GROWTH PROFILE AND LIPID COMPOSITION OF LOCALLY ISOLATED BENTHIC DIATOM *Amphora subacutiuscula* (SCHOEMAN, 1972) UNDER DIFFERENT CULTIVATION CONDITIONS

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by

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ALA	α-linoleic acid
ANOVA	Analysis of variance
ARA	ARachidonic acid
ATP	Adenosine triphosphate
BCA	bicinchoninic acid
bp	Base pairs
BSA	Bovine serum albumin
C16	16 carbons
C18	18 carbons
CO_2	Carbon dioxide
d	Day
DHA	Docosahexaenoic acid
DI	deionized water
DUFA	Diunsaturated fatty acid
DW	Dry weight
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl ester
g nitrate L^{-1}	Gram of nitrate per liter
g tryptone L^{-1}	Gram of tryptone per liter
g yeast extract L^{-1}	Gram of yeast extract per liter
g	Earth's gravitational acceleration
GCMS	Gas chromatography mass spectrometer

gL^{-1}	Gram per liter
h	Hour
HPLC	High-performance liquid chromatography
L	Liter
LSU	Large subunit
mg EPA g-1 biomass	Milligram of EPA per gram of biomass
MUFA	Monounsaturated fatty acid
NJ	neighbour-joining
PCB	Polychlorinated biphenyls
PCFA	Principal component factor analysis
PCR	Polymerase chain reaction
ppt	Part per thousand
PUFA	Polyunsaturated fatty acid
rDNA	Ribosomal deoxyribonucleic acid
SEM	Scanning electron microscopy
SGR	Specific growth rate
TAG	Triacylglycerol
TEM	Transmission electron microscopy
TEP	Transport exopolymer particles
TFA	Total fatty acid
TL	Total lipid
TRYP	Tryptone
UV	Ultraviolet
ω-3	Omega-3

ω-6	Omega-6
YE	Yeast extract

PROFIL TUMBESARAN DAN KOMPOSISI LIPID DARI DIATOM BENTIK *Amphora subacutiuscula* (Schoeman, 1972): DI BAWAH KAEDAH PENGKULTURAN YANG BERLAINAN

ABSTRAK

Diatom bentik adalah pengeluar EPA semulajadi, namun demikian jumlah EPA yang dihasilkan adalah rendah dalam persekitaran semula jadi, terutamanya penderitaan daripada pembatasan resapan cahaya. Kekurangan ini telah mendorong penyelidikan untuk memperbaiki kandungan EPA dalam biojisim yang dikultur serta mod pengkulturan heterotrofik mempersembahkan suatu peluang yang baru. Sebahagian daripada ini, komposisi kimia kasar dan komposisi asid lemak pada strain pemencilan tempatan telah ditentukan sebagai sebahagian daripada kajian ini untuk mencari satu spesies untuk digunakan sebagai makanan dalam akuakultur tropika terutamanya diet yang kuat mempengaruhi ke atas kemandirian dan komposisi biokimia kasar *Artemia* sp. yang sedang membesar. Walau bagaimanapun, tiada kajian setakat ini yang telah dijalankan untuk mengkaji hubungan antara pindahan PUFA bentik diatom dari mod yang berbeza penanaman ke atas *Artemia* sp.. Dalam kajian ini, diatom bentik pemencilan tempatan ini telah dikenal pasti sebagai *Amphora subacutiuscula* berdasarkan filigeni dan dinding sel silika klasifikasi. *A. subacutiuscula* EPA pengeluaran ini telah disaring untuk mengkaji keupayaan heterotrofik serta mengenalpasti sumber karbon pilihannya. Faktor penghad yang lain seperti pemakanan (karbon, nitrogen dan silikat) dan persekitaran (pH,

suhu dan kemasinan) telah diambil kira untuk pengeluaran EPA. Di bawah kajian saringan keupayaan berheterotrofik, *A. subacutiuscula* mempunyai keupayaan untuk tumbesaran heterotrofik dengan menggunakan glukosa dan sukrosa sebagai sumber karbon. Penyelidikan ini adalah unik di mana sebelum ini, sumber karbon pilihan dan keupayaan untuk menjalani heterotrofik tidak pernah dilaporkan atau tidak diketahui. Faktor pemakanan seperti sumber karbon (glukosa), silikat, nitrogen kompleks (ekstrak yis) dan sumber nitrogen ringkas (nitrat) memberi kesan yang ketara ke atas kandungan EPA *A. subacutiuscula*. Suhu merupakan faktor alam sekitar tunggal yang paling mempengaruhi kandungan pemakanan dan metabolit *A. subacutiuscula* terutamanya kualiti kandungan asid lemak. Di bawah kajian pengoptimuman, kandungan EPA dan jumlah lipid pada heterotrofik *A. subacutiuscula* yang baru diasingkan telah dipertingkatkan dengan ketara sehingga 34.3 mg EPA g^{-1} biojisim dan 57% berbanding 5.89 mg EPA g^{-1} biojisim dan 8% apabila ia dikultur di bawah mod fototrofik. Satu percubaan pemakanan dengan menggunakan *A. subacutiuscula* sebagai makanan kepada *Artemia* sp. telah menunjukkan bahawa biojisim heterotrofik *A. subacutiuscula* adalah lebih berkesan dalam meningkatkan pertumbuhan (kadar pertumbuhan spesifik, 117.3% $hari^{-1}$), kemandirian (94%) serta kandungan nutrien *Artemia* sp. yang sedang membesar. Di bawah pertumbuhan heterotrofik, *A. subacutiuscula* berupaya menghasilkan biojisim yang berkualiti untuk kegunaan industri akuakultur dan juga sebagai sumber EPA alternatif, begitu juga sebagai

makanan tambahan akuakultur dan makan tambahan yang berkhasiat untuk manusia.

GROWTH PROFILE AND LIPID COMPOSITION OF LOCALLY ISOLATED BENTHIC DIATOM *Amphora subacutiuscula* (SCHOEMAN, 1972) UNDER DIFFERENT CULTIVATION CONDITIONS

ABSTRACT

Benthic diatoms are the natural EPA producers but the amount is notably low under their natural environment, mainly suffer from the limitation of light diffusion. This drawback has prompted research to improve the EPA content and the heterotrophic mode of cultivation presents a new opportunity as a practical solution. The gross chemical composition and fatty acid composition of the locally isolated strain was determined to find a species as feed in tropical aquaculture, especially the diet that strongly influences survival and gross biochemical compositions of ongrown *Artemia* sp.. However, no studies to date have been conducted to investigate the relationship between PUFA transferred from different cultivation mode of benthic diatom to *Artemia* sp.. A locally isolated benthic diatom, identified as *Amphora subacutiuscula* based on phylogeny and silica cell wall classification was used in this study. This EPA producer *A. subacutiuscula* was screened for its heterotrophic capability and the carbon source preference. Other limiting factors, such as nutritional (carbon, nitrogen silicate) and environmental (pH, temperature and salinity) were taken into account for EPA production. Under a screening study, *A. subacutiuscula* possesses heterotrophic capabilities by utilizing glucose and sucrose as the carbon source. This investigation is unique as the carbon source preference and its ability to undergo heterotrophic were previously unknown and not reported.

Nutritional factors such as carbon source (glucose), silicate, complex nitrogen (yeast extract) and simple nitrogen source (nitrate) were found to significantly affect the EPA content of *A. subacutiuscula*. Temperature was the sole environmental factor that extensively affected nutritional and metabolite contents of *A. subacutiuscula* especially the quality of fatty acid methyl ester.Under the optimization studies, the EPA content and total lipid of this newly isolated heterotrophic *A. subacutiuscula* was enhanced significantly to 34.3mg EPA g^{-1} biomass and 57% as compared with 5.89 mg EPA g^{-1} biomass and 8% when it was cultivated under phototrophic mode. A feeding trial using *A. subacutiuscula* as a feed to *Artemia* sp. showed that the heterotrophic biomass was a superior diet in improving the growth (specific growth rate, 117.3% day^{-1}), survival (94%) and nutritional content of the ongrown *Artemia* sp.. The heterotrophic growth of *A. subacutiuscula* enables the production of higher quality of microalga biomass for application in aquaculture industries by serving as an alternative EPA source, supplemental aquaculture feeds and also as a nutritional supplement for humans.

CHAPTER 1

INTRODUCTION

1.1 Polyunsaturated fatty acid and its potential benefits

Polyunsaturated fatty acids (PUFAs) are defined as fatty acids containing two or more ethylenic bond (Arts *et al*., 2009). In Algal, its fatty acid can be saturated or unsaturated (1 to 6 double bonds). The double bonds are virtually always in the *cis* configuration and in polyunsaturated fatty acid (PUFA) the double bonds are allylic (three carbon atoms apart). Among the various fatty acid constituents of algal lipids, perhaps the most important are the PUFA, and within this group the essential fatty acids (EFA). PUFA are gaining increasing importance as valuable pharmaceutical products and ingredients of food owing to their beneficial effect on human health (Stoll *et al*., 1999; Davis and Kris-Etherton, 2003; Richardson, 2004). In aquatic animal, PUFA have been shown to be essential for a variety of mollusc, crustacean, fish larvae and also for other marine animals (Volkman *et al*., 1992; Brown *et al*., 1997; Sargent *et* al., 1999; Brown, 2002; Sorgeloos *et al*., 2003).

Docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) are the omega-3 PUFA that important in the development and functional of brain, retina, and reproductive tissue in humans as well as in animals. In human they can used in the treatment of various diseases and

disorder, including cardiovascular problems, a variety of cancers, and inflammatory diseases. Most marine animals have little or no capability to transform PUFA such as linoleic acid (LA) or linolenic acid into longer and more unsaturated fatty acids, they must obtained from the food chain.

DHA and EPA have been known to be essential fatty acids for good survival besides improved quality and growth of the marine organism. They are present in high concentration in neural and visual membranes, and insufficiency in the diet may result in serious consequences for a wide range of physiological and behavioural processes (Mourente *et al*., 1993; Reitan *et al*., 1994; Copeman *et al*., 1999; Dhont and Stappen, 2003). These include impaired pigmentation and vision at low light intensities, leading to low hunting capabilities and impaired development of neuroendocrine system.

1.2 Benthic diatom

Diatoms are unicellular photosynthetic eukaryotes within the class Bacillariophyceae and generally between 2 to 200 µm (Hasle *et al*., 1996). Diatoms are typically divided into two categories based on valve symmetry, namely the centric and pennate diatoms. The centric diatoms are mostly planktonic and can be found floating on the water surface, while pennate diatoms are often found as benthic forms growing on sediments or attached to submerged substrates (Lebeau and Robert, 2003a). Diatoms are so

diverse that, scientists estimated about 100,000 extant diatom species on the planet (Round *et al*., 1990; Hasle *et al*., 1996).

Pennate or benthic diatoms are used in numerous applications. In aquaculture, diatoms serve as feedstock for mollusks and crustaceans (Scipione and Mazzella, 1992), and act as larval settlement inductors and food for the early juvenile stages of gastropods such as abalone (Couteau *et al*., 1994; Kawamura *et al*., 1995; Brown *et al*., 1997; Gallardo and Buen, 2003). In biotechnology, most studies surrounding diatoms have been focusing on the diatoms' intracellular metabolites such as eicosapentaenoic acid (EPA) production, total lipid content, amino acid content, pigment production, antibiotics and antioxidant properties (Kyle and Gladue, 1991; Wen and Chen, 2001a; 2001b; 2002; Chen *et al*., 2007).

1.3 Cultivation of benthic diatom

Naturally, diatoms are obligate phototrophs. They are able to assimilate both artificial and natural sunlight as the energy source, and inorganic carbon (usually carbon dioxide, CO_2) as the carbon source (Huang *et al*., 2010). To date, three main phototrophic cultivation systems have been developed for microalgae cultivation. They are the open pond system, closed photobioreactors with natural sunlight and closed

photobioreactor with artificial illumination (Wen and Chen, 2003; Lebeau and Robert, 2003a).

Under phototrophic cultivation systems, valuable products such as lipids and PUFAs have been produced. However the amount of these particular products produced are significantly low. Most phototrophic diatoms are affected by light limitation due to mutual shading of cells (Chen and Chen, 2006) which they cannot fully overcome as light penetration is inversely relative to cell concentration (Chen and Johns, 1995; Wen and Chen, 2003) resulting in lower biomass and productivity. Cohen and Heimar (1994) reported that the EPA productivity of *Porphyridium cruentum* in the open pond was between 0.5 mg L^{-1} day^{-1} to 1.0 mg L^{-1} day^{-1} during winter and summer. It is estimated that the EPA productivity in open ponds can only reach 4 - 8 mg L^{-1} day^{-1} in the optimum conditions (Ratledge, 1997).

Closed photobioreactors have been employed to overcome the problems encountered in open pond systems (Tredici, 1999; Molina Grima *et al*., 2003; Wen and Chen, 2003). These systems are made of transparent materials and illuminated with natural light. Contamination can be avoided due to the closed system design, but the growth of algae is still limited by the bioreactor configuration and cultivation conditions (Wen and Chen, 2003). Enclosed photobiorectors in general are similar to conventional fermenters, with a requirement of light and carbon dioxide.

These systems have been employed for EPA production from *Nannochloropsis* sp. (Zittelli *et al*., 2000) and *Phaeodactylum triocornutum* (Molina Grima *et al*., 2001). However, it is difficult to scale up production and require higher capital cost (Wen and Chen, 2003).

A heterotrophic cultivation refers to the condition where microalgae are cultivated in darkness and use organic substrates such as glucose and acetate as energy and carbon source (Chojnacka and Marquez-Rocha, 2004). Under the heterotrophic cultivation, the requirement of light can be eliminated, therefore offering a possibility to greatly increase cell density and productivity (Chen, 1996; Huang *et al*., 2010; Wen and Chen, 2003). Besides that, heterotrophic growth can be a cost-effective alternative to phototrophic growth (Chen, 1996).

However, not all species are able to undergo heterotrophic growth. Chen and Chen (2006) reported that a diatom suitable for heterotrophic growth should have four essential characteristics: (1) ability to divide and metabolize in darkness, (2) ability to grow in economical and easy handled medium, (3) ability to adapt quickly in a newly introduced environment, and (4) capability to survive and withstand hydromechanical stress inside a fermentor. Heterotrophic cultures of *Nitzschia laevis* can attain higher cellular EPA content than that in phototrophic cultures (Tan and Johns, 1996; Wen and Chen, 2000a; 2000b). The same result was reported by Chu *et al.* (1996), where the cultures of *Nitzschia inconspicua* in heterotrophic culture system

resulted higher cell densities than in phototrophic cultures. As a result, the production of the metabolite of interest may be enhanced.

Aside from phototrophic and heterotrophic cultivation, some microalgae are able to undergo mixotrophic cultivation. The photoheterotrophic or mixotrophic cultivation is a combination of phototrophic and heterotrophic that is able to promote higher cell densities. Photoheterotrophic or mixotrophic cultivation refers to the cultivation of diatoms on organic carbon source in the presence of light. Under this mode of cultivation, microalgae are able to undertake both phototrophic and heterotrophic mechanisms. It is believed that the mixotrophic cultivation has the potential to overcome the limitations imposed by the phototrophic cultivation and carries the advantages of the heterotrophic mode of cultivation. Unfortunately, only algal strains that are not sensitive to photoinhibition are suitable for mixotrophic cultivation, limiting the usage of mixotrophic cultivation on algal cultivation (Lee, 2004). When the suitable strains are grown in mixotrophic cultivation, the biomass and metabolite of the tested microalgae increased. For instance, the EPA content of *Navicula saprophila* grown mixotrophically was 19.2 mg EPA g^{-1} biomass, which was higher compared to 13.6 and 17.3 mg EPA g^{-1} biomass under the phototrophic and heterotrophic mode, respectively (Kitano *et al*., 1997).

Other than the cultivation mode, nutritional and environmental factors are reported to be significantly influencing the diatom in terms' of cell growth

and biosynthesis of products. The major nutritional factors consist of carbon, nitrogen, phosphorus and silicate sources since silicates are required for cell wall formation. The major environmental factors include the culturing salinity (especially for marine strains), temperature and pH. A detailed investigation is needed to establish the most suitable medium and environmental condition for specific species.

1.4 Nutritional and environmental factors towards heterotrophic benthic diatom

Carbon is the most important element for heterotrophic cultivation of diatoms as it serves as an energy source for cells to grow. Microalgae must obtain energy from at least one organic carbon source which is usually supplied in the form of monosaccharides (glucose), disaccharides (fructose, galactose), polysaccharides (sucrose, lactose), starch, acetate, glycerol and alcohol (Droop, 1974; Tan and John, 1996; Vazhappily and Chen, 1998; Wen and Chen, 2003; Perez-Garcia *et al*., 2011) depending on the microalgal species used. Glucose was employed as a single carbon source in the heterotrophic culture of *Chlorella zofingiensis* (Ip and Chen, 2005), *Nitzschia laevis* (Wen and Chen, 2000a; 2001a; 2001b; 2002; Chen *et al*., 2007); *N. inconspicua* (Chu *et al*., 1996), whereas acetate was used as a carbon source in the heterotrophic cultivation of *Navicula saprophila* (Kitano *et al*., 1997).

There are two types of nitrogen sources: simple nitrogen sources such as nitrate, urea and ammonium and the complex nitrogen sources such as yeast extract, tryptone and corn steep liquor which are added to the culture medium for heterotrophic microalgae growth. The complex nitrogen sources were reported to be superior compared to the simple nitrogen sources; it may provide amino acids, vitamins and growth factors that indirectly promote growth of most algal species under the heterotrophic mode (Vogel and Todaro, 1997; Aasen *et al*., 2000; Wen and Chen, 2001a). In the culture of *N. laevis* for EPA production, a combination of 0.62 g nitrate L^{-1}, 1.6 g tryptone L^{-1} and 0.8 g yeast extract L^{-1} was identified as the best nitrogen sources (Wen and Chen, 2001a). On the other hand, different species may react differently towards the nitrogen supplied. Of the nitrogen sources (nitrate, ammonium and urea) tested, the highest biomass and lutein yield was attained when urea was used as a nitrogen source in the culture of *Chlorella protothecoides* (Shi *et al*., 2000).

The cell walls of diatoms, the frustules, are composed of silica. In order to form its silica frustules, it is necessary for silicon to be supplied into the culture medium to grow diatoms (Wen and Chen, 2003; Chen and Chen, 2006). A few studies testing the effects of different concentrations of silicate towards lipid and EPA content found that the lipid content of diatom increased with decreasing silicate (Taguchi *et al*., 1987). Under the conditions of limited silicate concentrations, Wen and Chen (2000a) reported an increase of EPA content in heterotrophic *N. laevis*.

Besides nutritional factors, environmental factors such as temperature (Wen and Chen 2003), salinity and pH also play an important role affecting the lipid accumulation and fatty acid composition as well as the EPA content of diatoms. Jiang and Chen (2000) reported a low specific growth rate and higher DHA content was achieved at 15℃ compared to 30℃ when *Crypthecodinium cohnii* was cultured under heterotrophic cultivation. Low temperatures also enhanced PUFA formation in *N. laevis* and *Crypthecodinium cohnii* (de Swaaf *et al*., 1999; Wen and Chen, 2001a; 2001b). However, different species may respond differently (Wen and Chen 2003). Therefore, the effect of chemical and environmental factors should be studied carefully for individual species.

Just as temperature, salinity and pH are also important factors affecting the heterotrophic cultivation of diatoms. In the culture of *N. laevis*, EPA yield was the highest at 14 ppt of the artificial seawater (Wen and Chen, 2001b). Jiang and Chen (2001) found that pH had significant effects on cell growth and fatty acid profile and proportion of omega 3 PUFAs. Between a range of pH 4 to pH 10, pH 7.2 was found to be the best for cultivation of *Crypthecodinium cohnii*.

1.5 *Amphora subacutiuscula* as EPA feedstock

Amphora subacutiuscula, a benthic diatom which was isolated and identified in this study containing various biologically active compounds and is thus of economic interest in aquaculture for the feeding of bivalve, mollusc and gastropod larvae or post-larvae. To date, most benthic diatom cultivation still heavily depends on wild harvested biomass. Their EPA and lipid content plus the biomass produced were significantly very low. The research undertaken and presented in this thesis investigated the use of the benthic diatom *A. subacutiuscula*, as an alternative feedstock to supply lipid, fatty acid methyl ester, EPA, biodiesel production as well as feed for *Artemia* sp.. This study also aimed to enhance the EPA content by investigating factors affecting the culture of *A. subacutiuscula* as well as the factors affecting the production of EPA.

Amphora subacutiuscula was selected as the studied strain mainly due to their greater specific gravity (de Jonge and van Beusekom, 1992) and more economical production costs contributed by its ease of harvesting and dewatering process. In addition, the heterotrophic benthic diatom produced in this study has an advantage to grow without depending on a substrate for attachment, which takes shorter time to grow and at the same time high nutrition biomass can be produced. The high EPA and fatty acid methyl ester content extracted from *A. subacutiuscula* in this study may serve as an alternative for commercial fish oil and fossil fuel, which are believed to be in

shortage in the near future. Besides that, the locally isolated *A. subacutiuscula* may serve as feed for *Artemia* sp. which was then to be used as food for fish and gastropod larvae or juvenile.

Among the live diets used in the larviculture of fish and shellfish, nauplii of *Artemia* constitute the most widely used food item. The cysts of *Artemia* are made available in cans and upon 24hrs incubation in seawater, these cyst release free swimming nauplii that can directly be fed as a nutritious live food source to the larvae of a variety of marine as well as freshwater organisms. The freshly hatched *Artemia* nauplii develop into second larval stage within a matter of hours. It is important to feed first-instar nauplii to the predator rather than starved second-instar meta-nauplii which have already consumed 25 to 30% of their energy reserves within 24 h after hatching.

However, as the fish larvae grew up, the mouth opening tends to be bigger and therefore bigger size of *Artemia* is needed. *Artemia* juveniles and adults are used as a nursery diet not only for their optimal nutritional value but also for energetic advantages as well. The improvement of both *Artemia* growth and its biochemical composition are key issue for the suitable use of *Artemia* biomass in the rearing processes. Different kinds of diets are frequently used for the on-growing of *Artemia* such as live microalgae, dried algae, bacteria, yeast and waste products from food industry, but best results are undoubtedly obtained with live microalgae. The selected microalgal

species is a crucial issue for the improvement of *Artemia* growth, to modify their growth rate and its biochemical composition.

The rapid growth of aquaculture has stimulated the increasing demand for live feed, especially diatom as the primary producer in the aquatic ecosystems. The supply and availability of feed has been and remains a major challenge that plagues the growth, development and productivity of the aquaculture industry. This is also further emphasised by limited knowledge of the nutritional properties of the feed. The quality and availability of feed are critical in determining the success of the targeted aquaculture industries. The high mortality rate is generally linked to the quality and quantity of the feed. The search for new or alternative feeds has undoubtedly been investigated. However, the fundamental nutritional and environmental influences on the growth performance and behaviour of the diatom need to be investigated as well as their macronutrient and micronutrient requirements before being used as an alternative feed for aquatic organisms. Many researcher hypothesize that the nutritional composition of the microalgal diet strongly influences survival and changes in the gross biochemical compositions of ongrown *Artemia* sp.. However, no studies to date have been conducted to investigate the relationship between PUFA from different cultivation mode of benthic diatom transferred to *Artemia* sp..

1.6 Objectives of the study

1. To isolate and identify the local strains of diatom with heterotrophic capability.
2. To study and compare the effects of different cultivation modes on the biomass and biochemical composition of *A. subacutiuscula*.
3. To evaluate the nutritional and environmental requirement of *A. subacutiuscula* towards biomass and EPA accumulation.
4. To study the growth performance and nutrient uptake of heterotrophically cultivated *A. subacutiuscula*.
5. To evaluate the nutritional value of *A. subacutiuscula* as a feed to *Artemia* sp. in prolonging survival of *Artemia* sp..

1.7 Scope of the thesis

The fundamental objective of this study was to identify the species of the locally isolated benthic diatom. Apart from that, the detailed ultrastructure of the strain was used to identify the species.

Secondly, wild isolated *A. subacutiuscula* was screened for its ability to undergo heterotrophic growth. Five different carbon sources were introduced to identify the preferred carbon source for the heterotrophic growth of *A. subacutiuscula*.

Laboratory based experiments were conducted to investigate the effects of cultivation modes, nutrients (carbon sources, silicate, simple and complex nitrogen sources) availability and environmental factors (salinity, initial pH and growth medium temperature) on the growth as well as on enhancing the EPA content of *A. subacutiuscula*. The results were used to optimize the cultivation process, production of total lipid, fatty acid methyl ester and Eicosapentaenoic Acid (EPA) content of the resulting biomass. Simultaneously the nutrient uptake of the strain was discussed.

Lastly, after the optimization studies, biomass of *A. subacutiuscula* which cultivated under different modes of cultivation was used to feed *Artemia* sp. and the effects of *A. subacutiuscula* on the growth rate, survival rate and biochemical composition of *Artemia* sp. were investigated.

CHAPTER 2

LITERATURE REVIEW

2.1 The pennate benthic diatom genus *Amphora*

Diatom are generally divided into two groups: the centric diatom and pennate diatom. Centric diatoms (centrales), which are radially symmetrical, while the pennate diatoms (pennales) are bilaterally symmetrical. Due to the differences noted in pennate diatoms, Round *et al.* (1990) divided the diatoms into three classes: Coscinodiscophyceae, Fragilariophyceae and Bacillariophyceae.

Pennate benthic diatom can be found during low tides in the intertidal areas, growing on sediments (5mm from the surface) or attached to rocks, as well as in the soil (Round *et al*., 1990; Lebeau and Robert, 2003a). The benthic diatom genus *Amphora* Ehrenberg has a widely distribution in the marine, brackish and freshwater environment (Archibald and Schoeman, 1984; Sala and Maidana, 2003; Sala *et al*., 2006, 2007). Morphological classification analysis of the genus is necessary in the identification process. However, to prevent taxonomic confusion, molecular analysis must always be included to the classic description.

According to Sar *et al*. (2004), under light microscopy, *Amphora* from the Halamphora group are easily misidentified due to their similarity in their valve outline. The detail electron microscopy images and description might be useful

to reduce the current taxonomic confusion. Moreover, *Amphora* is a very large and heterogeneous genus represented in the aquatic ecosystem.

In 1972, *Amphora subacutiuscula* was sampled from a maturation pond of the Walvis Bay and was described as a new species by Schoeman, but was restricted to a brief description and line drawing as the iconotype. They are lacking of some importance details such as morphology of striae (dorsal, ventral and girdle), raphe and conopeum. Little is known about the morphology of it. Following this idea, references must be made on classic identification incorporating with the modern analysis to solve the above bottomneck.

2.2 Importance and application of benthic diatom

Benthic diatoms play a major role in building and maintaining the Earth's atmosphere by producing oxygen and consuming carbon dioxide. Hence, they play an importance role and application in biotechnology, aquaculture, pharmaceutical, biomedical and biofuel industry. In addition, benthic diatoms are also used as a biodepollution agent besides as an alternative feedstock of lipid and PUFAs production.

2.2.1 Biotechnological application of biomass

Benthic diatom biomass may be used for various biotechonological applications, mainly in a small scale to a higher scale for commercial outlets. Some specific examples of biotechnological applications of biomass are presented as below.

2.2.1.1 Silica from the cell wall

The siliceous exoskeleton of benthic diatoms exhibits a wide range of morphologies. Their various forms and interesting complex structures, create an interest in the emerging field of nanotechnology (Lebeau and Robert, 2003a; 2003b; Jamali *et al*., 2012). Silica nanoparticles are proven to be important for several biotechnological and biomedical applications such as drug delivery, cell labeling, biosensor design, ultrasound medical imaging and as a targeting and therapeutic platform for drug or enzyme-release systems (Dolatabadi and de la Guardia, 2011). In addition, it can be used as an insecticide (Korunic, 1998; 2013), where the benthic diatom cell walls which are made of silicon dioxide, are able to absorb the waxes on the skin of the insect. As a result, the insect dies from desiccation. Benthic diatom exoskeletons can also be used in abrasive products, deodorants, decolouring agents, filter agents as well as the microfabrication of nanomaterials (Parkinson and Gordon, 1999).

2.2.1.2 Application in aquaculture

A high demand for benthic diatom production arises due to the popularity of abalone culture in the aquaculture industry. Benthic diatoms play an important role in abalone culture as they act as inductors for larval settlement and as food for the early juvenile stages (Kawamura *et al*., 1995). In the Philippines, *Navicula* sp. is used to improve larval settlement in the tropical abalone, *Haliotis asinina* (Gallardo and Buen, 2003). Besides that, benthic diatom of the genus *Coconeis* sp. was reported by Zupo (2001) to play an important role in sex reversal of the shrimp *Hippolyte inermis* Leach 1915.

The blue green pigment, marennine, produced by pennate diatom, *Haslea ostrearia*, was used for oyster greening in the oyster farming industry in France (Turpin *et al*., 1999). Benthic diatoms are also an essential food source to feed different groups of commercially important aquatic organisms. For example, they are used to directly feed all life stages of filter-feeder molluscs (Brown *et* al., 1997) and larval or juvenile stages of some fish (Reitan *et* al., 1997) and crustacean species (Piña *et* al., 2006); or indirectly to feed or enrich copepod, rotifers and Artemia, which in turn are commonly used as major live prey for the rearing of many marine and freshwater larval species (Couteau, 1996; Couteau and Sorgeloos, 1997; Sorgeloos *et al*., 2001; Ritar *et al*., 2004; Iglesias *et al*., 2007; Aragão *et al*., 2004;Seixas *et al*., 2008). Diatoms may also provide adequate levels of amino acids and vitamins for aquaculture food chains (Brown, 1991; Lebeau and Robert, 2003b).

2.2.2 Pharmaceutical and biomedical application

Antibacterial, antitumoral and antifungicidal compounds have been recorded in all algal classes, notably in diatoms. Rowland *et al*. (2001) reported that antitumoral compounds against human lung cancer and anti-HIV were extracted from pennate diatom, *Haslea ostearia*. An amino acid which acts as a moisturizing agent in human skin was extracted from diatom (Lebeau and Robert, 2003a). Active dermatological compounds such as aspartic acid and isoleucine from *Chaetocerous calcitrans*; serine, glutamic and tyrosine from *Thalassiosira* sp. (Derrien *et al*., 1998) were used in cosmetic product. Domoic acid, a toxin produced by *Nizschia navisvaringica* (Kotaki *et al*., 2000) and *Pseudo*-n*itzschia multiseries* (Wright *et al*., 1989) has been used as an antihelminthic compound in traditional medicine and also exhibits insecticidal properties (Lincoln *et al*., 1990).

2.2.3 Biofuel industries

Biodiesel is another potential product from diatom which recently received much attention worldwide. Due to the world energy crisis, many countries have started to find greener and sustainable resources to resolve this problem. Finding alternative energy resources is a pressing mission for many countries, especially countries lacking conventional fuel resources. Algae can be used to produce biofuel called algae fuel or third generation biofuel (Schenk *et al*., 2008;

Demirbas, 2010). Compared to second generation biofuels, algal fuels have a higher yield: they can produce 30 -100 times more energy per hectare compared to terrestrial crops (Demirbas, 2010). High lipid content was recorded in diatoms under stressful conditions (Hu *et al*., 2008). In order to optimize the lipid production with the aim to produce more biodiesel to fulfill the fuel demand, some diatom strains were selected for lipid enhancement studies through genetic manipulation (Dunahay *et al*., 1995).

Microalgae have higher potential in biodiesel production compared to other oil crops. This is mainly because the cultivation of microalgae does not need much land compared to terrestrial plants (Chisti, 2007). Moreover, microalgae grow extremely fast and many strains are rich in oils. Oil levels of 20 – 50% are common in microalgae and levels as high as 55% in heterotrophic *Chlorella protothecoides* (Xu *et al*., 2006a; Perez-Garcia *et al*., 2011). These criteria enable possible industrial production of biofuel from microalgae in the near future.

2.2.4 Biodepollution agent

Diatoms are potentially useful in the bioremediation of water. In aquaculture, wastewater from ponds must be treated due to its high phosphate and nitrogen content. Lefebvre *et al*. (1996) showed that diatoms can be used to treat fish farm effluent. After the treatment, the resulted diatom biomass may be

given back to bivalves as feed. Moreover, this method is more efficient compared to when effluent is provided direct to the cultured bivalves (Lefebvre *et al*., 2000). Additionally, diatoms are also largely used in heavy metal removal on top of serving as an important bioindicator to measure impacts of river pollution. For example, Maznah and Mansor (2002) showed that the diatom community structure and the specific sensitivity of certain diatom species could be related to the degree of water quality.

2.2.5 Sources of lipid and PUFAs

An alga synthesizes fatty acids as building blocks for the formation of various types of lipids (Hu *et al*., 2008). The most commonly synthesized fatty acids have chain lengths that range from C16 to C18 (Hu *et al*., 2008). The polyunsaturated fatty acid (PUFA) such as arachidonic acid (ARA) and eicosapentaenoic acid (EPA) are also produced by benthic diatom.

Under heterotrophic conditions, the production of saturated fatty acids are favoured while highly polyunsaturated fatty acids are mainly produced under phototrophic conditions (Suen *et al*., 1987; Wen and Chen, 2000b). Higher oxygen level in phototrophic culture could lead to the high degree of fatty acid unsaturation because the desaturase enzymes have a requirement for molecular oxygen (Ratledge, 1992; Wen and Chen 2000b). However, the production of EPA and DHA is higher in heterotrophic growth of benthic diatom

(Barclay *et al*., 1994; Gladue and Maxey, 1994; Tan and Johns, 1996; Kitano *et al*., 1997, 1998*;* Wen and Chen, 2000a,2000b, 2001, 2003; Chen *et al*., 2007).

Microalgae or diatoms are an excellent source of PUFA as their fatty acid profile is simpler than in fish oil. Production conditions can be controlled and the algal species can be selected according to the required PUFA of interest (Lebeau and Robert, 2003b).

2.3 Polyunsaturated fatty acid

Algal lipids consist of various fatty acid constituents., and the most important are the polyunsaturated fatty acids (PUFAs). PUFAs are fatty acid that contain more than one double bond in their backbone. There are at least 4 independent families of PUFAs, which include the ω-3 series, ω-6 series, ω-9 series and ω-7 series. All of them are derived from α-linolenic acid (ALA, 18:3, ω-3), *cis*-linoleic acid (LA, 18:2, ω-6), oleic acid (OA, 18:1, ω-9) and palmitoleic acid (PA, 16:1, ω-7).

LA is converted to γ-linolenic acid (GLA, 18:3, ω-6) by enzyme Δ^6 desaturase and GLA is elongated to form dihomo-GLA (DGLA, 20:3, ω-6), the precursor of the 1 series of prostaglandins (PGs). DGLA can also be converted to arachidonic acid (AA, 20:4, ω-6) by the enzyme Δ^5 desaturase (d-5-d). AA forms the precursor of 2 series of PGs (Wen and Chen, 2003; Singh, 2005; Das,

2006). These pathway is called the ω-6 pathway, where the ω-6 PUFA were produced (Figure 2.1).

ALA is converted to eicosapentaenoic acid (EPA, 20:5, ω-3) by d-6-d and d-5-d. EPA forms the precursor of the 3 series of PGs, TXs and the 5 series of LTs (Wen and Chen, 2003; Singh, 2005; Das, 2006). These pathway is called the ω-3 pathway, where the ω-3 PUFA were produced (Figure 2.1). LA, GLA, DGLA, AA, ALA, EPA and docosahexaenoic acid (DHA, 22:6, ω-3) are all PUFAs, but only LA and ALA are essential fatty acids (EFAs).

As demonstrated in Figure 2.1, dietary LA, ALA and OA are metabolized by the enzymes Δ^6 and Δ^5 desaturases and elongases. As a result, these 3 series compete with one another for the same set of enzymes, though the enzymes seem to prefer ω-3 to ω-6 and ω-6 over ω-9. Hence under normal physiological conditions the metabolites of ω-9 are formed only in trivial amounts in the cells (Das, 2006). According to Das (2006), the activities of Δ^6 and Δ^5 desaturases are slow in humans ($\Delta 5 > \Delta^6$). As a result, the conversion of LA and ALA to their respective metabolites may be inadequate under medical compromise patients (diabetes, hypertension, hyperlipidemia and metabolic syndrome X). It is necessary to supplemented AA, EPA and DHA (to bypass Δ^6 and Δ^5 desaturases). Generally, supplement of AA is not necessary since; it can be obtained from the diet.

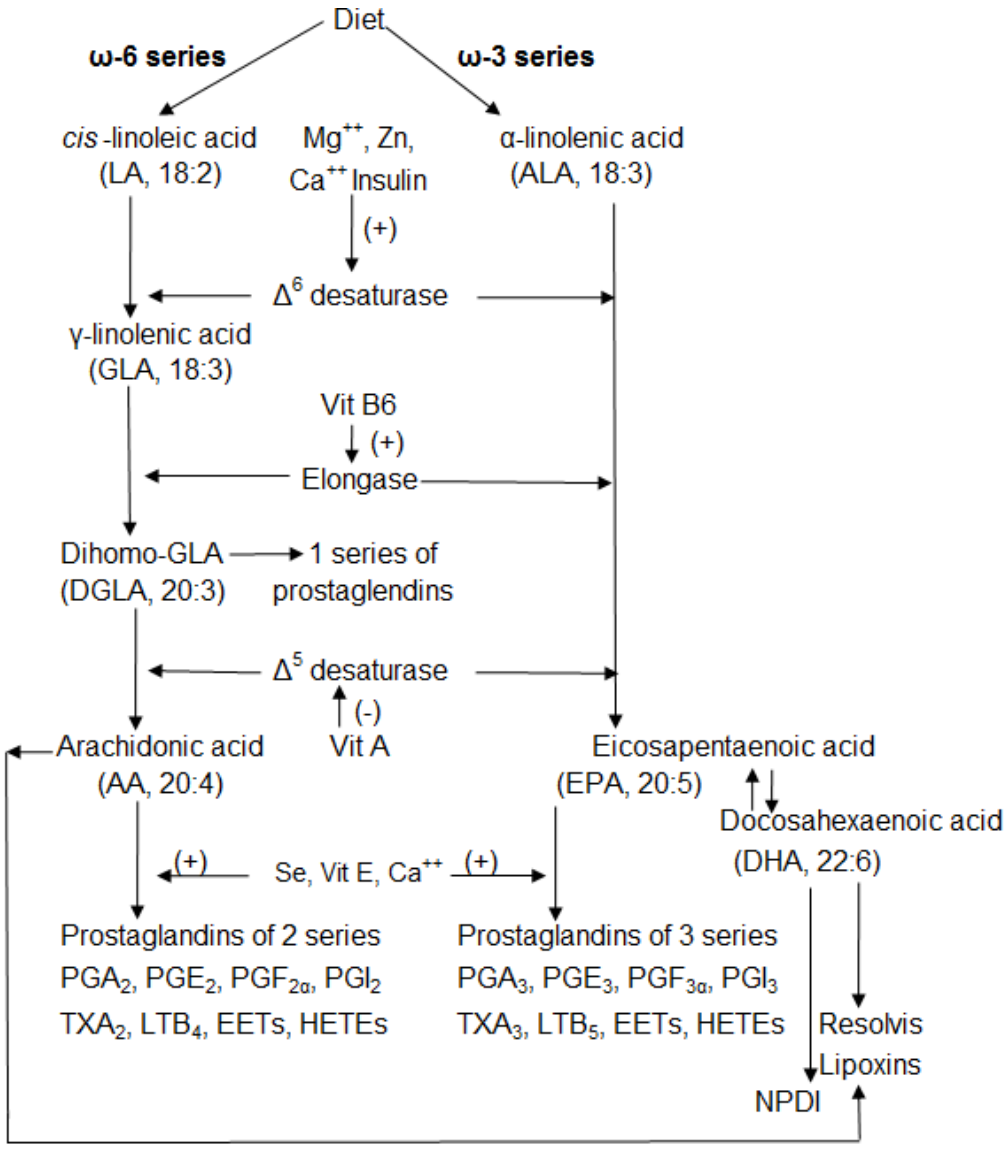

Figure 2.1: Scheme showing the metabolism of essential fatty acids and co- factors that enhance the activity of Δ^6 and Δ^5 desaturase and elongase.

Key: (+): indicates enhancement of the activity of enzyme or increase in the formation of the product; (-): indicates either in the inhibition of the activity of the enzyme or decrease in the formation of the product; EETs: epoxy-eicosatrienoic acids (5,6-, 8,9-, 11,12- and 14,15-EETs); HETEs: hydroxyeicosa-tetraenoic acids (19- and 20-HETEs); PGA: prostaglandin A; PGE: prostaglandin E; PGF: prostaglandin F; PGI: prostaglandin I; TXA: thromboxane A; LTB: leukotriene B; Se: selenium; Vit E: vitamin E; Ca^{++}: calcium; Vit A: vitamin A; Vit B6: vitamin B6 Mg^{++}: magnesium; Zn: zink; NPDI: neuroprotectin Di. (modified from Das, 2006).