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Growth profile of *Nannochloropsis* sp. with combination effect of Indole 3-Acetic Acid (IAA) and 6-Benzyl Amino Purine (BAP)

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Microalgae is potential microorganism that grow in the water and has many benefits. *Nannochloropsis* sp. is a potential green algae that is widely used because it is easily cultivated and contains high nutrition. *Nannochloropsis* sp. commercially used as food ingredients, agricultural fertilizers, biomass energy, and the pharmaceutical industry. IAA and BAP are plant growth regulator that affect the growth and biochemical content of several species of microalgae. The Objectives in this study to determine the growth profile of *Nannochloropsis* sp. on culture media by treating a combination of IAA and BAP in various concentrations. The methods in this study are preparation and sterilization of tools and culture media, making Conway fertilizer, making stock solutions of IAA and BAP, determining starter time, and microalgae cultivation with a combination treatment of IAA and BAP. The variation of IAA and BAP concentrations consisted of 0; 0.1; 1; 10 mg/L. Data analysis is used quantitatively with a theoretical approach. We showed that the highest growth was in the treatment of IAA 0.1 + BAP 1 mg/L with an OD value of 1.358 at the end of the exponential phase. Our result showed that combination IAA and BAP can increase growth of *Nannochloropsis* sp.

Keywords: BAP, IAA, Growth, *Nannocloropsis* sp.

INTRODUCTION

Microalgae were a group of unicellular organisms that include plant-like protists (Miazek et al., 2017). They were live in waters and photosynthesize to produce biomass (Nur, 2014). Microalgae are classified as potential microorganisms because they have the ability to use light energy efficiently and high adaptability to the environment so that it has many benefits (Endrawati and Riniatsih, 2013). *Nannochloropsis* sp. is a unicellular green microalgae that is commercially used as food ingredients, agricultural fertilizers, biomass energy, and pharmaceutical industry because it contains protein, carbohydrates, lipids, and various kinds of minerals (Darsi et al., 2012). In addition, they had advantages that are easily cultivated. *Nannochloropsis* sp. has high nutrition compared to other microalgae. Nutritional content of *Nannochloropsis* sp. includes protein 52.11%; carbohydrates 16.00%; fat 27.65%; EPA 30.50%; total ω3-HUFA 42.70%; vitamin C 0.85%; and chlorophyll-α 0.89% (Dayanto et al., 2013). Growth of *Nannochloropsis* sp. influenced by several factors, one of the main factors is the composition of culture media.

The right composition of culture media can increase the growth and biochemical content of microalgae (Jati et al., 2012). Research by Park et al., (2013) stated that the addition of growth regulators (ZPT) of auxin and cytokinin in culture media can increase microalgae growth by

controlling internal biochemical pathways. PGR is a synthetic organic compound that has the same structure and effect as phytohormone. These organic compounds are not small amounts of active nutrients and give a biochemical, physiological and morphological response (Yuliantina, 2013). The addition of a growth regulator for auxin and cytokinin to microalgae culture media can increase biomass and its biochemical content.

Indole 3-acetic acid (IAA) was the most abundant natural auxin. IAA plays a role in regulating cell growth and elongation. IAA can stimulate cell division, biomass production, and pigment biosynthesis in microalgae (Koslova et al., 2017). In addition, IAA is also more effective than IBA and NAA in increasing the growth and composition of microalgae *Chlorella pyrenoidosa* and *Scenedesmus quadricauda* cells. The addition of 10 mg / L IAA in the *Chlorella sorokiniana* IAM C212 cultivation media produced the highest dry weight of 4.68 g / l (Ozioko et al., 2015). According to Salama et al., (2014) the concentration of IAA 10^{-8} to 10^{-5} M can increase growth, biomass, lipid content, and carbohydrate *Scenedesmus obliquus* (Salama et al., 2014). 6-Benzyl Amino Purine (BAP) is a plant growth regulator type of cytokinin which acts to stimulate cell division and accumulation of photosynthetic pigments (Lizawati et al., 2009; Han et al., 2018). The study of Du et al., (2017) showed that the addition of BAP 1 mg / l can increase the growth of *Chlorella pyrenoidosa* with cell density of 23.58 x 10^6 on the 12 days of cultivation and lipid content of 14.70 mg / l. Addition of cytokinins to *Chlorella vulgaris* has an effect on the accumulation of photosynthetic pigments (Piotrowska et al., 2009). The study of Kokkiligadda et al., (2017) shows that the addition of 4 mg/L BAP can increase lipid content and biomass 1.26 times higher in microalgae. The objective of this study was to determine the growth profile of *Nannochloropsis* sp., by treating a combination of IAA and BAP in various concentrations.

MATERIALS AND METHODS

The research was conducted on 10th of September to 2nd of November, 2018 at the Laboratory of Plant Biosciences and Technology, Department of Biology, Faculty of Sciences, Sepuluh Nopember Institute of Technology, Surabaya.

The tools used include 500 ml glass bottles, volume pipettes, drop pipettes, measuring cups, glass preparations, glass cover, heat resistant plastic, plastic hoses, 0.22 µm filter syringes, cuvettes, culture racks, aerators, microscopes compound, spectrophotometer, autoclave, refractometer, pH meter, and Laminar Air Flow (LAF). While the ingredients used in this study are *Nannochloropsis* sp. with a density of 10^7 cells/ml from the Natural Feed Laboratory of the Brackish Aquaculture Center (BPBAP) Situbondo, sea water, Indole 3-acetic acid (IAA), 6-Benzyl Amino Purine (BAP), Conway fertilizer, aquades, and 70% alcohol .

This study consisted of 5 stages: preparation and sterilization of tools and culture media, making Conway fertilizer, making stock solutions for IAA and BAP, determining the start time, and microalgae cultivation. Sea water used as a culture medium is regulated by salinity 30-32 ppt using a refractometer and pH 7-8 using pH meter. All glassware and seawater are sterilized using an autoclave at 121°C with a pressure of 1 atm for 15 minutes. While the plastic hose is sterilized by immersion in sodium hypochlorite (NaClO) solution for 10-15 minutes, then washed and put in heat-resistant plastic to be sterilized using autoclave. Conway fertilizer is made with a composition of Na_2EDTA (45 g/L), $NaNO_3$ (100 g/L), H_3BO_3 (33,6 g/L), Na_2HPO_4 (20 g/L), $MnCl_2.4H_2O$ (0.36 g / L), $FeCl_3.6H_2O$ (1.3 g/L), $ZnCl_2$ (2.1 g/L), $CoCl_2.6H_2O$ (2 g/L), $(NH_4)6Mo_7O_{24}.4H_2O$ (0.9 g/L), $CuSO_4.5H_2O$ (2 g/L), vitamin B12 (1 ml/L), and vitamin B1 (1 ml/L). Making IAA stock solution using 1 N NaOH solvent while BAP using 1 N HCl with a concentration of 100 mg/L and 1000 mg/L. Next, the stock solution is sterilized with a syringe filter (0.22 µm) in Laminar Air Flow (LAF). The start time is 300 ml by inoculating 10% of the total culture volume and adding 1 ml/L of Conway fertilizer. After that it was cultivated until the death phase to make a growth curve and determine the start time. *Nannochloropsis* sp., cultivated at 500 ml using 10% seeds, aged starter, IAA and BAP with variations in concentration each consisting of 0; 0.1; 1; 10 mg/L, and 1 ml/L Conway fertilizer. Culture is regulated with ambient temperatures of 25-27°C, light intensity 900-1000 lux, and photoperiod bright: dark 24: 0. Furthermore, cell density measured every 24 hours starting from the first day to the death phase using a UV Vis spectrophotometer with a wavelength of 680 nm. Measured Optical Density (OD) is used as the y axis and time (observation day) as the x axis to make the growth curve.

RESULTS

Observation results of growth of *Nannochloropsis* sp. for 14 days shown in Figure 1. Based on the graph of the growth curve can be determined the growth phases of *Nannochloropsis* sp. and the age of the starter. The growth of microalgae normally consists of the lag phase, the exponential phase, the stationary phase, and the death phase (Ru'yatin et al., 2015). Based on Figure 1 the growth lag phase of *Nannochloropsis* sp. on days 1 to 2, the exponential phase on days 2 to 10, the stationary phase on days 9 to 11 and the phase of death on days 11 to 14. While the time of the start of *Nannochloropsis* sp. in the half exponential phase that is on day 6. Starter age microalgae will be used for cultivation in combination treatment media IAA and BAP. This is because in this phase active microalgae cells multiply through division. In this phase the enzymes and metabolites needed for cell division are available. In addition, absorption of nutrients occurs rapidly for growth (Prayitno, 2015).

BAP 1 mg/L is suspected because of the main role of the combination of IAA and BAP concentrations that work optimally. IAA and BAP are classified as growth regulating substances which are non-nutrient organic compounds that are active in small amounts and give a biochemical, physiological, and morphological response (Yuliantina, 2013). Its activities in growth depend on the type, chemical structure, genotypic concentration, and physiological phase of the organism (Yulizar et al., 2014; Lestari, 2011; Allaf, 2013). Several studies have stated that the addition of a growth regulator of auxin and cytokinin to microalgae culture media can increase the growth rate, biomass and biochemical content (Koslova et al., 2017). IAA is a growth regulator for auxin which plays a role in regulating cell growth and elongation. IAA can stimulate cell division, biomass production, and pigment biosynthesis in microalgae thus increasing growth (Koslova et al., 2017). IAA with a concentration of 0.1 mg/L produced the highest growth when combined with BAP 1 mg/L. However, in the study of Ozioko et al., (2015) the addition of single IAA in *Chlorella sorokiniana* IAM C212 culture media had the highest growth with a concentration of 10 mg/L which resulted in dry weight of 4.68 g/L. Likewise in the study of Park et al., (2013) showed that a single IAA 10 mg/L could increase the size of *Chlamydomonas reinhardtii* microalgae cells.

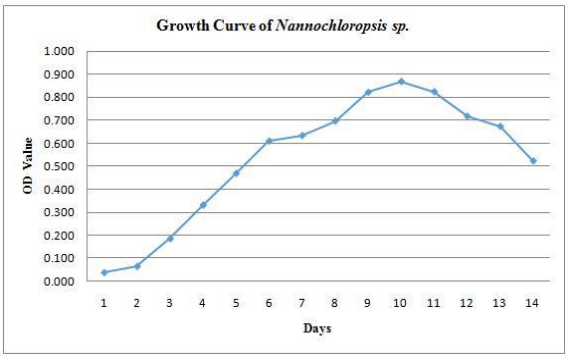
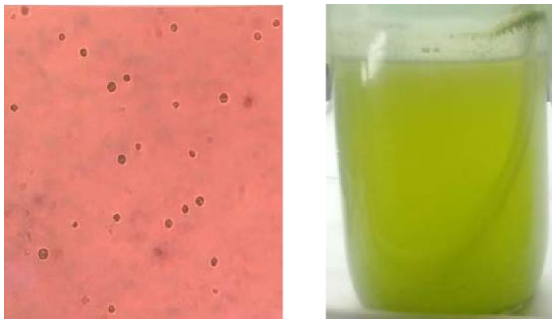


Figure 1;Growth Curve of *Nannochloropsis* sp.



A **B**

Figure 2; (A) *Nannochloropsis* sp. (400x); (B) Culture of *Nannochloropsis* sp.

This shows that IAA works more optimally in low concentrations when combined with other growth regulators, including BAP, cytokines. In addition, each species of microalgae has a different response to the growth regulator given. BAP (concentration of 1 mg / L) produced the highest growth when combined with IAA 0 mg / L. The study of Du et al., (2017) also shows that the addition of BAP 1 mg / l can increase the growth of *Chlorella pyrenoidosa*. BAP is a growth regulating type of cytokinin which acts to stimulate cell division and accumulation of photosynthetic pigments (Lizawati et al., 2009; Han et al., 2018).

The lowest growth with the treatment of IAA 10 + BAP 0.1 mg/L is thought to be due to high IAA concentrations when combined with BAP so that it no longer stimulates growth but inhibits growth. Provision of plant growth regulators that are too high can inhibit growth because microalgae also produce endogenous fitohormones so they become unbalanced (Nursetiadi, 2016; Koslova et al., 2017). While the concentration of BAP 0.1 mg / L is thought to not meet the needs of microalgae to stimulate higher

growth.

The role of IAA which is classified as a growth regulator of auxin in stimulating the growth of microalgae cells is to induce cell growth and elongation by loosening the cell wall at low pH, thus triggering cell elongation faster through signaling TIR/AFB. Auxin stimulates proton H^+ ATPase activity in the plasma membrane. Proton (H^+) from the cytoplasm will enter through the proton H^+ATPase pump to the cell wall matrix and result in acidification of apoplast (pH 4.5-6). The H^+ ion on the cell wall activates the enzymes that play a role in deciding some of the cross-bonds of hydrogen in the cellulose molecule chain that make up the cell wall. This process also induces hyperpolarization of the plasma membrane and is regulated by the Small Auxin Up-RNA (SAUR) protein. Potassium pump activation also occurs so that potassium ions are pumped into the cytoplasm (Pamungkas et al., 2009; Majda and Robert, 2018). Increased potassium concentration stimulates absorption of water into the cytoplasm to maintain turgor pressure in the cell, so that the cell undergoes expansion because the cell wall experiences tensile stress. After experiencing a stretch that causes cell elongation, the cell wall will re-stiffen due to metabolic activity of Ca^{2+} absorption from outside the cell to perfect the arrangement of calcium pectat in the cell wall (Hasanah and Setiari, 2007).

The role of BAP which is classified as a cytokinin growth regulator is to stimulate cell division by increasing the rate of protein synthesis (Lizawati et al., 2009). The process of cell division is influenced by enzymes, namely Cyclin-dependent kinase. CDK influences the transition of G1 to S and G2 phases to M. CDK in collaboration with several types of cyclin in the cell division cycle. The transition of phase G1 to S is regulated by cyclin-D (CYCD). CYCD work is influenced by the presence of hormones and sucrose. Hormones and sucrose will form active complexes of CYCD and CDKA. This complex activates the E2F promoter to activate transcription genes in phase S. While the transition phase G2 to M is influenced by CDK-CYC. Increased activity of the CDK-CYC complex will accelerate the transition from the G2 to M phase (Arif et al., 2007). Therefore, if the cytokinin works optimally the cells divide faster.

Based on Figure 3 the growth curve of *Nannochloropsis* sp. can be determined the phases of growth. The growth of microalgae normally consists of the lag phase, the exponential phase, the stationary phase, and the death phase (Ru'yatin et al., 2015). The highest growth was in the treatment of IAA 0.1 + BAP 1 mg/L, while the lowest growth was in IAA 10 + BAP 0.1 mg/L. Phase lag or adaptation phase in the treatment of IAA 0.1 + BAP 1 mg/L on days 1 to 2, while the lag phase in the treatment of IAA 10 + BAP 0.1 mg/L on days 1 to 3. Phase lag is the phase when microalgae growth is slow, however, it increases in cell size. Photosynthesis takes place in this phase, but cell division has not yet occurred so that the density has not increased. Microalgae cells physiologically prepare themselves to divide at a certain age by producing enzymes and other metabolic compounds needed for cell division (Prayitno, 2015). Some parameters that affect the time of the lag phase are the type and age of microorganism cells, the size of the inoculum and the condition of the growing media. If cells grow in a nutrient-deficient medium, the phase time is adapted longer, because cells must produce enzymes that are in accordance with the types of nutrients available (Ru'yatin et al., 2015).

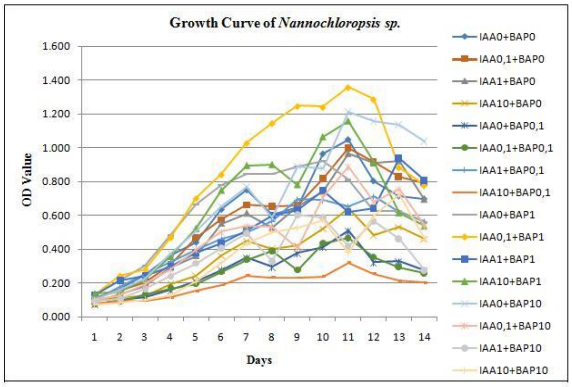


Figure 3; Growth Curve of *Nannochloropsis* sp. with Addition of IAA and BAP with Variations in Concentrations of Each 0; 0.1; 1; 10 mg/L

Exponential phase on treatment of IAA 0.1 + BAP 1 mg/L on day 11 with OD value 1.358, while exponential phase at treatment IAA 10 + BAP 0.1 mg/L on day 11 with OD value 0.321. The exponential phase is a phase when the growth of the microalgae population increases significantly because active microalgae cells multiply through division. In this phase the enzymes and metabolites needed for cell division are available. The growth phase with the highest CO_2 uptake and biomass formation occurs at the exponential phase. In this phase also, absorption of nutrients occurs quickly so that nutrients in the media are reduced. Availability of nutrients that decrease in

the media quickly becomes one of the microalgae growth factors entering the stationary phase (Prayitno, 2015). Increased microalgae cell density is also followed by increasing salinity in culture media. Salinity of *Nannochlorosis* sp. culture media increased from an average of 30 ppt to 41 ppt. Salinity has an effect on cell organisms in maintaining osmotic pressure with their environment (Ru'yatin et al., 2015). Microalgae cultures at lower or higher levels of salinity can change growth rates and cause varying compositions in microalgae cells. The stress of salinity can also reduce photosynthetic activity because electron transport is limited (Chavan et al., 2014). *Nannochloropsis* sp. can grow on sea water media with salinity 0-35 ppt (Suryanto, 2009).

Stationary phase at 0.1 BAP IAA treatment 1 mg/L on days 9 to 12, while stationary phase at IAA 10 BAP treatment 0.1 mg/L on days 7 to 12. Stationary phase is the phase when the population density increase is balanced with the mortality rate so that the rate of population growth is small. The number of cells tends to remain because cells have reached a saturation point (Ru'yatin et al., 2015). In this phase there is an accumulation of toxic compounds caused by metabolism of microalgae, lack of nutrients, and changes in environmental conditions (Hadiyanto and Azim, 2012).

The death phase in the treatment of 0.1 BAP IAA 1 mg/L on days 12 to 14, while the death phase in the treatment of IAA 10 BAP 0.1 mg/L on days 12 to 14. The death phase is the phase when there is a decrease in population density microalgae. In this phase, in the culture media there is competition for living and nutrition because the number of cells increases with the volume of media and nutrients remain. In addition, there is a decrease in water quality and accumulation of metabolites (NO^{2-} and NH_4) so that the cell death rate is higher than the rate of cell growth (Ru'yatin et al., 2015; (Lavens and Sorgeloos, 1996).

Based on the results of the study, the addition of 0.1 BAP and IAA 1 mg/L can increase the growth of *Nannochloropsis* sp. The composition of culture media with the addition of IAA and BAP can be used as a reference for mass cultivation of *Nannochloropsis* sp. In addition, known growth patterns can be used to determine the right harvest time. Harvesting is done to obtain the highest amount of biomass that can be used for various benefits. Harvesting must be done in the final phase of the exponential growth of microalgae (Ru'yatin et al., 2015).

CONCLUSION

Based on the results of the study it can be concluded that *Nannochloropsis* sp. cultivated in the media with the addition of IAA 0.1 + BAP 1 mg/L resulted in the highest growth with a value of OD 1.358 in the exponential final growth phase.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

DE designed and performleed the experiments. AA wrote the manuscript. DE, SN, and AER, performed microalgae treatments, experiments, cultures, and data analysis. AA and DE designed experiments and reviewed the manuscript. All authors read and approved the final version.

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