SELECTION OF CULTURE CONDITIONS THROUGH MANIPULATION OF PHYSICAL AND CHEMICAL PARAMETERS FOR PRODUCTION OF HIGH-VALUE METABOLITES IN MARINE MICROALGA *Tetraselmis tetrathele* (WEST) BUTCHER 1959 BIOMASS

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 Doctor of Philosophy

June 2021

Science has always been a passion of mine. Dedicating my life to improve the lives of others is very pleasing to me. Whatever I had gone through before has led me to be the person that I am today. Special dedication of this grateful feeling to …

my beloved father and mother,
Mr. Abd Wahab Ahmad and Mrs. Norsiah Harron;

my beloved son,
Muhammad Faiz Firdaus;

my loving brothers and sisters; and

all whom I love

for their patience, unconditional love and encouragement 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 Doctor of Philosophy

SELECTION OF CULTURE CONDITIONS THROUGH MANIPULATION OF PHYSICAL AND CHEMICAL PARAMETERS FOR PRODUCTION OF HIGH-VALUE METABOLITES IN MARINE MICROALGA *Tetraselmis tetrathele* (WEST) BUTCHER 1959 BIOMASS

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June 2021

Supervisor: **Assoc. Prof. Natrah Fatin Mohd Ikhsan, PhD**
Institute: **Bioscience**

Microalgae cultivation is one of the crucial aspects in the commercialization of microalgae to produce a high amount of biomass and metabolites. In the aquaculture industry, microalgae biomass is used as feed and growth enhancers, and it is also considered a renewable and sustainable resource. Commercially, *Tetraselmis* sp. is one of the most commonly used microalgae in the aquaculture field. Although there are several studies on the benefits and culture conditions of *Tetraselmis* sp., little information is known about the understanding of theoretical and technical knowledge on mass culture, which can affect biomass productivity and the quality of microalgae biomass produced. The present study aims to obtain key insights of three growth factors considered as major contributors on the effect of microalgae growth: (1) ammonium nitrogen concentration (chemical parameter), (2) light intensity, and (3) culture temperature (physical parameters) for mass production of an indigenous species, *Tetraselmis tetrathele* under tropical conditions. Bubble column reactors (BCRs) were used to mimic indoor and outdoor conditions in enhancing the growth characteristics of cells, the effect of physiological processes, and the composition of metabolites. Overall, this study revealed that although the growth performance of *T. tetrathele* decreased under 35 °C, this indigenous species showed excellent self-adaptation capabilities to cope with high ammonium nitrogen (0.87 g L^{-1}) and varying light intensities (up to 1,500 μmol m^{-2} s^{-1}) by protecting microalgae from photodamage. These characteristics have significant implications for the selection of optimal conditions when designing more efficient microalgae culture systems in tropical conditions. The knowledge obtained from this work can be useful in assessing the applicability of this strain culture and also enhancing the understanding of the physiology of microalgae to sustainably maximize microalgae cultivation. Besides, these findings are particularly useful for relevant stakeholders to efficiently expand commercialization by selecting

high-quality biomass production with specific metabolites of interest in *T. tetrathele*.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMILIHAN KEADAAN PENGKULTURAN MELALUI MANIPULASI PARAMETER FIZIKAL DAN KIMIA UNTUK PENGHASILAN METABOLIT TINGGI-NILAI DALAM BIOJISIM MIKROALGA MARIN *Tetraselmis tetrathele* (WEST) BUTCHER 1959

Oleh

NURUL FARAHIN BINTI ABD WAHAB

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Penyelia: **Prof. Madya. Natrah Fatin Mohd Ikhsan, PhD**
Institut: **Biosains**

Pembiakan mikroalga adalah salah satu aspek yang penting dalam pengkomersilan mikroalga untuk menghasilkan biojisim dan metabolit. Dalam industri akuakultur, biojisim mikroalga digunakan sebagai bahan makanan dan penggalak pertumbuhan, dan ia juga dianggap sebagai sumber boleh diperbaharui dan mapan. Secara komersilnya, *Tetraselmis* sp. adalah salah satu daripada spesies mikroalga yang sering digunakan dalam bidang akuakultur. Walaupun terdapat banyak kajian mengenai manfaat dan keadaan pengkulturan *Tetraselmis* sp., hanya terdapat sedikit maklumat mengenai pengetahuan teknikal dan teori terhadap kultur spesies tersebut yang boleh mempengaruhi produktiviti biojisim dan kualiti biojisim mikroalga yang terhasil. Kajian ini bertujuan mendapatkan pemahaman penting mengenai tiga faktor pertumbuhan yang dianggap sebagai penyumbang utama terhadap kesan pertumbuhan mikroalga: (1) kepekatan ammonia nitrogen (parameter kimia), (2) keamatan cahaya, dan (3) suhu kultur (parameter fizikal) untuk pengeluaran besar-besaran sejenis spesies tempatan, *Tetraselmis tetrathele* dalam keadaan tropika. Reaktor turus gelembung (BCRs) digunakan untuk menyerupai keadaan dalam dan luar untuk meningkatkan ciri-ciri pertumbuhan sel, kesan proses fisiologi, dan komposisi metabolit. Secara keseluruhannya, kajian ini menunjukkan bahawa walaupun prestasi pertumbuhan *T. tetrathele* berkurang di bawah suhu 35 °C, namun spesies tempatan ini menunjukkan keupayaan adaptasi kendiri untuk menghadapi tahap ammonia nitrogen yang tinggi (0.87 g L^{-1}) dan keamatan cahaya yang berubah-ubah (sehingga 1,500 µmol m^{-2} s^{-1}) dengan melindungi mikroalga daripada kerosakan disebabkan oleh cahaya. Ciri-ciri ini mempunyai kesan yang signifikan terhadap pemilihan keadaan optimum apabila mereka bentuk sistem pengkulturan mikroalga yang lebih berkesan dalam keadaan tropika. Pengetahuan yang diperoleh daripada kajian ini berguna untuk menilai kebolehgunaan kultur strain ini dan juga meningkatkan pemahaman mengenai fisiologi mikroalga untuk memaksimumkan pembiakan mikroalga

secara mapan. Selain itu, dapatan kajian ini sangat berguna untuk pihak berkepentingan yang berkaitan untuk mengembangkan pengkomersilan secara berkesan dengan memilih penghasilan biojisim berkualiti tinggi dengan metabolit khusus iaitu *T. tetrathele*.

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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 Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure **Page**

LIST OF APPENDICES

LIST OF SYMBOLS AND ABBREVIATIONS

cm	Centimeter
°C	Degree celsius
DNA	Deoxyribonucleic acid
ETR	Electron transport rate
et al.	*et alia*
GCMS	Gas Chromatography Mass Spectrometry
g	Gram
g-dw L^{-1}	Gram dry weight per liter
gL^{-1}	Gram per liter
HPLC	High Performance Liquid Chromatpgraphy
h	Hour
α	Light-limited slope of RLC
*E*k	Light-saturation index
*ETR*max	Light-saturated rate of RLC
L	Liter
F_{m}	Maximal fluorescence measured in darkness
F'_m	Maximal fluorescence under light condition
F_v/F_m	Maximum quantum efficiency defined by $(F_m-F_0)/F_m$
µM	Micro molar
µmol m^{-2} s^{-1}	Micromole per square meter per second
mg	Milligram
mg L^{-1}	Milligram per liter
mm	Millimeter
F	Minimum fluorescence under light condition

F_0	Minimum fluorescence measured in darkness
min	Minute
M	Molar
%	Percentage
PPFD	Photosynthetic photon flux density
PS I	Photosystem I
PS II	Photosystem II
±	Plus minus
PCR	Polymerase Chain Reaction
pH	Potentiometric hydrogen ion concentration
rETR	Relative electron transport rate
RLC	Rapid light curve that are measured by plotting the ETR or rETR against the actinic PPFD
s	Second
μ	Specific growth rate
SD	Standard deviation
UPM	Universiti Putra Malaysia

CHAPTER 1

INTRODUCTION

1.1 Background of study

The rising growth and life expectancy of the global population have increased the demand for energy, healthy food, water, drugs, and other resources. This has caught the attention of the United Nations Committee on Science, Technology, and Innovation to provide scientific grounding to tackle various sustainability challenges, in line with the 2030 Agenda for Sustainable Development, which includes 17 Sustainable Development Goals (SDGs) proposed during the general assembly in September 2015. Microalgae, hailed as the "Green Gold", have emerged as a promising source for innovative and sustainable development to solve the global food and energy crisis (Wolkers *et al*., 2011). Microalgae have gained global attention of academicians and engineers over the past half-century for numerous reasons. The increase in the global demand for microalgae-based products is projected to amount to USD 53.43 billion by 2026, as compared to USD 32.60 billion in 2017 (Rahman, 2020). The growing market demand has resulted in mass microalgae cultivation for use in several applications, such as food and animal feed production (Lim *et al*., 2017), pharmaceuticals (Ambati *et al*., 2019), wastewater treatment (Schulze *et al*., 2017), and bioenergy production (Chia *et al*., 2018; Qu *et al*., 2020). Owing to the increasing market demand for microalgae, microalgae cultivation becomes one of the crucial aspects to be focused on for commercialization by producing a high amount of biomass and metabolites to fulfill the demands.

The environmental conditions, such as nutrients, light, and temperature, are some of the main basic requirements for microalgae growth, which subsequently affect the quality of biomass produced. Microalgae biomass consists of numerous beneficial compounds that are useful in various markets. Microalgae are known to produce carotenoids, which are responsible for light harvesting in photosynthetic metabolism. Carotenoids play an important role in alleviating certain cancers, premature aging, cardiovascular disease, and arthritis (Ambati *et al*., 2019), and also as a coloring agent in chewing gums, candies, and beverages (Adarme-Vega *et al*., 2012). Besides, carotenoids also have antiaging, antiobesity, and antioxidant properties, which are considered as better alternatives for synthetic compounds (Gong and Bassi, 2016). The specific content of metabolites is strain-dependent and can be heavily influenced by the culture conditions employed. During photosynthesis, the energy converted from sunlight is stored as lipid or carbohydrate within the algae, which is then extracted from algae for energy supply. For example, the lipid content of microalgae is usually in the range of 20%–50% of the cell dry weight, sometimes exceeding 50%, and can also be as high as 80% under certain conditions (Brindhadevi *et al*., 2021; Japar *et al*., 2021), thus reducing the requirement of other resources for the production of the same amount of oil. Many marine microalgae are rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and also other fatty acids classes. Fatty acids with 14–20 carbons are used for the production of biodiesel, and polyunsaturated fatty acids (PUFAs) with more than 20 carbons are used as health food supplements and as feed for early larvae of crustacean and fish, early and late larvae mollusk, brine shrimp, copepods, and rotifers, especially DHA and EPA (Adarme-Vega *et al*., 2012; Sun *et al*., 2018).

Among the local marine microalgae, *Tetraselmis* sp. is recognized as one of the few species of microalgae that has been explored extensively due to its high amount and quality of intracellular content, such as PUFAs for human consumption and aquaculture feed (Farhadian *et al*., 2008), polysaccharides for antibiotic development research (Kermanshahi-pour *et al*., 2014), high vitamin E content (Carballo-Cárdenas *et al*., 2003), and antioxidant for pharmaceutical and cosmeceutical purposes (Farahin *et al*., 2019). This genus is recognized as “novel food” and even approved for human consumption of biomass by the European Union (Mantecón *et al*., 2019). In culturing purposes, this strain displays high potential for commercial-scale production of biomass due to its high growth rate, ability to grow in high ammonium nitrogen (Farahin *et al*., 2021) and seawater with high salinity, and also to outcompete contaminants (Pereira *et al*., 2016). Apart from elucidating the capabilities of microalgae with high-density cultures, a better understanding of the factors influencing microalgae growth and biomass must be acquired (Borowitzka and Vonshak, 2017).

1.2 Problem statement

The enhancement of cultivation conditions using various techniques contributes to the growth and production of numerous compounds in microalgae. The effect of abiotic stress on metabolites, such as lipid, fatty acids, and pigment production from microalgae and corresponding growth rates, has been considered in previous studies (Go *et al*., 2012; Roleda *et al*., 2013; Michels *et al*., 2014a; Imaizumi *et al*., 2016). Nitrogen in the form of either ammonium (NH_4^+) or nitrate (NO_3^-) is an essential nutrient for the growth of microalgae, which subsequently contributes to the biomass produced. Several previous studies pointed out that different strains of microalgae require different levels of nitrogen uptake (Raven *et al*., 1992; Feng *et al*., 2020). Meanwhile, ammonium, which is the most predominant source of nitrogen, exists in urban, agricultural, and aerobic digested effluents with various concentrations, ranging from the concentration as low as 0.01 gL^{-1}-N to the concentration as high as 2.0 gL^{-1}-N (de la Noüe *et al*., 1992; Cai *et al*., 2013; Reddy *et al*., 2017). Furthermore, ammonium nitrogen at a certain level of concentration can be toxic and inhibits the productivity of microalgae. Thus, further elucidation of the ammonium nitrogen tolerance in microalgae is needed.

Besides, commercial-scale production of biomass from this microalgae has remained uneconomical due to the challenges of achieving high biomass productivity (Gonçalves *et al*., 2016; Pereira *et al*., 2018). Light is vital in microalgae growth because this source allows microalgae to produce biomass and metabolites, as well as fix inorganic carbon into organic molecules (Huerlimann *et al*., 2010; Liu *et al*., 2019). Microalgae growth depends heavily on the degree of light penetration, and different light intensities are required for different species and also culture cell density and depth. Several studies reported that some species of microalgae could tolerate very high light intensity. The differences in light intensity tolerance are due to different cellular concentrations; moreover, the thylakoid structure of each species has different compositions of light-harvesting pigments (Conceição *et al*., 2020). However, high illumination of microalgae culture negatively affects its photosynthetic process due to photoinhibition, resulting in diluted culture with low biomass concentrations. Hence, to better solve the above-mentioned problems, it is essential to determine microalgae adaptability to varying light intensities to control microalgae growth, especially in mass culture.

Other than light, temperature is also recognized as another key factor that controls the photosynthetic rates of microalgae and autotrophic organisms, which are thermally sensitive (Davison, 1991; Veeramani and Santhanam, 2015). According to the National Oceanic and Atmospheric Administration (NOAA) (2020), the past five years recorded the highest temperature range, and the average global temperature is now about 1.2 °C above the preindustrial level (World Meteorological Organization, 2021). These climatological conditions have affected the yield and quality of microalgae biomass. Microalgae are highly susceptible to high temperature stress, which impairs their cell functions (Mathur *et al*., 2014). Therefore, the ability to withstand and/or acclimate to the environmental temperature variation is essential for the adaptation and survival of microalgae.

Tetraselmis tetrathele (West) Butcher 1959, a marine green microalga within the *Chlorophyta* isolated from Port Dickson, Malaysia, is recognized as one of the few potential species that can produce a large amount and high quality of intracellular content, such as PUFAs for human consumption and aquaculture feed (Juario and Storch, 1984; Fábregas *et al*., 2001; Farhadian *et al*., 2008; Michels *et al*., 2014b), polysaccharides for antibiotic development research (Kermanshahi-pour et al., 2014), high content of vitamin E (Carballo-Cárdenas *et al*., 2003), and antioxidant for pharmaceutical and cosmeceutical purposes (Farahin *et al*., 2019). Only a few studies focused on the cultivation of the *Tetraselmis* genus for high biomass production and its relationship with photosynthetic performance under stress conditions. The irradiance curves assessed by the variable chlorophyll fluorescence method provide in situ reaction (Michels *et al*., 2014b). Based on this information, the determination of the photosynthetic system capacity and energy captured for light energy processing can be performed. Thus, to maximize the biomass yield of this microalga, this study determined the effect of high ammonium nitrogen, high light intensity, and culture temperature on the growth rate, photosynthetic activity, and production of pigments and fatty acids of *T. tetrathele* without the limitation of nutrients and carbon dioxide supplies. The knowledge obtained from this work could be useful in assessing the applicability of this strain culture under the abiotic stress mentioned and also enhancing the understanding of the physiology of microalga to sustainably maximize the production of microalgae cultivation.

1.3 Research objectives

The main objective of this study was to investigate the range of suitable culture conditions of *T. tetrathele* for its applications under tropical conditions. For this study, bubble column reactors were used to mimic indoor and outdoor conditions in enhancing the growth characterization of cells to study the effect of physiological processes and metabolite compositions.

The specific objectives of this study using *T. tetrathele* are as follows:

1) To evaluate the tolerance capability in high ammonium nitrogen on growth, physiological response, and metabolite production.
2) To determine the growth performance of the microalga and its metabolites under high light intensity.
3) To investigate the effect of different temperatures on the growth rate and metabolite compositions.

With respect to the first objective, this study specifically examined the growth rate and photosynthetic efficiency (F_v/F_m) of *T. tetrathele* in different NH_4^+-N concentrations, and quantified the production of pigments and PUFAs profiles (with NH_4^+ and NO_3^- as nitrogen sources) under six-day batch cultures. Meanwhile, with respect to the second objective, this study determined the effect of high light intensity on the growth rate, photosynthetic performance, and production (i.e., pigments and fatty acids) of *T. tetrathele* in semi-continuous cultures. Thirdly, with respect to the final objective, this study proceeded to investigate the effect of temperature on the growth rate, photosynthetic and physiological effects, and compositions (i.e., pigments, lipids, and fatty acids) of *T. tetrathele* under semi-continuous cultivation at the temperature of 25– 35 °C.

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