BIOCHEMICAL COMPOSITION AND ANTIOXIDANT CAPACITY OF MARINE MICROALGAE *Chlorella salina* Butcher AND *Isochrysis maritima* Billard and Gayral ISOLATED FROM PENANG COASTAL WATERS

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By

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LIST OF SYMBOLS

Symbol	Description
%	Percent
±	Plus minus
γ	Gamma
α	Alpha
°C	Degree celcius
°N	Degree north
°S	Degree south
‰	Part per thousand

LIST OF ABBREVIATIONS

cell mL^{-1}	Cell per mililtre
DHA	Docohexanoic acid
DO	Dissolved oxygen
DPPH	2,2-diphenyl-1-picrylhydrazyl
EPA	Eicosapentanoic acid
FA	Fatty acid
FAME	Fatty acid methyl ester
g	Gram
g L^{-1}	Gram per litre
GC	Gas chromatography
GPS	Global positioning system
h	Hour
L	Litre
m	Meter
M	Molar
mL	Millilitre
mm	Millimeter
mm^3	Millimeter cubic
mg mL^{-1}	Milligram per millilitre
µm	Micrometer
MUFA	Monounsaturated fatty acid
M	Molarity
n3	Omega 3

n6	Omega 6
PBR	Photobioreactor
Ppt	Part per thousand
PUFA	Polyunsaturated fatty acid
rpm	Revolutions per minute
SEM	Scanning electron microscope
SFA	Saturated fatty acid
SGR	Specific growth rate
sp	Species
TEM	Transmission electron microscope

KOMPOSISI BIOKIMIA DAN KEUPAYAAN ANTIOKSIDA MIKROALGA MARIN *Chlorella salina* Butcher DAN *Isochrysis maritima* Billard dan Gayral DIPENCIL DARI PERAIRAN PULAU PINANG

ABSTRAK

Chlorella salina dan *Isochrysis maritima* berjaya dipencil, ditulenkan dan dikultur di dalam medium Conway. Morfologi dan struktur-ultra strain telah dipantau menggunakan mikroskop elektron pengimbas (SEM) dan mikroskop elektron transmisi (TEM). Perubahan pada profil pertumbuhan, analisis analitikal, pigmen, kandungan biokimia serta komposisi asid lemak telah dikaji semasa kultur pertumbuhan kelompok. *C. salina* dan *I. maritima* dikultur menggunakan kaedah pengkulturan kelompok biasa dengan peningkatan skala daripada kultur permulaan 100 mL kepada kultur eksperimen 1 liter. Kultur dengan skala isipadu lebih besar menunjukkan kepekatan biojisim lebih tinggi (hanya pada *I. maritima*), kadar pertumbuhan spesifik lebih tinggi dan masa penggandaan yang lebih cepat. Berat kering, berat kering tanpa abu bagi kedua-dua spesies serta kandungan kelembapan *I. maritima* dipengaruhi secara signifikan oleh fasa pertumbuhan (p<0.05) manakala jumlah kandungan abu didapati tidak terjejas. Alga hijau, *C. salina* mengandungi kedua-dua pigmen, klorofil *a* dan klorofil *b* manakala hanya klorofil *a* dikesan pada *I. maritima*. Kandungan protein menurun bagi kedua-dua pencilan manakala karbohidrat dan lipid terkumpul, masing-masing pada *C. salina* dan *I. maritima*, selari dengan peningkatan usia kultur. Secara keseluruhan, proksimat kandungan biokima *C. salina* didapati mengikut urutan di mana kandungan protein adalah paling tinggi, disusuli oleh karbohidrat dan terendah adalah lipid manakala bagi *I. maritima*, kandungan tertinggi adalah protein, disusuli oleh lipid dan terendah adalah karbohidrat, tanpa mengira fasa pertumbuhan. Corak variasi yang jelas pada profil

asid lemak dipamerkan oleh alga hijau, *C. salina* dengan pecahan asid lemak utama mengikut urutan di mana kandungan PUFA paling tinggi, diikuti oleh SFA dan terendah adalah MUFA sepanjang fasa pertumbuhan, manakala bagi *I. maritima* kandungan SFA yang tertinggi, disusuli oleh PUFA dan terendah adalah MUFA semasa pertumbuhan eksponensial, seterusnya semasa fasa pegun kandungan PUFA dan SFA hampir setara dan MUFA kekal paling rendah. Kelimpahan relatif asid lemak tidak tepu (PUFA) yang penting dikesan pada *C. salina* terutamanya asid linolik (LA) dan asid α-linolenik (ALA) dengan kehadiran rendah asid γ-linolenik (GLA) dan asid docoheksanoik (DHA) manakala kadungan ALA, GLA dan DHA agak tinggi pada *I. maritima* dengan kehadiran minor asid arakidonik (ARA) dan asid ekosapentanoik (EPA). Potensi mikroalga marin ini sebagai sumber antioksida semulajadi telah dinilai menggunakan ujian penjerapan radikal bebas DPPH pada kepekatan ekstrak berbeza. Keputusan menunjukkan nilai maksimum 43.37 ± 2.20% dan 11.64 ± 2.61% dicapai semasa fasa pegun pada kepekatan tertinggi (25 mg mL^{-1}), masing-masing pada *C. salina* dan *I. maritima*, menunjukkan kedua-dua strain merupakan penjerap radikal yang lemah. Kajian semasa ini memberi pengetahuan yang lebih mendalam dan pemahaman yang lebih baik mengenai profil pertumbuhan, variasi komposisi nutrisi serta keupayaan antioksida berkaitan dengan fasa pertumbuhan mikroalga marin tropika.

BIOCHEMICAL COMPOSITION AND ANTIOXIDANT CAPACITY OF MARINE MICROALGAE *Chlorella salina* Butcher AND *Isochrysis maritima* Billard and Gayral ISOLATED FROM PENANG COASTAL WATERS

ABSTRACT

Chlorella salina and *Isochrysis maritima* were successfully isolated, purified and cultured in Conway medium. Morphology and ultra-structures of strains were observed using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Changes in growth profile, analytical analyses, pigments, biochemical compounds as well as fatty acid compositions were investigated during a batch culture growth. *C. salina* and *I. maritima* were cultivated in conventional batch culture method with up scaling from 100 mL starter culture to 1 liter experimental culture. Larger scale volume cultures displayed higher biomass concentration (only in *I. maritima*), higher specific growth rates and faster doubling times. Dry weight, ash free dry weight of both species and moisture content of *I. maritima* were significantly influenced by growth phase (p<0.05) while total ash content was unaffected. The green algae, *C. salina* contained both pigments of chlorophyll *a* and chlorophyll *b* whereas only chlorophyll *a* was detected in *I. maritima*. Protein content in both isolates decreased whereby more carbohydrate and lipid accumulated in *C. salina* and *I. maritima*, respectively as culture aged. In total, the proximate biochemical compound of *C. salina* was found in the sequence where protein had the highest content, followed by carbohydrate and the lowest content was lipid whilst *I. maritima* had highest protein content followed by lipid and least amount of carbohydrate regardless of the growth phases. A clear define pattern of variations in fatty acid profile was exhibited in the green algae, *C. salina* with major fatty acids fraction where highest content was PUFA followed by SFA and lowest

was MUFA throughout the growth phases whereas *I. maritima* had highest content of SFA followed by PUFA and least amount of MUFA during the exponential phase and subsequently had similar content of PUFA and SFA while MUFA remained as lowest content obtained in the stationary phase. Relative abundance of important PUFA detected in *C. salina* namely linoleic acid (LA) and α-linolenic acid (ALA) with low occurrence of γ-linolenic acid (GLA) and docohexanoic acid (DHA) while *I. maritima* relatively high in ALA, GLA and DHA with minor presence of arachidonic acid (ARA) and ecosapentanoic acid (EPA). The potential of these marine microalgae as natural antioxidant source were evaluated using DPPH free radical scavenging test at different extract concentrations. The results showed maximum values of 43.37 ± 2.20% and 11.64 ± 2.61% were achieved during stationary phase at highest concentration (25 mg mL^{-1}) in *C. salina* and *I. maritima*, respectively, indicating both strains were weak radical scavenger. The present study provides deeper knowledge and better understanding on growth profiles, variation of nutritional compositions and antioxidant capacity associated with the growth phases of tropical marine microalgae.

CHAPTER 1

INTRODUCTION

Microalgae are microscopic photosynthetic organisms that are found in both marine and freshwater environments. These organisms constitute a polyphyletic and highly diverse group of prokaryotic and eukaryotic. Their photosynthetic mechanism is similar to that of higher plants. Indeed, they are generally more efficient in converting solar energy into organic compounds, mainly because of their simple cellular structure (Chacón-Lee and González-Mariño, 2010). These groups play a key role in the productivity of oceans and constitute as primary producer in marine food chain by producing organic material from sunlight, carbon dioxide, and water. Besides, they also form the oxygen necessary for the metabolism of the consumer organisms.

As the basis of natural food chain, exploitation of this photosynthetic microorganism has a long history in aquaculture industry. Asia accounted 89% of world aquaculture production in 2012 which was dominated by China (FAO, 2012). Various microalgae species have been widely cultivated for aquaculture organism including mollusks, crustaceans, fish larvae and brine shrimp (Renaud et al., 1991; Brown et al., 1997). Likewise, more than 40 species of microalgae for instance, *Chaetoceros, Isochrysis, Tetraselmis, Chlorella, Spirulina* and *Skeletonema* are used in aquaculture industries worldwide, depending on the requirements of local seafood production (Pulz and Gross, 2004). Since the mid-1990s, aquaculture has been the major driving growth in total fish production as global wild capture production has leveled off. The contribution of aquaculture production has continued to grow in the

new millennium from 47.3 million tonnes in 2005 to 63.6 million tonnes in 2011 (FAO, 2012).

It is well know that microalgae show considerable metabolic flexibility in response to changes in environmental factors, hence the microalgae cultivation needs to be controlled and maintained at optimal conditions in order to ensure maximum growth, high survival rate and high biomass production. Nutrients, light, temperature, salinity and pH are among major processing aspects that influence overall biomass productivity and the biochemical compositions (Renaud et al., 2002; Araujo and Garcia, 2005). The chemical composition of microalgae cells are also known to vary during their growth phase cycle.

The nutritional quality of microalgae biomass is correlated to the biochemical composition which have essential role in the diet of marine animals, either directly or indirectly (through enrichment of zooplankton). Microalgae lipids have been used as a dietary source for metabolic energy and essential components for aquaculture organism, with fatty acid content being the central factor in the selection of microalgae species (Huerlimann et al, 2010).

Due to their high content in protein, carbohydrates, lipids, fatty acids, richness in minerals and vitamins as well as bioactive compounds (Becker, 2007), microalgae have received increasing interest as natural source of valuable compounds. Moreover, the value of the microalgae as natural source is further enhanced by the relative ease of purification of target compounds (Li et al., 2007).

Microalgae may serve as continuous and reliable source of natural products because they can be cultivated on a large scale.

Currently, extracts from microalgae biomass have gained a firm position in the market. There is increasing demand for products derived from microalgae which related to the taxonomic and physiology of the strains. Microalgae combine properties typical of higher plants (efficient oxygenic photosynthesis) with biotechnological attributes of microbial cells (fast growth in liquid culture ability to accumulate nutritional requirements and metabolites). This particular combination support the use in applied phcology processes and represents the basis of microalgae biotechnology (Del Campo et al., 2007). Successful algal biotechnology mainly depends on choosing the right algal with relevant properties for specific culture conditions and products.

To date, commercial industries are focusing on few taxa, dominate mainly by *Spirulina, Dunaliella, Chlorella, Haematococcus* and *Anabaena*. These potential microalgae are able to synthesize a wide range of valuable metabolic compounds, making them a potentially important source for animal feed, health food, pharmaceutical and nutraceutical, cosmetics industries, food additives and colouring products, bio-fertilizer as well as environmental applications. Besides, the interest of microalgae production has increased due to its potential for bio-fuel and biodiesel production (Ulloa et al., 2012)

Microalgae have also drawn major interest as prospective and valid sources of antioxidant for the nutraceutical industries (Cerón et al., 2007). Due to

phototrophic life, microalgae are exposed to high oxygen and radical stress. This has resulted in the development of numerous efficient protective systems. These protective chemicals contents have been proposed as important substances in the fight against various oxidation-associated conditions (Hajimahmoodi et al., 2010) and important lipid peroxidation for food preservation (Rodriguez-Garcia and Guil-Gerrero, 2008). Moreover, the use of synthetic antioxidants nowadays has decreased due to health risk (Li et al., 2007; Natrah et al., 2007). Therefore, the search for natural antioxidant activity as alternatives to synthetic products is a great potential to discover.

Malaysia‟s aquaculture industry particularly fish production is expending in order to meet the demand in the country and to supply for the export purpose, thus the demand for microalgae culture is also rising. Despite that, research and development on microalgae had received little attention and still very low in number in comparison to macroalgae (seaweed) which has been commercialized and became an economically important natural source for Malaysia. Microalgae strains used as live feed in Malaysia are usually imported and the biochemical composition has not been characterized in detail. Moreover, the influence of growth phase on biochemical composition has not been clearly investigated in attempt to harvest the highest productivity of a target compound. Besides, microalgae cultivation in Malaysia mostly concentrates for aquaculture farming solely.

Therefore, the present study was undertaken in search of tropical indigenous microalgal isolates which can be applied in hatcheries as they are more tolerant to local environmental conditions. Most of the imported strains might not withstand the local conditions and eventually producing low biomass concentration and poor

nutritional values. This study also examined the variation of biochemical composition as a function of culture period for cells to be harvested in order to achieve maximum yield of a specific compound. In addition, the exploitation of this untapped resource is further extending in search for other beneficial application of microalgae biotechnology possibilities, particularly in function of antioxidant properties.

The main objectives of this study were aimed at:

1. To determine the growth profile of *Chlorella salina* and *Isochrysis maritima* based on cell count, growth rate and doubling time.
2. To examine the growth phases associated with changes in the proximate biochemical components (protein, carbohydrate and lipid) as well as the essential fatty acids composition of *C. salina* and *I. maritima*.
3. To screen for the radical scavenging activities of the crude extracts of tropical marine microalgae, *C. salina* and *I. maritima* as potential source of natural antioxidant properties.

CHAPTER 2

LITERATURE REVIEW

2.1 General overview of marine microalgae

Microalgae are heterogenus groups of organism that exert profound impact in today‟s world and have been doing so for billion years. In addition, the algae lack body and reproductive features of the land plants that represent adaptation to terrestrial life. Some of the most significant classes are green algae (Chloropyhceae), brown algae (Phaeophyceae), red algae (Rhodophyceae) and diatoms (Bacillariophyceae) (González-Mariño, 2010; Markou et al., 2012). Cyanobacteria, which have at times been called „blue-green algae‟ are included in this definition even though they are prokaryotic organisms (Pulz and Gross, 2004; Tomaselli, 2004; Brodie and Lewis, 2007; Gong et al., 2011). The interest for these groups of phototrophic organisms lies in their potential utilization, in a similar way to heterotrophic microorganisms, to produce biomass for food, feed and biochemical using solar energy.

Microalgae can be found all over the world. They are mainly distributed in all regions of water bodies, be it freshwater, marine or brackish, as planktonic algae, but are also found on the surface, attached to the bottom or living within sediments of all type of soils, known as benthic algae (Lee, 2008; Graham et al., 2009). Although they are generally free-living, a certain number of microalgae live in symbiotic association with a variety of other organisms (Tomaselli, 2004). Table 2.1 summarizes the different types of habitat colonized by different algae divisions.

Table 2.1: Distribution of algal divisions (Barsanti and Gualtieri, 2006)

Division	Common Name	Habitat			
		Marine	Freshwater	Terrestrial	Symbiotic
Cyanophyta	Blue-green algae	Yes	Yes	Yes	Yes
Prochlorophyta	n.a.	Yes	n.d.	n.d.	Yes
Glaucophyta	n.a.	n.d.	Yes	Yes	Yes
Rhodophyta	Red algae	Yes	Yes	Yes	Yes
Heterokontophyta	Golden algae	Yes	Yes	Yes	Yes
	Yellow-green algae				
	Diatoms				
	Brown algae				
Haptophyta	Coccolithophorids	Yes	Yes	Yes	Yes
Cryptophyta	Cryptomonads	Yes	Yes	n.d.	Yes
Chlorarachniophyta	n.a.	Yes	n.d.	n.d.	Yes
Dinophyta	Dinoflagellates	Yes	Yes	n.d.	Yes
Euglenophyta	Euglenoids	Yes	Yes	Yes	Yes
Chlorophyta	Green algae	Yes	Yes	Yes	Yes

Note: n.a., not available; n.d., not detected

Furthermore, some algae not only tolerate extreme environmental conditions but require such conditions to thrive (Pulz and Gross, 2004). These are called extremophilic algae and can be found in a remarkable range of habitats, from snowfileds to the edge of hotsprings, and from volcanic and geothermal sites to artic environment that show no obvious sign of vegetation (Brodie and Lewis, 2007). Some of them thrive under adverse conditions for example in distilled water or under conditions of high salinity, acidity, temperature and some in presence of heavy metals (Tschermak-Woess, 1988).

The profound diversity of size ranging from 0.2-2 μm in diameter (picoplankton) up to filamentous form with size of 100 μm and higher (Lee, 2008;

Markou et al., 2012), ecology and colonized habitats, cellular structure, levels of organization and morphology, pigments for photosynthesis, reserve and structural polysaccharides, and type of life history reflect the varied evolutionary origins of this heterogeneous assemblage of organisms, including both prokaryote and eukaryote species (Barsanti and Gualtieri, 2006).

Microalgae represent almost untapped resources for human, due to their enormous biodiversity which is estimated between 200,000 to few thousands species, much more diverse than higher plants (Pulz and Gross, 2004; Natrah et al., 2007; Klein et al., 2011). There are more than 40,000 species known to date (Bhola et al., 2011) and thanks to modern molecular techniques, new species are still being discovered. Specifically, only several hundreds have been studied or kept in culture collection around the world (Görs et al., 2010; Klein et al., 2011). Nevertheless, not all groups of microalgae suitable and can be used, depends on the contents of target product, growth production, ease of cultivation and other fundamental factors.

The classification into divisions is based on various properties such as pigmentation, chemical nature of photosynthetic storage product, the organization of photosynthetic membranes, and other morphological features (Chacón-Lee and González-Mariño, 2010). Barsanti and Gualtieri (2006) stated that there is no easily classification system acceptable to all existing algae because taxonomy is under constant and rapid revision at all levels following accumulating new genetic and ultra-structural evidence.

2.2 Structural and morphological features of microalgae

A typical microalga is often surrounded by a cell wall composed of polysaccharides that are partially produced and secreted by the Golgi body (Tomaselli, 2004). Some are naked, lacking the cell wall. The plasma membrane surrounds the remaining part of the cell. This membrane is a living structure responsible for controlling the influx and outflow of substances in the protoplasm. The nucleus is bounded by a double nuclear membrane which contains DNA molecules, the genetic material of the cell, distributed among the chromosome and undergoes division by mitosis (Tomaselli, 2004; Barsanti and Gualtieri, 2006).

The chloroplasts have membrane sacs called thylakoids that carry out the light reactions of photosynthesis. The thylakoids are embedded in the stroma where the dark reactions of carbon fixation take place. The stroma has small ribosomes, DNA, and in some cases the storage product. Chloroplasts are surrounded by the two membranes of the chloroplast envelope. Some algal division besides this double membrane, one or two membranes of endoplasmic reticulum are present. Sometimes chloroplasts have a dense proteinaceous area, the pyrenoid, which is associated with storage-product formation (Lee, 2008).

Reproduction system of microalgae may be vegetative by division of a single cell or fragmentation of a colony while asexual by production of spore. Aplanospores are aflagellate spores that begin their development within the parent cell wall before being released while autospores are aflagellate daughter cells lack the capacity to develop in the spores and will be released from the ruptured wall of the parent cell. Both vegetative and asexual allow stability of an adapted genotype within a species

from generation to the next whereas or sexual mode involves chromosome or gene association, and meiosis, resulting in genetic recombinant (Tomaselli, 2004; Barsanti and Gualtieri, 2006).

Microalgae may have different types of cell organization: unicellular, colonial and filamentous. Most of the unicellular species are non-motile, but gliding and swimming motility may occur. In motile forms, motility is essentially due to the presence of flagella (Tomaselli, 2004). The phytoplankton must remain floating near the water surface to obtain sufficient illumination for photosynthesis. Most algae can keep afloat and regulate their orientation and depth in two ways: a dynamic solution, obtaining lift by swimming; and a static solution, by buoyancy control or through adaptations so as to reduce sinking rates. In many cases the two solutions function together (Barsanti and Gualtieri, 2006).

2.3 Microalgae cultivation

2.3.1 Growth aspects of the microalgal cells

The main factors controlling marine microalgae productivity and chemical composition are particularly the nutrients quantity and quality, lights, temperature, salinity and pH as well as stage of harvest (Brown et al. 1993; Fidalgo et al., 1998; Gatenby et al., 2003; Khoeyi et al., 2011). The optimal parameters as well as the tolerate ranges are species specific. Indeed, the various factors may be interdependent and a parameter that is optimal for one set of conditions is not necessarily optimal for another (Lavens and Sorgeloos, 1996).

2.3.1.1 Culture medium

In the production of microalgae biomass, the composition of the culture medium is a fundamental factor. For growth to occur, cells require a wide range of mineral nutrients in addition to light, water and carbon (Graham et al., 2009). Mineral nutrients basically are a combination of elements such as nitrogen, phosphorus and micronutrients which consist of various trace metals and the vitamins thiamin (B_1), cyanocobalamin (B_{12}) and sometimes biotin. However the specific types of these nutrients, their concentrations and ratios vary between the media.

Culture medium can be prepared for freshwater, marine and also for soil samples predominantly based on the published recipes, which are well known and widely used in the phycological and aquaculture communities. Various specific recipes for algal culture media are described by Andersen et al. (2005). The complexity and cost of culture media often hinder their use for large-scale culture. The relationship between the nutrients used and the composition of the biomass is known. On the other hand, the culture medium affects the specific growth rate and the maximum level of biomass production (López-Elias et al., 2008). Deficiency in the medium with respect to a given nutrient can cause the alga to adapt its metabolism to new external conditions (Sánchez et al., 2000) thereafter changes biochemical compositions of cells (Harrison et al., 1990).

2.3.1.2 Light

Light conditions are the main factors affecting phytoplankton physiology and the most important factor affecting microalgae photosynthesis kinetics productivity (Ugwu et al., 2007). Light quantity and quality determine the amount of energy available to photosynthetic organisms to conduct their metabolic activities (Khoeyi et al., 2011). In nature, the light regime is discontinuous and intensity varies daily (Ma et al., 1997). Microalgae do not make distinction between natural solar light and artificial light (fluorescent light) as they can be grown both outdoor and indoor (López-Elias et al., 2005; Hu et al., 2008), but they are much more sensitive to light intensity and photoperiod of light and dark cycles (Rocha et al., 2003).

According to Seyfabadi et al. (2010), light are limited in most microalgae cultivation mainly large scale systems because light is easily absorbed and scattered by the cells. Changes in light quantity bring about the differences in microalgae biomass and biochemical composition, and therefore, show different adaptation to different conditions. Numerous studies with microalgae of various groups suggest that pigments, unsaturated fatty acids, carbohydrates, and protein content all change in response to increased or decreased of light intensity (Harrison et al., 1990; Renaud et al., 1991; Fábregas et al., 2002; Solovochenko 2008). The growth rate of photoautotrophic algae is nonlinear function of irradiance levels. At low intensity growth rate increase linearly with irradiance levels, however at high intensity, growth rate becomes light saturated (Fábregas et al., 2004).

2.3.1.3 Temperature

Temperature is an important physical factor since it controls the basic rates of all chemical reactions in the microalgae cell and biomass production (Zhu et al., 1997; Sandnes et al., 2005; Sayegh and Montagnes, 2011). The temperature-growth range is ecologically important as it defines the range over which the algae can be metabolically active and determines the distribution of the algae (Teoh et al, 2010). In the marine environment, an increase of 10°C would double the rate of abiotic chemical reactions (Wolfe et al., 1998). Additionally, a decrease in growth temperature below an optimal level generally increases the degree of unsaturation of lipids in membrane systems (Hu, 2004). However, Thompson et al. (1992) and Durmaz et al. (2009) found that the response to temperature was species specific and that there was no overall consistent relationship between temperature and fatty acid unsaturation.

The optimal temperature for phytoplankton cultures is generally between 20°C and 24°C, although this may vary with the composition of the culture medium, the species and strain cultured. Most commonly cultured species of microalgae tolerate temperatures between 16°C and 27°C (Lavens and Sorgeloos, 1996).

2.3.1.4 pH

Microalgae are sensitive to the hydrogen ion concentration (pH) changes, where adjustment is essential for keeping optimal growth of cultures (Khalil et al., 2010). The pH range for most cultured algal species is between 7 and 9, with the

optimum range between 8.2 – 8.7 (Barsanti and Gualtieri, 2006). Khalil et al. (2010) mentioned that pH is important for the character of metabolism of microorganism and hence for the biosynthesis of the bioactive products as secondary metabolites. pH shift can cause a multitude of bioenergetics and biochemical changes in photosynthetic organisms (Alyabvey et al., 2011). Complete culture collapse due to the disruption of many cellular processes can result from a failure to maintain an acceptable pH. The kind of buffer used is not only dependent on the pH range of interest but also taking into account the interference with other medium components (Rocha et al., 2003). In the case of high-density algal culture, the addition of carbon dioxide allows to correct for increased pH, which may reach limiting values of up to pH 9 during algal growth (Barsanti and Gualtieri, 2006).

2.3.1.5 Salinity

Marine phytoplankton is typically tolerant to changes in salinity. Their adaptability to salinity is based on their tolerance extent either as halophilic where salt is require for optimum growth or as halotolerant which have mechanism response that permit their existence in saline medium (Roa et al., 2007). This is due to their capability of accumulating molecules as osmoregulatory substances in response to an increase in salinity of the environment (Hu, 2004).

The halotolerant unicellular green algae, *Dunaliella*, is an example for its ability to survive extreme salt stress or hypersaline environments and serve as a useful model to comprehend the strategies of cell response to high salt concentration (Liska et al., 2004). Most species grow best at a salinity that is slightly lower than that of their native habitat. The literature suggests that, within fairly wide tolerance

range, different levels of salinity are associated with small changes in the gross chemical composition of marine microalgae. The optimum salinity for maximum lipid production was found to be within the range of 30-35 ppt for the two species *Isochrysis* sp. and *Nannochloropsis oculata* (Renaud et al., 1994b).

2.3.1.6 Mixing

In photoautotrophic cultures, mixing does take part in ensuring efficient use of lights and nutrients to enhance the photosynthetic productivity (Erisken, 2008). Mixing is necessary to prevent settling of the microalgae, keeping the cells well dispersed in the medium and to improve gas diffusion between culture medium and air (Lavens and Sorgeloos, 1996). According Ricmond (2004), at low population density mixing has no effect on photosynthetic rate. As the cell densities became higher, increasing stirring resulted in significantly enhanced photosynthesis.

Barsanti and Gualtieri (2006) mentioned that microalgae in the ocean itself seldom experience turbulence hence mixing must be gentle as not all algae species can tolerate vigorous mixing. Different method of mixing is achieved depending on the volume, from stirring manually by hand in small scale cultures of test tubes and flasks, aerating in tanks to bigger scale cultures using paddle wheel or pump in raceways and ponds (Lavens and Sorgeloos, 1996; Barsanti and Gualtieri, 2006). Leupold et al. (2013) added, each microalgae species has its own hydrodynamical growth optimum and that has to be taken in consideration when cultivation microalgae and designing production systems.

2.3.2 Growth dynamics

Batch culture is the most common and widely used system in microalgae cultivation due to its simplicity, practical and low cost. The population shows a typical pattern of growth according to sigmoid curve (Figure 2.1). Basically there are six reasonably well defined phases of algal growth in batch cultures, starting with the lag and accerelation phase, followed by the exponential phase, continued with the retardation (early stationary), stationary phase and finally the death phase (Barsanti and Gualtieri, 2006).

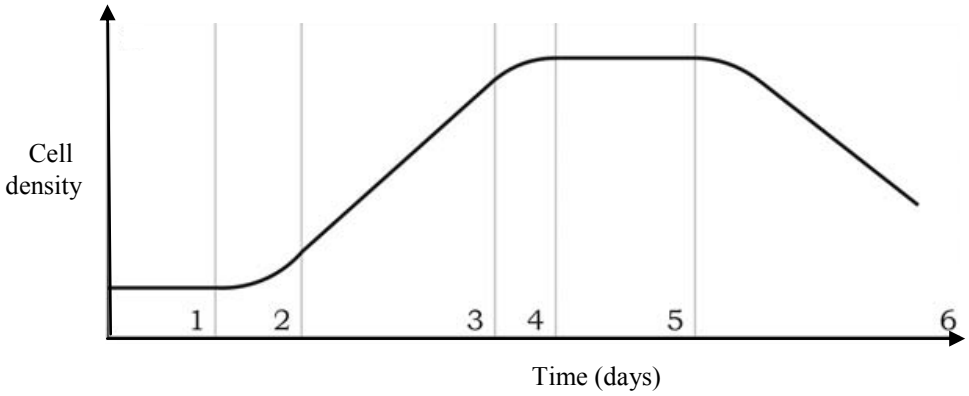

Figure 2.1: General pattern of microalgae growth curve in batch cultures

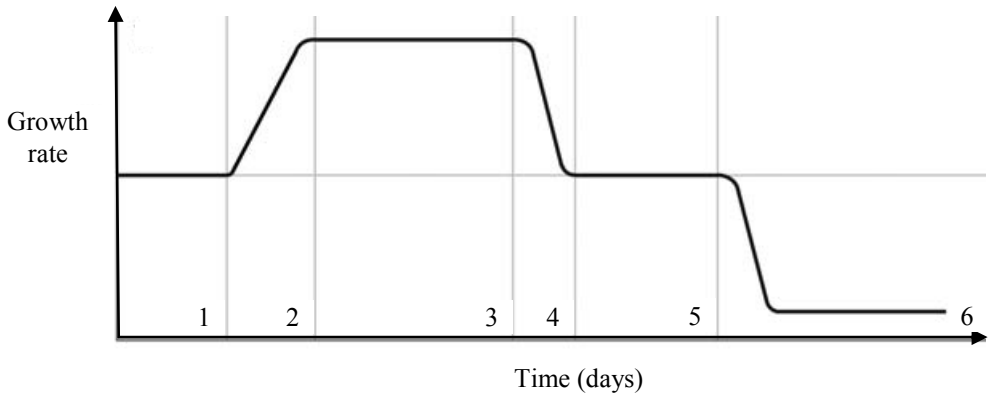

Figure 2.2: Corresponding pattern of microalgae growth rate 1) Lag; 2) Acceleration; 3) Exponential; 4) Retardation; 5) Stationary; 6) Decline

After the inoculums, growth does not necessarily start right away, even most cells may be viable, but not in condition to divide. The growth lag could also be the period of physiological adaptation and adjustment of cells due to changes in nutrient or new culture conditions. During lag phase is the specific growth rate where the minimum level may often be observed. This lag or induction phase is relatively long when an algal culture is transferred from a plate to liquid culture. Meanwhile, an inoculum taken from healthy exponentially growing culture is unlikely to have any lag phase when transferred to fresh medium under similar growth conditions, thus reduce the time required for upscaling (Lee and Shen, 2004; Barsanti and Gualtieri 2006).

Once the cells have adjusted to the new environment and begin to grow and multiply in a short period of accelerating growth phase, they eventually enter the exponential or logarithmic growth phase. At the latter phase, cell density continuously increases while the growth rate reached its maximum value. Growth rate is one important way of expressing the relative ecological success of a species or strain in adapting its natural environment or the experimental environment exposed upon it.

Declining or retardation growth normally occurs in the cultures when specific requirements for cell division is limiting or is inhibiting reproduction. In this phase, culture biomass is often very high and exhaustion of nutrients, carbon dioxide, or photo inhibition and other factors begin to limit the growth and eventually the growth rate decreases.

Cultures enter stationary phase when limiting factor and the growth rate are balanced, which results in a relatively constant cell density and the net growth is zero. The cells may undergo dramatic biochemical changes. The nature of the changes depends upon the growth limiting factor. Nitrogen limitation may result in the reduction in protein content and relative changes in lipid and carbohydrate content (Renaud et al., 1991; Chu et al., 1996). Light limitation due to mutual shading of cells will result in increasing pigment content of most species and shifts in fatty acid composition (Yingying and Changhai, 2009).

When vegetative cell metabolism can no longer be maintained, the death or „crash" phase characterized by a negative growth rate, generally very rapid (Cui et al., 2006). During this phase water quality deteriorates, due to nutrients depleted to a level incapable to sustain growth. Cultures of some species will lose their pigmentation and appear washed out or cloudy, whereas other species may lyses but the culture colour will be maintained. Cell density decreased rapidly and the culture eventually collapses (Lavens and Sorgeloos, 1996; Lee and Shen 2004; Barsanti and Gualtieri, 2006).

2.4 Nutritional values of microalgae

The nutritional value of microalgae is related to their biochemical composition (Renauld et al., 1991; Fidalgo, 1998; Yinying and Changhai, 2009). The composition of algae, as with any higher plants, is not a constant factor (Richmond, 2004). The content varies within species, culture of batch or continuous system, culture conditions which can change significantly under different physical and chemical treatments; particularly in their gross composition and fatty acids content (Brown et al., 2002). Two important characteristic to evaluate the potential of a species are growth rate, in terms of cell numbers or biomass and biochemical composition (Araújo et al., 2005). Microalgae have a great potential to produce a wide range of important biochemical compounds mainly protein, carbohydrate, lipid and especially essential fatty acids. (Seyfabadi et al., 2010).

The high protein content of various microalgae species is the main reasons to consider them as unconventional source of protein. Spolaore et al. (2006) mentioned that many metabolic studies have confirmed the capacities of microalgae as a novel source of protein; the average quality of most of the algae examined is equal or even superior to that of other conventional high quality plant proteins. Basically the amount of protein in microalgae depends on the concentration of nitrogen uptake. Among the greatest biochemical changes are associated with low levels nitrogen in the culture medium causing a large decrease in microalgal protein (Renauld et al., 1991; Ördög et al., 2012).

Carbohydrate in microalgae can be found in the form of starch, glucose, sugars and other polysaccharides (Becker, 2004). Carbohydrate productions serve as two main purposes in algae; act as structural components in the cell wall and also as storage compounds inside the cell (Markou et al., 2012). The same authors further suggest that microalgae carbohydrate can be used by several biomass technologies for biofuel productions. The yields of carbohydrate content depend on the microalgal species and on the cultivation parameters and environmental conditions.

Lipids are major sources of metabolic energy and important materials for the formation of cell structure and tissue membranes. They are crucial in the physiology and reproductive processes and reflect the biochemical and ecological conditions of the marine environment (Bergé and Barnathan, 2005). According to Huerlimann et al. (2010) the total lipid content varies between species ranging from very low to very high, 4.5% to 80%, respectively under certain conditions. Algal lipids are composed of glycerol, sugar or bases esterifies to saturated or unsaturated fatty acids (Spolaore et al., 2006). Microalgae are gaining interest because their ability to produce considerable amount of polyunsaturated fatty acids (PUFA). Among all the fatty acids, omega 3 (n3) and omega 6 (n6) are of particular interest. Nevertheless, human and animal lack requisite enzymes to synthesis PUFA and must be obtain from food and are, therefore, often know as essential fatty acids (Milledge, 2011).

2.5 Antioxidant properties

Recently, the use of photosynthetic microorganisms such as microalgae has received increasing attentions due to their diverse phytometabolic contents with various chemical structures and biological activities (Hajimahmoodi et al., 2010). In particular, microalgae have drawn major interest as prospective and valid sources as natural antioxidant (Matsukawa et al., 2000; Goiris et al., 2001; Herrero et al., 2006; Cerón et al., 2007). An antioxidant is generally defined as substance that effectively reduces, prevents or delays the adverse effects cause by free radicals (Klein et al., 2011).

During the photosynthesis process, microalgae absorb light which converted carbon dioxide (CO_2) into carbohydrate, and at the same time generating molecular oxygen. Microalgae are exposed to free radicals and other oxidizing agents when oxygen is activated by ultraviolet radiation (UV) (Rodriguez-Garcia and Guil-Guerrero, 2008).

This has resulted in the development of antioxidant protective mechanism in the microalgae cells. Free radicals can be produced during every step in a chain reaction, thus it is important for an antioxidant to prevent the chain initiation step by scavenging the initiator radical (Goh et al., 2010).

Hence, the scavenger capacity of microalgal which able to produce protective chemicals contents including protection against lipid-membrane peroxidation of polyunsaturated fatty acids and protein, DNA damage and UV light effects (Cerón et

al., 2007) in the cells, also have been proposed as important substances in the fight against various oxidation-associated conditions related to health like chronic diseases, inflammation, ageing or skin UV-exposure (Hajimahmoodi et al., 2010) as well as important lipid peroxidation for food preservation (Wang et al., 2009; Rodriguez-Garcia and Guil-Guerrero, 2008).

On the other hand, many synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG) have long been used in industries to retard the oxidation process, however, the use of these synthetic antioxidants are under strict regulation due to potential health hazards and suspected activity as promoters of carcinogenic (Mirinda et al.,1998; Natrah et al., 2007; Sheih et al., 2009, Goiris et al., 2012). The search for new source of natural and safe antioxidants as alternatives to synthetic products is therefore of great interest among researchers and industrialist.

2.6 Commercial application derive from microalgae

2.6.1 Aquaculture industry

Over-harvesting and fishing of wild populations have reached critical thresholds in recent years, has lead to an ever increasing focus on aquaculture production (Borowitzka et al., 1997) and play vital contributions to human nutrition in reveling nutritional deprivation (Muller-Fugea, 2000). Fish oils are well known sources of polyunsaturated fatty acids (PUFAs), however issues have been raised such as fish oil content varies with species, locality and water depth (Khozin-Goldberg et al., 2011), peculiar taste and unpleasant odour (Vazhappilly and Chen

1998; Pulz and Gross, 2004) as well as accumulation of pollutants and toxins in fish (Patil et al., 2007; Milledge, 2011).

Microalgae are critical component of the aquaculture, especially in mariculture, being the food source for larvae of many species of mollusk and fish. In addition, microalgae serve as a food source for zooplankton (artemia, rotifers, copepode, brine shrimp) which in turn are fed to late larval, juvenile fish and crustaceans (Fidalgo 1998; Brown 2002; Huerlimann et al., 2010; Seyfabadi et al., 2010). The importance of algae in aquaculture is not surprising as algae are the natural food source of these animals. Although several alternatives for algae exist such as yeasts and microencapsulated feeds (Ferreira et al., 2009), live algae are still the best and preferred food source (Borowitzka et al., 1997).

Microalgae production and its use in aquaculture has been extended and optimized in hatcheries. Most algae species in aquaculture have been selected in basis to their mass-culture potential, of proper cellular size, digestibility and overall the essential nutritional value (Brown et al., 1993; Gatenby et al, 2003; Alonso et al., 2012; Courtois de Viçose et al., 2012). Live microalgae (nanoplankton) which were used to enrich foods of young larvae, ornamental fish, shell fish and bivalves (Olivera et al., 1999; Cho et al., 2007) in hatchery tanks are known as „green water technique". The „green water technique" has positive role in the larvae rearing ponds mainly providing nutrition for fish as well as improving the water quality by oxygen production, pH stabilization (Muller-Feuga, 2000) therefore increase the survival rates.

For most species, microalgae requirements differ, depending on whether they are for broodstock, larval or post-larval rearing (Gatenby et al, 2003). The larval stages require high bacteriological and biochemical quality, but in small amounts and for a short time. Post-larvae accept lower quality, but remain sensitive to the biochemical composition and require amounts nearly a hundred times greater, depending on the length of the nursery stage (Muller-Feuga, 2000).

Furthermore, lipids are highly significant at various early stages of marine fish larvae, affecting the spawning and the egg quality of many fish species, mussels and oysters (Navarro et al., 2001; Pronker et al., 2008; Ronquillo et al., 2012). Various polyunsaturated fatty acids (PUFAs) synthesized by algae are important for the growth of fish, shrimps and molluscs. Deficiency in these acids stems, at times, from an indiscriminate use of algal species in hatcheries and seems to be the major cause of the low survival rates often encountered. The content of highly unsaturated fatty acids (HUFAs), in particular eicosapentaenoic acid (EPA) (20:5n3), arachidonic acid (AA) (20:4n6) and docosahexaenoic acid (DHA) (22:6n3) is of major importance, particularly promoting regular growth and enhance larval survival rates hence its inclusion in the daily diet as a standard nutritional requirement is urged (Fidalgo et al., 1998; Carvalho and Malcata, 2005; Patil et al., 2007). Because of the requirements for essential long chain PUFAs, thus fish farming depends on marine lipids.