Effect of Nitrogen Deficiency and UV Light on *Dunaliella salina* β-carotene Production

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ABSTRACT
Green alga (*Dunaliella salina*) could produce high amount of β-carotene under extreme environmental conditions in order to defend its cells from extreme environmental stresses. β-carotene is widely used in various industries as antioxidant, provitamin A, food coloring, etc. In the present study, nitrogen deficiency and UV-C radiation are combined such as normal, UV, N-, and UV N- and used to culture *Dunaliella salina* to determine the best condition for cell growth and β-carotene production for 6 days. The cells were counted with a haemocytomenter, and β-carotene was extracted every three days. Result showed that normal culture *Dunaliella salina* had highest cell growth but lowest β-carotene production, while UV N- culture had lowest cell growth but highest β-carotene production. Environmental stress such as nitrogen deficiency and UV light can increase β-carotene production, but reduce cell growth as the side effect.

Keywords: *β-carotene, Dunaliella salina, nitrogen deficiency, UV-C.*

1. INTRODUCTION

β-carotene is the richest provitamin A carotenoid in food and human body tissues. Due to its high bioactivity, β-carotene is widely used in medicine. In the human body β-carotene plays an important role related to the supply of provitamin A, good growth, and vision. β-carotene function as an anticancer and antioxidant. In the cosmetic industry β-carotene is used as a bioactive ingredient in facial creams that can protect the skin from oxidation and UV radiation. Besides being used because of its high bioactivity, β-carotene is also widely used in industry because of its physical properties. The orange-red color of β-carotene is widely used as a colorant in the food and pharmaceutical industries [1].

Several methods can be used to produce β-Carotene. The physicochemical method is the oldest method for producing β-Carotene. This method extracts natural β-carotene from plants, this method is starting to be abandoned because of the high production costs [1]. The method used in the industry until now is the chemical synthesis method with the wittig reaction. This method only produces All-trans-β-carotene, while 9-cis-β-carotene which is direct precursor of 9-Cis retinoic acid is not produced in this method. 9-cis retinoic acid acts as a hormone, it controls normal reproduction and maintenance of epithelial tissue [2]. In addition, several studies also state the presence of carcinogenic activity in this synthetic β-carotene. This is the main reason to look for other methods to produce β-carotene [1].

Therefore, a microbiological biosynthetic method was developed. This method is a biosynthetic method of β-carotene by microorganisms [1]. The microalgae that has become the center of attention for producing β-carotene is *Dunaliella salina* because of its potential to produce β-carotene in large quantities when subjected to extreme environmental stresses such as light intensity and high salinity as well as extreme temperatures and pH or lack of nutrients [3].

Nitrogen availability is one of the most potential environmental factor affecting β-carotene production in *Dunaliella salina* [4]. Nitrogen is a macronutrient needed by plants to carry out protein synthesis and cell division in microalgae, a lack of nitrogen will increase the synthesis of enzymes that increase the rate of pigmentation [5].

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Lamers et al [4] reported nitrogen defficiency can increase β-carotene production in *Dunaliella salina* up to three times in low light intensity condition and two times on high light intensity condition. Another research studying about nitrogen defficiency on *Dunaliella salina* also showed similar result. Pisal and Lele [6] reported nitrogen defficiency increase β-carotene production in *Dunaliella salina* from 1.65 pg/cell (normal condition) to 7.05 pg/cell (nitrogen defficiency condition) [6].

Dunaliella salina does not have a cell wall, it is only coated with a membrane [7]. Therefore, genetic improvisation through UV radiation is possible. UV-C radiation plays an important role on the induction of expression of genes, this affect photosynthesis and growth of which resulted in pigment and starch accumulation [3].

Sharma et al [8] using 50 mJ/cm2 UV-C radiation, to induce carotenoid biosynthesis in *Dunaliella salina*. Total carotenoids and β-carotene in *Dunaliella salina* were doubled in 24 h [8]. Another research studying about the effect of UV-C light on *Dunaliella salina* also showed similar result. Dunaliella salina irradiated by 0.4 mmol photon/m^2s UV-C light for 22 minutes and grown Johnson's medium containing 2.5 M NaCl. Total carotenoid and β-carotene in mutant *Dunaliella salina* were two times higher compared to the wild type culture [3].

2. MATERIAL AND METHODS

2.1. Sample Collection

Dunaliella salina obtained from Micro, Small and Medium Enterprises (MSME) Klaten.

2.2 Vitamin and walne nutrient preparation

Nutrient and vitamin are prepared using a standard 1 mL/L, so the concentration of each ingredient is multiplied by 1000.

Normal walne nutrient: 100 mg $NaNO_3$, 45 mg Na_2EDTA, 33.6 mg H_3BO_3, 20 mg $NaH_2PO_4.2H_2O$, 1.3 mg $FeCl_3.6H_2O$, 0.36 mg/L $MnCl_2.4H_2O$ were dissolved in 1000 ml aquadest.

Nitrogen deficiency walne nutrient: 0.03 mg $NaNO_3$, 45 mg Na_2EDTA, 33.6 mg H_3BO_3, 20 mg $NaH_2PO_4.2H_2O$, 1.3 mg $FeCl_3.6H_2O$, 0.36 mg/L $MnCl_2.4H_2O$ were dissolved in 1000 ml aquadest.

Algae vitamin: 0.1 mg B1 vitamin, and 0.005 mg B12 vitamin were dissolved in 100 ml aquadest.

2.3 Mutagenesis by UV-C

Dunaliella salina were spread in petridish and exposed to 0.14 mW/cm2s UV-C irradiation for 15 sec. Petridish were stored in a box for 2 hour to prevent light-induced DNA repair.

2.4 Culture method

125 ml *Dunaliella salina*, 125 ml sea water (30 ppt), 0.25 ml walne nutrient, and 0.25 ml algae vitamin were poured into 300 ml erlenmeyer. 2 pieces of 21 watt lamps were installed on the front, back and top of the Erlenmeyer with a distance of 3 cm. 2.4 L/min aerator were installed into the erlenmeyer and cultured for 6 days.

Table 1. Cultivation condition

Condition code	UV-C irradiation	$NaNO_3$ content in walne nutrient
Normal	No	100 mg/L
UV	Yes	100 mg/L
N-	No	0.03 mg/L
UV N-	Yes	0.03 mg/L

2.5 Cell density measurement

Cell density was monitored daily using Neubauer haemocytometer. 1mL of well-mixed culture supension was taken and diluted 10 times. Then the diluted sample placed on a hemacytometer to count the cell number.

2.6 Specific growth rate calculation

Specific growth rate of *Dunaliella salina* from different cultivation conditions were calculated by the following equation [12]:

$$\text{Specific growth rate } (\mu) = \frac{\ln X2 - \ln X1}{t2 - t1} \quad (1)$$

where X1 is the cell concentration at the beginning of the log phase, X2 is cell concentration at the end of the log phase, t2–t1 is the log phase time interval *Dunaliella salina*.

2.7 Cell dry weight measurement

30 mL of sample was extracted and filtered with whatman no 93 filter paper and dried in oven at 100˚C to measure dry weight of the sample.

2.8 β-carotene content measurement

β-carotene was extracted every 3 days. 30 ml sample was centrifuged at 4˚C, 3000 rpm for 10 min. Supernatant was discarded, then 8 ml aquadest, 8 ml ethanol, and 4 ml n-hexane (contain BHT 200 ppm) were added to pellet and centrifuged at 4˚C, 3000 rpm for 10 min. Supernatant was collected and checked for absorbance at 450 nm wavelength. Absorbance data was connected to standard curve to obtain β-carotene content

(mg/L) in the sample, and β-carotene content (%w/w) were calculated by the following equation:

$$\beta - \text{carotene content}\left(\%\frac{w}{w}\right) = \frac{\beta\text{-carotene content}\left(\frac{mg}{L}\right)}{\text{Cell dry weight}\left(\frac{mg}{L}\right)} \quad (2)$$

3. RESULT AND DISCUSSION

3.1 Growth of Dunaliella salina

Growth of *Dunaliella salina* was observed through cell density and specific growth rate. In Fig.1 Normal cultivation condition can obtain its highest cell density (241.83×10^6 cell/mL) at day 5, UV cultivation condition obtain highest cell density (224.17×10^6 cell/mL) at day 5, N- cultivation condition obtain its highest cell density (118.33×10^6 cell/mL) at day 3, and UV N- cultivation condition obtain its highest cell density (118.17×10^6 cell/mL) at day 5. The day that the cultivation condition obtain its highest cell density considered as the end of log phase (t2) and day 0 considered as the beginning of the log phase (t1), this would be used for measuring specific growth rate.

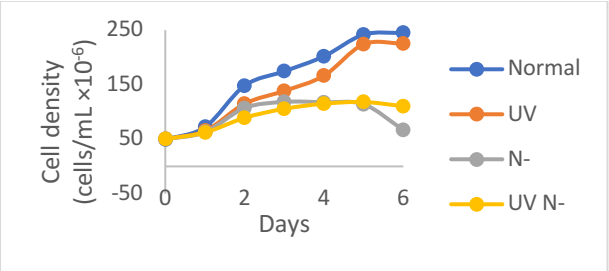

Figure 1 Cell density (×10-6) cells/mL of Dunaliella salina cultured at different cultivation conditions

Normal cultivation conditions give the highest specific growth rate compared to other cultivation conditions (Table 2). UV is cultivation condition with second highest specific growth rate, then followed by N- cultivation condition and the last one is UV N- is cultivation condition with the smallest specific growth rate. Nitrogen is a basic component that supports growth and biomass production of *Dunaliella salina.* Cell division is difficult to occur when nitrogen supply is not sufficient [6]. This result the growth of *Dunaliella salina* with N- cultivation condition having slower growth rate compared to normal condition. UV irradiation causes damage to cell DNA which affects the cell cycle and reduces photosynthetic efficiency [9]. This result growth of *Dunaliella salina* with UV cultivation condition having a slower growth rate compared to normal condition. UV N- cultivation condition combining nitrogen defficiency and UV-C irradiation causing double growth inhibition as result this cultivation condition result the slowest growth rate compared to other cultivation condition.

Table 2 Specific growth rate

Cultivation Condition	Specific growth rate (day^{-1})
Normal	0.319
UV	0.300
N-	0.215
UV N-	0.171

3.2 Color Change of Dunaliella salina

In Figure 2 color change of UV and normal cultivation condition are green to dark green, indicate photosynthetic growth of *Dunaliella salina* and blocked synthesis of β-carotene. While color change of UV N- and N- cultivation condition are green to yellow-orange, indicate carotenogenesis and uninterrupted synthesis of β-carotene [10].

Days	Cultivation condition			
	UV N-	N-	UV	Normal
0				
1				
2				
3				
4				
5				
6				

Figure 2 Color change of *Dunaliella salina*

3.3 β-carotene accumulation

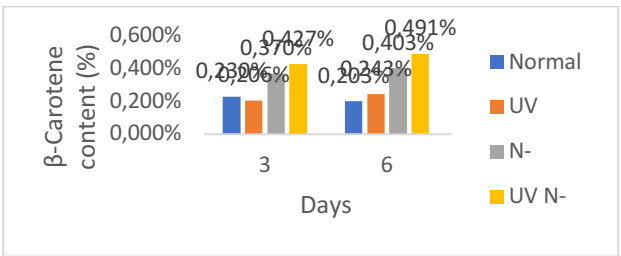

Figure 3 β-carotene content (%w/w) based on dry weight of *Dunaliella salina* cultured at different cultivation condition

β-carotene content in each cultivation condition were measured every three days. The highest β-carotene content found in UV N- cultivation condition at day 3 (0.427%) and day 6 (0.491%). While the lowest β-carotene content found in UV cultivation at day 3

(0.206%) and normal cultivation at day 6 (0.203%). In Figure 3 β-carotene content in N- and UV N- much higher than normal and UV cultivation condition. This indicate nitrogen deficiency can significantly increase the β-carotene produced by *Dunaliella salina*.

There are two enzymes that play a role in the isoprenoid synthesis pathway (Figure 4), 1-deoxyxylulose-5- phosphate synthase (DXS enzyme) and Phytoene synthase (PSY enzyme). DXS enzyme function is to catalyze biosynthesis of isopentenyl diphosphate (IPP) from 1-deoxyxylulose 5-phosphate (DXP). PSY enzyme function is to catalyze biosynthesis of phytoene from Geranylgeranyl Diphosphate (GGPP) [11].

β-carotene are the final biosynthetic products of the isoprenoid synthesis pathway (Figure 4). The biosynthesis of isopentenyl diphosphate (IPP) is catalyzed by 1-deoxyxylulose-5- phosphate synthase (DXS enzyme). Biosynthesis of carotenoids from IPP through a common route including their conversion into dimethylallyl diphosphate (DMAPP), geranyldiphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP), which is the substrate for formation of phytol (chlorophyll side chain) and phytoene (Figure 4). Phytoene formation is catalyzed by phytoene synthase (PSY enzyme). Phytoene formation is considered the first regulatory step in carotenogenesis, which is β-carotene formation. Under nitrogen deficiency conditions, DXS enzyme was lower than normal condition, but PSY enzyme which involved in carotenoid production was same with normal condition, resulting lower growth rate and carotenoid accumulation in nitrogen deficiency condition [11].

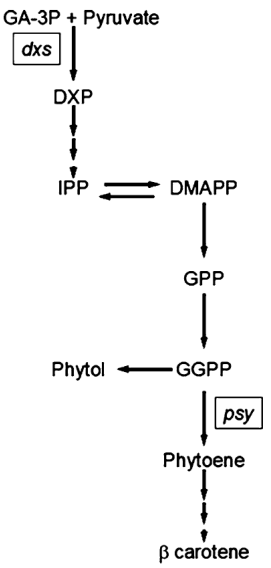

Figure 4 Pathway of photosynthetic pigment biosynthesis in algae [11].

UV light also increase the amount of β-carotene produced by *Dunaliella salina* but this is unsignificant compared to the nitrogen deficiency. Shows in Figure 3 UV and normal cultivation condition are giving a similar amount of β-carotene, and slightly increase amount of β-carotene in UV N- compared to N- cultivation condition. UV-C plays an important role on the induction of expression of genes that are encoding the antioxidant. During stress condition alga cytosol contained several strong oxidative agents, such as super peroxide free radical. This oxidative agent stimulates alga cell to produce more carotenoid to protect their cell or tissue from further damage by the stress condition [3].

4. CONCLUSION

Environmental stress such as nitrogen deficiency and UV light increase the β-carotene produced by *Dunaliella salina* but reduce the growth rate of *Dunaliella salina* as the side effect. Higher increase of β-carotene production result higher reduction in *Dunaliella salina* growth rate. Nitrogen defficiency stress gives higher increase the amount of β-carotene produced by *Dunaliella salina* than UV light stress with 2.1 mJ/cm^2 dosage. Combined nitrogen defficiency and UV light stress gives higher increase the amount of β-carotene than nitrogen defficiency and UV light respectively. The results of this study can be applied in the β-carotene production from *Dunaliella salina* for application in the pharmaceutical, cosmetic, and food industry. Further studies are required to indentify the optimum nitrogen content and UV light dosage that can lead to the highest β-carotene production of *Dunaliella salina.*

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