GROWTH AND BIOCHEMICAL COMPOSITION OF *Isochrysis maritima* Billard and Gayral 1972 UNDER PHOTOAUTOTROPHIC AND HETEROTROPHIC CONDITIONS: ASSESSMENT FOR AQUACULTURE FEED

by

MOHAMMAD BASRI BIN ESHAK

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LIST OF ABBREVIATIONS

ALA	Alpha linolenic acid
ARA	arachidonic acid
$CHCl_3$	chloroform
DHA	docosahexanoic acid
EPA	eicosapentaenoic acid
FAME	fatty acid methyl ester
g	gram
H_2SO_4	sulphuric acid
HCl	hydrochloric acid
L	litre
LA	linoleic acid
mg	milligram
mL	millilitre
MUFA	monounsaturated fatty acid
NaOH	sodium hydroxide
PUFA	polyunsaturated fatty acid
rpm	rotation per minutes
SFA	saturated fatty acid
SGR	specific growth rate

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PERTUMBUHAN DAN KOMPOSISI BIOKIMIA *Isochrysis maritima* Billard dan Gayral 1972 DI DALAM KEADAAN KULTUR FOTOAUTOTROFIK DAN HETEROTROFIK: PENILAIAN UNTUK PEMAKANAN AKUAKULTUR

ABSTRAK

Kesan keadaan fotoautotrofik dan heterotrofik terhadap pertumbuhan dan komposisi biokimia *Isochrysis maritima* yang dipencilkan daripada Teluk Aling, Pulau Pinang telah dikaji. Dalam keadaan fotoautotrof, *I. maritima* telah dikultur dalam sumber nitrogen yang berbeza (nitrat, nitrit, dan urea) dan dituai pada fasa pertumbuhan yang berbeza (fasa eksponen, fasa pegun awal dan fasa pegun lewat) bagi mengkaji kesan sumber nitrogen dan fasa pertumbuhan kepada pertumbuhan dan komposisi biokimia. Hasil kajian menunjukkan urea pada fasa pegun lewat merupakan sumber nitrogen yang paling sesuai, yang mencatatkan pertumbuhan sel yang tinggi (10.9 ± 0.63 x 10^6 cell.mL^{-1}) dan komposisi biokimia yang tinggi termasuk protein (32.1 ± 3.17%), karbohidrat (37.0 ± 0.36%) dan asid lemak (jumlah SFA = 32.92 ± 2.59%; jumlah MUFA = 6.26 ± 0.83%; jumlah PUFA = 15.55 ± 1.49%) dan seterusnya dikultur dalam media Walne yang lebih besar (50 L). Dalam keadaan heterotrophic, *I. maritima* telah dikultur dalam pelbagai sumber karbon organik (glukosa, sukrosa, laktosa, fruktosa dan natrium asetat) pada kepekatan yang berbeza (0.02 M, 0.05 M dan 1.0 M) dengan urea sebagai sumber nitrogen bagi menentukan sumber karbon dan kepekatan yang sesuai untuk memaksimumkan pertumbuhan spesies ini. Glukosa pada kepekatan 0.02 M menunjukkan pertumbuhan sel yang paling tinggi dan kemudiannya digunakan untuk

kajian dalam 50 L media Walne. Keputusan untuk 50 L media Walne bagi kedua-dua keadaan trofik menunjukkan bahawa sel-sel heterotrofik dengan 0.02 M glukosa sebagai sumber karbon mencatatkan kepadatan sel maksimum yang lebih tinggi (36.90 ± 0.25 x 10^6 cell.mL^{-1}) sebanyak 4.5 kali ganda berbanding dengan sel-sel fotoautotrof (8.29 ± 0.70 x 10^6 cell.mL^{-1}). Kandungan karbohidrat juga mencatatkan peratusan lebih tinggi dalam sel-sel heterotrofik dalam semua peringkat pertumbuhan (eksponen, 40.8%; pegun awal, 48.3%; pegun lewat, 47.6%) tetapi tidak ada kesan yang ketara dalam kandungan protein antara kedua-dua keadaan trofik. Jumlah SFA, MUFA dan PUFA adalah lebih tinggi dalam keadaan heterotrofik berbanding fotoautotrofik. Walau bagaimanapun, dengan PUFA sebagai elemen yang paling penting dalam pemakanan akuakultur, peratusan yang rendah dalam EPA (0.28 ± 0.06%) dan DHA (3.22 ± 0.26%) dalam sel-sel heterotrofik berbanding sel fotoautotrofik (EPA : 0.44 ± 0.11%; DHA: 8.58 ± 0.73%) ditambah dengan nisbah omega–6/3 PUFA yang tinggi (2.11–10.32) membuktikan bahawa spesies ini dalam keadaan heterotrofik tidak sesuai untuk pemakanan akuakultur berbanding keadaan photoautotrophic (0.35–0.38). Kesan suhu penyimpanan (25°C, 4°C, 20°C) kepada komposisi biokimia pati *I. maritima* telah dikaji untuk tempoh 8 minggu. Di antara semua suhu yang dikaji, *I. maritima* memberikan hasil yang terbaik selepas disimpan pada –20°C. Pada suhu ini, pati *I. maritima* menunjukkan kandungan biokimia yang malar sepanjang tempoh penyimpanan kecuali jumlah PUFA yang tinggi pada minggu ke-8.

GROWTH AND BIOCHEMICAL COMPOSITION OF *Isochrysis maritima* Billard and Gayral 1972 UNDER PHOTOAUTOTROPHIC AND HETEROTROPHIC CONDITIONS: ASSESSMENT FOR AQUACULTURE FEED

ABSTRACT

The effects of photoautotrophic and heterotrophic conditions on growth and biochemical composition of *Isochrysis maritima*, isolated from Teluk Aling, Penang were investigated. Under photoautotrophic condition, *I. maritima* was cultivated in different nitrogen sources (nitrate, nitrite, and urea) and harvested at different growth phases (exponential, early stationary and late stationary) to examine the impact of nitrogen sources and growth phases on growth and biochemical composition. The results revealed that urea at late stationary was the most suitable nitrogen source, which recorded high cell growth ($10.9 \pm 0.63 \times 10^6$ cell.mL^{-1}) and biochemical composition including protein ($32.1 \pm 3.17\%$), carbohydrate ($37.0 \pm 0.36\%$) and fatty acids content (total SFA = 32.92 ± 2.59 %; total MUFA = $6.26 \pm 0.83\%$; total PUFA = $15.55 \pm 1.49\%$) and was further investigated onto higher volume of Walne's medium (50 L). Under heterotrophic condition, *I. maritima* was cultivated in different organic carbon sources (glucose, sucrose, lactose, fructose and sodium acetate) at different concentrations (0.02 M, 0.05 M and 1.0 M) with urea as nitrogen source to determine the suitable carbon sources and concentrations to maximize the growth of this strain. Glucose at 0.02 M concentration showed higher cell growth and was further investigated onto mass cultivation at 50 L culture medium. Results in 50 L Walne's medium for both trophic

conditions revealed that heterotrophic cells fed with 0.02 M glucose shows higher maximum cell density (36.90 ± 0.25 x 10^6 cell.mL^{-1}) by 4.5-fold compared to photoautotrophic cells (8.29 ± 0.70 x 10^6 cell.mL^{-1}). Carbohydrate content recorded slightly higher in heterotrophic cells at all growth stages (exponential, 40.8%; early stationery, 48.3%; late stationary, 47.6%) but there was no significant effect in protein content between both trophic conditions. The total saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) were higher in heterotrophic condition compared to photoautotrophic. Despite that, since PUFAs were the most essential element in feed nutrition, low eicosapentaenoic acid (EPA) (0.28 ± 0.06 %) and docosahexanoic acid (DHA) (3.22 ± 0.26 %) content in heterotrophic cells compared to photoautotrophic cells (EPA: 0.44 ± 0.11 %; DHA: 8.58 ± 0.73 %), plus with high omega–6/3 PUFA ratio (2.11–10.32) recorded proved that this species cultivated under heterotrophic condition was not suitable for aquaculture feed as compared to photoautotrophic condition (0.35–0.38). The effect of storage temperature (25°C, 4°C, –20°C) on the biochemical composition of *I. maritima* concentrates was investigated for 8 weeks period. From all temperatures studied, *I. maritima* gave the best result after storage at –20°C. At this temperature, *I. maritima* concentrates showed constant biochemical compositions reading throughout the storage period except for total PUFA which significantly higher at week-8.

CHAPTER 1

1.0 INTRODUCTION

In recent years, algae have been extensively utilised in different sectors including research and development, food industries, pharmaceutical, aquaculture and waste-water management (Spolaore *et al.*, 2006; Priyadarshani and Rath, 2012). Algae have received increasing interest because of their ability to synthesise valuable biomass compounds, such as lipids, proteins, carbohydrates, pigments, etc. (Markou and Nerantzis, 2013). As the primary source of polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), microalgae cultivation has been proven to be one of the profitable biotechnology exploitation in food and feed industries (Pulz and Gross, 2004; Mansour *et al.*, 2005). In addition, the ability of algae, especially microalgae, to accumulate substantial amount of lipid has been considered as the next generation of feedstock for biofuel production. However, their commercial cultivation for the sole purpose of biofuel production is not economically feasible and sustainable (Norsker *et al.*, 2011; Razon and Tan, 2011).

All algae are eukaryotic. Cyanobacteria, which are often referred to blue-green algae, was traditionally included among the algae, however, this prokaryotic bacteria which lack membrane-bounded organelles (plastids, mitochondria, nuclei, Golgi bodies, and flagella), are now known to be unrelated to any of the other algal groups, which are all eukaryotes and possess organelles. Based on size, algae can be classified into macroalgae and microalgae. Macroalgae are bigger in size which can be up to 60 meter long such as *kelp* (Barsanti and Gualtieri, 2006) whereas microalgae are much smaller

which can be down until 2 μm, for example *Selenastrum* sp.. Microalgae are too tiny to be seen with the naked eye. However, they may appear in color due to the presence of chlorophyll *a* and other pigments within their cells. For example the chlorophyll *a* and *b* will give a green color to the algae (Hoff and Snell, 2001).

Marine microalgae, *Isochrysis* sp. have received overwhelming interest in research study due to its ability to provide resources in form of novel genetic material. These resources contribute to the production of various nutritional and pharmaceutical products, proteins (essential amino acids), energy, other key nutrients such as vitamins, essential PUFAs and pigments which are transferred through the food chain (Brown, 2002). The most commonly used *Isochrysis* strains were *Isochrysis galbana* and *Isochrysis* sp. (T–Iso) or sometimes refer to *Isochrysis* affinis *galbana*. *Isochrysis* strains has been widely used as a mariculture feed due to its high content of long chain polyunsaturated fatty acid (PUFAs) especially DHA and its ease of cultivation (Jeffrey *et al.*, 1994; Lin *et al.*, 2007; Nalder *et al.*, 2015). This species is also able to tolerate cultivation under relatively higher culture temperatures which can be advantageous especially in unpredictable tropical condition (Babuskin and Radhakrishnan, 2014). Plus, with high growth rate, lacks a cell wall, and robust in outdoor culture conditions (Devos *et al.*, 2006; Lin *et al.*, 2007; Liu *et al.*, 2013) make this species a popular microalgal species in aquaculture.

Various studies have attempted to enhance the production of both cell biomass and biochemical composition of algae. These include the manipulation of chemical variables such as nitrogen (Arumugam *et al.*, 2013) and phosphorus concentrations (Sun and Wang, 2009), physical variables such as temperature (Durmaz *et al.*, 2009) and

salinity (Rao *et al.*, 2007), and cultivation modes including photoautotrophic, heterotrophic and mixotrophic modes (Chojnacka and Noworyta, 2004). Microalgae are frequently cultivated in photoautotrophic condition (Gouveia and Oliveira, 2009), which involves light as an energy source and inorganic carbon as a carbon source. Although the metabolites production is relatively high, this cultivation condition is frequently associated with the production of low biomass concentration due to light limitation (Chen and Chen, 2006).

The dependence of photoautotrophic cultivation on light energy prevents the cells to reach high density due to self-shading or photo-inhibition problem. In order to eliminate the requirement for light, microalgae was introduced to heterotrophic cultivation for possibly increasing cell density (Chen, 1996; Huang *et al.*, 2010; Pahl *et al.*, 2010). Heterotrophic cultivation involves utilisation of organic carbon as the energy and carbon sources for growth under dark conditions (Wen and Chen, 2003; Chojnacka and Marquez-Rocha, 2004; Chen *et al.*, 2011). Although this condition has an advantage in terms of microalgae growth, not all microalgae have the capability to grow in dark condition (Perez-Garcia *et al.*, 2011). Cell density has been reported to be enhanced with the elimination of light and utilisation of organic carbon source. Thus, this culture mode was said to be a cost-effective and also an alternative for the mass cultivation of microalgae (Chen, 1996; Pahl *et al.*, 2010). In addition to its ability to improve the cell biomass, heterotrophic cultivation also received much attention due to its ability to increase fatty acid content compared to photoautotrophic cultivation (Miao and Wu, 2006; Xiong *et al.*, 2008; Liu *et al.*, 2011; Wang *et al.*, 2012). A study by Wen and Chen (2000) on the heterotrophic production of EPA by the diatom *Nitzschia laevis* indicated

that the production of PUFA including DHA and EPA were higher under the dark condition. A study by Liu *et al.* (2011) on *Chlorella zofingiensis* also showed an increment in monounsaturated fatty acid (MUFA) (37.4%) in heterotrophic cultivation compared to photoautotrophic cultivation (20.1%).

Nutrients inconsistency in live food plus the culture 'crash' risk through failure of culture system or contamination problem are the main problems in production of live feed (Lucas and Southgate, 2003). Alternatives to fresh microalgal cultures such as microencapsulated diets, bacteria and yeast cultures, and dried or concentrated microalgae have been extensively studied (Knauer and Southgate, 1999). Algal concentrates have good potential as supplements or replacements of fresh cultures for the use in bivalve aquaculture (Knauer and Southgate, 1999; McCausland *et al.,* 1999; Brown and Robert, 2002; Ponis *et al.,* 2003), penaeid hatcheries (D'Souza *et al.*, 2002), and rotifer cultures (Guevara *et al.*, 2011; Seychelles *et al.*, 2009). Recent studies on the applicability of microalgal concentrates are limited and it is difficult to determine the extent of their use in industry (Welladsen *et al.*, 2014). According to Heasman *et al.* (2000), algal concentrates should have a shelf life of at least 2 months to span a typical 4–6 week hatchery rearing cycle and became acceptable for commercial use.

1.1 Objectives

The present study was undertaken in search of tropical indigenous microalgae isolates that possess great composition of polyunsaturated fatty acid (PUFA) that later can be exploited for various applications especially aquaculture. Subsequently, the chosen species which was *Isochrysis maritima* was cultivated photoautotrophically in different nitrogen sources and heterotrophically in different carbon sources. The best nitrogen and carbon sources were then utilised in higher volume cultivation for both trophic conditions to compare the cell biomass and biochemical compositions of this species at different growth stages for aquaculture feed. We also aimed to examine the effect of storage period on biochemical compounds of *I. maritima* concentrates after storage under three different temperatures. Our main objectives in this study were:-

1. To study the effects of different nitrogen sources and growth phase on biomass and biochemical composition of *I. maritima* under photoautotrophic condition

2. To study the heterotrophic cultivation potential of *I. maritima* under different carbon sources

3. To compare the biochemical composition of *I. maritima* between photoautotrophic and heterotrophic conditions at higher cultivation volume

4. To evaluate the biochemical composition of *I. maritima* concentrates under long-term preservation at different temperatures

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Microalgae

Microalgae are defined as eukaryotic microorganisms such as green algae (Chlorophyceae) and red algae (Rhodophyceae) with rapid growth rate and live in rough conditions due to their unicellular and simple multicellular structure (Brennan and Owende, 2010; Mata *et al.*, 2010; Abishek *et al.* 2014). For most phycologists, they described microalgae as microscopic organisms which contain chlorophyll *a* and thallus which are not differentiated into roots, stem and leaves including oxygenic photosynthetic bacteria (cyanobacteria) (Richmond, 2004; Lee, 2008). However, the simplest and general definition of microalgae was given by Priyadarshani and Rath (2012) which described microalgae as microscopic unicellular organisms with ability to convert solar energy into chemical energy via photosynthesis.

Microalgae have different types of cell organization such as unicellular (coccoid), colonial and filamentous (Richmond, 2004; Barsanti *et al.*, 2008; Graham *et al.*, 2009) and the prominent cell organization is unicellular type. Some unicellular and colonial algae like *Chlamydomonas* sp. and *Platydorina* sp. are propelled by flagella to aid them in locomotion (Graham *et al.*, 2009). The taxonomic classification of microalgae described by biologist was based on pigmentation, life cycle, and basic cellular structure. There are nine divisions of microalgae including Chlorophyta, Cyanophyta, Dinophyta, Glaucophyta, Heterokontophyta, Euglenophyta, Rhodophyta, Crytophyta and Haptophyta (Barsanti *et al.*, 2008; Lee, 2008).

Microalgae are preferably distributed in aquatic and sub-aerial environment which are more exposed to the atmosphere rather than immersed in water. Aquatic microalgae can be found anywhere from freshwater lakes to thermal springs due to their tolerance for a wide range of pH, temperature, turbidity, oxygen and carbon dioxide concentration. They can either be planktonic that live throughout the lit regions of water bodies or benthonic which live by attaching to the bottom of sediments. Benthic microalgae can be found on stones (epilithic), on sand (epipelic), on other algae or plants (epiphytic) or on animals (epizoic) (Barsanti *et al.*, 2008). All microalgae that attach to rocks or other substrates are also known as periphyton (Graham *et al.*, 2009; Pandit *et al.*, 2014).

The utilization of microalgae in commercial application for aquaculture feed, biofuel raw materials and wastewater treatment has recently gained enormous research interest. The ability to convert carbon dioxide from surrounding into biofuels stocks, as well as food, feeds, and high value products became the main reason of microalgae study being popular among worldwide researchers (Chisti, 2007; Bilad, 2014). Report also stated that microalgae are more efficient than terrestrial plants in converting sunlight into biochemical energy (Stephenson *et al.*, 2011; Chisti, 2013). Besides, microalgae are able to grow extremely fast and can double their biomass within a day (Chisti, 2008). The capability of microalgae to be cultivated in non-arable land (Markou and Nerantzis, 2013) throughout the year (Chen *et al.*, 2011) might also be the key factor to the popularity of microalgae application in industries.

2.1.1 *Isochrysis maritima*

Marine microalgae, *Isochrysis* sp. has received overwhelming interest in research study due to its ability to provide resources in form of novel genetic material. These resources contribute to the production of various nutritional and pharmaceutical products, proteins (essential amino acids), energy, other key nutrients such as vitamins, essential PUFAs and pigments which are transferred through the food chain (Brown, 2002). *Isochrysis* sp. has been widely used as a mariculture feed due to its high content of long chain polyunsaturated fatty acid (PUFAs) (Jeffrey *et al.*, 1994) which is important to the growth of aquaculture species.

Isochrysis maritima is a motile golden brown microalga in the genus *Isochrysis* together with *Isochrysis galbana* and *Isochrysis littoralis*. Below are the taxonomic classifications for this species (Billard and Gayral, 1972):-

Empire : *Eukaryota*

Kingdom : *Chromista*

Subkingdom : *Hacrobia*

Phylum : *Haptophyta*

Class : *Prymnesiophyceae*

Subclass : *Prymnesiophycidae*

Order : *Isochrysidales*

Family : *Isochrysidaceae*

Genus : *Isochrysis*

Isochrysis maritima possess pairs of apical sub equal homodynamic flagella (6 and 8 μm) and generally move in a backward direction. It also contains single yellow-green chromatophore and stigma. The size of this species is about 3 x 6 μm for young cells and 6 x 7 μm for older cells (Billard and Gayral, 1972). This species is not fully studied yet, so details regarding this species remain unknown.

2.2 Microalgae growth profile in batch culture

Batch culture is the most common method for cultivation of microalgae due to its simplicity and low cost (Barsanti and Gualtieri, 2006). In a simple batch culture system, a limited amount of complete culture medium and microalgae inoculum are placed in a culture vessel to be incubated at certain culture conditions for growth. The microalgae culture will grow rapidly under optimal conditions until the rate of cell division begins to decline, indicating the transition process from the exponential phase to the stationary phase. At that point, the culture is completely harvested (Lavens and Sorgeloos, 1996) and the washed container was refilled with sterilized and enriched medium, and inoculated to begin a new culture. The culture vessels can be from simple conical flask or an environment controlled fermentor (Richmond 2004).

In batch culture, there are five reasonably well defined phases of microalgae growth. It is started with lag phase, followed by exponential phase, declining growth rate phase, stationary phase and death phase (Lavens and Sorgeloos, 1996) according to typical pattern of sigmoid growth curve as shown in Figure 2.1.

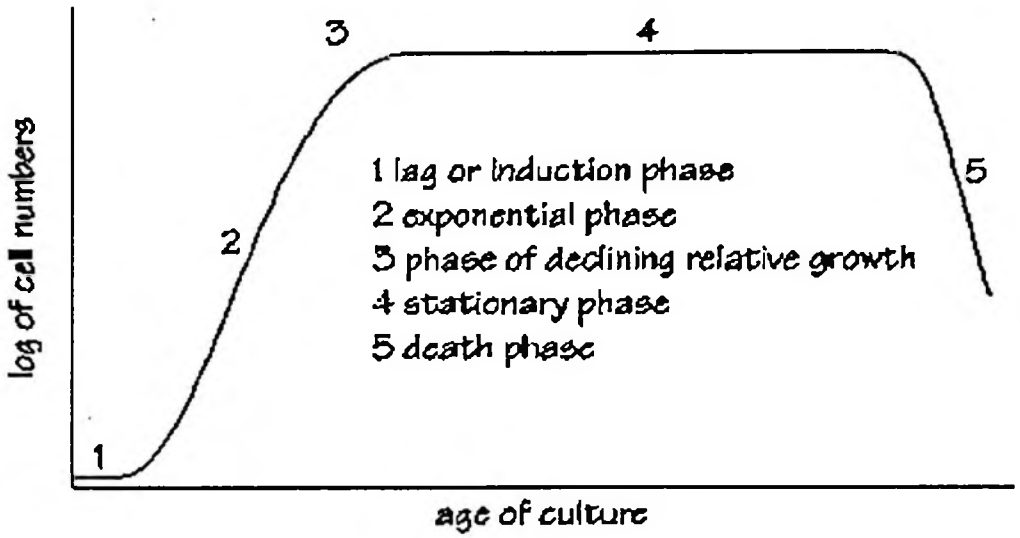

Figure 2.1: General pattern of microalgae growth in batch cultures (Lavens and Sorgeloos, 1996).

2.2.1 Lag phase

Lag phase is the first phase of microalgae in batch culture where the cell growth rate is zero. The lag in growth is attributed to the physiological adaptation of the cell metabolism to growth, such as the increase levels of enzymes and metabolites involved in cell division and carbon fixation as the result of the changes in nutrient or culture condition (Richmond, 2004, Barsanti and Gualtieri, 2006). The condition of the inoculum has a strong influence on the duration of the lag phase. This phase can be relatively long when microalgae culture is transferred from a plate to liquid culture. Inoculum that is taken from healthy exponentially growing culture is unlikely to have any lag phase when transferred to the fresh medium under similar growth conditions, which can reduce the time required for upscaling (Barsanti and Gualtieri, 2006).

2.2.2 Exponential phase

The cells start to enter the exponential (or logarithmic) growth phase after they adapt to the new environment. The cells will multiply rapidly and directly proportional to time as long as essential nutrients and light energy are available (Richmond, 2004). In this stage also, the specific growth rate of a microalgae population is determined (Wood *et al.*, 2005). Specific growth rate is a good way of showing the relative ecological success of a species or strain in adjusting its natural environment or the experimental control environment introduced on it. The duration of exponential phase in cultures depends upon the size of the inoculum (Richmond, 1986), the growth rate, the capacity of the medium (Fogg, 1975; Richmond, 2004) and the cultivation conditions to support algal growth such as light, temperature and pH (Fogg, 1975; Richmond, 1986; Richmond, 2004). Factors that limit cell density whether in natural environment or cultured control environment are called carrying capacity (Graham *et al.*, 2009).

Cell count and dry weight are common methods of biomass determination. Alternatively, another parameter can be measured as a proxy for cell number if it can be proven to be linearly correlated with cell number (Wood *et al.*, 2005) including In-vivo fluorescence and optical density. The concentration of chlorophyll, protein, carbohydrate, and lipid in the culture are also included (Richmond, 1986; Wood *et al.*, 2005). The correlations between fluorescence or turbidity and cell count can be set but they will become less precise as experimental conditions are assorted. For example cell fluorescence may vary with different temperatures. So, a study with several temperatures needs to be done in order to determine correlations for each temperature. Correlations also become inaccurate as cultures move into stationary phase (Wood *et al.*, 2005).

Once the growth phase has been plotted, careful determination of the exponential phase of growth is needed. Two points, N_1 and N_2, at the extremes of this linear phase are taken and substituted into the equation

$$\mu = \frac{(\ln X_2 - \ln X_1)}{(t_2 - t_1)}$$

where X_2 and X_1 are the cell density (cell.mL^{-1}) at time t_2 and t_1 (day), respectively.

2.2.3 Declining growth phase / early stationary phase

Declining growth phase or early stationary phase usually occurs in the cultures when specific requirements for cell division are limited. In this phase, culture density is normally very high. According to Fogg and Thake (1987), there are several factors contributing to this phase including exhaustion of nutrients, low supply of carbon dioxide and oxygen, changes of pH, reduction of light intensity and auto-inhibition by toxic producing algae.

2.2.4 Stationary phase

Stationary phase occur when limiting factor and the growth rate are equal, where the cell concentration remains constant at its maximum value. The biochemical composition of cells may experience dramatic changes. The final yield acquired in this phase depends on the nature of the limiting factor (Fogg and Thake, 1987). For example, nitrogen limitation may result in the reduction of protein content, relative changes in lipid and carbohydrate content (Weldy and Huesemann, 2007) meanwhile light limitation will result in increasing pigment content of most species and shifts in fatty acid composition (Sun and Wang, 2009). Pigment content especially carotenoid also

increase when nitrogen deficiency occurs as it prevents cell multiplication without undermining the ability of microalgae to assimilate carbon (Fogg and Thake, 1987).

2.2.5 Death phase

The death phase is the final stage in the microalgae growth phase. This phase generally occurs when the cell metabolism can no longer be maintained due to the several limiting factors which lead to the decrease in cell density and the culture eventually collapses. Some culture species will lose their pigmentation and appear cloudy, whereas some species may undergo lysis but the colour of culture does not change. This is an important reason why colour is not reliable in estimating culture health. Occasionally cell growth of some species can recur after a culture has apparently died. In this occurrence, most cells including bacteria will die and release nutrients back into the media (Bold and Wynne, 1985). Then, the very few remaining cells or more likely germination of cysts or temporary cysts will be able to begin this secondary growth.

2.3 Factors affecting microalgae growth and biochemical contents

Microalgae growth and biochemical composition can be affected by several factors including physical and chemical factors (Pruvost *et al.*, 2002; Sun and Wang, 2009; Guides *et al.*, 2010). Physical factors involve light, pH, salinity and temperature whereas chemical factors involved the nutrient requirements for microalgae growth such as nitrogen, carbon and phosphorus.

2.3.1 Light

Light plays an important role in cultivating microalgae since they are photoautotrophic organisms (Grima *et al.*, 1999; Pruvost *et al.*, 2002) which possess photosynthetic pigments like many other plants. Each photosynthetic pigment such as chlorophyll *a*, chlorophyll *b*, carotene, and phycocyanin will absorb certain colour and give the colour to the algae. Microalgae absorb light as energy and convert the inorganic carbon into organic carbon and water (Carvalho and Malcata, 2005).

The quantity and quality of light specify the total energy available for photosynthetic organisms to run their metabolic activities (Khoeyi *et al.*, 2011). The requirements of light for microalgae culture vary with the culture depth and the density of the algal culture (Lavens and Sorgeloos, 1996). Culture with higher depths and cell density needs to be supplied with high light intensity to ensure the light is adequate enough to penetrate through cell self-shading (Pruvost *et al.*, 2002). Excess high light intensity such as direct sunlight may result in photoinhibition (Grima *et al.*, 1996; Marxen *et al.*, 2006).

Microalgae are unable to discriminate between natural and artificial light, but they are sensitive to light intensity and photoperiod (Rocha *et al.*, 2003). A lot of studies with microalgae of various groups show that growth, pigments and biochemical compositions including lipid, fatty acids, carbohydrates, and protein content are affected by changes in light intensity (Harrison *et al.*, 1990; Sun and Wang, 2009). Study by Gómez-loredo *et al.* (2016), showed a better growth of *Isochrysis galbana* after cultivating under 62.0 μmol photons m^{-2} s^{-1} compared to lower light intensity. Besides,

microalgae density and biochemical contents are also reported to be affected by light duration or photoperiod (Dunstan *et al.*, 1993; Tzovenis *et al.*, 2003; Bahadar and Bilal Khan, 2013).

2.3.2 pH

Other than light, pH also influences the growth of microalgae and its metabolites (Wen and Chen, 2003; Çelekli and Dönmez, 2005; Meseck *et al.*, 2006). Most of microalgae species require pH range between 7 and 9, with an optimum close to neutrality (Lebeau and Robert, 2003). pH usually decrease in microalgae growth due to carbon dioxide in the medium is consumed by the microalgae. For this reason, and because precipitates could form when adding the enrichment medium, initial batch pH should be as low as growth congeniality allows (Støttrup and McEvoy, 2003). An increased pH in the medium can be rectified by the addition of carbon dioxide or sodium bicarbonate (Lebeau and Robert, 2003; Meseck *et al.*, 2006).

2.3.3 Salinity

Microalgae vary in their adaptability toward salinity. They are categorized into two groups based on their tolerance extent which are halophilic (salt requiring for optimum growth) and halotolerant (having response mechanism that permits their existence in saline medium) (Rao *et al.*, 2007). In both case, the microalgae produce some metabolites to protect from salt injury and also to balance the osmotic pressure that exist due to the surrounding (Richmond, 1986). *Dunaliella* (Rao *et al.*, 2007) and *Nannochloropsis* (Chini Zittelli *et al.* 1999; Hu and Gao, 2006) are examples of microalgae that can survive in a wide range of salinity. A study by Liu *et al.* (2013) also

stated that *Isochrysis galbana* was able to tolerate a wide range of salinity from 5 to 45 ‰ as indicated by the little difference in the final cell density.

There are few reports on the effect of salinity upon the growth and biochemical composition of microalgae. Report by Cho *et al.* (2007) indicated that *Chlorella ellipsoidea* showed the highest specific growth rate at salinity 10 ‰. Meanwhile, carbohydrates content increased while lipids and protein decreased at high salinity level (Araújo and Garcia, 2005). On the other hand, changes in salinity may also affect the fatty acid composition of microalgae. Chini Zittelli *et al.* (1999) reported that salinity at 30 ‰ showed an increment of PUFAs. in *Nannochloropsis* sp. by more than 13% and EPA content by 0.5% of the dry biomass compared to 20 ‰ salinity. Study by Al-Hasan *et al.* (1990) also proved the effect of salinity on microalgae when high salinity gave rise to a slightly higher EPA content in *Navicula* sp.

2.3.4 Temperature

The influence of temperature towards microalgae growth has been investigated in numerous studies. Generally, the optimum temperature for microalgae growth is between 20 °C and 24 °C (Lavens and Sorgeloos, 1996). However, the result might be different with the content of the culture medium and the species cultured (Renaud *et al.* 1995; Durmaz *et al.*, 2008). The effects of temperature on the cell cultures related to the temperature dependence of the structural components of the cells (particularly lipids and proteins), as well as to the temperature coefficients of reaction rates (Sandnes *et al.*, 2005). Consequences of these primary effects are significant to the changes in metabolic

regulatory mechanisms, specificity of enzyme reactions, cell permeability and cell composition (Richmond, 1986).

Besides growth, changes in temperature have major effects on the biochemical compounds for certain microalgae (Renaud *et al.* 2002). A study by Araújo and Garcia (2005) on *Chaetoceros* cf. *wighamii* showed that the carbohydrate and lipid content were higher in lower temperature (20 °C and 25 °C). Similar results were obtained by Durmaz *et al.* (2008) on *Diacronema vlkianum.* In addition, Zhu *et al.* (1997) also reported that polyunsaturated fatty acid content in *Isochrysis galbana* TK1 especially C18:3 (n–3) and C22:6 (n–3) were higher in low temperature.

2.3.5 Mineral Nutrients

The primary nutrients for microalgae are carbon, nitrogen and phosphorus like any other true plants. Silicate is essential for the growth of diatoms which exert this mineral nutrient for production of an external shell (Lavens and Sorgeloos, 1996). Hence, any changes involving all of these essential nutrients might affect the growth and biochemical compounds of microalgae as well (Hu, 2004; Guschina and Harwood, 2009).

2.3.5 (a) Carbon

Carbon is an essential nutrient in microalgae cultivation. According to Singh *et al.* (2011), about 45% of microalgae total biomass is built up by carbon. Microalgae can utilize both organic and inorganic carbon sources (Rashid *et al.*, 2014). Inorganic carbon like carbon dioxide is used by autotrophic microalgae in the presence of light

(photoautotrophic) meanwhile heterotrophic microalgae consumed organic carbon such as glucose, sucrose and fructose as a food source (Wen and Chen, 2003).

A lot of studies proved that the manipulation of carbon sources and concentration would affect microalgae growth and its content (Wen and Chen 2000; Hu and Gao, 2003; Fang *et al.*, 2004). According to Rashid *et al.* (2014), the growth rate and biomass productivity of microalgae increased at high CO_2 level (1–15%). But, the usage of CO_2 in microalgae cultivation might decrease the pH level at early stage of cultivation since the interaction of CO_2 with water will lead to the formation of carbonic acid (Zhang *et al.*, 2012). Another study by Alkhamis and Qin (2013) also recorded that algal dry weight increased as glycerol concentrations which act as carbon source increased from 0 to 200 µmol on *Isochrysis galbana* but the highest algal production occurred at 50 µmol glycerol.

Glucose is the major carbon source used in heterotrophic cultivation (Wen and Chen, 2000). Other carbon sources such as ethanol, glycerol, sucrose, fructose and starch are able to utilize depending on the microalgae species used (de Swaaf *et al.*, 2003; Wen and Chen, 2003). The optimal carbon concentration for most microalgae ranges from 1 g/L to 25 g/L (Rashid *et al.*, 2014). However, the optimal carbon concentration may vary depending on the species used. For example, the optimal glucose concentration for *Scenedesmus acutus* was 1 g/L meanwhile 40 g/L or *Nitzschia laevis* (Wen and Chen, 2003). On the other hand, the effect of carbon sources on lipid content and fatty acid composition varied depending on the species (Fang *et al.*, 2004).

2.3.5 (b) Nitrogen

Nitrogen content in total biomass of microalgae can range from 1% to more than 10 % in each cell (Grobbelaar, 2004). Nitrogen is an essential element of all structural and functional proteins in microalgae cells (Hu, 2004). There are various forms of nitrogen sources that exist in different oxidation states that can be utilized by microalgae. Among them, nitrate and ammonium are the most common nitrogen sources used by microalgae (Richmond, 2004).

The manipulation of the nitrogen source can cause important changes in the growth and biochemical composition of microalgae species (Fidalgo *et al.*, 1995). A study by Wen and Chen (2001) on optimization of nitrogen sources for heterotrophic production of EPA by the diatom *Nitzschia laevis* showed that nitrate and urea recorded the highest result in terms of growth and EPA content. Other study also suggested nitrate as one of the best nitrogen sources for the growth and astaxanthin production in the microalgae *Haematococcus pluvialis* (Borowitzka *et al.*, 1991). Meanwhile, *Isochrysis* strain was reported to grow well in nitrate and urea as nitrogen source compared to ammonia (Feng *et al.*, 2011; Alkhamis and Qin, 2015).

On the other hand, previous study also suggested that nitrogen limitation is capable to alter the growth and biochemical contents of microalgae. Nitrogen limitation puts algal cells under unfavourable environmental or stress conditions, which normally causes carbon partitioning from carbohydrate or protein into lipid (Harwood and Jones, 1989). For example, *Monallantus salina* was reported to produce as much as 72% lipids in nitrogen deficient conditions (Shifrin and Chisholm, 1981). Besides, Chen and Johns

(1991) indicated that limiting nitrogen in the culture medium induced *Chlorella vulgaris* to produce more total lipid. Moreover, the same result was also obtained by Hu *et al.* (2008) in their study on cyanobacteria.

2.3.5 (c) Phosphorus

Phosphorus is one of the essential nutrients for microalgae growth. It is an important element in energy conversion and conduction of information in microorganisms (Rashid *et al.*, 2014) involving synthesis of acid nucleic, phospholipids, and many esters such as ATP and NADP (South, 1987; Wen and Chen, 2003; Chen and Chen, 2006). Plus, it is also important in the synthesis of PUFA and antioxidant products such as astaxanthin and β-carotene. Phosphorus is commonly supplied for microalgae growth in the form of orthophosphate (PO_4^{3-}) (Chen and Chen, 2006).

The manipulation of phosphorus concentration was proven effectively to alter the microalgae density and its contents. A study by Brinda *et al.* (2004) reported that high biomass and astaxanthin were recorded when microalgae *Haematococcus pluvialis* was cultured in the phosphorus deficient medium. A significant change was also observed involving cell density, biochemical composition, and the activity of extracellular carbonic anhydrase in *Isochrysis galbana* when the concentration of phosphorus in the medium was increased from 5 μmol/L to 1000 μmol/L (Sun and Wang, 2009).

The effects of phosphate on PUFA biosynthesis vary in different species. It has been proven by the study from Yongmanitchai and Ward (1991), which indicated that EPA composition in *Phaeodactylum tricornutum* was higher at high phosphorus concentration. Nevertheless, Stinson *et al.* (1991) proved otherwise in the culture of

Pythium irregulare. They discovered that a higher initial phosphate concentration resulted in a lower EPA yield.

2.4 Microalgae cultivation conditions

Microalgae can be grown in different cultivation conditions either involving organic or inorganic carbon sources and with or without light utilization. In microalgae cultivation, the microalgae growth conditions significantly affected the growth and biochemical compositions of the microalgae (Chojnacka and Marquez-Rocha, 2004). Chen *et al.* (2011) reported that there are four major types of cultivation conditions which are photoautotrophic, heterotrophic, photoheterotrophic and mixotrophic. However, only photoautotrophic and heterotrophic cultivation conditions will be discussed in this section since photoheterotrophic and mixotrophic are not involved in this study.

2.4.1 Photoautotrophic

Photoautotrophic condition is the most frequent employed cultivation condition for microalgae growth (Gouveia *et al.*, 2009; Gouveia and Oliveira, 2009; Yoo *et al.*, 2010). This condition involved light as an energy source and inorganic carbon (CO_2) as carbon source to form various forms of chemical energy such as polysaccharides, proteins, lipids and hydrocarbons through photosynthesis process (Chen and Chen, 2006; Huang *et al.*, 2010). Microalgal photosynthesis process is similar to all true plant. The process is divided into light cycle and dark cycle. Light cycle involve oxidation of water (H_2O) using light energy to produce molecular oxygen (O_2), ATP and NADPH. The ATP and NADPH produced will be utilized in the dark cycle to reduce CO_2 thereby

forming organic compounds in a process known as carbon fixation (Graham *et al.*, 2009).

Photoautotrophic cultivation is popular for outdoor scale-up cultivation system due to several advantages. The dependence of this system towards light source can be solved by using natural sunlight. Besides, the ability of microalgae to absorb CO_2 as carbon source will be the major advantage for microalgae growth as long as the cultivation takes place in highly saturated CO_2 environment such as power plant and factory. Moreover, the contamination risk is also low in this system compared to others (Chen *et al.*, 2011).

Even though microalgae can utilize light efficiently compared to true plant, photoautotrophic growth of microalgae is lower compared to other trophic conditions. This might be due to the light limitation in the result of self-shading at high cell densities (Pruvost *et al.*, 2002). The excessive light will also cause photoinhibition, especially during sunny days (Andersen, 2005; Marxen *et al.*, 2006; Huang *et al.*, 2010). Regardless of high metabolites production, the inability of photoautotrophic to produce high biomass concentration has always been the main limitation in this conventional cultivation method.

2.4.2 Heterotrophic

In order to eliminate the requirement for light, microalgae was introduced to heterotrophic cultivation and subsequently serve the possibility of increasing cell density (Chen, 1996; Huang *et al.*, 2010; Pahl *et al.*, 2010). Heterotrophic cultivation involves utilization of organic carbon as the energy and carbon sources for growth under dark conditions (Wen and Chen, 2003; Chojnacka and Marquez-Rocha, 2004; Chen *et al.*, 2011). Although this condition has an advantage in terms of microalgae growth, not all microalgae have the capability to grow in dark condition (Perez-Garcia *et al.*, 2011). According to Wen and Chen (2003) and Chen and Chen (2006), certain characteristics need to be owned by microalgae to involve in heterotrophic production including:-

1) The ability to undergo cell division and metabolize in the absence of light

2) The ability to be cultivated on inexpensive and easily sterilized media

3) The ability to adapt efficiently to the new cultivation condition

4) The capacity to restrain hydromechanical stresses in fermentor

With the elimination of light as the organic carbon source and afterward be able to reach high cell density, this culture mode was said to be a cost-effective and also an alternative for a mass cultivation of microalgae (Chen, 1996; Pahl *et al.*, 2010). Despite all the advantages by performing this condition for microalgae production, nevertheless, Pahl *et al.* (2010) described several disadvantages including:-

1) Restricted number of microalgae species which can grow in dark condition

2) High probability of contamination by bacteria

3) Growth inhibition at low organic substrate concentrations

4) The inability to generate some light-induced products, such as pigments.

Besides to improve in cell biomass, heterotrophic cultivation also received much attention due to its ability to increase fatty acid content compared to photoautotrophic cultivation (Wang *et al.*, 2012). A study by Wen and Chen (2000) on heterotrophic production of eicosapentaenoid acid (EPA) by the diatom *Nitzschia laevis* indicated that the production of PUFA including DHA and EPA are higher in dark culture. On the other hand, Liu *et al.* (2011) reported that *Chlorella zofingiensis* also showed an increment in MUFA (37.4%) in heterotrophic cultivation compared to photoautotrophic (20.1%).

Amongst the reported influencing factors of heterotrophic microalgae, carbon sources have been the important nutritional factors due to their role in providing the energy and carbon skeleton for cell growth (Xiong *et al.*, 2008; Heredia-Arroyo *et al.*, 2011). In order to grow in the dark, microalgae need the energy which is usually obtained from any organic carbon source such as acetate or glucose. Other organic carbon sources such as fructose, sucrose, lactose and starch were also used to promote the growth and PUFA production, depending on the microalgae species used (Wen and Chen, 2003; Fang *et al.*, 2004).