UNIVERSITI PUTRA MALAYSIA

CULTURE OF MARINE MICROALGAE, Tetraselmis tetrathele (WEST) BUTCHER, IN ANNULAR PHOTOBIOREACTOR FOR APPLICATION IN FORMATION OF NANOCOSMECEUTICALS

NURUL FARAHIN BINTI ABD WAHAB

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By

NURUL FARAHIN BINTI ABD WAHAB

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

September 2015

SPECIAL DEDICATION OF THIS GRATEFUL FEELING TO MY…..

Beloved father and mother;
Mr. Abd Wahab Ahmad & Mrs. Norsiah Harron

Loving brothers and sisters

and to those I loved for the understanding, encouragement and unconditional love and support throughout the course of this work.
I love you.

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

CULTURE OF MARINE MICROALGAE, *Tetraselmis tetrathele* (WEST) BUTCHER 1959, IN ANNULAR PHOTOBIOREACTOR FOR APPLICATION IN THE FORMATION OF NANOCOSMECEUTICALS

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

There has been a remarkable surge of interest on natural products and their application in the cosmeceutical industry. Cosmeceutical are cosmetic-hybrids intended to enhance health and beauty of the skin. Topical delivery of antioxidants from natural marine sources is one of the approaches used to reduce the reverse sign of skin aging. The marine prasinophyte *Tetraselmis tetrathele* is one of the important microalgae used as feed in aquaculture due to its high nutritional values and able to be mass produced because of its eurythermal and euryhaline characteristics. This indigenous microalga also contains bioactive compounds such as flavonoids, polyphenols and polyunsaturated fatty acids (PUFA), which makes it an appropriate raw material for various product developments in cosmeceutical industries. The antioxidant activity of *T. tetrathele* (UPMC-A0007) was determined by culturing it in f/2 media for 56 days in 120 L annular photobioreactor. Microalgae biomass was collected six times throughout the culture period to quantify total phenolic (TPC) and antioxidant contents. The antioxidant activities of *T. tetrathele*'s crude extract were determined by diphenylpicrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays. Two groups of cell size; small sized-cells ($3.0\text{-}5.0\times10^{-11}$ g/cells) and big sized-cells ($5.5\text{-}8.0\times10^{-11}$ g/cells) were observed. The total phenolic content of small sized-cell (2.99 ± 0.14 mg GAE/g) was 1.6 times higher compared to big sized-cell. These results suggest that *T. tetrathele* could be a valuable source of phenolic content and antioxidant. The effective antioxidant production can be achieved by controlling the cell size in during culture process. Identification of phytochemical constituents was achieved by GC-MS analyses. Generally, six main chemical compounds identified were responsible for the bioactivity in both small sized-cells and big sized-cells.

Compositions from ternary phase diagrams were selected as pre-formulated emulsions. Topical nanocosmeceutical formulations from palm kernel oil esters (PKOEs) : 1% of crude extract *T. tetrathele*/Tween 80/water systems were chosen due to the presence of large isotropic liquid region which are suitable for the production of nanoemulsion. Particle size analysis showed that the mean particle sizes of these formulations (T1, T2 and T3) ranged from 102.3 to 249.5 nm. Zeta potential analysis for all emulsions showed negative values from -33.2 to -71.7 mV. Stability studies showed that, after four hours of stirring at room temperature (25°C), the formulations were stable during centrifugation test at 4000 rpm for 15 minutes. In addition, T1, T2 and T3 were stable with no separation at different storage temperatures (4, 25 and 45°C) for the duration of eight weeks. However, between eight to ten weeks, only T1 and T2 were stable at 4, 25, and 45°C. This study illustrated that T1 and T2 formulations are considered to be the most suitable formulation for nanocosmeceutical product because they were stable after undergoing thaw cycles test, storage at room temperature (25°C) and 45°C for more than eight weeks. Moreover, the particle size ranged between 165 to 199 nm which resulted in low occurrence of Ostwald ripening.

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

PENGKULTURAN MIKROALGA MARIN, *Tetraselmis tetrathele* (WEST) BUTCHER 1959, DI DALAM FOTOBIOREAKTOR ANULUS UNTUK APLIKASI DALAM FORMULASI NANOKOSMESEUTIKAL

Oleh

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Terdapat peningkatan permintaan luar biasa terhadap produk yang berasaskan semulajadi dan juga aplikasinya dalam industri kosmeseutikal. Kosmeseutikal merupakan kosmetik-hibrid yang bertujuan untuk meningkatkan tahap kesihatan dan juga kecantikan kulit. Penghantaran antioksidan secara topical daripada sumber marin semulajadi adalah salah satu pendekatan bagi mengurangkan tanda penuaan. Prasinofit marin *Tetraselmis tetrathele* merupakan salah satu mikroalga penting yang digunakan sebagai makanan dalam akuakultur kerana ianya mempunyai kandungan nutrisi yang tinggi dan mudah untuk dihasilkan dalam kuantiti yang banyak kerana cirinya yang boleh bertoleransi dengan pelbagai suhu dan juga saliniti. Mikroalga asli ini juga mempunyai sebatian bioaktif seperti flavanoid, poliferol dan asid lemak tidak tepu (PUFA) yang menjadikannya sebagai bahan mentah yang sesuai dalam perkembangan pelbagai produk industri kosmeseutikal. Aktiviti antioksidan bagi *T. tetrathele* (UPMC-A0007) yang dikultur dalam media f/2 selama 56 hari di dalam 120 L fotobioreaktor anulus telah ditentukan. Biojisim mikroalga telah dikumpulkan sebanyak enam kali sepanjang tempoh pengkulturan bagi mengukur jumlah sebatian fenolik (TPC) dan kandungan antioksidan. Aktiviti antioksidan daripada ekstrak mentah *T. tetrathele* ini ditentukan oleh *diphenylpicrylhydrazyl* (DPPH), *ferric reducing antioxidant power* (FRAP) dan *2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)* (ABTS) esei. Dua kumpulan saiz sel; sel bersaiz kecil ($3.0\text{-}5.0\times10^{-11}$g/sel) dan sel bersaiz besar ($5.5\text{-}8.0\times10^{-11}$g/sel) telah dikenalpasti. Kumpulan sel bersaiz kecil (2.99 ± 0.14 mg GAE/g) menunjukkan 1.6 kali lebih tinggi kandungan sebatian fenolik berbanding kumpulan sel bersaiz besar. Keputusan ini menunjukkan bahawa *T. tetrathele* boleh dijadikan sebagai sumber bagi kandungan sebatian fenolik dan antioksidan. Penghasilan antioksidan secara efektif boleh dicapai dengan mengawal saiz sel semasa proses pengkulturan dijalankan. Pengenalpastian juzuk fitokimia telah dibuat menggunakan analisis GC-MS. Secara umumnya, enam sebatian kimia utama telah dikenalpasti bagi mewakili sebatian utama yang bertanggungjawab untuk aktiviti-biologi dalam kedua-dua saiz sel tersebut.

Komposisi daripada gambarajah fasa segitiga telah dipilih sebagai emulsi pra-formulasi. Formulasi nanokosmeseutikal yang berasaskan minyak ester isirong kelapa

sawit (PKOE) : 1% daripada ekstrak mentah *T. tetrathele* / Tween 80 / air telah dipilih kerana gambarajah fasa segitiga menunjukkan kawasan isotropi yang besar, dan mencadangkan bahawa kawasan ini sesuai digunakan bagi penyediaan nanoemulsi. Analisis saiz partikel menunjukkan bahawa saiz zarah purata formulasi (T1, T2 dan T3) adalah antara 102.3 kepada 249.5 nm. Potensi zeta adalah antara -33.2 hingga -71.7 mV. Kajian kestabilan menunjukkan bahawa, selepas empat jam proses pengacauan pada suhu bilik (25°C), formulasi didapati masih stabil selepas proses pengemparan pada 4000 rpm selama 15 minit. Selain itu, kajian kestabilan di bawah suhu yang berbeza (4, 25 dan 45°C) juga menunjukkan bahawa T1, T2 dan T3 didapati stabil selama lapan minggu. Walau bagaimanapun, di antara lapan hingga sepuluh minggu, didapati hanya T1 dan T2 sahaja yang masih stabil pada suhu 4, 25 dan 45°C. Kajian ini menunjukkan bahawa formulasi T1 dan T2 dianggap sebagai formulasi yang paling sesuai bagi tujuan produk nanokosmeseutikal kerana tahap kestabilannya setelah menjalani ujian kitaran pencairan, penyimpanan pada suhu bilik (25°C) dan 45°C selama lebih daripada lapan minggu. Manakala purata saiz zarahnya antara 165 hingga 199 nm mengurangkan berlakunya proses kematangan *Ostwald*.

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

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
Cm	Centimeter
°C	Degree celcius
GAE	Gallic Acid Equivalent
G	Gram
H	Hour
HLB	Hydrophilic Lipophilic Balance
LFCs	Lead Functional Components
µm	Micro meter
µM	Micro Molar
Ml	Mililiter
Mm	Milimeter
mV	Milivolt
Min	Minute
Nm	Nano meter
OD	Optical Density
O/W	Oil-in-Water
PKOEs	Palm Kernel Oil Esters
ROS	Reactive Oxygen Species
Rpm	Rotational per minute
S	Second
sp.	Species
SPSS	Statistical Package for the Social Sciences
TEAC	Trolox Equivalent Antioxidant Activity
TEM	Transmission Electron Microscopy
Tween 80	Polyoxyethylene Sorbitan Monostearate
v/v	Volume per volume
W/O	Water-in-Oil
w/w	Weight per weight
XG	Xanthan Gum

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background of study

In recently years, the use of microalgae as a natural source for nutrition (Varfolomeev and Wasserman, 2011), food supplement (Graziani *et al*., 2013), cosmetic (Balboa *et al*., 2014) and pharmaceutical products (Pangestuti and Kim, 2011) has received increasing attention. Microalgae are unicellular photosynthethic microorganisms containing chlorophyll, and can be found in both saline and fresh water environments. They are diverse group of photosynthethic microorganisms that convert sunlight, water and carbon dioxide to synthesise chemical energy such carbohydrates, lipids and protein into algal biomass (Demirbas and Fatih, 2011; Gong *et al*., 2011). These microorganisms are potentially source of diverse phytometabolic contents with various chemical structure (arachidonic acid, linolenic acid, sterol, α-tocopherol and phycobilin) and biological activities for many application purposes such as antioxidant, anticancer and protection to the skin (Servel *et al*., 1994; Plaza *et al*., 2009; de Jesus Raposo *et al*., 2013). The chemical composition and productivity depend on growth phase, harvesting time, nutrients contents and light intensity. Due to that, microalgae biomass is able to provide additional physiological and pharmacological benefits for human health as a biochemical product. Recently, the market produces about 5000 t of microalgae dry matter/year and generates a turnover of aproximately US$ 1.25×10^9/year (El Gamal, 2010; Hajimahmoodi *et al*., 2010).

Previous studies have reported that plants, fruits and macroalgae are well known for their high content of antioxidant properties with diverse chemical structure and biological activities (Kaur and Kapoor, 2002; Ismail *et al*., 2004; Duan *et al*., 2006; Zhang *et al*., 2007; Kuda and Ikemori, 2009; Tsantili *et al*., 2011; Lester *et al*., 2012). However, higher plants and macroalgae have certain limitations such as time to grow, space, water resources and usage of herbicides. Furthermore, plant and macroalgae can be limiting resource in developing countries. These resources which become one of the main food sources in poverty stricken countries will be depleted in order to meet the application of biotechnology research such as pharmaceutical and cosmetic purposes (Balasubramanian *et al*., 2011).

Microalgae have received increasing attentions as an alternative antioxidant source because they are the fastest-growing plants in the world and hence they have higher productivity compared to land plants (Chisti, 2007; Demirbas, 2010). Microalgae do not require high quality land and herbicides since they can grow in almost all aquatic environment even in sewage and salt water (Scott *et al*., 2010). Microalgae can also produce antioxidant substances against oxidative and radical stressors. Therefore, several researchers tried to extract antioxidant compounds from various algae biomass (Li *et al*., 2007; Goh *et al*., 2010; Hajimahmoodi *et al*., 2010; Custódio *et al*., 2012; Manivannan *et al*., 2012). Marine microalgae produce a wide variety of chemically active metabolites in their surroundings such as antioxidant activities as an aid to protect themselves against other settlings organisms (Servel *et al*., 1994; Vo *et al*., 2012). Therefore, marine microalgae are believed to be a promising supply to provide not only novel biologically active substances for the growth of pharmaceuticals but also essential compounds for human nutrition and aquaculture food mainly for live feeds for larval culture (Huerlimann *et al*., 2010; Pangestuti and Kim, 2011). Among marine microalgae, *Tetraselmis tetrathele* (West) Butcher 1959, is a green four-flagelated prasinophyte, which has good nutritive characteristics (especially in relation to polyunsaturated fatty acid composition) and thus it can be useful for health food, aquaculture and nutrition applications (de La Peña and Villegas, 2005; Natrah *et al*., 2007). *Tetraselmis tetrathele* could be a potential microalga for the future as it can be easily mass produced due to its euryhaline and eurythemal characteristics. Therefore, *T. tetrathele* is suitable for large scale production as it is necessary to consider its cost-effective production (Ronquillo *et al*., 1997).

The potential and invention of algal biomass became a reality in Germany in the 1940s, and towards the end of World War II. At that time, microalgae were grown in bigger quantities for various purposes, such as the production of lipids for energy using flue gasses, anti-microbial substances and the production of various bio-chemicals (Chaumont, 1993; Ugwu *et al*., 2008; Grobbelaar, 2012). Then, after industrialization has begun, mass cultivation of algae was implemented by some group of workers in Carnegie Institute at Washington for reduction of CO_2. Between early 1970s and late 1970s, this work was continued by many research groups, most notably in Stanford (USA), Tokyo (Japan), Essen (Germany), Israel, Czech Republic and Taiwan (Burlew, 1953). As a matter of fact, the intention of growing algae depended on the needs of the people nowadays.

Algae are grown either in open culture systems (lakes, ponds) or closed systems (photobioreactors). Closed systems offer better control over contamination, mass transfer, and other cultivation conditions even though the open pond systems seem to be favoured for commercial cultivation of microalgae at present due to their low capital costs (Li *et al*., 2008). Photobioreactor has been proposed as it gives the advantages of closed systems over open ponds ranging from laboratory to industrial scale such as high biomass productivity and and cell density, reduced contamination and better use of CO_2 (Shen *et al*., 2009). Photobioreactor is defined as a closed (mostly closed) vessel for phototrophic production where energy supplied lights. Commonly, laboratory-scale photobioreactors are artificially illuminated (either internally or externally) using fluorescent lamps or other light distributors. For example, closed photobioreactors have attracted much attention because they allow a better control of the cultivation conditions than open systems. With closed photobioreactors, higher biomass productivities are obtained and contamination can be simply prevented. With the availability of suitable culture systems, culture conditions such as nutrient availability, light intensity, pH, temperature and salinity can then be optimized for high quality and maximum biomass production (Singh and Sharma, 2012).

The term cosmeceuticals, coined by Dr. Albert Kligman, may be defined as a hybrid of drugs and cosmetics (Kligman, 2005). Cosmeceutical is a category of multifunctional products that rely on science and technology to deliver clinically proven active ingredients to the skin. Cosmeceutical-based products are formulated with pharmaceutical-type ingredients, have a unique ability to treat or beautify skin from inside out. Due to that, this product has the largest growing segments of skin care market based on the sales report and becomes makes the popular among consumers nowadays. In industry, the effectiveness of the products is one of the major concerns. The advancement of nanotechnology enables nanoemulsions to be used as a nanocarrier to effectively deliver the active components in the products to its targeted cells (Teo *et al*., 2010; Ng *et al*., 2013).

An emulsion is defined as a dispersion consisting of mixture of two insoluble materials; water and an oil phase stabilized against separation using an emulsifier surfactant (Abd Gani *et al*., 2011). It is system that most commonly used for cosmetics and pharmaceuticals. Microemulsions and nanoemulsions are two common types of colloidal dispersions that can be created from these components. These two kinds colloidal dispersion have some important differences eventhough there are many structural similarities. Nanoemulsions are composed of two phases but having extremely small size in the ranges of 20-500 nm (Mason *et al*., 2006; Solè *et al*., 2010; Bernardi *et al*., 2011). Due to extremely small size, nanoemulsions are becoming the subject of many studies on their wide range of potential uses and applications especially to be used as delivery system in cosmeceuticals. The characteristic of being absorbed by the skin makes nanoemulsions are sought after in the pharmaceutical industry (Mou *et al*., 2008; Salim *et al*., 2012a; Salim *et al*., 2012b; Ng *et al*., 2013).

Palm kernel oil ester (PKOE) is a long chain fatty acid synthesized from palm kernel oil, through enzymatic transesterification process. PKOE is rich in oleyl laurate, C30:1 (54.1%). Palm kernel oil ester will be used in this project as it has shown novel characteristics such as exhibiting superb behavior without the 'oily feeling', colourless and low viscocity. This statement had been approved by Salim *et al*. (2012a) that it is the best ingredient to be used in formulation of cosmeceutical and pharmaceutical industry. Since *T. tetrathele* (isolate UPM-A007) is an indigenous species, the combination between this species and the use of palm kernel oil ester to the nanoemulsions formulation, it will be add on the novel application of palm oil commodities in cosmeceutical industries in Malaysia.

1.2 Statement of problem

Microalgae are biologically diverse collection of microorganisms amenable to fermentation and mass culture. As well as cyanobacteria and nearly dozen eukaryotic classes, microalgae produce a wide array of compounds with biological activities (Arad and Yaron, 1992; El Gamal, 2010). Production of secondary metabolite by microalgae varies with environmental conditions. However, productivity of microalgae varies due to the differences in culture system, geographic locations, culture strategies (batch or continuous culture), algae species etc. Microalgae might become economic sources of new drug and other specialty chemicals when these processes are better understood. Moreover, production can be optimized in a controlled system.

Phytochemicals (e.g. phenolic acid, flavanoids and polyunsaturated fatty acids) are important nutrients produced by microalgae as secondary metabolites, especially when microalgae are in stress conditions. It may play a critical role in age-related disease or even cancer (Kobayashi *et al*., 1997; Rao *et al*., 2007; Sánchez-Saavedra and Voltolina, 2006). Hydrophilic and hydrophobic of bioactive compounds can present a problem in delivery for oil-in-water (o/w) systems. Nanoemulsion is kinetically stable (metastable dispersion state) and believed to be stable against creaming or sedimentation, flocculation and coalescence. However, nanoemulsion is also a very fragile system. Mechanical energy created to the mixture in the systems may be destroyed by spontaneous process such as coalescence or Ostwald ripening (Sonneville-Aubrun *et al*., 2009; Teo *et al*., 2010). Slightest sign of destabilization with ease to appear. They become opaque and creaming may be able to be seen as they are transparent and usually very fluid. Therefore, stability of the nanoemulsion is a critical factor to be analysed. The accomplishment of developing long time stability of cosmetic products (three years of shelf life) is often difficult and costly in the development of new formulation (Ng *et al*., 2013). The properties such as particle stability, rheology, appearance, colour, texture and shelf life will be affected by the size and polydispersity of nanoemulsion. (Bernardi *et al*., 2011).

Until now, there has been little attempt to prepare nanoemulsions using PKOE (Salim *et al*., 2012a). Currently there are no published reports on the use of palm kernel-based wax esters loaded with microalgae extracts as nano-delivery carrier in cosmeceuticals. *Tetraselmis tetrathele* is selected as the study organism because of its hardy characteristic, ease of culture, fast growing, good in nutrition and it is an indigenous species**.** The procedure for producing nanoemulsions comprised of nano-size droplets emulsions is difficult. On the other hand, one of the main problems with nanoemulsions is stabilization of developed system. The solubility and instability of actives in the nanoemulsions system also contribute to the difficulty in producing a stable nanoemulsions system.

Hence, the present study focused on the following objectives:

1. To mass produce *T. tetrathele* culture in annular photobioreactor.
2. To determine the phytochemical constituents of *T. tetrathele* extracts
3. To develop and characterize the physicochemical properties of palm kernel esters containing *T. tetrathele*-based nanoemulsions.

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