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Growth and Total Carotenoid, Chlorophyll a and Chlorophyll b of Tropical Microalgae (*Isochrysis* sp.) in Laboratory Cultured Conditions

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Abstract: A study was conducted to determine the total content of carotenoids, in *Isochrysis* sp. under stress condition (nitrogen starvation). *Isochrysis* sp. was cultured in f/2 medium at optimum light intensity (1200 Lux) with constant aeration (4.5 L min^{-1}) at 8 pH. Salinity of the water was maintained between 20-24 g L^{-1} and temperature range was 20-24°C. The culture of *Isochrysis* sp. showed a short lag phase lasting about 24 h followed by exponential phase starting from day 2 until day 10. In day 4, specific growth rate (μ) for *Isochrysis* sp. was 1.6059 day^{-1}, the highest rate compared to other day. Stress parameter (NO_3^- starvation) was introduced into the mass culture on day 4 by eliminating NO_3^- in f/2 media. There was considerable declining ($p<0.05$) in cell count was observed in the absence of nitrogen proving the dependency of carotenoid production over the nitrogen limitation which probably due to the main role played by the nitrogen in various metabolic activities of the cell. The highest total carotenoid per cell was recorded at day 10 with 0.001932347 mg mL^{-1}, whereas the lowest total carotenoid content per cell was observed at day 4 with 0.000100649 mg mL^{-1}. The finding reveals that it is best to harvest the carotenoid on day 10, when the maximum carotenoid can be obtained.

Key words: Carotenoids, *Isochrysis* sp., mass culture, metabolic activities and exponential phase

INTRODUCTION

Carotenoids are sky-scraping in demand in global market owing to its widespread industrial application food processing, pharmaceutical and medicinal purposes. Currently, the worldwide carotenoids market is forecast to accumulate $1172.6 million in 2011 (http://www.bccresearch.com/report/FOD025B.html). The increasing demand for functional foods and nutraceuticals will also trigger an increase in use of carotenoids by the food industry globally. This has stimulated research and development of carotenoid from naturally occurring sources especially microalgae because they are among the fastest growing autotrophs on earth, which utilize commonly available material for growth, high productivity (Ausich, 1997). Besides, microalgae can be produced in controlled condition with a low cost due to its ability to grow in a wide variety of environments favors to an exceptional biochemical production (Moreau *et al*., 2006).

Although, the classical source of carotenoids is plants, they are also available in animals and microorganisms (Lopez-Ruiz *et al*., 1995). However, the great interest in studying these compounds is due to their physiological and biological functions which have been extensively and in detail revised by Van den Berg *et al*. (2000). In addition to the provitamin, A activity of some carotenoids, they also have other functions, such as antioxidants and enhancers of the immune response (Hughes *et al*., 2000). Recently, epidemiological studies have indicated an association between high vegetable intake and a lower risk of chronic degenerative diseases such as certain types of cancer, cardiovascular diseases (Machlin, 1995) and age-related macular degeneration (Bone *et al*., 2000). According to a report by Business Communications Company, (http://www.bccresearch.com/report/FOD025B.html), the worldwide market value of commercially-used carotenoids was US $886.9 million in 2004 and it was expected to break the billion dollar barrier

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by 2011. Microalgae are the untapped resources with more than 50,000 species exist but only a limited number, of around 30,000, have been studied and analyzed (Richmond, 2000).

Significant attention has recently been drawn to the use of microalgae for deriving functional food, as microalgae produces a great variety of metabolites that are essential for human health including proteins, enzymes, bioactive compounds and carotenoids (Sandmann, 2001). In microalgae, carotenoids function as accessory pigments in the photosystems, as structural components of light harvesting complexes, as well as photoprotective agents and also playing role in photoaxis (Hagen *et al*., 1993; Taylor, 1996; Eskling *et al*., 1997). However, most carotenoids are not easily detected *in situ*, since their presence is masked by other pigments, especially the chlorophylls. The ability of carotenoids to fulfill light harvesting and protective functions in photosynthetic membrane is due to both, the properties of their excited electronic states and to their special organization in pigment-protein complexes. Since 1950s, a number of prymnesiophyte flagellates identified as *Isochrysis* sp. have been cultured and exchanged between academic, commercial and government facilities. The type species, *Isochrysis galbana*, was described from cultured material (Flagellate I) by Parke (1949) and amended by Hori and Green (1991). The *Isochrysis* sp. cells have no distinct cell wall as confirmed by Zhu *et al*. (1997) and only possess a plasma membrane covering. Cells are generally solitary, motile, 5-6 μm long, 2-4 μm wide and 2.5-3 μm thick in ellipsoid forms. there are two flagella, more or less equal, smooth, approximately 7 μm long, cells inserted with abbreviated haptonema; normally plastid usually single, parietal, yellow-brown with an immersed fusiform pyrenoid, the latter traversed by a pair of thylakoids, resembled that described for *I. galbana* previously (Green and Pienaar, 1977). The cells were fragile and plasmolysis occurred when the naked cells were exposed to a sudden change of osmotic pressure.

Isochrysis sp. has been utilized successfully as food source for a variety of bivalve mollusks (Enright *et al*., 1986) shrimp and marine fish larvae (De Pauw and Persoone, 1988). It is known as one of the most commonly used marine unicellular algae in many mariculture systems (Sukenik and Wahnon, 1991). Renaud *et al*. (1999) posited that, microalgae must possess a number of key attributes to be useful in aquaculture species. They must be of an appropriate size for ingestion, e.g. from 1-15 μm for filter feeders; 10-100 μm for grazers (Webb and Chu, 1983) and readily digested. They must have rapid growth rates, be amenable to mass culture and also stable in culture to any fluctuations in temperature, light, nutrients as may occur in hatchery system. Finally, they must have a good nutrient composition including an absence of toxins that might be transferred up the food chain.

Apart of its fulfill the requirements above, preference of *Isochrysis* sp. as feed is because of their ability to produce polyunsaturated fatty acid Docosahexaenoic Acid (DHA), one of the n-3 fatty acids that are essential for growth and development of mariculture organisms (Koven *et al*., 1989) and believed to prevent and treat pathologies such as coronary heart disease and atherosclerosis (Abd El-Baky *et al*., 2003), inflammatory problems and some cancers and play a role in infant nutrition (Connor and Neuringer, 1988). According to Volkman *et al*. (1993), prymnesiophytes (e.g. *Pavlova* sp. and *Isochrysis* sp.) and cryptomonads are relatively rich in DHA (0.2 to 11%) compared to eustigmatophytes, *Nannochloropsis oculata* 2 and chlorophytes, *Dunaliella tertiolecta*. Although, culturing method of microalgae for carotenoids production is widely exposed and comprehended, research findings for carotenoids in *Isochrysis* sp. is still scarce. At present, *Isochrysis* sp. is mainly used as mariculture feed due to its high content of long Polyunsaturated Fatty Acids (PUFA) which helpful in preventing heart and circulatory disease as well as facilitating brain development in infants (Kjell *et al*., 1994; Yongmanitchai and Ward, 1992). However, *Isochrysis* sp. is still not well-studied from biotechnological point of view, especially on carotenoids determination and exploitation compared to *Dunaliella salina* and *Haematococcus pluvialis*, current main producers of carotenoids.

Considering the importance of carotenoids, present work was initiated to investigate on isolation of carotenoids from *Isochrysis* sp. and quantity of total carotenoids present in the cell of *Isochrysis* sp. In addition, chemical stress (Nitrogen-depletion) will be introduced into mass culture to induce production of carotenoids. The study was aimed to quantify the percentage of total carotenoid present in a cell of *Isochrysis* sp; to promote excessive carotenoid production by introducing chemical stress (Nitrogen depletion) during mass culture and to prepare an optimum medium for the growth of *Isochrysis* sp.

MATERIALS AND METHODS

Microalgae: The marine microalga, *Isochrysis* sp. (100 mL) was obtained from Institute of Marine Biotechnology, University Malaysia Terengganu, Malaysia.

Culture media: The growth medium (f/2 media) was composed of (in g L^{-1}) $NaNO_3$ (22.5), $NaH_2PO_4.H_2O$ (1.5), $Na_2SiO.9H_2O$ (9), trace metal solution $FeCl_3.6H_2O$

(0.945), $Na_2EDTA.2H_2O$ (1.308), $CuSO_4.5H_2O$ (1.0), $Na_2MoO_4.2H_2O$ (0.63), $ZnSO_4.7H_2O$ (2.2), $CoCl_2.6H_2O$ (1.0), $MnCl_2.6H_2O$ (1.8) and Vitamin solution (vitamin B12 0.25 mL, Biotin 0.5 mL and thiamine HCl 100 mg). The media was prepared by adding each compound into 300 mL of deionized distilled water separately (stock solution). Then, to make 1 L of f/2 medium, 1 mL from each solution was added into 1 L of filtered sterilized seawater.

Growth condition: Microalgae was cultured in sterilized seawater (salinity of 36 ppt) enriched with f/2 medium (Guillard and Ryther, 1962) and maintained at 20-24°C, pH 8 and aerated at a rate of 4.5 L min^{-1}. Cultures were cultivated for 16 days in duplicate 100 mL of sterilized Erlenmeyer flask for preliminary culture. During mass culture, cell was cultivated for 14 days in a 30 L sterilized tank with initial 1600 mL of *Isochrysis* sp. and 8 L of f/2 medium were added into the tank. Later, the addition of f/2 medium into the tank was increased according to cell concentration. Cultures were illuminated with Philips fluorescent lamp (total 1200 lux) with continuous regime of light (daylight lamps).

Growth measurement: The sample was stirred and homogenized by homogenizer to ensure that the cell was fully dispersed. Cell concentrations were determined daily under microscope, with a Neubauer haemocytometer of 0.1 mm in depth. Each count was repeated three times using counter, average value is considered and instantaneous growth rates (μ) were calculated.

Stress parameter: The sample was cultivated in an optimized medium before inoculated under control and stress condition. When cells enter into late logarithmic phase (4th day cultures in complete f/2 medium), nitrogen compound (NO_3^-) in f/2 medium was eliminated, creating stress condition to the cells. The samples were withdrawn every 24 h and assayed for chlorophyll a, b and total carotenoids.

Pigment extraction: One hundred milliliter of algal cell was withdrawn from the culture per day (before and after the introduction of stress condition) and harvested by a gentle filtration vacuum through a 45 μm Whatman filter paper. The filter was folded and placed in a 10 mL falcon tube covered with aluminum foil to prevent penetration of light. 3 mL of N,N- dimethylformamide (DMF) was added and allowed to mix by vortex. The sample was left for 24 h under temperature of 4°C and meshed by homogenizer and centrifuged at 4000 rpm for 15 min. To separate extract and meshed filter paper, filtration was done again using 47 mm Whatman filter paper. The supernatant was taken and measured in spectrophotometer for carotenoid concentration.

Total carotenoid concentration: From the extraction, total carotenoids and chlorophyll levels were determined by UV/Visible Spectroscopy split beam spectrophotometer of samples in N,N-dimethylformamide (DMF) using the equation proposed by Wellburn (1994). The concentration of total carotenoid was calculated using following equation:

$$\text{Chlorophyll a (Ag mL}^{-1}) = 11.24\ A_{661.6} - 2.04\ A_{644.8}$$

$$\text{Chlorophyll b (Ag mL}^{-1}) = 20.13 A_{644.8} - 4.19\ A_{661.6}$$

$$\text{Total carotenoids (Ag mL}^{-1}) = (1000\ A_{470} - 1.90\ \text{Chl a} - 63.14\ \text{Chl b}/214)$$

One way ANOVA was conducted to determined significance day of culture toward cell count and followed by Non Parametric Test (Kruskal-Wallis H) test to determine the influence of cell culture. The positive/negative influence of days over the cell count was measured using regression analysis. Significant increase in carotenoid content was determined by One Sample t-test.

RESULTS

Table 1 shows three valuable data that are related to growth of *Isochrysis* sp. From this table, it can be observed that day 4 shows the highest growth rate with 1.6059 day^{-1} compared to the other days. *Isochrysis* sp. was experienced the slowest growth per day in day 15 with 0.7701 day^{-1}. Table 2 shows three valuable data that related to growth of *Isochrysis* sp. From this table, it can

Table 1: Growth rate trend of *Isochrysis* sp. in 16 days of preliminary culture

Day	No. of cell $\times 10^4$	Growth rate	Log10
1	27.5252		
2	32.6671	1.1879	0.0748
3	36.5838	1.1199	0.0492
4	58.7503	1.6059	0.2057
5	94.0839	1.6014	0.2045
6	131.5021	1.3977	0.1454
7	170.3330	1.2953	0.1124
8	212.5838	1.2480	0.0962
9	247.7505	1.1654	0.0665
10	300.9171	1.2146	0.0844
11	308.8333	1.0263	0.0113
12	307.7521	0.9965	-0.0015
13	306.5832	0.9962	-0.0016
14	297.7519	0.9712	-0.0127
15	227.7903	0.7649	-0.1164

be observed that day 2 shows the highest growth rate with 1.5339 day^{-1} compared to the others day. Isochrysis sp. was experienced the slowest growth per day in day 6 with 0.4297 day^{-1}.

Table 3 shows that there is no significance was observed at the p value of 0.448 indicating that the day of culture had no influence on cell count. Pigments in *Isochrysis* sp. in one cell according to days has been presented in Table 4.

Table 2: Growth trends of *Isochrysis* sp. in mass culture that lasting for 14 days

Day	No. of cells ×10^4	Growth rate	Log10
1	245.5121		
2	376.5822	1.5339	0.1858
3	564.5447	1.4991	0.1758
4	623.8355	1.1050	0.0434
5	560.4163	0.8983	-0.0466
6	240.8336	0.4297	-0.3668
7	223.9671	0.9300	-0.0315
8	174.1672	0.7776	-0.1092
9	153.6679	0.8823	-0.0544
10	140.6781	0.9155	-0.0384
11	134.3333	0.9549	-0.0200
12	133.6674	0.9950	-0.0022
13	121.6665	0.9102	-0.0409
14	116.3337	0.9562	-0.0195

Table 3: Kruskal-Wallis H test between day of culture and cell count

Test statistics	Cell count×10000
Chi-square	13.0
df	13.0
Significance	44.8

Table 4: Pigments in *Isochrysis* sp. in one cell according to days

		Conc. (μg mL^{-1} cell^{-1})		
Day	No. of cell ×10^4	Chl. b	Chl. a	Total carotenoids
1	245.5121	0.000482	0.001240	0.000131
2	376.5822	0.000481	0.000582	0.000201
3	564.5447	0.000349	0.000515	0.000130
4	623.8355	0.000457	0.000761	0.000101
5	560.4163	0.000374	0.000260	0.000163
6	240.8336	0.000732	0.000808	0.000430
7	223.9671	0.000416	0.000779	0.000927
8	174.1672	0.000943	0.001239	0.001411
9	153.6679	0.000743	0.001306	0.001765
10	140.6781	0.000728	0.001287	0.001932
11	134.3333	0.000560	0.001239	0.001344
12	133.6674	0.000648	0.000790	0.001157
13	121.6665	0.000484	0.000628	0.001155
14	116.3337	0.000443	0.000874	0.001000

Table 5 indicates relationship between chlorophyll a and b throughout the experiment. Table 6 indicates significant increase in carotenoid content in *Isochrysis* sp.

The temperature at which cultures are maintained should ideally be as close as possible to the temperature at which the organisms were collected. Most commonly cultured species of micro-algae tolerate temperatures between 16 and 27°C. Temperatures lower than 16°C will slow down the growth, whereas those higher than 35°C are lethal for a number of species.

The objective of preliminary culture for *Isochrysis* sp. was to observe its growth trend with complete nutrients and optimized conditions. Under laboratory cultured conditions, *Isochrysis* sp. showed a short lag phase that lasting about 24 h (Fig. 1). Subsequently, cells grew actively from day 3 until day 9, whereas specific growth rate for *Isochrysis* sp. showed the highest rate in 4th day of culture, 1.6059 day^{-1} compared to the other day. During this time, cell is doubling and the number of new microalgae appearing per day is proportional to the present population. On day 10 until day 13, the cells were entered stationary phase. At this phase, the growth rate slows as a result of nutrient depletion and accumulation of toxic products. In this phase, the microalgae begin to exhaust the resources that are available to them. In day 14 until day 15, it can be observed that the cells undergone dead phase where the microalgae was run out of nutrients and die off. Therefore, based on the highest growth rate stated in 4th day of preliminary culture, stress parameter (NO_3^- starvation, by eliminating NO_3^- in f/2 media) was introduced into the mass culture on day 4 (Fig. 2).

The influence of day during culture was tested using Kruskal Wallis H test which shows that there is no significance was observed at the p-value of 0.448, indicating that the day of culture had no influence on cell concentration. This is because during preliminary culture, the cells were provided with controlled parameter, whereas temperature, salinity, aeration rate, pH and light were maintained throughout the experiment.

After the sample has been extracted, it was stored at 4°C to prevent the degradation of carotenoids.

Table 5: Paired sample t-test

				Confidence interval				
	Mean	SD	SEM	Lower	Upper	t-value	df	Sig. (2-tailed)
Pair 1: Chl a-Chl b(mg mL^{-1})	-329760285 71E-4	0.0007127547	0.0001904917	-0.00007412926	0.0000817721	-1.731	13	0.107

Table 6: One Sample T-test for total carotenoid one-sample test

					Confidence interval	
	t-values	df	Sig. (2-tailed)	Mean difference	Lower	Upper
Total carotenoid	4.242	13	0.001	0.0004040621	0.000198302	0.000609822

Test value: 0

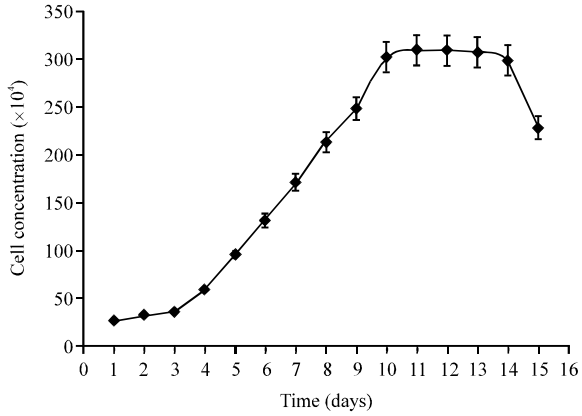

Fig. 1: Preliminary growth curve for *Isochrysis* species

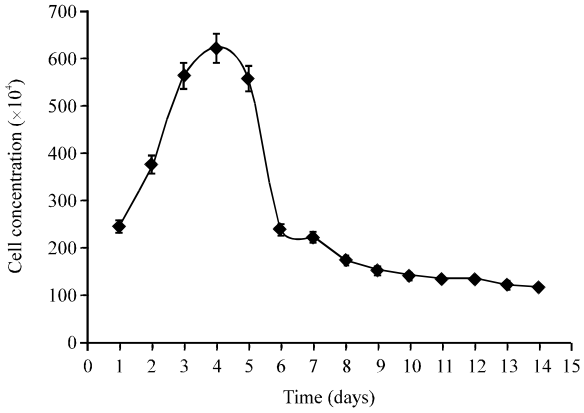

Fig. 2: Mass culture of *Isochrysis* sp. and the introduction of stress parameter at different days

The extracted pigments were then centrifuged for homogenization before read using spectrophotometer at following absorbance 662, 645 and 470 nm.

Figure 3 shows total carotenoid is slightly increased from day 5 onwards indicating that nitrogen starvation can influence production of carotenoid. Day 10 shows the highest production of carotenoid while day 1 shows the least carotenoid produced. The increase in carotenoid content of nitrogen-starved cells may be attributed to excessive formation of free radicals under stress. Carotenoid like β-carotene has antioxidant properties that quench excessive free radicals, restoring the physiological balance. Additional β-carotene is produced in order to protect the cells and to continue their growth. Hence, the carotenoid production is markedly increased under nitrogen starvation. Besides that, Fig. 3 also shows amount of chlorophyll before and after nitrogen starvation. Chlorophyll decreased with the decrease in availability of nitrate. This shows that chlorophyll

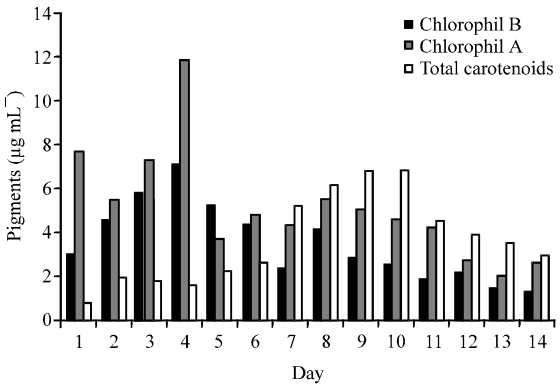

Fig. 3: Chlorophyll a and b and total carotenoid throughout mass culture, where nitrogen starvation was introduced in day 4

synthesis gets adversely affected, whereas carotenoid increases. The significant decreases in chlorophyll during nitrogen starvation may be because chlorophyll molecule contains four nitrogen atoms in its structure and, therefore, it becomes very difficult for the cell organelles to synthesize chlorophyll in the absence of nitrogen (Lichtenthaler, 1987). In order to identify the quantity of total carotenoid in a cell of *Isochrysis* sp., simple calculation was made as below:

$$\text{Pigments in one cell of } \textit{Isochrysis} \text{ sp.} = \frac{\text{Pigment for X day}}{\text{Volume of sample used in extraction} \times \text{No. of cell for X day}}$$

According to Fig. 4, the highest total carotenoid in one cell was recorded at day 10 with 0.0019 μg mL^{-1}, whereas the lowest total carotenoid content per cell was observed at day 4 with 0.0001 μg mL^{-1}. In nitrogen starvation, the cells become smaller and less dense compared to cells growing in full nutrients. Nevertheless, to gain maximum carotenoid production, it is suggested to harvest it at day 10 during nitrogen starvation provided with controlled parameter and optimized conditions as mentioned in this study. Apart of considering on total carotenoid, chlorophyll a and b were also estimated. From Fig. 4, it can be seen clearly that chlorophyll a is higher than chlorophyll b from the first day until the end of the research. This occurrence is due to the fact that chlorophyll a is the principal pigment in microalgae while chlorophyll b is the accessory pigment that collects the energy to pass on to chlorophyll a. Chlorophyll a absorbs well at a wavelength of about 450 nm but its primary absorption is at 675 nm in the long red wavelengths. Chlorophyll b absorbs most effectively at blue 470 nm with shorter peaks at 430 nm and 640 nm. Its main function is to collect energy but a secondary function is to regulate the antenna size. Chlorophyll a occupies the reaction

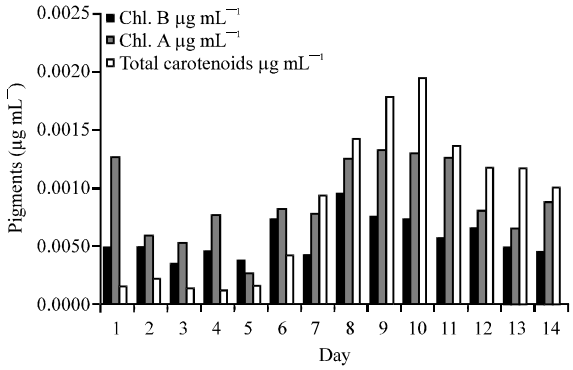

Fig. 4: Pigments in one cell of *Isochrysis* species

center of the antenna array. The array is made up of the core proteins surrounded by the peripheral proteins. Core proteins bind chlorophyll a and carotenoids. The peripheral proteins vary but land plants bind both alpha and beta on the peripheral proteins.

Paired sample t-test was conducted to study the relationship between chlorophyll a and b. Table 5 indicates that when chlorophyll a is increased, it has positive influence in chlorophyll b. As chlorophyll a, which responsible to absorb energy from the light, chlorophyll b, which is accessory pigment also increase by broaden the spectrum of available light and transfer the energy to chlorophyll a. One sample t-test was then conducted to observe the significant increase in carotenoid content (Table 6). From this data, it can be concluded that, from daily basis starting from day 4, there were considerable increase of carotenoid content after the introduction of stress parameter as $p<0.01$. As mentioned earlier, carotenoid content will increase in one cell of Isochrysis sp. as it is a defend mechanisms of the cell toward unbalance physiological changes created by stress factor (nitrogen limitation).

DISCUSSION

Microalgae's growing must be optimized if maximum growth is required in our system. The most important parameters regulating algal growth are nutrient quantity and quality, light, aeration and mixing, pH, salinity and temperature. In this experiment, *Isochrysis* sp. was cultured in f/2 medium, which a common and widely used general enriched seawater medium designed for growing coastal marine algae. It is enriched with $NaNO_3$, NaH_2PO_4, trace metal (consist of $FeCl_3.6H_2O$, Na EDTA.2H O, $CuSO_4.5H_2O$, $Na_2MoO_4.2H_2O$, $ZnSO_4.7H_2O$, $CoCl_2.6H_2O$, $MnCl_2.6H_2O$) and vitamin solution (consist of vitamin B12, Biotin and thiamine HCL) in adjustable quantity according to the usage of media. These compounds have different role in microalgae's growth. $NaNO_3$ and NaH_2PO_4 are macronutrients and must be provided in approximate ratio of 6:1 (Ramamurthy and Krishnamurthy, 2003). As well as all plants, light is the source of energy which drives conversion of inorganic carbon into organic matter, 1200 lux was used in this study as the tank was used can only accommodate up to 30 L of culture. Optimum light might required for carbon assimilation for *Isochrysis* sp. but too intense light may inhibit photosynthesis. Adequate mixing must be provided to ensure that all cells are collected and to prevent inaccuracy during cell count. The aeration for this experiment was maintained at rate of 4.5 L min^{-1}. According to Kaplan *et al.* (1985), agitation of the cultures had a strong effect both on growth rate and on algal yield, so enough aeration may require for optimum growth of microalgae. The excessive turbulence produced at high aeration rates may produce some cell damage especially to those naked flagellate species such as *Isochrysis* sp. The pH range for most cultured algal species was in between 7 and 9, with the optimum range being 8.2-8.7. Kaplan *et al.* (1985) studies the culture media for *Isochrysis* sp. was maintained at pH 8 because when *Isochrysis* sp. was cultured at pH 8, there was a marked pH effect on algal yield. It is easy to maintain the pH for marine culture media because large buffering capacity of natural seawater (due to a bicarbonate buffering system, HCO_3 being present at concentration 2.2 M). To maintain growth phase, it is necessary to control pH by means of the carbon supply to stabilize the carbonate buffer system and assure the CO_2 supply to the cells.

Marine phytoplanktons are extremely tolerant to changes in salinity. *Isochrysis* sp. exhibited resistance to a wide range of NaCl concentrations (Whyte, 1987). This may be because when microalgae are grown in saline environment, osmosis plays a role. If very high salinity is used in the medium, the external environment of the cell contains the hypertonic solution, i.e. higher concentration of the solute (NaCl) and lowers the concentration of the water, that present inside the cell.

In mass culture when stress factor (nitrogen limitation) was introduced, there was a significant declining of cell number of *Isochrysis* sp. due to the absent of nitrogen. Carotenoids production strictly depends on nitrogen limitation. Microalgae ceased to divide when nitrogen was not supplied in the growth medium as nitrogen is the primary requirement for all metabolic activities of the cell. Nitrogen limitation may result in the reduction in protein content and relative or absolute changes in lipid and carbohydrate content. According to Lapointe (1993), nitrogen is the key element for microalgae metabolism because it is a main component

of microalgal proteins and enzyme catalyst capacity and often limits microalgal growth and biomass. Suzuki *et al.* (1993) mentioned three points should be considered for the determination of photosynthetic pigments from particulate material: complete extraction of pigments, stability of the extracted pigments in solvents and method for separation and determination of pigments. In favor of pigment extraction, *Isochrysis* sp. was harvested during the stationary phase of algal growth by gentle filtration on Whatman 45 μm filter paper. In fact the extraction process should be executed without the presence of direct light to prevent photo inhibition that may cause deterioration of carotenoid sample. N,N-dimethylformamide (DMF) was used as an extracting solvent because it gives rise to higher extraction efficiency for phytoplankton, either culture or in nature, compared with 90% acetone (Suzuki and Ishimaru, 1990). Methanol is known to be an extracting solvent but can cause the formation of artifacts (e.g., esterification, epimerization and allomerization of chromatographic peaks (Zapata and Garrido, 1991) and the production of carotenoid derivatives (Khachik *et al*., 1998).

The results of present study reveal that it is best to harvest the carotenoid on day 10, when the maximum carotenoid can be obtained by introducing chemical stress (Nitrogen depletion) during mass culture and to prepare an optimum medium for the growth of *Isochrysis* sp.

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