The Increase in β-carotene Content in *Dunaliella salina* from the Application of Different Light Intensities

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**Abstract.** *Dunaliella salina* is an example of a natural source of β-carotene and it is made up of larger cells than others in the genus *Dunaliella*. It is therefore able to produce more β-carotene. *Dunaliella salina* is able to accumulate more β-carotene content when cultured in environmental stress conditions such as high salinity, low nutrients and high light. This study aims to determine the increase in β-carotene content in *D. salina* due to the application of difference light intensities and to further determine the maximal light intensity required to obtain the highest β-carotene content in *D. salina*. The treatments used were different light intensities of 700 Lux, 2,200 Lux, 3,700 Lux and 5,200 Lux with five repetitions respectively. The primary parameter that was measured was the β-carotene content of *D. salina*. The supporting factors in this research were the growth of *D. salina* and the water quality; i.e. temperature, pH, and salinity. The results showed that different light intensities can increase the β-carotene content in *D. salina* with the increased light intensity being the treatment. The maximum light intensity required to produce the highest amount of β-carotene content was 5,200 Lux with 0.0087 ml/L. Based on these results, further research is needed regarding light intensities to see if it can tolerate further stimulated growth in order to produce β-carotene more.

1. Introduction

Photosynthetic organisms have organic pigments that play a role in the process of photosynthesis such as chlorophyll, carotenoids and phycobilin. Carotenoids are commonly found in plants, algae, photosynthetic bacteria, non-photosynthetic bacteria, fungi and molds [1]. More than 400 carotenoids are found in nature and β-carotene is the most widely commercialized carotene [2]. There are two kinds of carotenoids used in aquaculture, natural and synthetic, which when used excessively can cause damage to the environment and the price of synthetic β-carotene is also more expensive than natural β-carotene [3].

β-carotene is a hydrocarbon (C_{40}H_{56}) with saturated chains that produce the color orange. It has two isomers, namely trans and cis. These molecules act physiologically as pro-vitamin A and free-radical scavengers [4]. β-carotene is widely used as an anti-cancer treatment, and it also prevents premature aging with the immunomodulators serving as a coloring additive in aquaculture and as an antioxidant [2]. Dunaliella has several advantages compared to natural sources of β-carotene. The advantages include continuous cultivation being easier due to the shorter culture time and a higher rate of cell growth. Dunaliella is easily cultivated because it is more resistant to changing environmental conditions.
A local isolate algae species of Brackish Water Aquaculture Institute (BBAP) Jepara, *D. salina* is a potential source of carotenoids as a feed additive or as a feed supplement in fish farming [5]. *Dunaliellallasina* is an unicellular green alga, which can grow in a variety of environmental conditions such as a broad range of low temperature, salinity, pH, and high light [6]. *Dunaliellallasina* can accumulate more of a concentration of β-carotene when cultured under conditions of environmental stress. The intensity of the light will affect the cellular responses of *D. salina*; the cells will release chlorophyll and other pigments will increase as phycobiliprotein and β-carotene are a form of self-defense [7]. The results of the study [8] showed that when cultured in salinity stress conditions and low nitrogen levels, *D. salina* is capable of producing β-carotene, which makes up 60.4% of the total carotenoids. Based on the above, we have conducted research to determine if the increase in the content of β-carotene can be due to different light intensities. This study aims to determine the increase in the content of β-carotene in *D. salina* when due to different light intensities and to determine the best light intensity that can produce the highest content of β-carotene in *D. salina*.

2. Materials And methods

2.1 Materials

The tools used in this study were a glass tube, aerator, test tubes, test tube rack, measuring cup, pipette, micro-pipette, binocular microscope, haemocytometer, hand counters, spectrophotometers, fluorescent light 40-watt, rack culture, refractometer, pH meter, lux meter, autoclave, cover glass, paper label, vortex, centrifuge, cuvettes, dark plastic, styrofoam and aluminum foil. The materials used in this study were *D. salina* seeds and medium Walne derived from the Brackish Water Aquaculture Institute (BBAP) Situbondo, sea water, methanol, acetone, alcohol, soap, wipes and distilled water.

2.2 Method

The research activities were carried out at the Laboratory of Education in the Faculty of Fisheries and Marine Resources of Universitas Airlangga in Surabaya. The method used in this study was an experimental method using a completely randomized design (CRD) with four treatments and five replications. The treatments were the difference in the intensity of light on the culture of *D. salina*. The intensities of the light given were 700 Lux (A), 2200 Lux (B), 3,700 Lux (C) and 5.200 Lux (D) respectively.

2.2.1 Preparation and light intensity settings

The culture medium used was medium Walne obtained from the Brackish Water Aquaculture Institute (BBAP) Situbondo. There were 20 experimental units, which used 20 glass tubes. The prepared culture space was created by installing a shelf culture and every part of the sides was covered with dark plastic. The lighting treatment was created using 40-Watt fluorescent lamps placed on racks. The values obtained determined the light intensity on through the distance between the lamp and a bottle of the culture media. The measurement of light intensity was done using a Lux meter [9]. The light intensity measurements were conducted using a Lux meter, exposing the sensor lens on the lamp to the light until the needle on the scale showed the desired number.

2.2.2 Sterilization

Sterilization included the sterilization of the equipment, materials and culture rack. The equipment was washed with fresh water and dried in the sun. The equipment used for culturing was sterilized using an autoclave at 121 ºC for 15 minutes. Sterilization using an autoclave was also done for the equipment made of heat-resistant glass. The media sterilization was done by inserting the media into sea water in a glass bottle and then covering it using coated cotton guaze and aluminum foil [10]. Glass bottles containing the medium were sterilized using an autoclave at a temperature of 121ºC for 15 minutes.
2.2.3 Fertilizer preparation for culture D. salina

Fertilizer was used as a culture medium in the laboratory; we used Walne fertilizer obtained from Brackish Water Aquaculture Institute (BBAP) Situbondo. The composition of the fertilizer was Na$_2$EDTA 45 g, NaH$_2$PO$_4$.H$_2$O 20 g, FeCl$_3$.6H$_2$O 1.5 g, H$_3$BO$_3$ 33.6 g, MnCl$_2$ 0.36 g, NaNO$_3$ 100 g trace metal solution 1 ml, vitamin 1 ml and 100 ml of distilled water. The fertilizer solution was sterilized using an autoclave and then stored in the refrigerator [10].

2.2.4 Environment and culture media - D. salina

The environment and culture can affect the growth of D. salina, therefore the same conditioned environment was needed for each treatment. The culture media used in this study was sterile sea water with a salinity of 30 ppt. The volume of the total culture of D. salina was 500 ml, and was a mixture of 464 ml of sterile sea water, D. Salina seeds 35 ml with a pure stock density of 719.825 x 10$^4$ cells / ml and with the desired density of 5x10$^5$ cells / ml, fertilizer from Walne by 0.5 ml and 0.5 ml of vitamin B12 [11].

The D. salina seeds used for the study were obtained from the Brackish Water Aquaculture Institute (BBAP) Situbondo. The delivery of the seeds of D. salina was carried out at low temperatures. The seed were packaged into a bottle and then put in a Styrofoam box that had been filled with ice, and then the seed were stored in the refrigerator. The seeds of pure stock were checked and counted using a haemocytometer under the microscope before inserting them into the culture bottle. The seeds were put in a culture flask at a density of 5x10$^5$ cells / ml. The numbers of seeds necessary for culturing were calculated using the following formula [12]:

$$V_1 = \frac{N_2XV_2}{N_1}$$

Information:

- $V_1$ = Volume of Dunaliella salina required (ml)
- $V_2$ = Volume of the desired culture media (ml)
- $N_1$ = Density of pure seeds or stock of Dunaliella salina (cells / ml)
- $N_2$ = Density of the seedlings of the Dunaliella salina desired (cells / ml)

2.2.5 Measurement growth of D. salina

The growth measurements were performed by calculating the density of the cells every day until harvesting. The sampling was done using a pipette. The cell density was calculated by direct counting using a haemocytometer and then counting the cells under a dissecting light microscope with a magnification of 400 times. The data obtained was calculated using the following formula[12]:

Phytoplankton Density (cell/ml) = nA+nB+nC+nD x 10$^4$

Note: nA, nB, nC, nD = Number of phytoplankton in block A, B, C, D

2.2.6 Harvesting D. salina

Phytoplankton harvesting should be done at the right time, at the peak of the population [10]. Harvesting D. salina was done partially on the 2nd, 4th, 6th, 8th, 10th, 12th and 14th [2]. This method intended to find differences in the growth phase changes in each treatment and to better know the progress of the formation of β-carotene in D. salina. D. salina was partially harvested by taking a 10 ml sample, putting it into a test tube and then extracting it to determine the content of β-carotene in D. salina [10].
2.2.7 Extraction

The analysis of the content of β-carotene was done by taking a 5 ml sample yield of *D. salina* using a pipette, and then centrifuging it at a speed of 5,000 rpm for five minutes [2]. The results of the centrifuge were then taken and added to 5 ml of distilled water and centrifuged at a speed of 5,000 rpm for five minutes. The results were then made into a pellet and added to 5 ml acetone: distilled water at a ratio (80:20 v/v) of 4 ml acetone and 1 ml of distilled water. The resulting mixture was homogenized using a vortex for two minutes and centrifuged at a speed of 5,000 rpm for five minutes. The resulting mixture was composed of two layers of liquid fluid; the colors were clear and yellowish respectively. The layers of clear liquid and yellowish liquid were taken to be read on a spectrophotometer with a wavelength of 480 nm.

2.2.8 Calculation the content of β-carotene

The extraction of the coating of yellowish liquid was then inserted into the corresponding cuvettes and read using a spectrophotometer with a wavelength of 480 nm [2]. The B-carotene calculation formula was as follows [13]:

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\text{Content of β-caroten (ml/L)} = \frac{A \times V \times 100}{A_{1cm}^{1%} \times 100}
\]

Note:
- \(A_{1cm}^{1%}\): coefficient of the absorbance of β-carotene 2273
- \(V\): sample volume
- \(A\): absorbance at a wavelength of 480 nm

3. Results and discussion

3.1 Result

The β-carotene content of *D. salina* was measured every other day, starting on day 2 through to day 14 in order to determine the development of the formation of β-carotene in *D. salina*. The data on the average β-carotene content of *D. salina* in all treatments has been presented in Table 1.

| Day | C (700 Lux) | D (5.200 Lux) | E (5.700 Lux) | F (6.200 Lux) |
|-----|-------------|---------------|--------------|--------------|
| 2   | 0.0012± 0.0002 | 0.0032± 0.0010 | 0.0054± 0.0013 |               |
| 4   | 0.0043± 0.0009 | 0.0054± 0.0021 | 0.0058± 0.0027 |               |
| 6   | 0.0049± 0.0012 | 0.0059± 0.0015 | 0.0074± 0.0012 |               |
| 8   | 0.0052± 0.0011 | 0.0068± 0.0011 | 0.0087± 0.0014 |               |
| 10  | 0.0043± 0.0008 | 0.0051± 0.0007 | 0.0057± 0.0010 |               |
| 12  | 0.0043± 0.0013 | 0.0043± 0.0005 | 0.0054± 0.0008 |               |
| 14  | 0.0039± 0.0021 | 0.0036± 0.0016 | 0.0044± 0.0022 |               |

Note: Different superscript letter notations in the same column show the comparison between treatments and that there is a very significant difference (P <0.05).
Figure 1. β-carotene content from *D. salina*

Based on Table 1 and Figure 1, the highest content of β-carotene was in treatment A (light intensity of 700 Lux) and B (light intensity 2,200 Lux), which occurred on the 10th day in 0.0052 ml / L and in 0.0053 ml / L. The C treatment (light intensity 3,700 Lux) and D treatment (the light intensity of 5,200 Lux) in relation to the β-carotene content highs occurred on the 8th day at 0.0068 ml / L and 0.0087 ml / L respectively. *D. salina* growth can be illustrated with the cell densities calculated every day for 14 days of maintenance by using a haemocytometer. The data and graph density showing the *D. salina* growth can be seen in Figure 2.

![Graph of β-carotene content from D. salina](image1)

Figure 2. Density of *D. salina*

Based on Figure 2, it can be seen that the density of *D. salina* follows the pattern of growth of phytoplankton culture in general: the adaptation phase, exponential growth, stationary period and death. In treatment A and B (light intensity of 700 Lux, and 2200 Lux), the 1st to the 2nd day was the adaptation phase, the exponential phase occurred on the 3rd day until the 10th day and the death phase started on the 11th day until the end of the culture on the 14th day. The density of *D. salina* in
treatments C and D (light intensity 3,700 and 5,200 Lux) showed that the 1st and 2nd days were the adaptation phase, the exponential phase occurred on day 3 to day 8 and the growth started to decrease in density (death phase) from day 9th until the end of the 14th day of culture.

3.2 Discussion

The results of the calculation of the β-carotene content of D. salina on the 2nd day through to the 14th showed that the β-carotene content of D. salina was the highest in treatment D (the light intensity of 5,200 Lux). This is presumably as the high light causes the enzyme in the cells of D. salina to work optimally for the formation of β-carotene. The high intensity light is capable of increasing the carotenoid hydroxylase enzyme (CH) and phytoene synthase (PSY), which is a precursor of phytoene. The increased carotenoid hydroxylase enzyme (CH) and phytoene synthase (PSY) causes the amount of phytoene to also increased [14]. The phytoene constituent component is β-carotene, thus the synthesis of β-carotene also increased.

β-carotene can be formed at the beginning of the growth stage along with chlorophyll-a, which serves as a photosensitizer that prevents damage from photooxidation in the process of chlorophyll release. This is as it is easily damaged because the chlorophyll will form a triplet which binds with oxygen to form a singlet oxygen (singlet oxygen) [15]. Singlet oxygen is an oxidant that will oxidize the chlorophyll, fatty acids, proteins and nucleic acids, leading to the death of the organism. β-carotene will immediately neutralize the singlet oxygen through a detoxification process so then cell death does not occur. If the environmental conditions are not appropriate then it will lead to the physiological conditions of the D. salina cells not being balanced, so then the synthesis of β-carotene increased as a form of self-defense [2]. The production of β-carotene in the D. salina cells serves as an antidote to the free radicals and harmful toxins that enter the cell.

On the 10th, 12th and 14th days, the average β-carotene content of D. salina showed similar results between the treatments. The average value of the content of β-carotene, which is almost the same on the 12th day and the 14th, is suspected to be caused by the cells in D. salina for all treatments when it is going through the death phase (Table 2). This causes the content of the β-carotene produced to also decrease. The production of the carotenoid pigments in D. salina are increasing in line with the increasing age of the cells; this will continue to decline until the cell death phase [15]. The production of β-carotene in this phase is used for cell survival when the nutrients in the medium begin to thin.

Light plays an important role in the formation of β-carotene in D. salina cells. The D. salina cells will respond to light, and then the cells will activate signal transduction from the cell nucleus to the plastid [16]. The activation of the signal transduction will cause a change in the gene expression in the nucleus and cytoplasm. The products of these genes will be sent to the chloroplast, and then the formation of β-carotene will begin, prompted by enzyme karotenogenesis. The carotenoid biosynthetic process begins with two molecules of GGPP (C20) that condense into phytoene due to the enzyme phytoene synthase (PSY) [17]. The biosynthesis of phytoene desaturation is followed by a reaction due to the enzyme phytoene desaturase (PDS) to produce the trans lycopene which is pink and 9,15,9'-tri-cis-ζ-carotene. This reaction is catalyzed by two desaturase and two isomerase. The first desaturase produces 9,9'-di-cis-ζ-carotene by the enzyme carotene isomerase (Z-ISO). The second desaturase produces 7,9,9'-cis-neurosporene and 7 ', 9'-cis-lycopene by the enzyme desaturase (ZDS). The carotene isomerase enzyme (CRTISO) will catalyze the formation of all-trans-lycopenes. Lycopene cyclase will undergo two reactions, namely by the enzyme β-cyclase (βLCY) to produce β-carotene and ε-cyclase enzyme (εLCY) which can produce α-carotene.

Phytoplankton growth consists of four phases; adaptation, exponential, stationary and death [10]. The adaptation phase in all treatment occurred on day 1 until the 2nd day; i.e. cell growth had not occurred significantly due to D. salina still adapting to the environment. The exponential phase of treatments A and B (light intensity of 700 Lux, and 2200 Lux respectively) occurred on day 3 to day 10 in treatments C (the light intensity of 3,700 Lux) and D (the light intensity of 5,200 Lux) until the 8th day. The stationary phase in all of the treatments did not appear, presumably because the stationary
phase lasts less than 24 hours while the observation cell density of *D. salina* was done once every 24 hours.

The death phase in treatment A (light intensity of 3,700 Lux) and B (light intensity 2,200 Lux) started on the 11th day, while in treatment C (light intensity of 3,700 Lux) and D (light intensity of 5,200 Lux), it started on the 9th day. This happened because in treatment C (light intensity of 3,700 Lux) and D (the light intensity of 5,200 Lux), there was more rapid cell division so then the number of cells increased. The higher the number of cells in the culture medium, the more nutrients are needed for growth. This resulted in a nutrient culture medium that decreased, so then in treatment C (light intensity of 3,700 Lux) and D (light intensity of 5,200 Lux), the phase of early death occurred. The quality of the water and nutrients in the media can lead to diminishing phytoplankton cells that cannot thrive, causing cell death [18]. Phytoplankton will utilize the nutrients in the culture medium until the peak cell densities require more nutrients, while the nutrient media will decline because there is no addition of nutrients [19]. This can cause cell death in the phytoplankton.

*D. salina* can reproduce in two ways: sexual and asexual [5]. Sexual reproduction can occur in response to environmental changes that are not appropriate, through the process of gametogenesis by producing isogametes that looks like zoospores [16]. *D. salina*’s sexual reproduction occurs by way of isogamy, as zygote developments will occur in meiotic division [20]. Red or green zygotes have an endomembrane that is smooth and very thin. The zygote will divide in meiosis after the resting phase and will be comprised of more than 16 cells released through a crack in the wall of the stem cells. This will split into 32 haploid daughter cells [16].

Changes in salinity are suspected of being the cause of stress in the *D. salina* cells in addition to the treatment of light intensity. Changes in salinity can damage the cell’s plasma membrane, thus triggering the activation of the enzyme protein kinase that may trigger the conversion of starch into glycerol in the chloroplasts [21]. Salinity changes that take place over a long period will cause the enzyme protein kinase to work to change gene expression through the transcription process so as to produce carotenoids.

4. Conclusion
Different light intensity affects the β-carotene content of *D. salina*. The higher the light intensity that is given, the higher the β-carotene content that is produced. The optimal light intensity needed to produce the highest β-carotene content is 5200 Lux paired with 0.0087 ml/L.

5. References
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