]. The differences in the growth rate in this study is considered because of the variation of nutrient ratio in treatments. The composition of inorganic fertilizers 3:3:1 produced the highest population growth rate, because the dose was similar with the control and was not excessive dose for growth of *Chlorella* sp.

Extra doses of inorganic fertilizer in the culture medium of *Chlorella* sp. does not always produce the higher growth rate. The excessive doses of the nutrient to the treatment of inorganic fertilizer composition 4:4:1.25 produced the lowest population growth rate among all treatments. Nutrient deficiencies in the media will inhibit the growth rate seen in the treatment of inorganic fertilizer composition 1:1:0.5.

3.2. Population Peak

Daily cell density showed an increase since the first day of observation, in which the initial density was 30×10^4 cells·mL⁻¹ and reached the maximum population at a certain time. In the first day of inoculation, the population of *Chlorella* sp. grew in the exponential growth phase without going through a stage of adaptation. The exponential growth phase generally occurs at the 7th day to the 9th day. The cell density began to decline after the culture of *Chlorella* sp. reached a maximum population and the observation was stopped at the 14th day (Figure 1).

The results showed that *Chlorella* sp. has high adaptability to the new environment. It could be seen from the value of the growth rate from the first day which was quite high at 49.2 %/day to 51.8 %/day. This happened because the inoculum of algae and the water media used were obtained from asimilar laboratory environment. Another supporting factor was that *Chlorella* sp. cells has been adapted to the research environment before the experiment was conducted.

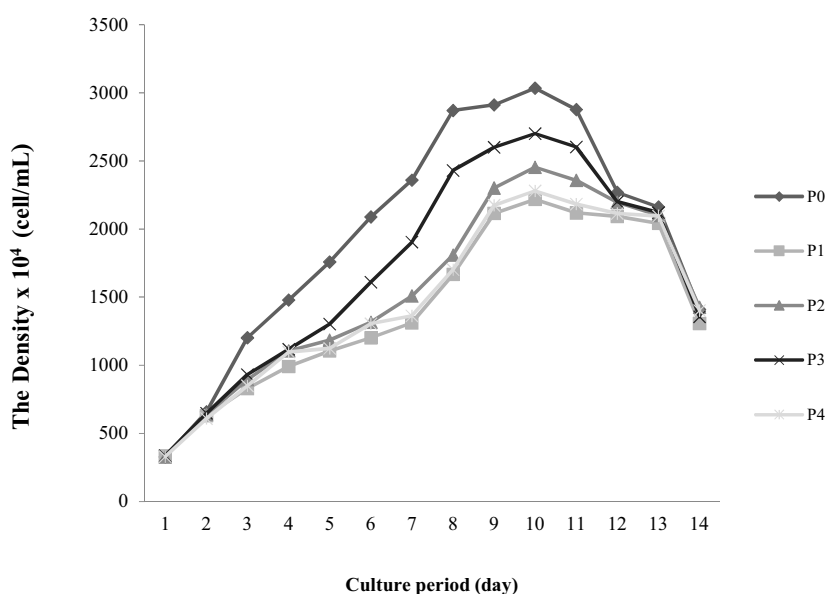
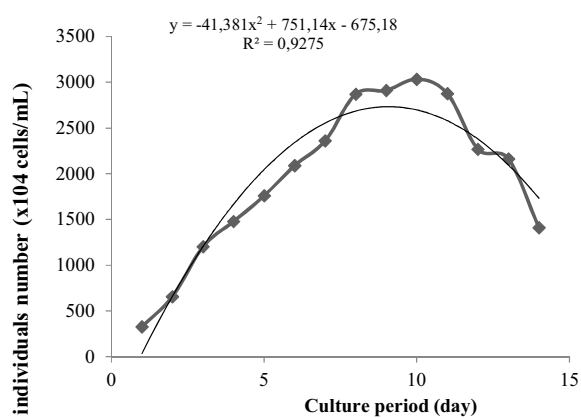


Figure 1. The density of *Chlorella* sp. with various ratio of urea:ZA:TSP in culture medium.

The peak population is the final process of the exponential phase of growth in the cycles of phytoplankton growth. The peak population is usually used to find out when the culture of microalgae can be harvested because at that time the population density reaches its maximum. The highest peak population density of *Chlorella* sp. was found in inorganic fertilizer with the composition of 3:3:1 at $2,348 \times 10^4$ cells·mL⁻¹ which was achieved at the day of 9.7, while the lowest peak population density for the inorganic fertilizer with the composition 1:1:0.50 at $1,924 \times 10^4$ cells·mL⁻¹ which was achieved at day of 11.2. The maximum population density of *Chlorella* sp. during the maintenance along with the treatment of a different inorganic fertilizer composition was presented in the Table 2.

Table 2. The maximum cells density of *Chlorella* sp. at the various ratio of urea:ZA:TSP in culture medium.

Fertilizer composition	Time to reach peak (day)	Density ($\times 10^4$ cells $\cdot$ mL $^{-1}$)
0.3:0.3:0.2	9.1	$2,733 \pm 857.018$
1:1:0.50	11.2	$1,924 \pm 621.157$
2:2:0.70	11.0	$2,088 \pm 678.043$
3:3:1.00	9.7	$2,348 \pm 775.449$
4:4:1.25	11.2	$1,989 \pm 635.349$

**Figure 2.** The curve of population density of *Chlorella* sp. cultured in Walne medium 0.3:0.3:0.2.

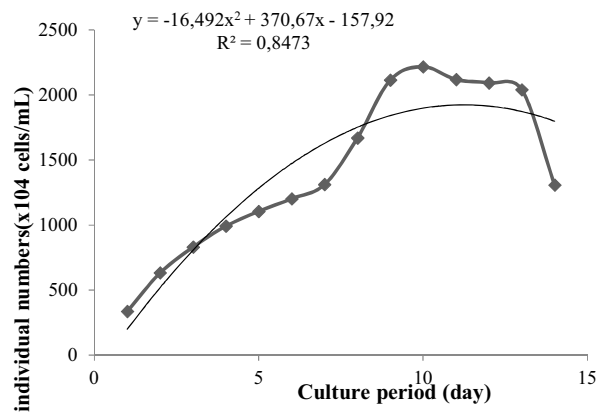


Figure 3. The curve of population density of *Chlorella* sp. cultured in medium with a ratio urea:ZA:TSP (1:1:0).

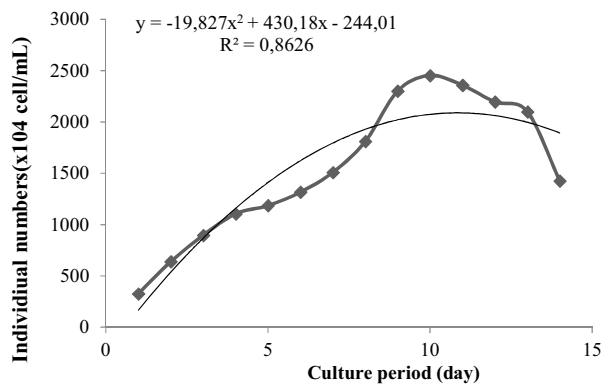


Figure 4. The curve of population density of *Chlorella* sp. cultured in medium with a ratio urea:ZA:TSP (2:2:0.70).

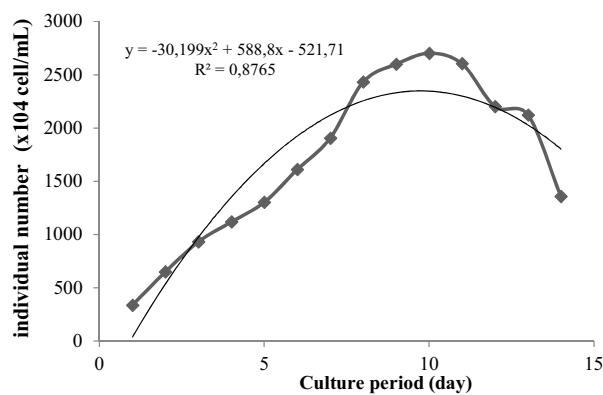


Figure 5. The curve of population density of *Chlorella* sp. cultured in medium with a ratio urea:ZA:TSP (3:3:1).

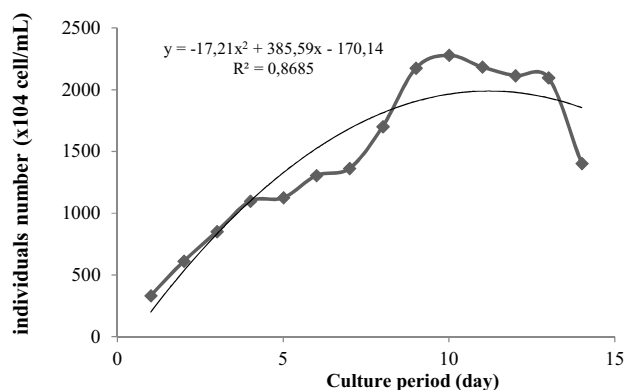


Figure 6. The curve of population density of *Chlorella* sp. cultured in medium with a ratio urea:ZA:TSP (4:4:1.25).

Population of *Chlorella* sp. decreased after reaching its maximum population. The decline was caused by the availability of nutrients in the culture medium decreased and eventually loss so that the cells deprived nutrients for growth. The growing population was occurred when nutrients concentration was enough, so that the cell division occurs rapidly (exponential phase), followed by the formation of autospores and enlargement of cells. Population growth decreased (stationer phase) after the nutrients have been in limited concentration or run out. Dead phase occurred when the nutrients run out, so the population growth stopped and there were many deaths cells in a population of *Chlorella* sp.

3.3. Doubling time

Doubling time is time required for microalgae to be double in density, while the growth rate is the rate of growth per unit time during the last exponential phase. Analysis of variance of the doubling time indicated that inorganic fertilizers did not affect significantly the population doubling time of *Chlorella* sp.

Inorganic fertilizer application in medium provided nutrients for the process of cell division that caused the population continue to increasing. Based on the observations it can be concluded that the inorganic fertilizers with specific composition are not always able to short the cell doubling time of *Chlorella* sp.

3.4. Water quality

Phytoplankton growth quietly related to the macro and micronutrients availability, and also it was affected by the environmental condition in the culture media. Environment factors that affect the phytoplankton growth are light, temperature, pH, water and carbon dioxide-free, which can stimulate or inhibit the growth of phytoplankton [12]. The light intensity during the study ranged from 3,020 lux to 5,770 lux. Optimum light intensity for growth of *Chlorella* sp. ranged between 2,000 lux to 8,000 lux [13].

Proper temperature for *Chlorella* sp. growth is 25 to 32 °C [6]. Water temperature of media during research was in the range of 26 to 29°C. Water temperature of media during research was in optimal range and stable because during the culture the laboratory was facilitated with the lamp and in indoors room with the air conditioner. Temperature is an important limiting factor for organism life, because every organism has limiting ability to tolerate temperature change in environment. Instead of that, temperature affects the stability of the enzyme catalyst in the process of photosynthesis by phytoplankton. Algal cell metabolism increases with rise in temperature to achieve optimal temperature and will fall back if the temperature has exceeded the optimum point.

Carbon dioxide is limiting factor to all of living creatures in aquatic [14]. Lack of free carbon dioxide would harm producers organisms (phytoplankton and water plants) so stunted. Too high carbon dioxide

content also has detrimental effects on aquatic organisms. Free carbon dioxide is important factor for *Chlorella* sp. growth because it is directly necessary for organic molecules synthesis through photosynthesis. Free carbon dioxide in water ranged from 0 mg L⁻¹ to 0.8 mg L⁻¹. Low free carbon dioxide content in water media was caused by most of the carbon dioxide up took by *Chlorella* sp. in photosynthesis, and no other aquatic organisms (zooplankton) that produces carbon dioxide lived in the culture media.

pH plays a role in determining the concentrations of free carbon in water and the balance between bicarbonate and carbonate. During this research, pH culture medium ranged from 7.7 to 8.1. Increased in medium pH was caused by the process of photosynthesis and decomposition of nitrogen from fertilizers that produced acid CO₂ and NH₄⁺ [15].

Phytoplankton including *Chlorella* sp. absorb nitrogen in the forms of nitrate ions (NO₃⁻), ammonium (NH₄⁺) and nitrite (NO₂⁻) [16]. The observation of high nitrate levels in the early observations ranged from 1.17 mg L⁻¹ up to 1.99 mg L⁻¹. Nitrogen levels continued to decline until the end of the observation ranging from 0.253 mg L⁻¹ up to 0.609 mg L⁻¹ because of the nitrate assimilation by *Chlorella* sp. for growth. When organic materials are aerated intensively, ammonification process will occur.

Result indicated that phosphate concentration in water at the beginning of observation was high, but decrease until the end of observation around 0.009–0.146 mg L⁻¹. Orthophosphate (PO₄³⁻) is dissolved in the form of inorganic phosphates, which can directly be used by *Chlorella* sp. The concentration of orthophosphate can be reduced more quickly in alkaline water media than in acidic water as the reduced levels of phosphorus are directly affected by pH and Ca concentration [15].

4. Conclusion

Culture of *Chlorella* sp. with inorganic fertilizer composition of 1:1:0.50; 2:2:0.70; 3:3:1; 4:4:1.25 produce highest population density of 1,924, 2,088, 2,348, 1,989 × 10⁴ cells·mL⁻¹ and achieved at day of 11.2, 11, 9.7 and 11.2, respectively. Walne medium produces highest population density of 2,733 × 10⁴ cells·mL⁻¹ at day of 9.1. The composition of inorganic fertilizers 3:3:1 produces the highest population density of 2,348 × 10⁴ cells·mL⁻¹ at day of 9.7. The composition of inorganic fertilizers 3:3:1 generates the highest growth rate of 25.9 %/day.

Suggestion

Inorganic fertilizer application provide nutrients to sustain life and reproduction of *Chlorella* sp. in organic fertilizers might be used as a substitute for Walne medium, since it is cheaper and sufficient nutritional content for the growth of *Chlorella* sp. inorganic fertilizer with the composition of 3:3:1 might be applied for the cultivation of *Chlorella* sp. in a mass scale as it is able to generate the highest rate of population growth.

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