PRODUCTION OF BETA-CAROTENE BY *DUNALIELLA SALINA*: EFFECT OF $NaNO_3$ CONCENTRATION AND LIGHT INTENSITY

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NUR ISMA IDAYU ISMAIL BACHELOR OF CHEMICAL ENGINEERING (BIOTECHNOLOGY) 2012

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We certify that the thesis entitled Production of Beta-carotene by Using *Dunaliella salina*: Effect of $NaNO_3$ Concentration and Light Intensity is written by Nur Isma Idayu Binti Ismail. We have examined the final copy of this thesis and in our opinion; it is fully adequate in term of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology).We herewith recommend that it be accepted in fulfillment of the requirement for the degree of Bachelor specializing in Chemical Engineering (Biotechnology).

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PRODUCTION OF BETA-CAROTENE BY USING *DUNALIELLA SALINA*: EFFECT OF $NaNO_3$ CONCENTRATION AND LIGHT INTENSITY

NUR ISMA IDAYU BINTI ISMAIL

A thesis submitted in fulfillment of the requirements
for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

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JUNE 2012

SUPERVISOR'S DECLARATION

"I hereby declare that I have checked this thesis and in my opinion, this thesis is adequate in term of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology)"

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"I hereby declare that this thesis entitled "Production of Beta-carotene by Using *Dunaliella salina* : Effect of $NaNO_3$ Concentration and Light Intensity" is the result of my own research except as cited references. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree".

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I dedicate this entire work to my family especially to my beloved parents for their patience and support to accomplish my study and also to all my friends for their companionship and unconditional love in good and bad time.

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ABSTRACT

Beta-carotene is used as vitamin A precursor and its antioxidant property has been used in dietary supplements for the prevention of diseases such as cancer and coronary heart disease. The purpose of this study is to study the effect of $NaNO_3$ concentration and light intensity to the growth and production of beta-carotene by *Dunaliella salina*. Four different $NaNO_3$ concentrations (100%, 75%, 50%, and 25% v/w) and light intensities (6000 lx, 8000 lx, 11000 lx and 17000 lx) were used. Cell growth was determined by cell counting method and analysis of total beta-carotene concentration in the samples was done using High Performance Liquid Chromatography (HPLC). From the result obtained, the $NaNO_3$ concentration and light intensity will affect the growth of *Dunaliella salina,* where the growth was reduced due to $NaNO_3$ starvation and it was also induced with the high light intensity until nitrate became the limiting source for the cells. Besides that, $NaNO_3$ concentration and light intensity will also affect the beta-carotene production of *Dunaliella salina,* where the production was increased as the $NaNO_3$ concentration was decreased and the light intensity was increased until the concentration of $NaNO_3$ has become the limiting factor of the experiment. In order to improve the research, further study to identify the optimal $NaNO_3$ concentration and light intensity required for maximizing the production of beta-carotene by *Dunaliella salina* shall be done.

ABSTRAK

Beta-karotena digunakan sebagai pelopor vitamin A dan sifat antioksidan telah digunakan dalam makanan tambahan pemakanan untuk mencegah penyakit seperti kanser dan penyakit jantung koronari. Tujuan kajian ini adalah untuk mengkaji kesan kepekatan $NaNO_3$ dan keamatan cahaya kepada pertumbuhan dan pengeluaran beta-karotena oleh *Dunaliella salina*. Empat $NaNO_3$ dengan kepekatan yang berbeza (100%, 75%, 50%, dan 25% v / w) dan keamatan cahaya (6000 lx, 8000 lx, 11000 lx dan 17000 lx) akan digunakan untuk mengkaji kesan parameter pada pertumbuhan dan pengeluaran beta-karotena oleh *Dunaliella salina*. Pertumbuhan sel ditentukan oleh kaedah pengiraan sel dan analisis kepekatan jumlah beta-karotena dalam sampel telah dilakukan dengan menggunakan Kromatografi Cecair Prestasi Tinggi (HPLC). Berdasarkan keputusan yang diperolehi, kepekatan $NaNO_3$ dan keamatan cahaya akan menjejaskan pertumbuhan *Dunaliella salina* di mana pertumbuhan telah dikurangkan disebabkan oleh kekurangan $NaNO_3$ dan ia juga disebabkan dengan keamatan cahaya yang tinggi sehinggalah nitrat menjadi sumber terhad untuk sel-sel. Selain itu, kepekatan $NaNO_3$ dan keamatan cahaya juga akan menjejaskan pengeluaran beta-karotena oleh *Dunaliella salina* di mana pengeluaran telah meningkat apabila kepekatan $NaNO_3$ telah menurun dan keamatan cahaya meningkat sehinggalah kepekatan $NaNO_3$ telah menjadi faktor penghad eksperimen. Dalam usaha untuk meningkatkan penyelidikan, kajian lanjut perlu dilakukan untuk mengenalpasti kepekatan $NaNO_3$ dan keamatan cahaya yang optimum diperlukan untuk memaksimumkan pengeluaran beta-karotena oleh *Dunaliella salina*.

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LIST OF SYMBOLS

^{o}C	Celsius
cm^3	Cubic centimeter
g	Gram
g/l	Gram per liter
m	Meter
m^2	Square meter
$m^2.s^{-1}$	Square meter per second
mg/ml	Milligram per milliliter
min	Minutes
ml	Milliliter
ml^{-1}	Per milliliter
$ml.min^{-1}$	Milliliter per minutes
mm	Millimeter
mm^2	Square millimeter
$m.s^{-1}$	Meter per second
nm	Nanometer
tonnes/year	Tonnes per year
US$/year	United State Dollar per year
W	Watt
w/v	Weight per volume
v/v	Volume per volume
β	Beta

µm	Micrometer
µmol.$m^{-2}s^{-1}$	Micromole per square meter second
%	Percent

LIST OF ABBREVIATIONS

ASW	Artificial Seawater
C	Carbon
CCAP	Culture Collection of Algae and Protozoa
CO_2	Carbon dioxide
O_2	Oxygen
HCl	Hydrochloric acid
HPLC	High Performance LiquidChromatography
K_2HPO_4	Dipotassium phosphate
KNO_3	Potassium nitrate
N	Nitrogen
NaCl	Sodium chloride
$NaNO_3$	Sodium nitrate
NO_3^-	Nitrate
NH_4^+	Ammonium

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

There are more than 25,000 species of microalgae which only 15 are in use. The technology in culturing microalgae becomes business oriented owing to their practical applications. Beta-carotene production by using green microalgae *Dunaliella* is a major success in applied algae biotechnology. Halotolerant algae of genus *Dunaliella* is a eukaryotic microorganism that can survive in a very high salinity condition. This microalga has the ability to adapt to sudden change in salinity, irradiance, nutrient availability, and light intensity (Lamers et al., 2008). According to Marín et al. (1998), recent researches on potential therapeutic properties of beta-carotene have also contributed to the interest in this microalga. Beta-carotene preparations have been shown to inhibit or prevent various types of tumors in humans and animals, including skin cancers such as melanoma, epidermoid cancers of head and neck, cancers of the gastrointestinal tract, carcinomas of secretory glands such as the pancreas and breast cancer (Tafreshi and Shariati, 2009). Besides that, beta-carotene is also effective in controlling cholesterol levels and reducing the risk of cardiovascular diseases, such as myocardial infarction and angina pectoris as well as coronary heart disease.

One type of this microalgae species, *Dunaliella salina* is known to store a large capacity of beta-carotene and glycerol. Beta-carotene content in *Dunaliella salina* may reach until 10% of its cell dry weight (Lamers et al., 2008; Zamani et al., 2011). This product is directed mainly at the natural food colorant, nutraceutical or health food market (Barzegari et al., 2010; Celekli and Donmez, 2006). *Dunaliella salina* is a suitable source for commercial production of beta-carotene and preferentially accumulates 9-cis-isomer which functions as a carbon sink. According to Raja et al. (2008), the natural 9-cis isomer plays a major role in quenching free oxygen radicals and preventing oxidative damage to the cell. The free radicals are highly reactive molecules and they can cause harmful reactions for example uncontrolled oxidation in the body damages fat, genetic materials, and cell membranes including cataracts and cardiovascular diseases. The 9-cis isomer is synthesized only by natural sources and it is a highly valuable metabolite because of its interesting physicochemical high liposolubility properties, which makes it being effectively preserved into animal tissues; furthermore, this isomer has proved to have a better antioxidant capacity than the all-trans isomer (Gómez et al, 2003).

There are many studies about the effect of salinity on the quantity and quality of beta-carotene production of *Dunaliella salina* (Hadi et al, 2008; Gómez et al, 2003; Pisal and Lele, 2005). Previous studies suggest that the stressful environmental conditions, like high irradiance and salinity, favor high production of carotenoids (Marin et al., 1998).

1.2 PROBLEM STATEMENT

Most of beta-carotene supplements in the market are synthetic which consisted of only all-trans isomer. Tafreshi and Shariati (2009) stated that, several studies have reported that the synthetic all-trans beta-carotene not only fails to reduce the incidence of cancer, but it may be carcinogenic. But in natural beta-carotene found in plants like carrot, avocado, broccoli, coffee, watermelon and microalgae like *Dunaliella salina*, it contains both all-trans beta-carotene and 9-cis beta-carotene. The 9-cis isomers in natural beta-carotene supplement is used as antioxidant since they have greater antioxidant effect and therapeutic effect in the

prevention of diseases such as cancer and coronary heart disease than the all-trans isomer (Levin and Mokady, 1994).

The commercial production of beta-carotene by *Dunaliella salina* has to face the dilemma that its production is enhanced by physiological stress (Sánchez-Estudillo et al., 2006). Giordano and Beardall (2009) stated that, beta-carotene accumulation is highest when the growth is lowest. But low growth of these microalgae will produce low biomass production. Under stress conditions such as nutrient limitation, high salinity and high light intensity, *Dunaliella salina* reacts by accumulating a large amount of beta-carotene as its secondary metabolite instead of producing chlorophyll as its primary metabolite.

Several strategies have been used to maximize the production of beta-carotene per unit time and per culture volume. These strategies are based on the observations that severe conditions, such as high salinities, low nutrient levels and high temperatures combined with high irradiance, retard growth and at the same time, induce beta-carotene production in the cell. The higher the stress intensity and as a result the slower the growth rate of the alga, the greater is the total amount of the light absorbed by the cell during one division cycle. This situation can lead to higher accumulation of beta-carotene per cell. However, these conditions at the same time decrease the cell number per unit culture volume by affecting cell viability. Moreover, prolonged nutrient starvation can lead to high mortality of the algae. Therefore, it is recommended by one group of authors that adjusting light and salinity likely is one of the best strategies to achieve optimal beta-carotene production in mass cultures of *Dunaliella salina* (Tafreshi and Shariati, 2009).

1.3 OBJECTIVE

The objective of this study is to study the effect of $NaNO_3$ concentration and light intensity to the growth and production of beta-carotene by *Dunaliella salina*.

1.4 SCOPE OF RESEARCH

The scopes of the research are as follows:

a) Study the effect of different $NaNO_3$ concentration and light intensity to the growth of *Dunaliella salina*.
b) Study the effect of $NaNO_3$ concentration and light intensity in the production of beta-carotene by *Dunaliella salina*.

1.5 RATIONALE AND SIGNIFICANT

Carotenes in *Dunaliella salina* contain a mixture of alpha-carotene and beta-carotene isomers which consist of 9-cis and all-trans beta-carotene. This natural 9-cis isomer is rapidly used up in quenching free radicals and preventing oxidative damage to cell (Borowitzka, 1992). Furthermore, the pre-cancerous tissues in people are reverted to normal with the natural beta-carotene supplement but not with synthetic supplements. So, this research is important to produce natural beta-carotene which contains 9-cis and all-trans isomers for better antioxidant effect. Besides that, microalgae are used in the production of beta-carotene because of its fast growth rate, the ability of to grow over wide salinity range and the ease of manipulation (Barzegari et al., 2010; Borowitzka and Siva, 2007).

CHAPTER 2

LITERATURE REVIEW

2.1 COMMERCIAL GREEN MICROALGAE

According to Lamers et al. (2008), microalgae are photosynthetic microorganisms with which many added-value compounds can be produced in food, feed, cosmetics applications and feedstock for the chemical industry, and they also have potential as sustainable energy carriers. The microalgae produce high value molecules such as fatty acids, pigments and stable isotope biochemicals. For most of the applications, the market is still developing and the biotechnological uses of microalgae will extend to a new area (Raja et al., 2008).

Referring to Norton et al. (1996), it has been estimated that between 22,000 and 26,000 species exist, of which only several species have been identified to be useful for commercial application. These species are *Spirulina*, *Chlorella*, *Haematococcus*, *Dunaliella*, *Botryococcus*, *Phaeodactylum*, and *Porphyridium*. *Dunaliella salina* has probably been the most successful microalgae for the mass cultivation especially due to its high salinity requirement that minimizes the number of competitors and predators. Algal biotechnology has made major advances in the past three decades, and microalgae like *Dunaliella* are cultivated for the production of carotenoids and glycerol (Hadi et al., 2008). In this study, the focus will be discussing on the production of carotenoids in *Dunaliella salina*. The photosynthetic

ability is the most important feature of microalgae which makes them the promising organisms for autotrophic cultivation on simple mineral media for various biotechnological purposes.

According to Pulz and Scheibenbogen (1998), many attempts have been made to cultivate them in simple systems such as shallow open ponds since microalgae are very efficient solar energy converter. There are around 110 commercial producers of microalgae in the Asia-Pacific region, with an annual production capacity reach 500 tonnes. About nine-tenths of algal cultivation plants are located in Asia. This biomass of microalgae market has a size of about 5,000 tons/year of dry matter and generates a turnover of US\$1.25 × 10^9/year (Raja et al., 2008). In spite of such attractive feature of cultivating microalgae, several phototrophic single-species cultivation has met only limited success. Contamination by bacteria and protozoa has made such propagation possible only if suitable selective environments can be measured (Margalith, 1999). For examples, *Spirulina platensis* is successfully cultivated in high alkaline water which pH higher than 9.2 and *Dunaliella salina* is being cultivated commercially in open ponds with highly saline brine. The fast-growing microalgae such as *Chlorella* species can be also grown in open ponds and this system demand comparatively low investment for construction and maintenance. Economically feasible production of added-value compounds with microalgae is possible because microalgae produce biomass and specific biomass ingredients directly from solar irradiation at high photosynthetic efficiencies and high volumetric and a real productivities (Lamers et al., 2008). Brennan and Owende (2010) stated that under natural growth conditions, phototrophic microalgae absorb sunlight, and assimilate carbon dioxide from the air and nutrients from the aquatic habitats. According to Margalith (1999), microalgae which utilize organic carbon substrates as their sole carbon and energy source may be employed for heterotrophic growth. To prevent growth inhibition in the culture, usually very low concentrations of organic compounds are employed. This may be circumvented by using a suitable fed-batch system but heterotrophic media used may invite rapid bacterial contamination, which again may be overcome only by rigorous aseptic operations. The natural conditions for commercial algae production have the advantage of using sunlight as a free natural resource. However, it can be limited by available sunlight due to diurnal cycles and the seasonal variations (Brennan and

Owende, 2010). To address the limitations in natural growth conditions with sunlight, artificial lighting means employing fluorescent lamps are almost exclusively used for the cultivation of phototrophic algae at pilot scale stages. The artificial lighting allows for continuous production, but at significantly higher energy input.

2.2 MODE CULTURE OF *DUNALIELLA SALINA*

Algae cultivation in open pond production systems has been used since the 1950s which can be categorized into natural water (lakes, lagoon and ponds) and artificial ponds or containers (Brennan and Owende, 2010). According to Borowitzka et al. (1984), pilot plant site of *Dunaliella salina* at Hutt Lagoon is a shallow salt lake near Geraldton in Western Australia. The pilot plant facility consists of a number of open ponds with a total area of about 3000 m^2, constructed of earth walls set on the lake bed, and a shore compound housing the laboratory, workshop, water storage tanks and generator house. Water sources for the pilot plant are natural brines from the lake and fresh water from a well sunk on the shore. The current state of the corresponding production technologies are based on either open pond systems or closed photobioreactors (Campo et al., 2007). The potential of scientific and technological advances for improvements in yield and reduction in production costs for carotenoids from microalgae. Many types of system have been designed for the growth and handling of this microalgae on a large scale. Within the open pond modes, the best choice is the open shallow pond which made of leveled raceway 2-10 m wide and 15-30 cm deep and the running is as simple loop or meandering systems. Each unit covers an area of several hundred to a few thousand square meters. Turbulence is usually provided by rotating paddle wheels, which create a flow of the algal suspensions along the channels at a rate of 0.2–0.5 m s^{-1}. The adequate supply of carbon dioxide is very critical, and it is usually controlled through a pH-stat, so warranting both provision of carbon and optimum pH of the culture simultaneously.

Production of microalgae based on closed photobioreactor is designed to overcome some of the major problems associated in open pond system such as pollution and

contamination risk. However, compared with the cultivation of open pond system, this closed photobioreactor system cost is substantially higher. Tubular, flat plat and column photobioreactors are the examples of closed system. Brennan and Owende (2010) stated that this closed system is more appropriate for sensitive strains because it makes the control of potential contamination becomes easier and this system is usually used for the production of high-value pharmaceutical and cosmetic products. Photobioreactor consist of an array of straight glass or plastic tubes which aligned horizontally or vertically. The microalgae culture will be re-circulated either with a mechanical pump or air-lift system to allow CO_2 and O_2 exchange between the medium and aeration gas as well as to provide mixing.

2.3 *DUNALIELLA SALINA*

On 1938, Dunal had discovered the alga which gave the reddish colour of salt water during production of sea salt in southern France (Oren, 2005). According to Tafreshi and Shariati (2009), *Dunaliella* species belong to the phylum *Chlorophyta*, order *Volvocales* and family *Polyblepharidaceae*, and are unicellular, photosynthetic and motile biflagellate microalgae morphologically distinguished by the lack of a rigid cell wall. García et al. (2007) stated that, there are 23 species of the genus *Dunaliella* found in saline environments and exhibiting optimal growth at different salt concentrations with varying abilities to turn orange-red under particular culture conditions. The famous species of *Dunaliella* are *Dunaliella salina, Dunaliella tertiolecta, Dunaliella primolecta, Dunaliella viridis, Dunaliella bioculata, Dunaliella acidophyla, Dunaliella parva* and *Dunaliella media*. Borowitzka and Siva (2007) have assessed current issues of the taxonomy of *Dunaliella. Dunaliella* cells are found to be ovoid, spherical, pyriform, fusiform or ellipsoid with length from 5 to 25 µm and width from 3 to 13 µm (Oren, 2005; Ramos et al., 2011). These motile cells are also biflagellate with the flagella inserted at the anterior end of the cell the flagella length also varying between species. The cells also contain a single cup-shaped chloroplast which mostly has a central pyrenoid surrounded by starch granules. The genus of *Dunaliella* have been in the subject of numerous studies as a result of several factors such as the ease of culturing, the ability of several species to grow over wide salinity ranges and at extreme salinities, the accumulation of extremely

high levels of beta-carotene in *Dunaliella salina*, and lastly the wide tolerance to heavy metals and pesticides by some species. Besides chlorophylls a and b, the members of *Dunaliella* contain valuable carotenoid pigments such as alpha-carotene and beta-carotene, violaxanthin, neoxanthin, zeaxanthin and lutein (Ye et al., 2008).

Teodoresco was the first to describe *Dunaliella salina* in 1950 and named it after Dunal (Borowitzka and Siva, 2007) and this alga often found in natural marine habitats which make the water reddish in colour (Giordano and Beardall, 2009). The vegetative cell division of *Dunaliella salina* commences with the nuclear division followed by a furrowing of the cell at the anterior or flagella end of the cell and then at the opposite or posterior of the cell. The furrowing generally proceeds faster at the flagella end and the posterior furrowing proceeds with the division of the chloroplast and pyrenoid which then produce two daughter cells. The massive accumulation of beta-carotene by the strains under suitable growth conditions has led to interesting biotechnological applications and this pigment is primarily composed of the isomers 9-cis and all-trans (Gómez et al., 2003). The 9-cis beta-carotene occurs only in natural sources and is the most attractive from a commercial point of view. The result from the previous study showed that the 9-cis beta-carotene has higher antioxidant potency than that of the all-trans isomer (Levin and Mokady, 1994). According to Ye et al. (2008), *Dunaliella* have been exploited commercially to yield dried biomass and natural beta-carotene in several countries such as Israel, China, USA, Australia and Mexico since 1980s. The synthesis of beta-carotene increases with the unbalances physiological condition of cell due to stress factor (Pisal and Lele, 2005). This alga accumulates large amounts of beta-carotene as droplets in the chloroplast, to prevent chlorophyll photo-damage, when culture conditions include high light intensities, high temperature, high salinity and deficiency of nutrient (Lamers et al., 2008; Tafreshi and Shariati, 2009). The figure of *Dunaliella salina* in different culture condition was shown in **Figure 2.1**.

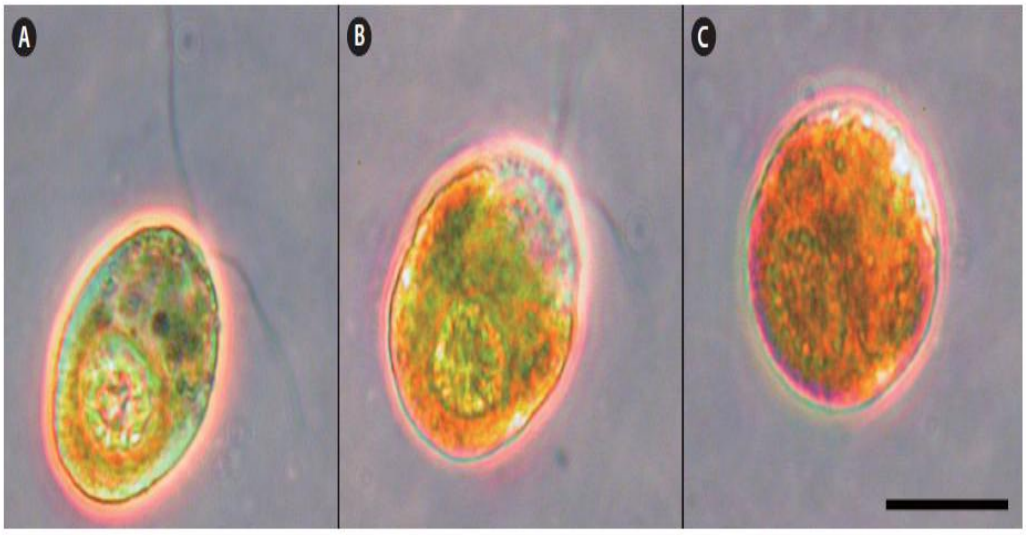

Figure 2.1: *Dunaliella salina* cells in different culture condition. (A) Green cell from a non-stressed culture. (B) Stressed cell turning orange. (C) Orange cell from a culture exposed to nutrient stress due to beta-carotene accumulation (Ramos et al., 2011)

2.4 HALOTOLERANCE

According to García et al. (2007), halotolerant algae of genus *Dunaliella* is microorganisms that live in a very saline environment and can even survive in saturated salt solution. These organisms have the ability to adapt to sudden changes in salinity, irradiances and nutrient depletion in their habitats. *Dunaliella salina* has the potential to overcome these difficulties and is widely employed for the production of valuable fine chemicals such as carotenes. Previously, many researchers have manipulated on the stress factors and alternative work systems for massive production of beta-carotene (Ye et al., 2008). Under suitable conditions, some strains of *Dunaliella* could accumulate 10% or more beta-carotene of the total dry organic matter in weight (Hejazi and Wijffels, 2003; Lamers et al., 2008). High light intensity leads to an increase in the ratio of 9-cis to all-trans isomer (Liu et al., 1996). Several physiological adaptations have been developed by *Dunaliella* including the lack of a rigid cell wall, a variable intracellular concentration of glycerol changes in photosynthetic pigments balance and structural modifications in the chloroplast.

2.5 CAROTENOIDS

Carotenoids (C_{40}) are a group of natural fat-soluble pigments which are found in plants, algae, and photosynthetic bacteria (Stahl and Sies, 2005). They are responsible for the color in some species of yeast, bacteria and fungi as well as many vegetables and fruits. According to Lamers et al. (2008), the colors of these pigments range from yellow to red and some found in tomatoes (lycopene), maize corn (zeaxanthin) and carrot (beta-carotene).They play an important role in photosynthesis and there are about 600 different compounds of carotenoid that have been identified so far, of which 50 can be found in the human diet (Tourniaire et al., 2009). Carotenoids represent one group of valuable molecules for several industries such as pharmaceutical, chemical, food and feed industries. It is not only because they can act as vitamin A precursors, but also for their coloring, antioxidant and possible tumor-inhibiting activity. (Frengova and Beshkova, 2009). **Figure 2.2** shows several health promoting functions attributed to carotenoids.

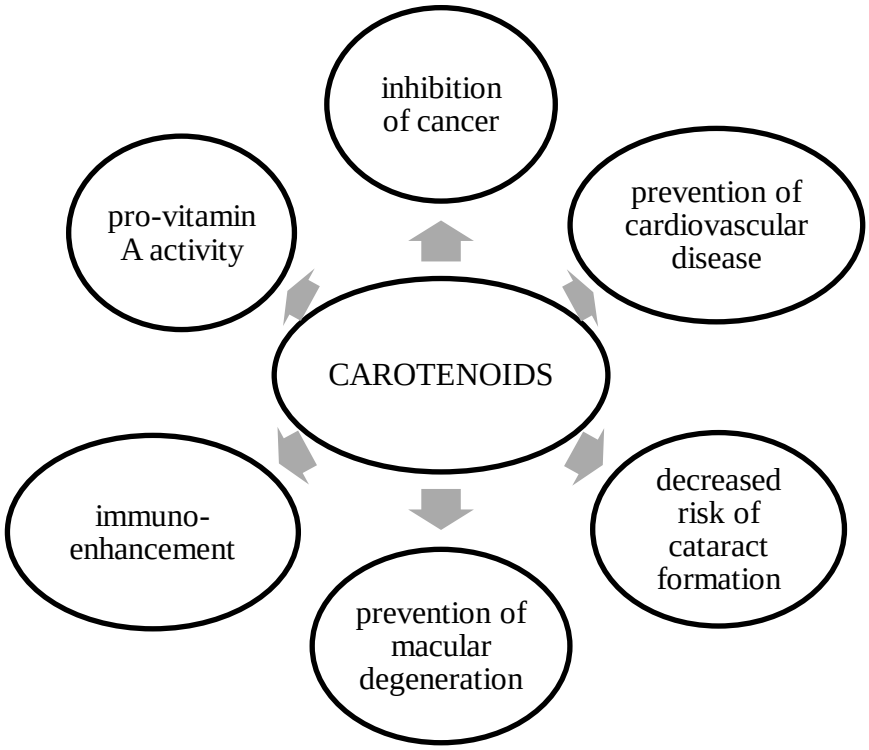

Figure 2.2: Health promoting functions attributed to carotenoids (Dutta et al., 2005)

According to Dutta et.al (2005), carotenoids are isoprenoids compounds which are biosynthesized by tail to tail linkage of two C_{20} geranylgeranyl diphosphate molecules and produce the parent C_{40} carbon skeleton from which all individual variation are derived. The skeleton can be modified by three factors which are cyclization at one end or both ends of the molecule to give different end groups, changes in hydrogenation levels and the addition of oxygen containing functional groups. They are separated into two groups where the first group contains hydrocarbons such as alpha-carotene and beta-carotene, the second group consists the oxygenated carotenoids such as lutein, zeaxanthin and astaxanthin (Ginka and Dora, 2009). The structure of these carotenoids found in *Dunaliella* was shown in **Figure 2.3**. According to Kleinegris et al. (2010), carotenoids protect the cell from damage by light and oxygen. It functions as accessory pigment in light harvesting, but in addition, they are important for protecting photosynthetic organisms from destructive photooxidation which can occur in the presence of light, oxygen and chlorophylls (Campo et al.,2007; Lamers et al., 2008). In plants the presence of carotenoids is often masked by chlorophyll. In photosynthetic organisms, carotenoids exert an essential function in the photosynthetic apparatus.

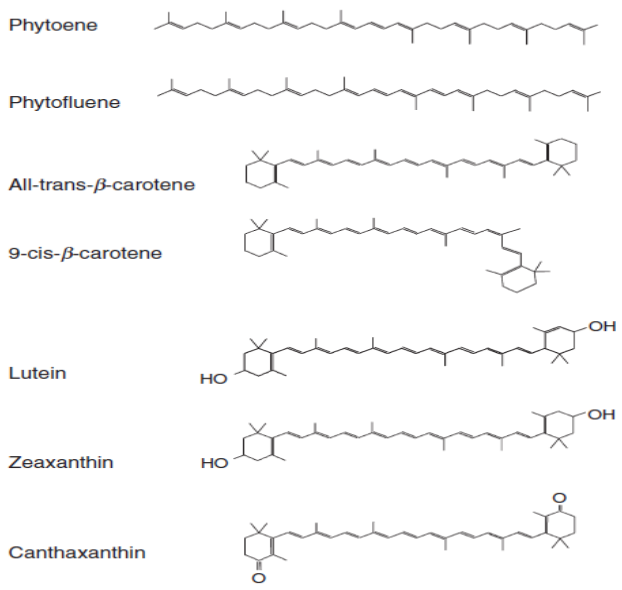

Figure 2.3: Structures of the biotechnologically most important carotenoids (Tafreshi and Shariati, 2009)

2.6 BETA-CAROTENE

As a member of carotenoids, beta-carotene is found in various structures as the configuration of each double-bond in beta-carotene can exist in trans or cis naturally (Ye et al., 2008). The all-trans, 9-cis, 13-cis and 15-cis stereo isomers of beta-carotene have been identified in natural sources (**Figure 2.4**). Beta-carotene accumulated within oil globules in the integranal thylakoid space of chloroplast and the 9-cis and all-trans isomer are in approximately equal amount, make up approximately 80% of the total (Gómez et al, 2003; Prieto et al., 2011). The beta-carotene is a terpenoid pigment that is highly valuable due to its nutritional benefit as a precursor of vitamin A for animals and for its antioxidant properties and it is also used for chemoprevention of some types of cancer (Gómez et al., 2003). Animals cannot synthesis carotenoids and therefore these pigments needed to be supplemented to them. Prieto et al. (2011) and Campo et al. (2007) stated that beta-carotene is a terpenoid pigment of increasing demand and a wide variety of market applications such as food colorants, as pro-vitamin A in food and animal feed, as an additive to cosmetics and multivitamin preparations and as a health food product under the antioxidant claim. Due to its capacity of accumulating large amounts of carotenoids in oil globules can be enhanced, *Dunaliella salina* is used worldwide as the main source of natural beta-carotene for industries (Lamers et al., 2008).

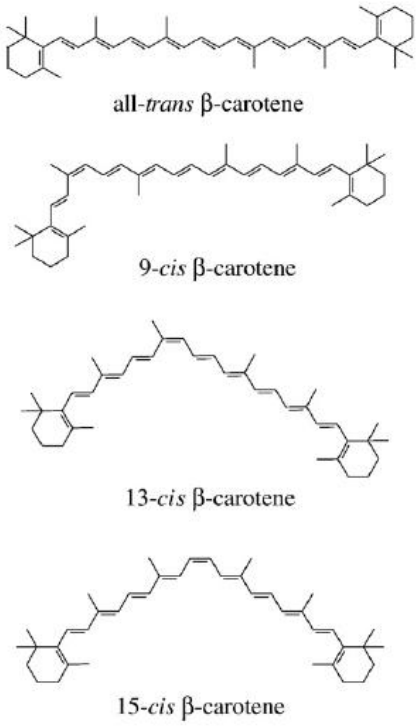

Figure 2.4: Chemical structure of beta-carotene isomer (Ye et al., 2008)

Besides *Dunaliella salina*, there are also bacteria *flavoubacterium*, fungus *B. trispora* and mutant of yeast which are *X. dendrorhous* and *R. glutinis* that have been used in the production of beta-carotene. Bacteria have the obvious advantage of faster growth rate over algae and fungi, but there are very few bacterial sources of beta-carotene available and most produce beta-carotene only as a minor product. Production of beta- carotene by *B. trispora* is dependent upon sexual mating of two compatible strains during fermentation, and the growth of *B. trispora* in fermenters becomes viscous and needs considerable energy input to keep the broth aerobic and well mixed (Bhosale and Bernstein, 2004).

2.7 EFFECT OF NITROGEN STARVATION ON GROWTH AND PRODUCTION

Several studies have shown that the high carotenoid accumulation, mainly in the form of beta-carotene in *Dunaliella salina* is enhanced by suboptimal growth conditions such as

nutrient limitation, temperature, salinity and light intensity (Marín et al., 1998). Nutrient limitation is known to change the total lipid content of many microalgae (Gordillo et al., 1998). According to Pisal and Lele (2005), nitrogen is one of the major requirements in growth media for any cell culture. The absence or depletion of nitrogen is considered as a stress by these microalgae. As stated by Tafreshi and Shariati (2007), the best nitrogen source *for Dunaliella salina* is nitrate whether it is from $NaNO_3$ or KNO_3. Low nitrate concentration negatively affected growth, but enhanced carotenoid accumulation (Marín et al., 1998; Zamani et al., 2011). In a study, *Dunaliella salina* was cultivated under nitrogen-sufficient and nitrogen-limited conditions under moderate light levels to induced carotenogenesis (Sánchez-Estudillo et al., 2006). In a paper to study this nitrogen deficiency effect, Marin et al. (1998) grew *Dunaliella salina* in normal media which contain nitrates source from $NaNO_3$ and also in media containing less concentration of $NaNO_3$. These microalgae stop to divide when the $NaNO_3$ concentration was decreased because nitrogen is the primary requirement for the cell metabolism. Beta-carotene content will increase and this increment is because of the excessive formation of free radicals under the stress condition due to nitrogen depletion. In order to protect the cell itself and to continue their growth, additional of beta-carotene is produced. The antioxidant properties of beta-carotene quench the excessive free radicals and restore the physiological balance.

Instead of NO_3^-, NH_4^+ can also be used as the sole nitrogen source of *Dunaliella salina*. These microalgae cultured on NH_4^+ rather than on NO_3^- increased not only the amount of chlorophyll per cell but also increase the cell size. However, the apparent concentration of beta-carotene per cell was also smaller (Giordano and Beardall, 2009). This property has made the $NaNO_3$ became the nitrogen source in this study.

2.8 EFFECT OF LIGHT INTENSITY ON GROWTH AND PRODUCTION

Light intensity also plays an important role in the production of beta-carotene in microalgae. According to Giordano and Beardall (2009), this factor is one of the features that make *Dunaliella* a very important organism for biotechnology by carotenogenesis. The

biosynthesis of beta-carotene appears to be dependent on the overall amount of light received by the organism over one cell division. According to Hadi et al. (2008), the microalgae were cultured in a growth chamber under different light radiation by using cool-white fluorescent tubes at certain photoperiod. The growth of cell decreased as the light intensity was increased but the beta-carotene content increased dependently on the light intensity. It showed that the carotenogenesis or beta-carotene formation can be greatly enhanced at higher irradiation. The formation and accumulation of beta-carotene protect the microalgae from damage occurred during the excessive irradiances by preventing the formation of reactive oxygen species, quenching the triplet-state chlorophyll, reacting with single oxygen and also functioning as a light filter (Hadi et al., 2008). To date, few researches have addressed the effect of different light intensities of beta-carotene content in *Dunaliella salina*. However, it remains difficult to compare the exact results of these studies due to the various light regimes that have been applied and also different carotenogenic subspecies of occasionally unknown genetic background are used such as *Dunaliella salina* and *Dunaliella bardawil* (Gómez et al., 2003 ; Lamers et al., 2008). Maximal growth rates of *Dunaliella salina* have been reported as occurring at 127 μmol m^{-2} s^{-1} (Giordano and Beardall, 2009).

CHAPTER 3

METHODOLOGY

3.1 CULTURE OF MICROALGAE

The strains of *Dunaliella salina* (Dunal) Teodoresco 1905 in this experiment were purchased from Culture Collection of Algae and Protozoa (CCAP) and cultivated in a 1000 ml Schott bottle containing 2ASW (Artificial Seawater) medium. The recipe for stock solution medium was listed in **Table 3.1** and for the complete 2ASW medium in **Table 3.2** according to the CCAP. The algal samples were grown in a growth chamber equipped with Phillips cool white fluorescent lamps for photoperiod of 12:12 (light/dark cycle).

Table 3.1: Stock solution recipe for 2ASW medium

Stock	**Component**	**Per 1000 cm^3**
(1) extra salt	$NaNO_3$	30.00 g
	$NaHPO_4$	1.20 g
	K_2HPO_4	1.00 g
(2) vitamin solution	Biotin	0.0002 g
	Calcium Pantothenate, B_{15}	0.02 g
	Cyanocabalamin, B_{12}	0.004 g
	Folic Acid	0.0004 g
	Inositol	1.0 g
	Nicotinic Acid, B_3	0.02 g
	Thiamine HCl	0.1 g
	Thymine	0.6 g
(3) soil extract*		

*See appendix A

Table 3.2: Recipe for 2ASW complete medium

	per 1000 cm^3
Extra salts stock solution (1)	3.75 cm^3
Vitamin stock solution (2)	2.50 cm^3
Soil extract (3)	25.00 cm^3
Tricine	0.50 g
NaCl	35.00 g

3.2 PREPARATION FOR DIFFERENT $NaNO_3$ CONCENTRATIONS

Four culture mediums with four different concentrations of nitrate will be prepared separately. The $NaNO_3$ concentrations used are 100, 75, 50, and 25% (w/v) of the nitrate concentration of 2ASW medium (Marin et al., 1998) which corresponding to 30g/L, 22.5g/L, 15g/L and 7.5g/L.

3.3 EFFECT OF LIGHT INTENSITY

The microalgae will be aerated and batch cultured under radiation from fluorescent lamps with different light intensity of 6000 lux (20W), 8000 lux (25W), 11000 lux (36W) and 17000 lux (55W) in the growth chamber with a photoperiod of 12 hours light and 12 hours darkness (Hadi et al., 2008).

3.4 GROWTH MEASUREMENT

In order to monitor the growth of *Dunaliella salina*, samples were taken at 7^{th} day and cell concentrations determined by direct counting, using a light microscope (magnification 10X) with a 0.1mm deep counting chamber or improved Neubauer (Pisal & Lele, 2005; Prieto et al., 2011) and reported as the number of cells ml^{-1} of the culture medium (Zamani et al., 2011). **Figure 3.1** shows the hemocytometer used to calculate the cell density. Hemocytometer consist of two chambers and eight $1mm^2$ areas (**Figure 3.2**). The cell was diluted with ultra-pure water then suspended onto the chamber and special designated cover slip would be used to cover the hemocytometer. Only cells found on the big square would be calculated. The total amount of cell density (cells/ml) could be calculated by using this formula:

$$\text{Concentration of total cells: } N_T \times \frac{1}{g} \times 10^4 \times dilution\ factor$$

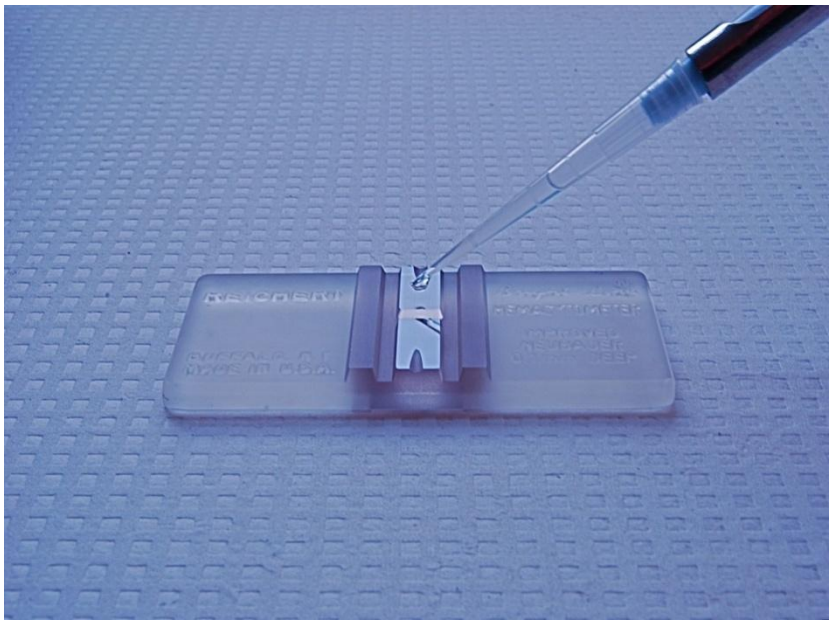

Figure 3.1: Hemocytometer

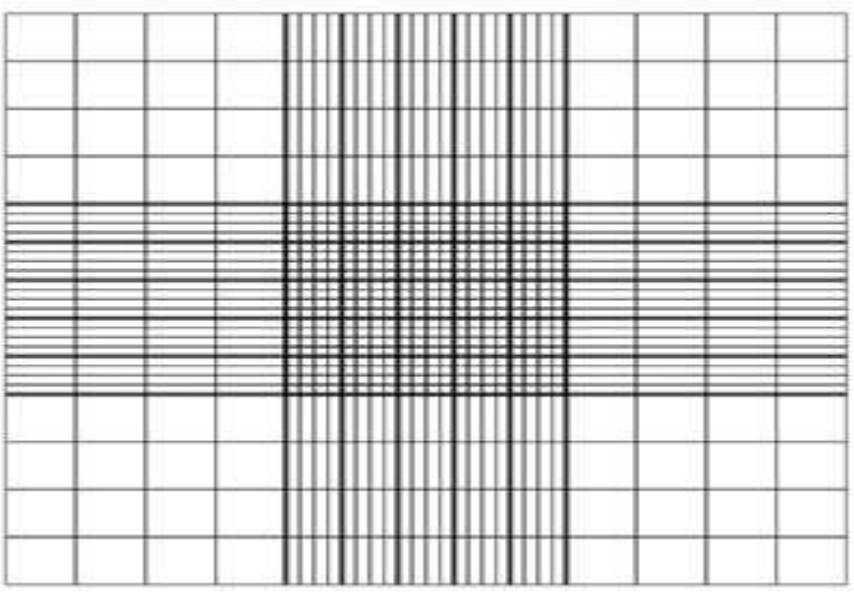

Figure 3.2: Hemocytometer counting chamber

3.5 MICROALGAE HARVESTING AND EXTRACTION OF CAROTENOID

For the determination of carotenoid concentrations, the algal cells in culture medium were harvested by centrifugation at 6000*g* for 15 min (El-Baky et al., 2004). The supernatant was removed and the cells were frozen at -20 °C. The pigments were extracted in 90% acetone for 24 hours in the dark and cool place (Borowitzka and Siva, 2007; Marin et al., 1998). The extract was centrifuged at 5000*g* for 10 min. The cells were removed and supernatant containing beta-carotene was analyzed using HPLC.

3.6 IDENTIFICATION OF BETA-CAROTENE

Concentrations of total beta-carotene in the samples were determined using the High Performances Liquid Chromatography (HPLC) (Agilent Technologies 1200 Series, USA manufactured). HPLC separation was carried out at a flow rate of 1.0 ml min^{-1} on 250 mm x 4.6 mm (i.d) packed with C18 column, 5µm particle size and eluted with 75:20:5 (v/v) methanol: acetonitrile: tetrahydrofloran. All solvents were filtered with 0.45µm Nylon membrane and degassed with ultrasonicator prior to use. The injection volume used was 10µL. The wavelength of 400 nm was utilized for the detection and the reference wavelength used was 600 nm. The temperature was at 30°C and the beta-carotene peak came out at retention time of 2.6 min. Crystalline beta-carotene with HPLC grade (≥95%) Sigma-Aldrich Malaysia (C 4582) was used as the standard with several dilutions (0.4 mg/ml to 0.02 mg/ml) in 90% acetone. Integrated areas of the peaks were converted to beta-carotene concentrations through the use of calibration curves generated from HPLC by injecting known quantities of authentic standards.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Effect of $NaNO_3$ concentration and light intensity to the growth of *Dunaliella salina*

Appendix B.1 showed the average cell density (cells/ml) cultured under different $NaNO_3$ concentrations and light intensities. The data were then plotted as shown in **Figure 4.1**. For the low light intensity which was at 6000 lx, it can be observed that the cell density decreased as the $NaNO_3$ concentration in the medium was decreased. This result explained that *Dunaliella salina* cell number ceased to divide when the available nitrate as the nitrogen source was depleted (Ben-Amotz and Avron, 1983). This showed that at low light intensity, the microalgae stop duplicate as the $NaNO_3$, which function as the major component for the cell metabolism, become scarce. The depletion of nitrogen source causes an imbalance in C to N ratio. Limitation of growth by the diminished supply of nitrogen has effects on the ability of *Dunaliella salina* to acquire carbon and allocation of carbon to cellular components. The carbon allocation in cells will affect the ratio of protein and carbohydrate by the substantial rearrangements with a large amount of carbon being inverted in protein synthesis. Change in nitrogen source from $NaNO_3$ will affect the metabolism or the management of the biological processes of the cells (Giordano et al., 2000) which may lead to lethal photodynamic reaction of the microalgae and decline its growth (Marín et al., 1998).

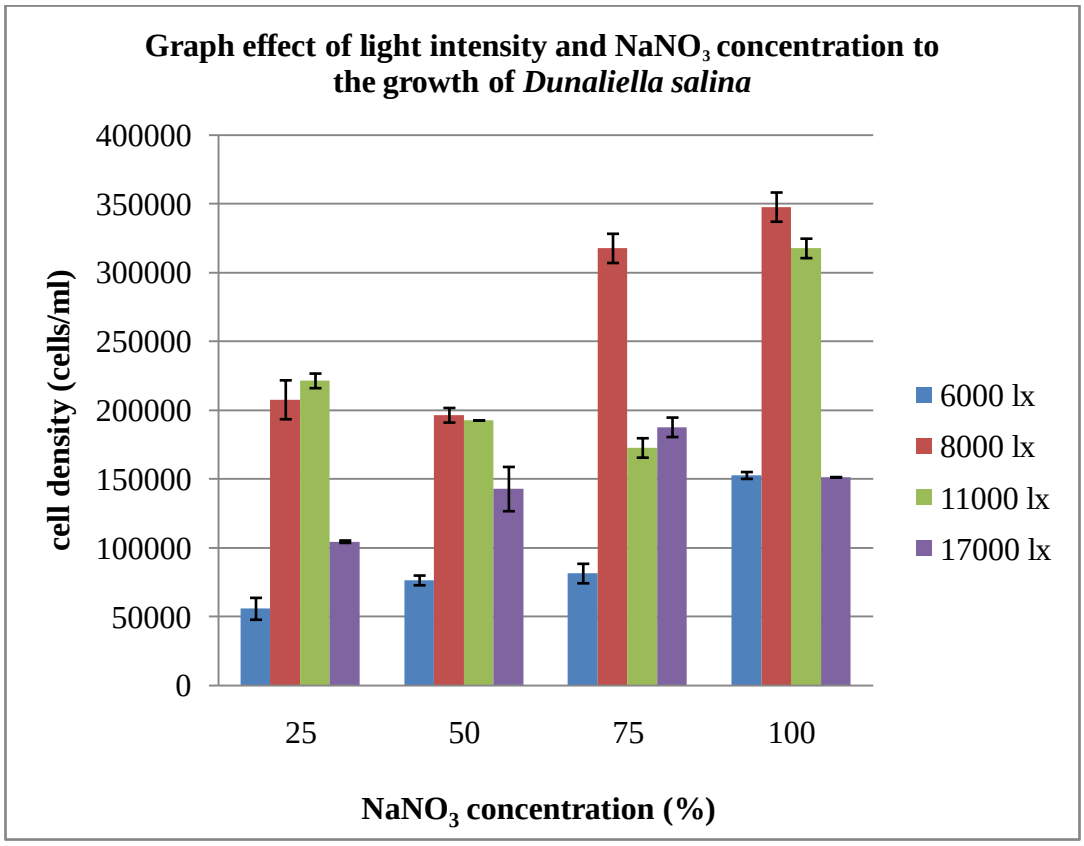

Figure 4.1: Effect of light intensity and $NaNO_3$ concentration on the growth of *Dunaliella salina*

At the light intensity of 8000 lux, even though the cell density was found to be increased compared with the lowest light intensity in this experiment, the cell density was also decreased as the $NaNO_3$ concentration in the medium was decreased. Cousel et al. (2008) stated that the higher light intensity promotes cell growth when nutrients are available. Based on the result obtained from this experiment, this followed the expected result from Cousel and co-workers which suggested that an increase in light intensity resulted in faster growth rate but once the cell division was inhibited due to lack of nutrient such as nitrogen, carotenogenesis was enhanced at high light compared to nutrient deprived cell under low light conditions and result in lower cell density.

For the light intensity of 11000 lx, the cell density reduced greatly when the concentration of $NaNO_3$ was reduced from 100% to 75%. *Dunaliella salina* grew better under

nitrogen sufficient condition compared with a nitrogen limited condition (Sánchez-Estudillo et al., 2006). The cell density seemed to be slightly increased as the NaNO3 concentration was reduced to 50% and 25%. According to a research studying the effect of light on the growth of *Dunaliella salina* (Giordano and Beardall, 2009), the maximal growth rate of this species has been reported to be at light intensity of 127 µmol m^{-2} s^{-1} which corresponding to 11000 lx. The result obtained in this experiment found that it was the same as studied by the author where the cell density was found to be highest at the given light intensity in good nutrient condition. Though, the authors also noted that there was complex interaction between light and other factors studied such as temperature and salinity in determining the growth rate of the microalgae.

For the highest light intensity which was at 17000 lx, the cell density increased as the $NaNO_3$ was decreased from 100% to 75%. But as the $NaNO_3$ concentration was further decreased, the amount of cell density also decreased. Marín et al. (1998) stated that, this is because of the prolonged nutrient starvation always leads to high mortality of the cells; hence, it will decline the population of this microalgae. According to Hadi et al. (2008), the growth of the cell decreased as the light intensity was increased. Due to the very high light intensity, *Dunaliella salina* could not tolerate with the highest light intensity used in this experiment. This light intensity would inhibit the growth of *Dunaliella salina* where the cell density was found to be decreased in amount. This high light intensity approaches level that causing light saturation of photosynthesis in *Dunaliella salina* (Cousel et al., 2008).

As the conclusion, the $NaNO_3$ concentration and light intensity will affect the growth of *Dunaliella salina* where the growth was reduced due to $NaNO_3$ starvation and it is also induced with higher light intensity until nitrogen became the limiting source for the cells.

4.2 Effect of $NaNO_3$ concentration and light intensity to the total beta-carotene production in *Dunaliella salina*

Appendix B.2 showed the data for the total beta-carotene production at different $NaNO_3$ concentration and light intensity. The data was plotted in **Figure 4.2** which shows

the effect of different light intensities and $NaNO_3$ concentrations on beta-carotene production. At the lowest light intensity which was 6000 lx, the production of beta-carotene seemed to not change markedly with limitation of $NaNO_3$ concentration. This mostly due to the fact that beta-carotene synthesis continues during early stationary phase (Ben-Amotz and Avron, 1983). According to Sánchez-Estudillo et al. (2006), the beta-carotene production should be effected by the cell growth of *Dunaliella salina*. At this light intensity the cell density was very low with the reduction of $NaNO_3$ concentration. Slower growth of these microalgae would produce low biomass production. Even though the production of beta-carotene was enhanced when the growth rate was lower, *Dunaliella salina* still needed enough cell density to produce beta-carotene in large amount (Sánchez-Estudillo et al., 2006).

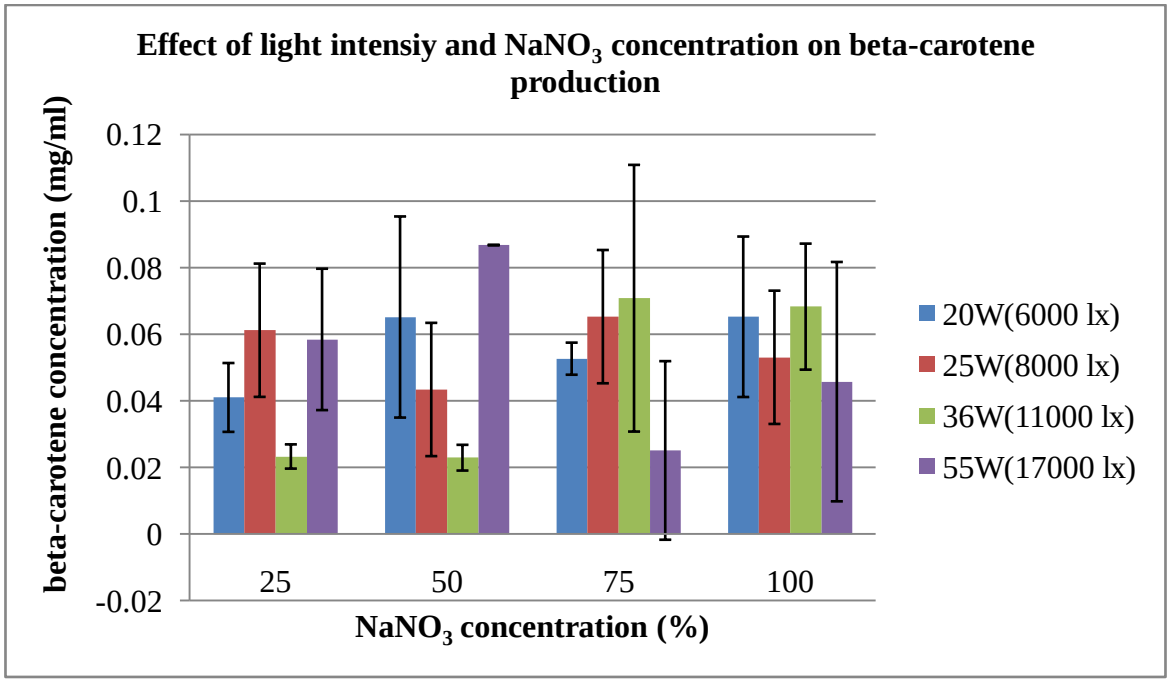

Figure 4.2: Effect of light intensity and $NaNO_3$ concentration on beta-carotene production

At the light intensity of 8000 lx, the production of beta-carotene also appearred not changing obviously with the limitation of $NaNO_3$ concentration, similar to that at light intensity of 6000 lx. According to Hejazi and Wijffels (2003), at low light intensity, no increase of beta-carotene content of the cells was observed. This due to beta-carotene content of the cells

followed different patterns due to the differences in the cell growth rate at different light exposures. Accumulation of beta-carotene in the production stage was lower than during the growth stage however the beta-carotene productivity per cell was higher in the production stage than during growth phase. It can be conclude that an increase in beta-arotene content of the cell under stress condition was not necessarily coupled with the increment in total production of beta-carotene. Zamani et al. (2011) stated that low nitrate or nitrogen source will effectively affect the growth of *Dunaliella salina* but enhanced the beta-carotene production. The concentration of beta-carotene should increase as the concentration of $NaNO_3$ was decreased. This was due to the stress condition produced when the nitrogen source from $NaNO_3$ was depleted in the medium of culture.

At the light intensity of 11000 lx, the beta-carotene production was just slightly increase as the $NaNO_3$ concentration was decreased to 75%. This occurs due to the nitrogen deficiency effect in the culture medium (Marín et al., 1998). This condition has enhanced the accumulation of beta-carotene as the secondary metabolite of the microalgae to protect themselves. Beta-carotene produced will quench the excessive free radical and restore the physiological balance of the microalgae. At lower concentrations of $NaNO_3$, the production of beta-carotene was significantly reduced. This happened because of the cell density of *Dunaliella salina* was higher at that time and it will reduce the accumulation of beta-carotene (Giordano and Beardall, 2009). It has been stated that since the production of beta-carotene by *Dunaliella salina* has to face a dilemma when its production was enhanced by its physiological stress, its production was highest when the growth was lowest (Sánchez-Estudillo et al., 2006).

At light intensity of 17000 lx, beta-carotene production was slightly decreased as the $NaNO_3$ concentration was decreased to 75% in the medium. This happened because of the cell density of *Dunaliella salina* was slightly higher at that time and it will reduce the accumulation of beta-carotene (Giordano and Beardall, 2009). As the $NaNO_3$ concentrations were further decrease to 50% and 25%, the beta-carotene production seemed to inversely increase. This was due to the stress resulting from the very high light intensity may have interfered with the effect of nutrient starvation in the medium (Marín et al., 1998). So, the

further stress condition facing by *Dunaliella salina*, the higher the accumulation of beta-carotene produced.

As the conclusion, the $NaNO_3$ concentration and light intensity will affect the beta-carotene production of *Dunaliella salina* where the production was increased as the $NaNO_3$ concentration was decreased and the light intensity was increased until the concentration of $NaNO_3$ has become the limiting factor of the experiment that will affect the growth of *Dunaliella salina* and beta-carotene production.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

$NaNO_3$ concentration and light intensity will affect the growth of *Dunaliella salina*, where the growth was reduced due to $NaNO_3$ starvation and it is also induced with the high light intensity until nitrogen became the limiting source for the cells. Besides that, the $NaNO_3$ concentration and light intensity will also affect the beta-carotene production of *Dunaliella salina,* where the production was increased as the $NaNO_3$ concentration was decreased and the light intensity was increased until the concentration of $NaNO_3$ has become the limiting factor of the experiment that will affect both the growth of *Dunaliella salina* and beta-carotene production.

5.2 RECOMMENDATIONS

There are several recommendations in order to improve the research. Firstly, further study need to be done to identify the optimal $NaNO_3$ concentration and light intensity required for maximizing the production of beta-carotene by *Dunaliella salina*. Secondly, a large volume of microalgae culture needed to be prepared before running the experiment in order to keep the experiment in good progress.

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APPENDIX A

culturecollection
of algae and protozoa

ecipes

SE1 (Soil Extract 1)

used in media for marine algae

Preparing the soil

Site selection for a good soil is very important and for most purposes a soil from undisturbed deciduous woodland is best. Sites to avoid are those showing obvious signs of man's activity and particular care should be taken to avoid areas where fertilizers, crop sprays or other toxic chemicals may have been used.

A rich loam with good crumb structure should be sought. Stones, roots and larger invertebrates should be removed during an initial sieving through a 1 cm mesh. The sieved soil should be spread to air dry and hand picked for smaller invertebrates and roots. It should be turned periodically and picked over again. When dry it may be sieved through a finer mesh (2-4 mm) or stored as it is prior to use.

Medium

Soil is prepared as above. Air-dried soil and twice its volume of supernatant distilled water are autoclaved together at 15 psi for 2 hours and left to cool. The supernatant is then decanted and filtered through Whatman No 1 filter paper, then distributed to containers in volumes suitable for making up batches of media. The aliquots and their containers are autoclaved for an appropriate length of time (e.g. 1 litre or less for 15 minutes) and are then kept in a cool place (e.g. a refrigerator) until required.

APPENDIX B

Table B.1: Average cell density (cells/ml) of different $NaNO_3$ concentrations and light intensities

$NaNO_3$ concentration (w/v) / Light intensity (lx)	25%	50%	75%	100%
6000	55625	76250	81250	15200
8000	288750	196250	357500	280000
11000	192500	192500	172500	317500
17000	104375	142625	187500	151250

Table B.2: Beta-carotene (mg/ml) production at different $NaNO_3$ concentration and light intensity

$NaNO_3$ concentration (w/v) / Light intensity (lx)	25%	50%	75%	100%
6000	0.04102	0.06516	0.05265	0.06523
8000	0.06119	0.04340	0.06527	0.053064
11000	0.02329	0.02294	0.0708	0.06827
17000	0.05844	0.08677	0.02512	0.04575