Biotechnological approaches to enhance fucoxanthin production in a model diatom *Phaeodactylum tricornutum*

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Dissertation submitted in partial fulfillment of a
Philosophiae Doctor degree in Biology

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Abstract

Diatoms are a major group of algae in the phytoplankton community and can be found in waters worldwide wherever sufficient nutrients and lights are present. Marine diatoms, such as *Phaeodactylum tricornutum*, can accumulate high levels of lipids and have the potential to be engineered into cell factories for sustainable bio-based industries. They are expected to be a promising resource for future clean energy supply and sustainable production of bioactive compounds such as value-added unsaturated fatty acids and carotenoids.

The aim of this study was to explore rational biotechnological approaches to increase the yield of valuable products, especially fucoxanthin. Following a summary of diatoms' morphology, ecology and reproduction methods, a review of bioactive compounds of microalgae, especially marine diatoms, is given. Firstly, we described the potential of microalgae as a large-scale industrial production source of bioactive compounds including carotenoids such as fucoxanthin, astaxanthin and valuable lipids. Ultraviolet C (UVC) light radiation was used as a physical mutagen to induce mutations in the model diatom species *Phaeodactylum tricornutum* and selected positive mutants with enhanced lipids and carotenoid accumulation. Adaptive laboratory evolution (ALE) was also applied to improve phenotypical performance in *P. tricornutum*. Liquid chromatography-mass spectrometry (LC-MS) was applied to quantify the major pigments in the wild type, UVC mutants and ALE strains. It was reported for the first time that ALE was successfully applied to diatoms, increasing both the productivity of valuable carotenoids and biomass production. The final fucoxanthin content was doubled compared with the wild type and the growth rate in the final cycle was approximately two-fold higher than the growth at the beginning of ALE. For the next study, *P. tricornutum* was mutated with chemical mutagens ethyl methanesulfonate (EMS) and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG). EMS exhibited higher efficiency in creating positive mutants with high carotenoid content than did NTG with a similar lethality rate. Furthermore, as we found that both chlorophyll *a* and the lipids had significant correlations with total carotenoids, we established a high-throughput screening method for

selecting high carotenoid accumulation strains: five mutants were selected using this screening method. These five mutants were then cultivated in repeated batch cultures over two months for strain stability validation: four out of five mutants remained stable while one strain faded. In general, four mutants out of approximately 1,000 isolated strains exhibited at least 33% enhancement of fucoxanthin production and the high-throughput screening method significantly increased the screening efficiency.

Útdráttur

Stór hluti svifþörunga eru kísilþörungar og finnast þeir í vötnum um allan heim þar sem næg næringarefni og ljós eru til staðar. Sjávarþörungar, eins og *Phaeodactylum tricornutum*, geta innihaldið mikið magn fituefna og eru því möguleikar á að nýta þá til framleiðslu á verðmætum lífefnum. Vonir eru bundnar við þá til framleiðslu á lífeldsneyti og ýmsum lífvirkum efnum eins og ómettuðum fitusýrum og karótenóíðum.

Markmiðið þessa verkefnis var að kanna mögulegar aðferðir í líftækni til að auka framleiðslu verðmætra efna í þörungnum, sérstaklega fucoxanthin. Fyrst var framkvæmd ítarlega heimildarvinna um form, vistfræði og æxlun kísilþörunga, síðan er yfirlit yfir lífvirk efni í smáþörungum, sérstaklega saltvatns-þörungum. Að lokum var þörungurinn *Phaeodactylum tricornutum* rannsakaður nánar. Honum var stökkbreytt með útfjólubláu ljósi og ræktaður í margar kynslóðir (þróaður) með það fyrir augum að fá fram afbrigði með aukinn vaxtarhraða og meiri myndun karótenóíðum. Útfjólublátt ljós (UVC) var notað til að valda stökkbreytingum og afbrigði af *P. tricornutum* voru síðan valin til áframhaldandi ræktunar. Afbrigðin voru ræktuð í margar kynslóðir til að þróa nýjar svipgerðir af *P. tricornutum*. Við þetta náðist aukin vöxtur í *P. tricornutum* ásamt aukinni myndun verðmætra kartenóíða. Að lokum var *P. tricornutum* stökkbreytt með EMS (ethyl methanesulfonate) og afbrigði með auknu fucoxanthin innihaldi valin með nýrri afastamikilli aðferð sem gerði mögulegt að velja jákvæð afbrigði meðal þúsunda afbrigða. Fimm afbrigði voru valin með þessari aðferð og ræktuð áfram í tvo mánuði til að kanna stöðugleika þeirra. Fjögur þeirra sýndu stöðugleika og jókst fucoxanthin innihald þeirra um 33%.

Dedication

To my parents Taoyuan Yi, Honghua He and to all my friends in Iceland that give me unconditional love and support

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Abbreviations

64PPs - pyrimidine pyrimidone photoproducts
ACCase - acetyl-CoA carboxylase
ACP - malonyl-acyl carrier proteins
ALA - α-linolenic acid
ALE - adaptive laboratory evolution
AXA - amarouciaxanthin A
C. reinhardtii - *Chlamydomonas reinhardtii*
CHLG - chlorophyll synthase
CL - chrysolaminarin
CPDs - cyclobutane pyrimidine dimers
CPDs - cyclobutane pyrimidine dimers
CRISPR/Cas9 - Clustered regularly interspaced short palindromic repeats and their associated proteins
DA - domoic acid
DCW - dry cell weight
DGDG - digalactosyldiacylglycerol
DHA - docosahexaenoic acid
DIC - differential interference contrast
DPA - diphenylamine
DXP - 1-deoxy-D-xylulose 5-phosphate
DXR - DXP synthase
ELISA - enzyme-linked immunosorbent assay
EMS - ethyl methanesulfonate
EPA - eicosapentaenoic acid
FABB - fatty acid synthase ketoacyl-acyl carrier protein synthase I
FABFs - ketoacyl-acyl carrier protein synthase II
FAD - fatty acid desaturase
FAE - fatty acid elongase
FCPs - fucoxanthin-chlorophyll-binding proteins
FDA - Food and Drug Administration
FRAP - ferric-reducing ability of the plasma

FXOH - fucoxanthinol
GC-MS - gas chromatography–mass spectrometry
GMOs - genetically modified organisms
H. pluvialis - *Haematococcus pluvialis*
HAT - hydrogen atom transfer
HBI - highly branched isoprenoid
HIV - human immune deficiency viruses
HPLC-DAD - high performance liquid chromatography involving diode array detector
HTA - hexadecatrienoic acid
IBD - Inflammatory Bowel Diseases
IL-1beta - interleukin-1beta
KAS - ketoacyl-ACP synthase
KEGG - Kyoto Encyclopedia of Genes and Genomes
LC-MS - liquid chromatography–mass spectrometry
LEDs - light emitting diodes
LHCs - light-harvesting complexes
MAT - malonyl-CoA: ACP transacylase
MGDG - monogalactosyldiacylglycerol
MICs - minimum inhibitory concentrations
MNs - meganucleases
MRSA - multidrug-resistant *Staphylococcus aureus*
MS - mass spectrometry
NASH - nonalcoholic steatohepatitis
NMR - nuclear magnetic resonance
NTG - *N*-methyl-*N*-nitro-*N*-nitrosoguanidine
P. tricornutum - *Phaeodactlyum tricornutum*
PA - palmitoleic acid
PBRs - photobioreactors
PC- phosphatidylcholine
PCA - principal component analysis
PEPCK - phosphoenolpyruvate carboxykinase
PFE - pressurized fluid extraction

PG - phosphatidylglycerol
PI - phosphatidylinositol
PM - phenotype microarray
PUAs - polyunsaturated aldehydes
SCF - supercritical fluids
SET - single electron transfer
SFE - Supercritical fluid extraction
SQDG - sulfoquinovosyldiacylglycerol
TAGs - triacylglycerides
TALEN - transcription activator-like effector nuleases
TE - thioesterase
TLC - thin layer chromatography
TM - targeted mutagenesis
TNF-alpha - tumor necrosis factor-alpha
UGPase - UDP-glucose pyrophosphorylase
UVC - Ultraviolet C
WT - wild type

Acknowledgements

It has been such a unique journey for me to finish my Phd project and definitely I am not getting through it alone. There are so many people in my mind that help me prevail over all the obstacles to reach the destination.

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1 Introduction

Diatoms are a major group of unicellular algae and have played a vital role in the global ecosystem for millions of years. Diatoms are also the largest group of phytoplankton and account for approximately 40% of marine carbon fixation and are responsible for the majority of global biosilicification through cell wall construction (frustules) [1-3]. They tend to flourish in natural high-nutrient concentrations, as well as in artificial environments. They have drawn increasing attention in recent years due to their practical applications [4]. Lipid content in certain diatom species can reach more than 60% of total dry weight. Fast growth rate combined with high lipid production make diatoms the leading candidate as a resource for biofuels [4]. Diatoms also contain a high content of valuable carotenoids. Fucoxanthin, as the dominant carotenoid in diatoms, has various physiological benefits and has attracted enormous attention in the food, cosmetics, and pharmaceutical industries [5]. Currently, fucoxanthin is mainly produced by brown seaweed, which contains less than 1mg/g dry weight. Diatoms usually contain 1-2.5% dry weight of fucoxanthin, which is several times higher than the fucoxanthin content of seaweed [6]. Nevertheless, the current benefit-cost ratio of using diatoms for producing valuable chemicals is still too low [7]. The aim of this project was to utilize different biotechnological approaches to increase the growth rate and yield of valuable products, especially fucoxanthin. Increased growth and yield performance are key to the successful utilization of diatoms in industrial processes.

1.1 Marine diatom background

1.1.1 Morphology

Diatoms are unicellular, microscopic algae and most known species' cell walls are made mainly of silica. They are ubiquitously distributed in aqueous environments. They are one of the most common phytoplanktons in the world and exist in fresh water as well in oceans, at both high temperatures and low, and in various pH conditions. They play an important role in the marine system as they consume atmosphere carbon

dioxide and convert it into carbohydrates, which form the primary food source for marine zooplankton [1,8].

Most diatom species, except *Phadactylurm tricornutum* Bohlin and endosymbiotic species, are formed with distinctive silica cell walls that enclose the protoplasts, obtained from its ability to assimilate silicon from the surrounding environment. The silica cell wall protects the protoplast and is also involved in the diatom's gas exchange, nutrients uptake, and cellular production secretion. The functions of the silica cell wall are partially associated with its unique structure. A complete frustule is constituted by two valves: the older valve with its cingulum is called an epitheca, while the younger valve is called a hypotheca [9]. Intriguingly, the forming of the frustule is semiconservative: when one cell divides into two, each offspring inherits one valve from the parent cell and the new valve has already been formed before the siblings totally separate from each other. Scientists have typically focused on two aspects of the morphology of the diatom frustule: valves and girdles, where the valves have more variability than the girdles. The outlines of the valves are mainly classified as centric or pennate, ranging from oval to circular shapes, as well as some elongated types which are linear or rhombic, etc. The girdle shape is much less complex; usually rectangular or square. The size of the frustule ranges from 1μm to 1mm and can change slightly during its lifespan. Centric diatoms are radically symmetrical and are often considered to be planktonic but can also attach to objectives [1,8]. Pennate diatoms are bilaterally symmetrical and may have one or more raphes. The raphes are believed to secrete a viscid layer that helps diatoms to adhere to substances, so a large number of rapid diatoms are categorized as benthic [1,2,10].

The diatom used in this thesis was *Phaeodactylum tricornutum*. This species is a widely used model diatom which the whole genome has been sequenced and is also considered as a potential candidate for large-scale biodiesel production and other commercial applications. It is widely accepted that it has three morphotypes: oval, triradiate and fusiform (Figure 1.1), with occasional reports of a fourth morphotype: cruciform. Spatially resolved force-indentation curved analysis shows that the frustule compositions of the three morphotypes are different; the ovoid form is the only one to have silicon in its cell walls [11]. The girdle regions of the oval and fusiform cells are 5 times softer than in the valve cell, suggesting that these regions have low silica and high organic compound content [1,12].

The arms of triradiate are also softer than the core region which is thought to be due to the existence of organelles in the arm region. Morphotype diversity is important for diatom adaptation to environmental changes. The transformation from one morphotype to another may be triggered by a range of condition changes, such as salinity, pH, and temperature changes. Low salinity can cause the conversion of fusiform and triradiate cells to oval or even round cell morphotypes [1,12]. The different proportion of each morphotype also reflects cell physiology and culture conditions. This is partially due to different morphotypes having different cell compositions. For instance, the content and composition of exopolysaccharides, proteins, and lipids vary across the three different morphotypes. The fatty acids C14:0, C16:2, C16:3 contain observably higher amounts of fusiform than the oval cells [13]. Further difference between the morphotypes indicatesantimicrobial resistance against Staphylococcus aureus of fusiform cells are much higher than oval cells [12].

1.1.2 Biology of marine diatoms

Diatoms are unicellular microalgae, though they can also form chains, colonies, and either float with the current or attach themselves to another substance. The silicified cell wall encloses cell organelles and is a complicated structure. The cell wall is transparent and porous, allowing light to come through freely, making gas exchange and compound secretion possible [14].

The attachment type and the growth form are highly related to substrata type. The diatom genera that grow on rocks are adapted to resist the drag force of currents and generate prostrate forms that have tight attachment to the substrate. Solid current-exposed surfaces favor the establishment of flat cells or those that have strong adherence to the substratum (*Cocconeis, Ceratoneis, Tabellaria*), while in low currents stalked and filamentous forms may grow into tufts (Synedra) [15]. Diatoms attaching to plants are less tightly adhered as they utilize the hydrodynamic protection of the host. Common epiphytes are *Gomphonema*, *Cocconeis*, or *Tabellaria* [1].

Diatoms are sensitive to environmental and biological variations such as salinity, light, water velocity, temperature, and nutrients. Most diatoms have the capacity to adapt to a wide range of temperatures and light intensities. However, some diatom species, such as *Aulacoseira roseana*,

grow better in low light conditions, while other species, such as *Melosira varians*, respond to high light levels with a massive growth rate, especially in combination with moderate water temperature [1,16]. The preferred water temperature for most diatoms is between 15 and 25 °C while some species such as *Nitzschia palea* can tolerate water temperatures higher than 30 °C and a few can grow well under 4 °C (*Aulacoseira islandica*)[17].

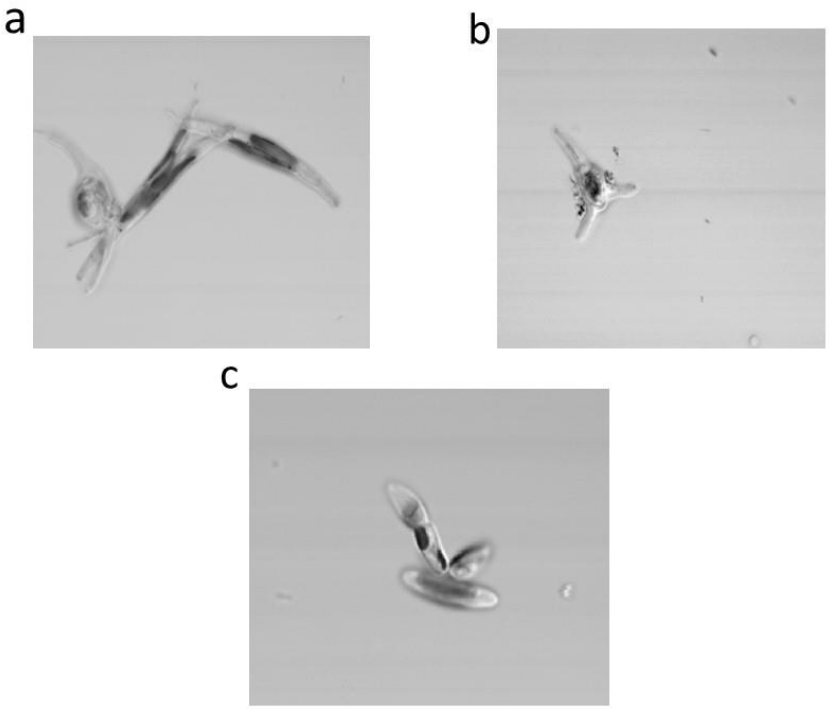

Figure 1-1 *Phaeodactylum tricornutum morphotype under confocal microscopy. (a) fusiform morphotype; (b) triradiate cell; (c) oval cell.*

Water pH is determined by overall water geochemistry conditions and various diatom taxa have different pH value preferences. Calcium concentration (associated with the carbonic buffer system and therefore also associated with pH value) is presumed to play an important role in determining the species' distribution. The diatom's diversity is usually lower in extreme pH conditions than in moderate conditions. In moderately to highly alkalescent environments (pH value between 9.5 to 10.5) some diatom taxa have a symbiotic relationship with filamentous cyanobacteria. Genera such as *Eunotia, Stenopterobia, Frustulia* prefer to grow in acidic

conditions while species such as *Denticula, Epithemia, Nitzschia* develop best in moderately to highly alkaline environments [1,18].

Salinity is the foundational element of water hardness and iconic compositions and can be the determining factor for diatom taxa distribution. It may impact diatom cellular osmotic pressure, along with nutrient uptake and other processes. A few diatom genera can tolerate less than 30-40 g/l salinity and they are categorized as brackish water species: *Amphora coffeaeformis, Navicula salinarum, Cyclotella quillensis.* Other genera flourish under moderate salinity (between 5-20 g/l range) conditions and are recognized as halophilic. The remaining diatoms are freshwater species that require lower mineral content to thrive in pure freshwater [19].

Diatoms are sensitive to nutrient content and ratio changes. Benthic genera might be more responsive than the planktonic genera to eutrophication. Nutrient excess favors a number of diatom taxa such as *Nitzschia palea* but inhibits the growth of genera that thrive in lower nutrient concentrations such as *Cymbella microcephala* and *Achnanthes biasolettiana.* Nitrogen restriction is beneficial for *Epithemia* and *Rhopalodia* growth because they typically have a symbiotic relationship with nitrogen-fixer cyanobacteria. The diatom species' optimal growth conditions and their tolerances of nitrogen and phosphate have been well studied, though it should be noted that these values can be simultaneously affected by other determining factors such as pH, salinity and light [6,20,21]. Herbivore grazing alters both diatom taxa biomass (high intensity grazing restricts biomass growth) and its growth form. It has been observed that diatom communities are closely correlated with the morphology and density of grazers' mouthparts. The community growth form may change if there is an alteration in the grazers' mouthpart morphology or density[22]. Mayflies may feed on filaments or stalked forms that are located on the outer parts while caddisflies and snails tend to feed on encrusted and prostrate forms. Hence, the abundance and the type of grazers determine the growth form of the diatoms [6,22].

1.1.3 Diatom reproduction

Diatoms are peculiar in many ways: their large diversity lineage, their complex frustules and raphe systems [23]. One of the most unique aspects of a diatom is its life cycle, in which size and sex play very important roles and one complete life cycle is extraordinarily long compared with those of

other algae species. The integrated life cycle in most diatom species is characterized as two phases: the vegetative phase and the rejuvenation phase. In the vegetative phase, which diatoms spend most of their diploidic life going through, mitotic division usually takes months or even years and the cell size decreases gradually during this process (Figure 1.2). During the vegetative growth phase, cells are constrained by two silica cell walls (two valves), where the slightly larger valve contains girdle elements and this valve overlaps the smaller one. Because of the cell walls' constitution, during mitosis, two siblings inherit each valve from their parental cells, with one sibling remaining the same size and the other shrinking (Figure 1.2). As a result, average length and diameter reduce in diatom populations over time. Cell shape also changes during the reproduction process, except for the circular valves since the oval cells' girdles are very stiff. Mostly, the cells decrease their valves' outline complexity, with the elongated diatoms reducing more in length than in width. The rejuvenation phase only takes a few days and cells are divided through sexual reproduction. The cell size increases to its maximum value through the formation of auxospore [1,23,24].

Sexual reproduction only occurs if two conditions are both fulfilled. Firstly, cell must reach its' optimal size which is 30 - 40% of the maximum size and is most suitable for forming gametangia [25]. Secondly, environmental factors such as temperature, pH, salinity, osmotic pressure and nutrients must be suitable for sexual reproduction. It has been suggested that other factors also play an important role in sexual induction [1,26].

P. tricornutum is a distinctive diatom species in which the frustules do not become smaller during asexual reproduction [27]. This enables continuous cultivation and revokes the need for sexual reproduction. It is uncertain whether *P. tricornutum* can reproduce sexually and till now no evidence has been provided to support the existence of a sexual reproduction capacity even in laboratory settings [27].

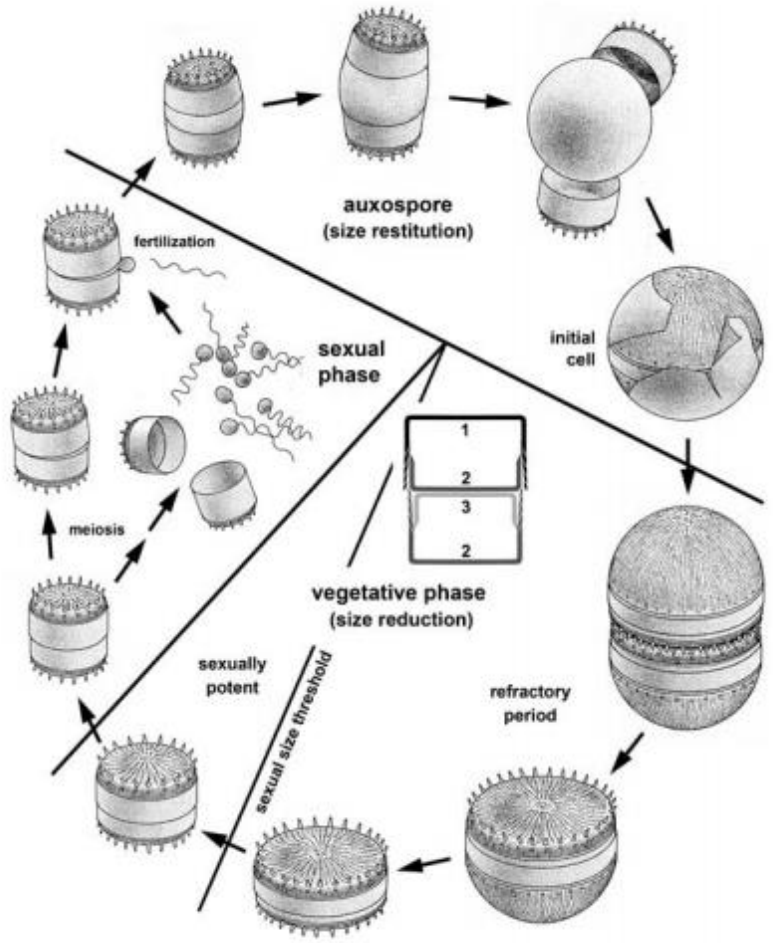

Figure 1-2 *The life cycle of one centric diatom with vegetative, sexual and auxospore phases. Cell size reduces during vegetative phase due to internalized cell division. The inset diagram exhibits a vegetative cell dividing into sections, with two newly created cell walls binding with old ones. The vegetative cells become smaller and once they reach the sexual size threshold they become sexualized when environmental and nutrient conditions are fulfilled. Meiosis occurs when gametes are formed, at which point diatoms are divided into large egg cells and small male sperms. The auxospore that originated from the zygote expands to maximum size and then forms an "initial cell." (Figure from G. M. David[1])*

1.1.4 Diatom evolution

Marine diatoms began to flourish approximately 100 million years ago, forming organic compounds to provide a food source for marine organisms [28]. The evolutionary history of diatoms is quite complex and unique and is very different from that of land plants. A eukaryotic heterotroph engulfed a cyanobacterium 1.5 billion years ago and formed the photosynthetic plastids of the plantae which is the ancestor of land plants, green and red algae [29]. The genome inside cyanobacterium was subsequently transferred into the eukaryotic heterotroph's nucleus and approximately 1/10 of the plantae genome originated from cyanobacterium. A second endosymbiosis occurred 500 million years later, when another eukaryotic heterotroph engulfed a red alga. The red-alga endosymbiont transferred into the plastid of Stramenopiles which formed the ancestor of brown macroalgae, plant parasites and diatoms. More than 170 red algal genes have been detected in the diatom genome, with most genes encoded for plastid components [10] (Figure 1.3).

Recent discoveries have provided additional information on diatom genomes [14] [30]. Chlamydiae are groups of intracellular bacteria that only exist in symbionts or pathogens. One discovery revealed that some Chlamydiae genes are found in red algae and plants, but not in cyanobacteria, suggesting that Chlamydiae are also involved in primary endosymbiosis. One study reveals that at least 587 genes in the *P. tricornutum* genome are also discovered at bacteria diverse lineages along with Chlamydiae. Some of these genes add novel functions to diatoms [30]. Viruses also seem to be involved in diatoms' gene transfer; however the process has not yet been fully studied [31].

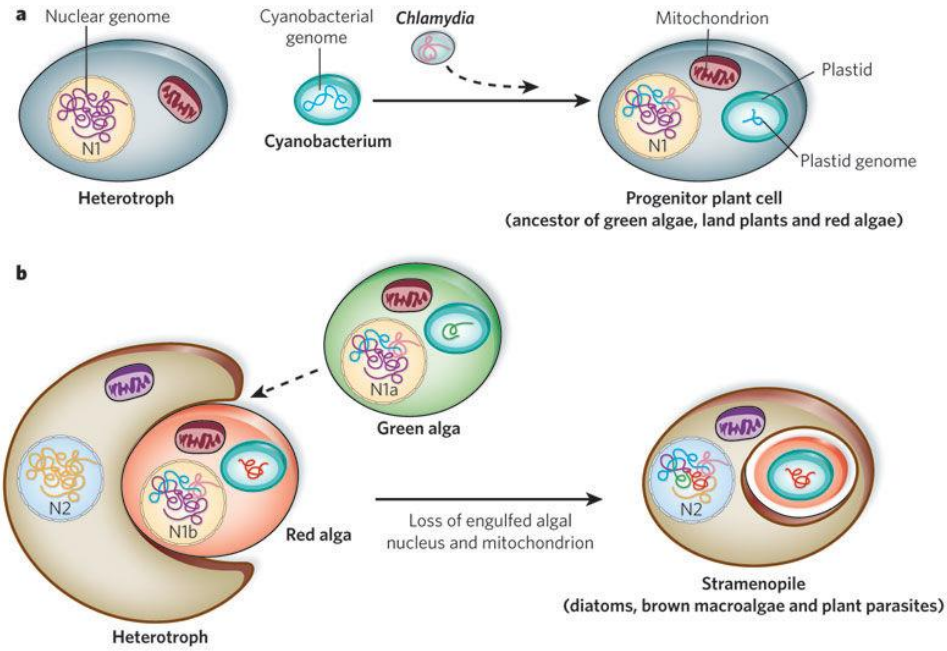

Figure 1-3 *Endosymbiosis in diatoms. Representation of (a) primary and (b) secondary endosymbioses of the origin of plastid and the effect on the diatom genome. (Figure from Armbrust E.V[28].)*

1.1.5 Objectives and a preview of the thesis

Diatoms have drawn increasing attention for commercial application purposes due to their fast growth rate and high content of valuable products. Nevertheless, diatoms have not been widely applied for large-scale cell factories production due to the low feasibility. There is still a gap in the knowledge on how to improve the growth rate and the yield of diatoms biomass. **The primary objective of this study was to explore rational biotechnological methods that can increase the yield of value-added products, especially fucoxanthin using the model species *Phaeodactylum tricornum*.** This goal was achieved by fulfilling the following objectives:

Objective 1: To adapt *P. tricornutum* strains to selected light stress conditions and determine its production potential using Adaptive laboratory evolution (ALE)

Objective 2: To physically and chemically mutate *P. tricornutum* in order to achieve high growth rate, neutral lipid and carotenoid accumulation

Objective 3: To unravel the metabolic pathways of major carotenoids and develop high-throughput screening method to select positive mutants from mutagenesis

Firstly, a basic introduction to diatoms is given in this thesis. Following this, the effects of physical mutagenesis and ALE on marine diatom model *P. tricornutum* are demonstrated. In addition, the outcome of chemical mutagenesis on diatoms is shown and one high-throughput screening method to select positive mutants of fucoxanthin hyper-production strains generated by mutagenesis was developed.

The content of the chapters of this thesis are as follows:

Chapter 1: This chapter introduces the background of the marine diatom: its morphology, ecology, reproduction and evolutional history. The bioactive compounds in diatoms, especially carotenoids and lipids are summarized. This chapter is based on two published reviews.

Chapter 1.2 is a full reprint of the book chapter Fu, W.; Nelson, D. R.; Yi, Z.; Xu, M.; Khraiwesh, B.; Jijakli, K.; Chaiboonchoe, A.; Alzahmi, A.; Al-Khairy, D.; Brynjolfsson, S.; Salehi-Ashtiani, K, Bioactive Compounds

From Microalgae: Current Development and Prospects. In Studies in Natural Products Chemistry, Atta-ur-Rahman, Ed. Elsevier: 2017; Vol. 54, pp 199-225.

Chapter 1.3 is a full reprint of the journal paper Yi, Z.; Xu, S. M.; Di, X.; Brynjólfsson, S.; Fu, W., Exploring Valuable Lipids in Diatoms. 2017; Vol. 4.

Chapter 2: This chapter demonstrates the effect of physical mutagen UVC and adaptive laboratory evolution on improving the phenotypic performance of strain *P. tricornutum*. It addresses Objective 1 of this study.

Chapter 2 is a full reprint of the journal paper Yi, Z. Q.; Xu, M. N.; Magnusdottir, M.; Zhang, Y. T.; Brynjolfsson, S.; Fu, W. Q., Photo-Oxidative Stress-Driven Mutagenesis and Adaptive Evolution on the Marine Diatom Phaeodactylum tricornutum for Enhanced Carotenoid Accumulation. Mar Drugs ***2015,*** *13 (10), 6138-6151.*

Chapter 3: This chapter explores chemical mutagenesis on improving the performance of *P. tricornutum* and sets out a high-throughput screening method for selecting positive mutants from mutagenesis. It addresses Objectives 2 and 3 of this study.

Chapter 3 is a full reprint of the published research article Yi, Z. Q.; Su, Y. X.; Xu M. N.; Bergmann, A.; Ingthorsson, S; Rolfsson, O.; Salehi-Ashtiani, K.; Brynjolfsson, S.; Fu, W. Q., Chemical Mutagenesis and Fluorescence-based High-throughput Screening for Enhanced Accumulation of Carotenoids in a Model Marine Diatom Phaeodactylum Tricornutum. Mar. Drugs 2018, 16(8), 272.

Chapter 4: Conclusion and prospects.

1.1.6 List of publications

Zhiqian Yi, Yixi Su, Maonian Xu, Andreas Bergmann, Saevar Ingporsson, Ottar Rolfsson, Kourosh Salehi-Ashtiani, Sigurdur Brynjolfsson, Weiqi Fu. Chemical mutagenesis and fluorescence-based high-throughput screening for enhanced accumulation of carotenoids in a model marine diatom *Phaeodactylum tricornutum*. *Mar. Drugs* 2018, 16(8), 272; https://doi.org/10.3390/md16080272

Zhiqian Yi, Maonian Xu, Manuela Magnusdottir, Yuetuan Zhang, Sigurdur Brynjolfsson and Weiqi Fu, Photo-Oxidative Stress-Driven Mutagenesis and Adaptive Evolution on the Marine Diatom *Phaeodactylum tricornutum* for Enhanced Carotenoid Accumulation. *Mar. Drugs* **2015**,13(10), 6138-6151; doi:10.3390/md13106138

Zhiqian Yi, Maonian Xu, Xiaxia Di, Sigurdur Brynjolfsson and Weiqi Fu, Exploring Valuable Lipids in Diatoms, *Front. Mar. Sci*., 30 January 2017. (Review)

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1.1.7 Authors' contribution

Research Paper 1

The first research paper presented in this thesis demonstrates the effect of physical mutagen UVC and adaptive laboratory evolution on improving strain *P. tricornutum* phenotypes [33]. In this work I performed the research experiments, while Maonian Xu and Yuetuan Zhang contributed to the pigment extraction. The majority of data was analyzed by myself and Manuela Magnusdottir, with Maonian Xu, Yuetuan Zhang and Weiqi Fu also contributing to the data analysis. The paper was mainly drafted by me; Maonian Xu contributed toward the pigments analysis section and Weiqi Fu contributed to part of the introduction and discussion. The study was conceived by Sigurdur Brynjolfsson and Weiqi Fu; the project was designed by Weiqi Fu. All the listed authors edited the manuscript and approved its final version for publication.

Physical mutagenesis is a general method for generating mutants with desired phenotypes. Nethertheless, few studies have been published which have conducted physical mutagenesis on marine diatoms. In this paper, the physical mutagen UVC was applied to induce mutations in *P. tricornutum*. Firstly, different exposure times for the lethality of *P. tricornutum* were examined and 10 min, 15 min and 20 min exposure times achieved 36%, 14.4% and 8.9% survival rates. For further UV mutagenesis experiments, 10 min and 15 min exposure times were chosen. Nine mutated strains were selected for further measurement: 4 out of 9 mutants reached approximately twofold of total neutral lipids content in WT and all 9 mutants had a higher lipid content than WT. The LC-MS was applied to quantify the specific pigments inside the mutants and WT. The top mutated strain UV85 yielded 1.7 fold more fucoxanthin content than wild type cells and most strains exhibited increased fucoxanthin content. However, chlorophyll *a* and beta-carotene content reduced in some strains. It appeared that the enhancement of fucoxanthin was postively correlated with the increase of neutral lipids, although it was not proportional for the correlation.

Adaptive laboratory evolution (ALE) has been proved to improve strain performance and develop new phenotypic functions in a few bacteria and algae species [34]. In this study, we utilized LED light as a photo-oxidative stressor to carry out ALE on *P. tricornutum*. Adaptive evolution was executed in a semi-continuous culture and the diatom biomass was kept the same at the beginning of each cycle. It appeared that the growth rate of the

ALE strain enhanced gradually: from 0.14 gDCW/L/day growth at Cycle 1 to 0.29 gDCW/L/day at Cycle 11. The specific pigments were also measured with LC-MS. After 11 cycles, both beta-carotene and chlorophyll *a* did not have significant changes compared with the first cycle, while fucoxanthin content increased approximately one time by Cycle 11 compared to Cycle 1. On the contrary, the neutral lipid content decreased slightly from 24.6% at Cycle 1 to 19.3% at Cycle 11. It is reported here for the first time that ALE has been successfully applied on marine diatoms to increase phenotypic performance and the results show that ALE is an effective method for enhancing both the growth rate and carotenoid accumulation in marine diatom *P. tricornutum*.

Research Paper 2

The second research paper presented in this thesis explores the utilization of chemical mutagens to improve *P. tricornutum* and validates a high-throughput screening approach for selecting positive mutants from mutagenesis [35]. I perfomed most of the research experiments, Maonian Xu and Andreas Bergman contibuted to the pigment extraction process, Yixi Su and Saevar Ingthorsson contributed to confocal microscope monitoring; and the data was analyzed by myself, Yixi Su, Maonian Xu, Andreas Bergmann, Kourosh Salehi-Ashtiani and Weiqi Fu. The draft was mainly written by me; Yixi Su contributed to part of the introduction, computational modeling and supplementary sections; Maonian Xu contributed to the PCA and OPLS-DA sections and Andreas Bergmann contributed to part of the materials and methods section; Weiqi Fu contributed to part of the introduction and discussion. This project was supervised by Sigurdur Brynjolfsson, Ottar Rolfsson, and Weiqi Fu; the research was conceptualized and designed by Weiqi Fu. All the authors validated and edited the manuscript and approved it for final publication.

Many studies on the chemical mutagenesis of microalgae to produce positive mutants with high carotenoid accumulation have been conducted by selecting mutants that can tolerate carotenogenic pathway inhibitors such as norflurazon and nicotine [36-38]. In this study, diphenylamine (DPA) was applied in order to enhance the growth stress of isolated positive mutants. The effect of different doses of DPA on *P. tricornutum* growth was explored. The diatom growth reduced significantly from 30 µM to 60 µM. Particularly, the wild type still grew while the DPA concentration was under 40 µM, but the cell numbers decreased when the DPA exceeded 40 µM. Accordingly, 40 µM DPA was chosen for the subsequent screening experiments. Both EMS and NTG were applied to mutate *P. tricornutum*

and the efficiency of each mutagen was checked. The cell lethality of 0.1 M EMS and 0.2 M EMS in ditaoms was 42.3% and 71.5%, respectively, while 0.1 mM and 0.2 mM NTG was 36.9% and 65.8%, respectively. Under similar lethality, EMS created more positive mutants than NTG, which implied that EMS had better mutagenesis efficiency when creating high carotenoid accumulation strains. As carotenoid fluorescence was relatively low and chlorophyll *a* and neutral lipid fluorescence was high and could be easily detected, the correlations of both chlorophyll *a* and lipid fluorescence intensity with total carotenoid content was examined in order to develop an effective method for screening hyper-fucoxanthin production strains. The results exhibited that both chlorophyll *a* and lipids had positive correlations with carotenoid content and the correlation in the exponential growth phase was higher than in the stationary phase. Five positive mutants screened by the high-throughput screening were chosen for the stability test and four mutants exhibited stable total carotnenoid content while one mutant dropped to nearly the same level as WT. The specific pigments in the five mutants were detected through LC-MS. For fucoxanthin, four of the five mutants had a 69.3%, 64.2%, 63.8%, 53.2% increase than WT, respectively. For chlorophyll *a*, four of the five mutants had higher accumulation than WT as well: the content was 81.9%, 79.1%, 33.7% and 10.2% greater than that of WT. In terms of neutral lipids, all the mutants had higher lipid content than WT. Based on the metabolite profiles, PCA was utilized for sample grouping and OPLS-DA S-plot demonstrated the differences in production between mutants and the WT group. Both OPLS-DA and PCA results confirmed the phenotypic differences between the mutants and the wild type. Correlated enzymatic reactions between fucoxanthin synthesis and pigmentation or lipid metabolism through a genome-scale metabolic model of *P. tricornutum* were analyzed and highly correlated reactions with fucoxanthin production were found. The integration of the computational results with liquid chromatography–mass spectrometry data revealed key compounds underlying the correlative metabolic pathways. In general, four mutants out of approximately 1,000 isolated strains exhibited at least a 33% enhancement of fucoxanthin production. The high-throughput screening method significantly increased the screening efficiency and may be applied to other algal species in order to obtain carotenoid-hyperproducing stains.

Chapter 1.2 is a full reprint of the book chapter: Fu, W.; Nelson, D. R.; Yi, Z.; Xu, M.; Khraiwesh, B.; Jijakli, K.; Chaiboonchoe, A.; Alzahmi, A.; Al-Khairy, D.; Brynjolfsson, S.; Salehi-Ashtiani, K., Chapter 6 - Bioactive Compounds from Microalgae: Current Development and Prospects. In Studies in Natural Products Chemistry, Atta ur, R., Ed. Elsevier: 2017; Vol. 54, pp 199-225. I am a contributing author for this book chapter and I am the main author of section: 1.2.3 Primary Screening for Bioactive Compounds from Microalgae and part of the introduction which forms the basis of this book chapter.

1.2 Bioactive compound: Current Development and Prospects (Book chapter)

Microalgae make up the largest and likely most diverse group of photosynthetic organisms in freshwater and marine systems. As new technologies are emerging for the study of bioactive compounds from microalgae, this group is drawing attention as a promising source of natural products that have wide applications in the food and pharmaceutical industries. Algae-derived bioactive compounds are attractive resources for drug screening, given their tremendous structural diversity and biological availability. In this chapter, we first discuss medicinally important products, such as carotenoids, including β-carotene, fucoxanthin, astaxanthin, and lutein, as well as essential fatty acids that originate in microalgae. We then briefly introduce screening assays for antioxidant, anti-microbial, anti-viral, anti-cancer and immunomodulatory effects, and explore biosynthesis of natural products, which have been widely used in food and cosmetics for their antioxidant effects and nutritional value, and we discuss the potential use of fucoxanthin and its derivatives as anti-cancer agents. In addition, we describe health benefits of the essential fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Further, this chapter emphasizes that microalgae provide a rich source of compounds for therapeutic drug screening and describes examples of screening assays for detection of biological activities of algae-derived compounds.

1.2.1 Introduction

Microalgae have drawn great attention as a promising source for the sustainable production of various bioactive compounds. These include fatty acids, phycobiliproteins, chlorophylls, carotenoids, and vitamins that can be widely used in pharmaceuticals, cosmetics, and food additives and ingredients [39]. The natural bioactive compounds from microalgae are attractive as research targets, and may have great possibilities for commercialization in the near future due to their potential therapeutic activities. These properties include anti-oxidant, anti-viral, anti-bacterial, anti-fungal, anti-inflammatory, anti-tumor, and anti-malarial effects. However, natural products from microalgae remain largely unexplored compared to those obtained from land plants, although algae have many advantages over terrestrial plants. For instance, algae can grow rapidly, are

easier to cultivate, and do not compete directly with crops for agricultural land. These and other advantages highlight the importance of developing microalgae for the production of medicinally important natural products as well as for pharmaceuticals discovery and development, among other bio-products [40].

Compared with other well-studied organisms, microalgae are underexploited and very few have been described and studied among the tens of thousands of microalgal species that exist worldwide [40-42]. Although land plants are highly exploited for their production of natural products, the diversity of compounds produced by algal species are estimated to be over 10 times greater than those produced by land plants. These include a plethora of secondary metabolites and an array of environment-specific basal metabolites [40]. For this reason, algal-derived natural products (specifically including bioactive compounds) are a great untapped resource for multi-faceted usages spanning health, materials, and other industrial sectors [40].

In this chapter, we focus on microalgae-derived compounds with potential benefits to human health. To this end, this chapter first covers the common pathways for biosynthesis of bioactive compounds such as carotenoids and their derivatives in several representative species of green algae and diatoms. Then, we discuss the biological activities of the bioactive compounds with a focus on human nutrition and health. We also review the structural properties of these compounds, chemical characteristics, and their dose-activity relationship. Last, because microalgae are rich reservoirs of natural products, following a critical review and speculations for future directions, we conclude that more efforts should be invested into algal research with a focus on developing bioactive compounds for human health.

1.2.2 Microalgae and Its Derived Natural Products

Model and Key Algal Genera

Microalgae represent one of the most diverse groups of microorganisms in freshwater and marine systems [43]. Microalgae refer to eukaryotic microorganisms in taxonomy. However, in its broadest definition, microalgae include prokaryotic cyanobacteria, green algae, diatoms and many other eukaryotic groups [40,44]. Among microalgae, one particularly large group is the green microalgae that belong to the phylum Chlorophyta

and include common genera such as *Chlorella, Dunaliella, and Haematococcus*. Diatoms of the phylum Heterokontophyta constitute another large group and include widely occurring genera such as *Phaeodactylum* [45]. Species of microalgae belonging to distant lineages, including spirulina *Arthrospira platensis*, a cyanobacterium and *Chlorella pyrenoidosa*, a green alga or a Chlorophyte, have already been used to produce therapeutic compounds to treat human diseases and improve health [46,47]. Of these, algae from the green and brown lineages are emerging organisms of choice for the discovery of new medicinally important compounds. The main model organism for a broad range of algal studies, *Chlamydomonas reinhardtii*, a green, motile alga, has proved to be capable of producing a variety of *trans*-proteins through its chloroplast [48,49] and has spurred several studies using algal chloroplasts for the incorporation of *trans* genes into microalgae [48].

Green algae have been used for the production of natural pigments [50,51]. Although *Haematococcus* has been studied less extensively than *Chlamydomonas*, *Haematococcus* has the capacity to produce a unique variety of pigments, the foremost of which is astaxanthin [50,52-54]. Astaxanthin can be found in a wide variety of nutritional supplements due to its high antioxidant capacity and subsequent health benefits to a variety of tissue types [55-57]. The widespread interest in astaxanthin production in *Haematococcus* has resulted in several studies aiming to increase its yields via various methods [58-63]. The major algal species used to produce astaxanthin belongs to the *Haematococus* genus, but some *Chlorella* species, such as *Chlorella zofingiensis*, also produce astaxanthin. *Chlorella* and *Haematococcus* utilize different pathways to synthesize astaxanthin [54,59,64-66]. Other species able to produce astaxanthin include *Chlamydomonas nivalis*. Accumulated astaxanthin in the latter has been shown to contribute to UV- and oxidative protection in their natural habitats [67].

Green algae, in addition to *Chlamydomonas, Chlorella*, and *Haematococcus*, also include the halo-tolerant alga, *Dunaliella salina*. *Dunaliella* has been successfully utilized for decades to produce β-carotene. Extracts of *Dunaliella* have shown significant cytotoxicity to neuroblastoma cells but not healthy cells [68]. *Dunaliella* is also of interest for "molecular farming" due to its ease of culturing and extremophilic capacity [69].

Diatoms as a major group of microalgae are rich sources of fucoxanthin [70]. As a predominant pigment in diatom cells, fucoxanthin shares similar structural properties with the xanthophyll carotenoids like astaxanthin, and has been shown to have inhibitory effects on cancer cells by having pro-apoptotic activities [71,72]. In addition, fucoxanthin has attracted a great level of additional attention because of demonstrated antioxidant, anti-inflammatory, anti-obesity, antidiabetic, and antimalarial activities that have been shown to be effective in treating many diseases [73,74].

Finally, the use of microalgae to produce poly-unsaturated long-chain fatty acids (PUFAs) is of increasing interest [75,76]. While nutritional supplements with PUFAs have shown to confer significant health benefits [77,78], traditional production of PUFAs by extraction from fish oils usually involves unsustainable fishing methods that deplete global fisheries and also introduce the risk of heavy metal contamination [76,79-82]. The alternative, namely extracting PUFAs from algae, has shown great promise from a great variety of algal species and many species naturally synthesize PUFAs to a high degree. In general, accumulation of dietary PUFAs occurs naturally in organisms adapted to cold environments, as longer fatty acids with higher desaturation confer fitness benefits in such climates [83]. Species of *Crypthecodinium*, *Nannochloropsis*, *Phaeodactylum*, *Monodus*, *Nitzchia*, and *Isocrysis* are among the top candidates for industrial production of PUFAs intended for health supplements [84]. In addition, the microalgae *Nannochloropsis gaditana* and *C. debaryana* were reported as potential resources for partially oxidized n-3 PUFAs, e.g., oxylipins, which also display anti-inflammatory affects [85].

Bio-prospecting for New Algae

All algae that are currently used for production of medicinally relevant compounds were originally isolated from environmental samples. However, it is estimated that a very small fraction of extant algal species have been cultured for production of medicinals [86]. Recently, several groups have been conducting bio-prospecting expeditions to discover new commercially relevant species of algae for the production of medicinals and other bio-compounds. Although most of these studies have focused on the production of fuel precursors [84,87-90], a few studies have used bio-prospecting to find strains that over-accumulate medicinal compounds [91,92].

Isolation of new species is often accompanied by a number of challenges including a lack of knowledge regarding metabolic requirements of the species for growth, nutritional requirements (e.g., phosphorus, sulfur, and nitrogen sources), as well as pH and other growth parameters such as temperature and culture density. Understanding the chemical interactions of new strains or those not well characterized is essential for optimizing their productivity. One high-throughput phenotyping assay that can achieve this is the Biolog® phenotype microarray (PM) (Biolog, Hayward, CA, USA). Importantly, it provides a fast, reliable, and quantitative method to characterize phenotypes. An example of phenotype data output in the form of growth rate measurements is shown in **Figure 1.4**. The assay examines cellular metabolism in response to thousands of metabolites, signaling molecules, and effector molecules. Information gleaned from the Biolog PM assays can then be used for functional metabolic profiling [93]. One recent study established a reliable method for characterizing metabolic phenotypes of microalgae that can be used to expand existing network models or guide the reconstruction of new algal metabolic models [93]. The study presented the implementation of the PM platform for metabolic phenotyping of microalgae using *C. reinhardtii* as a model organism, then used the results to expand a well-curated existing metabolic network model of *C. reinhardtii* [93]. This technology may be applied in combination with other assays for large-scale screening of unexploited microalgae or algal mutants that produce bioactive compounds.

Medicinally Important Natural Products

Several groups of bioactive compounds from microalgae are shown in **Table 1.1**, including examples of carotenoids, essential fatty acids, and polysaccharides.

Carotenoids can be chemically divided into two groups, deoxygenated carotenes and oxygenated xanthophylls. In addition, a distinction can be made between primary and secondary carotenoids. For example, β-carotene is one of the typical primary carotenoids and is a component of the photosynthetic apparatus, which makes it necessary for photosynthesis. On the other hand, astaxanthin is a representative example of secondary carotenoids, for it only accumulates to large amounts under particular environmental stimuli [94,95]. Carotenoids constitute a key group of microalgal bioactive compounds. The halo-tolerant microalga *D. salina* contains the highest amount of β-carotene (up to 10% of dry weight)

compared to other algae in a closed tubular photobioreactor setting [96]; while *Isochrysis sp.* contains the second highest amount [97].

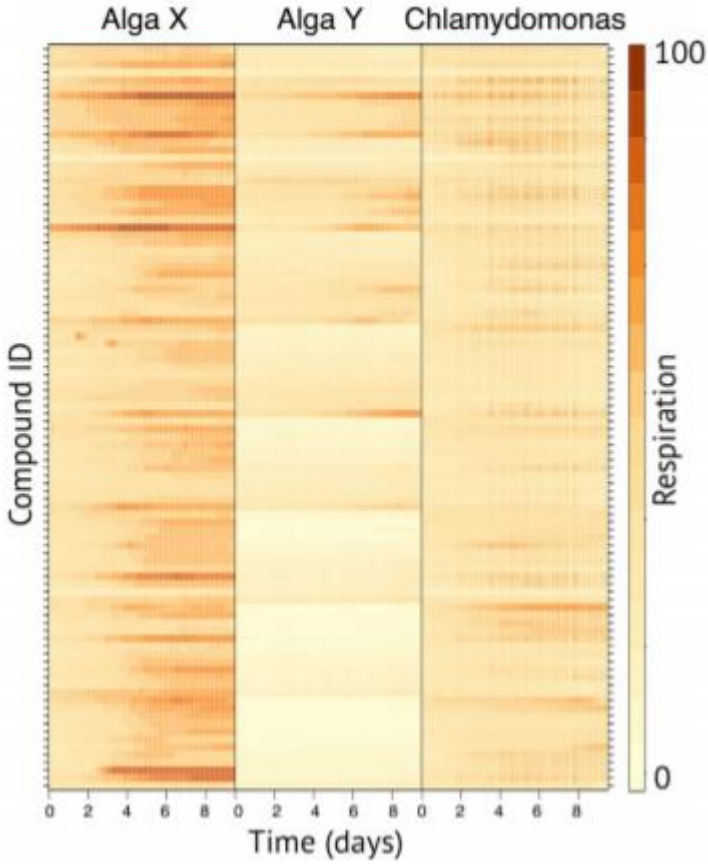

Figure 1-4 *Comparison of three different algal strains on a BioLog Phenotype Microarray plate testing for respiration on carbon substrates as an example. The three algae tested used different carbon sources for heterotrophic growth as evidenced by their ability to reduce a tetrazolium dye by metabolically produced NADH. Algal strains X and Y are new isolates undergoing further characterization, and Chlamydomonas indicates the model species C. reinhardtii strain CC-503. Compound IDs can be found at http://www.Biolog.com. Plates shown are PM1, a carbon source assay plate.*

Astaxanthin is commercially produced in the green alga *Haematococcus sp.*, particularly the species *H. pluvialis* [98]. As for fucoxanthin in microalgal species, the content in *Phaeodactylum tricornutum* is as high as 16.5 mg/g freeze-dried diatom weight, which is ten times higher than that

in brown algae and demonstrates the potential of *P. tricornutum* as a commercial source of fucoxanthin [99]. The content of fucoxanthin in the diatom *Odontella aurita* could reach 18.5 mg/g dry weight under nutrient depletion conditions [100].

In addition to carotenoids and other bioactive compounds, microalgae are also a great source of essential polyunsaturated fatty acids, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Particularly in diatoms, EPA and DHA could account for 17.5-30.2% and 0.7-6.1% of total fatty acids, respectively [101], and total lipid content could reach 57.8% of dry diatom cell weight [102]. In addition, the fatty acids oxylipins derived from the microalgae *C. debaryana* and *N. gaditana* also display anti-inflammatory activity [85].

Table 1-1 Selected examples of carotenoids and essential fatty acids from microalgal production species.

Substances	Microalgal species	Dry weight (%)	References
Carotenoids			
Lutein	*Dunaliella salina*	0.4-0.8%	[103-105]
β-Carotene	*D. salina*	10%	[96]
Fucoxanthin	*Chaetoceros* sp., *Cylindrotheca* sp., *Odontella* sp., *Phaeodactylum* sp., *Isochrysis* sp.	1.5-2.0%	[99,100]
Astaxanthin	*Haematococcus* sp., especially *H. pluvialis*	1-8%	[98]
Essential fatty acids			
Eicosapentaenoic acid (EPA)	*Phaeodactylum tricornutum, Monodus subterraneus, Porphyridium cruentum, Amphora* sp.	0.7-6.1% of total lipids	[101,102]
Docosahexaenoic acid (DHA)	*Spirulina platensis, Rhizosolenia setigera, Thalassiosira stellaris, Crypthecodinium cohnii, Isocrysis.*	17.5-30.2% of total lipids	[84,101,102]

Some diatoms are known to be producers of marine toxins, yet these toxins may also show health benefits when administered appropriately. Among these, domoic acid (DA) was the causative substance leading to the 1987 human fatalities as well as long-term health complications, which occurred after victims ingested cultivated mussels that had fed on diatoms [106]. Subsequently, diatoms in the genus *Pseudonitzschia* were identified as a source of this marine toxin, along with other producers such as the red alga *Chondria armata*. In addition, developmental defects and lower viabilities have been found in diatom grazers, such as copepods [107]. Interestingly DA was used in small doses in Japan to treat intestinal pinworm infestations [108]. Yet generally, reports on human health are rare, except for one study showing the anti-proliferative activity of organic extracts from the diatom *Skeletonema costatum* towards human non-small-cell bronchopulmonary carcinoma line (NSCLC-N6) [109]. Identified active compounds include

several polyunsaturated aldehydes (PUAs), such as 2*E*,4*Z*,7-octatrienal and 2*E*,4*Z*,7*Z*- decatrienal [110].

1.2.3 Primary Screening for Bioactive Compounds from Microalgae

Probing microalgae for new bioactive chemicals includes the process of efficient organism separation into axenic unialgal cultures, scale up techniques for biomass production, effective extraction and isolation for bio-assaying and structural analyses, and chemical determination of potential bioactive compounds [111]. This section will cover the extraction methods as well as screening assays that have been applied in microalgae for bioactive compounds.

Extraction Methods

The extraction capability has to be considered when a solvent is chosen. Usually, the higher the efficiency of the extraction process, the wider the range of compounds in the extract. The microalgae's enormous biodiversity and various bioactive compounds make the selection of appropriate extraction techniques a vital step. Conventional solid-liquid and liquid-liquid extraction methods that consume large amounts of organic solvents are labor-intensive and highly dependent on the proficiency of operators. Therefore, conventional extraction techniques are not exactly reproducible [112]. Advanced extraction techniques have been developed to overcome these drawbacks by offering automation, low organic solvent consumption, eco-friendliness, high selectivity and efficiency. These new techniques, such as supercritical fluid extraction, pressurized fluid extraction, ultrasound-assisted extraction, and microwave-assisted extraction, have had tremendous impacts on modern bioactive compound screening [112].

Supercritical fluid extraction (SFE) utilizes supercritical fluids (SCF) to enhance extraction efficiency. A supercritical fluid is any substance that is heated and pressurized above its critical points [113]. By far, carbon dioxide is the most widely applied SCF for analytical and extraction purposes. SC-CO_2 has been widely used for nonpolar bioactive chemicals recovery, and most SC-CO_2 extracted compounds in microalgae are antioxidants and lipids. *D. salina* is known for its high β-carotene content and there are various reports illustrating carotenoid recovery from *Dunaliella* biomass by means of SFE [114]. Carotenoids have been extracted from *Nannochloropsis gaditana*, *Synechococcus* sp. and *D.*

salina by utilizing ethanol as a modifier to SC-CO_2 in a microscale supercritical extraction apparatus [115].

Pressurized fluid extraction (PFE) subject solvents to enough high temperatures and pressures to keep them in a liquid state. Commercial instruments perform batch extractions using PFE with temperatures usually in the range of 100 to 200°C and a pressure that is just high enough to keep the solvent in liquid phase. PFE is a versatile approach regardless of the polarity of extracted bioactive compounds. For instance, *Synechocystis* sp. extracts were obtained by PFE to screen for bioactive compounds with anti-microbial and antioxidant activities [116]. Hexane, ethanol and water were chosen as the solvents to compare their efficiency. Gas chromatography–mass spectrometry (GC-MS) and high performance liquid chromatography involving diode array detector (HPLC-DAD) analysis demonstrated ethanol was the most suitable solvent to extract these compounds [117]. Advanced extraction methods hugely benefit bioactive compound screening; and each method has its own applicability; therefore, it is crucial to select the optimal extraction technique for a given extraction.

With regard to advanced solvent-based extraction, solvent combinations commonly used contain methanol/ethanol/isopropanol with a less polar solvent like hexane or chloroform in various ratios. A single unmixed solvent like acetone or hexane has also shown good extraction capability [118]. From reported data, the selection of extraction solvent is rather matrix-dependent, and it is recommended to optimize extraction conditions for each case or each compound. For example, using maceration [119], several extraction variables has been taken into account including time, solvent properties, temperature and solvent to diatom ratio, for the extraction of fucoxanthin from *P. tricornutum*. The optimal solvent for fucoxanthin extraction has been found to be ethanol with a temperature limit of 40 ^{o}C, while the solvent to diatom ratio is favored at 6 to 1 for a 12 h time period. The utility of ethanol for fucoxanthin recovery has also been reported in supercritical CO_2 extraction, where the addition of ethanol as a co-solvent could give rise to 90 times higher fucoxanthin yield [120]. Microwave-assisted extraction of fucoxanthin from diatom *Cylindrotheca closterium* is reported to be the best method when compared with maceration and ultrasound-assisted extraction, due to microwave's capability to break frustule [121]. In that study, no degradation of fucoxanthin was found even when the extraction temperature reached 56 ^{o}C. However, Kim et al[99] found no significant difference among

extraction methods in the recovery of fucoxanthin from freeze-dried diatom *P. tricornutum* when comparing maceration, ultrasound-assisted extraction, Soxhlet extraction and pressurized liquid extraction, but they also emphasized the importance of ethanol for high extraction yields. Our lab also uses short-duration microwave-assisted extraction with methanol coupled with vortexing to achieve relatively complete recovery of intracellular small molecule metabolites. We note that these methods work well for small molecules but would not be ideal for the extraction of protein-based macromolecules.

Screening Assays

The process of screening natural products can follow one of two approaches, either an "isolate first" or a "test first" approach. Both approaches have been successful in discovering natural bioactive products, and a fusion approach has been promoted recently where extractions or fractions of extracts are tested for bioactive chemicals, and then bioassays are used if the extracts show strong biological activity [122]. For instance, liquid chromatography–mass spectrometry (LC-MS) and/or nuclear magnetic resonance (NMR) can be applied to profile the extracts or fractions for chemical characterization, and if new compounds are discovered, they should be purified and analyzed by using biological assays. Ideally, those assays should be rapid, cost-effective, sensitive, easy to operate, reliable and repeatable. Phenotypic assays are usually the most appropriate option for primary screening of microalgae bioactive products [123,124].

Anti-oxidant assays

Extracts of microalgae including carotenoids and many other bioactive compounds are well known for their antioxidant properties [125]. A number of assays applied in food industry for evaluating antioxidant capacity of food components were also used to perform antioxidant measurements in microalgae [126]. The ferric-reducing ability of the plasma (FRAP) assay [127] was used to detect antioxidants in green algae as well as in diatoms. However, FRAP can only test antioxidant action via the single electron transfer (SET) mechanism through ferric to ferrous ion reduction but not through radical quenching. To assess antioxidant levels of microalgae that are accumulating phenols and carotenoids [126], the trolox equivalent antioxidant capacity or TEAC assay was used to measure antioxidant activity by hydrogen atom transfer (HAT) as well as SET [126].

On the other hand, the 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH-induced) oxidation of linoleic acid (or AIOLA) assay was developed to evaluate an antioxidant's capability to prevent oxidation of linoleic acid caused by alkylperoxyl radicals, generated from the azo compound AAPH [126]. Though many of these methods are quick and easy to perform, there are drawbacks for each assay as discussed previously [128]. Standardization of methods is suggested to establish a universal antioxidant capacity assay in microalgae.

Anti-microbial assays

The most common assay for detecting anti-microbial compounds in microalgae is the agar diffusion assay. This assay helps in the discovery of anti-microbial compounds that act on gram-positive bacteria, gram-negative bacteria and fungi. In a previous study, a total of 54 extracts from 20 samples of cyanobacteria were inspected for their anti-microbial ability [129]. In total, 78% exhibited anti-microbial activity and 45% exhibited anti-fungal ability. Bioassay-guided fractionation of the extracts then led to the isolation of three chemicals that showed anti-fungal, anti-microbial, cytotoxic, anti-viral and anti-inflammatory effects [129]. Samples extracted from *Synechocystis* sp. were investigated against microorganism panels including *Staphylococcus*, *Aspergillus niger*, *Escherichia coli* and *Candida albicans*. Volatile compounds and fatty acids with anti-microbial activity were identified with the aid of GC-MS and HPLC-DAD, and include phytol, palmitoleic and oleic acids [116].

Anti-viral assay

Many assays could be utilized for screening anti-viral agents, such as turbidity on a microplate or plaque formation on plates. Bioactive products extracted from microalgae have been shown to exhibit anti-viral activity against a wide range of retroviruses, including herpes viruses, togaviruses, rhabdoviruses, and human immune deficiency viruses (HIV) [130]. Cyanovirin-N is one polypeptide isolated from the terrestrial species *Nostoc ellipsosporum*, which interacts with the viral envelope and has strong anti-viral activity against drug-resistant HIV-1 [45]. In addition, steroids and glycolipids extracted from algae have bioactivity against HIV. Many researches have also utilized sulfated polysaccharides to combat viruses [130]. It has been reported that sulfated polysaccharides inhibit HIV replication at a concentration of 10 ng/ml without any side effect on host cells [130]. Polysaccharide calcium spirulan extracted from *Spirulina* sp.

exhibits wide activity against enveloped viruses such as herpes simplex virus, influenza and HIV. Nostoflan extracted from *Nostoc flagelliforme* also exhibits activity against herpes simplex viruses possibly due to inhibition of virus−cell interaction [131].

Anti-cancer assay

The activity of anti-cancer agents can be measured in a cell toxicity screen that utilizes common targeted cancer cells together with vital stains, such as the dyes MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and sulforhodamine B, as well as an enzymatic assay for the detection of lactate dehydrogenase released by dying or dead cells [132-134]. Anti-proliferative property is one of the most remarkable activities of microalgae-extracted bio-products. Compounds exhibiting cytotoxic effects on cancer cells are not only utilized as potential anticancer drugs but also can guide the design and manufacturing of synthesized analogs. Moreover, while the process for developing new drugs is facing increased difficulties with respect to needed time, effort, and financial resources, many natural bioactive compounds are being used in pre-clinical studies and clinical trials as candidate anticancer agents. For instance, dolastatin 10, which has potent cytotoxic effects on cancer cells, is a microtubule-disturbing compound extracted from cyanobacterium *Symploca sp*. The structural elucidation of dolastatin 10 lead to a synthetized analog, namely auristatin PE, that conjugates to an antibody and eliminates cancer cells [135]. Other intriguing anticancer compounds extracted from cyanobacteria include iejimalide A which is a V-ATPase inhibitor, curacin A which has anti-mitotic effects, and debromoaplysiatoxin and lyngbyatoxin which are protein kinase C activators, but none of these compounds has been approved by the Food and Drug Administration (FDA, USA) yet [136].

Immunomodulatory assay

There are multiple choices for immunomodulatory assays and one good option is treating macrophages that are pre-stimulated with lipopolysaccharide [137], and then detecting the end inflammatory products. For example, proinflammatory cytokines such as interleukin-1beta (IL-1beta) and tumor necrosis factor-alpha (TNF-alpha) [138] may be detected and analyzed to assess the immunomodulation potential of compounds, based on the objectives of the study. It is intriguing to note that polysaccharides extracted from *P. tricornutum* displayed immune

suppression effect in a rat paw assay while in a phagocytic test it exhibited proinflammatory effects [139]. Besides these extracts, many secondary metabolites isolated from microalgae exhibit various immune-modulatory effects [140]. In conjunction with specific cell types such as human monocytic leukemia cell line THP-1 [141], the enzyme-linked immunosorbent assay (ELISA) and the Western blot assay, are both quantitative and extended methods to measure cytokines, prostaglandins, and metabolite dynamics or genes expression in immunological analysis [142].

Bioassay-guided isolation and structure elucidation process

Acquiring active products should follow certain bioassay-guided isolation and structure elucidation processes [40]. Firstly, lyophilized biomass is sequentially extracted with aqueous soluble or lipid soluble solvents. Then the extracts are fractionated and evaluated in bioassays. Active fractions are subjected to HPLC that leads to the isolation of pure active compounds. Known compounds are identified by means of previous datasets or online databases, while newly discovered compounds are structurally elucidated by integrating results from mass spectrometry (MS), NMR and other spectroscopic techniques [40]. With this tremendous progress in modern experimental techniques, screening for new bioactive compounds is becoming more time- and cost- effective.

1.2.4 Biosynthesis and Biological Activities

Biosynthesis

Natural products from microalgae include compounds with tremendous structural diversity and complexity [143]. The production of therapeutic compounds from microalgae has its basis in acquired fitness-conferring ecophysiological adaptations that have evolved over the past billion or so years. These adaptations include the evolution of biosynthetic pathways to produce pigments to harvest light and protect against excess sunlight, and to generate terpenoids, isoprenyls, and a host of signaling molecules to interact competitively with neighboring species [144,145]. Other molecules include sugars and polymers that help protect against temperature and osmolyte fluctuations as well as maintain cell turgor in the face of mechanical stress in addition to lipids that provide extended energy storage [146].

The biochemical pathways responsible for biosynthesis of these compounds have evolved so that various microalgal lineages now produce myriads of diverse compounds due to time-course dependent habitation and co-habitation effects. Although secondary metabolites from plants have been more thoroughly characterized than those from algae, the diversity of algal-derived secondary metabolites is estimated to be an order of magnitude greater than that of land plants [40].

Successful approaches have been developed to increase production of PUFAs in plants by a combination of molecular and traditional breeding techniques that could be applied to microalgae [76]. Lipid biosynthetic pathways in microalgae have recently been mapped out in more detail due to the availability of new genomes, so more genetic targets are available for researchers wishing to increase PUFA output through molecular techniques [90,147]. Apart from engineering approaches, a more comprehensive conceptual framework for the genetic architecture behind lipid accumulation would shed insight into optimal nutritional modes of cultivation for increased yields of lipid products [90].

Carotenoid biosynthesis has evolved in lineage specific pathways in microalgae (Figure 1.5). However, even land plants have been shown to acquire genes that may impact pigment production via the process of horizontal gene transfer [148]. Similarly, certain fungal species were found to laterally pass genes required for carotenoid biosynthesis to aphids [149]. Thus, it is conceivable that the hyper-plastic genomes of microalgae have passed carotenoid biosynthetic genes among themselves many times during their evolution [150].

The biochemical pathways responsible for β-carotene biosynthesis have been mapped in a variety of species [68,97,151-153]. Comparative analyses reveal that the production of astaxanthin in several different green algal species is achieved through a variety of species-unique biosynthetic pathways, divergent from the intermediate β-carotene. Even more diversity is seen in β-carotene pathway regulation [68,151,154,155]; however, we should expect this diversity to apply to any compound that is involved in eco-physiological stress response. Thus, a key concept for the production of desired compounds in algae is regional specific-enhancement due to variable environmental conditions.

Biological Activities

Carotenoids are primarily a major class of fat-soluble pigments and antioxidants, and the intake of some carotenoids is associated with lowered risk of diseases through their involvement in cell signaling pathways [156]. In the case of β-carotene, due to its antioxidant activity and the nutritional value as pro-vitamin A [157], it has been widely applied into food products and cosmetics [158]. However, it should be noted that β-carotene at high concentrations could also act as a pro-oxidant under high partial pressures of oxygen [159]. Many carotenoids including lutein, β-carotene and lycopene (Figure 1.5) have shown positive impacts on skin photoprotection against sunlight and UV light [160,161]. Claims that β-carotene is a remedy against lung cancer and cardiovascular diseases are not unequivocally established and instead, it was found that β-carotene was associated with increased incidences of lung cancer for smokers, yet no direct link for the observed adverse associations was identified [162]. We note that this observation relates to one type of cancer and one type of carotenoids and should not be overly generalized. For instance, crocin, a plant derived carotenoid, has been shown to have anti-proliferative activities *in vitro* and in animal studies [163,164].

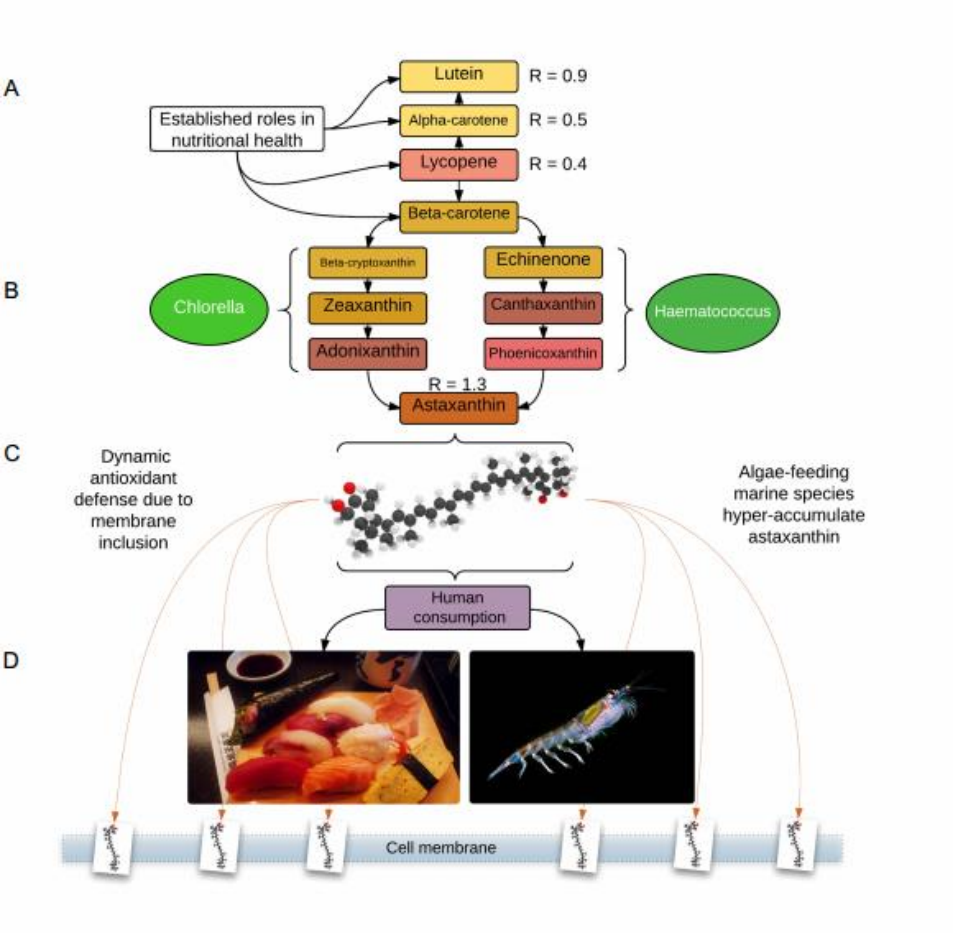

Figure 1-5 *Pathways of carotenoid metabolism from lycopene to lutein and astaxanthin. Reactivity index indicates antioxidant activity toward peroxide radicals. Relevant carotenoid biosynthesis pathways from (A) microalgae, (B) lineage-specific pathways, (C) the molecular structure of astaxanthin (red¼oxygen, black¼carbon, and white¼hydrogen); and (D) dietary sources of astaxanthin and its bioactivity.*

Astaxanthin is known for its exceptional antioxidant activity (Figure 1.5) that is much greater than tocopherols and β-carotene [53]. Astaxanthin is unique among carotenoids for being able to span the lipid bilayers and provide superior anti-oxidant defense [58,165-168]. Astaxanthin's anti-oxidant capacities have been tested via *in vitro* lipid peroxidation and radical scavenging models as well as *in vivo* vitamin E-deficient rat model [169,170]. It has also been reported that astaxanthin inhibited pro-inflammatory gene expression in lipopolysaccharide-stimulated macrophages by suppressing activation of nuclear factor kappa B (NF-κB)

which is involved in several cancer related processes, presumably as a result of scavenging intracellular reactive oxygen species by astaxanthin [171]. Multiple bioactivities of astaxanthin have been reviewed, including anticancer, cardiovascular disease prevention, antiviral and immuno-modulating activities [64,172,173]. Recent studies display the potential of astaxanthin in the treatment of nonalcoholic steatohepatitis (NASH) and also cognition deficits using mouse models [174,175]. In summary, astaxanthin was more effective at treating NASH than was vitamin E in mice and also has several-fold greater antioxidant activity than do vitamin E or β-carotene.

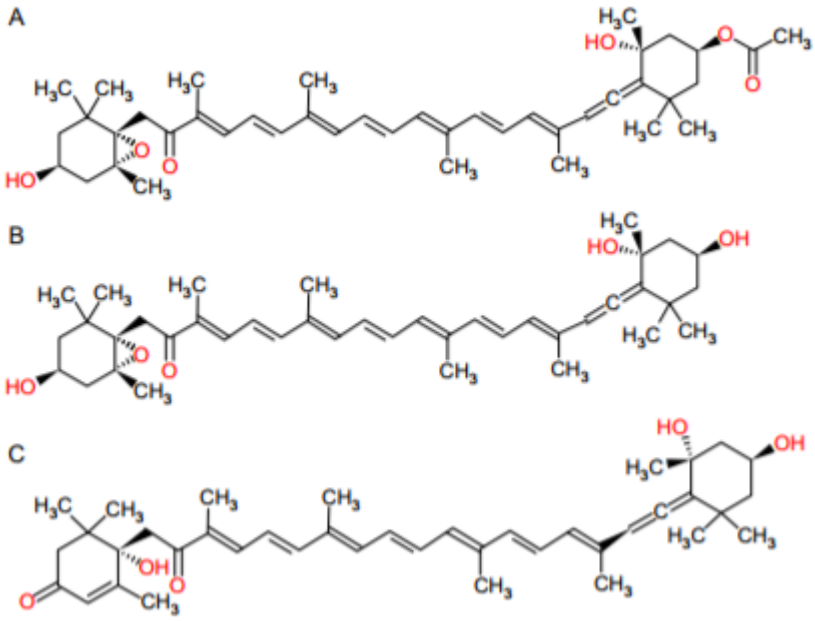

Figure 1-6 *(A) Carotenoid fucoxanthin, (B) its derivatives/metabolites fucoxanthinol, and (C) amarouciaxanthin A (chemical structures retrieved from ChemSpider with IDs 4444651, 9448555, and 10470395, respectively).*

Since the bioactivities of fucoxanthin have been reviewed in recent publicationss [100], here the discussion of its bioactivities focuses on the usage concentrations, potential targets and safety. Using *in vitro* cell model and *in vivo* mouse model revealed that the active form of fucoxanthin in blood circulation is its gastrointestinal metabolite Fucoxanthinol (FXOH).

FXOH is further transformed into amarouciaxanthin A (AXA) (Figure 1.6) in liver [176]. The accumulation of AXA preferentially takes place in adipose tissue, while FXOH, which is generally in low levels in adipose tissues, mainly accumulates in lung, liver and heart organs [177]. The anti-cancer activity of fucoxanthin has been highlighted [178] and shown effective against various cancer cell lines through the activation of a number of anti-inflammation and apoptosis signaling pathways; for example, treatment using fucoxanthin has shown similar effects as astaxanthin that resulted in the inhibition of NF-κB, which is otherwise able to induce cell proliferation after activation [179]. However, the *in vivo* bioavailability data poses a central question: will those active FXOH or AXA accumulate in target animal tissues in a sufficient amount to exert anti-cancer activity? As an example, an IC_{50} value of 2.0-4.6 μM [176] and an effective dose of 10-20 μM [180] has been used in *in vitro* prostate and breast cancer cell lines; however, the concentrations of FXOH and AXA in plasma were 47 and 82 nM after oral administration of fucoxanthin at an amount of 160 nmol (0.105 mg) for one week as shown in an *in vivo* mouse model [177]. Therefore, more *in vivo* anticancer studies are needed to find an effective dose with careful interpretation of the activities of FXOH from *in vitro* assay data. Similarly, antioxidant activity of fucoxanthin and its metabolites most likely occurs in their accumulation sites: lung, liver, heart and adipose tissues, and this occurs preferentially via radical-scavenging activity [181,182]. Fucoxanthin and its metabolites have shown anti-obesity effects, primarily by promoting energy expenditure in adipose tissue and modulating blood glucose and insulin levels [183]. Apart from the work on the pharmacokinetics of fucoxanthin, researchers have also done intensive studies on the safety of fucoxanthin and FXOH through studying their toxicity and mutagenicity. Both single doses (1000 and 2000 mg/Kg) and repeated doses (500 and 1000 mg/Kg) of fucoxanthin for 30 days have been orally administrated to mice, and no apparent adverse effects were found except hypercholesterolemia, which needs to be investigated further [184]. No mutagenic activity for fucoxanthin or FXOH has been found using both *in vivo* mouse model (oral administration at doses of 500, 1000 and 2000 mg/Kg) and *in vitro* Ames assay, respectively [185].

As for the health benefits of EPA and DHA, both compounds have been well recognized for the prevention of cardiovascular diseases, by alleviating adipose tissue inflammation and insulin resistance [186,187]. Also EPA and DHA derived lipids such as oxylipins, have played an

extremely important role in the resolution of inflammation. For instance, such PUFAs produced in microalgae can relieve Inflammatory Bowel Diseases (IBD) symptoms when consumed in diet [85,188]. Furthermore, anti-bacterial activity of fatty acids, namely EPA, hexadecatrienoic acid (HTA) and palmitoleic acid (PA), from the diatom *P. tricornutum* has also been found against multidrug-resistant *Staphylococcus aureus* (MRSA) using a disc diffusion assay [12,189]. Greater anti-bacterial activity has been discovered in cultures with higher contents (about 76%) of fusiform morphotypes [12]. With a bioactivity-guided fractionation approach, EPA in an aqueous methanol fraction was found to be the major compound exhibiting anti-bacterial activity, with an IC_{50} value of 10-20 μM and a minimum bactericidal concentration (MBC) of 40-80 μM, while the reference compound ampicillin had an IC_{50} value of 0.25-0.5 μM and a MBC of 320-640 μM [189]. However, EPA was not detected in a gelatin nanofiber-based formulation containing *P. tricornutum* trifluoroethanol extract, which also demonstrates anti-bacterial activity to MRSA as well as *Escherichia coli* [190]. Anti-bacterial activity of *P. tricornutum* ethyl acetate extract against *Listeria monocytogenes* has been reported by Krivosheeva et al. [191], but the composition of that extract has not been resolved. Shin et al. [192] found weak anti-bacterial activity of EPA against *Bacillus subtilis*, *L. monocytogenes*, *S. aureus* and *Pseudomonas aeruginosa* using an agar disc diffusion assay with minimum inhibitory concentrations (MICs) of 0.5 to 1.35 mg/mL. The potential target for EPA's and DHA's anti-microbial activity may be bacterial fatty acid synthesis; for example, bacterial enoyl-acyl carrier protein reductase (FabI) [193]. However, this hypothesis has not been proven using either EPA or DHA, rather, the former study used a broth dilution method to show that monounsaturated (e.g., oleic and palmitoleic acids) and di-unsaturated (e.g., linoleic acid) fatty acids inhibited FabI and exhibited good anti-bacterial activity towards *S. aureus* with IC_{50} values ranging from 20 to 41 μM. With regard to those potent anti-bacterial fatty acids, the proportions of both oleic and linoleic acids in diatoms are quite low: 0.1-1.1% and 0.5-2.9%, respectively, with palmitoleic acid being an exception with a proportion of 14.6-34.4% [101].

Other algal fatty acids have been shown to exhibit potential anti-cancer activity. For example, palmitic acid has been shown to be a potent selective inhibitor of topoisomerase II; which is consistent with the extensive use of topoisomerase "poisons" in anticancer therapies [194]. In the case of palmitic acid, exogenous palmitic acid was able to selectively induce

apoptosis in cancer cells but healthy cells remained relatively unharmed. As microalgae are adept at biochemical responses to ecological stress, one would expect them to produce a variety of compounds that could disrupt competitors' replication machinery [195]. Any such compounds would be strong candidates for anti-cancer therapies.

Compared to small molecules, microalgal macromolecules such as carbohydrates are underrepresented. Studies in bioactive microalgal macromolecules are quite limited to the immuno-modulating activities of polysaccharides, such as diatom chrysolaminarin (CL) [196] and other sulfated polysaccharides in non-diatomaceous microalgae [197]. The diatom chrysolaminarin (CL) is a water-soluble β-1,3-D-glucan consisting of C-2 and C-6 branches [196] with relatively small molecular weights ranging from 1 to 10 kDa [198]. CL from the diatom *Chaetoceros muelleri* has been found to stimulate the immunity of Atlantic cod through incorporating CL into fish feed as a dietary supplement. This supplement enhanced cod survival rate and reduced the amount of bacterial pathogens [198]. CL isolated from the diatom *Synedra acus* showed anti-proliferation activity against human colon cancer cell lines HCT-116 and DLD-1 with IC_{50} values of 54.5 and 47.7 μg/mL, respectively, and it had no cytotoxicity to those cells in concentrations up to 200 mg/mL [199]. In non-diatom microalgae, many sulfated polysaccharides, such as those produced by *Chlorella vulgaris* and *Tetraselmis suecica*, have also been reported to have anti-inflammation activities [197].

1.2.5 Perspectives

Although production of biofuels from algae has seen a periodic rise and fall in popularity during recent years, bioactive compounds from microalgae are likely to be associated with steadier trends as the beneficial uses of these compounds are coming to light. With greater consumer preferences towards consuming naturally derived supplements over their chemically manufactured counterparts, more demand on natural resources can be expected, which may negatively impact the environment if they are not produced in a sustainable manner. For example, because PUFAs are currently extracted mainly from fish oils, the increased demand on PUFAs is likely to make their production unsustainable as the fishing industry itself is facing an uncertain future. It is therefore necessary to emphasize the lower environmental impact associated with algal cultivation as an alternative source of compounds such as PUFAs in support of

environmentally responsible practices. As most algae-derived bioactive compounds are secondary metabolites, their cellular levels in wild type producer strains may be quite low. Therefore, direct large-scale production and extraction of bioactive compounds from unmodified cultivated microalgae is not likely to be economical. Likewise, synthesizing such products chemically is often problematic due to the compound's structural complexity and synthesis cost, often turning pharmaceutical industries away from producing such compounds [200]. On the other hand, progress in developing light-driven "cell factories" in microalgae, using mutagenesis, adaptive evolution, genetic engineering, and synthetic biology approaches makes algal cell factories become viable options that can be explored. The availability of complete genome sequences of a number of microalgal species, molecular genetic and genome editing tools, genome-scale metabolic models, and omics data analysis tools will provide the needed resources to optimize strains for increased production of the intended bioactive compounds [41,42,201,202].

1.2.6 Concluding remarks

In this chapter we have described the significance of microalgae as diverse sources of bioactive compounds. We have used astaxanthin, fucoxanthin, EPA, and DHA as examples of promising algae-based compounds with health and commercial values, and emphasized the enormous potential that microalgae hold for discovery of new bioactive molecules. With continued changes in lifestyle, personal diet, and their ensuing health-consequences, a trend in increased use of dietary supplements has been observed globally [203]. It is thus reasonable to expect a strong market pull towards developing these compounds, particularly as knowledge of their effectiveness is becoming available to the informed public. Furthermore, as described in this chapter, the significance of algae as sources of novel bioactive compounds goes beyond dietary supplements and extends to therapeutics. As such, assays for determining biological properties, such as anti-microbial, anti-viral, anti-cancer and immunomodulatory effects, together with extraction methods were presented and discussed. Further development of targeted compounds needs to be boosted through exploitation of novel algal strains in conjunction with the use of high-throughput assays and synthetic biology tools. Last, we note that it is the scientific community's responsibility to conduct and report carefully controlled studies to unambiguously establish the efficacy of these bioactive compounds, distinguishing the obtained data from anecdotal

information propagated by irresponsible commercial, or profiteering entities.

Chapter 1.3 is a full reprint of the review: Yi, Z.; Xu, S. M.; Di, X.; Brynjólfsson, S.; Fu, W., Exploring Valuable Lipids in Diatoms. 2017; Vol. 4. I am the co-first author for this review and I am contributing of the introduction and biosynthesis of fatty acids and lipids part. I also conduct the majority of the editing and reference work for this review.

1.3 Exploring Valuable Lipids in Diatoms (Paper 1)

Diatoms are one major group of algae in oceans that accounts almost half of marine primary food production and have also been identified as a promising candidate for biofuel production for their high level accumulation of lipids. They have gained increasingly attention for their potential applications in pharmaceuticals, cosmetics, nutrient supplements and biofuels. This review aims to summarize the recent advances in diatom lipid study. Chemical structures and bioactivities of different lipid classes are discussed with a focus on valuable lipids such as fatty acids, polar lipids, steroids, and oxylipins from various diatoms species. Further, current extraction and fractionation approaches are compared and recent analytical techniques and methods are also reviewed with an emphasis on lipid class composition and fatty acid profiling. Biosynthetic pathways and key catalyzing enzymes are illustrated for a better understanding of fatty acid metabolism. Past engineering attempts towards generating appropriate diatom strains for lipid production are discussed with examples using mutagenesis, environmental stimulants and genetic modification methods. Some possible future directions and applications of diatom-derived lipids are also proposed.

1.3.1 Introduction

Diatoms, representing one major group of photosynthetic algae, are unicellular eukaryotes that live within cell walls made of silica (SiO_2) [204]. They play an essential role in global carbon and silicon recycling in ocean and their photosynthetic activity accounts for almost half of marine primary production. Over 8,000 species are recorded worldwide in fresh water and oceans and it was estimated that there are 20,000 to 200,000 extant diatom species in the world [205-207].

Diatoms have attracted increasing attention for their potentials in developing a variety of bioactive compounds and fine chemicals for industrial applications [15]. For example, diatoms are rich in pigments such as carotenoids that have been widely applied in food supplements and feeds, pharmaceutical ingredients and cosmetics [161,208]. The major carbon storage compound in diatoms is lipids, among which triacylglycerides (TAGs) and fatty acids usually make up 15% to 25% of dry biomass [209]. TAGs could be easily transesterificated to biodiesels

using chemical catalysts while value-added bioactive fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have essential physiological functions in preventing myocardial infarction and high cholesterol, softening blood vessel and optimizing blood pressure for human wellness [210].

Diatom feedstock is a promising candidate for developing a variety of value-added bio-products towards a sustainable bio-economy. However, diatoms are clearly underexploited from an engineering perspective and there has been less focus on development of valuable lipid products. In this review, attempts have been made to emphasize valuable lipid products in diatoms. Chemical structures of lipids and their bioactivities as well as analytical methods and biosynthetic pathways are described for a better understanding of lipid products. Various existing and emerging techniques on generating appropriate diatom strains are also discussed. We envision it is feasible to produce valuable lipid products in diatoms with the rapid development of cell factories in the near future.

1.3.2 Lipids and Fatty Acids in Diatoms

Diatoms are a rich source of natural products (or bioactive compounds) that may be developed as candidate marine drugs. A large variety of lipids can be generated in diatoms, in addition to other types of bioactive compounds such as pigments [211], halogen-containing compounds [212], toxic domoic acid and isomers [213], attractants and deterrents [214], and long chain polyamines with biomineralizing functions [215]. Lipids are the major constituents of diatom cells and the average lipid content in diatoms could achieve to 25% of dry weight [4], although the production of lipids in diatoms can vary on culture conditions. In this section, we will give an overview on valuable lipids from diatoms, referring to the multiplicity of structural groups and biological roles.

Fatty Acids

Most fatty acids in diatoms (as shown in **Table 1.2**) vary from C14:0 to C22:6. The most common fatty acids are myristinic acid (14:0), palmitic acid (C16:0), palmitoleic acid (C16:1*n*-7), DHA and EPA [216,217]. The number of double bonds in fatty acid chains is usually only two or three, and rarely more than six. Many known microalgal species have similar fatty acid profile, but fatty acid content in each species varies and it mainly depends on the strains and culture conditions [216-218]. EPA was one of

the most characterized fatty acids in diatom lipids [218], while small portion of rare C24–C28 polyunsaturated fatty acids (PUFAs) were also found in diatoms and other microalgae [219].

EPA and DHA are the most valuable FAs found in many species of diatoms with relatively high levels [220]. Some fatty acids in diatoms show intriguing biological activities, particularly the unsaturated fatty acids. For instance, 16:3*n*-4 and 16:1*n*-7, which are usually minor components of fatty acid fractions, are highly active against Gram-positive bacteria [221]. Omega-3 fatty acids such as EPA and DHA have been found to have effects in alleviating a number of health conditions (e.g., arteriosclerosis, hypertension, inflammation, microbial, viral and tumor activity). EPA and DHA play important roles in protecting cardiovascular system [222], vision [223], and treating psychiatric disorders [224]. DHA as an essential fatty acid has effects on preventing age-associated declines in cognition, such as Alzheimer's disease, multiple sclerosis and Parkinson's disease [225]. DHA is also an active ingredient to manage behavioral disorders and enhance retinal function and visual acuity [225].

The omega-6 and omega-3 fatty acids are important fatty acids in cell membranes [226]. Although humans and other mammals can synthesize saturated fatty acids and some monounsaturated fatty acids from carbon groups in carbohydrates and proteins, they lack necessary enzymes to insert a *cis* double bond to the n-6 or the n-3 position of fatty acid [227]. Humans can synthesize EPA and DHA from α-linolenic acid (18:3*n*-3) (ALA) through a series of desaturation (addition of a double bond) and elongation reactions [226]. However, due to low conversion efficiency, it is recommended to obtain EPA and DHA from additional dietary sources. Long-chain omega-3 PUFAs in particular have anti-inflammatory properties and a variety of seafood is recommended for daily intake of omega-3 PUFAs [226,228]. For example, fatty fish is used to produce omega-3 PUFAs commercially.

Triacylglycerols (TAGs)

Diatoms are considered to be sources for sustainable production of biofuels as they accumulate a large amount of TAGs efficiently. However, the contents of TAGs in diatoms vary within the species and the genera [229]. Myristinic acid (14:0) and Palmitoleic acid (16:1) were found to be the main contents reported in diatoms even though a variety of fatty acids has

been found in TAGs in diatoms [230]. C16 fatty acids account for nearly 100 % of the total fatty acids at the *sn*-2 position of TAGs in diatoms, which include monounsaturated fatty acid palmitoleic 16:1 at a major level and unsaturated fatty acids C16:2 and C16:3 at a minor level [231]. Various stressors that induce changes in metabolic activities may enhance TAG contents in diatoms and the details will be elucidated in the later section.

Polar lipids

The polar lipids from diatoms mainly consist of monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylglycerol (PG), and other minor constituents, such as 1-deoxyceramide-1-sulfate and phosphatidylsulfocholine which existed as rare sulfoforms [232] (Figure 1.7). Glycolipids are important components that locate mostly in chloroplasts and have been demonstrated to display antiviral, antibacterial and anti-inflammatory activities [233]. Phospholipid molecules that are the universal components in cell membranes may be utilized as an ingredient in functional foods, cosmetic and pharmaceutical industries for their roles as carriers of PUFAs.

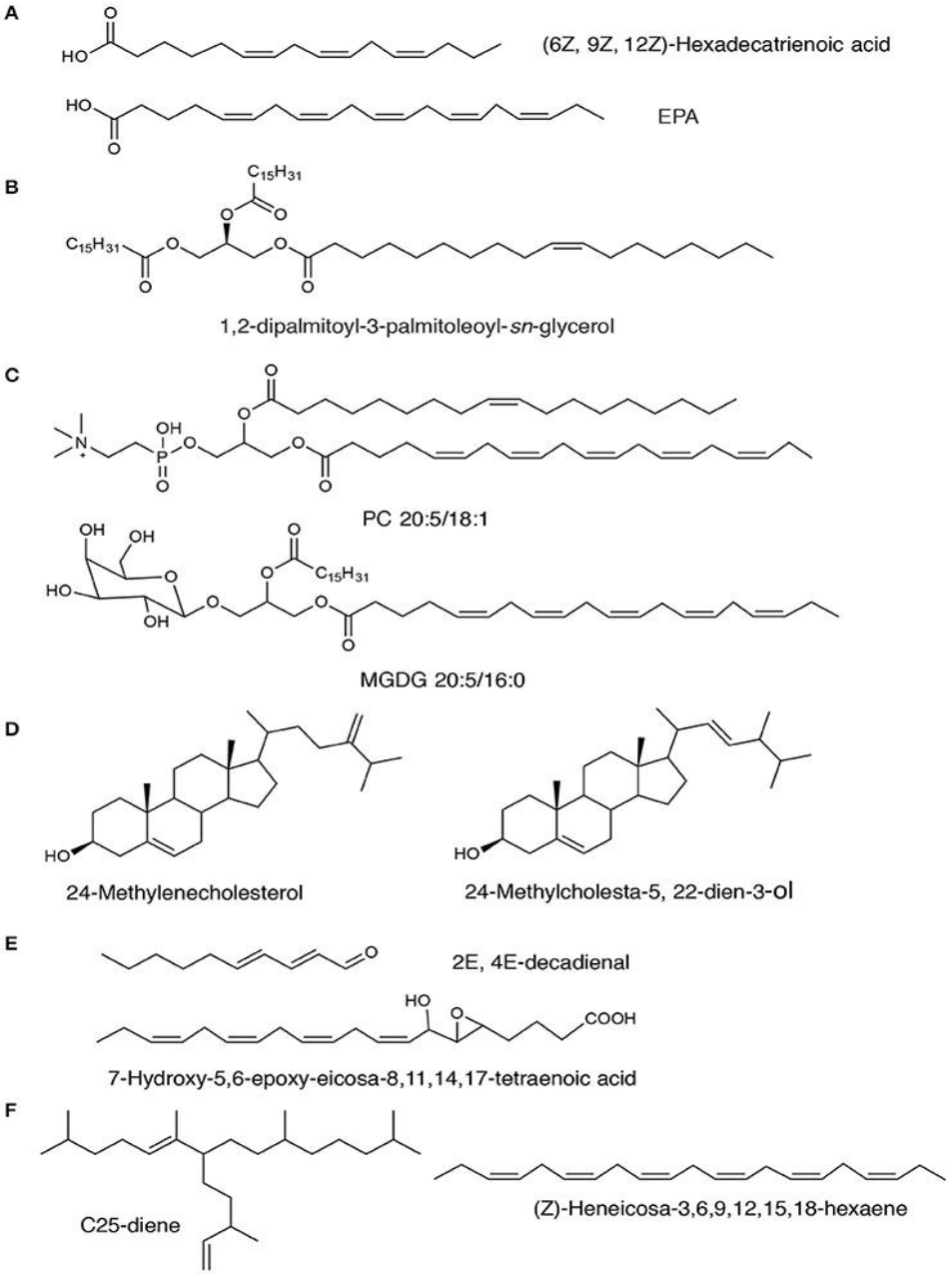

Figure 1-7 *Chemical structures of representative diatom-derived lipids. The compounds are classed into subgroups corresponding to structures. (A) Fatty acids; (B) Neutral lipids: TAG; (C) Polar lipids: PC and MGDG compounds; (D) Sterols; (E) Oxylipins; (F) Isoprenoids: C25 HBI.*

Steroids

A number of studies have shown that Δ^5-series with 24-methylencholestrol (**Figure 1.7**) was the most common sterol, while diatomsterol (24-methylcholesta-5,22*E*-dien-3β-ol) also frequently present in more than a half of the known pennate diatoms [234]. Sterols with methylation at C-23 are also produced in various species of diatoms in addition to dinoflagellates [235,236]. The majority of diatoms that have been explored mainly contain C_{28} sterols, although C_{27} or C_{29} sterols are proven to be the major sterol constituent in some phylogenetic groups. Other minor sterols such as stanols or Δ7-sterols have also been found in a small number of species. Although there is still not sufficient information about chemical profiling of sterols in diatoms, sterols and their derivatives were found to have important bioactivities such as cytotoxic, anti-inflammatory, antitrypanosomal and antimycobacterial properties [237].

Oxylipins

Oxylipins that derived from the incorporation of oxygen into the carbon chains of PUFAs usually act as chemical mediators in many ecological and physiological processes in marine and freshwater diatoms [238,239]. approximately 30% of marine diatoms produce an array of oxylipin metabolites [238,239]. Biosynthesis of oxylipins (**Figure 1.7**) is condition-dependent and immediately activated upon predation [240-242]. Particularly, oxylipin production starts by the oxidation action of iron non-heme enzymes lipoxygenases (LOXs) on the precursor PUFAs and membrane phospholipids upon loss of cell integrity. A LOX provides a specific and precise incorporation of a hydroperoxide group into the carbon chains and mediate the further transformations, resulting in generation of a group of important oxylipins [239].

Polyunsaturated aldehydes (PUAs) that were extracted from blooming diatoms are the well-studied metabolites among the diatom-oxylipin family. Studies on bioactive aldehydes have showed that these compounds may inhibit the growth of different marine invertebrates such as diatom predators and induce different responses in marine ecosystem [243,244]. This property may explain the dynamics behind some blooms and also explain why oxylipin profiles can be used as an additional taxonomic identification tool, providing a functional feature to species characterization and morphological traits. The structure-activity relationship of oxylipin has been studied and it has showed that the double

bond geometry *E*/*Z* has no effects on inhibiting the development of embryos of sea urchin egg and the biological activity is primarily due to the chain length, the α,β,γ,δ-unsaturated aldehyde element, and the side chain polarity of PUAs [245]. Moreover, the biological importance of PUAs has been increasingly noted for their antimitotic and proapoptotic properties [246], anti-inflammatory activity [247], and antimicrobial properties [248].

Additionally, a series of volatile alicyclic olefins, which are possibly phytoplanktonic pheromones, were identified in some diatom species. Three rearrangement products of diene-hydroperoxides from lipoxygenase metabolites, bacillariolides I-III, which are formed at the cyclization of epoxyalcohols, were extracted and isolated from marine diatom *Nitzschia pungens* [249,250].

All together these studies provide additional information on the chemical diversity of oxylipins, which is particularly important in diatom taxonomy and physiology. Many of these compounds are well-characterized metabolites in diatoms. However, their biological functions are still not well understood and more efforts are needed to further study on their impacts.

Isoprenoids

Many classes of hydrocarbons including alkanes and alkenes are known in diatoms [251]. All-(*Z*)-heneicosa-3,6,9,12,15,18-hexaene and n-21:6 hydrocarbon were found as major hydrocarbons in diatoms. A series of C_{25} highly branched isoprenoid (HBI) alkenes from the diatoms *Haslea ostrearia*, *Rhizosolenia setigera*, and *Pleurosigma intermedium* have been reported [252,253]. Some representative HBI alkenes structures can be found in **Figure 1.7**. These hydrocarbons share the same parent carbon skeletons but exhibit differences in their degree of unsaturation (ranging from two to six double bonds). The main characteristic of this acyclic skeleton is an alkyl side chain at C-7 of the main chain. A large number of HBI isomers were produced in different diatoms that were cultivated under a series of growth conditions and determined by a combined spectroscopic and chromatographic analysis approach using NMR spectroscopy and chiral gas chromatography, respectively [254]. Alkenes from the *Haslea* genus exhibit configurational but not geometric isomerism and the stereoisomeric centres (C-22) were identified. In contrast, HBIs isolated

from *P. intermedium* and *R. setigera* show evidence of homochirality, although they clearly exist as a mixture of geometric isomers [254]. As previously mentioned, C_{30} isoprenoids (triterpenoids) were also found in diatoms such as *R. setigera* [253,255,256]. In addition, one previous study has indicated that the extent of unsaturation in the series of isoprenoids depends on the growth temperature and an increase in growth temperature from 18 to 25 °C can enhance the degree of unsaturation of HBI and *Z* to *E* isomerization [257]. However, the increased salinity has no effects on unsaturation of HBI, but can decrease the unsaturation on C_{30} compounds [257]. Therefore, the diatom C_{25} and C_{30} isoprenoids may be used to as taxonomic markers and also an effective tool for tracking carbon flow in shrinking sea ice for understanding the dynamics of climate change and marine ecosystems well [257].

Table 1-2 *Valuable lipids in diatoms*

Lipids Class	Valuable Compositions	Representation	Bioactivities	Diatom Sources
Fatty acids[a]	HTA	16:3 (n-3)	Anti-bacterial	All the diatoms produce fatty acids but the content in each species varies.
	Palmitoleic acid	16:1 (n-7)	Anti-bacterial	
	Oleic acid	18:0	Antimicrobial	
	Remenic acid	18:2 (n-7)	Antimicrobial	
	EPA DHA	20:5 (n-3) 22:6 (n-3)	Anticancer, antimalarial, anti-bacterial, anti-inflammatory	*Skeletonema menzelii* and *P. tricornutum* have the potential for commercial production of EPA and DHA.
TAG[b]	TAG		Biofuels	TAGs in *Thalassiosira weissflogii* and *C. cryptica* may account for 82% and 88% of total glycerolipids, respectively
Polar lipids[c]	Glycolipids		Anti-inflammatory, antitumor, antibacterial, antiviral	Glycolipids were characterized by mass spectrometry in diatom species, such as *T. pseudonana* and *Stephanodiscus sp.*
	Phospholipids		Valuable ingredient in functional foods, cosmetic, pharmaceutical industries	Phospholipids were identified in diatom species such as *P. tricornutum* and *Cylindrotheca fusiformis*
Steroids[d]	24-methylenecholesterol		Anti-inflammatory, antitrypanosomal, anti-mycobacterial	The sterol 24-methylenecholesterol accounts for 90% of total sterols in *Synedra acus*.
Oxylipins[e]	PUAs		Antimitotic, anti-inflammatory, antimicrobial	Some diatoms are a rich source of PUAs, such as *Thallasiosira rotula*, but some do not produce oxylipins, such as *Skeletonema pseudocostatum*.

[a] Ref. [217,221-225,258,259] [b] Ref. [204] [c] Ref. [204,233,260,261] [d] Ref. [237,262] [e] Ref. [243,244,246-248]

1.3.3 Chemical Characterization

Extraction and Fractionation of Lipids

It has been shown that chloroform/methanol extraction could result in higher overall lipid yields than other solvents or solvent pairs by dissolving more polar substances, such as polar lipids [263]. In order to reduce the solvent toxicity from chloroform, alternative solvents or solvent pairs have also been tested, such as methyl-*tert*-butyl ether [264], hexane [265] and hexane/isopropanol [266]. Hexane extraction results in decreased yields of polar lipids due to its non-polar property. However, the use of hexane would reduce the amount of chlorophyll, which is a major contaminant for lipid extraction [265]. Different aforementioned solvents have been studied for lipid extraction, and it has been found that chloroform-methanol (1:1) is optimal for a total lipid recovery [267]. Therefore, the selection of the extraction solvent is up to the property of target lipid class as well as the safety concern.

Early lipid class composition was analyzed by thin layer chromatography (TLC), mainly by comparing the migration distance of analyte spots with standards [220]. On the advent of solid-phase extraction (SPE) cartridges, lipid classes in diatoms were fractionated using a silica-based SPE method [260]. This method is followed by many later studies [265,267,268]. Generally, the cartridge needs to be conditioned with hexane, and neutral lipids, glycolipids and phospholipids will be eluted stepwise using chloroform, acetone and methanol according to the polarity of each fraction. The use of hexane instead of chloroform for the conditioning and elution of neutral lipids has also been reported in order to avoid toxicity of chloroform [265]. It should be noted that phosphatidylcholines, the choline-containing phospholipids, might be retained in the SPE column, which could be verified by comparative profiling of each fraction with raw lipid extracts [265]. Also, each class of lipids could be checked by TLC qualitatively [269].

Fatty Acid Profiling

Gas chromatography (GC)-based fatty acid profiling is a routine and well-established method [270]. Fatty acids released from lipid saponification, are converted to their corresponding methyl esters and then subjected to GC separation. Fatty acids could be separated by flame ionization using retention times, and identification could be further confirmed by mass

spectrometry by comparing the mass to charge ratios (m/z). To reduce the time-consuming sample preparation steps, a more efficient fatty acid profiling method [271] was developed, where lipid extraction and saponification steps are removed. This approach remains to be assessed in diatom samples. The ratio of polyunsaturated fatty acids to triacylglycerol in lipid samples can also be determined using ^{1}H nuclear magnetic resonance by checking the chemical shift of diagnostic proton [265].

Lipidomics for Diatom Lipid Analysis

Lipidomics is actually the metabolomics of biological lipids. It is advanced by the rapid development of sensitive and high resolution of mass spectrometry as well as lipid identification database, which allows for high-throughput analysis and accurate lipid profiling (Yamada et al. 2013). Processing of lipidomic data can also be achieved with the development of bioinformatics [272], and data could be visualized in multiple ways, such as principle component analysis plot and heat map, which enables sample classifications and a holistic overview [273]. However, the problem is the separation efficiency and ionization of various lipid classes, which could not be fully achieved by ultra-high performance liquid chromatography. For example, isobaric molecules, which have the same m/z values and retention times, have the separation issues [274]. To this end, ion mobility mass spectrometry becomes an emerging technique to characterize lipid isomers with added separation dimension on conformational structure [274,275].

1.3.4 Biosynthesis of Fatty Acids and Neutral Lipids in Diatoms

Marine diatoms are excellent producers of highly valuable polyunsaturated fatty acids, such as EPA and DHA. Therefore, it is of particular interest to investigate their biosynthetic pathways, which could be further modified and optimized for industrial production. **Figure 1.8** shows the biosynthesis pathway of long chain fatty acids in the model diatom *P. tricornutum*. Acetyl-CoA serves as the building block for the elongation of acyl chain by two carbons per elongation step. The elongation of the acyl chain takes place from the malonyl-acyl carrier proteins (ACP) catalyzed by ketoacyl-ACP synthase (KAS). Malonyl-ACPs are formed from acetyl-CoA by carboxylation and transfer of malonyl group to ACP catalyzed by acetyl-CoA carboxylase (ACCase) and malonyl-CoA: ACP transacylase (MAT), respectively. Fatty acids, including 16:0, 18:0 and 18:1, are released from

acyl carrier proteins by thioesterases and converted to corresponding acyl-CoAs. EPA and DHA are synthesized from 18:1 after a series of desaturation and elongation reactions mainly following the major route shown in **Figure 1.8**.

Interestingly, in diatom *P. tricornutum*, the fatty acid EPA partitions into triacylglycerol, but DHA does not, even though *P. tricornutum* has a high content of DHA [276]. The biosynthesis of TAG is also affected by other catabolic metabolites, such as branched-chain amino acid degradation pathway [277]. Thus, the accumulation of TAG should be understood at a system level, rather than only limited to the fatty acid synthesis pathway.

In general, for the application of diatom lipids in food or pharmaceuticals, the incorporation of long chain PUFA into TAG is preferred, so that PUFA-containing lipids could be concentrated in oil bodies [276], while the biofuel industry would be in favor of the production of saturated short to medium length chain fatty acid-containing TAGs, which have low cloud points and resistance to oxidation [278]. So engineering strategies may be optimized in different scenarios.

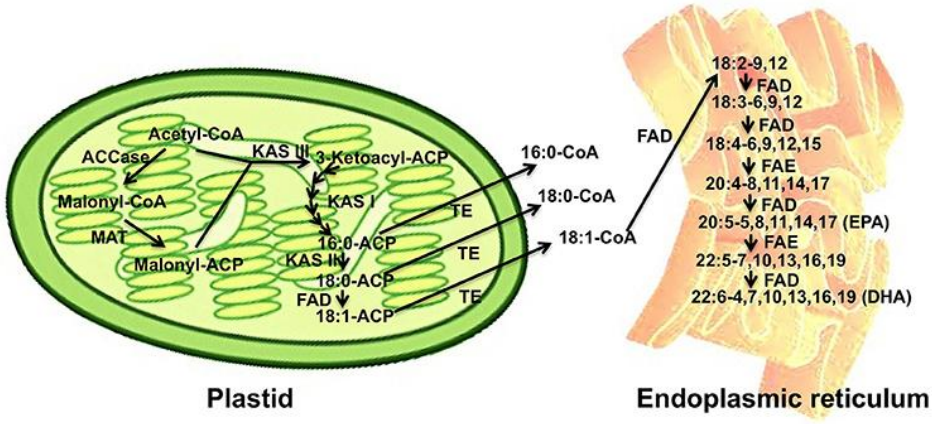

Figure 1-8 *A scheme of long chain fatty acid biosynthesis pathway in the model species P. tricornutum and compartmentation of biosynthesis processes in plastid (left) and endoplasmic reticulum (right). Major biosynthetic routes for EPA and DHA biosynthesis are illustrated as examples, and for other routes one can refer to the previous studies [279,280]. Abbreviations: ACCase, acetyl-CoA Carboxylase; MAT, malonyl-CoA acyl carrier protein transacylase; KAS I, II and III, isoform I, II and III of ketoacyl-acyl carrier protein synthase; FAD, fatty acid desaturase; TE, thioesterase; FAE, fatty acid elongase. These enzymes in each group of TEs, FADs, or FAEs in the Fig. 2 are not identical but substrate-specific.*

Mutagenesis

Mutagens were usually classified as physical mutagens and chemical mutagens [281]. Physical mutagens involve in different kinds of irradiation such as UV, γ and heavy ion beams. Due to easy implementation, UV has been known to be the most widely applied physical mutagen [282,283]. UV light has strong genotoxic effect which induces DNA damage such as cyclobutane pyrimidine dimers (CPDs) and pyrimidine pyrimidone photoproducts (64PPs) [284]. EPA production in *P. tricornutum* was increased by 33% through UV mutagenesis [285]. Chemical mutagens that were most frequently used in microalgae were alkylating agents such as methylnitronitrosoguanidine (MNNG) and ethyl methanesulfonate (EMS) [286]. To date, only EMS has been reportedly applied on diatoms to achieve higher lipid production. Novel strains created by EMS exhibited 2-2.5 fold enhancement in total lipid content in *P. tricornutum* than wild type without compromising growth and biomass production [287]. However, certain positive mutants have lower expression of phospholipids and some mutants accumulated long chain fatty acids such as nervonic acid (C24:1) and erucic (C22:1) acid. All selected positive mutants exhibited higher

activity of fatty acid synthase ketoacyl-acyl carrier protein synthase I (FABB) and ketoacyl-acyl carrier protein synthase II (FABFs) [287].

Environmental stimulants

Environmental stresses play a crucial role in diatom biomass and lipid production. Under favorable growth environment, diatoms grow rapidly with relatively low lipid contents. Environmental stresses usually reduce diatom growth rate, but result in higher lipid accumulation. Nonetheless, the mechanism has not been well understood. Nitrogen is vital to diatom's proper physiological functions, and it is a key component in metabolism of amino acids, nucleic acids, and photosynthetic pigments and its uptake is at the highest level among all nutrients [288]. Accordingly, pigments reduction has been observed in Antarctic sea ice diatoms under nitrogen deficiency [289]. Nitrogen deprivation is a common method to enhance lipid contents in diatoms. Nitrogen deprived *P. tricornutum* cells accumulated higher lipid level regardless of the stoppage of their cell divisions and slight increase of cell density. In order to achieve higher lipid productivity, diatoms were first cultured under optimal growth conditions to achieve high biomass and then transferred to nitrogen starvation condition to get high lipid content for massive production [288]. Phosphorus is another vital component in phospholipids and nucleic acids and it has an essential role in physiological processes such as respiration and photosynthesis [290]. Changes of the growth rate, cell size and pigment composition and lipid content in microalgae have been studied under phosphorus deprivation [290]. *P. tricorntum* had a twofold increase in fatty acid content but reduced in a half in biomass production under phosphorus starvation environment [290]. In most diatom species, silicon is one key component of cell wall structure and therefore a limiting micro-nutrient for diatom biomass production [291]. The acetyl-CoA carboxylase activity was enhanced by twofold and fourfold respectively after 4h and 15h growth without silicate in *Cyclotella cryptica* [291]. Light irradiation is also well known to modulate lipid content in algae and under photo-oxidative stress microalgae are capable to produce extra TAGs [292,293]. Under UV-A and UV-B radiation, lipid accumulation could be induced in a very short time (<=1h) in *Nitzschia palea* [294].

Genetic modifications

Various genetic modification approaches have been applied on diatoms [293,295]. Over-expressing *P. tricornutum* heterologous thioesterase

increased 72% of total fatty acid content [296]. Two heterologous thioesterase genes were inserted into *P. tricornutum* and ratios of C12 and C14 fatty acids to total fatty acids increased [297]. *T. pseudonana* exhibited increased lipid yields from the knockdown of a multi-functional lipase without affecting diatom growth [298]. Knocking down the genes encoding the decarboxylation enzyme phosphoenolpyruvate carboxykinase (PEPCK) and UDP-glucose pyrophosphorylase (UGPase) resulted in down-regulated decarboxylation and up-regulated lipid synthesis in *P. tricornutum* [299]. Meganucleases (MNs) and transcription activator-like effector nuleases (TALEN) were utilized to induce targeted mutagenesis (TM) of lipid metabolic genes and generated one strain with 45-fold enhancement in triacylglycerol accumulation in *P. tricornutum* [300]. Recently, Clustered regularly interspaced short palindromic repeats and their associated proteins (CRISPR/Cas9) system has been successfully applied in *P. tricornutum* and *T. pseudonana* to create stable mutants [301,302], but to our knowledge there was no reports on editing lipid metabolic genes using CRISPR/Cas9 system yet.

1.3.5 Conclusions and Perspectives

Diatoms are a promising oil feedstock and energy source as they accumulate a large amount of lipids consisting of TAGs and diverse fatty acids. Many valuable lipids and lipid derivatives such as essential fatty acids, steroids and oxylipins can be produced in diatoms in natural growth environment. Diatoms are excellent candidates for the mass production of oils or fatty acids since these microorganisms usually have high essential fatty acid content and fast growth rate among many other advantages. As a major group of algae, diatom-derived algal oils may be preferred and gain great attention in global market for their potentials in sustainable production in comparison with fish oils.

Further study may emphasize on the lipid productivity improvement in diatoms. Advanced metabolic engineering tools such as CRISPR/Cas9 system may be applied in creating strains that accumulate specific lipid species such as EPA and DHA. With the understanding of biosynthetic pathways of lipids in model and other diatom species, synthetic biology tools could be developed and applied for high-throughput strain development. In addition, research efforts should be made to achieve simultaneous production of a group of valuable and interrelated compounds as diatoms can generate a variety of valuable products.

Chapter 2 is a full reprint of the research article : Yi, Z. Q.; Xu, M. N.; Magnusdottir, M.; Zhang, Y. T.; Brynjolfsson, S.; Fu, W. Q., Photo-Oxidative Stress-Driven Mutagenesis and Adaptive Evolution on the Marine Diatom Phaeodactylum tricornutum for Enhanced Carotenoid Accumulation. Mar Drugs ***2015,*** *13 (10), 6138-6151. And I am the first author of this manuscirpt which forms the basis of this chapter.*

2 Photo-oxidative stress-driven mutagenesis and adaptive evolution on the marine diatom Phaeodactylum tricornutum for enhanced carotenoids accumulation (Paper 2)

Marine diatoms have recently gained much attention as they are expected to be a promising resource for sustainable production of bioactive compounds such as carotenoids, and biofuels as a future clean energy solution. To develop photosynthetic cell factories, it is important to improve diatoms for value-added products. In this study, we utilized UVC radiation to induce mutations in the marine diatom *Phaeodactylum tricornutum* and screened strains with enhanced accumulation of neutral lipids and carotenoids. Adaptive laboratory evolution (ALE) was also used in parallel to develop altered phenotypic and biological functions in *P. tricornutum* and it was reported for the first time that ALE was successfully applied on diatoms for the enhancement of growth performance and productivity of value-added carotenoids to date. Liquid chromatography–mass spectrometry (LC-MS) was utilized to study the composition of major pigments in the wild type *P. tricornutum*, UV mutants and ALE strains. UVC radiated strains exhibited higher accumulation of fucoxanthin as well as neutral lipids compared to their wild type counterpart. In addition to UV mutagenesis, *P. tricornutum* strains developed by ALE also yielded enhanced biomass production and fucoxanthin accumulation under combined red and blue light. In short, both UV mutagenesis and ALE appeared as an effective approach on developing desired phenotypes in the marine diatoms via electromagnetic radiation-induced oxidative stress.

2.1 Introduction

Diatoms form a major lineage of unicellular algae and play an essential role in the marine ecosystems [14]. Diatoms account for the production of a large portion of the total energy of the oceans and are also responsible for the global silicon cycle [303]. They have been receiving increasing level of attention as promising feedstocks for sustainable production of biofuels, pharmaceutical ingredients, cosmetics, and food. The marine pennate diatom *Phaeodactylum tricornutum* is a model species to study diatom physiology and diatom-based biotechnological applications as its genome has been sequenced, annotated and published [14]. In addition, this particular diatom, i.e., *P. tricornutum*, usually exists in three different morphotypes in liquid cultures: fusiform, oval, and triradiate, making it an ideal model to study cellular mechanisms involved in morphological transformation [304]. It grows rapidly and accumulates a large amount of neutral lipids, which can be converted to biodiesel, accounting for as much as 20% of its total dry weight in normal non-stress growth conditions [305].

Fucoxanthin, one type of xanthophyll pigment, is the major carotenoid in *P. tricornutum*, and works as an accessory pigment in the chloroplasts together with chlorophyll *a* and *c* forming a complex with the chlorophyll *a*/*c* binding proteins (FCPs) [306,307]. Fucoxanthin absorbs blue-green to yellow-green parts of light spectrum; the golden brown or olive-green color of diatoms is due to high amount of fucoxanthin [308]. Fucoxanthin exhibits varied bioactive properties acting as an antioxidant and chemo preventive agent against obesity, cancer, inflammation, angiogenesis and diabetes; it exerts protective effects on numerous organs such as skin, liver, brain blood vessel, bones, and eyes [308].

To develop diatom-based "cell factories", and to produce bio-based commodities including fuels and value-added products such as carotenoids, multiple approaches and strategies have been established to harness diatom's potential as a feedstock [309]. Nutrients depletions such as nitrogen and phosphate deficiency lead to accumulating neutral lipids in diatom *P. tricornutum* [305,310]. Using both meganucleases and TALE (transcription activator-like effector) nucleases to precisely and stably modify the genome of *P. tricornutum*, investigators have been able to enhance triacylglycerol accumulation by 45-fold through disrupting the UDP-glucose pyrophosphorylase [300]. In green microalgae and diatoms, accumulation of carotenoids has been closely linked to the growth

conditions such as light quality, salinity, pH, and nitrate limitation. Optimization of these growth parameters can promote high-levels of carotenoid production [311-313].

Mutagenesis and selection of mutants has been utilized as an effective approach to increase strain performance of microbes for decades [309]. Algal mutants could be obtained by physical mutagens such as UV and gamma radiation and by chemical mutagens such as EMS (ethyl methanesulfonate) and NTG (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine) for enhanced production of carotenoids or biomass [309,314]. Ultraviolet (UV) light has a potent genotoxic effect that induces DNA damage, promotes mutations and can even cause cancer in animals [315]. UV induced random mutagenesis has an advantage of not being classified as a genetically modified method since in many countries including EU genetically modified organisms (GMOs) may encounter regulatory hurdles [316]. UV light can be generally divided into three categories based on its spectrum: UVA (from 320nm to 400nm), UVB (from 290nm to 320nm) and UVC (<290nm). UV light causes specific DNA damage such as pyrimidine pyrimidone photoproducts (64PPs) and cyclobutane pyrimidine dimers (CPDs) [315]. The wavelength resulting in highest formation of 64PP and CPD is around 260 nm, which is the same as the absorption peak wavelength of DNA. Consequently, UVC promotes more DNA damage and more random mutations than UVA and UVB. Therefore, UVC would be a suitable radiation source to mutate *P. tricornutum* cells. In this study, *P. tricornutum* cells were exposed to UVC light, subsequently followed with 96-well microplate selection. Strains selected from UV radiation were studied and the major pigments and neutral lipids in cells were analyzed.

Adaptive laboratory evolution (ALE) has been established to develop new phenotypic and biological functions and improving strains performance [317]. It has been proven successful in improving strain function in adaptation to specific conditions and enhancing their tolerance to abiotic stresses in different green algal species [318,319]. However, no study has been reported on applying ALE in diatoms. We have set out for the first time to utilize ALE to improve both growth performance and carotenoid accumulation without compromising growth for high-level carotenoid productivity in *P. tricornutum* culture. Schematic process was shown in Figure 2.1.

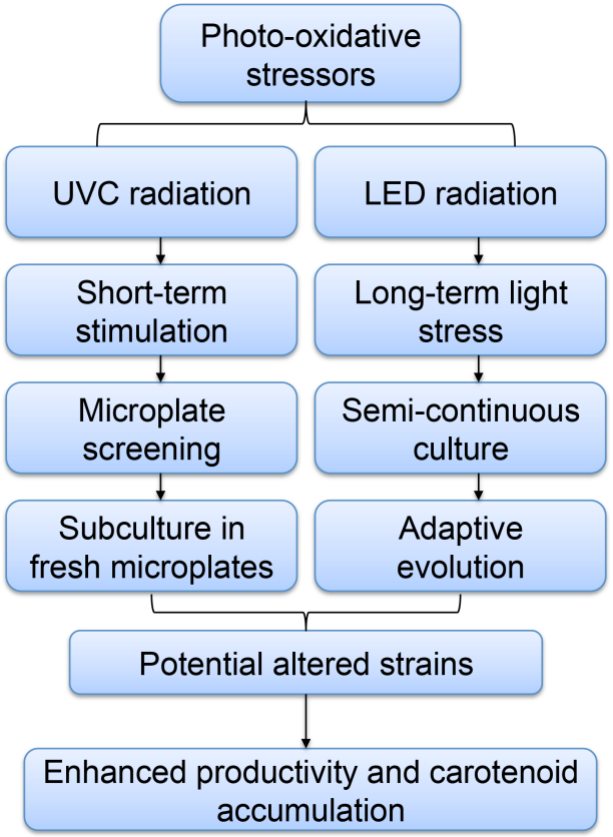

Figure 2-1 *Schematic process for improving diatoms for value-added products.*

2.2 Results and Discussion

2.2.1 Screening of *P. tricornutum* mutants

The lethality effect of UV exposure time was studied by subjecting *P. tricornutum* to UVC radiation for different time periods. The survival rate was dependent on the UV exposure time, and the prolonged exposure time led to lower survival rate (Figure 2.2a), which was consistent with previous publications [314,316,320]. It was found that 15.0 min of UV exposure time resulted in an approximately 14.4% survival rate while 36% and 8.9% survival rates were achieved upon 10.0 min and 20.0 min UV treatments, respectively (Figure 2.2a). To increase the chance of obtaining mutants, exposure time of 10.0 min and 15.0 min was chosen for further UV mutagenesis experiments. A total of 11 mini-pools of strains that showed a

higher growth rate which were seeded in twenty 96-well microplates were selected and re-cultivated in 48-well microplates. Mutants designated as UV85 to UV99 were exposed to UVC for 15.0 min while UV103 and UV105 were treated for 10.0 min (Figure 2.2b). UVC irradiation is known to induce random mutagenesis in microbes [315,316], and mutants clearly displayed varied growth rates (Figure 2.2b). In comparison with wild type, UV103 and UV105 had higher specific growth rates, UV93, UV98 and UV99 had approximately identical growth rates, and the rest of the strains exhibited lower growth rates. On average, the specific growth rate for 15.0 min UVC treated strains was 0.34 day^{-1} while the growth rate for 10.0 min UVC treated strains was 0.66 day^{-1}. The specific growth rate of wild type was 0.46 day^{-1}, implying that long UVC exposure may induce serious growth suppression on diatoms and the cells may exhibit reduced growth performance after recovering from UVC damage.

2.2.2 UVC treatment induced accumulation of neutral lipids

Nine of the eleven selected mini-pool strains were studied further and most UVC treated strains gained enhanced neutral lipid contents than the wild type (Figure 2.3). UV85 to UV99 were exposed to UVC radiation for 15.0 min, and UV103 and UV105 were exposed for 10.0 min. Strains UV86, UV93, UV94, and UV95 reached approximately two folds of the amount of the neutral lipid content in wild type. According to the gravimetric method (See details in Experimental Section), the neutral lipid content in wild type was approximately 23.3% of the total dry cell weight (DCW). Therefore, total neutral lipids content in mutants may reach up to 50% of dry weight. On average, 15.0 min UVC irradiated strains accumulated 42.6% of the dry weight while 10.0 min UVC treated strains achieved 34.8% of dry weight as neutral lipids.

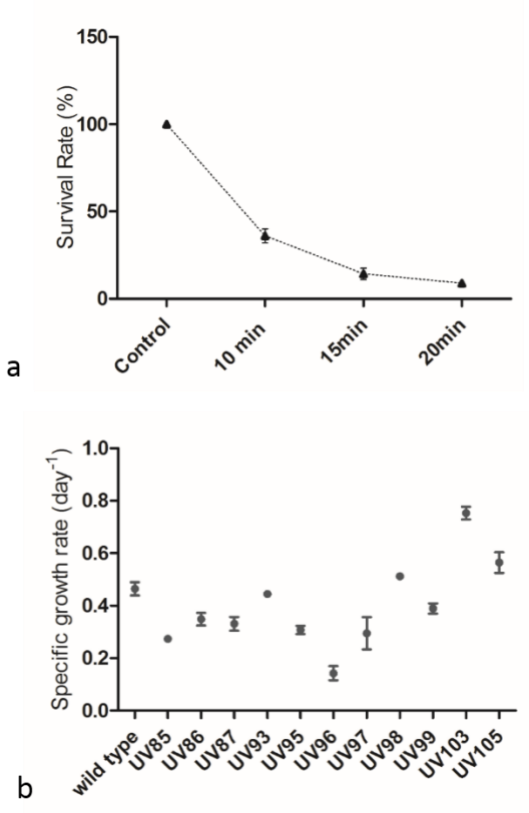

Figure 2-2 *(a) The lethality curve of P. tricornutum under different UV exposure times; (b) The growth of screened UVC treated strains. Each well of strain had been sub-cultured in biological triplicates and each triplicate had been technically measured twice. The seed culture of wild type for comparison was taken from an Erlenmeyer flask culture under logarithmic growth.*

Neutral lipids in microalgae that can be easily converted to biodiesel have gained much attention as an alternative to fossil diesel [305]. Previous research has indicated that neutral lipid content increases under several abiotic stresses such as nitrogen deprivation, phosphorus limitation, salinity stress and light stress[9,305,321]. In this study, the increased lipid accumulation in *P. tricornutum* cells as demonstrated by enhanced Nile Red fluorescence intensity may be attributed to the mutations generated by the UVC radiation. Strains in 15.0 min UV exposure group had accumulated more neutral lipids, i.e., 42.6% in average than strains in 10.0 min UV exposure group with an average content of neutral lipids of 34.8% while the average specific growth rates were reversed, i.e. higher in 10.0

min UV treated group (0.66 day^{-1}) than in 15.0 min UV treated group (0.34 day^{-1}). Combining these data with the fact that neutral lipids usually accumulate under stress conditions in algae[9], it appears that oxidative stress caused by UVC may also contribute to the increased accumulation of neutral lipids. It has been reported that applying a low dosage of UVC radiation over 24 hour increased lipid droplets amount in both *D. salina* and *H. pluvialis*[322], and this study shows that short time and high dose UVC exposure can also accumulate neutral lipids.

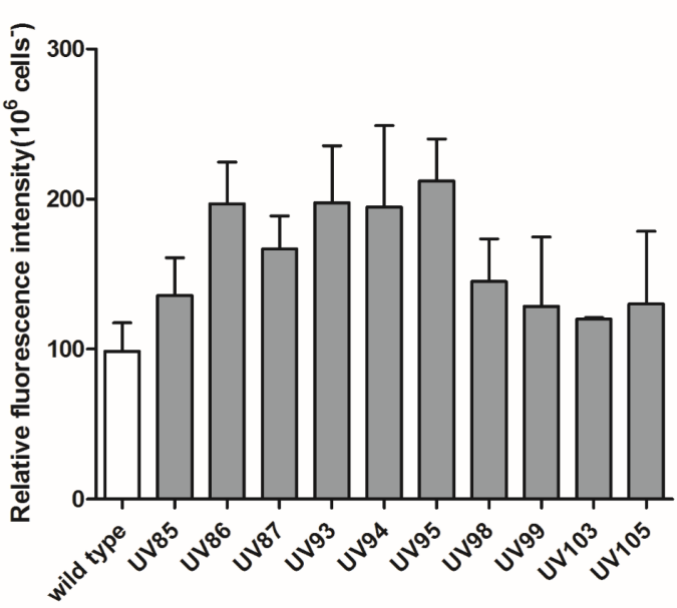

Figure 2-3 *Analysis of neutral lipids in UVC treated strains using Nile red based fluorescence. Strains were incubated with Nile Red (Details in Experimental Design and Methods) in dark room for 20min and fluorescence intensities were measured. The intensity differences between stained samples and unstained samples correlate with the content of neutral lipid.*

2.2.3 Carotenoids and chlorophyll *a* contents in wild type *P. tricornutum* and its UV-mutants

One of the major species of carotenoids in diatoms is fucoxanthin [308]. It was tentatively identified by both chromatographic and mass spectroscopy information in comparison with reference values from a previous study [323]. Figure 2.4 indicated that retention times of major fucoxanthin ions were 6.11 min and 6.29 min, comparable to the reference value at 6.36 min

as suggested previously [323]. The base peak in the mass spectra of fucoxanthin presented a mass to charge ratio (m/z) of 641.4192 identified as $[M+H-H_20]^+$, which suggested the depletion of a H_2O molecule from the protonated mother ion with a m/z of 659.4135. Further fragmentation eliminating a molecule of CH_3COOH gave rise to a product ion at m/z = 581.3992.

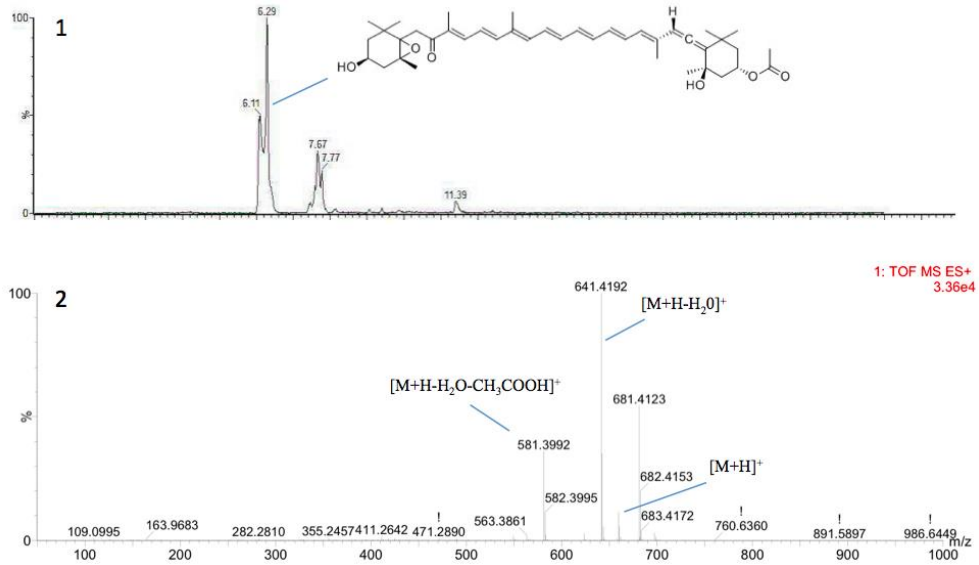

Figure 2-4 *Identification of fucoxanthin by LC-MS [1]: 1) extracted ion chromatogram at m/z 641.4; 2) mass spectra of fucoxanthin eluted out at 6.29 min.*

Production of carotenoids and chlorophyll *a* in wild type cells and mutants are shown in Figure 2.5. Beta–carotene was quantified with standards with known concentrations to ensure the quality of extraction and LC-MS analysis processes. The relative fucoxanthin and chlorophyll *a* contents in UV-treated mutants were normalized to the original levels in wild type cells. UV radiation stimulated the accumulation of fucoxanthin in most selected mutants (Figure 2.5a), and the top strain UV85 yielded an increase of 1.7 times of fucoxanthin content in comparison with wild type cells. However, β–carotene and chlorophyll *a* contents (Figure 2.5b and 2.5c) decreased in some UV-mutants. In these mutants, it appeared that the increase in accumulation of neutral lipids was positively correlated with the enhancement of fucoxanthin production in cells though the correlation was not proportional. It has been found that every selected mutant had higher fucoxanthin content than wild type. This phenomenon may be explained as

that fucoxanthin plays an essential role in coping with photo-oxidative stress [308] and could be directly induced with light stress treatment. The autogenous up-regulation of fucoxanthin might offer a better defense barrier against photo-damaging effects. It is likely that the majority of the mutants exhibiting elevated fucoxanthin level were induced by UVC oxidative stress and only a small fraction of mutants were mediated through causative mutations.

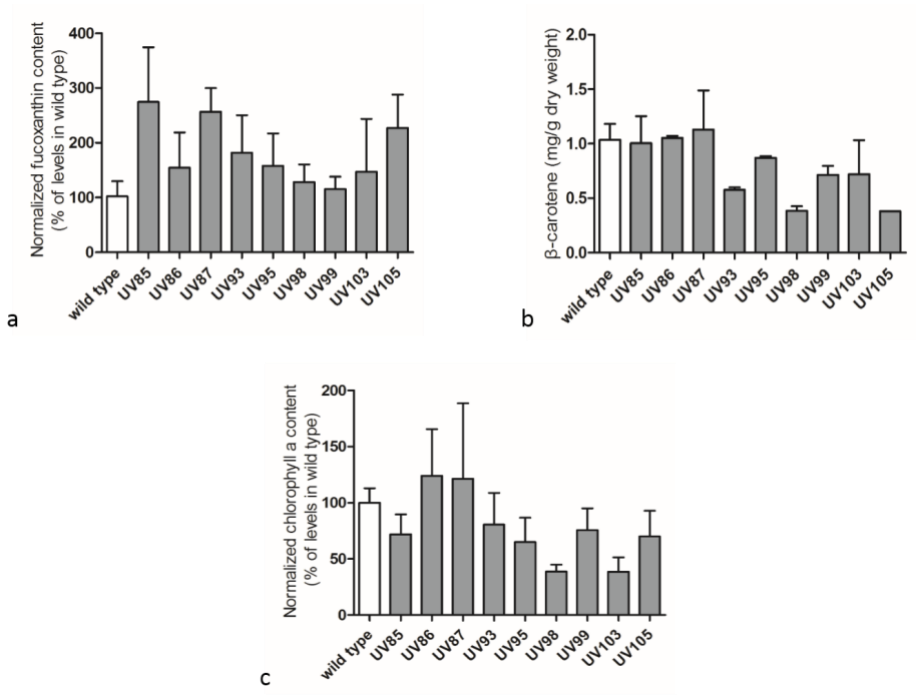

Figure 2-5 *Comparison of fucoxanthin, β-carotene and chlorophyll a contents between wild type cells and UV-mutants. (a) Fucoxanthin; (b) β-carotene; (c) chlorophyll a. Data were averaged from biological triplicates; error bars represent standard deviation.*

2.2.4 Adaptive laboratory evolution (ALE) increased growth rate and fucoxanthin content

Adaptive laboratory evolution (ALE) was performed in a semi-continuous culture mode and cell density was kept the same at the beginning of each ALE cycle by removing part of diatom culture and adding fresh medium [317]. The growth rate of *P. tricornutum* cells increased gradually over ALE cycles, from a rate of 0.14 gDCW/L/day at Cycle 1 to 0.29 gDCW/L/day at Cycle 11 (Figure 2.6).

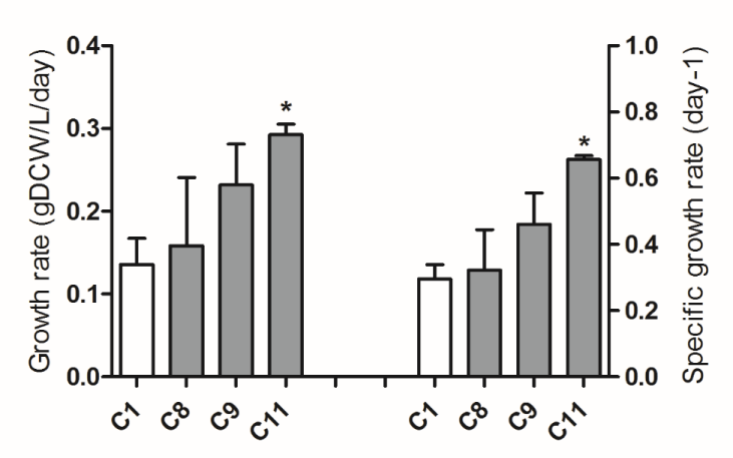

Figure 2-6 *P. tricornutum growth rate and specific growth rate over cycles during adaptive laboratory evolution (ALE). The average growth rates correspond to biomass produced per day in one cycle. The results were averaged from three biological replicates and error bars represent standard deviation. Asterisk represents statistically significant difference between C11 and C1 ($p < 0.05$).*

The neutral lipid content in cells slightly decreased from 24.6% at Cycle 1 to 19.3% at Cycle 11 (Figure 2.7). After 11 cycles of ALE, β-carotene, fucoxanthin and chlorophyll *a* contents were measured by UPLC-UV-MS (See details in Experimental Section). Both chlorophyll *a* and β-carotene did not show any significant changes during the whole ALE process while the fucoxanthin content in cells increased progressively (Figure 2.8). The fucoxanthin content at the end of the 11th ALE cycle was 2.1 times of initial level at the first cycle.

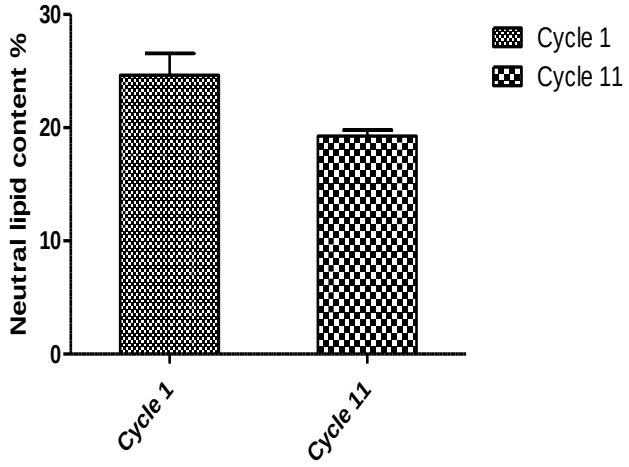

Figure 2-7 *Effects of ALE on the accumulation of neutral lipids in cells. The neutral lipid content was measured by gravimetric method discussed in the Experimental Section. Each value was averaged from three triplicate experiments. The error bar represents the standard deviation.*

Adaptive laboratory evolution has been developed and used during the last few decades to adapt microorganisms to defined conditions [317]. ALE promotes phenotype changes and the strains' adaptation to environment therefore improves strains performance via particular metabolic pathways and concentrates energy assimilated on growth through down-regulation of stress-response pathway or defense pathway [317,324]. Light emitting diodes (LEDs) that offers longer lifetimes and higher energy efficiency are becoming one of the most prevalent light sources in the world [325] and the quality LEDs that emit narrow spectra may be an effective tool to study the light effects on green algae as well as diatom [326]. It has been reported that ALE combined with LED has successfully enhanced the accumulation of carotenoids and the growth rate in green microalgae *C. vulgaris* and *Dunaliella salina* [313,318,319]. The average LEDs intensity in present study was commanded through the duration of the on/off cycle (0.1ms) [318]. Since the peak light intensity of 250 μmol $m^{-2}s^{-1}$ was much higher than the average intensity [312,318,319], this flash light device is likely to have produced additional light-induced oxidative stress to the ALE samples.

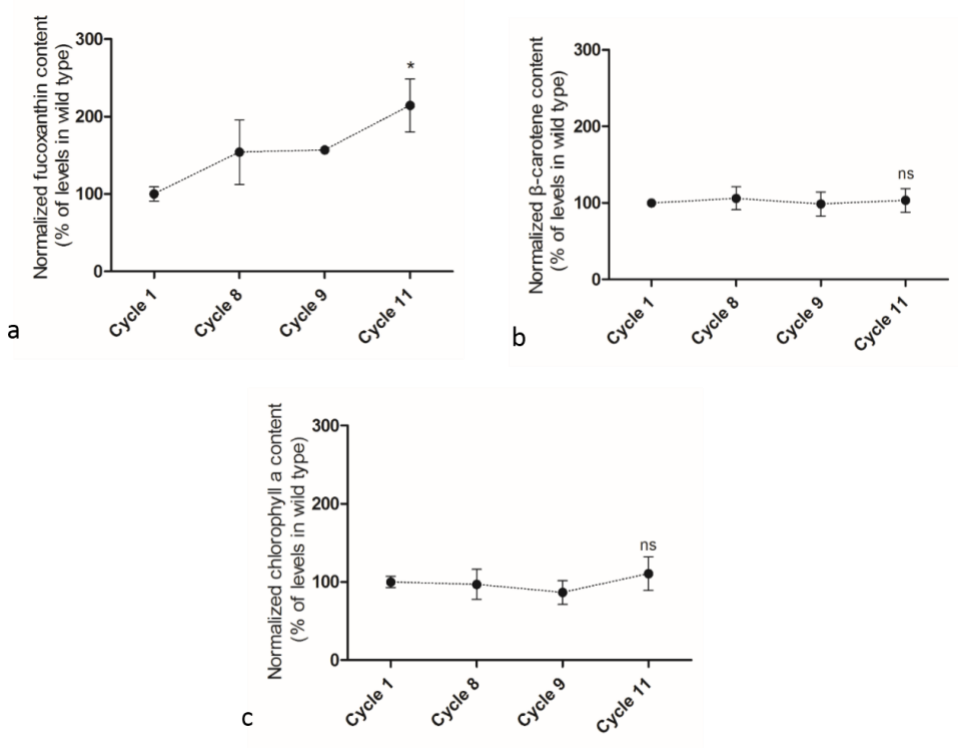

Figure 2-8 *Changes of fucoxanthin, β-carotene and chlorophyll a contents during adaptive laboratory evolution. (a) fucoxanthin; (b) β-carotene; (c) chlorophyll a. Data were averaged from three biological replicate and error bars represented standard deviation. Asterisk means significant difference ($p < 0.05$) and 'ns' indicates no significant difference between C11 and C1.*

It is the first time that ALE has been manipulated on diatom in this study. As a fundamental structural pigment, chlorophyll *a* was maintained stable during the whole ALE process, consistent with the ALE results reported for *D. salina* [318]. The final fucoxanthin content was approximately two folds higher than the initial level and the final growth rate was also approximately two fold higher. The mechanisms to synthesize and accumulate large amounts of fucoxanthin are still not clear. Elevated light stress accumulated carotenoid content in *D. salina* and *C. vulgaris* [318,319], and it may be speculated that light-induced oxidative stress also plays an important role in the fucoxanthin biosynthesis in *P. tricornutum* in this ALE process. It is possible that adapted strains with increased fucoxanthin content might have higher light utilization efficiency, consequently improving the growth rate. On the contrary, the neutral lipid

content decreased slightly, from 24.6% at Cycle 1 to 19.3% at Cycle 11. Linking the UV growth rate and neutral lipid results, it implies that neutral lipid content is not positively correlated with growth rate as well as fucoxanthin content. The strains with improved growth performance and fucoxanthin accumulation may come from the metabolic up-regulation of accessory pigments as well as the aggregative mutants created during the ALE process that may be fixed over time [317,318], though the latter is likely to be the minor contributor as no more than 20 generations were achieved in diatoms over 11 cycles. The results showed that adaptive laboratory evolution is an efficient method to promote both carotenoids accumulation and growth rate in the marine diatom *P. tricornutum*.

2.3 Experimental Design and Methods

2.3.1 Diatom culture and growth conditions

Phaeodactylum tricornutum strain (CCAP 1055/1) was purchased from the Culture Collection of Algae and Protozoa (CCAP), Scotland, U.K. The cultures were grown at 22±2 ℃ in the modified f/2 medium without silicate in which additional 10 mM nitrate and 3 mM magnesium sulfate were added. Culture pH was maintained at 8.0±0.5, unless otherwise indicated. For Erlenmeyer flask and microplate cultivation, continuous illumination was provided by the fluorescent lamps with a light intensity of 25 $\mu E/m^2/s$ as measured with a quantum sensor (SR. NO. Q40526 of QUANTUM, Model LI-1400, LI-COR biosciences, Lincoln, NE, USA) on the surface of an empty Erlenmeyer flask and microplate. To ensure the diatom cultures were maintained under exponential growth phase, the initial concentration was at an optical density of OD_{600}=0.1±0.05 and the final density was controlled under an OD_{600} of 0.9.

2.3.2 UV mutagenesis

The ultraviolet C (UVC) exposure was selected as the mutagenic agent (Figure 2.1). The germicidal tubular UV lamp was purchased from Light Tech (Stock Code: LTC30T8, USA) and the UV Output power was 13.4 Watts with a peak wavelength of 254 nm. Ten milliliters of *P. tricornutum* culture in exponential growth phase with a cell concentration of $5x10^6$ cells/ml were distributed evenly on a round petri dish. The cultures were then directly exposed to UV lamp at a distance of 20.0 cm for either 10 or 15 minutes. Cultures after UV radiation were immediately kept at dark conditions over night to prevent photo reactivation and then transferred to

96-well microplates for growth experiments. The OD_{600} for each well was measured every 24 h by SpectraMax M3 Multi-mode Microplate Reader (Molecular devices, Sunnyvale CA, USA) to evaluate the growth rate. For all wells in a 96-well microplate, OD_{600} decreased at the beginning and most wells did not show any significant growth after one week cultivation. Eleven mini-pools of strains that had relatively higher growth rates were selected from twenty 96-well microplates and successfully transferred and re-cultured in 48-well plates in triplicate for 4.0 days prior to growth measurements.

2.3.3 Growth measurements and calculations

The cell number was counted using Leica DMIRB microscopy and bright-line hemacytometer (Hausser Scientific, Horsham, USA) every 48 hr in triplicates and cell concentration (cells/ml) was used to calculate growth rate. The biomass dry weights were measured by collecting cell sample suspension (usually 10.0 ml) on a cellulose membrane (pore size, 0.45μm), washing samples with deionized water twice, and drying the collected samples at 60 °C overnight before weighing. The specific growth rate is the growth rate related to the population size, and specific growth rate $\mu = \ln(N2/N1)/\Delta t$, where N2 and N1 are the final cell number and the initial cell number, respectively and Δt represents the interval time.

2.3.4 Gravimetric method for determining total neutral lipid content

Fifty milliliters diatom cultures were centrifuged at 4000×*g* for 10.0 minutes and cell pellets were collected by removing the supernatant. Cell pellets were re-suspended with 4 ml methanol and chloroform (1:1 v:v) thoroughly and sonicated 20 min in an ice bathing. After sonication, 1.0 ml of 5.0% NaCl saline was added and mixed by vortex for one minute. The mixture was then centrifuged at 2000×*g* for 10 minutes and the lower layer was collected. The extraction process was repeated twice with additional 2ml chloroform added each time. Total chloroform layers were rotary evaporated in a pre-weighted glass tube by miVac Quattro (Genevac, Ipswich, England). Differences between the after-weighted and pre-weighted were counted as the neutral lipid mass in these cell culture.

2.3.5 Nile red staining for neutral lipid detection

Two microliters Nile Red (0.1mg/ml in acetone) was added into 200 µl diatom samples in 96-well microplates, mixed properly and Samples were incubated for 20 minutes at room temperature in dark room. SpectraMax M3 Multi-mode Microplate Reader (Molecular Devices, Sunnyvale, USA) was used to measure fluorescence intensity of each well. The excitation wavelength and emission wavelength were set as 530 nm and 580 nm, respectively. Fluorescence intensity was detected immediately after 30 seconds of vigorous shaking. The relative fluorescence difference was utilized to screen different strains according to the neutral lipid content in each stain. All samples were prepared in triplicates.

2.3.6 Adaptive laboratory evolution (ALE)

ALE was implemented with a 5-day cycle by a semi-continuous culture system [318,319]. The initial biomass density at beginning of each cycle was set at a fixed density (approximately 0.7 gDCW/L) by removing part of the culture and refilling with fresh medium. The initial and final optical density at 600 nm was measured for each cycle. Bubble cylindrical column photobioreactors (PBRs) were with 30 cm height, 4cm diameter, 300±5 mL working volume and input gas was 135 mL/min air with 1% CO_2 [318,319].

2.3.7 Artificial light setup

The artificial light supply was setup with 75% red LED light (Part number: SSL-LX5093SRC, LUMEX, Taiwan, China) and 25% blue LED light (Part number: VAOL-5LSBY2, LUMEX, Taiwan, China) with a photon flux of 30.0 µE/m^2/s based on (Al, Ga) InPsystem. The average photon flux was provided with flashing light at a duty cycle of 12% and at a frequency of 10 kHz [318,319]. The red and blue lights were centered at 660nm and 470nm respectively with 20nm bandwidth at half peak height for both output spectra.

2.3.8 Chlorophyll and carotenoid analysis

The procedures used here were described previously [323,327]. An aliquot of 0.50 ml cell culture was centrifuged at 2000×g for 10.0 minutes. The cell pellet was collected and re-suspended with 3.0 ml of ethanol and hexane mixture (2:1 v/v) containing 0.1% butylated hydroxytoluene by vortex mixing until the solution was colorless followed by the addition of 2.0 ml of de-ionized water and 4.0 ml of hexane. The samples were vigorously

mixed and the mixture was centrifuged again at 2000×*g* for 5.0 min. An aliquot of upper hexane layer was transferred to another glass tube, evaporated with miVac Quattro (Genevac, England) at room temperature and re-dissolved with methyl tertiary butyl ether (MTBE): acetonitrile (ACN) (1:1 v/v). An aliquot of 5.0 µl sample was taken and analyzed by ultra high performance liquid chromatography, coupled with UV and mass spectrometer (UPLC-UV-MS) [323,327].

UPLC separation was performed on ACQUITY UPLC (Waters, Milford, USA) using an HSS T3 1.8µm column (2.1× 150mm; Waters, UK) by reversed phase chromatography. The mobile phases constituted Phase A: ACN+methanol+MTBE (7:2:1, v/v/v) and Phase B: 10 mM ammonium acetate. Elution flow rate was kept consistent at 0.45 ml/min and with a gradient of 60% Phase A at 0 min, 75% Phase A at 4.0 min, 100% Phase A at 12.0 min, 98% Phase A at 22.0 min, 60% Phase A from 23.0 and 27.0 min. UV detection was carried out by a TUV detector (Waters, Milford, USA) at 450 nm.

2.4 Conclusions

In this study we explored photochemically induced mutagenesis and adaptive laboratory evolution on the growth and pigment accumulation in the marine diatom *P. tricornutum.* UVC induced stress-driven mutagenesis enhanced neutral lipids and fucoxanthin accumulation and created mutants with different growth rates. Adaptive laboratory evolution (ALE) promoted both growth performance and fucoxanthin levels in *P. tricornutum.* These results indicated that both methods may be promising tools in modifying diatoms for the accumulation of value-added carotenoids. UVC mutagenesis and ALE may be combined as an effective strategy in further developing *P. tricornutum* strains for industrial applications.

Chapter 3 is a full reprint from the research article: Yi, Z. Q.; Su, Y. X.; Xu, M. N.; Bergmann, A,; Ingthorsson, S.; Rolfsson O.; Salehi-Ashtiani, K.; Brynjolfsson, S.; Fu, W. Q., Chemical Mutagenesis and Fluorescence-Based High-Throughput Screening for Enhanced Accumulation of Carotenoids in a Model Marine Diatom Phaeodactylum tricornutum. Mar. Drugs 2018, 16, 272. I am the first author of this manuscirpt and I conducted all the experiment. I analysed majority of the data and wrote major part of the manuscript.

3 Chemical mutagenesis and fluorescence-based high-throughput screening for enhanced accumulation of carotenoids in a model marine diatom *Phaeodactylum tricornutum* (Paper 3)

Diatoms are a major group of unicellular algae that are rich in lipids and carotenoids. However, sustained research efforts are needed to improve the strain performance for high product yields towards commercialization. In this study, we generated a number of mutants of the model diatom Phaeodactylum tricornutum, a cosmopolitan species that has also been found in Nordic region, using the chemical mutagens ethyl methanesulfonate (EMS) and N-methyl-N′-nitro-N-nitrosoguanidine (NTG). We found that both chlorophyll a and neutral lipids had a significant correlation with carotenoid content and these correlations were better during exponential growth than in the stationary growth phase. Then, we studied P. tricornutum common metabolic pathways and analyzed correlated enzymatic reactions between fucoxanthin synthesis and pigmentation or lipid metabolism through a genome-scale metabolic model. The integration of the computational results with liquid chromatography-mass spectrometry data revealed key compounds underlying the correlative metabolic pathways. Approximately 1000 strains were screened using fluorescence-based high-throughput method and five mutants selected had 33% or higher total carotenoids than the wild type, in which four strains remained stable in the long term and the top mutant exhibited an increase of 69.3% in fucoxanthin content compared to the wild type. The platform described in this study may be applied to the

screening of other high performing diatom strains for industrial applications.

3.1 Introduction

Diatoms are a major group of unicellular algae, and they play a vital role in global ecosystems[328]. They provide nearly half of the primary food source for marine ecosystems[328], and various products from marine diatoms such as pigments, polyunsaturated fatty acids (PUFAs) and neutral lipids (biodiesels) have attracted extensive attention, as their commercialization has been exploited in recent years[283,328,329]. Phaeodactylum *tricornutum* Bohlin, a model diatom species, has been widely studied due to its ease of cultivation and fully sequenced genome[328]. Although *P. tricornutum* is not usually considered as a widely distributed species, it has been found in many European countries including Finland [330].

One of the most valuable metabolites in *P. tricornutum* is fucoxanthin, a xanthophyll pigment that is one of the most abundant carotenoids. It is localized primarily in the chloroplast and binds to chlorophyll *a*/*c* to form fucoxanthin-chlorophyll-binding proteins (FCPs), which play a major role in light-harvesting systems[331,332]. The remarkable biological traits of fucoxanthin are based on its molecular structure, which contains one allenic bond and a few oxygenic functional groups such as hydroxyl, carbonyl and epoxy moieties[308]. This pigment has numerous health-promoting effects such as antioxidant, anti-inflammation, anticancer, anti-obesity, and antidiabetic activities[308].

Biotechnological methods such as genetic modifications and mutagenesis have been developed to improve strain characteristics and the production of valuable alga-derived products[6,283,333,334]. Briefly, genetic engineering is a rational approach where selected genes are manipulated. The approach has been applied to diatoms to increase the yield of value-added products such as carotenoids and fatty acids[333]. On the other hand, mutagenesis leads to random changes in the genome, resulting in unpredictable outcomes: While in most cases, randomized mutagenesis is likely to create mutants with lower yields than the wild type (WT), rare random mutations with a positive effect can be isolated if an effective screening strategy is used[335]. Thus, diverse mutagenesis experiments have increased the yields of target products in different studies[286,336].

Since the 1990s, most studies on engineering microalgae to overproduce carotenoids were conducted by screening for mutants that could resist carotenogenic pathway inhibitors such as nicotine, norflurazon, glufosinate, diphenylamine (DPA) and compactin[37,38,337,338]. The herbicide-resistant mutants are expected to possess mutated enzymes with altered expression and enzymatic properties that enable the synthesis of desired pigments in the presence of inhibitors and certain herbicides as nicotine and DPA were known to inhibit lycopene cyclase[286]. An advantage of random mutagenesis is its simplicity, as it requires little knowledge of the biosynthetic pathways of the desired products and very few technical operations[286]. More importantly, the resulting mutants are not subject to regulations for genetically modified organisms (GMOs)[339] in the food industry in Europe and many other regions. In situations where limited molecular tools are available for microalgal genetic engineering, random mutagenesis can be an important approach for developing improved algal strains[339].

Depending on the mutagen properties, mutagenesis can be divided into physical and chemical mutagenesis. Physical mutagens contains electromagnetic radiation such as X rays, UV light and particle radiation as β and α particles[340]. Among chemical mutagens, alkylating agents such as ethyl methanesulfonate (EMS) and *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (NTG) are the most widely used for creating positive algal mutants with high lipid or carotenoid content [286]. The genomes have been randomly modified by mutagenesis, which sometimes leads to various phenotypic effects. The diploid nature of vegetative *P. tricornutum* cells could lead to carotenoid content fluctuations in mutants because the allele for a particular gene in one chromosome chain may be positively mutated while the other one might not [300,341].

Conventional methods for screening mutated strains with high carotenoid content require manual inspection of every colony, which is time consuming and inefficient[342]. After many mutated strains have been created, efficient screening of the desired phenotypes is the critical step and a major bottleneck in mutagenesis applications. In the green alga *Dunaliella salina salina* (Dunal) Teodoresco, a flow cytometry-based approach has been used to examine correlations between lipidic composition and carotenoids for establishing a high-throughput screening method [342], while little information is presently available for diatoms.

Among abundant computational approaches, genome-scale network reconstructions are a crucial part in connecting genome information to phenotypes. One promising strategy to deciphering undiscovered potential correlations between certain creatures is metabolic network reconstruction, which could be utilized to analyze system level reactions [343]. A comprehensive genome-scale metabolic model of *P. tricornutum* was recently published [343]. It is based on genomic, genetic, and biochemical knowledge and includes information on connections between genes and reactions as well as reaction stoichiometry. Genome-scale models (GEMs) enable exploration of the complex diatomic metabolism via quantitative predictions.

In this study, we are aiming at creating a high-throughput method to increase screening efficiency of selecting fucoxanthin-hyperproducing strains from mutagenesis. We first applied DPA as an inhibitor of the carotenogenic pathway and tested its effects on *P. tricornutum* growth. Then, we compared the mutagenesis effects of EMS as well as NTG on *P. tricornutum*. Under similar lethality rate, EMS showed a higher efficiency for creating positive mutants with higher carotenoid content. In addition, as we found that both chlorophyll *a* and total neutral lipid fluorescence intensity had significant correlations with carotenoid metabolism, we established a high-throughput screening method (**Figure 3.1**); 5 mutants were selected from 1,000 isolated strains based on this method. Mutants were cultivated over two months to validate strain stability, and liquid chromatography-mass spectrometry (LC-MS) was applied to detect specific lipophilic compounds. Finally, 4 of 5 selected diatom mutants exhibited higher fucoxanthin production than the WT strain.

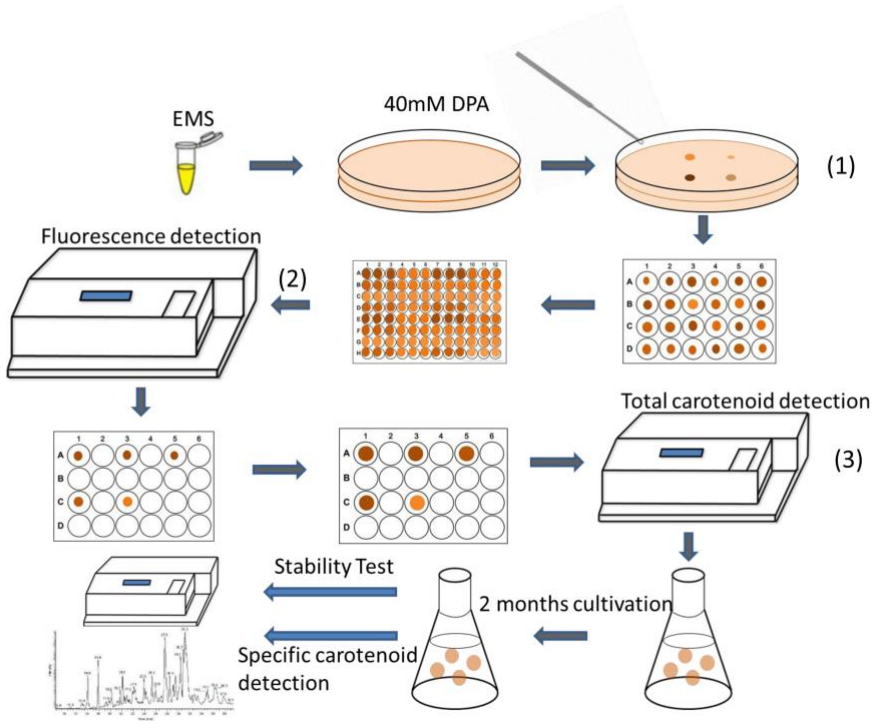

Figure 3-1 *Schematic process for high-throughput screening of targeted mutants. The detailed description was in the Experimental Section. There were three main screening steps for this method: (1) select colonies with large size and deep color for microplate cultivation; (2) pick out strains with relatively high chlorophyll a and Nile red fluorescence intensity; (3) select strains with high total carotenoid content following with pigment extraction.*

3.2 Results

3.2.1 Effect of different doses of DPA on *P. tricornutum* growth

Herbicides have been widely applied in mutagenesis experiments to create mutants with higher yields of targeted products [286]. DPA can inhibit carotenoid synthesis [286], and the purpose of applying DPA in this study was to enhance the selective pressure for isolating positive mutants, as DPA-resistant mutants will likely have higher fucoxanthin contents. It was found that 10 μM DPA treatment of the WT reduced the specific growth rate from 0.645 day^{-1} to 0.431 day^{-1} (**Figure 3.8**). When DPA was applied in a range from 30 μM to 60 μM, the diatom specific growth rate decreased significantly. Particularly, the WT still grew when the DPA concentration

was below 40 μM, but cell numbers declined once the DPA concentration exceeded 40 μM. Consequently, 40 μM DPA was chosen for the subsequent screening experiments.

3.2.2 Effects of EMS and NTG on creating positive mutants

We examined the ability of both EMS and NTG to create *P. tricornutum* mutants. As fucoxanthin was the dominant carotenoid in *P. tricornutum*, the total carotenoid amount could be utilized as a good indicator for fucoxanthin content. The total carotenoid content of mutants in the 0.1 M EMS group varied from 8.8 to 11.1 mg/g DW (**Figure 3.2a**). Two mutants in the 0.1 M EMS group had higher carotenoid content than WT (10.3 mg/g), but neither mutant's content exceeded that of the WT by more than 10%. For the 0.2 M EMS group, total carotenoid content varied from 8.0 to 11.8 mg/g DW. Four mutants had higher carotenoid content than WT, and 2 mutants among these had more than 10% total carotenoids greater than WT. For the 0.1 mM NTG group, total carotenoid content varied from 9.1 to 10.9 mg/g DW; 3 mutants had higher total carotenoids than WT, but all the differences between mutant and WT carotenoid content were less than 10% (**Figure 3.2b**). In the 0.2 mM NTG group, the carotenoid content varied from 8.8 to 11.1 mg/g DW. Three mutants had higher carotenoid content than WT, but the differences between mutant and WT content were all under 10%. The cell lethality of 0.1 M and 0.2 M EMS in diatoms was 42.3% and 71.5%, respectively, while 0.1 mM and 0.2 mM NTG caused 36.9% and 65.8% death rates. It implied that EMS had better efficiency than NTG at similar lethality rates of creating carotenoid hyper-production mutants in *P. tricornutum* at both concentrations. EMS was chosen for the following mutagenesis procedures.

3.2.3 Correlations of both chlorophyll a and lipids with carotenoid metabolism

As carotenoid fluorescence was relatively low and chlorophyll *a* fluorescence was higher and is easily detected[342], we tested the correlations between chlorophyll *a* fluorescence intensity and total carotenoid content in order to develop an effective and quick method to screen fucoxanthin-rich mutants (**Figure 3.3**). During the exponential growth phase, chlorophyll *a* exhibited a good linear correlation with total carotenoid content with 0.8687 coefficient value. The relationship between chlorophyll *a* content and carotenoids and their corresponding coefficient of determination are provided (**Supplementary Table 3.1**). During the

stationary growth phase, the correlation was not as good as in the exponential state.Nile red, as a lipophilic dye that integrates into intracellular lipids, can irradiate strong fluorescence under excitation at 530 nm[344]. Nile red fluorescence intensity correlates linearly with cellular neutral lipid content[344]; therefore, Nile red fluorescence was utilized to explore the relations between neutral lipid composition and total carotenoids. In the exponential growth phase, Nile red fluorescence intensity and total carotenoids also had a moderately linear correlation with coefficient value 0.6356. Nevertheless, the correlation between Nile red fluorescence intensity and total carotenoids was much lower in the stationary phase (**Supplementary Table 3.1**).

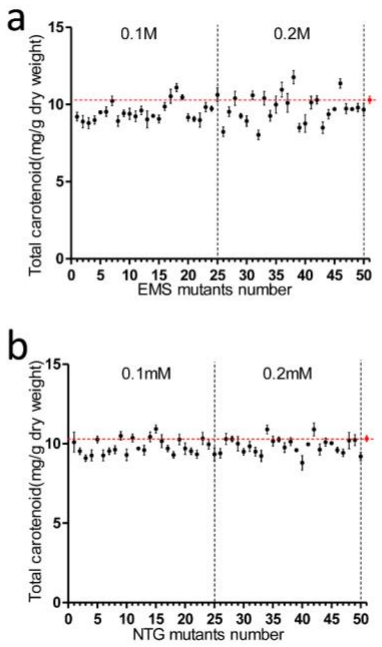

Figure 3-2 *Analysis of total carotenoid content in EMS and NTG mutants. (a) EMS mutagenesis, from Y axis to left dotted line are No. 1 - No. 25 mutants treated with 0.1 M EMS, No. 26 - No. 50 mutants were treated with 0.2 M EMS. (b) NTG mutagenesis, mutants No.1 to No.25 were treated with 0.1 mM NTG while No. 26 to No. 50 mutants were treated with 0.2 mM NTG. The strain designated as No.51 is the untreated wild type; the transverse dotted line represented wild type total carotenoid concentration. Each data point corresponds to the average value from triplicate experiments.*

3.2.4 Detection and analysis of major pigments and lipids in the diatom strains

Five positive mutants screened by the high-throughput process were selected for LC-MS analysis, and 7 pigments were quantitated. As shown in **Figure 3.4a,** EMS7, EMS13, EMS30 and EMS67 strains exhibited significantly higher fucoxanthin content than WT, while EMS3 had similar content as WT (based on one-way ANOVA analysis for fucoxanthin content in each strain). Among these 5 mutants, EMS67 had the highest fucoxanthin accumulation, 69.3% higher than that of WT, while EMS7, EMS13 and EMS30 fucoxanthin contents were 53.2%, 63.8% and 64.2% greater than that of WT, respectively. For chlorophyll *a*, 4 of 5 mutants had greater accumulation than WT; EMS7, EMS13, EMS30 and EMS67 had 33.7%, 10.2%, 79.1% and 81.9% more than WT, respectively, while EMS3 displayed similar content as WT. For beta-carotene, all 5 mutants showed higher accumulation than WT; EMS67 had 101.5% more beta-carotene than WT. EMS30 and EMS67 had 129.5% and 49.1% more neoxanthin than WT, respectively. For diadinoxanthin, EMS67 had 89.1% more accumulation than WT while EMS3 and EMS13 had 34.6% and 18.1% less accumulation than WT, respectively. For zeaxanthin, EMS3 had 17.4% more accumulation than WT while EMS7, EMS13, EMS30 and EMS67 had 14.8%, 12.5%, 23.1% and 22.7% more than WT, respectively. For chlorophyll *c*, EMS13 had 129.6% more accumulation than WT while EMS30 and EMS67 had 21.4% and 24.5% less accumulation than WT, respectively. As shown in **Figure 3.4b**, both EMS30 and EMS67 strains had higher chlorophyll *a* fluorescence intensity than WT. The Nile red fluorescence intensity of EMS30 was close to that of WT, but the Nile red fluorescence signal of EMS67 was much stronger than that of EMS30.

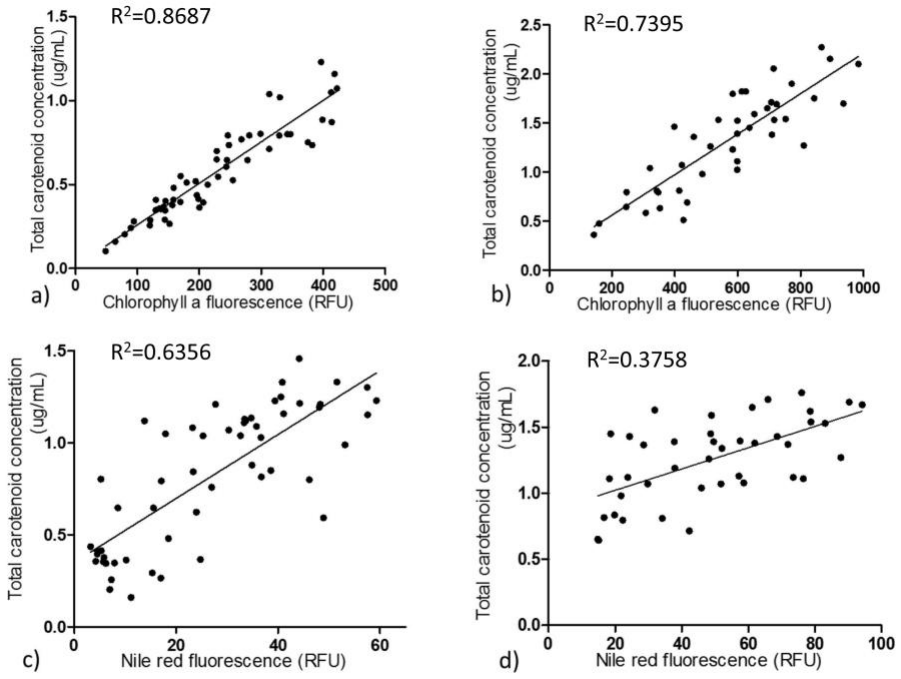

Figure 3-3 *Correlation of chlorophyll a fluorescence and Nile red fluorescence with total carotenoid content. Correlation between chlorophyll a fluorescence intensity and total carotenoid content in P. tricornutum in exponential (a) and stationary (b) growth phases, respectively. Correlation between Nile red fluorescence intensity and total carotenoid content in exponential (c) and stationary growth (d) phases, respectively. Each dot represents the averaged value of each strain from biological triplicates. Chlorophyll a and Nile red fluorescence were measured in 96 well plates by a fluorescence spectrophotometer. All four of these correlations are significant ($p<0.01$).*

With regard to neutral lipid content, all the strains had higher neutral lipid content than WT (**Supplementary Figure 3.4**). For EMS7, EMS13, EMS30 and EMS67, the lipid content was 59.4%, 41.3%, 44.8% and 62.7% greater than that of WT, respectively. PCA was used to summarize the metabolite profiling data and cluster samples, including WT, EMS30 and EMS67 (**Figure 3.5a**). Higher intragroup variations were found in treated groups, while less variation was found in the WT group. The first component explained 25% of the chemical variation, mainly that between the WT and EMS67 groups, and the second component explained 18% of the variation, mainly that between the EMS30 and the WT groups. In OPLS-DA plots (**Figure 3.5b** and **Figure 3.5c**), the horizontal axis

indicated intergroup variation. OPLS-DA was performed well based on its goodness-of-fit parameter ($R^2 > 0.9$) and predictive ability parameter ($Q^2 > 0.9$). The vertical axis indicated the intragroup variation, and in both OPLS-DA plots, the WT group showed less variation than the treated groups. Markers identified as contributing to intergroup differentiation are labeled in the S-plots and reported in Supplementary Table 3.2 and most of the lipophilic markers identified were fatty acids. Both the PCA and OPLS-DA plots result confirmed phenotypic differences between WT and mutants.

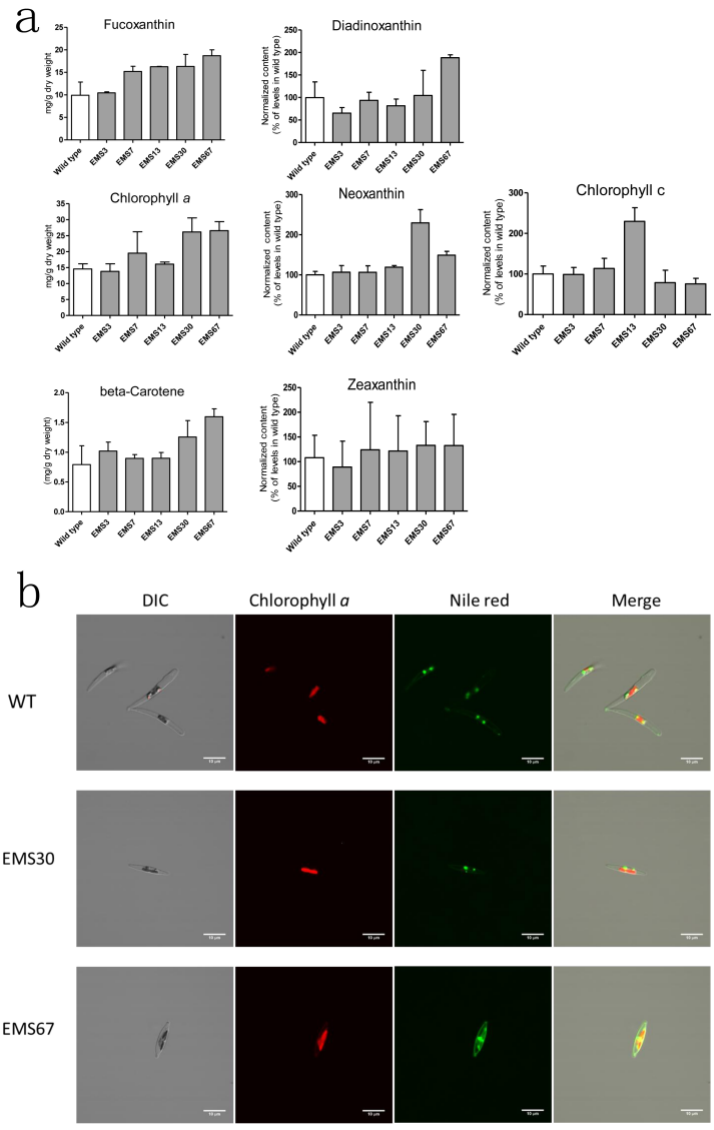

Figure 3-4 *Analysis of pigments and lipids in wild type and selected positive mutants. (a) Both wild type and mutants were analyzed during the exponential growth phase. Pigments were extracted and determined using ultrahigh performance liquid chromatography-mass spectrometry (UPLC-MS), Reported values are the averages of three biological replicates. (b) Chlorophyll a and Nile red fluorescence observed through a confocal microscope. Intergroup comparison was conducted by one-way ANOVA.*

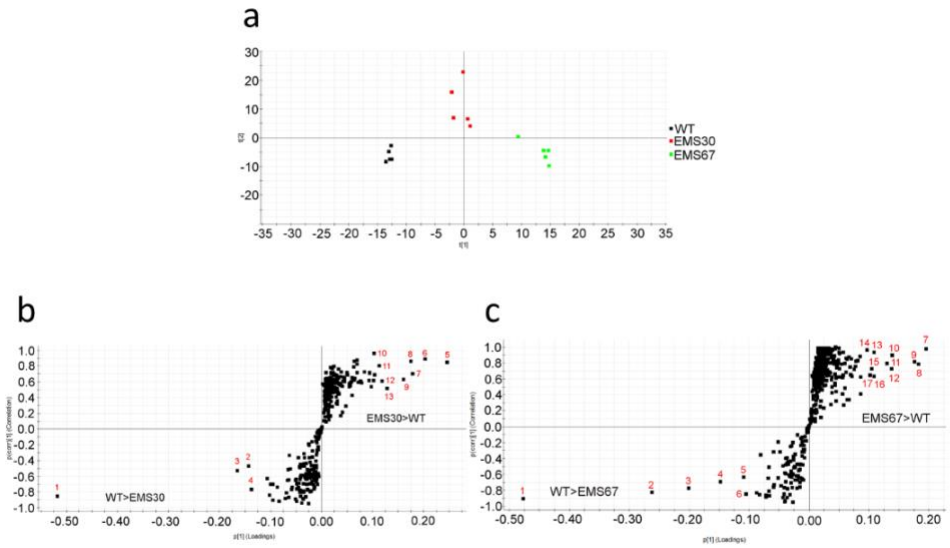

Figure 3-5 *Phenotypic differentiation of WT and positive mutants. (a) Principal component analysis (PCA) was used for sample grouping based on their metabolite profiles. (b) OPLS-DA S-plot showing the differences in production between WT and EMS30 groups: dots in the left lower quadrant are compounds contributing to the differentiation of WT from EMS30 with a potentially higher production in WT; dots in the right upper quadrant are compounds contributing to the differentiation of EMS30 from WT with a potentially higher production in EMS30. (c) OPLS-DA S-plot showing the differences in production between WT and EMS67 groups: dots in the left lower quadrant are compounds contributing to the differentiation of WT from EMS67 with a potentially higher production in WT, and dots in the right upper quadrant are compounds contributing to the differentiation of EMS67 from WT with a potentially higher production in EMS67.*

3.2.5 Assessment of selected mutant stability for carotenoid accumulation

After our three-step selection (**Figure 3.1**), 5 positive mutants were chosen for stability analysis. The accumulation of total carotenoids in the selected strains before and after two months of repeated batch cultivation was also quantified. As shown in Figure 6a, before continuous cultivation, all five selected mutants had higher carotenoid content than the WT. EMS7 had the

lowest content among the 5 mutants but had 22.5% more carotenoids than WT, while EMS30 and EMS67 had 47.4% and 46.7% more than WT, respectively. After two months of repeated batch cultivation (with approximately 16 generations had passed) 4 strains had almost identical carotenoid content as they did previously, with changes less than 10% (**Figure 3.6b**). Nevertheless, total carotenoid content in EMS3 dropped from 13.3 mg/g DW to 10.2 mg/g DW, nearly to the same level as WT.

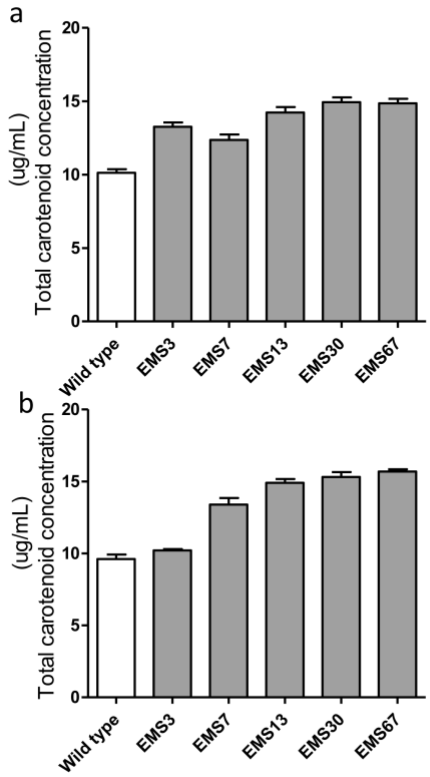

Figure 3-6 *Stability evaluation of carotenoid accumulation in selected mutants. All strains were in the exponential growth phase, and the total carotenoids of mutants were measured both at the beginning of two months of Erlenmeyer flask cultivation (a) and at the end of two months of repeated batch culture in Erlenmeyer flasks (b). Each value was averaged from biological triplicates.*

3.3 Discussion

To date, there are still gaps between diatom research development and its fully commercial applications [6,345]. It is essential to enhance the production of valuable compounds in diatoms towards commercialization. In this study, we utilized both EMS and NTG chemical mutagens to mutate *P. tricornutum* and designed an efficient screening process to select for desired phenotypes.

It has been reported that fatty acids and particular lipid compositions are closely linked with carotenoid accumulation in *Dunaliella salina* and *Haematococcus sp.* [342,346-348]. In addition, the correlation between Nile red fluorescence intensity and total carotenoid content was established in *D. salina* [342] with a coefficient of 0.74 in the exponential growth phase. The mechanism causing the correlation between lipid metabolism and carotenoid synthesis is yet to be explored, although studies have demonstrated that inhibition of carotenoid synthesis did not interfere with lipid metabolism [346-348]. Biochemical research shows that pyruvate is a precursor of both lipids and carotenoids and that pyruvate is converted to acetyl-CoA via the pyruvate dehydrogenase complex (PDC) in lipid metabolism or converted to 1-deoxy-d-xylulose 5-phosphate (DXP) via DXP synthase (DXS) [349]. In addition, carotenoids are lipophilic and synthesized in oil-rich chloroplasts; lipid globules also participate in carotenogenesis-related steps as transportation or modifications [346]. Although phytoene synthase and phytoene desaturase protein abundance and mRNA expression remained constant while beta-carotene was over-expressed under active lipid biosynthesis in *Dunaliella*, their enzymatic activities were significantly increased because the enzymatic activities were not necessarily related to protein and mRNA amount [346].

The intrinsic membrane antenna proteins in diatoms are fucoxanthin-chlorophyll-binding proteins (FCPs), which are located on thylakoids and serve both photosystems I and II [331]. The FCPs share homology with light-harvesting complexes (LHCs), but there are still large differences between them in terms of pigment composition and pigment ratio. The molar ratio of chlorophyll a to carotenoid in FCPs of diatoms is ~ 1, but the ratio of chlorophyll a to carotenoid is close to 2 in LHCs [332,350]. Diatom thylakoids were enlarged, and the expression of chlorophyll a was transcriptionally increased to fully utilize irradiated photons under low light conditions in exponential growth [331]. In this study, the molar ratio

of chlorophyll a to carotenoids was relatively stable (varied from approximately 1.27 to 1.42) in the exponential growth phase in various mutants despite the large differences between carotenoid contents. This result implies that fucoxanthin metabolism may synergize with chlorophyll *a* accumulation to achieve appropriate ratios for optimal photosynthetic efficiency at utilizing luminous energy, while mutants that could not reach this ratio and failed to grow as quickly in colonies would not be selected for the following screening. As pigmentation and pigment composition are extremely sensitive to environmental conditions such as light intensity and quantity, pH, temperature, and nutrient availability [351], it is essential that the same growth conditions and collection times are strictly maintained for all strains and particularly for chlorophylls, as they are the most labile compounds. Chlorophyll a fluorescence could change dramatically as a result of external stimulants or internal growth phases [331], indicating that extreme attention should be paid to maintaining samples properly and measuring the fluorescence at identical designated times. Nevertheless, previous UV mutagenesis results [283] disclosed that most selected positive UV mutants had similar or lower beta-carotene and chlorophyll a content than WT, which was different that the situation found in the EMS positive mutants. Both beta-carotene and chlorophyll a had higher expression levels in EMS mutants than in WT (Figure 4a). The differences in UV and EMS mutants could be explained by their different mutagenesis mechanisms: UV promotes dipyrimidine sites forming cyclobutene pyrimidine dimers and pyrimidine-pyrimidone products that induce DNA damage [284], while EMS alkylates guanine, which induces mispairing alkylated G with T, causing G/C to A/T transitions [352].

The comprehensive genome-scale network reconstruction was structured on biochemical and genetic information from literature and has provided a scheme to study and evaluate the unexplored metabolic capabilities in diatoms [343]. To explore possible key enzymatic reactions that were involved in the correlations, we simulated the metabolism of P. tricor*nutum* with the published iLB1025 model [343]. Randomized flux distributions within the model were estimated and then identified the enzymatic reactions linearly correlated with fucoxanthin production were then identified. We explored reactions across six compartments: cytosol, mitochondrion, extracellular space, chloroplast, peroxisome and thylakoid. Reactions in chlorophyll *a* and lipid metabolism correlated with flux in fucoxanthin production (**Supplementary Table 3.3**). In porphyrin and chlorophyll metabolism, 13 reactions of a set of 25 reactions that we

analyzed had linear correlations with fucoxanthin synthesis; 10 of these 13 were positively correlated, while the other 3 had negative correlations. Based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway maps, these 13 reactions belong to the chlorophyll *a* biosynthetic pathway. Among 439 total analyzed lipid metabolic reactions, 12 reactions that mostly belong to fatty acid elongation reactions were linearly correlated with fucoxanthin production. Partially correlated lipid and chlorophyll synthetic reactions were exhibited, and the highly correlated reactions are labeled in red (**Supplementary Figure 3.3**). We also examined the correlated reactions in nucleotide metabolism, starch and sucrose metabolism, fructose and mannose metabolism and biosynthesis of steroids. The highly correlated reactions are also summarized (**Supplementary Table 3.3**). In addition to the overlapping precursors, certain interactions between the translational or transcriptional regulation of these metabolites could also play an important role in the correlations. It would be intriguing to study the impacts of genetic or metabolic manipulations of these predicted highly correlated reactions on the production of fucoxanthin.

The metabolic pathways for synthesizing chlorophyll *a* and fucoxanthin share a few precursors, from glyceraldehyde-3-phosphate (GA3P) and pyruvate to geranylgeranyl pyrophosphate (GGPP). In fucoxanthin metabolism, GGPP is first converted to prephytoene-PP and then to phytoene under catalysis by phytoene synthase. For chlorophyll *a* metabolism, GGPP is converted to phytyl-PP by catalysis via geranylgeranyl reductase (GGDR), and then phytyl-PP is combined with chlorophyllide to synthesize chlorophyll *a* under chlorophyll synthase (CHLG) catalysis [353]. Lipid and fucoxanthin metabolism share the early precursors GA3P and pyruvate; DOXP synthase catalyzes the conversion of pyruvate and GA3P into DOXP in fucoxanthin metabolism, while pyruvate dehydrogenase converts pyruvate into acetyl-CoA in lipid metabolism [354].

As chlorophyll *a* and neutral lipid content could be determined spectrophotometrically in a high-throughput fashion, these findings enable the high-throughput screening of fucoxanthin-hyperproducing strains in diatoms by the development of fluorescence-based approaches for estimating fucoxanthin content. A comparison of **Figure 3.4a** and **Figure 3.6b** shows that the LC-MS data of fucoxanthin were consistent with the total carotenoids extracted with methanol. Four of 5 selected positive

mutants showed stability in total carotenoid accumulation over 2 months repeated batch cultivation. The fading of fucoxanthin in one of the mutants may be because *P. tricornutum* is diploid [300,341] and the alleles for a particular gene are not mutated simultaneously. Therefore, it is suggested that the production stability of all selected mutants of *P. tricornutum* should be checked over long-term repeated cultivation.

The fluorescence-based high-throughput screening method developed here demonstrated efficiency advantages over conventional screening methods. In this study, 5 mutants were selected from approximately 1,000 seeded mutated strains by fluorescence-based screening. This method combined fluorescence detection and agar plate and microplate cultivation, which enables the possibilities of large-scale mutagenesis screening, a key factor in creating prominent mutants. Different from one-by-one traditional spectrophotometer methods, the fluorescence detection that was established and based on a correlation between chlorophyll *a* fluorescence and total carotenoid content enabled an indirect and nondestructive approach to estimating fucoxanthin content in diatom cells. In general, this method could significantly increase the screening efficiency to obtain fucoxanthin-hyperproducing strains of diatoms. Furthermore, this screening method may be applied in other algal species that have a broad prospect in creating strains hyperproducing carotenoids. This high-throughput screening method may be attempted on any species whose carotenoid content has a satisfying correlation with chlorophyll a and/or neutral lipid content.

3.4 Material and Methods

3.4.1 Cells and chemicals

The *Phaeodactylum tricornutum* (CCAP 1055/1) strain was from the Culture Collection of Algae and Protozoa (CCAP), Scotland, the U.K. All the chemicals were purchased from Sigma-Aldrich unless otherwise specified. Bidistilled water was generated using a Milli-Q System (Millipore, Bedford, MA, USA).

3.4.2 Diatom culture and growth

Diatoms were cultivated at 22.0 ± 2 °C in modified f/2 medium in which the pH was maintained at 8.0 ± 0.5. Cultures with a volume of 50 ml were grown in 250 ml Erlenmeyer flasks under continuous radiation with a light

intensity of 30 μE/m^2/s by daylight lamp (Osram, TEKNE, BL1, 73061-48, Munich, Germany) unless otherwise indicated. The light intensity was measured by a quantum sensor (Model LI-1400, LI-COR biosciences, Lincoln, NE, USA) to ensure persistent and steady illumination. The optical density at 625 nm (OD_{625}) was used to determine the dry weight (DW) of the biomass[355,356]. The correlation of the biomass DW and OD_{625} was demonstrated (**Figure 3.7**).

3.4.3 EMS and NTG mutagenesis

For EMS mutagenesis, the *P. tricornutum* strain at a cell density of 1x10^6 cells/ml was treated with either 0.1 M or 0.2 M EMS; for NTG mutagenesis, *P. tricornutum* was exposed to either 0.1 mM or 0.2 mM NTG. Both treatments were sustained for 1 h in dark at room temperature. After each treatment, *P. tricornutum* cells were washed thrice with 5% sodium thiosulfate to remove remaining mutagens, followed by being washed twice with fresh f/2 medium. The cells were kept in a dark room overnight to prevent light-reactivation and then seeded in f/2 agar plates under fluorescent lamp irradiation. After approximately 15 days of cultivation, single colonies with deep color and large sizes were selected for further cultivation.

3.4.4 Herbicide test

The herbicide DPA was dissolved in f/2 medium at different concentrations: 10 μM, 20 μM, 30 μM, 40 μM, 50 μM and 60 μM. *P. tricornutum* were seeded in 48 well plates at an initial density of 1x10^6 cells/ml, and cells were illuminated with 30 μE/m^2/s from a daylight lamp. After four days of cultivation, cells were collected, and cell numbers were counted with hemocytometer.

3.4.5 Chlorophyll a fluorescence and Nile red staining measurement

Both chlorophyll *a* and Nile red fluorescence were measured with a SpectraMax M3 Multi-mode Microplate Reader (Molecular devices, Sunnyvale, CA, USA). For chlorophyll *a* fluorescence detection, the excitation wavelength was set at 440 nm, and emission was measured at a wavelength of 680 nm. Nile red is a high-affinity lipophilic dye that binds lipids and emits fluorescence under excitation. Its staining method was mentioned in a previous article.

3.4.6 Confocal imaging

For imaging, fluorescence was measured using an Olympus FV1200 Confocal microscope. Differential interference contrast (DIC), chlorophyll *a* fluorescence, Nile red fluorescence images were acquired. Strains were in the exponential growth phase, and the settings for the observation for each strain were identical: the excitation laser wavelength for chlorophyll *a* and Nile red was 488 nm, and the optical emission filter allowed light between 560 nm and 620 nm for Nile red and between 655 nm and 750 nm for chlorophyll *a*.

3.4.7 Spectrophotometer for pigment detection

Spectrophotometric method was applied to estimate chlorophyll *a* and total carotenoid content based on pigment extraction [357]. Samples were transferred to a 1 mL EP tube and centrifuged at 10,000 RPM for 20 min, and then supernatants were discarded. Next, 1 mL 100 % (v/v) methanol was added to each tube, whose contents were then vigorously pipetted and vortexed. Samples were sonicated for 1 h and centrifuged again, and supernatants were collected. The optical absorbances at 665 nm, 652 nm and 470 nm were measured [357]. The equations for chlorophyll *a* content and total carotenoid content are below:

$$Ca=15.65*A_{665}-7.34*A_{652} \quad (1)$$

$$Xcarotenoids=((1000*A_{470})-(2.86*Ca))/221 \quad (2)$$

Ca represents chlorophyll *a* content, Xcarotenoids represents total carotenoid content and A_{665}, A_{652}, and A_{470} represent the optical absorbance at each wavelength, respectively.

3.4.8 LC-MS determination and analysis of major pigments

As described in former studies [358], we also used an ACQUITY UPLC coupled to a SYNAPT G2 HDMS system (Waters, Milford, USA), which was equipped with an HSS T3 1.8 µm column (2.1 × 150 mm; Waters, UK). The same applies to flow rates and the used gradient and mobile phases, respectively (Phase A: ACN:MetOH:MTBE = 70:20:10 [v:v:v]; Phase B: water with 10mM Ammoniumacetate).

Ten concentration steps (1.7 - 400mg/L) of standards of β-carotene (CAS-no.: 7235-40-7), fucoxanthine (CAS-no.: 3351-86-8) and chlorophyll a (CAS-no.: 479-61-8) were created by diluting pure substances in pure isopropanol in order to calibrate those three substances as well as to validate the accuracy of our detection according to the formerly described methods [358]. In the beginning of daily LC-MS-batches 10 measurements of pooled samples were performed to equilibrate the column. Through this we were given proof that retention time shifts and decreased sensitivity have not occurred within daily batches. Between measurements runs of pure isopropanol were performed to prevent carry-over-effects. Identifications of carotenoids other than the calibrated ones were realized using m/z-ratios and retention times formerly described in studies using the same or similar LC-MS-methods [283,358].

3.4.9 Metabolic modeling analysis

The *i*LB1025 genome-scale reconstructed metabolic model of *P. tricornutum* was applied to predict metabolic reactions correlated with fucoxanthin production [343]. The model was analyzed by randomly sampling fluxes from the system. Random sampling generated numerous flux vectors on behalf of the system's feasible states where single flux vector elements amount to the fluxes in individual reactions [359]. The correlation coefficients between fluxes in fucoxanthin production and other metabolic reactions in the system were calculated for the selection of the most correlated metabolic reactions. The CosMos algorithm was used to identify the correlation, and the computational analysis was executed in the MATLAB (9.1, The MathWorks, Natick, MA, USA) environment applying the COBRA toolbox version 2.0 [343,359].

3.4.10 Data processing and analysis

MassLynx v4.1 (Waters corp., Milford, USA) was used to identify and quantify cellular compounds. Principal component analysis (PCA) and OPLS-DA (orthogonal partial least squares discriminate analysis) were conducted by software SIMCA 14. PCA was used to summarize the metabolite profiling data and reveal the grouping of samples. OPLS-DA was used for group-group comparisons, and OPLS-DA S-plots were applied to visualize the metabolites that contribute most significantly to the intergroup variations [360]. Before applying the PCA, data were normalized through summing, log transformation, and scaled to a mean

of zero and unit standard deviation. Intergroup comparison was applied by one-way ANOVA.

3.4.11 High-throughput screening method

The high-throughput screening method comprised three steps of screening. After chemical mutagenesis, mutants were cultivated evenly in f/2 agar plates with 40 μM DPA. After 15 days under 30 $\mu E/m^2/s$ continuous illumination, colonies with darker color and larger size were selected and cultured in f/2 medium in microplates. Strains were re-seeded every 7 days, and the initial biomass density was kept at approximately 0.06 (OD_{625}). For the secondary screening, 200 μL medium of each strain was transferred to 96 well microplates in triplicate after 36 h cultivation of each initial seeding to keep strains in identical exponential growth phases. The optical density, chlorophyll *a* fluorescence intensity and Nile red fluorescence values were averaged from triplicate measurements. The equations we utilized are listed below: $RFU_{chlo\ a}$ represents the relative fluorescence intensity of chlorophyll *a*, and RFUnr represents the Nile red fluorescence intensity:

$$RFU_{chlo\ a} \geq 2309*OD_{625} - 24.3 \quad (3)$$

$$RFUnr \geq 167.1*OD_{625} - 0.21 \quad (4)$$

Strains screened via equations (3) and (4) were picked out and cultivated in 48 well plates for the next screening step. Total carotenoids and chlorophyll *a* contents, which had been extracted with 100% methanol, were calculated based on previously described equations [357]. Positive mutants whose total carotenoids were at least 15% higher than those of WT were selected for further cultivation in Erlenmeyer flasks. After 2 months repeated batch cultivation, pigment content was re-examined, followed by methanol extraction to evaluate the strain stability. Specific pigment content of strains grown in Erlenmeyer flasks was determined through LC-MS. The schematic process is demonstrated in Figure 3.1.

3.5 Supplementary documents:

3.5.1 Supplementary figures:

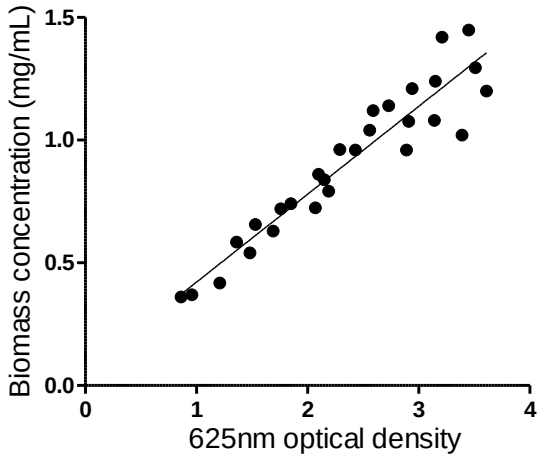

Figure 3-7 *Correlation between optical density at 625 nm and diatom dry biomass concentration. Diatom cell samples were harvested on a cellulose membrane (pore size, 0.45 µm) and washed twice with deionized water dried at 60 ℃ overnight, dried at 60 ℃ overnight.*

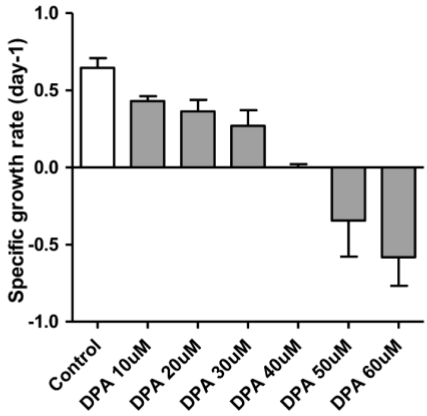

Figure 3-8 *Effects of DPA concentration on P. triconrnutum growth. P. tricornutum were incubated with different concentrations of DPA: 10 μM, 20 μM, 30 μM, 40 μM, 50 μM, and 60 μM for 4 days, respectively. Specific growth rate (μ) is defined as the rate of increase of biomass of a cell population per unit of biomass concentration and was measured in biological triplicates for each treatment.*

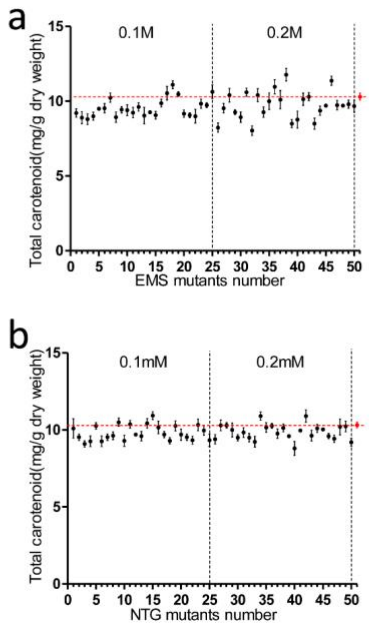

Figure 3-9 *Analysis of total carotenoid content in EMS and NTG mutants. (a) EMS mutagenesis, from Y axis to left dotted line are No. 1 - No. 25 mutants treated with 0.1 M EMS, No. 26 - No. 50 mutants were treated with 0.2 M EMS. (b) NTG mutagenesis, mutants No.1 to No.25 were treated with 0.1 mM NTG while No. 26 to No. 50 mutants were treated with 0.2 M EMS. The strain designated as No.51 is the untreated wild type; the transverse dotted line represented wild type total carotenoid concentration. Each data point corresponds to the average value from triplicate experiments*.

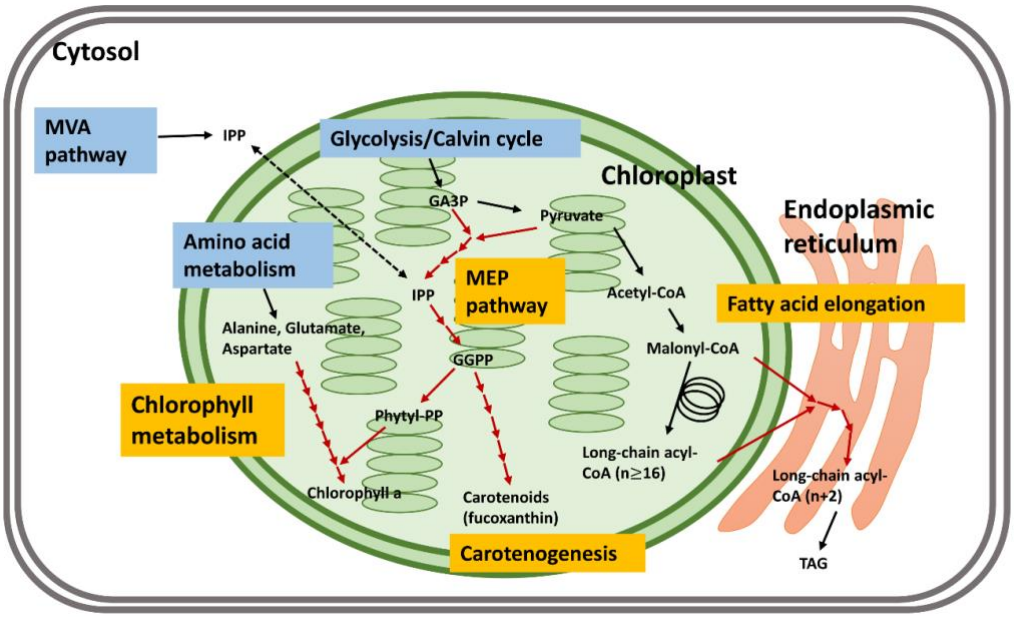

Figure 3-10 *Intersectional metabolic pathways for carotenogenesis, chlorophyll a production and lipid metabolism.*

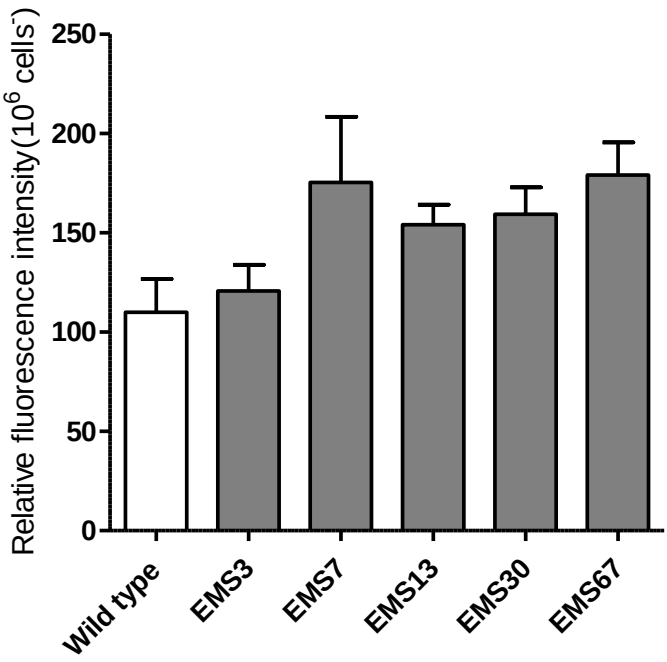

Figure 3-11 *Relative Nile red fluorescence intensity for selected strains. Strains were incubated with 1ug/mL Nile red for 20min in dark room. Each fluorescence value was averaged from triplicates.*

3.5.2 Supplementary tables

Table 3-1 *Equations for the correlations of total carotenoids with chlorophyll a or lipids content (indicated by Nile red fluorescence intensity).*

	Equations
a)	$y=0.0025x+0.0123$ ($R^2=0.8687$) (x: relative chlorophyll a fluorescence intensity; y: total carotenoid content)
b)	$y=0.0021x+0.1501$ ($R^2=0.7395$) (x: relative chlorophyll a fluorescence intensity; y: total carotenoid content)
c)	$y=0.018x-0.3253$ ($R^2=0.6356$) (x: relative Nile red fluorescence intensity; y: total carotenoid content)
d)	$y=0.0081x+0.8609$ ($R^2=0.3758$) (x: relative Nile red fluorescence intensity; y: total carotenoid content)

Table 3-2 *Enzymatic reactions with the highest correlation with fluxes in the fucoxanthin production. The data was obtained through conducting various flux analysis in published iLB1025 model.*

Reaction	Correlation	KEGG ID	EC	Catalyst
Nucleotide metabolism: Pyrimidine metabolism				
TDSR_c	1.00	R02016	1.8.1.9	Thioredoxin-disulfide reductase
OMPDC_c	1.00	R00965	4.1.1.23	Orotidine-5''-phosphate decarboxylase
MDUMT_c	1.00	R02101	2.1.1.45	5,10-Methylenetetrahydrofolate:dUMP C-methyltransferase
ASPCT_c	1.00	R01397	2.1.3.2	Aspartate carbamoyltransferase
DHR_c	-1.00	R01993	3.5.2.3	Dihydroorotase
ORPRT_c	-1.00	R01870	2.4.2.10	Orotate phosphoribosyltransferase
DHRDH_c	1.00	R01867	1.3.98.1	Dihydroorotate dehydrogenase (Fumarate)
UTAL_c	0.98	R00571	6.3.4.2	UTP:ammonia ligase (ADP-forming)
DCDT_c	0.81	R02024	1.17.4.1	2'-Deoxycytidine diphosphate:oxidized-thioredoxin 2'-oxidoreductase
ATDCM_c	-0.61	R01665	2.7.4.14	ATP:dCMP phosphotransferase
DCMAH_c	0.61	R01663	3.5.4.12	dCMP aminohydrolase

ITCY_c	0.26	R00962	2.7.1.48	ITP:cytidine 5''-phosphotransferase
CMP_c	0.26	R00511	3.1.3.5	Cytidine-5''-monophosphate phosphohydrolase
DURIPP_c	0.15	R02484	2.4.2.1	Deoxyuridine phosphorylase
UPRT_c	-0.15	R00966	2.4.2.9	Uracil phosphoribosyltransferase
NTD1_c	0.15	R02102	3.1.3.5	2''-Deoxyuridine 5''-monophosphate phosphohydrolase

Nucleotide metabolism: Purine metabolism

DGOTO_c	0.64	R02019	1.17.4.1	2'-Deoxyguanosine 5'-diphosphate:oxidized-thioredoxin 2'-oxidoreductase
DAOTO_c	0.56	R02017	1.17.4.1	2'-Deoxyadenosine 5'-diphosphate:oxidized-thioredoxin 2'-oxidoreductase
AIAL_c	0.21	R04559	4.3.2.2	1-(5''-Phosphoribosyl)-5-amino-4-(N-succinocarboxamide)-imidazole AMP-lyase
PRAIS_c	0.21	R04591	6.3.2.6	Phosphoribosylaminoimidazolesuccinocarboxamide synthase
PRAIC_c	-0.21	R04209	4.1.1.21	Phosphoribosylaminoimidazole carboxylase

Reaction	Correlation	KEGG ID	EC	Catalyst
ITPA_c	0.20	R00719	3.6.1.5	ITP-apyrase
DAMPH_c	-0.17	R02088	3.1.3.5	2"-Deoxyadenosine 5"-monophosphate phosphohydrolase
ATDAM_c	0.17	R01547	2.7.4.3	ATP:dAMP phosphotransferase
FPGFT_c	0.16	R04325	2.1.2.2	Phosphoribosylglycinamide formyltransferase
PPRGL_c	0.16	R04144	6.3.4.13	Phosphoribosylamine-glycine ligase
PRFGS_c	0.16	R04463	6.3.5.3	Phosphoribosylformylglycinamidine synthase
PRDPAR_c	0.16	R01072	2.4.2.14	5-Phosphoribosylamine:diphosphate phospho-alpha-D-ribosyltransferase
PRFGCL_c	0.16	R04208	6.3.3.1	Phosphoribosylformylglycinamidine cyclo-ligase
GPAR_c	0.15	R01229	2.4.2.7	GMP:diphosphate 5-phospho-alpha-D-ribosyltransferase
PUNP3_c	0.15	R02147	2.4.2.1	Purine-nucleoside phosphorylase (Guanosine)
GMP5N	0.15	R01227	3.1.3.5	GMP-5"-nucleotidase
XPPRT_c	-0.14	R02142	2.4.2.8	XMP:pyrophosphate phosphoribosyltransferase
PUNP7_c	-0.14	R02297	2.4.2.1	Purine-nucleoside phosphorylase (Xanthosine)

X5NT_c	-0.14	R02719	3.1.3.5	XMP-5''-nucleotidase
Nitrogen metabolism				
NITR_c	0.86	R00794	1.7.1.1	Nitrate reductase (NADH)
NTRIR_h	0.34	R02016	1.8.1.9	Nitrite reductase (NADPH), chloroplast
NOR_c	0.25	R00790	1.7.7.1	Ferredoxin-Nitrite Reductase
Nucleotide sugar metabolism				
XYLE_c	1.00	R01473	5.1.3.5	UDP-xylose 4-epimerase
UDPRHMS_c	1.00			UDP-beta-L-rhamnose synthase
GDPMANST_c	1.00			GDP-mannose-3-sulftransferase
UDPGLDC_c	1.00			UDP-D-glucuronate decarboxylase
Starch and sucrose metabolism				
UGDH	1.00	R00286	1.1.1.22	UDP-glucose 6-dehydrogenase
Fructose and mannose metabolism				
GFUCS_c	1.00	R05692	1.1.1.271	GDP-L-fucose synthase
GMAND_c	1.00	R00888	4.2.1.47	GDP-D-mannose dehydratase
Reaction	**Correlation**	**KEGG ID**	**EC**	**Catalyst**
Biosynthesis of steroids: Terpenoid backbone synthesis				
GPPS_h	1.00	R01658	2.5.1.1	geranyl pyrophosphate synthase, chloroplast

CMK_h	1.00	R05634	2.7.1.148	4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase
GGPS_h	1.00	R02061	2.5.1.29	Geranylgeranyl diphosphate synthase
DXR_h	1.00	R05688	1.1.1.267	1-deoxy-D-xylulose-5-phosphate reductoisomerase
MECDPS_h	1.00	R05637	4.6.1.12	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase
GGDR_h	1.00	R02063	1.3.1.83	geranylgeranyl diphosphate reductase
HMBDPO_h	1.00	R08689	1.17.7.1	4-hydroxy-3-methylbut-2-en-1-yl-diphosphate:oxidized ferredoxin oxidoreductase
CMS_h	1.00	R05633	2.7.7.60	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase
FPPS_h	1.00	R02003	2.5.1.10	Farnesyl pyrophosphate synthase
DXPS_h	0.73	R05636	2.2.1.7	1-deoxy-D-xylulose 5-phosphate synthase
IDS1_h	0.55	R05884	1.17.1.2	isopentenyl-diphosphate synthase
IDIH_h	0.20	R01123	5.3.3.2	isopentenyl-diphosphate Delta-isomerase, chloroplast

Carotenoid biosynthesis

NOR_h	-1.00	R04800	1.3.5.6	Neurosporene oxidoreductase
PSY_h	1.00	R02065	2.5.1.32	phytoene synthase

		R07270		
ZDS_h	-1.00	R04798	1.3.5.6	zeta-carotene desaturase
BCAROXR_h	1.00	R07558 R07559		Beta-carotene, NADH:oxygen 3-oxidoreductase (zeaxanthin forming)
NSY_h	-1.00	R06948	5.3.99.9	Neoxanthin synthase
FXANS_h	1.00			Fucoxanthin synthase
PDS1_c	1.00	R04786	1.3.5.5	phytoene desaturase (1)
PDS2_h	1.00	R04787	1.3.5.5	phytoene desaturase (2)
LYCBC1_c	-1.00	R05341	5.5.1.19	Lycopene cyclase (gamma-carotene producing)
LYCBC2_h	1.00	R03824	5.5.1.19	Lycopene cyclase (beta-carotene producing)
DIADINX_h	1.00			Diadinoxanthin synthase
Reaction	**Correlation**	**KEGG ID**	**EC**	**Catalyst**

Porphyrin and chlorophyll metabolism

PPBNGD_h	-1.00	R00084	2.5.1.61	porphobilinogen deaminase
UPPS_h	1.00	R03165	4.2.1.75	Uroporphyrinogen-III synthase
MPOXR_h	1.00	R10068	1.14.13.81	Magnesium-protoporphyrin-IX 13-monomethyl ester,NADPH:oxygen oxidoreductase

PPBNGS_h	1.00	R00036	4.2.1.24	porphobilinogen synthase
PPPGO_h	1.00	R03222	1.3.3.4	protoporphyrinogen IX oxidase
CHLPAS_h	1.00	R06284	2.5.1.62	Chlorophyll A synthase
CPPPGO_h	1.00	R03220	1.3.3.3	coproporphyrinogen III oxidase (O2 required)
CHLPC1S_h	1.00			Chlorophyll C1 synthase
G1SAT_h	-1.00	R02272	5.4.3.8	glutamate-1-semialdehyde aminotransferase
GLUTRR_h	1.00	R04109	1.2.1.70	Glutamyl-tRNA reductase
CHLPC2S_h	1.00			Chlorophyll C2 synthase
MPML_h	1.00	R03877	6.6.1.1	Mg-protoporphyrin IX magnesium-lyase
MPMT_h	-1.00	R04237	2.1.1.11	S-adenosyl-L-methionine:Mg-protoporphyrin IX methyltransferase
DVPCHLDR_h	0.56	R06896	1.3.1.75	Divinylprotochlorophyllide vinyl-reductase
PCHLDOR_h	0.54	R03845	1.3.1.33	Protochlorophyllide oxidoreductase (light-dependent)
UPP3DC_h	0.19	R03197	4.1.1.37	uroporphyrinogen decarboxylase (uroporphyrinogen III)
UPP3DC_c	0.16	R03197	4.1.1.37	Uroporphyrinogen decarboxylase (uroporphyrinogen III)

Fatty acid biosynthesis

3OAS200_c	1.00	R09419	2.3.1.199	very-long-chain 3-oxoacyl-CoA synthase (20:0 forming)
3OAS220_c	1.00	R09419	2.3.1.199	very-long-chain 3-oxoacyl-CoA synthase (22:0 forming)
3OAS240_c	1.00	R09419	2.3.1.199	very-long-chain 3-oxoacyl-CoA synthase (24:0 forming)
3OAR200_c	1.00	R01779	1.1.1.330	very-long-chain 3-oxoacyl-CoA reductase (20:0 forming)
3OAR220_c	1.00	R01779	1.1.1.330	very-long-chain 3-oxoacyl-CoA reductase (22:0 forming)
3OAR240_c	1.00	R01779	1.1.1.330	very-long-chain 3-oxoacyl-CoA reductase (24:0 forming)
3HAD200_c	1.00	R02685	4.2.1.134	very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase (20:0 forming)
3HAD220_c	1.00	R02685	4.2.1.134	very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase (22:0 forming)
3HAD240_c	1.00	R02685	4.2.1.134	very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase (24:0 forming)
Reaction	**Correlation**	**KEGG ID**	**EC**	**Catalyst**
EAR200_c	1.00	R09449	1.3.1.93	very-long-chain enoyl-CoA reductase (20:0 forming)

EAR220_c	1.00	R09449	1.3.1.93	very-long-chain enoyl-CoA reductase (22:0 forming)
EAR240_c	1.00	R09449	1.3.1.93	very-long-chain enoyl-CoA reductase (24:0 forming)

Amino acid metabolism: aminoacyl-tRNA biosynthesis

CYSTL_c	1.00	R03650	6.1.1.16	Cysteine-tRNA ligase
ALATL_c	1.00	R03038	6.1.1.7	Alanine-tRNA ligase
LEUTL_c	1.00	R03657	6.1.1.4	Leucine-tRNA ligase
METTL_c	1.00	R03659	6.1.1.10	Methionine-tRNA ligase
TRPTL_c	1.00	R03664	6.1.1.2	Tryptophane-tRNA ligase
SERTL_c	1.00	R03662	6.1.1.11	Serine-tRNA ligase
GLUTL_h	1.00	R05578	6.1.1.17	Glutamate-tRNA ligase
GLUTL_c	1.00	R05578	6.1.1.17	Glutamate-tRNA ligase
ARGTL_c	1.00	R03646	6.1.1.19	Arginine-tRNA ligase
TYRTL_c	1.00	R02918	6.1.1.1	Tyrosine-tRNA ligase
ASNTL_c	1.00	R03648	6.1.1.22	Asparagine-tRNA ligase
LYSTL_c	1.00	R03658	6.1.1.6	Lysine-tRNA ligase
GLNTL_c	1.00	R03652	6.1.1.18	Glutamine-tRNA ligase
ASPTL_c	1.00	R05577	6.1.1.12	Aspartate-tRNA ligase
PHETL_c	1.00	R03660	6.1.1.20	Phenylalanine-tRNA ligase
THRTL_c	1.00	R03663	6.1.1.3	Threonine-tRNA ligase
GLYTL_c	1.00	R03654	6.1.1.14	Glycine-tRNA ligase
SERTL_h	-0.16	R03662	6.1.1.11	Serine-tRNA ligase, chloroplast

Amino acid metabolism: Cysteine and Methionine metabolism

MTRK_c	1.00	R04143	2.7.1.100	S-methyl-5-thioribose kinase
DKPPHL_c	1.00	R07395	3.1.3.77	2,3-Diketo-5-methylthiopentyl-1-phosphate phosphohydrolase
MTRI_c	1.00	R04420	5.3.1.23	S-methyl-5-thioribose-1-phosphate isomerase
Reaction	**Correlation**	**KEGG ID**	**EC**	**Catalyst**
DH5MTPOXR_c	1.00	R07364	1.13.11.54	1,2-dihydroxy-5-(methylthio)pent-1-en-3-one:oxygen oxidoreductase
MDRPD_c	1.00	R07392	4.2.1.109	5-Methylthio-5-deoxy-D-ribulose 1-phosphate dehydratase
TAL_m	0.21	R00996	4.3.1.19	threonine ammonia-lyase
THRS_c	0.20	R01466	4.2.3.1	threonine synthase
G3PAT140_h	1.00	R09380	2.3.1.15	glycerol-3-phosphate: acyl-ACP acyltransferase (14:0)
AGPATACP_PALM_PALM_h	1.00	R02241	2.3.1.51	1-Hexadecanoyl-sn-glycerol-3-phosphate O-acyltransferase (16:0) (ACP Substrate)
PAPA_EPA_PALM_h	1.00	R02239	3.1.3.4	Phosphatidate phosphatase(20:5(5Z,8Z,11Z,14Z,17Z)/16:0) chloroplast
CDPDAGS_EPA_PALM_h	1.00	R01799	2.7.7.41	CDP-diacylglycerol synthase

				(20:5(5Z,8Z,11Z,14Z,17Z) /16:0) chloroplast
G3PAT160_h	1.00	R09380	2.3.1.15	glycerol-3-phosphate: acyl-ACP acyltransferase (16:0)
AGPATACP_MYRS_PALM_h	1.00	R02241	2.3.1.51	1-Tetradecanoyl-sn-glycerol-3-phosphate O-acyltransferase (16:0) (ACP Substrate)
AGPATACP_EPA_PALM_h	1.00	R02241	2.3.1.51	1-5,8,11,14,17-Eicosapentaenoyl-sn-glycerol-3-phosphate O-acyltransferase (16:0) (ACP Substrate)
PAPA_MYRS_PALM_h	1.00	R02239	3.1.3.4	Phosphatidate phosphatase(14:0/16:0) chloroplast
SQDGS_MYRS_PALM_h	1.00			Sulfoquinovosyldiacylglycerol synthase(14:0/16:0)
SQDGS_EPA_PALM_h	1.00			Sulfoquinovosyldiacylglycerol synthase(20:5(5Z,8Z,11Z,14Z,17Z)/16:0)
SQDGS_PALM_PALM_h	1.00			Sulfoquinovosyldiacylglycerol synthase(16:0/16:0)
SQDGS_HDE_TTC_c	1.00			Sulfoquinovosyldiacylglycerol synthase(16:1(9Z)/24:0)
ASQ_EPA_PALM_2OEPA_h	1.00			Sulfoquinovosyl diacylglycerol-2-O-acyl transferase (20:5(5Z,8Z,11Z,14Z,17Z) /16:0)(SQDG-2-O-20:5(5Z,8Z,11Z,14Z,17Z))

Phosphoglycerolipid metabolism

PGPP_EPA_PALM_h	1.00	R02029	3.1.3.27	Phosphatidylglycerol phosphate phosphatase (20:5(5Z,8Z,11Z,14Z,17Z)/16:0) chloroplast
PGD3TDS_EPA_HDE3T_h	1.00			Phosphatidylglycerol sn-2 palmitoyl delta 3 desaturase (20:5(5Z,8Z,11Z,14Z,17Z)/16:1(3E)) chloroplast
AGPATCOA_HDE_TTC_c	1.00	R02241	2.3.1.51	1-9-hexadecenoyl-sn-glycerol-3-phosphate O-acyltransferase (24:0) (CoA Substrate)
PAPA_HDE_TTC_c	1.00	R02239	3.1.3.4	Phosphatidate phosphatase (16:1(9Z)/24:0)
Reaction	**Correlation**	**KEGG ID**	**EC**	**Catalyst**
PGPS_EPA_PALM_h	1.00	R01801	2.7.8.5	Phosphatidylglycerol phosphate synthetase (20:5(5Z,8Z,11Z,14Z,17Z)/16:0) chloroplast
PAPA_PALM_PALM_h	1.00	R02239	3.1.3.4	Phosphatidate phosphatase (16:0/16:0)
UDPSQS_h	1.00	R05775	3.13.1.1	UDP-sulfoquinovose synthase
CDIPT_HDE_PALM_c	0.18	R01802	2.7.8.11	CDP-diacylglycerol: myo-inositol 3-phosphatidyl transferase (16:1(9Z)/16:0)
CDPDAGS_HDE_PALM_c	0.18	R01799	2.7.7.41	CDP-diacylglycerol synthase(16:1(9Z)/16:0)
DAGK_HDE_PALM_c	0.14	R02240	2.7.1.107	Diacylglycerol kinase(16:1(9Z)/16:0)

ACPT_EPA_EPA_c	1.00			Betaine lipid synthase (20:5(5Z,8Z,11Z,14Z,17Z) /20:5(5Z,8Z,11Z,14Z,17Z)
BPNT_c	1.00	R00188	3.1.3.7	3",5"-Bisphosphate nucleotidase (pap)

Table 3-3 *Tentative identification of different lipophilic compound expression between WT and EMS30.*

Marker No.	Group	Supposed metabolite	RT (min)	m/z	formula	adduct	ppm
1	WT	eicosasphinganine	9.54	312.3287	C20H43NO2	[M+H-H2O]+	6
2	WT	Octadecanoic acid	7.8	284.2979	C18H39NO2	[M+H-H2O]+	9
3	WT	Docosanamide	11.03	340.3603	C22H45NO	[M+H]+	8
4	WT	DGCC(16:0/20:5)	10.82	756.5796	C46H79NO8	[M+H-H2O]+	2
5	EMS30	Antheraxanthin	13.47	607.3956	C40H56O3	[M+Na]+	28
6	EMS30	PA(P-16:0/20:4)	13.48	663.4583	C39H69O7P	[M+H-H2O]+	24
7	EMS30	Ganoderic acid V	13.48	551.3312	C32H48O6	[M+Na]+	5
8	EMS30	Hoslundin	14.74	429.0912	C23H18O7	[M+Na]+	8
9	EMS30	PG(22:6/21:0)	15.82	887.5728	C49H85O10P	[M+Na]+	5
10	EMS30	Spectinomycin adenylate	14.73	684.205	C24H36N7O13P	[M+Na]+	7
11	EMS30	4,4'-Sulfonylbisacetanilide	14.72	355.0719	C16H16N2O4S	[M+Na]+	1
12	EMS30	Benzyl Dimethylphosphonoacetate	12	281.0555	C11H15O5P	[M+Na]+	2
13	EMS30	PG(22:6/21:0)	15.49	887.5737	C49H85O10P	[M+Na]+	5

Table 3-4 *Tentative identification of different lipophilic compound expression between WT and EMS67.*

Marker No.	Group	Supposed metabolite	RT (min)	m/z	formula	adduct	ppm
1	WT	eicosasphinganine	9.54	312.3287	C20H43NO2	[M+H-H2O]+	6
2	WT	Docosanamide	11.03	340.3603	C22H45NO	[M+H]+	8
3	WT	N-Docosylacetamide	12.24	368.3927	C24H49NO	[M+H]+	11
4	WT	Ixocarpanolide	13.48	495.2662	C28H40O6	[M+Na]+	11
5	WT	4,4'-Sulfonylbisacetanilide	15.27	355.0704	C16H16N2O4S	[M+Na]+	1
6	WT	Phe Cys Gly Leu	13.5	439.2021	C20H30N4O5S	[M+H]+	7
7	EMS67	Hoslundin	14.74	429.0921	C23H18O7	[M+Na]+	8
8	EMS67	PG(22:6/21:0)	15.82	887.5728	C49H85O10P	[M+Na]+	5
9	EMS67	PG(22:6/21:0)	15.49	887.5737	C49H85O10P	[M+Na]+	5
10	EMS67	PA(P-16:0/20:4)	13.48	663.4583	C39H69O7P	[M+H-H2O]+	24
11	EMS67	4,4'-Sulfonylbisacetanilide	14.72	355.0719	C16H16N2O4S	[M+Na]+	1
12	EMS67	Antheraxanthin	13.47	607.3956	C40H56O3	[M+Na]+	28
13	EMS67	Chlorophyllide	14.02	615.2473	C35H34MgN4O5	[M+H]+	3
14	EMS67	Spectinomycin adenylate	14.73	684.205	C24H36N7O13P	[M+Na]+	7
15	EMS67	Palmitamide	5.9	256.2659	C16H33	[M+H]+	9
16	EMS67	Benzyl Dimethylphosphonoacetate	12	281.0555	C11H15O5P	[M+Na]+	2
17	7	Anthemis glycoside A	14.96	832.2451	C39H49NO21	[M+H-2H2O]+	26

4 Conclusions and prospects

4.1 Mutagenesis

In this study, both physical mutagen UV and chemical mutagen EMS were explored to produce fucoxanthin-rich mutants of *P. tricornutum*. For UV mutagenesis, 15 min UV irradiation resulted in diatoms with 85.6% lethality and 10 min UV irradiation produced 64% lethality. The average specific growth rate for the 15 min irradiation group was 0.34 day^{-1} while the specific growth rate was 0.46 day^{-1} for the wild type group, implying that UV light may suppress diatom growth and prolonged UV irradiation may cause permanent growth suppression even after recovering from UV damage. Fucoxanthin hyper-producing strains created by UV mutagenesis also had a higher neutral lipid content than the WT group.

For chemical mutagenesis, EMS had better efficiency for generating positive strains of high fucoxanthin than NTG in *P. tricornutum* on account of the fact that more positive mutants were created by EMS than by NTG at similar lethality rates. Both 0.1M EMS and 0.2M EMS were applied to mutant *P. tricornutum*, 2 mutants out of 25 had at least 10% higher total carotenoid content than WT in the 0.2M EMS group while none of the mutants had over 10% total carotenoid content in the 0.1M EMS group. Therefore, in our study 0.2M EMS had the best mutagenesis efficiency for generating positive mutants.

4.2 Adaptive Laboratory Evolution

Adaptive laboratory evolution has been applied to adapt microorganisms to constrained environments in the last decades. It promotes phenotypical changes and enables adaptation to defined conditions. Consequently, ALE improves strains' performance via cellular regulation and control or modification of genetics. Before the experiment, it was assumed that diatoms would make an appropriate target for adaptive evolution due to their fast growth and strong adaptivity to the environment. The experimental results confirmed our assumption: both the growth rate and fucoxanthin content increased with the semi-continuous PBR culture. The

growth rate of *P. tricornutum* increased from 0.14 gDCW/L/day at Cycle 1 to 0.29 gDCW/L/day at Cycle 11. The fucoxanthin content increased 110% from the start of Cycle 1 by the end of Cycle 11 while chlorophyll *a* did not show any significant change during the whole ALE process. Light emitting diodes (LEDs) irradiate narrow spectra which could be utilized to study certain light effects on algae or land plants. The LEDs' intensity was controlled in an on/off duty cycle (0.1 ms) and the peak light intensity was 250 $\mu E/m^2/s$, which generated extra oxidative light stress for the strains. The mechanisms required to synthesize and accumulate large amounts of fucoxanthin are still not fully understood. Elevated light stress enhanced carotenoid content in *C. vulgaris* and *D. salina [318,319]*, so light-induced oxidative stress could also be assumed to play an important role in enhancing fucoxanthin production in this study. Nevertheless, the neutral lipid contents of the ALE samples decreased slightly from 24.6% total dry weight at the beginning of Cycle 1 to 19.3% at the end of Cycle 11. Strains with increased growth rate and enhanced fucoxanthin content may be mainly due to accessory pigments' metabolic up-regulation since aggregative mutants generated by ALE only achieved less than 20 generations in 11 cycles. This study exhibited that ALE could be an efficient method for increasing both biomass growth and fucoxanthin content.

4.3 High-throughput screening method

During the exponential growth phase, chlorophyll *a* had a good linear correlation with total carotenoid content (R^2=0.8687). At the stationary growth phase, the correlation between chlorophyll *a* fluorescence intensity and total carotenoid content was not as strong as in the exponential phase (R^2=0.7395). In the exponential growth phase, Nile red fluorescence had a moderately linear correlation with total carotenoids (R^2=0.6356), while during the stationary growth phase, Nile red fluorescence had a weaker correlation with total carotenoid content (R^2=0.3758).

The computational model predicted that enzymatic reactions could be involved in the positive correlations. In porphyrin and chlorophyll metabolism, the enzymatic reactions which had the strongest correlations with fucoxanthin synthesis belong to the chlorophyll *a* biosynthetic pathway. Among lipid metabolism, the 12 reactions which mostly belong to fatty acid elongation process were closely associated with fucoxanthin production.

Four positive mutants out of the five which were selected by fluorescence-based screening retained almost identical carotenoid contents after two months' repeated batch cultivation while 1 positive mutant (EMS3) dropped down to nearly the same level as WT in the stability test.

The high-throughput screening method significantly increased the efficiency of selecting fucoxanthin hyper-production strains in *P. tricornutum* and may be applied to other algal species with a broad spectrum in order to screen positive strains.

4.4 Prospects

Forward genetics is an approach used to identify the functions of genes or set of genes for certain phenotypes. Both UVC and EMS were proven to be suitable for producing hyper-fucoxanthin mutants in *P. tricornutum*. Moreover, new sequencing technologies accelerate the process immensely and the rapid identification methods used for mutated alleles will soon be applied routinely worldwide [361]. A straightforward extension for this study would be sequencing the selected positive mutants and the ALE strain. Afterwards, the genomes of these mutants would be compared, and mutated alleles and key genes which are responsible for enhancing fucoxanthin production would be identified. Emerging genome-editing tools as Zinc-finger nucleases (ZFN), meganucleases (MNs), transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) were successfully applied with *Phaeodactylum tricornutum* to obtain the desired alterations [300]. It would be of interest to combine these emerging sequencing and genome-editing techniques with our high-throughput method when creating fucoxanthin hyper-production strains.

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