OPTIMIZATION OF PHOTOBIOREACTOR FOR ASTAXANTHIN PRODUCTION IN *CHLORELLA ZOFINGIENSIS*

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Abstract

Microalgae have been known to produce commercially valuable carotenoids beneficial to human health. One of the algal carotenoids that have received a lot of research attention is astaxanthin. Also known as "king of antioxidant", astaxanthin is 100 times more than the antioxidant capacity of that of vitamin E, and 10 times more than that of beta-carotene. *Chlorella Zofingiensis,* a strain of green microalgae, has been shown in recent years to be able to accumulate astaxanthin when exposed to environmental stress. This study investigated the astaxanthin production capacity of *Chlorella Zofingiensis* under nitrate, light and temperature stress under a semi-continuous turbidostatic flat-bed photobioreactor.

A total of 15 configurations consisting of different nitrate concentration and light intensities were tested in a batch system in phase 1 of the experiment. Growth rates and astaxanthin contents were monitored. Highest dry mass of 7.55 g/L was obtained at 0.5 g/L of nitrate and at light intensity of 300 umol photon.$m^{-2}s^{-1}$. It was found that low nitrate level, coupled with high light intensity, was the key to high cellular accumulation in *C. Zofingiensis*. Peak volumetric astaxanthin production was at 7.06 mg/L. Using the optimum nitrate/light intensity pair, it was further tested in a flat-bed photobioreactor in semi-continuous mode. Cellular astaxanthin level was at 0.69 mg/g, almost 50% lower than the batch system, but the growth of *C. Zofingiensis* biomass was more. Volumetric astaxanthin production of semi-continuous system was comparable to batch system.

Daily astaxanthin production for continuous system was 21% higher than that of the batch system.

Overall, the reported data suggests that *C. Zofingiensis* is an attractive candidate for the mass production of astaxanthin in continuous reactor, being plausible for selectively favoring the production of astaxanthin through the adequate management of growth conditions.

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Nomenclature

C_a	Chlorophyll a concentration	[mgL^{-1}]
C_b	Chlorophyll b concentration	[mgL^{-1}]
C_x	biomass concentration	[$g\ L^{-1}$]
g	gravitational acceleration	[ms^{-2}]
μ	specific growth rate	[h^{-1}]
μ_{max}	maximum specific growth rate	[h^{-1}]
ROS	Reactive Oxygen Species	
T	absolute temperature	[K]
t	time	[h]
λ	wavelength	[nm]
PFD	photon flux density in PAR range	[$\mu mol\ photons\ m^{-2}\ s^{-1}$]
PAR	photosynthetic active radiation, 400-700 nm	

Abbreviations

Asta	Astaxanthin
Chl	Chlorophyll
Carot	Carotenoids
FPR	Flat-plate reactor

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Chapter 1 Introduction

Microalgae and cyanobacteria, also known as blue-green algae, are found widely in our bio-sphere. They contribute approximately 40-50% of the oxygen in the atmosphere and they are the original source of fossil fuel. (M. Borowitzka 1997)They are also at the bottom of the food chain, directly and indirectly linked to our food security.

1.1 Photosynthesis

Microalgae and cyanobacteria are oxygenic photoautotrophic microorganism. They are able to use sunlight to metabolize carbon dioxide (CO_2) inside CH_2O under the liberation of oxygen (O_2). CH_2O are the building blocks for algal growth. The universal equation of photosynthesis is presented below:

Equation 1 - 1 $CO_2 + H_2O + photon \rightarrow CH_2O + O_2$

Essentially, microalgae convert light energy into chemical energy via the formation of chemical bonds. The basic unit of photosynthetic apparatus is the photosystem (PS). Photons are absorbed by carotenoids and chlorophyll pigments of the photosystem antenna complex. In Figure 1 - 1, the operation of PS is shown. The excitation energy is funneled through the pigment bed towards the reaction centre (P680), which is brought to

a higher energy level (P680*). Almost 95-99% of the excitations can be transferred to the reaction center. The transfer of energy is highly efficient because the excitations "fall" inside an "energy hold" with the reaction centre at the bottom. During the transport, the excitations lose some energy and this is the reason why reverse transport is not possible. Inside the reaction centre, the remaining excitation energy activates the reaction centre (P680 → P680*) by promoting an electron from the highest-energy filled orbital to the lowest-energy unfilled orbital. The electron is quickly transferred to an acceptor generating an oxidant and reductant, respectively, and this process is called charged separation (Richmond, 2004).

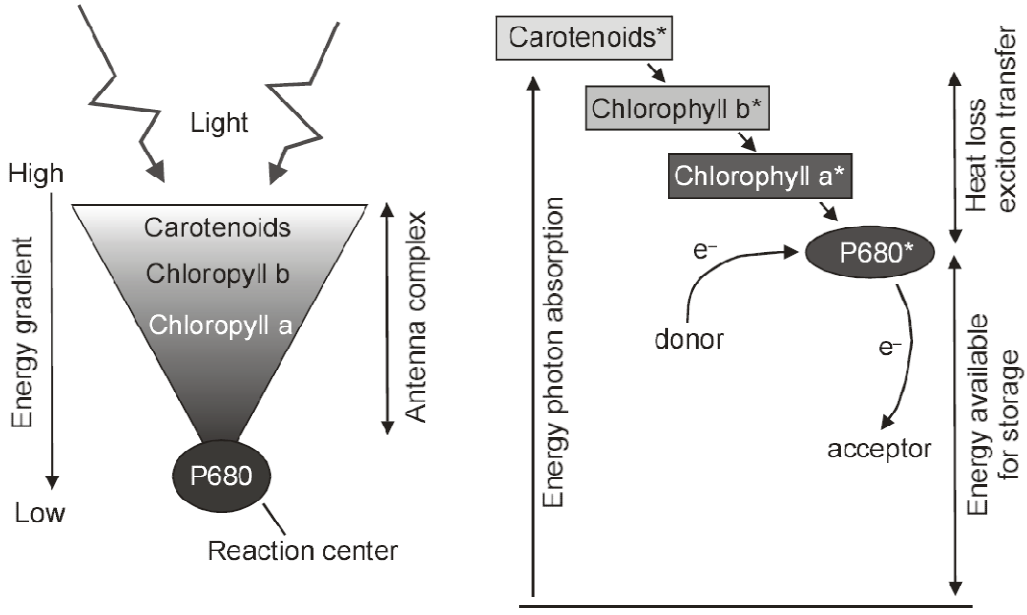

Figure 1 - 1 Schematic diagram of photosystem (Lawlor 2001)

1.2 Algal Pigmentations

Algae pigments are chemical compounds which reflect only certain wavelengths of visible light. This makes them appear "colorful". Flowers, corals, and even animal skin contain pigments, which give them their colors. More important than their reflection of light is the ability of pigments to absorb certain wavelengths.

All microalgae contain three major classes of photosynthetic pigments: chlorophylls, carotenoids (carotenes and xanthophylls) and phycobilins. The different division of microalgae is characterized by a specific pigment composition. A considerable diversity exists among the carotanoid and chlorophyll pigments. Chlorophylls and carotenes are generally fat-soluble molecules and can be extracted from thylakoid membranes with organic solvents such as acetone and methanol. The phycobilins and peridinin, in contrast, are water-soluble and can be extracted from algal tissues after the organic solvent extraction of chlorophyll in those tissues.

In

Figure 1 - 2, the characteristic absorption spectra of a Chlorohyta (green alga), Chrysophyta (diatoms) and a cyanophyta (cyanobacteria) are shown. The absorption peaks between 650–700 nm which is the red region, are caused by chlorophyll absorption. Carotenoids absorb most strongly in the 400-500 nm and transfer the excitation energy to

the chlorophylls, making photosynthesis efficient over a wider range of wavelengths, In addition to chlorophylls and carotenoids, cyanobacteria have pigments called phycobilisomes, which enables them to absorb 600-650 nm more strongly than other strains of microalgae.

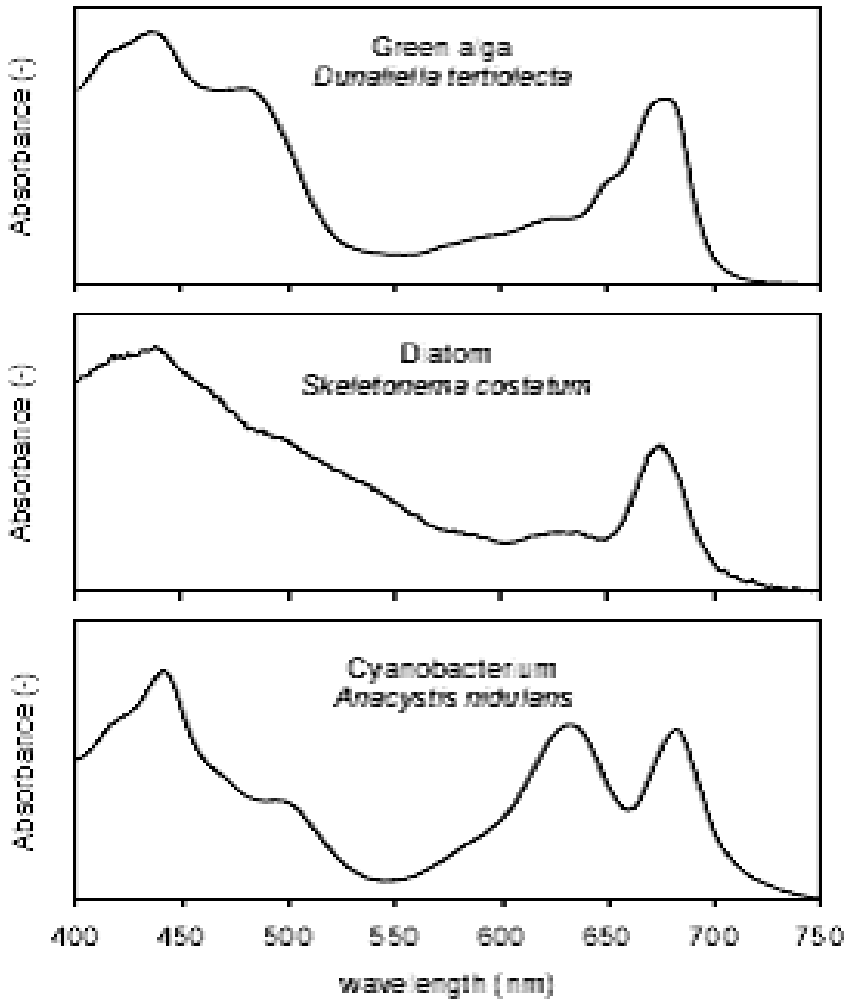

Figure 1 - 2 Characteristics absorption spectra of microalgae: a green alga - Dunaliella tiertiolecta; a diatom. (Kromkamp and Limbeek 1993) Skeletonema costatum and a cyanobacterium - Anacystis nidulans (Aubroit 1991)

1.3 Functions of Carotenoids

Carotenoids cannot transfer sunlight energy directly to the photosynthetic pathway, but must pass their absorbed energy to chlorophyll. For this reason, they are called accessory pigments. In addition to light harvesting, carotenoids have other functions in the cell. They protect the PS under unfavorable environmental conditions, such as high light intensity and high salinity. In the case of high light intensity, an overdose of excitation energy can lead to the production of toxic species (i.e. reactive oxygen species (ROS)) and damage of the PS. Carotenoids are able to scavenge these ROS. An overdose of excitation energy can be dissipated as heat by Carotenoids in the antenna complex, which in turn prevents the formation of ROS (Britton 1995, Miki 1991).

Scientists have paid special attention to carotenoids found in higher plants and algae, as well as other photosynthetic organisms such as animals, fungi and plants. Carotenoids are responsible for the red, orange and yellow color of plant leaves, fruits, flowers, fish flesh and crustacean shells. These Carotenoids (e.g. astaxanthin) are accumulated and exploited by commercial algal farming. These compounds with antioxidant ability are highly valued in the market, and it has been proven that adequate intake of carotenoids is able to prevent degenerative diseases. More details can be found in chapter 2. Beta-carotenoids, xanthophylls, astaxanthin, cantaxanthin, and lutein are the major carotenoids with commercial interest (Richmond, 1986).

Figure 1 - 3 The colorful world of carotenoids, from fruits to seafood. Daily intakes of carotenoids are known to reduce critical illnesses such as heart, liver and kidney diseases.

The carotenoids of interest, astaxanthin, are known to be the most powerful antioxidant available nowadays. Astaxanthin sells for approximately US$2,500 kg^{-1} with an annual worldwide aquaculture market estimated at US$200 million (Cysewski 2004). Projections for 2015 of global astaxanthin market rise to US$257 million (BCC Research 2008).

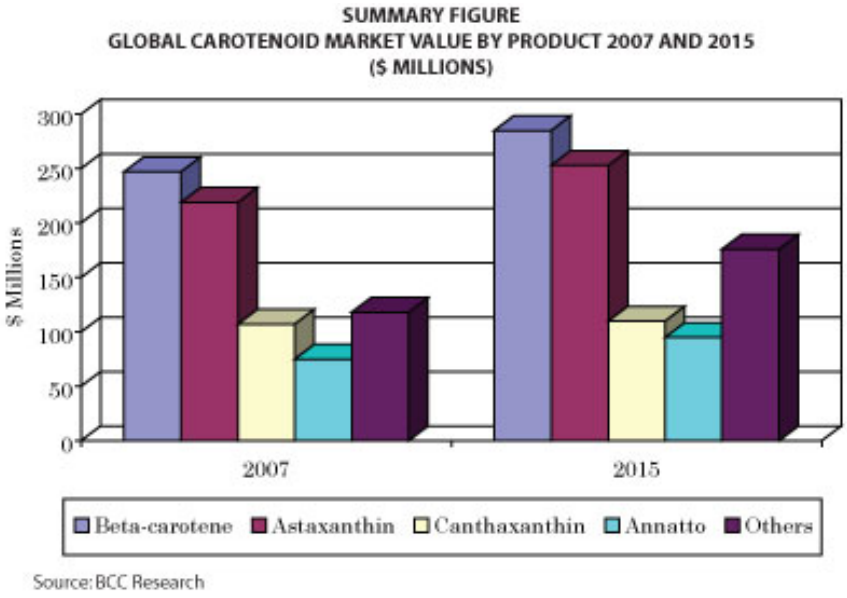

Figure 1 - 4 Global carotenoids market value by product in 2007 and 2015 (BCC Research 2008)

Most of the astaxanthin available in the market is synthetically-derived. However, consumer's demand for natural products provides an excellent opportunity for the natural carotenoids, and *Haematococcus pluvialis* represents the richest biological source of this pigment (Lorenz and Cysewski, 2003). It is now cultivated at large scale by several companies, and being used as commercial feed for salmon and rainbow trout to enhance their commercial value (Torrissen 1986). The Chlorophyte alga *Haematococcus pluvialis* is believed to accumulate the highest levels of astaxanthin in nature. Commercially grown *H. pluvialis* can accumulate 0.30 g of astaxanthin per kg of dry biomass (Burick 1991, Aflalo, et al. 2007).

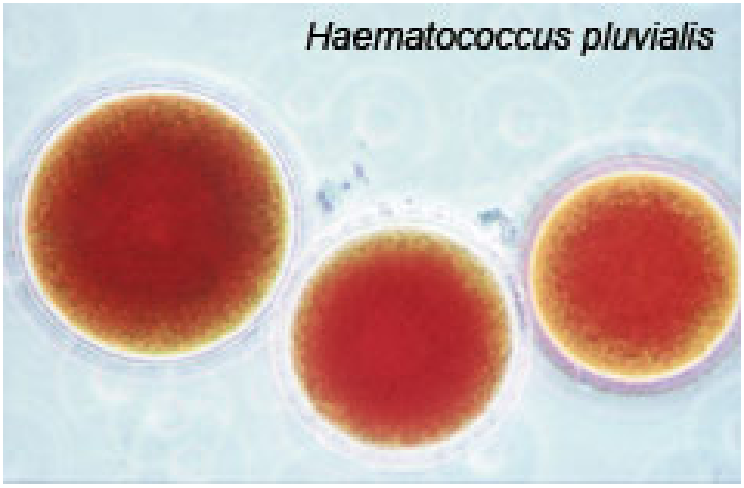

Figure 1 - 5 H. Plauvialis cyst (Bar, et al. 1995)

Figure 1 - 6 Commercial H. Plauvialis pond (Ausich 1997)

1.4 Commercial Exploration of Algae

In the early 1950's, the increase in the world's population and predictions of an insufficient protein supply led to a search for alternative and unconventional protein sources. Valuable biologically active substances from the algae stood out as a good candidate for this purpose.

Commercial large-scale culture of microalgae started in the 1960's in Japan with the culture of Chlorella by Nihon Chlorella. It was followed by the establishment of an Arthrospira harvesting and culturing facility in Lake Texcoco, Mexico. The first aquaculture field also appeared in the 1970's. By 1980, there were 46 large-scale factories in Asia producing more than 1000 kg of microalgae (mainly Chlorella) per month. The commercial production of Dunaliella salina, as a source of β-carotene, became the third major microalgal industry when production facilities were established by Western Biotechnology (Hutt Lagoon, Australia) and Betatene (Whyalla, Australia) (now Cognis Nutrition and Health) in 1986 (Lee 1997). These were soon followed by other commercial plants in Israel and the USA. The same as that of these algae, the large-scale production of cyanobacteria (blue-green algae) began in India at about the same time (Ausich 1997).

The Aquatic Species program conducted by United States National Renewable Energy Laboratory, Department of Energy (DOE) has the purpose of identifying potential algae species for the production of biodiesel at large scale. This program was initiated because the price of energy, specifically crude oil, was traded at historical high price and was threatening the livelihood of the average citizen. To strengthen energy security, DOE had looked into various energy production methods, and one of the most promising field was algal biotechnology. Over 200 laboratories over U.S. were involved in this project and

the speed of development was unprecedented. However, the program eventually failed as oil price plunged to historical low in 1995.

1.5 Valuable Products from Microalgae

Algae are major natural source for a vast array of high value compounds. Its applications include health food, aquaculture, fuel, cosmetics, medicine, etc. Although microalgae are a unique source for high-value compounds, their commercial application are still limited (Borowitzka, 1999). Table 1 - 1 contains a summary of the products and applications of algae.

	Product	**Application**
Biomass	Biomass Methane	Health food Functional food Feed addictive Aquaculture Soil conditioner Fuel
Coloring substances and antioxidants	Xantophylls (astaxanthin and canthaxanthin) Lutein B-carotene Vitamins C and E	Food and feed additives Cosmetic
Fatty Acids -FA	Arachidonic acid –AA Eicosapentaenoic acid- EPA Docosahexaenoic acid- EHA y-linolenic acid –GCA Linoleic acid – LA	Food additives
Enzymes	Superoxide dismutase- SOD Phosphoglycerate and Luciferin	Health food Research

	Restriction enzymes	Medicine
Polymers	Polysaccharides Starch Poly-B-hydroxybutyrics acid - PHB	Food additive Cosmetics Medicine
Special products	Peptides Toxins Isotopes Aminoacides (proline, arginine, aspartic acid) Sterols	Research Medicine

Table 1 - 1 Valuable products from microalgae (Cysewski 2004, Singh, Kate and Banerjee 2005)

Figure 1 - 7 Algal supplements, in the form of extracted pigments and dry powered form

1.6 Challenges Faced by the Algae Industries

1.6.1 Limited strains available on large scale farm

So far, the best choice with the lowest cost seems to be the open shallow pond. Open ponds are the oldest and simplest systems for mass cultivation of microalgae. In this system, the shallow pond is usually about 1 foot deep; algae are cultured under conditions similar to the natural environment. The pond is usually designed in a "raceway" or "track" configuration, in which a paddlewheel provides circulation and mixing of the algal cells and nutrients (Figure 2-2). The raceways are typically made from poured concrete, or they are simply dug into the earth and lined with a plastic liner to prevent the ground from soaking up the liquid. Baffles in the channel guide the flow around bends, so as to minimize space and loss.

Medium is added in front of the paddlewheel, and algal broth is harvested behind the paddlewheel, after it has circulated through the loop. Although an open pond culture system cost less to build and operate than enclosed photobioreactors, it has its intrinsic disadvantages. Since these ponds are open air systems, they often experience a lot of water loss due to evaporation. Thus, open ponds do not allow microalgae to use carbon dioxide efficiently, and biomass production is limited (Chisti, 2007). Biomass productivity is also limited by contamination with unwanted algal species as well as organisms that feed on algae. In addition, optimal culture conditions are difficult to

maintain in open ponds and recovering the biomass from such a dilute cell yield is expensive (Molina, Fernández and García, et al., 1999).Yet, only few strains are able to grow in adverse outdoor conditions and can out-grow other microorganisms.

1.6.2 Prohibitive cost

Microalgae are expensive to produce, although many efforts are under way addressed to achieve cost-efficient modes for mass cultivation of these organisms. Different systems have been designed for the growth and handling of microalgae on a large scale (Borowitzka 1999; Gudin and Chaumont 1980; Molina-Grima et al. 1999; Pulz 2001; Richmond 2004; Tredici 2004; Weissman et al. 1988). The more recently developed and technologically advanced closed systems, called photobioreactors, provide better options to grow virtually every microalgal strain, while protecting the culture from invasion of contaminating organisms and allowing exhaustive control of operation conditions. These photobioreactors are either flat or tubular and can adopt a variety of designs and operation modes. They offer higher productivity and better quality of the generated biomass (or product), although they are certainly more expensive to build and operate than the open systems.

1.6.3 Lack of industrial scale experiments

Most of the works done on algae are mostly lab scale or pilot scale testing. There is insufficient knowledge to adequately judge the economic viability. Scaling up of lab-

scale reactors often bring unforeseen operational problems and thus, brings uncertainty to the project. Productivity data are often extrapolated from small experiments, and not always presented clearly and consistently. Therefore, algal species that looked very promising when tested in the laboratory are not robust under conditions encountered in the real world. The risk involved leads to lesser investment into the field and this is the main reason why the number of algae on mass production remains little even after 50 years of algae exploration.

1.6.4 More research required on new and improved algal strains

Currently, only few strains are being used in microalgal biotechnology. The ideal strain should be amenable to fast growth outdoors at high cell densities, responding efficiently to strong light, and producing cells with a high content of desired products (Richmond, 2004). Research program focused on the evaluation of alternative microalgal strains with regard to their carotenoid profile and biotechnological potential is needed (Del Campo et al. 2000). The screening approach to the selection of producer strains of a specific carotenoid or adequate combination of several of them should be further pursued. Screening criteria must include species dominance, harvesting ease, and growth requirements in terms of temperature, water quality, pH, CO_2, tolerance to oxygen and light (Weissman et al. 1988).

1.7 Project Objectives and Scopes

As such, *C. Zofingiensis* was chosen as it accumulates both astaxanthin and lutein (Del Campo, et al. 2004). Despite being first discovered in 1970's, little research has been done on this strain. There are still a number of issues that have to be resolved through research and development before this strain can become an alternative source of astaxanthin on a commercial scale. As highlighted in previous sections, there is a dire need to introduce new strains with lower cost of production. The aim of this study was to investigate the performance and the feasibility of cultivating *C. Zofingiensis* under continuous culture with an air-lifting flat-bed photobioreactor.

The scopes and objectives of the project are as follows:

a) Determine the optimum nitrate/light intensity combination for maximization of astaxanthin accumulation

Fifteen different configurations of varying nitrate concentrations and light intensities were used in phase 1 of the experiment. Optimum growth and astaxanthin accumulation would be used for phase 2.

b) Study the change in pigment composition with time under both batch and continuous mode of operation

Chlorophyll a and b, total carotenoids and astaxanthin were monitored during the course of growth using spectrophotometry.

c) Compare photobioreactor performance under different temperature

This is phase 2 of the experiment. Three temperature settings were used and growth rate and pigment compositions were monitored

Chapter 2 Literature Review

2.1 Introduction:

Chlorella Zofingiensis was first discovered in the 1970's. However, it has not been studied extensively until recently, when Del Campo (Del Campo, et al. 2004) discovered that it can accumulate significant quantities of valuable substances such as carotenoids, astaxanthin and lutein.

2.2 Algae of interest: *C. Zofingiensis*

C. Zofingiensis belongs to the green algae group, Chlorophyceae. This group of green algae is abundant especially in freshwater. They can occur as single cells or as colonies. There are approximately 350 genera and 2650 living species of chlorophyceans. They come in a wide variety of shapes and forms, including free-swimming unicellular species, colonies, non-flagellate unicells, filaments, and more (Becker 1994). The main storage compound for green algae is starch, though oils can be produced under certain conditions. Some of the Chlorophyceae that have been researched extensively in recent years include *Botryococcus braunii* (found to produce the highest percentage of algal lipid), *Chlorococcum* (found to accumulate carotenoid up to 40% of cell dry weight (González 2007), *Nannochloris sp.* (has been employed in the aquaculture industries for their high

protein and nutrimental value since 1900), *Chlorella Vulgaris* (most studied and researched species of our time, contains the highest known source of chlorophyll content and known to reduce risk of cancer) (Apt and Behrens 1999).

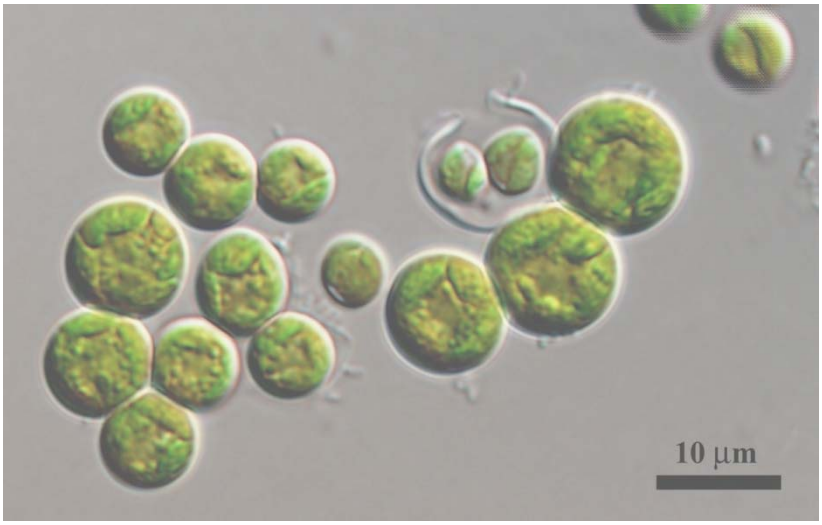

Figure 2 - 1 Microscopic image of *C. Zofingiensis*, showing size from 4-10 µm

2.3 Algal Fundamentals

Microalgae cells are a type of eukaryotic cell. They contain internal organelles such as chloroplasts, a nucleus, etc. The composition of the biomass is important in characterization of the microalgae species according to its function and product. Algal biomass contains three main components: carbohydrates, protein and lipids/natural oil. It also produces rare and useful substances such as antibiotics, carotenoids, steroids, etc. Not only do they have the capacity to produce high value compounds, they also have the ability to do it using only sunlight, carbon dioxide and seawater. For this reason

microalgae are called photoautotrophic microorganisms, i.e. they need light as their main supply of energy and they use CO_2 as carbon source for growth.

Photosynthesis, the most important process in algal metabolism, is a process that converts carbon dioxide into organic sugar, using the energy from the light. The overall equation of this process is stated below.

Equation 2 - 1 $6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2$

Light is first absorbed by the antenna pigments of photosystem (PS) II and I. The absorbed energy is transferred to the reaction center chlorophylls, P_{680} in photosystem II, P_{700} in photosystem I. Absorption of 1 photon of light by Photosystem II removes 1 electron from P_{680}. With its resulting positive charge, P_{680} is sufficiently electronegative that it can remove 1 electron from a molecule of water. When these steps have occurred 4 times, requiring 2 molecules of water, 1 molecule of oxygen and 4 protons (H^+) are released The electrons are transferred (by way of plastoquinone — PQ in the figure) to the cytochrome b_6/f complex where they provide the energy for chemiosmosis. Activation of P_{700} in photosystem I enables it to pick up electrons from the cytochrome b_6/f complex (by way of plastocyanin — PC in the Figure 2-2) and raise them to a sufficiently high redox potential that, after passing through ferredoxin (Fd in the Figure 2-2), they can reduce $NADP^+$ to NADPH (Lawlor 2001).

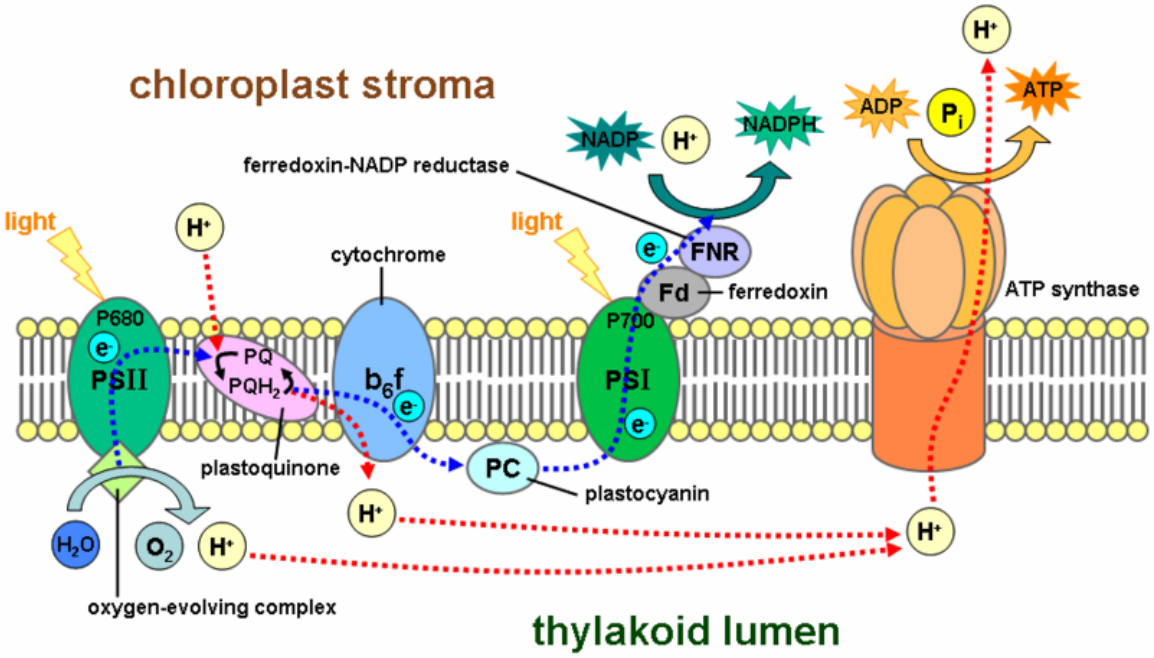

Figure 2 - 2 Schematic diagram of photosystem (Lawlor 2001)

2.4 Mechanism of Astaxanthin accumulation

The exact mechanism for astaxanthin accumulation in *C. Zofingiensis* is still non-conclusive. It is postulated that one of the mechanisms of astaxanthin accumulation is similar to commercial strain *H. pluvialis*. The process is summarized in Figure 2-3. Due to the involvement of ROS astaxanthin synthesis proceeds via cantaxanthin, the exceptional stress response is mediated by ROS through a mechanism which is not yet understood (S. Boussiba, 2000). He has suggested that astaxanthin is the by-product of a defense mechanism rather than the defending substance itself, although at this stage one cannot rule out other protective mechanisms. Further work is required for complete understanding of this transformation process. The biosynthetic pathways of astaxanthin will not be reviewed in details here as this dissertation focuses on the technical and

operational aspects of *C. Zofingiensis* cultivation for the purpose of astaxanthin production.

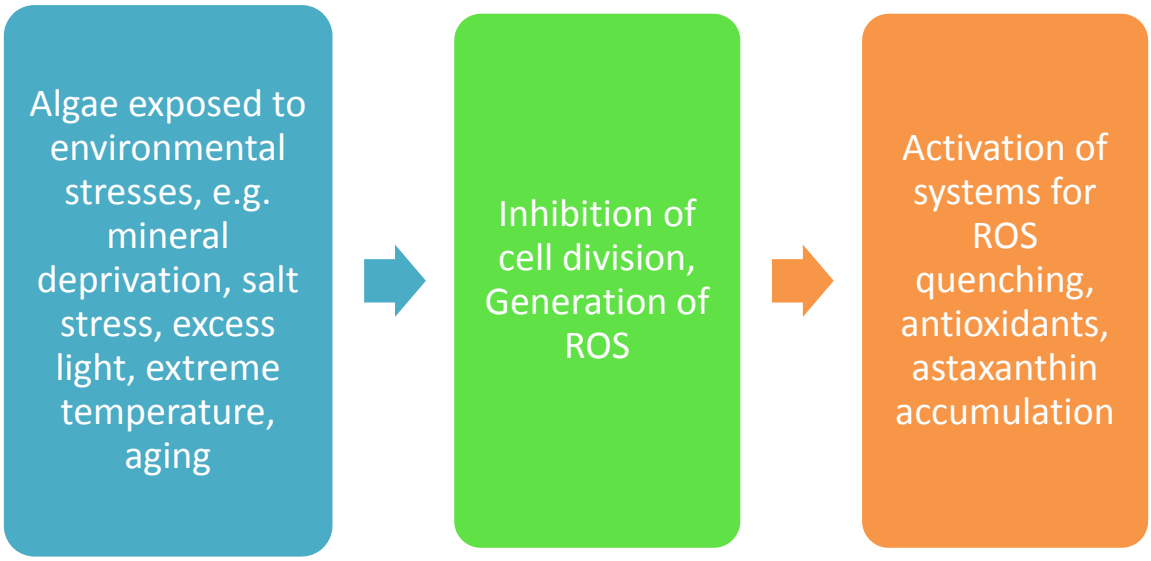

Figure 2 - 3 Suggested mechanism for astaxanthin accumulation (Boussiba and Vonshak 1991)

2.5 Factors Affecting Growth of Algae and Astaxanthin Accumulation

The standard growing conditions of *C. Zofingiensis* is similar to cultivation of other species of Chlorophyceae. Under standard batch condition, this algae has been shown to exhibit high values of both growth rate (about 0.04 h^{-1}) and standing cell population (over 7 g dry weight l^{-1}) under photoautotrophic conditions (Del Campo, et al. 2004).

The observation of astaxanthin accumulation has been investigated by Del Campo et Al (2004) and Ip and chen 2005 , and it has been reported that *C. Zofingiensis* accumulates a significant amount of valuable carotenoid, namely astaxanthin and lutein when grown photoautotrophically under stressed conditions. Secondary carotenoids, lutein and astaxanthin are produced as a defense mechanism against environmental injuries. *C. Zofingiensis* has only grown in batch system thus far, with standing cell population over 12.5 g/L dry weight, 3.27 mg/g of astaxanthin using acetate in feed (Del Campo et al. 2004). On another experiment, heterothrophic growth in the dark with glucose yields 23 g/l dry weight, 7mg/g of astaxanthin, highest recorded thus far (Chen and Chen, 2004). However, results obtained are still far from *Haematococcus pluvialis*, which has the highest cellular astaxanthin yield among all microalgae, at 8.6 mg/g, or over 16 g/L dry weight (S. Boussiba 2000).

There are many factors affecting the growth rate and astaxanthin accumulation in microalgae. Even though conditions for algae culture are carried out according to journal publications, it is important to determine the conditions for optimal growth as it has been reported that growth rate for the same species of algae culture can differ at different locations (Andersen 2005). The following section discusses the different factors, the inter-relation of these factors, and their effects on microalgae in general.

2.5.1 Temperature

Temperature is one of the most important environmental factor affecting the growth and development of living organisms. Photosynthetic systems always generate heat because of the inefficiency of photosynthesis in converting light energy into chemical energy (Bhosale 2004). The theoretical conversion of red light into chemical energy is 31%, with 69% is lost as heat. The amount of cooling depends on the incident light intensity and the cell concentration (i.e. how much light is absorbed), but regardless, cooling will be necessary especially for enclosed systems. In principle, temperature control is done using commercially available temperature controllers. Cooling is achieved with a heat exchange system. In the case of open system, heat is dissipated almost instantaneously to the surrounding (Andersen 2005).

In general, it is possible to describe the maximum growth rate solely as a function of temperature by applying the Arrhenius equation, given constant illumination (Goldman and Carpenter 1974).

Equation 2 - 2 $\hat{\mu} = Ae^{-E/RT}$

where A = constant, day^{-1}; E=activation energy, cal mol^{-1}; and T = temperature, Kelvin, ˚K.

According to van't IIoff rule, biological reactions should approximately double for each 10˚C rise in temperature. Restrictions to its general use are quickly apparent. Firstly, for each algal species, the Arrhenius relationship is applicable only in a definitely range of temperature. Secondly, there is evidence of a strong interaction between light intensity and temperature; for example, Sorokin has found that for a given temperature the activation energy decreases with increasing light energy (Andersen 2005). All microalgae follow a similar pattern of growths, as shown in Figure 2 - 4. At a fixed temperature, growth rate increases as light intensity increases. It starts to decrease when the maximum growth rate is reached.

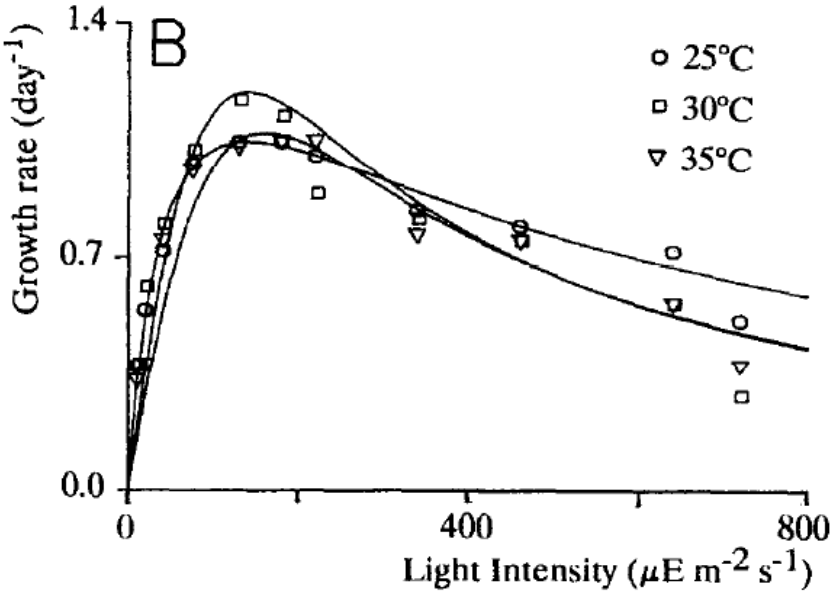

Figure 2 - 4 *Chlorella vulgaris*: observed growth rate versus irradiance level for 25, 30, 35˚C (Dauta, et al. 1990)

Changes in temperature also bring about changes in many biosynthetic pathways, including carotenoid biosynthesis. It is reported that temperature could control the

concentration of enzymes involved in carotenoid production, and changes in enzyme concentration ultimately control carotenoid levels in microorganisms (Hayman et al. 1974). Changes in the characteristics of the cells were observed with an increase in the growth temperatures, leading to changes in absorption efficiency associated with a variation in cell size and pigment levels. At higher growth temperature (33°C), the cellular accumulation of lutein and astaxanthin in *Muriellopsis sp.* was raised by six-fold, but the volumetric level was higher at 28°C (Del Campo et al. 1999). At higher growth temperatures, cell division is impaired but not protein synthesis. Thus the relative cell volume was reduced with decreased growth temperature and increased with an increase in growth temperature. Another study postulated that during induction of the cyst stage in algae elevated temperature provides non-growth conditions (Chen and Chen 2006). Moreover, since active oxygen derivatives can be generated endogenously from photosynthesis, it seems plausible to assume that relatively high culture temperature may also lead to enhanced formation of active oxygen in algal cells. However, there was a 15- to 20-fold increase in the cellular accumulation of carotenoid compared to a three-fold increase in volumetric production (Tjahjono, et al. 1994). *Mucor rouxii* also showed a threefold increase in carotenoid content when the temperature of mycelial cultures was raised to 40°C under aerobic conditions as compared to the amount obtained at the optimum growth temperature of 28°C (Mosqueda-Cano and Gutierrez-Corona 1995).

2.5.5 Nutrient

Algae require dissolved nutrients, similar to terrestrial flora. Nitrates and phosphates are two notable nutrients, as well as sodium and silicates. There are many culture medium formulae available but requirements for different microalgae vary. Moreover, the requirements also take into account the objective of the experiment, e.g. if high productivity of microalgae is the priority, high nitrate and phosphate would be essential for growth.

In general, a basic assumption governing the use of this model is that the growth rate of an alga is dependent solely-on the concentration and of a particular limiting nutrient according to the Monod model is described as

Equation 2 - 3 $$\mu = f(s) = \hat{\mu}\left[\frac{S}{K_S+S}\right]$$

Combining and Equation 2 - 2 and Equation 2 - 3, we obtain the following equation

Equation 2 - 4 $$\mu = f(s) = Ae^{-E/RT}\left[\frac{S}{K_S+S}\right]$$

where p = specific growth rate, day^{-1} ; G = maximum specific growth rate, day^{-1} ; S = limiting nutrient concentration, mg $liter^{-1}$; and K = half saturation coefficient, mg $liter^{-1}$.

To induce production of secondary carotenoids, manipulation of nitrate concentration has been a common technique to stimulate environmental stress on microalgae. It has been suggested that a high carbon to nitrogen ratio (C/N) may be efficient for inducing astaxanthin biosynthesis (Chen and Chen 2006, Chen and Johns 1991). Nitrogen limitation in the presence of excess organic carbon substrates such as acetate and glucose has proven effective in astaxanthin production in mixothrophic cultures (Ip and Chen, 2005).

2.5.2 pH

Algae grow best at neutral pH (7-8.5) and buffer is added to the medium for pH adjustment (Andersen 2005). As culture age, pH increases due to accumulation of minerals and oxidation of nutrients. Therefore, nutrient medium is pre-adjusted to pH 6.5 (Vonshak 1992) before feeding to the algae. Most of the research groups maintain culture under pH 7.5 for optimum growth (Esperanza, et al. 2007, Linden 2001, Weissman 1988).

2.5.3 Illumination

The specific growth rate for microalgae is dependent on the intensity of light. Growth of microalgae increases with increasing illumination. Upon reaching the peak growth rate, it declines with further increase in light intensity due to photo-inhibition. This pattern of

growth-light intensity relationship can be seen in almost all species of algae. (Chisti and Moo-Young 1989, Bar, et al. 1995, Fan, Vonshak and Boussiba 1994)

Carotenoid production and accumulation are reported to be positively affected by white-light irradiation in algae, fungi, and bacteria. However, the intensity and protocol of illumination varies with the microorganism. Irrespective of whether increases or decreases in illumination time and/or intensity lead to improvements in carotenoid yield, there are two aspects to the theory of photo-induction (Bar, et al. 1995, Bohne and Linden 2002, Dan Pelah 2004, Li and Huang 2009).

1. The first is that improvements of the volumetric production of carotenoid (mg/l) are generally associated directly with improved growth of the microorganism (Ausich 1997). Thus, the effect of light on growth of the microorganism plays an important role in establishing the authentic role of white-light illumination as a stimulant of carotenoid production.
2. Increases in the cellular accumulation (mg/g) of carotenoid are associated with increased activity of enzymes involved in carotenoid biosynthesis. In this case, it is important to assess the levels of biosynthetic enzymes, which in turn will establish the role of white-light illumination as a stimulant (Bhosale 2004).

H. pluvialis showed a remarkable increase in the concentration of astaxanthin with an increase in light intensity from 50 to 400 μmol photon $m^{-2}s^{-1}$ (Barbosa, Hadiyanto and Wijffels 2004). Shaish (1993) reported that induction of hyper-production by light could be replaced by reactive oxygen species (ROS) (Darley 1982), since exposure to white light ultimately leads to generation of active oxygen molecules, which possibly play an important role in the stimulation of carotenogenesis. The interrelation between ROS and carotenoid production under light stress is not clear. Boussiba (2000) has reviewed the complex regulatory mechanisms which function at both the gene and the protein level.

2.5.4 Mixing and Turbulence

For any algal reactors, efficient mixing should be provided in order to produce a uniform dispersion of microalgae within the culture medium, thus eliminating gradients of light, nutrient concentration (including CO_2) and temperature.

Gudin and Chaumount (1991) reported that the key problem of algae cultivation is cell damage due to shear stress (Gudin and Chaumont 1991). It has been reported that excessive mechanical shaking, causing turbulence induced by high revolution-per-minute (rpm) will cause permanent damage on cell structure which would affect the cell growth. Insufficient shaking will lead to algae settling and cell death. Few quantitative studies have been done regarding hydrodynamic stress in algae cultures cultivated in air-lifting photobioreactors (Carvalho, Meireles and Xavier 2006). The growth rates of some algae

have been reported to increase initially with increasing turbulence, probably due to the improved supply of light and CO_2. But upon an optimum level of turbulence, the growth decreases sharply with further increase of the superficial- gas velocity and this is believed to be due to cell damage.

Gas mixing systems, i.e., bubble column systems, cause less extensive damage to fragile microalgal species than mechanical pumping does. This is especially so for the case of air-lift units, in which mixing is achieved by fluid flow obtained from sparging air into a central draught tube (riser), where it decreases bulk liquid density hence causing the liquid to rise. The liquid then flows downward through the outer tube, thus creating a natural circulation. Although these systems appear to cause the least extensive degree of cell damage (Barbosa, et al. 2004), they are not completely devoid of shear stress: a cell-damaging hydrodynamic effect has been reported (Miro´n, et al. 1999) in bubble columns and air-lift reactors, which was associated with so intense turbulence patterns that the length scale of the fluid microeddies approached cellular dimension. Barbosa (Barbosa, Hadiyanto and Wijffels 2004) reported bubble formation at the sparger as the main event leading to cell death. Finally, the effect of "mutual shading", i.e., continuous cell movement from and to dark/light zones, has been claimed (Becker 1994) to be essential to guarantee high biomass productivity.

2.5.6 Gas Transfer

Because nearly 50% of the whole microalgal biomass is made up of carbon (Becker 1994), this element is a major component for cell growth. When grown photo-litotrophycally, all microalgae use inorganic carbon sources to synthesize organic compounds (Richmond, 2004). Though microalgae can take in inorganic carbon in various forms, CO_2 (aq), H_2CO_3, HCO_3^- and CO_3^{2-}, detailed studies on the influence of the carbon source upon microalgal productivity have indicated that, although HCO_3 is easily absorbed by cells, it is a poor source of carbon when compared with CO_2 (Goldman, Dennett and Riley 1981). In fact, it is possible to achieve a linear response in microalgal carbon biomass with mass input of carbon (which corresponds to an efficiency of virtually 100%) only if limited inputs of inorganic carbon and narrow pH ranges are permitted. Note that CO_2 in the open air accounts for only 0.03% (v/v) (Becker 1994), so fluxes of carbon transfer to the culture are small, even in the presence of extended interface areas or enhanced mixing. Consequently, CO_2-enriched air is the most commonly employed nutrient gas mixture.

Optimum biomass productivity was obtained by using either high bubbling rate (with small sized bubbles) with low inlet pressure of CO_2 or low bubbling rate with high inlet pressure of CO_2 (irrespective of bubble size). Although more efficient (47% vs 14%, in terms of assimilation efficiency), the former option could bring about problems of cell flotation and consequent washout.

Increasing awareness of the importance of CO_2 led to development of control systems, able to regulate the pH of the culture and thus, indirectly, control the amount of CO_2 supplied. The most common system employed for pH control is the on-off type, in which CO_2 is injected into the culture when pH is above a desired setpoint. Productivity of algae increases an average of 7-10% for chlorella species when this system is employed (Ana, 2006; González, 2007).

Parameters	*Experimental Conditions for Optimization of Growth and Accumulation of Astaxanthin*
Temperature	Fixed at 25°C°
pH	Nutrient medium pre-adjusted to pH6.5 and culture maintained at ph7.5
Illumination	3 different light intensity used, at 100, 300 and 600 μmol photon m^{-2} s^{-1}
Shaking	Continuous shaking at 130rpm
Nutrient	Various nitrate concentration at 1, 0.5, 0.25, 0.125, 0g/L

Table 2 - 5 Experimental conditions for phase 1

2.6 Chemical Structure of Astaxanthin

Astaxanthin exists in three main enantiomeric forms, termed 3s-3's, 3r-3's, and 3r-3'r, depending on the spatial orientation of the hydroxyl (OH) groups in chiral carbon number 3. Synthetic astaxanthin is produced as the free (un-esterified) xanthophyll and as a 1:2:1 mixture of the three stereo-isomers: 3s,3's, 3r,3's, and 3r,3'r.

Whether free or complexed, the atoms comprising an astaxanthin molecule can be oriented in different ways, producing different isomers. The most common geometric configuration in both synthetic and natural astaxanthin is the most thermodynamically stable all-E (all-trans) isomer.

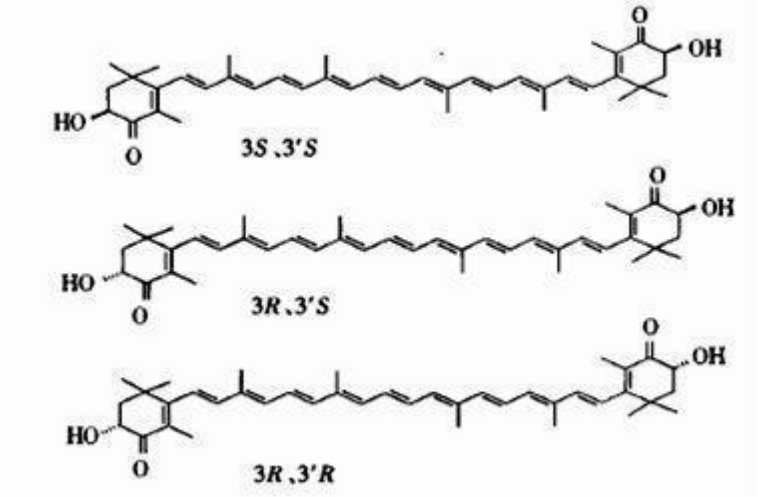

Figure 2 - 6 Astaxanthin enantiomer 3S, 3'S; 3R,3'S; 3R,3'R; Molecular formula - $C_{40}H_{52}O_4$ - Molar mass - 596,84 g/mol (Bar, et al. 1995)

The enhanced activity of astaxanthin may stem from its molecular structure. Astaxanthin belongs to the xanthophyll group of carotenoids, or the oxygenated carotenoids. The hydroxyl and keto functional groups present in the ending ionone ring of astaxanthin may be responsible for its uniquely powerful antioxidant activity and for its ability to span the membrane bi-layers as a direct result of its more polar configuration relative to

other carotenoids. Carotenoids with polar end groups like astaxanthin span the lipid membrane bi-layer with their end groups located near the hydrophobic-hydrophilic interface, where free-radical attack first occurs (Britton 1995).

2.7 Astaxanthin as an Antioxidant

Astaxanthin, unlike some carotenoids, does not convert to Vitamin A (retinol) in the human body. Too much Vitamin A is toxic for a human, but astaxanthin is not (Britton 1995). While astaxanthin is a natural nutritional component, it can be found as a food supplement. The supplement is intended for human, animal, and aquaculture consumption (McCausland, et al. 1999). Astaxanthin has 100-500 times the antioxidant capacity of Vitamin E and 10 times the antioxidant capacity of beta-carotene. Many laboratory studies also indicate astaxanthin is a stronger antioxidant than lutein, lycopene and tocotrienols. Currently, the primary use for humans is as a food supplement. Free radicals (e.g. hydroxyl and peroxyl radicals) are a highly reactive form of oxygen (e.g. singlet oxygen), and they can damage DNA, proteins and lipid membranes. They are produced in the body during normal metabolic reactions and processes. Due to our daily exposure to contaminants, chemicals, tobacco smoke, physiological stress or ultraviolet (UV) radiation, free radicals production is enhanced as a result (Guerin, et al. 2003). Phagocytes can also generate an excess of free radicals to aid in their defensive degradation of the invader. Oxidative damage has been linked to aging, atherogenesis, ischemia-reperfusion injury, infant retinopathy, macular degeneration and carcinogenesis (Papas 1999).

With the discovery of antioxidant, human being is able to rid some of the free-radicals produced. Dietary antioxidants, such as carotenoids, might help to prevent and fight several human diseases. Carotenoids are potent biological antioxidants that can absorb the excited energy of singlet oxygen onto the carotenoid chain, leading to the degradation of the carotenoid molecule but this process prevents other molecules or tissues from being damaged (Mortensen 1997). They can also prevent the chain reaction production of free radicals initiated by the degradation of poly-unsaturated fatty acids, which can dramatically accelerate the degradation of lipid membranes. Astaxanthin is very good at protecting membranous phospholipids and other lipids against peroxidation (Palozza 1992). Astaxanthin's antioxidant activity has been demonstrated in several studies. The antioxidant properties of astaxanthin are believed to have a key role in several other properties such as protection against UV-light photooxidation, inflammation, cancer, ulcer's *Helicobacterpylorii* infection, aging and age-related diseases, or the promotion of the immune response, liver function and heart, eye, joint and prostate health.

2.8 Industrial Production of Astaxanthin

There are 2 types of astaxanthin in the market nowadays: synthetic and natural astaxanthin, the chemical difference between natural and synthetic astaxanthin lies in the stereochemical orientation of the molecules in space, or enantiomers.

Synthetic astaxanthin is produced as the free (unesterified) xanthophyll and has a 1:2:1 mixture of the three stereoisomers: 3S, 3'S, 3R, 3'S, and 3R, 3'R. Synthetic astaxanthin fetches about US$2000 a kilogram on the market, while the natural product is sold for over US$7000 a kilogram. The industrial producers of synthetic astaxanthin are Hoffmann-La Roche AG and BASF AG.

Production of natural astaxanthin on a large scale is done on green alga *H. pluvialis.* *H. Pluvialis* has been the subject of intensive research, since it accumulates astaxanthin under certain stress conditions. It has shown to accumulate the highest levels of astaxanthin in nature; commercially more than 40 g of astaxanthin per kilogram of dry biomass. In the large-scale, outdoor system, the production of astaxanthin-rich *H. pluvialis is* a two-step process (Figure 2 - 7). First, vegetative cells must be produced under near-optimal growth conditions with careful control of pH, temperature and nutrient levels.

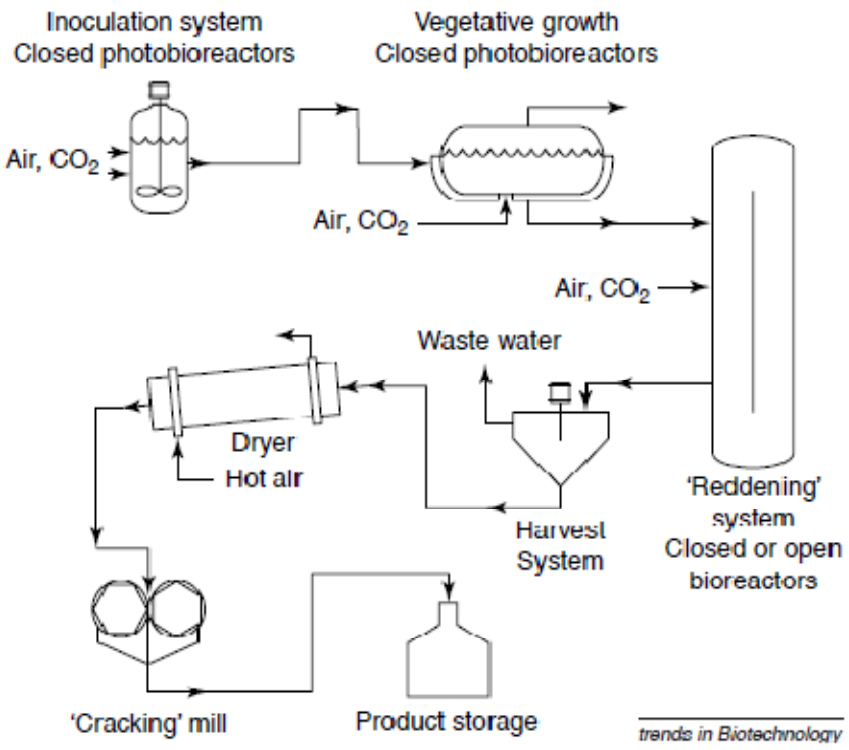

Figure 2 - 7 Typical process flow for the commercial production of natural astaxanthin by *H. pluvialis* (Burick 1991)

After a sufficient volume of vegetative cells is produced, the culture is subjected to environmental and nutrient stress. Commercial systems induce astaxanthin production by deprivation of nitrate and phosphate, increasing temperature and light, or by the addition of sodium chloride to the culture medium. Within 2 to 3 days after the culture is stressed, haematocysts are formed and begin accumulating astaxanthin; within 3 to 5 days following the formation of *H. pluvialis* (containing ~1.5–3.0% astaxanthin), they are ready for harvest. (Molina, et al. 2003) The change in a *H. pluvialis culture* is striking when haematocysts accumulate astaxanthin. Because haematocysts are considerably denser than water, harvesting of the haematocysts is accomplished by settling and

subsequent centrifugation. The haematocysts are then dried and cracked to ensure maximum bioavailability of the astaxanthin.

For feed-grade applications, ethoxyquin or other antioxidants are added to the paste before drying, to minimize oxidation of the carotenoids. Milling can then be used to crack cells, although the exact details of the techniques are proprietary to companies producing *H. pluvialis* algae.

2.9 Shortcomings of the Current Mass Production System

The success of the commercial mass production of astaxanthin by *H. pluvialis* is hampered by physiological and technical reasons, such as a slow growth rate and relatively low growth temperature, and by its susceptibility to contamination and the apparent requirement of a two-stage production process (Zhang and Lee, 1997; Lorenz and Cysewski, 2000; Guerin, et al., 2003). This raises the production costs in such a way that *H. pluvialis* astaxanthin cannot compete on price against the synthetic pigment (Guerin, et al. 2003).

Recently, Esperanza et al. (2007) proposed the use of a simpler one-step strategy for the production of *H. pluvialis* astaxanthin, in which cultures are operated under continuous mode, in only one stage. To promote astaxanthin accumulation, nitrate concentration in

the fresh medium entering the reactor was reduced as to generate moderate nitrogen limitation conditions, which supported active cell growth with simultaneous accumulation of the carotenoid. Besides representing an efficient and simple production process, the fact that the growing and dividing cell population exhibited active astaxanthin accumulation demonstrated that such ability neither required encystment nor was a property unique to non-growing cells, as assumed by others (Boussiba and Vonshak 1991, S. Boussiba, 2000, M. Borowitzka 1999, Harker, et al. 1996).

A preliminary assessment of the one-step system yielded a considerably high productivity, of 5.6 mg astaxanthin/L day (Del Campo, et al. 2000). Notwithstanding, productivity and efficiency of the one-step system versus the two-stage counterpart have recently been challenged by Aflalo (2007), on the ground that there is no real biotechnological advantage to vigorously growing cells for production of the secondary carotenoid astaxanthin. The identification and characterization of alternative microalgal species able to accumulate carotenoids of commercial interest, namely astaxanthin and/or lutein, is thus highly desirable (Del Campo, Moreno and Rodríguez, et al. 2000)

The early performance analysis of the one-step system was limited to only *H. pluvialis* strain and the effect of varying nitrogen supply to the cell suspension, keeping otherwise constant both the dilution rate and the illumination conditions (Del Campo, et al. 2004). A slightly different culture system, with improved aeration (threefold higher flow rate)

and subsequent superior culture's agitation as well as better and more homogenous illumination, has now been arranged in a setup which allowed simultaneous operation of four photobioreactors. The response analysis could then be extended to the influence of several factors and to a better evaluation of the capacities and potentialities of the system. (Del Campo, Moreno and Rodríguez, et al. 2000)

2.10 Method of Cultivation of *C. Zofingiensis*

Batch system is usually employed at the initial stage to determine the growth rate. Depending on the product and the objective of study, batch or continuous system can be used. Under standard batch-culture conditions, Del Campo (2004) found that this microalgal strain exhibits high values of both growth rate (about 0.04 h^{-1}) and standing cell population (7 g dry weight l^{-1}). Lutein, in a free (unesterified) form, was the prevalent carotenoid during early stages of cultivation (4 mg g^{-1} dry weight, or 20 mg l^{-1} culture), whereas esterified astaxanthin accumulated progressively to reach a maximum (1.5 mg g^{-1} dry weight, or 15 mg l^{-1} culture) in the late stationary phase. (Del Campo, et al. 2004)

Under low light irradiance and subjected to salt and low nitrogen stress, *C. Zofingiensis* grown under batch system accumulated higher amounts of total secondary carotenoids than those growing under high light and low nitrogen stress. (Dan Pelah 2004) Furthermore, *C. Zofingiensis* growing under conditions of salt stress and low light

accumulated higher amounts of canthaxanthin than astaxanthin. It is suggested that for canthaxanthin accumulation under salt stress, light is not a limiting factor, but for astaxanthin accumulation high light irradiance is mandatory.

For industrial application of algal production, continuous culture is preferred. Continuous culture is basically a method of prolonging the growth phase of a microorganism in batch culture, which involves feeding with fresh nutrients and at the same time removing spent medium plus cells from the system. Growth and environmental factors are kept constant. It is a good system to grow heterotrophic microorganisms at high cell densities and to study the growth and physiological behavior of microorganisms.

At present, most of the studies have been conducted on batch systems. Therefore, it is prudent to investigate the production of *C. Zofingiensis* under continuous systems. To facilitate this, different designs of photobioreactor are investigated for the purpose of scaling up in the future. Such studies would also facilitate future one-step production of astaxanthin using *C. Zofingiensis.*

2.11 Photobioreactor Design

The main parameter that affects reactor design is provision for light penetration, which implies a high surface-to-volume ratio; such penetration is crucial if one wants to

improve the photosynthetic efficiency, which is in turn an essential condition to reach high product and biomass productivities. (O. Pulz 2001) In order to achieve said high surface-to-volume ratio, several photobioreactor shapes have been developed that met with success. These shapes can be grouped in three basic types, tubular, flat plate and fermenter-type; the former two are specifically designed for efficient harvest of sunlight, whereas the latter requires artificial illumination. A summary of the developments thus far is presented in Table 2 - 8;

Reactor Type	**Light harvesting efficiency**	**Degree of control**	**Land area required**	**Scale-up**	**Productivity (g/L.d); species**
Vertical Tubular	Medium	Medium	Medium	Possible	0.5; P. cruentum
Horizontal tubular	Good	Medium	Poor	Possible	0.25; S. platensis 0.7; Nannochloropsis sp.
Helical	Medium	Good	Excellent	Easy	0.4; S. platensis
Flat-plate	Excellent	Medium	Good	Possible	0.85; Nannochloropsis sp. 2.15; S. platensis
Fermenter type	Poor	Excellent	Excellent	Difficult	0.03-0.05; several

Table 2 - 8 Review of existing PBR (Ana 2006)

For the purpose of scaling up, flat plate reactors are the most popular choices (M. Borowitzka 1997), considering that the light source required is free and readily available. Those reactor types are designed to enhance the highest possible area-to-volume ratio while ensuring reasonable working volume, mixing pattern and carbon dioxide level. Both reactor configurations may work with a separate unit for gas transfer, and several layouts have been already tested with success (Pulz 1992, Richmond, et al. 1993).

Flat plate reactors (FPR) are conceptually designed to make efficient use of sunlight; hence, narrow panels are usually built so as to attain high area-to-volume ratios. The greatest advantage of this system is its provision of an open gas transfer unit, which has proven efficient in overcoming the problem of oxygen buildup; and in the specific case of bubbled column FPR, the absence of a driving pump. However, such an open zone restricts effectiveness of contamination control, as compared with completely closed reactors, thereby limiting the strains suitable for this method of cultivation.

A 500-L FPR was developed by Pulz and Scheinbenbogen (1998), in which the culture was circulated from an open gas exchange unit through several parallel panels placed horizontally. The culture flew at a high linear speed (1.2 m s^{-1}), but hydrodynamic parameters usually lay in a safe operating range for the sake of cell integrity. Pusparaja et al. (1997) discussed a reactor encompassing an alveolar panel system oriented toward the sun, coupled with an open raceway for gas transfer. The use of such alveolar panels as

solar receptors increased volumetric productivity from 0.18 g L^{-1} d^{-1} in open ponds to 0.31 g L^{-1} d^{-1}. (Puspararaj, et al. 1997) Although the volumetric productivity attained inside the panels is higher, open raceways are the most often used cultivation systems for microalgae, so said combination may be of great practical significance. (Tredici and Materassi 1992, O. Pulz, 1992).

2.12 The Need for Investigation

Cultivation of *C. Zofingiensis* has always been using batch cultivation, which is not practical for scale-up. Continuous or semi-continuous culture and extraction have not been reported in the literature to date and thus there is scientific and commercial value in conducting a 1-step astaxanthin production system. Thus, the present study was addressed to verify the performance of the one-step system, as well as to analyze its behavior and production capability of algal biomass and astaxanthin under optimized temperature, illumination and nitrate concentration.

Chapter 3 Materials and Methods

3.1 Organism

The green microalga, *Chlorella Zofingiensis* (ATCC30412) was purchased from American Type Culture Collection (ATCC, Rockville, USA).

3.1.1 Initial Growth Conditions

C. Zofingiensis was inoculated using ATCC recommended culture medium, 5 g/L Proteose medium, a non-specific medium, for 2 weeks before sub-culturing was carried out. As culture was being removed from long-term maintenance at slow growth rates in a culture collection, an increment of 5^{o}C per transfer was necessary to coax the culture through a series of transfers. Stock cultures were kept as backup in case of unsuccessful transfer or contamination.

After 3 successful transfers, the medium was changed to CZ-M1 medium, a specific culture medium as described by Ip and Fung (2005b). The composition is listed in Table 3 - 1. CZ-M1 medium was adjusted to pH 6.5 and autoclaved before feeding in microalgae culture.

No	Chemical Name	Molecular formula	Amount
1	Sodium nitrate	$NaNO_3$	0.75 g
2	Potassium phosphate monobasic	$KH2PO_4$	0.175 g
3	Potassium phosphate dibasic	$K2HPO_4$	0.075 g
4	Magnesium sulfate heptahydrate	$MgSO_4.7H_2O$	0.075 g
5	Calcium chloride dihydrate	$CaCl_2.H_2O$	0.025 g
6	Sodium chloride	NaCl	0.025 g
7	Iron(III) chloride hexahydrate	$FeCl_3.6H_2O$	5.0 g
8	Zinc sulfate heptahydrate	$ZnSO_4.7H_2O$	0.287 mg
9	Manganese(II) sulfate monohydrate	$MnSO_4.H_2O$	0.169 mg
10	Boric acid	H_3BO_3	0.061 mg
11	Copper(II) sulfate pentahydrate	$CuSO_4.5H_2O$	0.0025 mg
12	Ammonium molybdate tetrahydrate	$(NH_4)6Mo_7O_{24}.H_2O$	0.00124 mg

Table 3 - 1 Composition of CZ-M1 medium (Ip and Chen 2005b)

C. Zofingiensis was maintained in 3L Erlenmeyer flask and was harvested during the late exponential growth phase (6 g/L). Separation was done using centrifugation at 1000g for

3 mins at 22°C. It was then washed with sterile water and resuspended in CZ-M1 medium with different nitrate concentration for subsequent test.

3.1.2 Maintenance of microalgae

Routine serial sub-culturing for the stock *C. Zofingiensis* was performed using aseptic microbiological technique and involved transferring an inoculum from a late exponential/stationary growth phase culture into fresh, autoclaved medium. The interval of transfer was approximately 10-14 days, as determined from *C. Zofingiensis* growth curve.

3.2 Phase 1: Optimization of Algal Growth

3.2.1 Experiment Design Overview

For the purpose of optimization of *C. Zofingiensis* for the cultivation in PBR, Phase 1 experiment involved varying both nitrate concentration and light intensity. Monitoring of growth rate and pigment profiles on samples was carried out. All tests were carried out in triplicates and repeated to ensure consistency. All microalgae samples were cultured with 250ml Erlenmeyer flask containing 100 ml of medium with biosilicone or sponge placed at the small opening (D=18mm) of the flask. Evaporation is negligible in these systems.

3.2.2 Chemicals

The components of the standard nutrient solution and the solvents were of analytical grade and were purchased from Sigma-Aldrich, Singapore. Standards of Chlorophyll a and b, astaxanthin and β-carotene were purchased from Sigma-Aldrich, St. Loius, Mo, USA. All other chemicals were of analytical grade and were acquired from Merck, Singapore.

3.2.3 Operating Conditions

The culture (5 days old) was introduced into Erlenmeyer bottle, each containing 100 ml of culture medium. Algal concentration was adjusted to 2 g/L, or 30% by volume. Five different nitrate concentrations were used; 1, 0.5, 0.25, 0.125, 0 g/L. The cultures were grown at 25°C and with orbital shaking at 130 rpm and illuminated with continuous cool white florescence light (2 x 50W) at 3 light intensities: 100, 300 and 600 μmol m^{-2} s^{-1}. Samples of concentrated culture were placed in the dark 24 hours before the experiment to induce synchronization and to avoid pre-adaption to light. Samples were also diluted to avoid self-shading. pH was measured and maintained at 7.5. CO_2 was manually bubbled when pH exceeded 7.5. Temperature measurement was done using thermometer placed inside the culture. Illumination can be measured by digital illumination meter (Nicetu LX 802).

3.2.4 Cryopreservation and Recovery of microalgae

Cryopreservation method was carried out according to Day and Brand (2006). A Nalgene 1°C Freezing Container (canister) that contains isopropanol as specified by the manufacturer is placed into a 4°C refrigerator at least a day before it was used for cryopreservation. 1.5 ml of microalgae in liquid culture medium was placed into a 2-ml cryovial. Then 0.5 ml of the 20% MeOH solution was added to the vial and the contents was quickly and gently mixed. At all time algal cultures should be kept in minimum light. The pre-chilled freezing canister was removed from the refrigerator, the cryovial was placed into one of the vial holder locations in the canister, and the lid is placed back onto the canister. The canister was then placed into a -80°C freezer. After at least 1.5 hours, in the -80°C freezer, the freezing canister was removed. The storage box was immediately removed from the rack in the liquid nitrogen dewer and the cryovial was transferred from the canister to the box. The box was then placed back into the rack, which was placed into the storage dewar for storage (Day and Brand 2006).

For recovery of living microalgae from the dewar a 400-ml volume of water was pre-warmed to approximately 37°C. The storage rack was removed from the liquid nitrogen dewar and the cryovial is removed from the rack and quickly inserted into the 37°C water bath. The cryovial is gently agitated during thawing and left in the water bath until all ice has just melted (generally under 2 minutes). The cryovial is immediately subjected to

centrifugation (as gentle as possible) to pellet the microalgae and the liquid was gently decanted. The vial was then filled with fresh culture medium and left undisturbed for several minutes. It was then again subjected to gently centrifugation, and the liquid was removed as before. Fresh culture medium was placed into the cryovial to suspend the microalgae and the culture was transferred to a larger volume of medium under normal culturing conditions.

3.3 Phase 2: Photobioreactor operation

3.3.1 Phase 2 Experiment Design Overview

Phase 2 of experiment involved semi-continuous cultivation of *C. Zofingiensis* in a turbidostat, air-lift flat-plate PBR (Figure 3-1 and 3-2) at different temperature. Using the optimum operating configuration determined in Phase 1 of the experiment, it would be further tested in a PBR. Two liters of microalgae was placed into each PBR and mixing of microalgae was induced by aeration from tubular diffuser. Again, both growth rate and pigment profile were monitored. All tests were carried out in triplicates and repeated to ensure consistency.

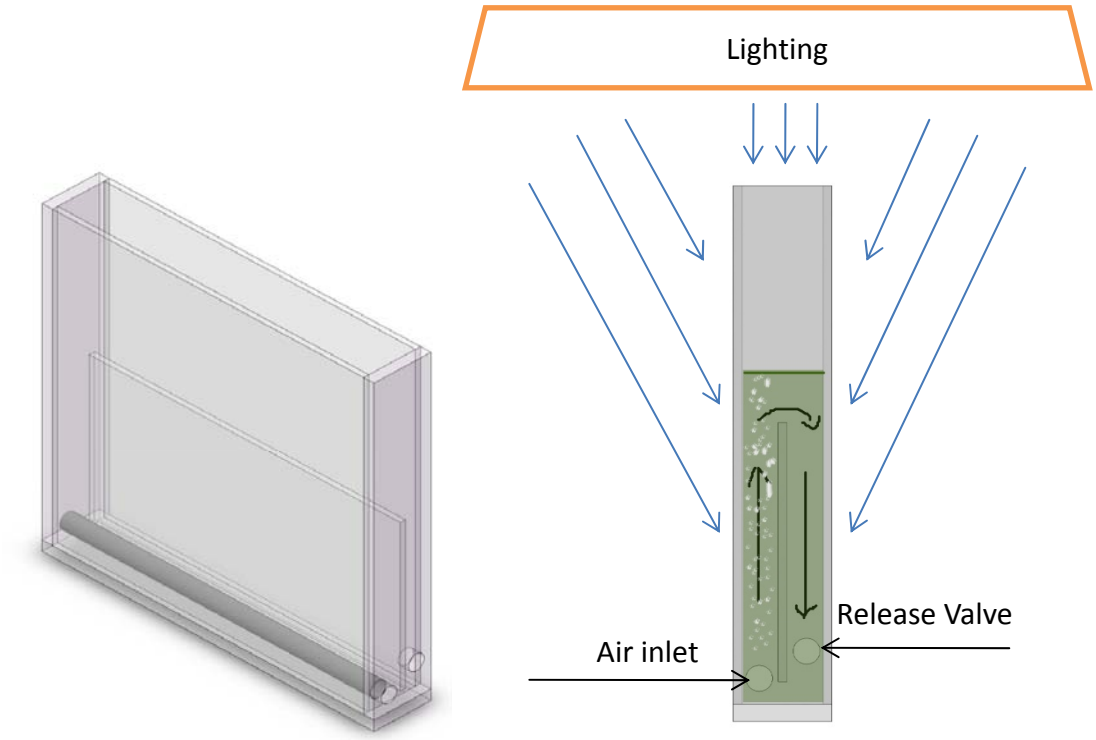

Figure 3 - 1 Left: Isometric view of the 3L flat-plate photobioreactor and experimental set-up from front view of reactor (arrows indicating direction of flow).

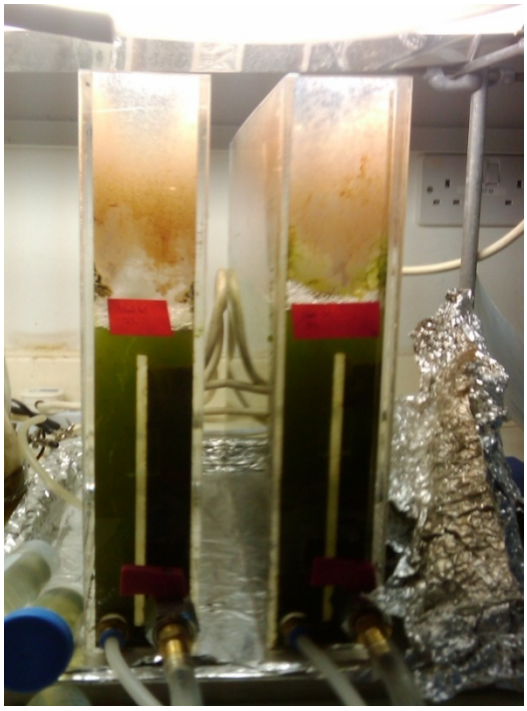

Figure 3 - 2 Picture of flat-bed algal photobioreactor

3.3.2 Operational Perimeters

The cells that entered late exponential/stationary growth phase from the stock culture were harvested by centrifugation (22°C at 1,000g for 5 minutes). The pelleted cells were rinsed twice in fresh CZ-M1 medium before using in phase 2 reactors. Culture of *C. Zofingiensis* was grown in semi-continuous mode. Concentration of microalgae was determined daily using spectrophotometer. Modified CZ-M1 medium with 0.125g/L nitrate concentration was used, as obtained from phase 1. Biomass concentration was diluted to about 50% of its concentration (at algal concentration 3.5g/L) at late exponential/stationary growth phase by manually removing part of the cultures and adding fresh medium. Reactors were operated at 3 different temperatures: 22, 25 and 28°C. pH was fixed at 7.5, and CO_2 was aerated to adjust the pH. Due to the loss of liquid medium from evaporation, reactors were topped up to 2 liter mark daily. Light intensity was maintained at 300 μmol m^{-2} s^{-1}.

Quasi-steady state was reached when the biomass increased in a repeated cyclic pattern over the day for three consecutive growth cycles. At quasi-steady state, growth parameters and pigment profiles were recorded. Experiment for each configuration would stop if repetitive peak dry weight concentration of *C. Zofingiensis* were observed in consecutive growth curve.

3.5 Physical and Analytical methods

3.5.1 Sample Preparation for Determination of Growth Parameters

The biomass concentration is measured as dry weight as it is one of the most direct and cost effective means to estimate biomass production. Dry weight can be conducted through sampling, separation, drying and weighing.

50 ml of *C. Zofingiensis* was taken from the batch reactor. 3 samples of 25ml were taken daily to reduce error or sampling. After sampling, cells were separated by centrifugation at 3600 g for 5 minutes at 4^{o}C, were subsequently washed with deionized water to rid of the salts and other contaminations. The sample was re-suspended in 50ml of deionized water and sample of 25 ml is filtered through 0.45 um (Whatman filter paper glass type). Drying temperature was set to 60^{o}C to avoid of over drying.

The rate of increase in biomass concentration is expressed by the specific growth rate (μ), which is calculated according to the following formula:

Equation 3 - 1 $$\boldsymbol{\mu = \frac{1}{x}.\frac{dx}{dt}}$$

where x is the biomass concentration. Thus μ represents the rate of in biomass growth per of biomass in units of d^{-1}. During exponential growth, the rate of increase in cells per unit

time is proportional to the number of cells present in the culture at the beginning of any unit of time. In other words, population growth follows this equation.

Equation 3 - 2 $$\frac{dn}{dt} = rN$$

where r (t^{-1}) is exponential growth rate of the population, the solution of which is

Equation 3 - 3 $$N_t = N_0 e^{rt}$$

where N_0 is the population size at the beginning of a time interval, N_t, is the population size at the end of the time interval, and r is the proportional rate of change. Equation 3 - 4 can be rearranged to give Equation 3 - 5.

Equation 3 - 6 $$r = \frac{\ln(\frac{N_t}{N_0})}{\Delta t}$$

Where Δt is the length of the time interval (t_1-t_0).

3.5.2 Ion chromatography (IC)

Nitrate concentration was determined using IC (Dionex ICS5000, Figure 3-3). Algae samples were filtered to prevent damage to the column and flow system. Filtrates were diluted 10 times by deionized water before putting into the autosampler.

Figure 3 - 3 Ion chromatography system with auto-sampler for measurement of nitrate concentration

3.5.3 Light Scattering (Turbidity)

Spectrophotometer (Figure 3-4) is employed to monitor the growth of algal culture. Results can be obtained very quickly and non-destructively. Optical density of the algal suspension was measured at an absorbance of 680 nm and 530 nm, using culture medium CZ-M1 as blank. Correlation between the dry weights of microalgae was taken to measure the growth rate of the algal sample.

Figure 3 - 4 Spectrophotometer for measurement of algal pigments

3.5.4 Sample Preparation for Determination of Astaxanthin Content

Quantification of astaxanthin and astaxanthin esters from *C. Zofingiensis* cells was carried out according to the procedure by Boussiba and Vonshak (1999). Samples collected are first centrifuged and frozen at -20^{o}C before putting into a DW3 freeze-drier (Heto Dry Winner, Denmark). The freeze drying took place under vacuum conditions below the "triple point" (6.2 mbar = 4.6 torr). The freeze-dried cells were powdered, grounded by motar and pestle, resuspended in a solution containing 5% (v/v) KOH and 30% (v/v) methanol, and heated in a water bath (70°C) for 5 min. After centrifugating the supernatant, which contained the chlorophylls, was discarded. The pellet was extracted twice with acetone at 70°C for 5 min. Standard curves for astaxanthin standards in acetone were made and astaxanthin concentrations were plotted as a function of absorbance and calibration equations were determined by linear regression. The absorbance of the combined extracts was determined at 550 nm. To allow the quantification of astaxanthin and astaxanthin esters separately from other carotenoids, the

measured concentration were subsequently multiplied by 3.2, a factor determined by measuring the absorbance of a purchased astaxanthin standard (Sigma, St. Louis) at two different wavelengths (A_{492}/A_{550}) (Equation 3-5). The amount of astaxanthin was then calculated by applying an absorption coefficient for astaxanthin in acetone according to Boussiba et al. (1992). The entire process is carried out in darkness.

Equation 3 - 7 $$C_{TA} = C_{A550} \times 3.2$$

where C_{TA} is the concentration of total astaxanthin (astaxanthin ester and astaxanthin) in mg/L; and C_{A550} is the concentration of astaxanthin measured at A_{550} (mg/L).

3.5.5 Sample Preparation for Determination of Chlorophyll And Total Carotenoids Content

Pigment extractions were carried out under dim light. 10 mg of freeze-dried algae was grinded and extracted by 30 ml of acetone. This process was repeated until cells turned colourless. Each analysis was carried out in triplicates.

For Chlorophyll a, b and total carotenoids (primary and secondary), the absorbance of the extracts was determined in a spectrophotometer (UV-1601, Shimadzu) at the wavelengths of 663.2, 646.8 and 470.0 nm, respectively. Chlorophyll and total carotenoid contents

were calculated by using the equation of Lichtenthaler (1987). Typical absorption spectra of chlorophyll a and b is shown in Figure 3-5.

Equations for the determinations of the concentrations of chlorophyll a (C_a), chlorophyll b (C_b), of total chlorophylls *(C_{a+b})* and of total carotenoids (primary and secondary) *(C_{x+c})* in 100% acetone as follows:

Equation 3 - 8 $$C_a = 12.25A_{661.6} - 2.04A_{644.8}$$

Equation 3 - 9 $$C_b = 21.50A_{644.8} - 5.10A_{661.6}$$

Equation 3 - 10 $$C_{a+b} = 7.05A_{661.6} - 18.09A_{644.8}$$

Equation 3 - 11 $$C_{x+c} = (1000A_{470} - 1.90C_a - 63.14C_b)/214$$

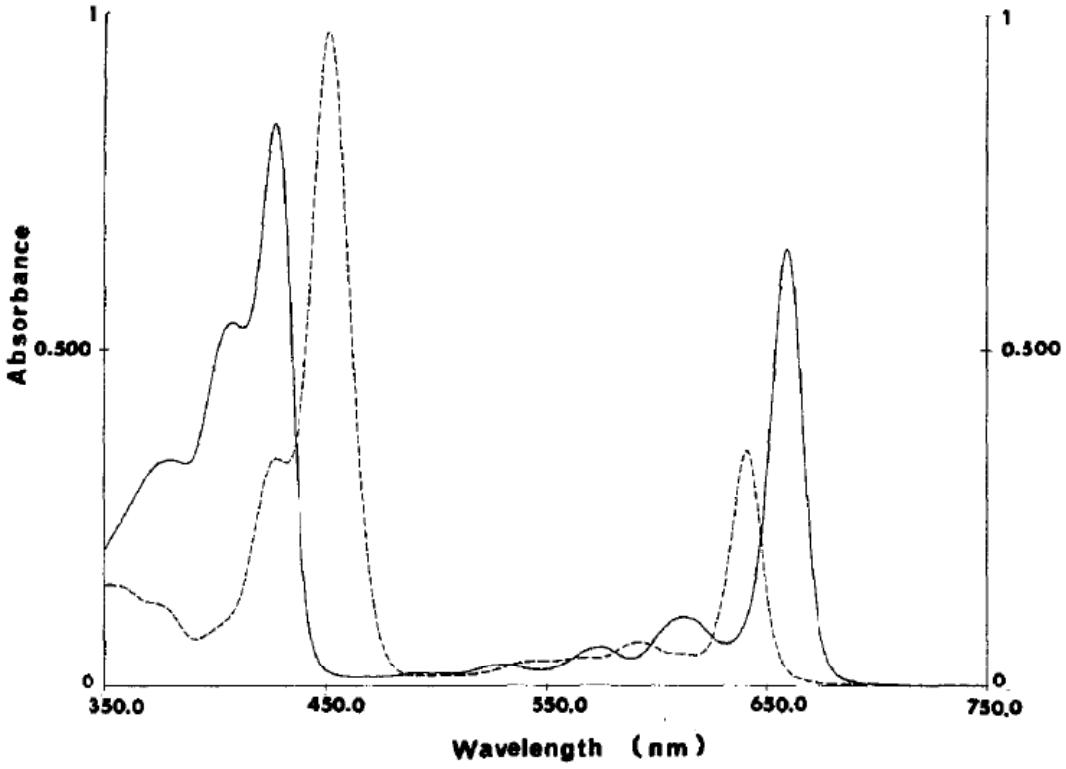

Figure 3 - 5 Typical absorption spectrum of chlorophyll a, b and total carotenoids.

Chapter 4 Results and Discussion

4.1 Experimental Results

4.1.1 Monitoring of Initial *C. Zofingiensis* Growth

After 3 successful transfers using bistro medium, dry weight concentration was recorded daily and the result of the initial growth curve as shown in Figure 4-1.

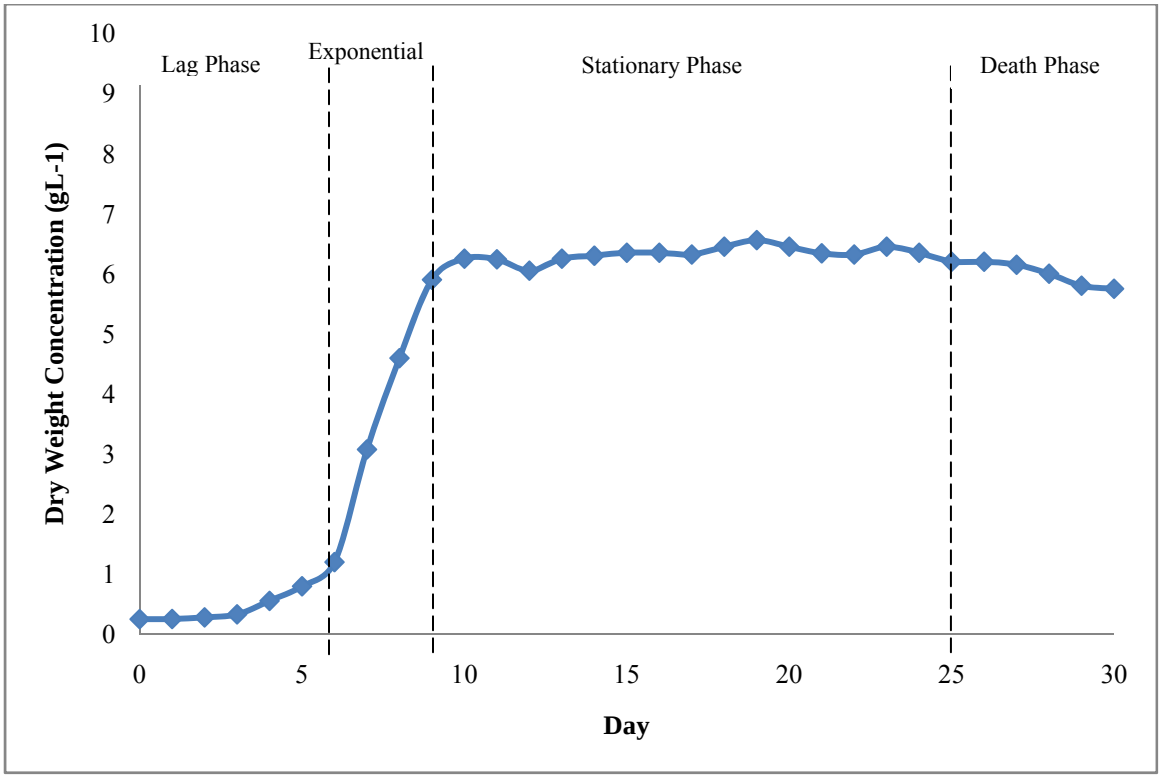

Figure 4 - 1 Initial Growth Curve of *C. Zofingiensis.*

The lag phase was found from 1-6th day; exponential phase from 6-9th day; stationary phase from 9-25th day; and death phase from 25th day onwards. The maximum growth rate was recorded at 1.077 per day with a maximum dry weight concentration of 5.9g/L recorded on Day 9, the last day of the exponential phase.

Due to concentration of microalgae samples, 10 times dilution was carried out. Figure 4 - 2 was plotted as indirect method of determining dry weight concentration of microalgae culture. A linear relationship was deduced and a R^2 value of 0.94 was found, indicating the line was a good fit, closely matching data points found. This graph would be used in monitoring the growth of culture.

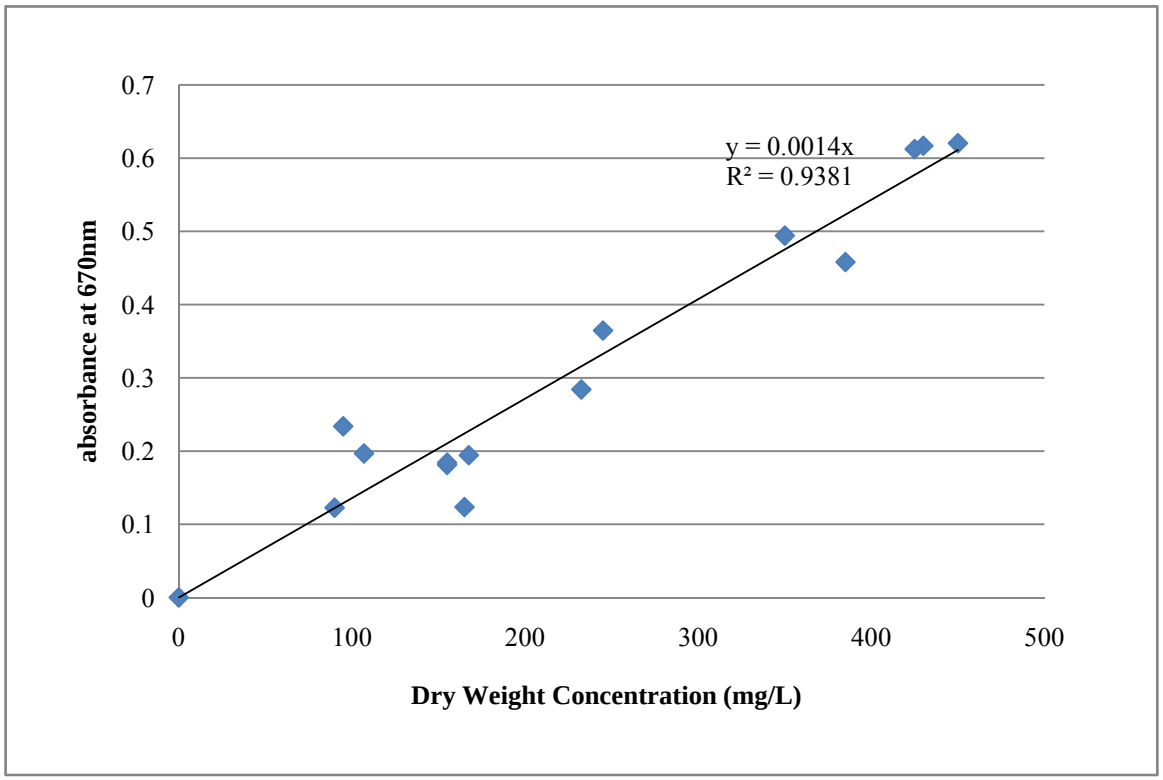

Figure 4 - 2 Absorbance of *C. Zofingiensis* was measured at 670 nm using a spectrophotometer and plotted against dry weight concentration at 10x dilution.

4.1.2 Phase 1: Batch Growth of *C. Zofingiensis*

4.1.2.1 Varying Nitrate Concentration and Light Intensity

When the profiles of growth curves for *C. Zofingiensis* became stable and reproducible, maximum dry weights for all configurations were determined. Samples testing were carried out in triplicates to reduce error. To recall, 15 different combinations of nitrate concentration and light intensity were employed in Phase 1.

As seen from Figure 4 - 3, it could be observed that nitrate concentration affected the growth of *C. Zofingiensis* severely. Two configurations clearly stood out from the rest, i.e. 0.5 g/L at 300 µmol m^{-2} s^{-1} and 1g/L at 300 µmol m^{-2} s^{-1} with peak dry weight concentration at 7.55 g/L. At nitrate concentration 1 and 0.5 g/L, it was observed that exponential growth phase was longer and lag phase was shorter compared to lower nitrate concentration. Lag phase was found to be longest (6 days) at low light intensity and low nitrate concentration. At no nitrate level, culture entered death phase after 5 days of growth as shown by decline in dry weight concentration regardless of light intensity.

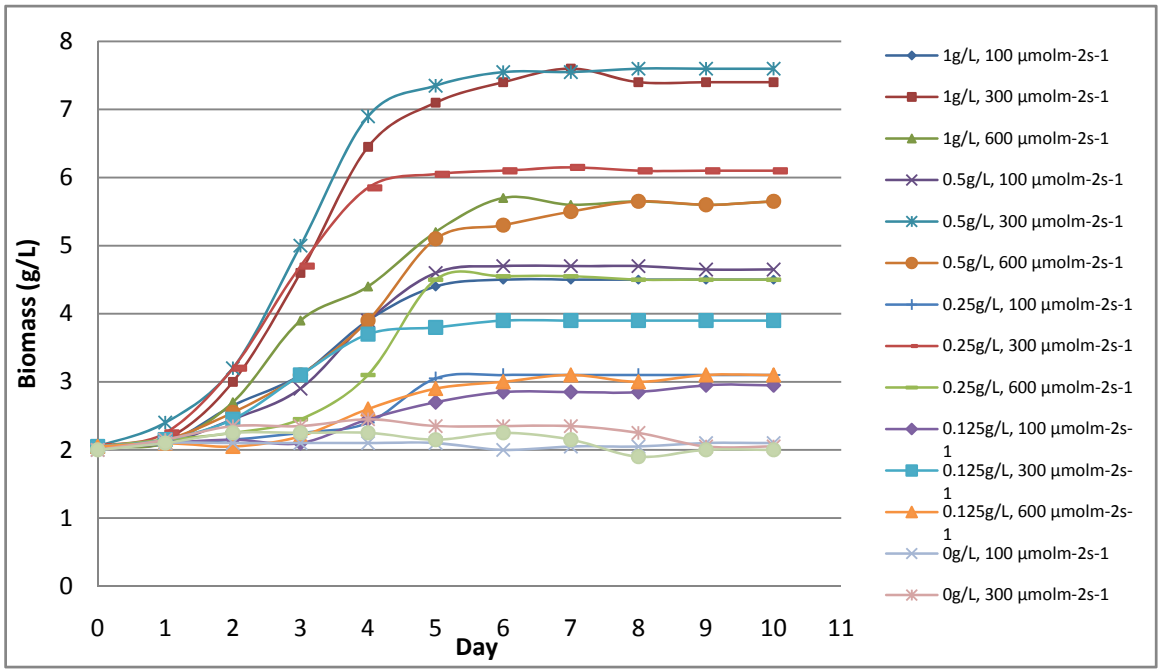

Figure 4 - 3 Growth curves at varying nitrate concentration at 25°C. Batch cultures of *C. Zofingiensis* were carried out at nitrate concentration 1, 0.5, 0.25, 0.125 and 0 g/L with light intensity at 100, 300, 600 µmol m^{-2} s^{-1}. A total of 15 configurations were tested.

Specific growth rate generally increased with increasing nitrate concentration. At nitrate concentration 1 g/L, a drop in specific growth rates across all light regimes were seen. The highest specific growth rates of *C. Zofingiensis* were found at 0.5g/L nitrate concentration, light intensity 300 µmol m^{-2} s^{-1}, at 0.45 day^{-1}. At 600 µmol m^{-2} s^{-1}, specific growth rate were 23% lower than at 300 µmol m^{-2} s^{-1} and 37% lower for 100 µmol m^{-2} s^{-1}.

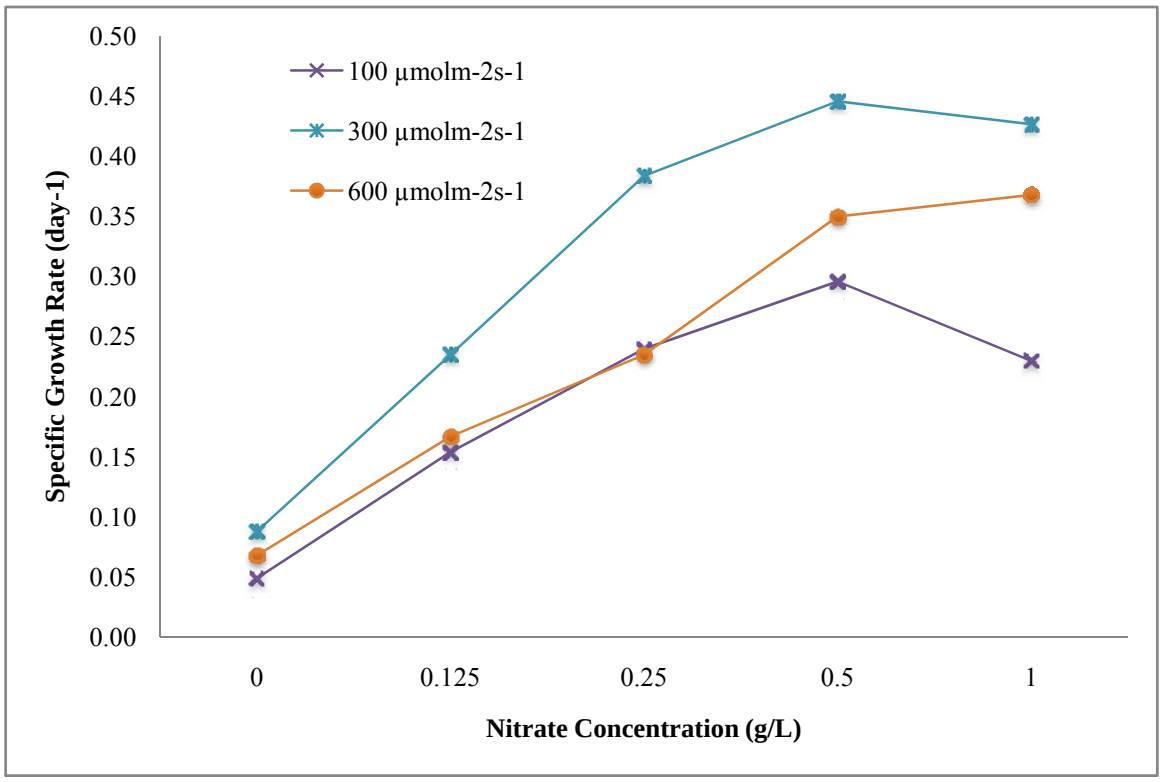

Figure 4 - 4 specific growth rate was plotted against nitrate concentration at light intensity 100, 300, 600 µmolm^{-2} s^{-1}. The initial culture concentration was 2g/L.

4.1.2.2 Physical Changes of *C. Zofingiensis*

Noticeable colour change was observed for *C. Zofingiensis*. With increasing light intensity, the colour of microalgae changed from green (Figure 4 - 6) to mixture of green and red (Figure 4 - 7) to red (Figure 4 - 8). At high light intensity, it could be seen that some of the algae biomass was black in colour.

At cellular level, it could be noticed that cells with red pigmentations were higher at 600 µmol m^{-2} s^{-1} than a light intensity of 300 µmol m^{-2} s^{-1}. At a light intensity of 100 µmol m^{-2} s^{-1}, significantly lower number of cells with red pigmentations was seen.

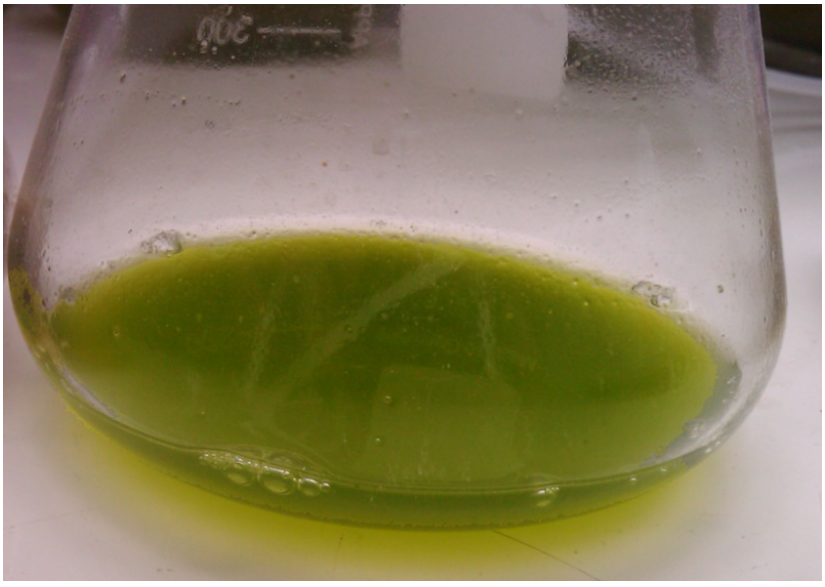

Figure 4 - 5 *C. Zofingiensis* at day 0.

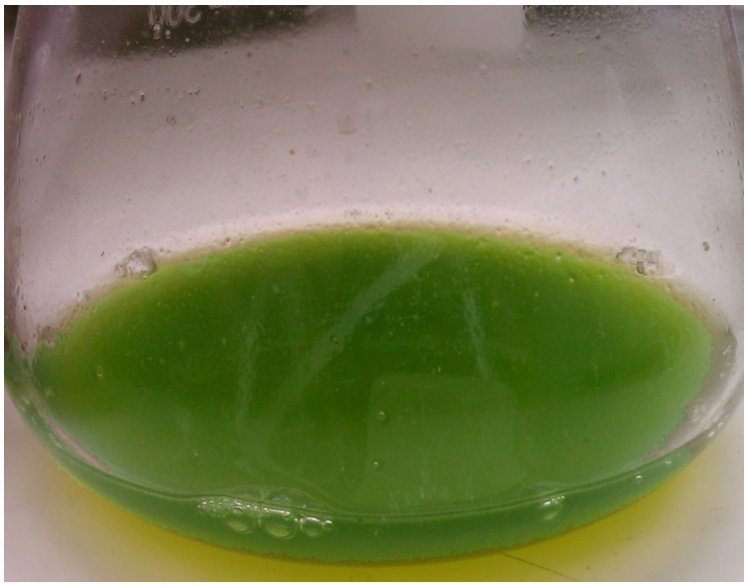

Figure 4 - 6 *C. Zofingiensis* at day 7 under 100 µmol m^{-2} s^{-1} and at 0.25 g/L of nitrate concentration, showing darkening of green colour.

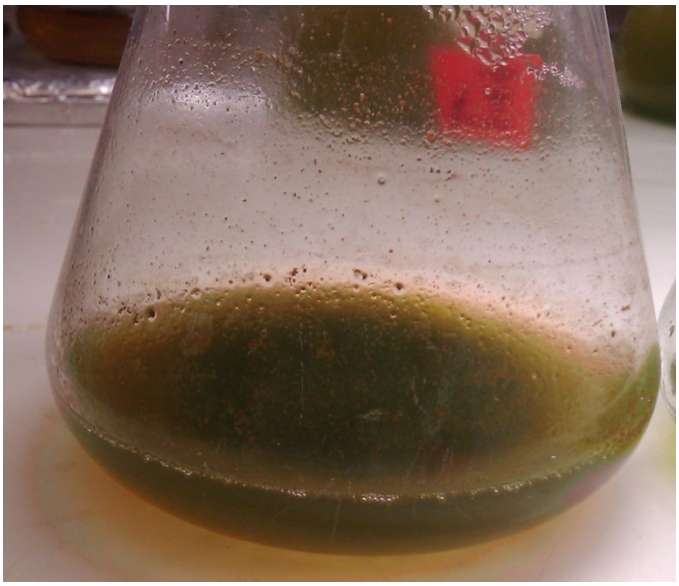

Figure 4 - 7 *C. Zofingiensis* at day 7 under 300 µmol m^{-2} s^{-1} and at 0.25 g/L nitrate concentration, showing mixture of green and red colour.

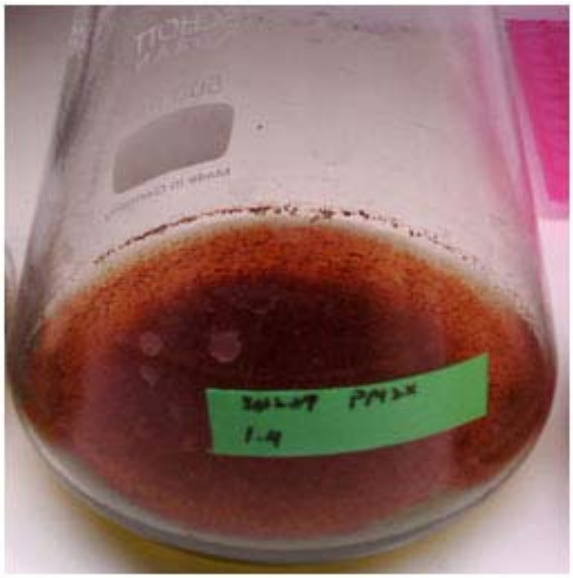

Figure 4 - 8 *C. Zofingiensis* at day 7 under 600 µmol m^{-2} s^{-1} and at 0.25g/L of nitrate concentration , showing mixture of red and black colour

4.1.2.3 Pigment Profiles in C. Zofingiensis

Results on chlorophyll a and b, total carotenoids and astaxanthin content in *C. Zofingiensis* under different configurations would be presented in this section.

Regardless of light intensity, cellular astaxanthin concentration decreased with increasing nitrate concentration (Figure 4 - 9a). At a nitrate concentration 1 g/L and low light intensity, astaxanthin content was the lowest, at 0.23 mg per gram of dry weight of *C. Zofingiensis*. Astaxanthin content increased with increasing light intensity (Figure 4 - 9). Astaxanthin content at high illumination was 20% higher than at mid- illumination, and 26% higher than at low-illumination.

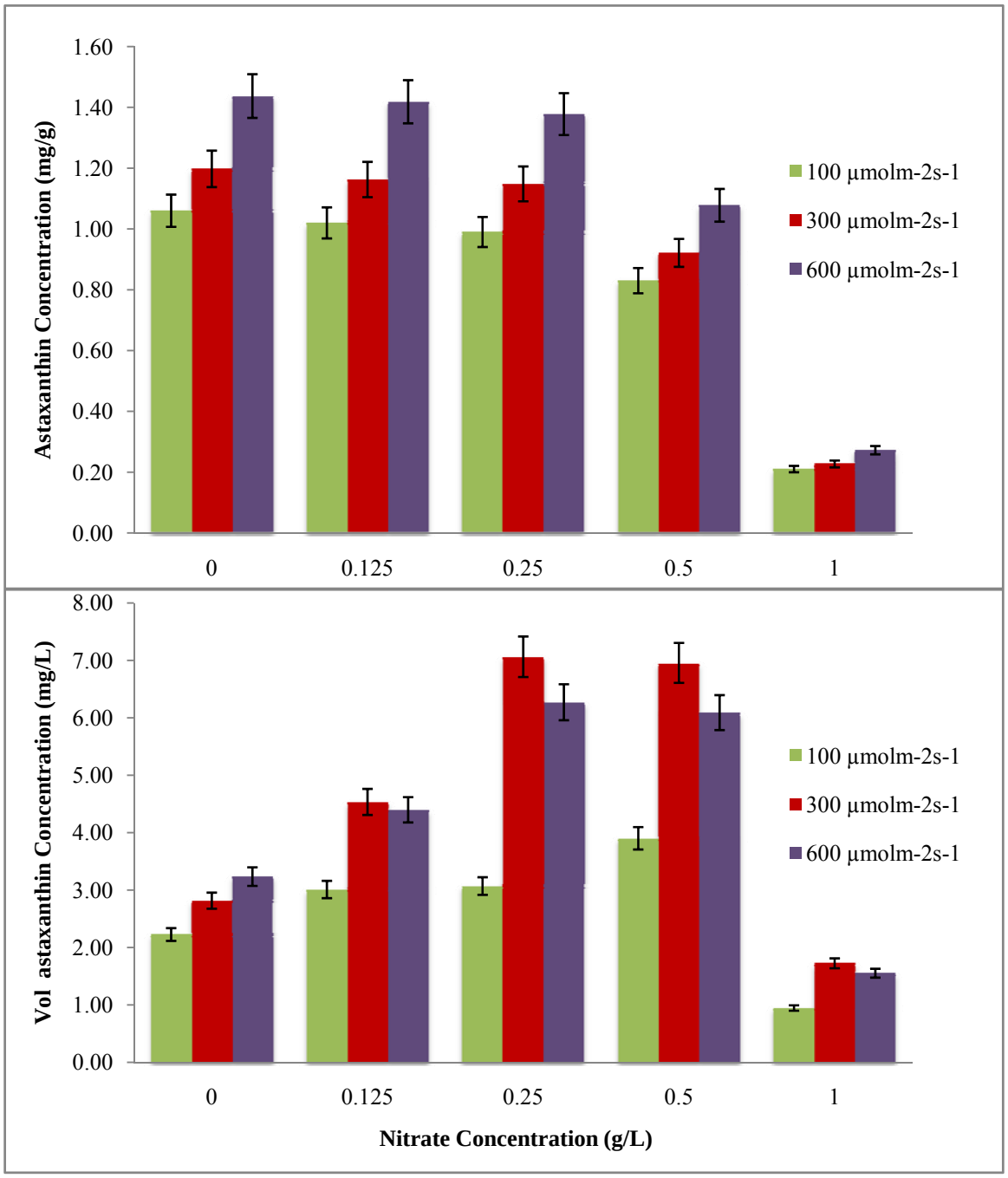

Figure 4 - 9a Astaxanthin concentration at day 10 was plotted against nitrate concentration at different light intensity. ; Figure 4 – 9b volumetric astaxanthin content plotted against nitrate concentration with changing light intensity

Multiplying astaxanthin content to dry weight concentration yielded volumetric astaxanthin concentration (Figure 4 – 9b). A different trend was observed. Volumetric astaxanthin production increased with increasing nitrate level, and was recorded highest at a nitrate concentration 0.25 g/L at mid light intensity. Similar yield was obtained at a nitrate concentration 0.5 g/L. At 1g/L, astaxanthin content was markedly lower than mid and high illumination. Production at mid illumination was the highest among the three illuminations used. At a nitrate concentration 0.25 and a light intensity of 300 μmol m^{-2} s^{-1}, a volumetric concentration of 7.06 mg/L was achieved.

Chlorophyll a and b

Chlorophyll a and b content increased with increasing nitrate concentration and decreased with increasing light intensity (Figure 4 - 10a). The highest chlorophyll content was observed at nitrate concentration 1 g/L with light intensity 100 μmol m^{-2} s^{-1} at 1.18 mg/g of microalgae. Comparing high light intensity to low light intensity, the decrease in chlorophyll level was about 35-40%.

Volumetric chlorophyll a and b concentration (Figure 4 – 10b) displayed similar results to the mass concentration with a clearer trend. Volumetric chlorophyll a and b level increased with increasing nitrate level, and was recorded highest at nitrate concentration 1 g/L at mid light intensity at 8.6 mg/L.

Total Carotenoids

Total carotenoids were seen to decrease slightly with increasing nitrate concentration and with increasing light intensity (Figure 4.11a). The highest total carotenoids concentration was 2.62 mg/g, observed at no nitrate level with light intensity 100 μmol m^{-2} s^{-1}. The lowest total carotenoids concentration was seen at high nitrate concentration and high light intensity.

Volumetric production of total carotenoids increased from nitrate concentration 0-0.5 g/L and decreased as nitrate concentration increased to 1 g/L (Figure 4 – 11b). Production at mid light intensity was the highest among all nitrate concentration and the highest concentration was 16.16 mg/L, seen at nitrate level 0.5 g/L and 300 μmol m^{-2} s^{-1}. The total carotenoids also included concentration of astaxanthin (a secondary carotenoid).

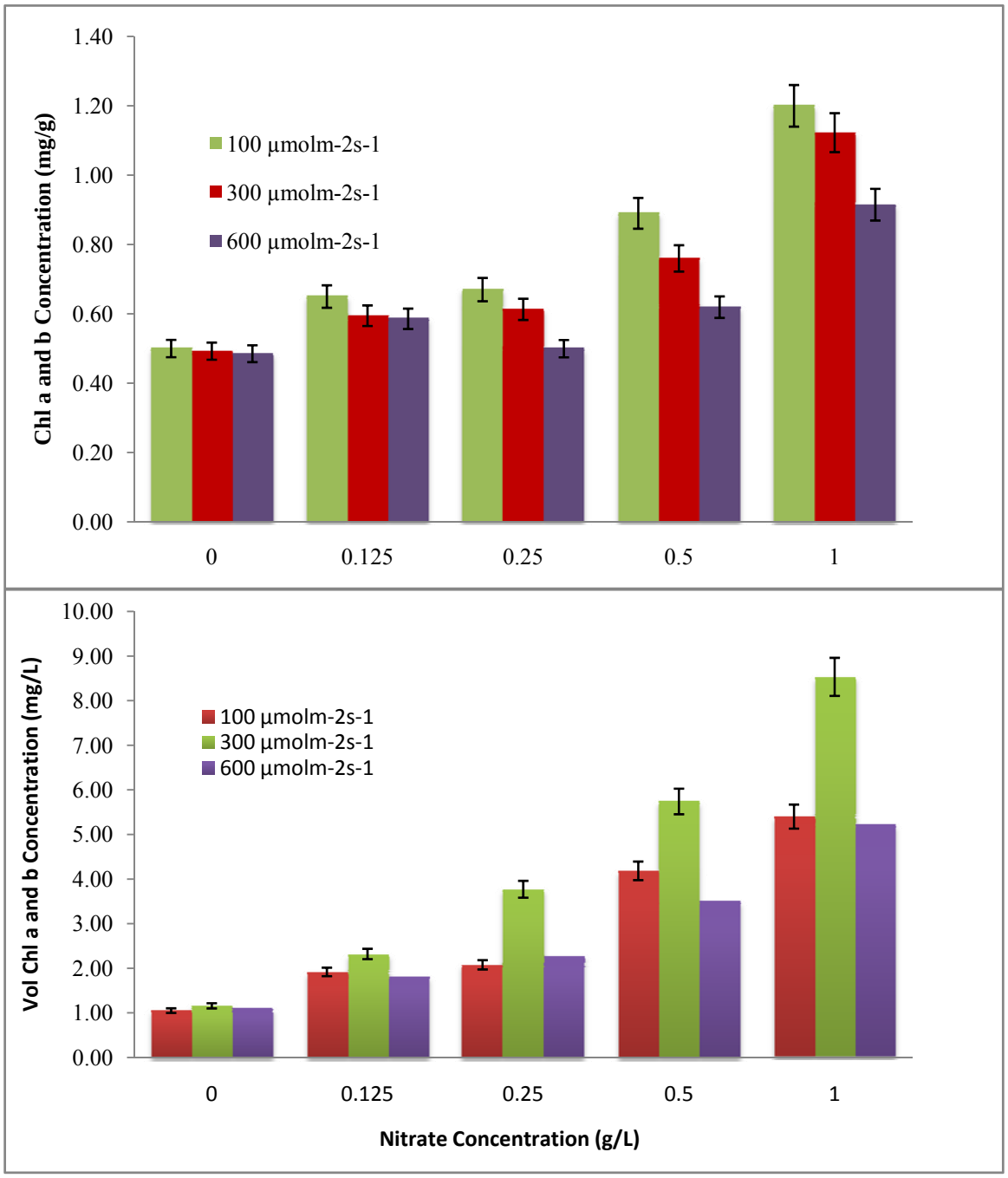

Figure 4 -10a Chlorophyll a and b concentration plotted against nitrate concentration with changing light intensity; Figure 4 – 10b Volumetric chlorophyll a and b concentration plotted against nitrate concentration with changing light intensity.

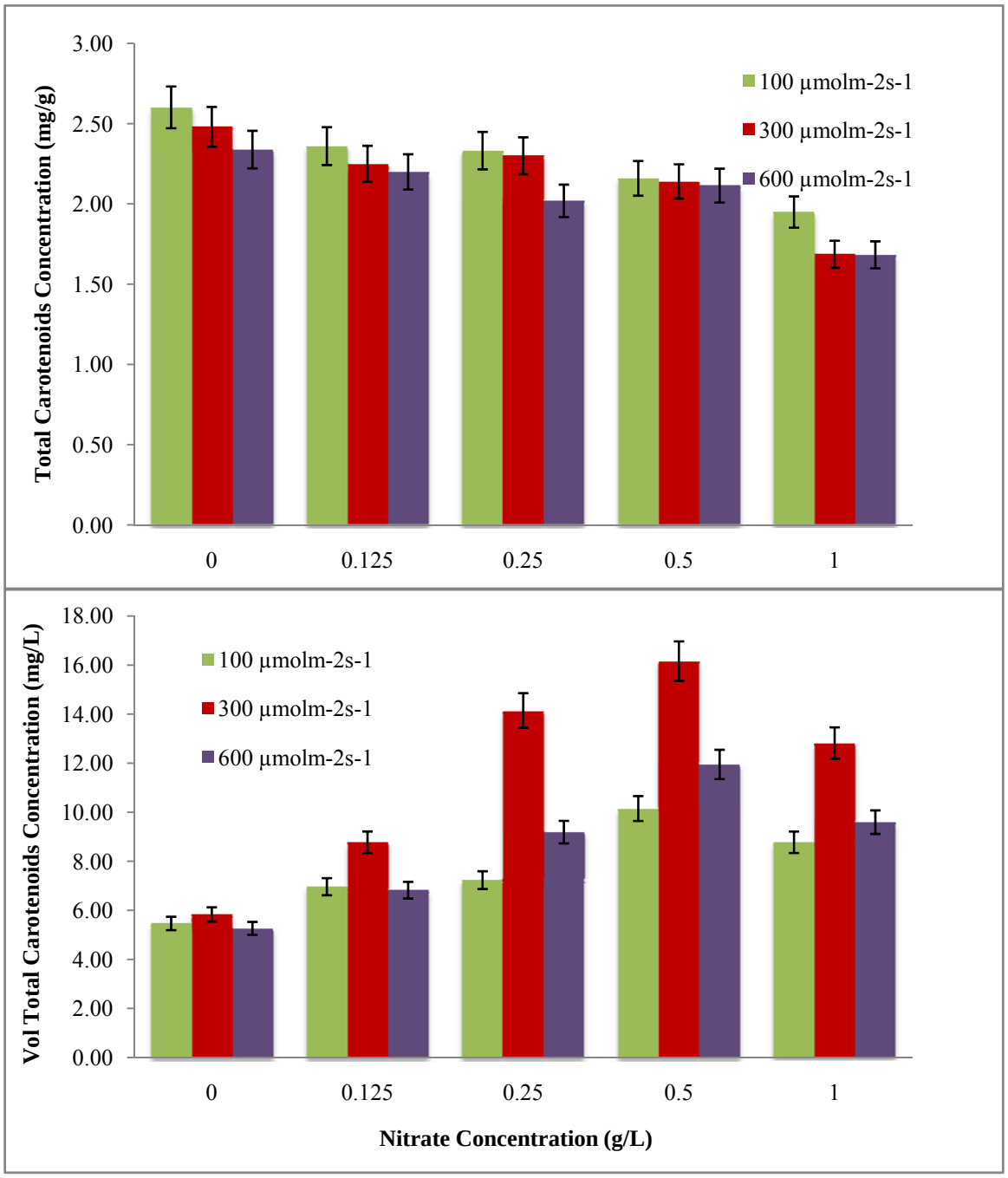

Figure 4 – 11a Total carotenoids concentration plotted against nitrate concentration with changing light intensity; Figure 4 – 11b Volumetric total carotenoids concentration plotted against nitrate concentration with changing light intensity.

4.1.2 Phase 2: Semi-Continuous Reactor Operation

4.1.2.1 Growth Rate with Changing Temperature

During the first growth cycle (Figure 4 - 12), all reactors exhibited the similar duration of lag phase (3 days) and exponential phase (4 - 5 days), with a maximum yield at approximately 8 g/L. It was followed by 4 days of stationary phase before diluting the culture to 3.5 g/L .

Reactors operated at 25 and 28˚C displayed shorter lag phase during the second cycle than samples operated at 22˚C. Exponential phase lasted for 4 days for all 3 temperatures. Yield obtained during the second cycle showed a 15% increment compared to the first cycle. Productivity at third cycle confirmed the maximum yield for all settings. Lag phase was not observed at the third and fourth cycle, and cycle duration was shortened from 8 days to 6 days. Lag phase was absent from the third cycle onwards.

Reproducible growth curves were seen from third growth cycle onwards. The optimum temperature for growth of *C. Zofingiensis* strain was 28˚C. Productivity at 28˚C was the highest, at dry weight concentration 9.24 g/L, followed by 25 and 22˚C.

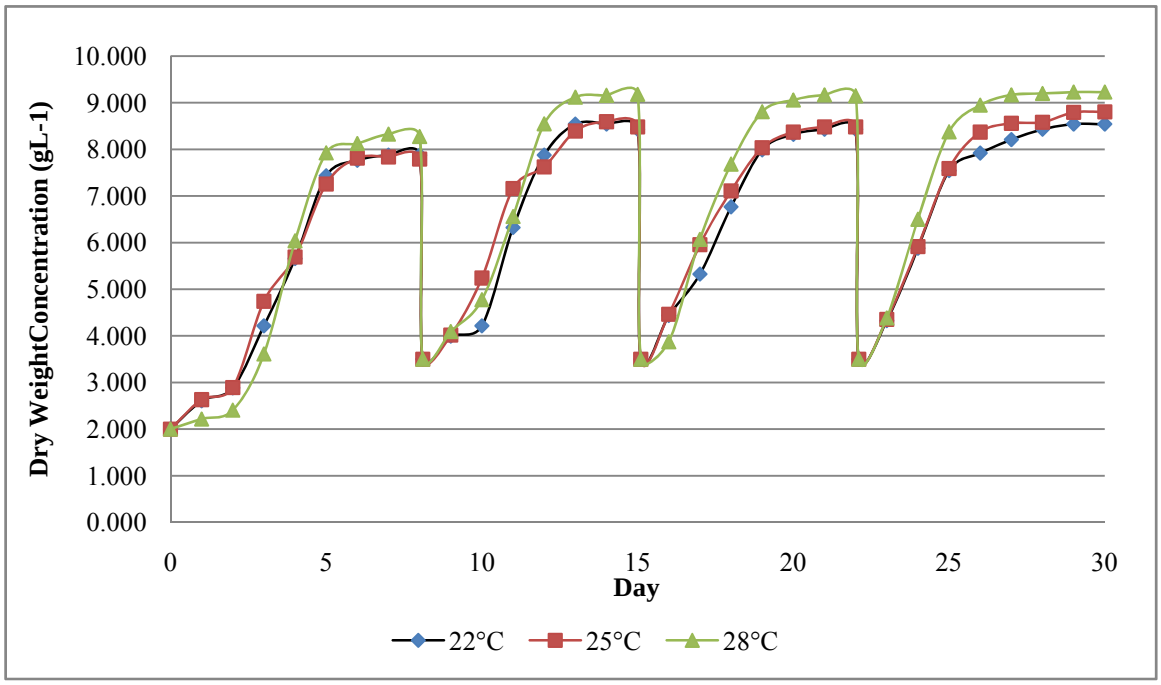

Figure 4 - 12 Growth curve of photobioreactor operated at turbidostatic mode for 30 days; subculture conducted on day 8, 16, 22 and 30.

4.1.2.2 Pigment Profile with Changing Temperature

Chlorophyll a and b, total carotenoids and astaxanthin were measured at the end of each cycle (Day 8, 16, 22, 30) and the results are presented in Figure 4-13.

Increment of temperature from 22 to 28˚C did not show significant changes in cellular pigment percentage. Percentage of chlorophyll a and b concentration fluctuated around 18-20% of total pigments. Volumetric chlorophyll a and b was about 4 mg/L. Total

carotenoids (excluding astaxanthin) decreased with increasing temperature and cell growth cycle, suggesting that some of the carotenoids could be converted to astaxanthin.

Astaxanthin (secondary carotenoids) concentration was seen to increase with increasing temperature and cell growth cycle. Astaxanthin level increased from 0.60 mg/g on day 8 to 0.69 mg/L on day 22, which has a 15% increment. Levels at day 22 and day 30 were approximately the same, suggesting that cellular astaxanthin concentration probably reached its maximum. Result from day 30, showed that astaxanthin concentration at 28˚C was higher compared to at 22 and 25˚C. Increment in temperature led to an increment of 12-15% in cellular astaxanthin concentration.

Generally, temperature changes from 22 to 28˚C did not show a sharp change in volumetric level of pigments (Figure 4 - 14). Though volumetric production at higher temperature did produce slightly better yield compared to lower temperature (12% difference). Yield improvement from increased growth cycles and cell aging displayed 27% improvement in cellular volumetric astaxanthin level.

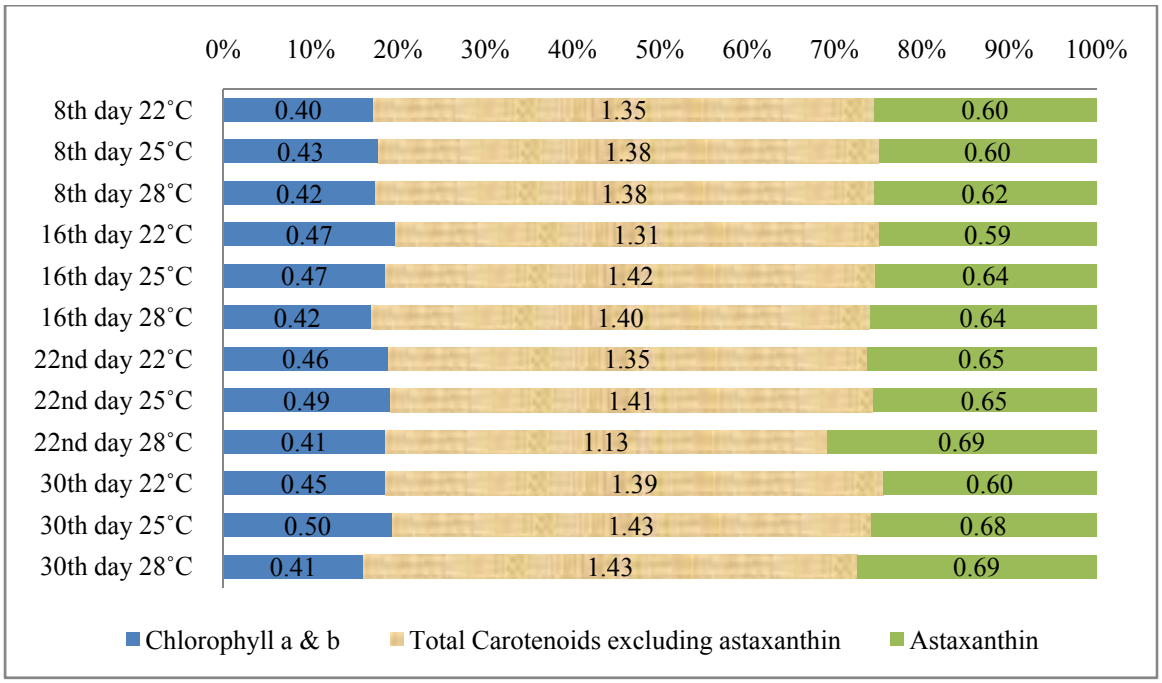

Figure 4 - 13 pigment concentration (mg/g) of *C. Zofingiensis* represented in percentage at day 8, 16, 22 and 30 for temperature 22, 25 and 28˚C.

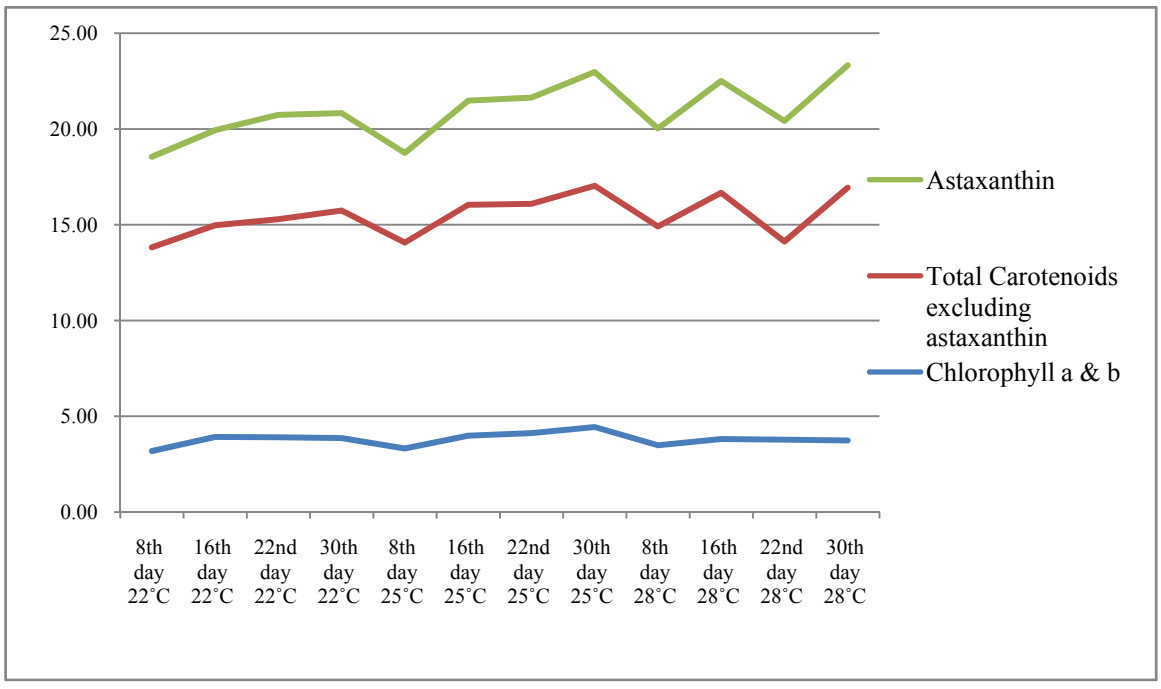

Figure 4 - 14 volumetric pigment concentration (mg/L) of *C. Zofingiensis* represented in percentage at day 8, 16, 22 and 30 for temperature 22, 25 and 28˚C.

4.2 Discussion

4.2.1 Nitrate Concentration Effect

Nitrate had shown to affect the growth of *C. Zofingiensis* and its pigment composition. Poor algal growth was observed in the nitrate-starved medium (Figure 4 - 3), whereas an addition of nitrate up to 0.5 g /L did show a significant growth improvement. The effect of nitrate concentration on the pigment content of *C. Zofingiensis* is shown in Figure 4 - 9a, Figure 4-10a and Figure 4-11a, where it could be seen that the cellular levels of total carotenoids decreased in response to increasing nitrate concentration in the medium. Using a different light source and intensity, Del Campo (2004) reported a maximum yield of 9.7 g/L. The maximum yield obtained for nitrate concentration 0.5 g/L at light intensity of 300 μmol photon m^{-2} s^{-1}, at 7.55 g/L, were lower to results from other research groups. (Bar, et al. 1995; Ip and Chen 2004; Del Campo, et al. 2004; Rise, et al. 1994) This might be caused by differences in lighting and method of cultivation. Nitrate concentration clearly indicates that nitrate is essential for stimulating cell growth. Under nitrogen-starved/limited conditions, it has been shown that the specific activity of nitrate reductase, an enzyme responsible for assimilation of nitrate in culture medium, is very low. It will greatly affect the normal cellular metabolism of the algae, resulting in extremely slow growth and low biomass (Darley 1982).

Similar to our findings, optimal growth of *H. pluvialis* was severely affected when grown in the absence of nitrate (Darley 1982). This further supports that nitrate was an important nitrogen source for the algal growth. As shown in Figure 4 - 4, it was noticed

that the growth of *C. Zofingiensis* was saturated at 0.50 g/L ofnitrate and excess nitrate supply (1 g/L) resulted in a significantly higher amount of chlorophylls and primary carotenoids inside the algal cells (Figure 4-10a, Figure 4-11a). Our experimental results showed that low nitrate concentration was favourable for astaxanthin accumulation. It was previously investigated that secondary carotenoids such as astaxanthin could be synthesized in the algal cells only when the chlorophylls and primary carotenoids were not sufficient for protecting the algae against environmental stresses such as high light intensity and nitrogen starvation (Rise, et al. 1994). Therefore, the high contents of chlorophylls and primary carotenoids at 1 g /L of nitrate might be a factor suppressing the biosynthesis of the secondary carotenoids and astaxanthin in *C. Zofingiensis*.

The increased astaxanthin pigment content was examined in nitrate-free medium in other green algae species such as *H. pluvialis* and *Chlorococcum* sp. (Rise, et al. 1994; Bar, et al. 1995; Orosa, et al. 2000). It was postulated that nitrate-free growth medium was faourable for astaxanthin formation. On the contrary, Boussiba (2000) reported that nitrate was essential for astaxanthin accumulation in *H. pluvialis* and was required for continuous synthesis of protein responsible for supporting the pigment formation.

4.2.2 Light Effect

It has been known that plants and algae develop secondary carotenoids to protect against potential damaging effects of oxidative stress by ROS, created as a by-product of photosynthesis (Bar, et al. 1995). Extreme environmental stresses both resulted in

overproduction of ROS, which may lead to the activation of stress-related genes involved in signaling, protection, and ROS scavenging. As such, light regulation of carotenoid biosynthesis is commonly observed in plants and algae. (Bohne and Linden 2002; Steinbrenner and Linden 2001; von Lintig, et al. 1997) The treated cells accumulated a much higher level of and astaxanthin (Figure 4 - 9a), which may protect *C. Zofingiensis* against high-light, as elucidated in higher plants and other green algae (von Lintig, et al. 1997; Zhang and Lee 1997).

Level of light irradiance did not show much effect on the level of chlorophyll a and b. Both cellular and volumetric chlorophyll a and b remained approximately the same constantly for different configurations. However, it had shown profound influence on the carotenoid profile, especially cellular astaxanthin concentration. The astaxanthin level was about 2-fold higher in cells of cultures at high irradiance (600 μmol photon m^{-2} s^{-1}) than in those at low irradiance (100 μmol photon m^{-2} s^{-1}). This could also be seen from the changes in physical appearance of *C. Zofingiensis* from green (Figure 4 - 5) to red (Figure 4 - 8). If the total cellular carotenoids concentration remained about the same percentage, the explanation to this would be some of the primary carotenoids were converted to astaxanthin (secondary carotenoid) as a strategy for survival. As explained earlier, secondary carotenoids had the effect of scavenging ROS from the cell to prevent damages to the cell. The above findings on the changes in pigment composition were consistent with other research done on *H. pluvialis, Chlorella sorokiniana* and *Chlorococcum* (Bar, et al. 1995; Aflalo, et al. 2007; Boussiba and Vonshak 1991; Del

Campo, et al. 2000; Yuan, et al. 2002). In general, increasing the light intensity enhances the accumulation of astaxanthin and suppressed the primary production of carotenoids and chlorophyll.

Light irradiance also had profound effect on cell production. It had been observed that specific growth rate at 300 μmol photon m^{-2} s^{-1} was higher than those recorded at 100 and 600 μmol photon m^{-2} s^{-1}. At high intensity, growth rate of *C. Zofingiensis* was lower than mid-light intensity. This might be due to photo-inhibition of algal growth, caused by excessive radiance on plants and microalgae. At high light intensities, chlorophyll can be damaged by the enhanced activity of electrons beyond that which it can process. This resulted in photo-inhibition by decreasing the photosynthetic capacity. It resulted in changes in pigment composition, with less chlorophyll and primary carotenoids generated. A prolonged exposure of plants or organisms to excessive radiation may result in the photodestruction of the photosynthetic pigments, since the discoloration (bleaching) of these pigments depends on oxygen and light; this phenomenon is normally called photooxidation, and it may cause the death of the cell or the organism (Powles, 1984; Hendrey et al., 1987). We postulate that astaxanthin produced in algae could function as photoprotective filters, reducing irradiation of the cell components, serve as antioxidants preventing accumulation of oxygen radicals, and/or act as a hydrophobic layer, reducing water losses upon dehydration or salinization (Bar et al. 1995; Boussiba 2000). In general, astaxanthin accumulates in certain algal strains in response to a variety of stress

conditions. The most thoroughly studied microalga in this respect is *H. pluvialis* (Boussiba 2000).

Combining the effects of irradiance on growth and pigment composition yields a more practical result. It is important to enhance growth of algae without experiencing photo-inhibition. From the experimental results, a maximum volumetric astaxanthin content of 7 mg/L was obtained from nitrate concentration 0.25 g/L at 300 μmol m^{-2} s^{-1}. The nitrate concentration was different from that reported by Del Campo (2004) and Ip and Chen (2005). Del Campo found the optimum nitrate concentration at 0.5g/L and irradiance of 700 μmol photon m^{-2} s^{-1}, with a volumetric yield of 8.24mg/L. Ip and Chen found similar volumetric yield, but the irradiance used was 500 μmol photon m^{-2} s^{-1}. Though the nitrate/lighting configurations used were different, the results showed similarity in yield and thus it could be said that an optimum configuration has to be determined even for the same algae culture.

4.2.3 Temperature Effect

Temperature plays a major role in photo-inhibition and light damage. Our results indicate that elevated temperatures from 22 to 28˚C promoted growth in *C. Zofingiensis*, and it was favourable enhanced the production of astaxanthin. In the present study, the specific growth rate increased with a rise in temperature from 22 to 28°C. Temperature above 28˚C was not tested in the current experiment, but results from other research group on

other species indicated that operating temperature beyond 30°C would lead to decrease in productivity. (Dauta, et al., 1974; Tjahjono, et al. 1994) This is consistent with the specific growth rate of *H. pluvialis* increased with a rise in temperature from 20 to 28°C; a further increase in temperature caused a decline in specific growth rate (Fan, et al., 1994).

The increased growth observed between 22 and 28˚C can be explained by increased activity of enzymes of the reductive pentose cycle. It is reported that the rate of photosynthesis decreased at 30˚C, and ceased completely at 34-36˚C. Algal cell respiration did not stop, suggesting that the cells were not dead until temperature was above 45˚C, where proteins start to breakdown. (Goldman and Carpenter, 1974) The data indicate that the algae themselves perceive and respond to elevated temperature accordingly.

By raising the cultivation temperature of *H. pluvialis* from 20 to 35˚C, Tjahjono (1994) obtained large amounts of astaxanthin. The amount of astaxanthin accumulation in *H. pluvialis* is usually around 30-35 mg/g of microalgae, operated at 25-28˚C. By altering the nutrient level, light intensity and slowly increasing operating temperature, Tjahjono (1994) reported an astaxanthin concentration of 42.6 mg/g. (Tjahjono, et al. 1994) Temperature had a stimulating effect on carotenoid accumulation in the cells, with the levels of astaxanthin being markedly enhanced; the astaxanthin level was reported highest from the current experiment. The extent to which *C. Zofingiensis* may acclimate

or adapt to elevated temperatures was not addressed in this study, but it has been proven that green algae have remarkable adaptable ability to high temperature. Our results showed that algae could be engineered and acclimated to different adverse environmental conditions to produce higher yield of algal products.

However, studies have indicated that growth of these algae is markedly reduced above 30˚C, suggesting a genetically fixed temperature limitation. (Harker, et al. 1996) As the algae exposed to the highest temperatures still respired, the possibility exists that they may recover, but recovery from thermal stress was also not addressed in this study.

4.2.4 Relationship between Algal Pigments

From the current study, it could be seen clearly that astaxanthin level increased with decreasing chlorophyll and other carotenoids level. Though the exact pathway for astaxanthin remains inconclusive, it is believed that astaxanthin, a secondary carotenoid, uses β-carotene (a primary carotenoid) as a pre-cursor for astaxanthin production (Misawa, et al. 1995). (Refer to Figure 4 – 15 for schematics of the carotenoid pathway) It is therefore consistent with our observation. During times of environmental stress, normal cell activities will cease and go into "shock", affecting the composition of algal pigments. It was previously studied that nitrate stress would not directly cause a decrease in chlorophyll a and b (Sanchez, et al. 1982), but it decreases the cell's metabolic

activities. Coupled with high light intensity, *C. Zofingiensis* showed resistance by producing astaxanthin, a type of secondary carotenoids for its survival.

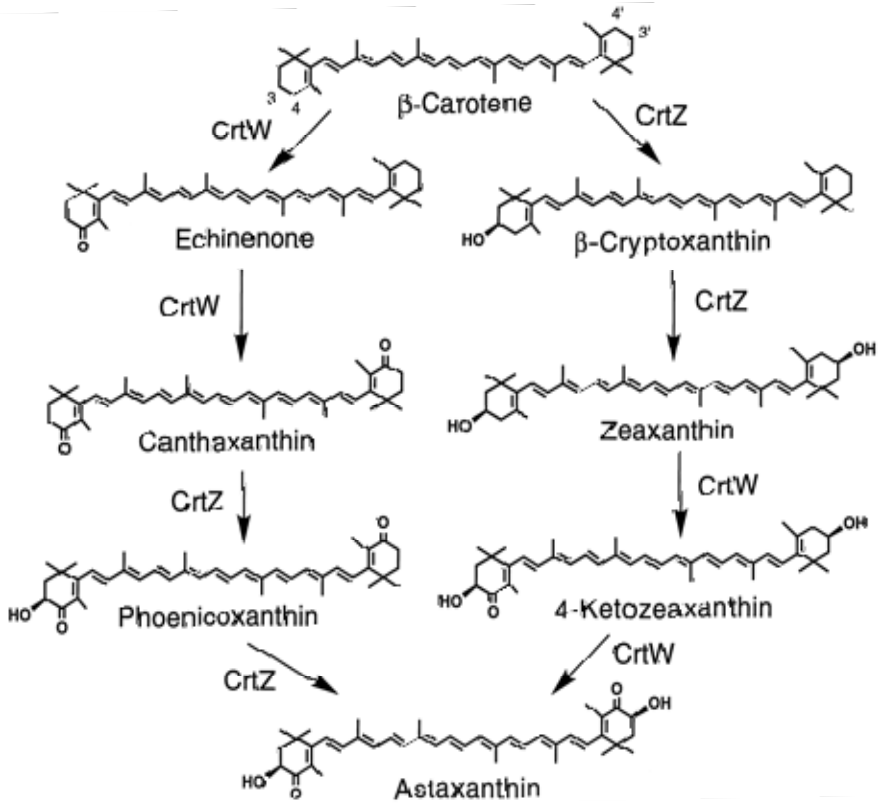

Figure 4 - 15 Postulated astaxanthin biosynthetic pathway deduced from in vivo complementation studies (Misawa, et al. 1995)

4.2.5 High Light and High Temperature: Practical Implication

The combination of high temperature and full sunlight is an ordinary occurrence that characterizes summer conditions in most parts of the world. Photo-inhibition of photosynthesis in situ may happen in full sunlight, even in the absence of any other stressing factor, despite the occurrence of a high temperature (Dauta, et al. 1990). When

the temperature rises above the optimum level, photosynthesis begins to decrease. At first the decline is gradual and reversible, but after a critical temperature is reached it becomes slow and irreversible (Darley 1982).

According to these authors, reversible inactivation of photosynthesis reflects damage to the chloroplasts that persists for some time after the plant is returned to favorable temperature conditions. Ögren (1988) verified the occurrence of photo-inhibition under full sunlight in the leaves of *Salix* sp. Laing et al. (1995), for *Phaseolus vulgaris*. (Laing and Greer 1995, Ögren 1988) These authors noticed an inhibition of 25% in the vegetative growth rate of *P. vulgaris* plants acclimated to 25°C at 1,300 μmol m^{-2} s^{-1}. They also found out there was a decrease in the rate of photosynthesis in the field, under high light and high temperature conditions.

4.2.6 Optimization of Reactor

It was observed that the performance of batch reactor system was more superior than reactor operated at turbidostatic semi-continuous mode. Overall, we noticed a significant decrease in the cellular astaxanthin concentration, from 1.56 mg/g to 0.69 mg/g of *C. Zofingiensis*, or a 2.2 times decrease. However, volumetric astaxanthin content in continuous reactor remained highly similar (6.73 mg/L in batch system; 6.23 mg/L in continuous system), due to higher yield achieved in continuous system. It can further hypothesize that the actual production of astaxanthin, given adjustment in light manipulation, mixing rate, reactor design and light/dark cycles, would result in higher

productivity than batch systems. The possible reasons for the low astaxanthin content would be discussed in the next section. Though these factors are not studied in the current experiment, future work on using *C. Zofingiensis* in PBR should address these issues.

4.2.7 Liquid Mixing Rate

In an air-lifting flat-bed reactor system, the determining factor in liquid mixing is its gas flow rate. Increasing the gas flow has its pros and cons for reactor operation. It results in an increase in gas bubble and irregularity in size of gas bubble. This lead to light dispersion and light transmission through the bubbles, allowing a further penetration of light along the reactor optical path (Miro´n, et al. 1999, Molina, et al., 1999, Fernández, et al., 2001). The exact effect is more pronounced in high density culture systems, where steep light gradient dictates across the reactor.

At a certain light regime, the effect of mixing results in a general improvement in productivity. The growth rates of some microalgae have been reported to increase initially with increasing turbulence, probably due to the improved supply of light or CO_2. As soon as the optimum level of turbulence has been reached, however, growth decreases sharply with further increase of the superficial-gas velocity (Barbosa, et al. 2004, Chisti and Moo-Young, 1989, Moo-Young and Chisti 1988). These observations are consistent

with our results. They found that a further increase will not lead to an additional increase in the mean liquid velocity, instead the additional energy input leads to increased fluctuation velocities (> 0.06 ms^{-1}) and to an increased turbulent dissipation (Moo-Young and Chisti 1988). Therefore other options to improve fluid dynamics have to be considered.

4.2.8 Hydrodynamic Stress and Cell Death

Cell damage can take place during bubble formation, bubble rising or bubble break-up. Very few quantitative studies have been done regarding hydrodynamic stress in microalgae cultures grown in gas-sparged photobioreactors (Carvalho, et al. 2006). It has been assumed until recently that the main cause for cell damage was the bubble bursting at the liquid surface. However, it is observed that bubble formation is responsible for cell damage and that cell death increases with increasing gas entrance velocity beyond a certain critical value, which is strain dependent. Barbossa (2000) concluded that the gas velocity at the sparger is an important parameter for reactor design and operation.

Chapter 5 Conclusions and Recommendations

5.1 Conclusions

In the present study, the use of nitrate, light intensity and temperature has been shown to effectively manipulate and enhance astaxanthin production by *C. Zofingiensis*. It was reported that *C. Zofingiensis* accumulate astaxanthin as main carotenoids (Orosa, et al., 2000). Astaxanthin is a typical secondary carotenoid which accumulates in lipid bodies located outside the chloroplast (Bar et al. 1995; Orosa et al., 2000). The role of secondary carotenoids and the pathway of biosynthesis in algal cells is not fully understood. *C. Zofingiensis* under nitrogen starvation and high light irradiance induced a drop in chlorophyll and other carotenoids (excluding astaxanthin) and the concomitant accumulation of secondary carotenoids (astaxanthin).

In the present study, different conbinations of nitrate concentration and light intensity were employed in the experiment. In terms of *C. Zofingiensis* yield, the batch system achieved a dry mass of 7.55 g/L, produced at 0.5 g/L of nitrate and at light intensity 300 mol photon.$m^{-2}s^{-1}$. It is lower than the yield reported by (Dan Pelah 2004, Del Campo, et al. 2004), which was reported at 12.5 g/L. Production at 0.5 g/L and 1 g/L gave similar result, except that chlorophyll a and b level were found to be 34% higher than nitrate

concentration 0.5g/L. The production of excess chlorophyll caused by high nitrate concentration could be seen more pronouncedly especially at low and no light level. Growth of *C. Zofingiensis* was severely affected by the lack of nitrate in the medium. Growth at 0.125 g/L displayed extremely low productivity (0.09 day^{-1}), and culture entered death phase after 4 days of culture for no nitrate level. Astaxanthin level was seen to decrease with increasing nitrate concentration. The optimum nitrate concentration for astaxanthin production was found at no nitrate level. However, volumetric astaxanthin yield revealed a different story. Production at nitrate concentration 0.5 and 0.25 g/L produced the highest amount of astaxanthin.

The production of astaxanthin might be further enhanced by improving the cellular astaxanthin content of the alga through applying high light intensity to the culture. It has been investigated that the production of reactive oxygen species, a harmful by-product generated at high light intensity, would increase the production of secondary carotenoids such as astaxanthin. Secondary carotenoids are known to protect the photosystem of microalgae by scavenging the ROS radicals.

From the experiment, a positive relationship was found between astaxanthin concentration and light intensity, which was consistent with journal publication. Though high light intensity induced high astaxanthin level in *C. Zofingiensis*, the volumetric astaxanthin content was low due to slow growth rate. Photo-inhibition was seen at 600

umol m^{-2} s^{-1}, and the effect was more evident at low nitrate concentration. The maximum volumetric astaxanthin obtained was found to be 7.06 mg/L, which was similar to the highest value in the culture for the level of the total astaxanthin under nitrogen and light stress reported by Bar, et al. (1995). Yet, the astaxanthin concentration in this experiment is still 17% lower than similar batch systems using acetate reported by Del Campo (2004). Though the nitrate/lighting configurations used were different, the results showed similarity in yield and thus it could be said that an optimum configuration had to be determined even for the same algae culture. Performance of such configuration was also 40% lower than the highest volumetric astaxanthin concentration obtained when *C. Zofigiensis* grown heterothrophically with glucose in the dark (Chen and Chen 2006). However, heterotrophic algae cultivation presents high production cost and other challenges, which has been discussed in chapter 2.

Therefore, when correlated with the volumetric level of carotenoids in the culture, the changes observed follow analogous trends, provided that the particular stress condition considered does not hamper growth severely, thus overcoming its positive effect on the cellular accumulation of carotenoids. Should this happen, as is in the case for nitrate limitation, a reduction in volumetric of carotenoids in the culture may occur. Alternatively, a stimulatory effect of a given factor on growth may add to the positive effect on astaxanthin accumulation or compensate/overcome an inhibitory effect, as is in the case for irradiance.

When evaluating or optimizing the yield of a particular metabolite by microalgal cultures, the nitrate concentration and light intensity have to be jointly and carefully considered. The highest volumetric productivity of astaxanthin in *C. Zofingiensis* cultures is recorded at nitrate concentration 0.25 g/L, when growth was optimal, despite the fact that the cellular astaxanthin level was not maximal, being significantly higher at 0.125 g/L or no nitrate. A similar situation is observed for the effect of irradiance on β-carotene accumulation. Moreover, the enhancing effect on growth of high irradiance more than compensated for the negative incidence of the latter on the astaxanthin level in cells. Maximal levels of astaxanthin in *C. Zofingiensis* strain (about 3–6 mg g^{-1} dry weight), were admittedly lower than those reported for β-carotene in *Muriellopsis* (Del Campo, et al. 2000) and for astaxanthin in *H. pluvialis*. Nevertheless, the fast growth exhibited by this strain of *C. Zofingiensis* and the high cell population achievable in culture can compensate for the above quoted drawback. As a matter of fact, the potential yield for astaxanthin achieved in batch cultures of *C. Zofingiensis* exceeded 15 mg /L. These values compare favorably with those found in the literature (Del Campo, et al. 2004, Zhang and Lee 1997)

Overall, the results in this study shown that *C. Zofingiensis* is an attractive candidate for the mass production of astaxanthin in continuous reactor, being plausible for selectively favoring the production astaxanthin through the adequate management of growth conditions. This microalgal strain also represents a unique model system for advancing

the knowledge of differential regulation during the synthesis astaxanthin in response to a different nitrate and light intensity.

5.2 Recommendations and Direction for Future Research

Future research could perhaps explore the following options for *C. Zofingiensis*:

1. Use of *C. Zofingiensis* for production of other valuable carotenoid pigments

C. Zofingiensis is also known to produce other types of secondary carotenoids under stress, which are also valuable commercially. Carotenoids such as lutein and canthaxanthin.

2. Employment of chemicals to induce environmental stress

Chemicals such as acetate, malonate, or other organic acids and salts could be added to enhance production of astaxanthin in *H. Plauvialis*. (Boussiba et al., 2000) Similar treatments could be done on *C. Zofingiensis* to enhance the production of astaxanthin. Thus far, only simple treatments such as salt stress, nitrate or light intensity have been conducted. There are still many methods to enhance the production of secondary carotenoids in *C. Zofingiensis.*

3. Manipulating light regimes, circulation rates, mass transfer and shear stress

As discussed in chapter 4, these factors are known to have profound effects on both cellular production and pigment composition. Moreover, these operational parameters cannot be controlled independently, as they are closely interrelated. Presently, the bottleneck for the development of microalgal biotechnology is the lack of cost effective large-scale cultivation systems. High volumetric productivities are required in order to reduce the size of cultivation systems and, consequently, reduce production and downstream processing costs. This entails high biomass concentrations and a high efficiency of light utilisation. Optimizations of these factors are essential especially for scaling up or for commercial production of astaxanthin.

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