ENHANCED SECONDARY METABOLITES ACCUMULATION IN *Tetraselmis suecica* VIA NUTRIENT SUPPLEMENTATION FOR BIOLOGICAL EVALUATIONS OF ANTI-OXIDANT, ANTI-OBESITY, AND ANTI-INFLAMMATORY PROPERTIES

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by

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In the name of Allah, the Most Gracious, and the Most Merciful

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS AND ABBREVIATIONS

α	alpha
β	beta
µ	micro
µm	micrometre
µmol	micromolar
%	percent
CO_2	carbon dioxide
mg/L	milligram per litre
mg/g	milligram per gram
mg/ml	milligram per millilitre
±	plus-minus
g	gram
TE/g	Trolox equivalent per gram
ppm	parts per million
IC_{50}	half-maximal inhibitory concentration
µg/ml	microgram per millilitre
g/L	gram per litre
R^2	R-squared, coefficient of determination
kg/m^2	kilogram per metre squared
κ	kappa
ppt	parts per thousand
°C	degree Celsius
v/v	volume per volume
<	less than
ml	millilitre
rpm	revolutions per minute

nm	nanometre
µg	microgram
µl	microlitre
mM	millimolar
m/z	mass-to-charge ratio

LIST OF APPENDICES

PENINGKATAN PENGUMPULAN METABOLIT SEKUNDER DALAM *Tetraselmis suecica* MELALUI PENAMBAHAN NUTRIEN UNTUK PENILAIAN BIOLOGI, SIFAT ANTI-OKSIDAN, ANTI-OBESITI, DAN ANTI-RADANG

ABSTRAK

Mikroalga adalah mikroorganisma yang berkeupayaan untuk mengadaptasi bagi menghasilkan pelbagai metabolit sekunder berfungsi dalam keadaan terkawal. Walau bagaimanapun, peningkatan pengumpulan metabolit sekunder melalui manipulasi penambahan karbon dan nitrogen dalam pengkulturan mikroalga agak kurang diberi tumpuan. Dalam kajian ini, mikroalga hijau marin, *Tetraselmis suecica* telah digunakan untuk menilai kesan penambahan gabungan sumber karbon yang berbeza (molase, gliserol, natrium asetat) dan nitrogen (urea, ammonium sulfat dan natrium nitrat) ke arah peningkatan pengumpulan metabolit sekunder untuk penilaian biologi. Secara ringkasnya, biojisim mikroalga dinilai, dan ekstrak mentah telah diekstrak menggunakan pelarut organik yang berbeza dengan kekutuban yang semakin meningkat (heksana, etil asetat, dan etanol). Jumlah kandungan fenolik ekstrak mentah telah dinilai. Berikutan itu, ekstrak daripada gabungan tambahan nutrisi mempunyai kandungan biojisim dan fenolik yang lebih tinggi telah dinilai untuk sifat anti-oksidan, anti-obesiti dan anti-radang secara in vitro. Pemprofilan kimia bagi ekstrak telah dijalankan menggunakan spektroskopi Inframerah transformasian Fourier (FTIR) dan Kromatografi Cecair dengan Spektrometri Jisim (LC-MS). Keputusan menunjukkan penambahan gabungan natrium asetat dan natrium nitrat menunjukkan peningkatan berat sel kering (1.095±0.03 g/L) dan jumlah kandungan fenolik (591.80±0.92 µg GAE/mg ekstrak) dalam ekstrak etanol berbanding kumpulan kawalan. Berbanding dengan ekstrak lain, ekstrak etanol mempamerkan kesan perencatan yang ketara (IC_{50}: 23.83 ug/ml) terhadap aktiviti lipase pankreas dan memberikan aktiviti antioksidan

dengan ketara melalui keupayaan pengurangan ferum plasma (FRAP) (152.03±5.84 FE µg/ml), dan 2,2′ -azino-bis-(3-ethylbenzothiazoline-6-sulfonic (ABTS) aktiviti penghapusan radikal (73.42±0.43%). Sebaliknya, ekstrak etil asetat mempunyai aktiviti penghapusan radikal melalui 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (IC_{50}: 123.00 µg/ml), mempamerkan sifat anti-radang melalui kesan perencatan terhadap denaturasi proteinase (45.27±0.02%) dan mengurangkan pengeluaran faktor nekrosis tumor-α (TNF-α) (665.71±55.56 µg/ml) dalam sel makrofaj RAW264.7 yang diaktifkan oleh lipopolisakarida (LPS). Analisa FTIR bagi ekstrak etil asetat dan etanol mendedahkan kehadiran kumpulan berfungsi amina, ester dan halocarbon. Selain itu, keputusan LC-MS mengesan kehadiran sebatian bioaktif utama dalam ekstrak etil asetat dan etanol seperti alfa karotena, canthaxanthin, rutin, quercitrin, apigenin-O-rutinoside, dan caffeoylglucoside sekaligus memberikan gambaran tambahan tentang sifat biologinya. Oleh itu, dapat disimpulkan bahawa metabolit berfungsi yang diperolehi daripada ekstrak etil asetat dan etanol *Tetraselmis suecica* yang dikultur melalui penambahan gabungan karbon dan nitrogen mempamerkan potensi sifat anti-obesiti, anti-oksidan dan anti-radang yang melayakkan kajian in vivo selanjutnya untuk aplikasi nutraseutikal dan terapeutik.

ENHANCED SECONDARY METABOLITES ACCUMULATION IN *Tetraselmis suecica* VIA NUTRIENT SUPPLEMENTATION FOR BIOLOGICAL EVALUATIONS OF ANTI-OXIDANT, ANTI-OBESITY, AND ANTI-INFLAMMATORY PROPERTIES

ABSTRACT

Microalgae are adaptive microorganisms with the capability to produce various functional secondary metabolites under controlled conditions. However, enhanced accumulation of secondary metabolites under the manipulation of carbon and nitrogen supplementations in microalgae cultivation are relatively underrepresented. In this study, a marine green microalga, *Tetraselmis suecica* was utilized to evaluate the effects of combined supplementation of carbon (molasses, glycerol, sodium acetate) and nitrogen (urea, ammonium sulphate and sodium nitrate) sources towards the secondary metabolites' accumulation for biological evaluations. Briefly, the microalgae were cultivated in media with or without supplementation of carbon and nitrogen sources. The biomass was obtained, and the crude extracts were extracted using different organic solvents with increasing polarity (hexane, ethyl acetate, and ethanol). The total phenolic contents of crude extracts were quantified. Following this, the extracts of combined supplementations with higher biomass and phenolic contents were evaluated for the in vitro antioxidant, anti-obesity, and anti-inflammatory properties. The chemical profiling of extracts was carried out using Fourier-Transform Infrared spectroscopy (FTIR) and Liquid Chromatography with Mass Spectrometry (LC-MS). The results showed that combined supplementation of sodium acetate and sodium nitrate exhibited enhanced dry cell weight (1.095±0.03 g/L) and total phenolic contents (591.80±0.92 µg GAE/mg extract) in ethanol extract compared to control. In comparison to other extracts, ethanol extracts exhibited a significant inhibitory effect (IC_{50}: 23.83 ug/ml)

against pancreatic lipase activity and considerably exerted antioxidant activities via ferric reducing ability of plasma (FRAP) (152.03±5.84 FE µg/ml), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) (ABTS) radical scavenging activity (73.42±0.43%). On the other hand, ethyl acetate extract possessed radical scavenging activity via 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (IC_{50}: 123.00 µg/ml), exhibited anti-inflammatory properties via inhibitory effect against proteinase denaturation (45.27±0.02%) and reduced tumour necrosis factor-α (TNF-α) production (665.71±55.56 µg/ml) in lipopolysaccharide (LPS)-activated RAW264.7 macrophages. The FTIR analysis of ethyl acetate and ethanol extracts of combined supplementation revealed the presence of functional groups of amines, esters, and halocarbons. Furthermore, LC-MS/MS results detected the presence of major bioactive compounds in ethyl acetate and ethanol extracts such as alpha carotene, canthaxanthin, rutin, quercitrin, apigenin-O-rutinoside, and caffeoylglucoside thus providing additional insights into their biological properties. Therefore, it can be concluded that functional metabolites derived from ethyl acetate and ethanol extracts of *Tetraselmis suecica* cultivated under combined carbon and nitrogen supplementations exhibit potential anti-obesity, antioxidant, and anti-inflammatory properties, worthy of further in vivo studies for nutraceutical and therapeutic applications.

CHAPTER 1

INTRODUCTION

1.1. Research background

Microalgae are unique unicellular microorganisms having the capability of carrying out photosynthesis by channelizing light and converting its energy into various valuable bioproducts (Enzing et al., 2014). They existed in various forms and shapes ranging from 3 to 10 µm (Morais et al., 2015). Microalgae constitute aquatic microorganisms living in a wide range of water bodies such as lakes, oceans, water sediments, rivers and ponds (M. I. Khan et al., 2018). Microalgae can be classified into two groups which are freshwater and marine microalgae based on their habitat.

Microalgae can be easily found in various environmental conditions including harsh and extreme habitats (Pulz & Gross, 2004). Owing to that, microalgae has emerged as a group of adaptive microorganisms whereby they can produce specific metabolites depending on their culture environment (Morais et al., 2015). The manipulation of the culture condition or inducing the stress condition for microalgae plays a vital role in order to produce desired bioactive compounds. There are few environmental factors that can be manipulated such as nutrient supplementation and deprivation, salinity levels, light intensity, temperature, radiation and also the combinations of those factors in a single cultivation system (M. I. Khan et al., 2018).

The versatility of microalgae in producing valuable compounds in various environmental condition has attracted more attention in research field whereby those compounds from microalgae can be used to develop many products such as pharmaceuticals, biofuels, cosmetics and health supplements (Khan et al., 2018). Other than environmental adaptive properties, microalgae also have drawn much attention among researchers because they have many advantages such as rapid growth and cost

effective. They also use non-arable lands which are unsuitable for agriculture purposes as compared to higher plants (Coêlho et al., 2019). It has been reported that over 15000 novel compounds were identified from diverse species of algae (Gil-Chavez et al., 2013; Patras et al., 2018). Microalgae can produce both primary and secondary metabolites. Among valuable compounds derived from microalgae that can be used to produce many products are pigments, lipids, vitamins, proteins, polysaccharides and chlorophylls (Brennan & Owende, 2010).

The growth and metabolic activities of microalgae are heavily influenced by nutritional factors present in their environment. Microalgae utilise nutrients during cultivation to grow and synthesize functional metabolites. Indeed, microalgae primarily assimilate essential macronutrients and micronutrients to compose their biomass and synthesize metabolites (Ramlee et al., 2021). Among important nutrients supplied into the microalgae cultivation are nitrogen, carbon, phosphorus, vitamins, and trace elements (de Carvalho et al., 2018). As nutritional factors play vital roles for microalgae growth, nutrients manipulation have drawn much interest in cultivating microalgae. Nutritional factors can be manipulated through nutrient supplementation and deprivation which nutrient supplementation consists of the addition of certain nutrients into the culture medium while nutrient deprivation limits or removes certain essential nutrients during the cultivation of microalgae (Chowdury et al., 2020). Several responses including biomass production, growth rate, biochemical composition, and metabolites production have been studied to investigate such correlation between nutrient alterations to the microalgae growth (S. Gupta & Pawar, 2018; Roy et al., 2023).

The bioactive compounds derived from microalgae possessed several biological properties that are useful to human health such as antioxidant, antimicrobial, anti-

diabetic, anti-inflammatory and anti-obesity (Morais et al., 2015). These biological properties confers a wide range of therapeutic application and high-demand commercialisation values (Eze et al., 2023). Generally, microalgal bioactive compounds can be classified into two groups: primary such as lipids and proteins (Katiyar & Arora, 2020) and secondary metabolites such as carotenoids (Sathasivam & Ki, 2018) and phenolic compounds (Jerez-Martel et al., 2017).

Green microalgae are known for its rich secondary metabolite content, exhibits variability in metabolite production under different nutritional conditions (Cardoso et al., 2020). *Tetraselmis suecica*, a green microalga, has gained attention for its potential as a prolific source of secondary metabolites with diverse bioactivities (K. H. Lee et al., 2021). The biological activities of extracts and biomass of *Tetraselmis suecica* were found to be useful in many industries especially in health industry.

The current study aimed to investigate the biological properties of secondary metabolites from *Tetraselmis suecica* under varying carbon and nitrogen supplementations including the effects of single and combined supplementations of carbon and nitrogen sources into the cultivation. The study seeks to understand how these nutritional factors influence the composition and bioactivity of the metabolites. These bioactive compounds produced were further characterized for the in-vitro biological properties specifically antioxidant, anti-obesity and anti-inflammatory activities. Furthermore, the chemical profiling analyses using the Fourier-Transform Infrared spectroscopy (FTIR) and Liquid Chromatography with tandem Mass Spectrometry (LC-MS) were determined.

1.2. Problem statement

Metabolic diseases such as diabetes and obesity are becoming more prevalent in Malaysia which raise public health concerns. According to the National Health and Morbidity Survey (2019), there is an exponential increase of diabetes and obesity prevalence in Malaysia (Ministry of Health, 2019). In this regard, the current pharmacologic treatments for these diseases in Malaysia are mainly directed towards the use the synthetic drugs such as metformin and the weight-loss drug, orlistat (Jovankić et al., 2023; Vazquez Arreola et al., 2022). However, the prominent drawback of these synthetic drugs may cause several undesirable side effects in the long term such as hepatotoxicity, flatulence, and gastrointestinal discomforts (Qi, 2018). Therefore, there is an urgent need to discover alternatives therapeutic strategy from natural bioresources that are fundamental for maintaining optimal health, encompassing a diverse array of essential components such as bioactive metabolites, vitamins, minerals, and polyunsaturated fatty acids (PUFAs).

Current approaches in managing metabolic diseases involve inculcating a healthy lifestyle and various drug-based therapies, yet their efficacy is limited, contributing to its rising prevalence. Thus, the alternative preventive and treatment strategies derived from natural bioresources are preferred over synthetic drugs due to reduced toxicity and lesser side effects (Nisar et al., 2018). Microalgae, integral to the human diet and widely consumed as a health daily supplement, have emerged as a promising natural source with the potential to enhance insulin sensitivity, inflammation, and various components of metabolic diseases (Ramos-Romero et al., 2021). These therapeutic effects are accredited to the diverse bioactive metabolites present in microalgae, exhibiting hepatoprotective, antioxidant, anti-obesity,

antihypertensive, anti-inflammatory, and immunomodulatory effects (Tamel Selvan et al., 2023).

Recent studies on microalgae cultivation are mainly focused on the nutrient deprivation instead of nutrient supplementation during the cultivation of microalgae as the nutrient-limited condition has been shown to improve the lipid production and fatty acids accumulation (Jayakumar et al., 2021; Marlen Trejo & Evelyn Zamudio Pérez, 2020). However, the limitation of nutrient sources such as phosphorus and nitrogen also decreased the microalgae growth rate and biomass production which could limit the further industrial utilisation of microalgae (Maltsev et al., 2023). In contrast, the supplementation of nutrients such as carbon and nitrogen sources into the microalgae cultivation can enhance the microalgae growth in terms of growth rate and biomass production (Anwar et al., 2018; Bashir et al., 2019). The accumulation of primary and secondary bio-compounds such as lipids, phenolics and pigments are improved by the supplementation of nutrient sources in microalgae (Roy et al., 2023). However, most of the studies were mainly focused on the effect of nutrient supplementation in microalgae cultivation on the microalgae growth and production of primary metabolites including lipid or proteins. Up to this date, there is lack of study focusing on the secondary metabolites' accumulation derived from microalgae cultivated under nutrient supplemented condition.

Owing to these considerations, the current study focused on the enhancement of secondary metabolites derived from a marine microalga, *Tetraselmis suecica* cultivated under combined supplementation of different types of carbon (molasses, glycerol, sodium acetate) and nitrogen sources (urea, ammonium sulphate, sodium nitrate). The study aimed to characterize the chemical and biological properties of crude extracts containing secondary metabolites. The biological activities including antioxidant, anti-

inflammatory and anti-obesity properties were investigated through *in vitro* bioassays. Meanwhile, in terms of chemical properties, the secondary metabolites derived from the supplemented cultivation conditions were identified through FT-IR and LC-MS analysis. The findings are useful to promote the use of secondary metabolites from microalgae as one of the alternative ingredients in the development of nutraceutical and pharmaceutical related products.

1.3. Research objectives

1.3.1. To determine the dry cell weight (biomass) and phenolics contents (extracts) of *Tetraselmis suecica* cultivated under varying carbon (molasses, glycerol, and sodium acetate) and nitrogen (urea, ammonium sulphate, and sodium nitrate) supplementations.

1.3.2. To evaluate the anti-oxidant, anti-obesity, and anti-inflammatory properties of crude extracts of *Tetraselmis suecica* via in-vitro bioassays.

1.3.3. To characterize secondary metabolites from crude extracts of *Tetraselmis suecica* by using Fourier-Transform Infrared spectroscopy (FTIR) and Liquid Chromatography-Mass Spectrometry (LC-MS/MS)

1.4. Research hypothesis

The accumulation of secondary metabolites in *Tetraselmis suecica* cultivated under varying carbon and nitrogen supplementations can be enhanced. These secondary metabolites such as phenolics, carotenoids, and flavonoids may potentially possess several biological activities such as anti-oxidant, anti-obesity, and anti-inflammatory.

CHAPTER 2

LITERATURE REVIEW

2.1. Overview of microalgae

Microalgae are unicellular and photosynthetic microorganism that can be found in various types of waterbodies such as lakes, ponds, oceans, rivers and wastewater which categorize them as freshwater and saltwater microalgae (Tan et al., 2020). It has been roughly estimated that over 72500 species of microalgae have been identified, but only up to 8000 species were studied and characterized (Guiry, 2012 & Khan et al., 2018). Microalgae, once overshadowed, are now at the forefront of research endeavours, capturing the attention of the scientific community across various sectors. The unique properties and bioactive compounds present in microalgae have ignited a growing interest, fostering exploration and innovation in fields ranging from biotechnology to healthcare (Randrianarison & Ashraf, 2017).

2.1.1. Introduction to microalgae

Microalgae are ubiquitous in nature and can be cultivated in non-agricultural lands whereby it can be cultivated in a large scale without competing the agricultural crops (Benedetti et al., 2018). Unlike plants, microalgae are capable to grow five to ten times faster than terrestrial plants (Zullaikah et al., 2019). Apart from that, microalgae have a rapid growth rate with intense biomass production compared to the plants and their growing condition is not limited to productive land where they can be cultivated in wastewater, even in arid and infertile lands (Zhu et al., 2013). Their easy cultivation and growth techniques prospect them as an alternative feedstock candidate in order to satisfy global demands in many industries (Hossain et al., 2020).

Microalgae have captured more attention as they can produce diverse of valuable industrial bioproducts which then can be used in many industries such as

aquaculture, cosmetic, health, and food (Bhalamurugan et al., 2018 & Borowitzka, 2013). The first glimpse of microalgae was reported in Chinese culture where *Nostoc* sp. was widely used as food supplements and herbal medicines in two thousand years back (Gao, 1998). The first commercial microalgae is *Chlorella* sp., which was produced in a mass production as a food substitute in 1960s followed by *Arthrospira* (now known as *Spirulina*) in 1970s (Enzing et al., 2014). Numerous studies has revealed that microalgae biomass exhibited numerous metabolites' composition such as proteins, lipids, carbohydrates, vitamins, carotenoids, phenolics, fatty acids, and others which bio-prospects them as a promising natural ingredients in functional food and pharmaceutical industry (Enzing et al., 2014; Rachana Singh et al., 2017).

The biosynthesis of the above-mentioned metabolites accounts a long journey from the upstream processing which comprises of the isolation, selection, cultivation of microalgae cells and harvesting of microalgae biomass, followed by the downstream processing which consists of the extraction of valuable microalgae bio-products (Mutanda et al., 2020). Each step requires a critical investigation in order to achieve the optimal accumulation of metabolites (Lee et al., 2018).

Microalgae are tolerant to a wide range of environmental conditions including extreme and harsh environment (Khan et al., 2018). Given to this unique survival skill, the variability of metabolites synthesis from microalgae are greatly depends on the manipulation of cultivation conditions (Rios Pinto et al., 2021; Sathasivam & Ki, 2018). This is because the microalgae growth and biomass production are highly relies on the microalgae cultivation conditions to produce specific useful bio-products (Cheng et al., 2019). From conventional to modern approaches, researchers have developed a large number of various microalgae cultivation techniques in generating their biomass on a par with a broad range of metabolites (Ramlee et al., 2021). The development of

various cultivation techniques seeks to offer feasible, sustainable and cost-effective methods in producing remarkable biomass yield with high quality of metabolites accumulation (Chew et al., 2017). Several cultivation parameters in growing microalgae which have drawn significant research interest are nutrient supplementation and deprivation, culture media composition, light intensity, growth cultivation modes, temperature, and others (Coêlho et al., 2019; Márquez-Rocha et al., 2020; Ramlee et al., 2021).

2.1.2. Cultivation of microalgae

The manipulation of microalgae cultivation conditions allows the accumulation of different useful metabolites. These metabolites produced due the manipulation of microalgae growing conditions, result in the improvement of biomass production (Ferreira de Oliveira & Bragotto, 2022). Several parameters such as cultivation modes, light intensity, temperature, pH, salinity, nutrient deprivation and supplementation have been studied and explored in achieving optimum microalgae cultivation condition (Chowdury et al., 2020). These environmental parameters are highly controlled in growing microalgae as different microalgae species require specific growing conditions depending on the environment in their primary habitat (Metsoviti et al., 2019).

Cultivation modes are divided into three; autotrophic, heterotrophic and mixotrophic cultivation. Basically, autotrophic only requires light as energy source and inorganic carbon as carbon source while heterotrophic does not require light as energy since this mode only uses external organic carbon such as acetate, glucose, and glycerol as energy and carbon source. On top of that, mixotrophic cultivation uses all light, inorganic and organic carbon to grow the microalgae (J. Márquez-Rocha et al., 2020). **Figure 2.1** illustrates the classification of microalgae cultivation mode with the essential requirements in producing beneficial primary and secondary metabolites.

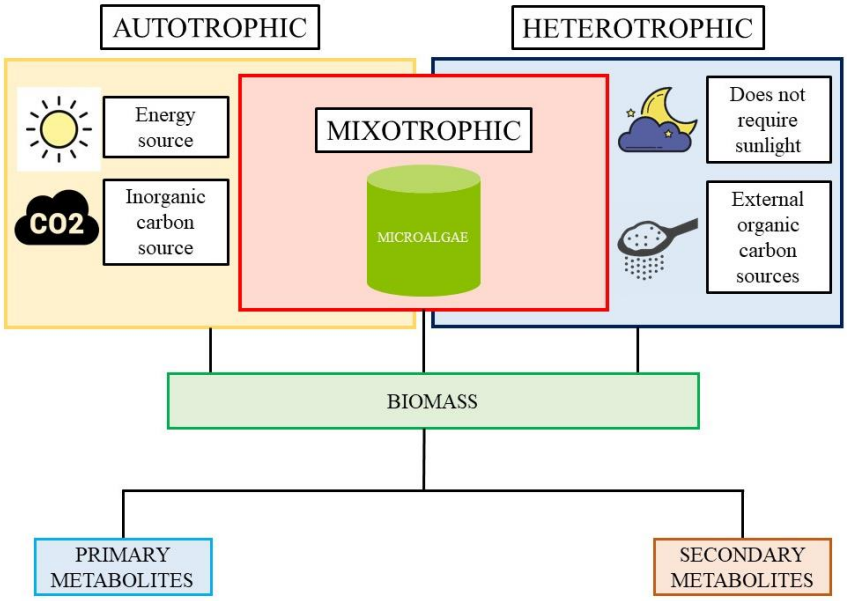

Figure 2.1: The cultivation modes of microalgae.

Light is the key player in microalgae cultivation as microalgae are photosynthetic microorganisms whereby they use light as a main source of energy to carry out photosynthesis prior to the synthesis of various metabolites (Maltsev et al., 2021). In other words, microalgae capture light energy and converting it into chemical energy which then produce the useful metabolites (Al-amshawee & Bin Mohd Yunus, 2020). Light also plays a crucial role in microalgae growth and biomass production through autotrophic and mixotrophic cultivation mode (Shandilya & Pattarkine, 2019). Microalgae exhibit a unique ability to harness energy in different modes, contributing to their adaptability and versatility. In autotrophic mode, microalgae utilize light directly as their primary energy source, engaging in photosynthesis. Conversely, in mixotrophic mode, they adeptly alternate between light and external carbon sources. There are two techniques in utilising light in microalgae cultivation; using natural light (sunlight) or artificial light (Ritchie & Sma-Air, 2023). Natural light usually being used in open pond cultivation whereby the light intensity cannot be controlled. Conversely, the utilisation of artificial light such as light-emitting diode (LED) and fluorescent light

is applicable in a controlled environment condition for growing microalgae to maximise the growth and productivity of the cells. Other than that, the duration of light illumination also a great importance in growing microalgae. For instance, the duration of light illumination can be manipulated through continuous lighting, light/dark cycle and also pulsed light illumination (Zarmi et al., 2020).

Temperature is often associated with the light intensity and quality as microalgae absorb heat from the light irradiance (Deb et al., 2017). High irradiance also can increase the photobioreactor temperature up to 50ºC (Huang et al., 2017). Extreme temperature could affect the microalgae growth and productivity as high temperature can suppress cell respiration and size while low temperature can decrease the cell growth rate (Khan et al., 2018). Different species of microalgae have their own optimal growing temperature, but the ideal temperature range for most microalgae species is between 20ºC to 35ºC (Barten et al., 2020; Gao et al., 2023).

The pH level has important significance in microalgae cultivation as it represents the solubility of CO_2 and minerals in the microalgae culture medium (Chowdury et al., 2020). The environmental pH varies from species to species as different species have their own optimal pH levels and most microalgae can grow in neutral condition between pH 6 to 8 (Yu et al., 2022). There are several factors influence the pH levels in the culture medium such as cells metabolic activity, nitrogen levels, and the amount of CO_2 assimilation (Li et al., 2020; Wang & Curtis, 2016). All of these factors affect microalgae growth rate, biomass and metabolites accumulation (Ramlee et al., 2021).

The biochemical composition and cells growth are influenced by the salinity levels of culture medium (Haris et al., 2022; Pandit et al., 2017). Open pond cultivation

often leads to intense evaporation due to seasonal change which also affect the sal concentration in the medium (Chowdury et al., 2020). When a salt stress is applied, microalgae metabolism changes to adjust themselves. The examples of metabolic changes are antioxidant accumulation, physiological changes, a shift from active cell state to energy storage (Osman et al., 2023).

Furthermore, nutritional factors have a great significance in microalgae cultivation. Nutrient sources are the main components in the culture medium and being divided into two groups: macronutrients and micronutrients. Macronutrients consisted of nitrogen, carbon, and phosphorus while micronutrients comprised of vitamins and trace elements (Yaakob et al., 2021). Each of them has their own functions in growing microalgae. Adjusted growing conditions such as nutrient repletion and depletion could accumulate different types of high-value metabolites from microalgae biomass, however stress condition also could affect microalgae growth and biomass yield (Chen et al., 2017). Previously published works are mostly focusing on the effects of stress conditions on metabolites production by depriving several macronutrients in microalgae cultivation (Maltsev et al., 2023).

2.2. Nutrient supplementations in microalgae cultivation

Nutrient supplementations fosters significant part in growing microalgae in many aspects such as enhancing growth rate, improving biomass production, and also generating novel metabolites accumulation (Yaakob et al., 2021). Indeed, microalgae assimilate nutrient such as carbon and nitrogen throughout the cultivation process which will influence the microalgae growth and biochemical composition (Ramlee et al., 2021).

2.2.1. Carbon

The regulation of microalgae metabolism, cells growth and biochemical composition are highly subjected to the addition of carbon sources into the microalgae cultivation (Ma et al., 2022). The presence of carbon sources helps to speed up nutrient uptake such as nitrogen and phosphorus during microalgae growth (P. L. Gupta et al., 2016). The abovementioned microalgae cultivation modes (autotrophic, heterotrophic and mixotrophic) require different kind of carbon sources namely organic and inorganic carbon. Microalgae in autotrophic mode assimilates inorganic carbon, specifically CO_2, through photosynthetic pathway with the help of light sources by converting the inorganic carbon into producing organic substances such as primary and secondary metabolites (Daneshvar et al., 2021). On the other hand, heterotrophic and mixotrophic microalgae utilise organic carbon for cells growth and metabolism. **Table 2.1** showed the previous studies regarding the use of organic carbon sources in microalgae cultivation and their biological significances.

Table 2.1: Previous studies related to carbon supplementation in microalgae cultivation.

Microalgae species	Type of nutrient supplementation	Response	Biological significance	References
Dunaliella tertiolecta	Carbon (sodium bicarbonate and sodium carbonate)	Cells growth, lipid, protein, phenolics, flavonoids, ascorbate, and pigments production	The supplementation of sodium bicarbonate enhanced the production of primary (lipid and protein) and secondary (phenolics, flavonoids, ascorbate, and pigments) metabolites.	(Roy et al., 2023)
Chlorella sorokiniana	Carbon (glucose, fructose, and sodium acetate)	Cells growth and lipid production	The supplementation of glucose gave the maximum lipid production.	(Qiao & Wang, 2009)
Dictyosphaerium sp.	Carbon (glucose)	Cells growth, lipid, and chlorophyll production	Increasing supplementation of glucose under mixotrophic cultivation enhanced growth, lipid, and chlorophyll production.	(Ogbonna & Ogbonna, 2018)
Dictyosphaerium sp.	Carbon (glycerol)	Cells growth, lipid, and chlorophyll production	Increasing supplementation of glycerol under mixotrophic cultivation enhanced growth and lipid production.	(Ogbonna & Ogbonna, 2018)
Chlorella vulgaris, Botryococcus braunii and Scenedesmus sp.	Carbon (glycerol)	Cells growth, lipids, and fatty acid production	The supplementation of crude glycerol improves biomass concentration and lipid production.	(Choi & Yu, 2015)
Chlorella vulgaris	Carbon (sodium bicarbonate)	Cells growth	Moderate amount (1200 mg/L) of sodium bicarbonate supplementation resulted highest biomass production.	(Yeh et al., 2010)

Isochrysis galbana, *Nannochloropsis oculate*, and *Dunaliella salina*	Carbon (glucose, xylose, rhamnose, fructose, sucrose, galactose)	Cells growth and lipid production	The supplementation of glucose gave the significant biomass and lipid production compared to other carbon sources.	(Gim et al., 2016)
Chlorella vulgaris and *Scenedesmus obliquus*	Carbon (molasses)	Cells growth, pigments, carbohydrate, protein, and lipid production	Increasing amount of molasses supplementation enhanced growth rate, carbohydrate, and protein production under heterotrophic and mixotrophic cultivation.	(El-Sheekh et al., 2014)
Micractinium reisseri	Carbon (sodium acetate)	Cells growth, lipid, carbohydrate, and pigments production	The supplementation of sodium acetate enhanced biomass (8.8-fold), lipid (38.85%), and pigments (30.83 mg/g) production.	(Cao et al., 2022)
Crypthecodinium sp.	Carbon (glucose and sodium acetate)	Cells growth, carbohydrate, protein, and lipid production	The supplementation of glucose improves the cells growth and protein content while sodium acetate enhanced total fatty acids and DHA accumulation.	(Li et al., 2022)
Haematococcus pluvialis	Carbon (sodium acetate)	Cells growth, lipid, carbohydrates, and pigments production	The supplementation of sodium acetate enhanced biomass concentration, total lipids, total carbohydrate and astaxanthin production.	(Yu et al., 2022)
Scenedesmus obliquus	Carbon (sodium acetate)	Cells growth and pigments production	The supplementation of sodium acetate improved biomass concentration, total carotenoid content and chlorophyll production.	(Cheng et al., 2021)

Chlorella vulgaris	Carbon (sodium acetate)	Cells growth and pigments production	The supplementation of sodium acetate enhanced cells concentration and chlorophyll production.	(Khan et al., 2020)
Chlorella pyrenoidosa	Carbon (glycerol)	Cells growth, lipid, and fatty acid production	The supplementation of increasing glycerol level improves cells growth, lipid, and fatty acid production	(Rana & Prajapati, 2021)

Based on **Table 2.1**, various organic carbon sources such as sodium acetate, sugar (glucose, sucrose, etc.), molasses, glycerol, and sodium bicarbonate were used. Different levels of concentration also have been utilized to evaluate the biological effects of the different types of carbon sources. For examples, the supplementation of glucose and glycerol in increasing levels influenced the cells growth, lipid and chlorophyll production in *Dictyosphaerium* sp. (Ogbonna & Ogbonna, 2018). However, excess level of carbon supplementation also affects the microalgae growth as outlined by Yeh et al. (2010) in which the moderate concentration (1200 mg/ml) of sodium bicarbonate supplemented in the cultivation media of *Chlorella vulgaris* recorded the highest biomass production. The supplementation of carbon sources not only influence the microalgae growth in terms of growth rate and biomass production, but it also affects the production of valuable bioactive compounds such as lipids, proteins, chlorophyll, and carotenoids. For instance, the supplementation of sodium acetate into the cultivation of *Scenedesmus obliquus* increased the level of total carotenoid content and chlorophyll production (Cheng et al., 2021). The sodium acetate supplementation also enhanced the production of astaxanthin and lipids in *Haematococcus pluvialis* (Yu et al., 2022). A by-product from biodiesel and sugar industries, such as glycerol and molasses can also being utilised as potential carbon sources in microalgae cultivation. These two carbon sources have been proven to improve microalgae growth performance and productivity in several microalgae such as *Chlorella pyrenoidosa*, *Chlorella vulgaris* and *Scenedesmus obliquus* (Choi & Yu, 2015; El-Sheekh et al., 2014; Rana & Prajapati, 2021).

2.2.2. Nitrogen

The composition of microalgae culture media is mostly comprised of nitrogen (A. Kumar & Bera, 2020). The presence of nitrogen sources in the media is vital as

nitrogen contributes to the microalgae growth rate and biomass production (Richmond, 2004). The most common form of nitrogen that are being assimilated by microalgae are ammonium, nitrite, nitrate and urea, but microalgae favours in utilising the nitrate and ammonium (Chowdury et al., 2020; Yaakob et al., 2021). **Table 2.2** shows the supplementation of various nitrogen sources in microalgae cultivation and its biological significances.

Table 2.2: Previous studies related to nitrogen supplementation in microalgae cultivation.

Microalgae species	Type of nutrient supplementation	Response	Biological significance	References
Nannochloropsis oceanica	Nitrogen (sodium nitrate)	Cells growth and protein content	The cells continued to grow gradually, and protein content increased under the supplementation of nitrogen compared to the starvation of nitrogen.	(Jia et al., 2015)
Isochrysis galbana	Nitrogen (sodium nitrate)	Cells growth	Increased concentration of nitrogen supplementation improves the cells density.	(Zarrinmehr et al., 2020)
Scenedesmus obliquus	Nitrogen (sodium nitrate, ammonium chloride, urea)	Cells growth and lipid production	Increased concentration of nitrogen supplementation enhanced the cells density and lipid production.	(An et al., 2020)
Entomoneis paludosa, *Nitzschia alexandrina*, and *Staurosira* sp.	Nitrogen (sodium nitrate)	Cells growth and lipid production	The supplementation of nitrogen increased growth rate, cell concentration and lipid content in all selected strains.	(Cointet et al., 2019)
Tetraselmis sp.	Nitrogen (potassium nitrate, sodium nitrate, ammonium nitrate, ammonium chloride, glycine, ammonium bicarbonate, ammonium acetate, urea, and yeast extract)	Cells growth	The biomass concentration was highest under yeast extract (organic) supplementation and sodium nitrate (inorganic).	(Kim et al., 2016)
Scenedesmus bijugatus	Nitrogen (calcium nitrate, potassium nitrate, ammonium nitrate, sodium nitrate, urea, and ammonium chloride)	Cells growth	The supplementation of potassium and sodium nitrate recorded the highest biomass growth.	(Arumugam et al., 2013)

Tetradesmus obliquus and *Coelastrella* sp.	Nitrogen (sodium nitrate and ammonium chloride)	Cells growth	The supplementation of sodium nitrate resulted 2-fold growth rate compared to ammonium chloride.	(Perera et al., 2022)

As depicted in **Table 2.2**, the supplementation of nitrogen directly affects the microalgae growth and lipid production. The nitrogen sources used can be classified into two groups: organic and inorganic nitrogen sources. The examples of organic nitrogen sources are urea, yeast and glycine while the most commonly used inorganic nitrogen sources are nitrate and ammonium (A. Kumar & Bera, 2020). Sodium nitrate is highly affecting the cells growth in several microalgae such as *Isochrysis galbana* (Zarrinmehr et al., 2020), *Entomoneis paludosa, Nitzschia alexandrina, Staurosira* sp. (Cointet et al., 2019), and *Scenedesmus bijugatus* (Arumugam et al., 2013). Although nitrogen depletion can increase the production of biomass and cells growth, but a study done by Jia et al. (2015) proved that nitrogen repletion specifically sodium nitrate can enhance the cells growth of *Nannochloropsis oceanica* compared to the nitrogen depletion. Based on the above-mentioned findings, it can be deduced that the supplementation of nitrogen can be one of the potential nutritional factors in maximising cells growth in microalgae.

2.2.3. Combined supplementation of nutrients

Other than single supplementation of carbon or nitrogen sources into the cultivation, the combined effects of these two major nutrient sources are explored as one of the microalgae cultivation strategies. **Table 2.3** listed the combined effects of nutrient sources in microalgae growing conditions.

Table 2.3: Previous studies related to combined nutrients supplementation in microalgae cultivation.

Microalgae species	Type of nutrient supplementation	Response	Biological significance	References
Scenedesmus sp.	Carbon (sodium carbonate, sodium acetate, sodium bicarbonate) and nitrogen (sodium nitrate)	Cells growth and total carotenoid content	The combined supplementation of sodium acetate and sodium nitrate reached the highest biomass production in terms of dry cell weight, while the combined supplementation of sodium carbonate and sodium nitrate depicted the highest total carotenoid content.	(Barajas-Solano et al., 2020)
Ankistrodesmus falcatus	Nitrogen (sodium nitrate), phosphorus (dipotassium phosphate) and iron (ferric ammonium citrate)	Cells growth and lipid production	Combined supplementation of nitrogen, phosphorus and iron increased the biomass productivity and lipid content of the extracts.	(Singh et al., 2015)
Chlorococcum sp.	Carbon (sodium acetate, glucose, fructose, sucrose, starch, glycogen, cellobiose, and glycerol) and nitrogen (sodium nitrite, sodium nitrate, urea, and ammonium)	Cells growth and lipid production	The combined supplementation of sodium nitrate and sucrose enhanced biomass and lipid production.	(Khanra et al., 2020)
Chlorella sp.	Carbon (glucose), nitrogen (ammonium chloride) and phosphorus (potassium dihydrogen phosphate)	Cells growth	An increased ratio of glucose and ammonium chloride resulted an increase in dry biomass.	(Gao et al., 2019)
Tetraselmis suecica	Carbon (glucose and sodium acetate) and nitrogen (sodium nitrate peptone, yeast extract, malt extract, meat extract, urea, and ammonium nitrate)	Cells growth	The combined supplementation of glucose, peptone, yeast, and malt extracts were found to exhibit the maximum cell concentration.	(Azma et al., 2011)

The supplementation of carbon combined with nitrogen sources are found to have synergistic effects on microalgae growth performance and metabolites accumulation. For examples, the combined supplementation of sodium acetate and sodium nitrate reached the highest biomass production in terms of dry cell weight, while the combined supplementation of sodium carbonate and sodium nitrate depicted the highest total carotenoid content in Scenedesmus sp. (Barajas-Solano et al., 2020). The combination of single carbon source specifically glucose and multiple nitrogen sources which are peptone, yeast, and malt extracts also recorded the maximum cell concentration in *Tetraselmis suecica* (Azma et al., 2011). Apart of that, lipid production by *Ankistrodesmus falcatus* and *Chlorococcum* sp. also improved in the microalgae extracts via the combination of multiple nutrient sources (Khanra et al., 2020; Singh et al., 2015).

Most of the previous studies are focusing on the supplementation of carbon and nitrogen sources and its effects on microalgae growth and primary metabolites accumulation such as lipids, proteins, and carbohydrates. However, there are limited studies on the accumulation of potential secondary metabolites derived from microalgae grown under supplementation conditions. Therefore, the present study is shifting the focus onto the accumulation of potential secondary metabolites such as pigments and phenolics derived from supplemented cultivation of *Tetraselmis suecica*.

2.3. The accumulation of secondary metabolites in microalgae and its applications

The quality of microalgae biomass captures more attention as it offers a wide range of beneficial bioactive compounds which made microalgae as the green gold of various industries. The microalgal derived bio-compounds array from simple primary metabolites to complex secondary metabolites (Sreenikethanam et al., 2022). All these

bioactive metabolites vary from species to species depending on cultivation conditions and extraction techniques (Chowdury et al., 2020). In contrast to primary metabolites, secondary metabolites are not directly engaged with the microalgae growth and development. Secondary metabolites such as phenolics, carotenoids and flavonoids represent bioactive compounds derived from microalgae which act as defensive mechanism and attractive agents. These metabolites also have beneficial biological properties such as anti-tumour, anti-oxidant, anti-cancer, and anti-inflammatory which is important in health industry (Ruiz et al., 2010; Sathasivam & Ki, 2018). There are several major groups of secondary metabolites found in microalgae such as phenolics, carotenoids, sterols, hydrocarbons, and vitamins (Park et al., 2022; Sreenikethanam et al., 2022). There are four major biosynthetic pathways of secondary metabolites which biosynthesize different metabolites groups; malonic acid pathway, mevalonic acid pathway, shikimic acid pathway, and Methylerythritol-phosphate (MEP) pathway. The first two pathways are synthesizing phenolic compounds while the latter two pathways are producing carotenoids and sterols (Dhaniaputri et al., 2022).

Given the unique ability of microalgae in surviving harsh environments, the development of various cultivation techniques through the manipulation of growing conditions to produce novel functional metabolites are getting wider in these recent years (Márquez-Rocha et al., 2020). The accumulation and industrial application of primary metabolites derived from microalgae cultivated under various cultivation techniques such as lipids, proteins and carbohydrates are well-known and well-studied. However, in-depth studies related to the capability of secondary metabolites derived from microalgae cultivated under manipulated growth factors are still underrepresented. The present study is emphasizing on the study of phenolics and