UNIVERSITI PUTRA MALAYSIA

SELECTION AND CHARACTERISATION OF TROPICAL MICROALGAE WITH HIGH LIPID CONTENT FOR ENHANCEMENT OF LEUCOCYTES VIABILITY IN BROWN-MARBLED GROUPER, Epinephelus fuscoguttatus (FORSSKAL, 1775)

YAU SOOK KUN

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By

YAU SOOK KUN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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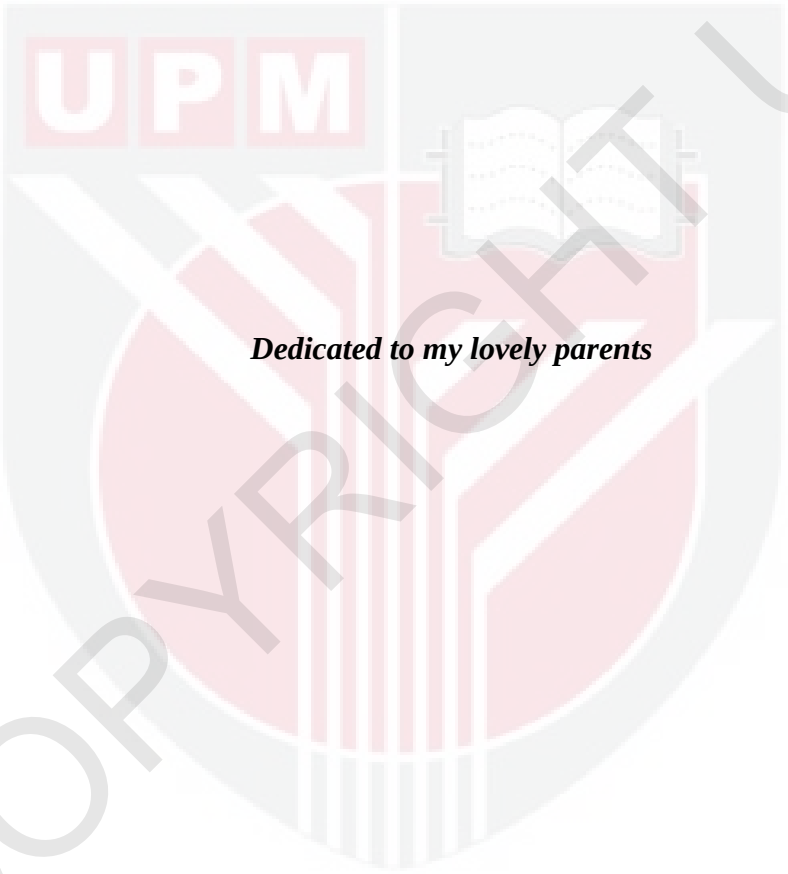

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Master of Science

SELECTION AND CHARACTERISATION OF TROPICAL MICROALGAE WITH HIGH LIPID CONTENT FOR ENHANCEMENT OF LEUCOCYTES VIABILITY IN BROWN-MARBLED GROUPER, *Epinephelus fuscoguttatus* (FORSSKAL, 1775)

By

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December 2015

Chair : Fatimah Md. Yusoff, PhD
Faculty : Institute of Bioscience

In an aquatic ecosystem, microalgae are important primary producers which photosynthesise and supply essential nutrients such as proteins, lipids, vitamins and pigments to the higher trophic level fish and shellfish species. Brown-marbled grouper, *Epinephelus fuscoguttatus* (Forsskal, 1775) is one of the important marine finfish species that is commonly cultured in many Asian countries due to the rapid depletion of wild catches from the sea and yet unstoppable market demand. However till now, the pathogenic disease outbreak frequently occurs in grouper intensive farming and is still the main limiting factor in mariculture production. Immunonutrition using lipid is an environmentally friendly, safe and potential alternative approach to harmful antibiotics and other chemicals, which should be preferred and practiced in sustainable fish farming for fish disease control and prevention. Therefore, this study aimed to screen and select the best microalgae oil strain for *in vitro* enhancement of immune response in brown-marbled grouper based on their growth performance and lipophilic contents. Green microalgae consisting of *Ankistrodesmus falcatus*, *Desmodesmus* sp. and *Scenedesmus* sp. as well as diatoms including of *Chaetoceros calcitrans*, *Cyclotella* sp. and *Skeletonema costatum* were isolated and cultured in Bold's Basal and Conway media respectively. After that, they were identified using a scanning electron microscope (SEM). Specific growth rate, total lipids, total carotenoids in lipid extract and fatty acid profiles of six microalgae species were characterised for screening and selection purposes. The selected microalgal lipophilic extract was further studied in immunonutrition of brown-marbled grouper *in vitro* using trypan blue exclusion (cell viability), bromodeoxyuridine (BrdU, lymphoproliferation) and respiratory burst assays.

In this study, an optimised SEM pretreatment protocol with shorter chemical pre-fixation times (24 hours for *Chaetoceros calcitrans*; 3 hours for other examined microalgae species) and ideal separation forces (3 min at 3213 x *g*) was developed for all the selected microalgae species. Hence, they were successfully identified based on their unique ultrastructural, physical and morphological appearances which were clearly shown in high quality SEM images. Among microalgae species studied, *Chaetoceros calcitrans* had significantly higher ($P < 0.05$) specific growth rate (0.23 day^{-1}) and total lipid (25.67% DW) and arachidonic acid contents (1.34% TIFA),

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whereas *Ankistrodesmus falcatus* showed the highest carotenoid (0.23 mg g^{-1} DW) and eicosapentaenoic acid contents (4.68% TIFA). In addition, two different microalgae lipids were rapidly grouped and identified based on their fatty acid biomarkers in principal component analysis (PCA) i.e. green microalgae lipid with MUFAs and C18-PUFAs as well as diatom lipid with SFAs and LC-PUFAs. Lipids derived from green microalgae and diatoms could be potentially used to fulfil the fatty acid requirements of farmed fish with and without C18-PUFAs bioconversion capability, respectively. Due to the outstanding traits (the highest total lipids with promising growth performance and the presence of LC-PUFAs), *Chaetoceros calcitrans* was selected for further evaluation of its immunonutrition value in brown-marbled grouper *in vitro*.

Chaetoceros calcitrans lipophilic extract (CCLE) that ranged from 6.25 to 50 µg mL^{-1} significantly ($P < 0.05$) enhanced leucocyte viability isolated from peripheral blood of brown-marbled grouper compared to the control. Meanwhile, 3.13 µg mL^{-1} of CCLE had the lowest total viable leucocyte count that was not significantly different ($P > 0.05$) compared to the control. Therefore, four concentrations including 6.25, 12.5, 25 and 50 µg mL^{-1} of CCLE were selected and further tested in lymphoproliferation. Accordingly, 50 µg mL^{-1} of CCLE exhibited the highest stimulation index in both peripheral blood T and B lymphocyte proliferations of brown-marbled grouper stimulated by PHA and LPS respectively, whereas 6.25 µg mL^{-1} of CCLE was the lowest. This study also demonstrated that T lymphocyte proliferation performed better than B lymphocyte proliferation regardless of incubation with or without CCLE. On the other hand, only 6.25 µg mL^{-1} of CCLE had significantly higher respiratory burst activity in spleen leucocyte of brown-marbled grouper ($P < 0.05$) than the control. In conclusion, CCLE showed a potential to be used as an immunonutrient for enhancement of fish immunity, especially brown-marbled grouper. It is recommended that further *in vivo* studies should be conducted to examine the effectiveness of CCLE in an animal model.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PEMILIHAN DAN PENCIRIAN MIKROALGA TROPIKA DENGAN KANDUNGAN LIPID TINGGI UNTUK PENINGKATAN DAYA TAHAN LEUKOSIT DALAM KERAPU HARIMAU, *Epinephelus fuscoguttatus* (FORSSKAL, 1775)

Oleh

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Di dalam ekosistem akuatik, mikroalga merupakan pengeluar utama yang penting untuk fotosintesis dan membekalkan nutrien penting kepada spesies ikan dan kerang bertahap trofik yang lebih tinggi seperti protein, lipid, vitamin dan pigmen. Kerapu harimau, *Epinephelus fuscoguttatus* (Forsskal, 1775) merupakan salah satu spesies ikan laut bersirip yang penting dan biasa dikultur di kebanyakan negara Asia disebabkan kekurangan hasil tangkapan dari laut dan permintaan pasaran yang sangat tinggi. Namun sehingga kini, wabak penyakit patogenik yang kerap berlaku dalam system penternakan intensif kerapu menjadi faktor utama yang menghadkan pengeluaran marikultur. Lipid immunonutrisi merupakan satu alternatif yang mesra alam, selamat dan berpotensi untuk menggantikan antibiotik berbahaya dan bahan kimia, dan patut diutamakan serta diamalkan dalam penternakan ikan mampan bagi kawalan dan pencegahan penyakit. Oleh itu, kajian ini bertujuan untuk memilih spesies mikroalga yang mengandungi minyak terbaik untuk peningkatan tindak balas imun secara *in vitro* dalam kerapu harimau berdasarkan prestasi pertumbuhan dan kandungan lipofilik mereka. Mikroalga hijau yang terdiri daripada *Ankistrodesmus falcatus*, *Desmodesmus* sp. dan *Scenedesmus* sp. serta diatom termasuk *Chaetoceros calcitrans*, *Cyclotella* sp. dan *Skeletonema costatum* diasingkan dan dikultur dalam media Bold's Basal dan Conway. Seterusnya, mereka dikenalpasti secara fizikal dengan menggunakan mikroskop imbasan elektron (SEM). Kadar pertumbuhan tertentu, jumlah lipid, jumlah karotenoid dalam lipid ekstrak dan asid lemak profil dalam enam spesies mikroalga yang dikaji ditentukan untuk tujuan pemeriksaan dan pemilihan. Ekstrak lipofilik dari mikroalga yang dipilih seterusnya dikaji dengan lebih lanjut dalam immunonutrisi kerapu harimau secara *in vitro* melalui kaedah pengecualian tripan biru (daya tahan sel), bromodeoxyuridine (BrdU, lymphoproliferasi) dan pemecahan respiratori.

Dalam kajian ini, rawatan awal SEM yang dioptimumkan dengan masa pra-penetapan kimia yang singkat (24 jam untuk *Chaetoceros calcitrans*; 3 jam untuk spesies mikroalga yang lain) dan kuasa pemisahan yang ideal (3 minit pada 3213 x *g*) telah dijalankan untuk semua spesies mikroalga yang dikaji. Dengan itu, mereka berjaya dikenalpasti berdasarkan ultrastruktur, fizikal dan morfologi yang unik yang

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jelas ditunjukkan dalam imej SEM berkualiti tinggi. Antara spesies mikroalga yang dikaji, *Chaetoceros calcitrans* mempunyai kadar pertumbuhan tertentu (0.23 hari^{-1}) dan jumlah lipid (25.67% DW) dan kandungan asid arakidonik (1.34% TIFA) yang signifikan lebih tinggi ($P < 0.05$), manakala *Ankistrodesmus falcatus* menunjukkan kandungan karotenoid (0.23 mg g^{-1} DW) dan asid eikosapetanoik (4.68% TIFA) yang paling tinggi. Seterusnya, dua lipid mikroalga yang berbeza dikumpulkan dan dikenalpasti dengan segera berdasarkan penanda biologi asid lemak mereka dalam analisis komponen utama (PCA) iaitu lipid mikroalga hijau dengan MUFAs dan C18-PUFAs serta lipid diatom dengan SFAs dan LC-PUFAs. Lipid yang diperolehi daripada mikroalga hijau dan diatom berpotensi untuk digunakan bagi memenuhi keperluan asid lemak untuk spesies ikan yang mempunyai keupayaan bio-penukaran C18-PUFAs (mikroalga hijau) dan sebaliknya (diatom). Disebabkan ciri yang sangat baik (jumlah lipid tertinggi dengan prestasi pertumbuhan yang memberangsangkan dan kehadiran LC-PUFAs), *Chaetoceros calcitrans* telah dipilih untuk dikaji dengan lebih lanjut untuk nilai immunonutrisi secara *in vitro* dalam kerapu harimau.

Julat ekstrak lipofilik daripada *Chaetoceros calcitrans* (CCLE) antara 6.25 hingga 50 μg mL^{-1} adalah signifikan ($P < 0.05$) untuk meningkatkan daya tahan leukosit yang diasingkan daripada darah periferal kerapu harimau berbanding dengan kawalan. Sementara itu, 3.13 μg mL^{-1} CCLE mempunyai jumlah kiraan leukosit berdaya tahan yang paling rendah dan perbezaan yang tidak ketara ($P > 0.05$) berbanding dengan kawalan. Oleh itu, empat kepekatan termasuk 6.25, 12.5, 25 dan 50 μg mL^{-1} CCLE dipilih dan seterusnya diuji dalam limfoproliferasi. Dengan itu, 50 μg mL^{-1} CCLE mempamerkan indeks rangsangan yang tertinggi dalam percambahan kedua-dua limfosit T dan B darah peripheral kerapu harimau yang dirangsangkan oleh PHA dan LPS masing-masing, manakala 6.25 μg mL^{-1} CCLE adalah paling rendah. Kajian ini juga menunjukkan bahawa percambahan limfosit T dengan prestasi yang lebih baik berbanding dengan percambahan limfosit B sama ada pengeraman dengan CCLE atau tidak. Sebaliknya, hanya 6.25 μg mL^{-1} CCLE mempunyai aktiviti pemecahan respiratori yang lebih tinggi dengan signifikan dalam leukosit limpa kerapu harimau ($P < 0.05$) berbanding dengan kawalan. Kesimpulannya, CCLE menunjukkan potensi untuk digunakan sebagai immunonutrien bagi meningkatkan imuniti ikan ternakan terutamanya kerapu harimau. Kajian lanjutan secara *in vivo* dicadangkan untuk mengkaji keberkesanan CCLE dalam model haiwan.

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I certify that a Thesis Examination Committee has met on 29 December 2015 to conduct the final examination of Yau Sook Kun on her thesis entitled "Selection and Characterisation of Tropical Microalgae with High Lipid Content for Enhancement of Leucocytes Viability in Brown-marbled Grouper, *Epinephelus fuscoguttatus* (Forsskal, 1775)" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

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Date: 16 February 2016

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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Signature:	_____	Signature:	_____
Name of Chairman of Supervisory Committee:	Fatimah Md. Yusoff	Name of Member of Supervisory Committee:	Mohd Salleh Kamarudin

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LIST OF ABBREVIATIONS

µg	microgram
µL	microliter
µmol	micromole
µM	micromolar
AA	arachidonic acid (C20:4n-6)
ANOVA	analysis of variance
ALA	α-linolenic acid (C18:3n-3)
BHT	butylated hydroxytoluene
BrdU	5-bromo-2'-deoxyuridine
C18-PUFAs	C18 polyunsaturated fatty acid
CCLE	*Chaetoceros calcitrans* lipophilic extract
Con A	concanavalin A
day^{-1}	per day
DHA	docosahexaenoic acid (C22:6n-3)
DMSO	dimethyl sulfoxide
DW	dry weight
ELISA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid (C20:5n-3)
FAMEs	fatty acid methyl esters
FBS	feotal bovine serum
g^{-1}	per gram
h	hour
kV	kilovolt
LA	linoleic acid (C18:2n-6)

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LC-PUFAs	long chain polyunsaturated fatty acids
LSD	least significant difference
LPS	lipopolysaccharide
M	molar
m^{-2}	per meter square
MA	myristic acid (C14:0)
mA	milliampere
mL^{-1}	per milliliter
MUFAs	monounsaturated fatty acids
nm	nanometer
OA	oleic acid (C18:1n-9)
ROS	reactive oxygen species
SA	stearic acid (C18:0)
s^{-1}	per second
SEM	scanning electron microscopy
SFAs	saturated fatty acids
SPSS	statistical package for the social sciences
PA	palmitic acid (C16:0)
PCA	principal component analysis
PHA	phytohemagglutinin
PBS	phosphate buffered saline
PUFAs	polyunsaturated fatty acids
TIFA	total identified fatty acids

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CHAPTER 1

INTRODUCTION

1.1 General background of the study

Microalgae are highly diverse photosynthetic microorganisms which primarily provide natural food and energy sources to the higher trophic-level consumers. In hatcheries and nurseries, microalgae are usually used as live feed for the entire growth stages of molluscs, larval stages of crustaceans, some fish species and zooplankton due to their appropriate size and high nutritive value (Brown *et al*., 1997). For instance, *Chlorella* sp., *Tetraselmis* sp., *Isochrysis* sp., *Chaetoceros* sp., *Nannochloropsis* sp., *Skeletonema* sp. and *Thalassiosira* sp. are those common strains consumed by farmed aquatic animal species either in unialgal or mixed microalgal diet (Hemaiswarya *et al*., 2011). Microalgae-derived lipids and carotenoids are highly valuable compounds which have been commonly studied in animal nutrition (Spolaore *et al*., 2006). A few years ago, single-celled microalgae lipids have been given much attention due to their potential to replace unsustainable fish oil in aquafeed formulation as they can *de novo* synthesise essential n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFAs), whereby most of the vegetable oils are incapable to synthesise them. In addition, the quantity (lipid content) and quality (fatty acid profile) of microalgae lipid can be improved by selecting species with suitable physical and chemical culture parameters. Carotenoid pigments found in microalgae lipid act as natural antioxidants which could not only prolong its shelf-life but also increase its nutritional value.

Dietary lipid not only provides concentrated energy and essential fatty acids to fish but also has been shown to affect the immune responses in freshwater and marine farmed fish such as channel catfish (Fracalossi & Lovell, 1994), rainbow trout (Kiron *et al*., 1995), Atlantic salmon (Thompson *et al*., 1996), grouper (Lin & Shiau, 2003; Cheng *et al*., 2006) and grass carp (Jin *et al*., 2013). They found that essential fatty acids are the main factor which could modulate the fish immunity by energy generation, regulation of plasma membrane's physical stability and fluidity, formation of eicosanoid precursors, gene expression and cell differentiation (Wesley Alexander, 1998; Calder, 2007). *In vitro* and *in vivo* investigations have proved that fatty acids such as eicosapentaenoic (C20:5n-3, EPA), docosahexaenoic (C22:6n-3, DHA) and arachidonic acids (C20:4n-6, AA), oleic (C18:1n-9, OA), linoleic (C18:2n-6, LA), conjugated linoleic (CLA), γ-linolenic (C18:3n-6, GLA), dihomo-γ-linolenic (C20:3n-6, DGLA), α-linolenic acids (C18:3n-3, ALA) affect the lymphocyte proliferation, cytokine production and natural killer (NK) cells activity (Calder *et al*., 2002). However unlike freshwater fish, marine fish are unable to bio-convert C18 polyunsaturated fatty acids (C18-PUFAs) to long chain polyunsaturated fatty acids (LC-PUFAs) due to their deficiency in delta-5 fatty acid desaturase (Tocher & Sargent, 1990). Thus, marine fish depend strongly on fish oil as the major source of n-3 LC-PUFAs in their daily diets for achieving optimum growth and health performance. In fact, fish oil derived n-3 LC-PUFAs is actually originated and

bio-accumulated from primary producers of microalgae in a marine food chain (Khozin-Goldberg *et al*., 2011). Furthermore, the immune response can be modulated by carotenoids (Rao & Rao, 2007) due to their free radicals scavenging activity (Sowmya & Sachindra, 2013). For instance, innate immune responses of rainbow trout have been enhanced by dietary β-carotene and astaxanthin which are derived from synthetic (Amar *et al*., 2000, 2001) and natural sources of *Dunaliella salina* and *Phaffia rhodozyma* (Amar *et al*., 2004).

Grouper is one of the most important and popular marine finfish species that was first cultured in Malaysia, Singapore, Thailand, Taiwan and Hong Kong in the early of 1970s and now it is widespread to other Southeast Asian countries (Seng, 1998). It is cultured in Kedah, Penang, Selangor and Johor (Peninsular Malaysia) as well as Sabah and Sarawak (Malaysian Borneo) (Pomeroy *et al*., 2002). It is considered an ideal candidate to be intensively cultured due to its favourable attributes such as successful captive spawning, good pellet feed acceptance, high tolerance to crowded conditions, excellent feed conversion ratio, good taste and high consumer demand (Boonyaratpalin, 1997; Sim *et al*., 2005). Brown-marbled grouper, *Epinephelus fuscoguttatus* is considered a luxurious aqua-protein due to its high market price at approximately RM40/kg (Ali *et al*., 2008). Furthermore, it is a fast growth marine finfish which grows faster than camouflage grouper, *Epinephelus polyphekadion* and humpback grouper, *Cromileptes altiveis* (James *et al*., 1998; Rachmansyah *et al*., 2009) that reaches market size of 0.5 kg in 9 to 12 months (Afero *et al*., 2010). Currently, due to the serious incidence of infectious disease outbreaks in intensive mariculture, research on regarding immunonutrition in grouper has been intensified to improve their health status and disease resistance. Several *in vitro* and *in vivo* studies showed that LC-PUFAs have enhanced the marine fish immunity (Wu *et al*., 2003; Li *et al*., 2012, 2013). Compared to *in vivo* study, *in vitro* study is a simpler approach with minimum interruptions such as environmental stress and complex cell interactions for investigating the interactions of nutrients with different immune cell types (Ryckaert *et al*., 2010; Li *et al*., 2012). Additionally, Li *et al*. (2012) reported that *in vitro* findings of fatty acid immunomodulation in fish could certainly mirror the fish *in vivo* conditions. Microalgae are LC-PUFAs rich plant lipid sources that possess high potential to enhance the immunity of brown-marbled groupers by fulfilling their essential fatty acid requirement.

1.2 Problem statements

In modern aquaculture, intensive farming is popularly adopted by aquaculturists with the inventions of culture facilities and technologies which can sustain a huge stocking density in a confined area in order to escalate the economic productivity and profitability. However, fish stocked at high density has overcrowded the enclosed ponds, tanks and net cages, exceeded the environmental carrying capacity and eventually caused the imbalance interactions between fish host, pathogens and the environment, poor water quality and defenceless farmed fish. At the same time, aquaculture practices such as handling, grading, transportation and anaesthetise are also very stressful to farmed fish. Stressors suppress the fish defense responses and hence it is highly susceptible to infectious diseases by pathogenic bacteria, virus,

fungi and parasite. In grouper intensive farming, the outbreak infectious diseases such as bacterial infections by *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Streptococcus* sp., *Pseudomonas* sp., *Pasteurella piscicida* and *Flexibacter* sp. (Punitha *et al*., 2008), viral nervous necrosis by nodaviruses (RNA viruses), sleepy disease by iridoviruses (DNA viruses) (Fukuda *et al*., 1999, Chua *et al*., 1994) and parasitic infection by *Cryptocaryon irritans* (Yambot *et al*., 2003) are serious problems which cause massive mortalities, severe economic losses and subsequently a constraint to the development of mariculture (Yeh *et al*., 2008).

There are a few methods such as sanitary prophylaxis, chemotherapy, disinfection and vaccination to be utilised frequently for treatment, control and prevention of fish infectious diseases (Harikrishnan *et al*., 2011a). Antibiotics such as amoxicillin, enrofloxacin, erythromycin, furazolidone and oxtetracycline are the most common chemotherapy which has successfully controlled the fish and shellfish diseases (Agnew & Barnes, 2007). However, use of antibiotics and other chemotherapeutics have been strongly criticised due to their detrimental effects such as bacterial resistance, immunosuppression and bioaccumulation of chemical residues in fish flesh tissues as well as negative impacts on environment and wildlife (FAO, 2003). In addition, antibiotic-resistance genes can be transferred from pathogens to human pathogenic bacteria which are harmful to human health and also the surrounding environment (Cabello, 2006). On the other hand, vaccination is an effective prophylaxis to control pathogenic infectious diseases, but it is expensive and yet stressful to fish (Ellis, 1988). Administration of vaccine through injection has the best observed results, but it is time-consuming and difficult to apply in hatchery due to the small fish size (Harikrishnan *et al*., 2011a). Vaccine is pathogen specific and the presence of different pathogens in farming sites has limited its efficacy (Robertsen, 1999; Ardo *et al*., 2008). Thus, it is impossible to immediately control all fish diseases using vaccines only (Sakai, 1999). Furthermore, the development of heterogenous species or multiple strain vaccines is extremely complex (Harikrishnan *et al*., 2011a). Hence, research on new alternatives to replace antibiotics, drugs and vaccines which are low-cost and harmless to human, animals and environment are important for sustainable aquaculture industry.

Immunonutrition is an environmentally friendly, useful and yet inexpensive approach to modulate fish immunity by consumption of macronutrients and micronutrients. Those nutritive compounds play different roles in immunomodulation i.e. 1) pathogen recognition (β-glucan and lipopolysaccharide), 2) phagocytosis and antigen presentation (AA and n-3 LC-PUFAs) and 3) adaptive immunity extension and memory formation (β-carotene and n-3 LC-PUFAs) (Pohlenz & Gatlin III, 2014). Immunonutrition has been frequently highlighted and applied in sustainable and eco-friendly aquaculture due to the increasing of consumer awareness toward safe and healthy fish foods (Kiron, 2012; Pohlenz & Gatlin III, 2014). A balanced nutrition is critical to ensure the immune development and function (Kelley, 2001). However, current information on minimum nutrient requirements of different fish species is according to their growth performance instead of immune performance (NRC, 2011). Therefore, it is important to determine the nutrient levels which ensure the optimum immune response in fish. Furthermore, many studies showed that fish oil replacement by n-3 LC-PUFAs-deprived vegetable oil has suppressed the marine fish

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immunity (Montero *et al*., 2002, 2008, 2010; Mourenta *et al*., 2005). Conversely, there is no study regarding the immunomodulatory effects of microalgae LC-PUFAs on brown-marbled groupers *in vitro*.

1.3 Objectives

1.3.1 To isolate, purify and identify the tropical microalgae from various ecosystems through the optimisation of SEM pretreatment protocol.

1.3.2 To determine the growth performance, total lipid content, total carotenoid content and fatty acid composition of tropical microalgae species.

1.3.3 To evaluate the *in vitro* immunomodulatory effects of selected microalgal lipophilic extract on brown-marbled grouper, *Epinephelus fuscoguttatus*.

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