THE PHYSIOLOGICAL RESPONSES AND EXPRESSION PATTERNS OF HEAT INDUCED GENES TO ELEVATED TEMPERATURE OF MARINE *Chlorella* FROM DIFFERENT LATITUDES

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ABSTRACT

The increased frequency of heat waves due to climate change threatens all organism. Microalgae form the basis of aquatic food webs, and high temperatures significantly impact their adaptation and survival. Algae respond to environmental changes by modulating their photosynthetic rates and biochemical composition, which is in turn regulated by their gene expression. In this study, the effects of elevated temperature on marine *Chlorella* originating from different latitudes were examined. Strains from the Antarctic, temperate zone, and the tropics were grown at various temperatures, ranging from 4 to 38, 18 to 38, and 28 to 40 °C, respectively. A pulse-amplitude modulated (PAM) fluorometer was used to assess their photosynthetic responses. In addition, the biochemical compositions, including lipid, protein, carbohydrate and fatty acids were profiled to evaluate changes induced by temperature treatments. Increasing the temperature from 35 to 38 °C for both Antarctic and temperate strains and from 38 to 40 °C for the tropical strain resulted in severe inhibition of photosynthesis, which in turn suppressed growth. The temperature causing severe stress for each strain was selected for stress and recovery treatments. At different time points, the expressions of the photosynthetic and fatty acid biosynthesis key genes were analysed during stress and recovery. All the strains demonstrated the ability to recover from different stress levels, however, the tropical strain recovered most rapidly, while the Antarctic strain reported the slowest recovery. The results confirmed that the thermal threshold for the analysed *Chlorella* strains temperature falls between 38 and 40 °C. The response of studied strains differed from one another. *Chlorella*-Ant and *Chlorella*-Trop exhibited photosynthetic genes during stress, revealing their remarkable capability for maintaining

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photosystem II's main component (psbC). In *Chlorella*-Temp, photosynthetic genes were suppressed, while during recovery, it was up-regulated.

Keywords: microalgae, stress, elevated temperature, photosynthesis, gene expression

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TINDAK BALAS FISIOLOGI DAN CORAK EKSPRESI GEN TERINDUKSI HABA PAROA SUHU TINGGI BAGI RUMPAI LAUT *Chlorella* DARIPADA LATITUD YANG BERBEZA

ABSTRAK

Peningkatan kekerapan gelombang panas akibat perubahan iklim menimbulkan ancaman kepada kebanyakan organisma. Mikroalga adalah keperluan kepada rantaian makanan akuatik, dan suhu tinggi mempunyai kesan yang besar terhadap penyesuaian dan kelangsungan hidup mereka. Alga bertindak balas kepada perubahan alam sekitar dengan mengubah kadar fotosintesis dan komposisi biokimia, yang dikawal oleh ekspresi gen mereka. Dalam kajian ini, kesan suhu tinggi pada rumpai laut *Chlorella* daripada latitud yang berbeza telah kaji Strain dari Zon Antartika, zon suhu sederhana, dan tropika dibiak pada pelbagai suhu, antara 4 hingga 38, 18 hingga 38, dan 28 hingga 40 ° C. Sebuah fluorometer termodulasi amplitud (PAM) telah digunakan untuk menilai tindak balas fotosintesis. manakala, komposisi biokimia termasuk lipid, protein, karbohidrat dan asid lemak diprofilkan bagi menilai perubahan yang disebabkan oleh rawatan suhu. Peningkatan suhu dari 35 hingga 38 ° C untuk kedua-dua strain Antartika dan suhu sederhana dan dari 38 hingga 40 ° C untuk strain tropika mengakibatkan perencatan teruk fotosintesis dan membantutkan pertumbuhan. Suhu yang menyebabkan tekanan ekstrem bagi setiap strain telah dipilih untuk melakukan rawatan tekanan dan pemulihan. Padatitik yang berbeza, titik-titik expresi gen utama biosintesis fotosintesis dan asid lemak dianalisis semasa tekanan dan pemulihan. Semua strain menunjukkan keupayaan untuk pulih dari tahap tekanan yang berbeza, bagaimanapun, tekanan tropika dapat pulih dengan lebih cepat manakala strain Antartika mempunyai pemulihan yang paling perlahan. Hasilnya menggariskan bahawa nilai haba untuk suhu strain *Chlorella* dianalisis antara 38 dan 40 ° C. Respon strain yang dikaji adalah berbeza bagi satu sama lain. *Chlorella*-Ant dan *Chlorella*-Trop menunjukkan ekspresi gen fotosintesis semasa

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tekanan dan ia menunjukkan keupayaan mengekalkan komponen utama fotosistem dalam *Chlorella*-Temp gen fotosintetik ditekan semasa demulihan ia adalah diatur nalk.

Katakunci: Mikroalga, stres, suhu tinggi, fotosintesis, ungkapan gen

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University of Malaya

TABLE OF CONTENTS

Abstract.. iii

Abstrak.. v

Acknowledgments.. vii

Table of Contents.. viii

List of Figure.. xii

List of Tables.. xiv

List of Symbols and Abbreviations.. xv

List of Appendices.. xvii

CHAPTER 1: GENERAL INTRODUCTION.. 1

CHAPTER 2: LITERATURE REVIEW.. 7

2.1 Climate change and Global Warming.. 7

2.1.1 Global Warming and Antarctic.. 8

2.1.2 Global Warming and Temperate.. 10

2.1.3 Global Warming and Tropics.. 12

2.2 Abiotic Stress and algae.. 14

2.2.1 Abiotic Stress Effects on Lipids and Membrane of Algae........ 15

2.2.2 Abiotic Stress Effects on Protein.. 19

2.2.3 Abiotic Stress Effects on Carbohydrate.. 22

2.2.4 Abiotic Stress Effects on Antioxidant Defence Mechanisms.... 24

2.2.5 Photosynthesis.. 27

2.2.5.1 Abiotic Stress Effects on Photosynthesis 28

2.2.5.2 Photosynthetic Parameters Measurement Principle.... 31

2.3 Algal Gene Regulatory Mechanisms and Gene Manipulation............. 33

2.4 *Chlorella*.. 35

2.5 *Chlorella* Responses to Elevated Temperature.. 37

University of Malaya

CHAPTER 3: MATERIALS AND METHODS 40

3.1 *Chlorella* Strains and Culture Conditions 40

3.2 Experiment Setups 40

3.2.1 Experiment 1: Growth and Biochemical Characterization of *Chlorella* strains 40

3.2.2 Experiment 2: Stress and Recovery Treatment 42

3.2.3 Experiment 3: Gene Expression Profiling and ROS measurement upon Stress and Recovery 44

3.3 Analytical Methods 45

3.3.1 Growth study 45

3.3.1.1 Monitoring of Growth by Optical Density (OD_{620}) 45

3.3.1.2 Specific Growth Rate 45

3.3.2 Photosynthetic Parameters Measurement 45

3.3.3 Biochemical studies 46

3.3.3.1 Chl-*a* Concentration and Carotenoid Content 46

3.3.3.2 Dry Weight Determination 47

3.3.3.3 Lipid Extraction 48

3.3.3.4 Fatty Acid Transesterification 48

3.3.3.5 Gas Chromatography 49

3.3.3.6 Determination of Protein Content 49

3.3.3.7 Determination of Carbohydrate Content 50

3.3.4 Detection of Reactive Oxygen Species (ROS) 51

3.3.5 Gene Expression Assay 52

3.3.5.1 RNA extraction 52

3.3.5.2 RNA Purification and quantification 53

3.3.5.3 cDNA synthesis 54

University of Malaya

3.3.5.4 Primer Design and Validation.................................... 55

3.3.5.5 Performing RT-qPCR.. 57

3.3.6 Statistical Analyses.. 58

CHAPTER 4: RESULT.. 59

4.1 Experiment 1: Growth and Biochemical Characterization of selected Marine *Chlorella* strains.. 59

4.1.1 Growth study.. 59

4.1.1.1 Optical Density (OD).. 59

4.1.1.2 Chlorophyll-*a* to Carotenoid Content.......................... 61

4.1.1.3 Specific Growth Rate (μ).. 63

4.1.2 Biochemical Composition.. 64

4.1.2.1 Protein Content.. 64

4.1.2.2 Carbohydrate Content.. 66

4.1.2.3 Lipid Content.. 68

4.1.2.4 Fatty Acid Composition.. 70

4.1.3 Photosynthesis properties.. 72

4.1.3.1 Maximum Quantum Yield (*Fv/Fm*)............................ 72

4.1.3.2 Photosynthetic Efficiency (α).................................... 75

4.1.3.3 rETRmax.. 77

4.2 Experiment 2: stress and recovery treatment....................................... 79

4.2.1 Growth and photosynthesis.. 80

4.2.2 Carotenoid to Chlorophyll Content............................... 83

4.3 Experiment 3: Gene expression studies and ROS measurment 85

4.3.1 Gene expression results.. 85

4.3.2 ROS level measurement... 89

University of Malaya

CHAPTER 5: DISCUSSION 92

5.1 Experiment 1: Growth, Photosynthetic Performance and Biochemical Compositions of the *Chlorella* Strains 92

5.1.1 Growth Study 92

5.1.2 Photosynthetic Performance 93

5.1.3 Biochemical Compositions 96

5.2 Experiment 2: Stress and Recovery Treatments 99

5.3 Experiment 3: Gene Expression Studies 101

CHAPTER 6: CONCLUSION 107

6.1 Conclusion 107

6.2 Appraisal of Study 110

6.3 Future Research Direction 111

References 112

List of Publications and Paper Presented 141

Appendix 142

University of Malaya

LIST OF FIGURES

Figure 2.1:	KEGG map of photosynthesis pathway…………………..……	30
Figure 2.2:	Image of *Chlorella* sp. using Optical microscope...…...……...	36
Figure 3.1:	The flowchart of the experiment 1…………………..……..	42
Figure 3.2:	The flowchart of the experiment 2…………………………..	43
Figure 3.3:	The flowchart of the experiment 3……………………......…	44
Figure 3.4:	Flowchart of RNA extraction…………………………..…...	54
Figure 3.5:	Summary of genomic DNA removal from RNA isolate….…..	51
Figure 4.1:	Optical density of *Chlorella* strain grown at different temperatures VS time (day) of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245)……………………………………………	60
Figure 4.2:	Ratio of chlorophyll *a* to carotenoid of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245).……………………...……	62
Figure 4.3:	Specific growth rate of *Chlorella*-Ant, *Chlorella*-Temp, and *Chlorella*-Trop under different temperatures …………..……..	64
Figure 4.4:	Protein content (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245)……………………………………..……..	65
Figure 4.5:	Carbohydrate content of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC373) and (c) *Chlorella*-Trop (UMACC 245)……………………………………………….	67
Figure 4.6:	Lipid content of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245)………………………..……………………	69
Figure 4.7:	Maximum quantum yield (F_v/F_m) of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245)……………………..……..	73
Figure 4.8:	Photosynthetic efficiency (α) of a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245)…………………………………….…..	76
Figure 4.9:	rETRmax of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245)………………………………………………………..	78

University of Malaya

Figure 4.10: Maximum quantum yield (F_v/F_m) of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245) during stress and recovery………………………………………………………….. 81

Figure 4.11: Optical density (OD) of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245) during stress and recovery………………..….. 82

Figure 4.12: Comparison of Chlorophyll-*a* to the carotenoid ratio of (a) *Chlorella*-Trop, (b) *Chlorella*-Temp and (c) *Chlorella*-Ant at different stress level and upon the corresponding recovery…… 84

Figure 4.13: Transcript abundance of *psbA psaB, psbC, rbcL, FAD3, accD* and *SAD* of *Chlorella*-Trop during stress and recovery……..... 86

Figure 4.14: Transcript abundance of *psbA psaB, psbC, rbcL, FAD3, accD* and *SAD* of *Chlorella*-Temp during stress and recovery……… 88

Figure 4.15: Transcript abundance of *psbA psaB, psbC, rbcL, FAD3, accD* and *SAD* of *Chlorella*-Ant during stress and recovery………… 89

Figure 4.16: ROS level of (a) *Chlorella*-Trop, (b) *Chlorella*-Temp and (c) *Chlorella*-Ant at different stress level and upon recovery. Error bars denote standard deviations from triplicate samples……..… 90

University of Malaya

LIST OF TABLES

Table 2.1: The list of several studies reporting gene expression of *Chlorella* strains in respond to abiotic stress……….……..………………. 34

Table 3.1: List of the selected temperature for each *Chlorella* strains……... 41

Table 3.2: Protein Standard Preparation……………………………………. 50

Table 3.3: Carbohydrate Standard Preparation…………………………….... 51

Table 3.4: Thermal cycler set up for cDNA synthesis.…………………….... 55

Table 3.5: List of genes and primers used for RT-qPCR amplification…….. 56

Table 3.6: PCR cycling condition…………………………………………... 57

Table 3.7: Dissociation curve condition (Melt curve stage)…….………….. 58

Table 4.1: Fatty acid profile of *Chlorella*-Trop, *Chlorella*-Temp and *Chlorella*-Ant incubated at three different temperatures……….... 71

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LIST OF SYMBOLS AND ABBREVIATIONS

ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CCAP	Culture Collection of Algae and Protozoa
Chl-*a*	Chlorophyll *a*
Cl	Chlorine
CO_2	Carbon Dioxide
CPDs	Cyclobutyl Pyrimidine Dimmers
DNA	Deoxyribo nucleic Acid
DW	Dry Weight
ETR	Electron Transport Rate
FAME	Fatty Acid Methyl Esters
F_v/F_m	Maximum Quantum Yield
H	Hour
HCl	Hydrochloric acid
H_2O_2	Hydrogen Peroxide
H_2SO_4	Sulfuric Acid
H_2SO_4-MeOH	Sodium Methoxide
KH_2PO_4	Potasioum dihydrogen phosphate
MAAs	Microsporine-like Amino Acids
MeOH-$CHCl_3$	Metanol-Chloroform
MUFA	Monounsaturated Fatty Acid
NaOH	Sodium Hydroxide
NPQ	Non-Photochemical Quenching
O	Oxygen Atom

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O_2	Dioxide Molecule
O_3	Ozone
O_{2-}	Superoxide Radicals
OD	Optical Density
OD_{620nm}	Optical Density at 620 nanometer
PAR	Photosynthetically Active Radiation
PSI	Photosystem I
PSII	Photosystem II
PUFA	Polyunsaturated Fatty Acid
rETRmax	Maximum Relative Electron Transport rate
SFA	Saturated Fatty Acid
UMACC	University of Malaya Algae Culture Collection
UVR	Ultraviolet Radiation
min	Minute
nm	Nanometer
%	percent
rpm	Rotation per minute
α	Alpha

University of Malaya

LIST OF APPENDICES

APPENDIX A Composition of Provasoli Medium……………………..…. 142

APPENDIX B Water PAM settings……………………………………… 144

APPENDIX C List of all the primers obtained from literature……..…… 145

APPENDIX D Primer design steps…………………………………..….. 146

APPENDIX E Result of running RT-qPCR product of the genes of interest on the Agarose gel 3%... 172

APPENDIX F BLAST results of the sequenced PCR product …………. 173

University of Malaya

CHAPTER 1: INTRODUCTION

1.1 Introduction

Global warming has received considerable attention in science, environment, and international economics and politics (Zhao and Su, 2014). It has been shown that climate changes are affecting the base of the food web and these effects are transmitted up through the food chain (Montes-Hugo et al., 2009). On a global scale, species respond to thermal stress with phenologlical changes and distributional range shifts that often involve local extinction (Jueterbock et al., 2014). Algae in the ocean and freshwater ecosystems are responsible for a high ratio of net global primary productivity (Beardall and Raven, 2004). Although algae can grow in a wide range of temperature (5–35 °C) (Rashid et al., 2014), and are able to easily tolerate temperatures of up to 15 °C lower than their optimal temperature, exceeding the optimum temperature by only 2 – 4 °C may result in total culture loss (Mata et al., 2010). Temperature is considered as a key factor for algae growth and it highly impacts their cellular chemical composition, the uptake of nutrients, carbon dioxide fixation, and the growth rates (Renaud et al., 2002).

Basically, microalgae use several strategies to survive under abiotic stress condition; for example, adjusting the saturation level of fatty acids in the membrane to regulate membrane fluidity, reducing protein synthesis to avoid misfolded proteins, accumulating compatible solute to maintain cell osmosity, producing enzymes to scavenge reactive oxygen species and adjusting the photosynthesis rate. Under optimal conditions of growth, algae synthesize fatty acids principally for esterification into glycerol-based membrane lipids, which constitute about 5–20% of their dry cell weight (DCW) (Hu et al., 2008). Fluctuations in temperature affect the fluidity of cytoplasmic and thylakoid membranes. In one hand, cold causes a reduction in the membrane fluidity (membrane rigidification), which can be compensated by desaturation of

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membrane lipids by fatty acid desaturases (FADs). On the other hand, heat causes the fluidization of the membranes. This is compensated by the synthesis of the membrane-stabilizing proteins and by the replacement of unsaturated fatty acids (UFAs) in membrane lipids by the de novo synthesized saturated FAs (Los et al., 2013). In polar microalgae, increases in unsaturated bonds promote a looser packing of lipids and decreased the solidification temperature (Lyon and Mock, 2014).

Under harsh conditions, there is an increase of cell maintenance costs due to the higher energy demand to maintain homoeostasis (Calow, 1991). Hence, carbohydrate metabolism is affected by abiotic stress resulting in a decrease of carbon storage and an increase in levels of dissolved sugars (Arbona et al., 2013). González and Ballesteros (2012) reviewed the effects of several environmental factors on the carbohydrate content of algae. Cultivation parameters such as limiting nutrients or starvation, inorganic carbon supply, sodium chloride concentration, irradiance and temperature can all affect the activity of enzymes associated with carbohydrate accumulation in algae (Vinocur and Altman, 2005). Interestingly, different algae have shown different patterns in carbohydrate homoeostasis when exposed to temperature changes; for example, in *Spirulina maxima,* elevated temperature significantly increased the carbohydrate content (Oliveira et al., 1999), while in six *Caulerpa spp*. isolated from the Gulf of Mexico, the total carbohydrate content increased during cold seasons (Rathore et al., 2009). In contrast, in a study on tropical microalgae from Australia, no consistent pattern was observed in carbohydrate content in response to temperature treatments (Renaud et al., 2002).

Elevated temperature causes disruption of weak interactions in protein structure, thus affecting the stability of protein conformational by altering the stability of the unfolded as well as of the native forms of protein (Daniel et al., 1996). Accumulation of unfolded proteins under heat stress (HS) would unleash a signalling cascade triggering the

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production of compatible solutes and molecular chaperones that cooperatively act to re-establish protein homeostasis. Heat shock proteins (HSPs) are the well-known molecular chaperones that comprise a group of highly conserved proteins that can be induced upon subjecting organisms to high-temperature stress (Liu et al., 2014). Among *HSP* families, the *HSP70* and *HSP90* are two most studied under different environmental stress conditions. It has been proposed that *HSP70* gene to be considered a good candidate for bio-monitoring assays because the *HSP70* gene is a sensitive biomarker for different classes of environmental stresses in green algae (Chankova et al., 2013). Also, HSP90s are one of the most abundant proteins, accounting for 1–2% of all cell proteins under non-stressed conditions (Rosic et al., 2011) and proposed as a potential biomarker in algae (Liu et al., 2014). In contrast, there are other proteins up-regulated during abiotic stress and trigger protein degradation pathway, ubiquitin is a well-studied protein consisting 76-residues protein found in most phyla, marks proteins for rapid degradation. Increased ubiquitin levels indicate higher levels of protein degradation, and thus increased protein turnover. Ubiquitin is induced by diverse types of stresses in the reflection of the need for more extensive protein turnover in stressed cells. Moreover, ubiquitination plays a main regulatory role in most eukaryotic cellular processes such as receptor endocytosis, intracellular signalling, cell-cycle control, transcription, DNA repair, gene silencing, and stress response (Shahsavarani et al., 2012). Expression of ubiquitin in the response to heat stress in algae was reported also (Vayda and Yuan, 1994; Kampen et al., 1995).

Environmental changes generally cause an imbalance between energy supply and consumption in photosynthetic alga that leads to an alteration of the photosynthetic apparatus and consequently photosynthetic temperature acclimation (Ras et al., 2013). Among various machinery of photosynthesis, the photosystem II complex (PSII) is particularly sensitive to heat, and even a short period of exposure to high temperatures

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irreversibly inactivates the oxygen-evolving complex of PSII (Allakhverdiev et al., 2007). Three photosynthetic related genes including *psbC*, *psaB* and *rbcL* are vastly studied to monitor photosynthetic respond in microalgae. For example, *psbC* encodes the chlorophyll–protein complex CP43, that is one of the interior transducers of excitation energy from the light-harvesting pigment proteins to the photochemical reaction centre II (Qian et al., 2008). It involved in splitting, acting as an oxygen-evolving enzyme of photosynthesis as well (Qian et al., 2009). *PsaB* encodes for the photosystem I (PSI) protein, which binds approximately 100 Chlorophyll *a* and 30 b-carotene molecules. As the photochemical reaction centre, this protein coordinates most of the electron transfer cofactors and acts as an inner antenna (Qian et al., 2009). Photosynthetic carbon fixation considered as a possible site of temperature sensitivity (Lilley et al., 2010). Ribulose-1, 5-bisphosphate carboxylase oxygenase (Rubisco) catalyses the first step of the Calvin–Benson–Basham cycle, the pathway responsible for the vast majority of carbon fixation by phytoplankton (Knopf and Shapira, 2005).

Understanding the biological principles and regulatory networks on which algae live on can explain their survival, reproduction, and distribution. In addition, knowledge on their responses toward stress is valuable if algae are to be used to meet the increasing need for feed, food, and biomass (Moreno-Risueno et al., 2010). The genus *Chlorella* (Chlorophyta, Trebouxiophyceae) is one of the most studied microalgae in the world and commercially cultivated by more than 70 companies globally (Spolaore et al., 2006). Annually, the biomass production of *Chlorella* transcends 2,000 tons (Pulz and Gross, 2004), and most of the biomasses are used for dietary supplements and nutraceuticals, with a minute fraction destined to the cosmetic market and aquaculture (Spolaore et al., 2006). *Chlorella* grows in brackish, fresh and also in marine water. Numerous species have been isolated from the temperate, tropical and Polar regions (Guccione et al., 2014). *Chlorella* is rich in protein with a balanced amino acid

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composition (Becker, 2007), and comprises a good content of vitamins, minerals, pigments (Jin and Hu, 2013) and short-chain polyunsaturated fatty acids, including oleic and linoleic acids (Petkov and Garcia, 2007).

1.2 Problem statement

Microalgae as the primary producers in the aquatic ecosystems are in general, adversely affected by global warming. The heat stress has been reported to inhibit growth and photosynthesis, and modify the biochemical composition of algae. Their various ecological niches may provide different responses to varying degree of temperature increase.

1.3 Research Question

1. Does elevated temperature affect the growth, photosynthetic performance and biochemical composition of *Chlorella* sp. from the Antarctic, temperate and tropical regions?
2. What is the expression pattern of the selected genes in *Chlorella* sp. from the Antarctic, temperate and tropical regions in response to heat stress?

1.4 Hypothesis

H_{0a} = There are no significant differences in growth among marine *Chlorella* strains from different latitudes in response to elevated temperature.

H_{1a} = There are significant differences in growth, among marine *Chlorella* strains from different latitudes in response to elevated temperature.

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H_{0b} = There are no significant differences in photosynthesis among marine *Chlorella* strains from different latitudes in response to elevated temperature.

H_{1b} = There are significant differences in photosynthesis among marine *Chlorella* strains from different latitudes in response to elevated temperature.

H_{0c} = There are no significant differences in biochemical composition among marine *Chlorella* strains from different latitudes in response to elevated temperature.

H_{1c} = There are significant differences in biochemical composition among marine *Chlorella* strains from different latitudes in response to elevated temperature.

H_{0d} = There are no significant differences in selected genes on their expression among marine *Chlorella* strains from different latitudes in response to elevated temperature.

H_{1d} = There are significant differences in selected genes on their expression among marine *Chlorella* strains from different latitudes in response to elevated temperature.

1.5 Objectives

The following are the objectives of the proposed study:

1. To investigate the effects of elevated temperature on growth, photosynthetic performance and biochemical composition of marine *Chlorella* sp. from the Antarctic, temperate and tropical regions.

2. To determine the expression patterns of selected genes in *Chlorella* sp. from the Antarctic, temperate and tropical regions in response to heat stress.

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CHAPTER 2: LITERATURE REVIEW

2.1 Climate Change and Global Warming

Climate change is a global concern and is primarily caused by natural and human activities. In the past century, human activities caused the emission of greenhouse gases (GHG), such as carbon dioxide (CO_2), nitrous oxide (N_2O), methane (CH_4), and halocarbons (a group of gases containing fluorine, chlorine, and bromine). These gases act as a heat trap and subsequently increase the atmospheric temperature. CO_2 is largely responsible for this increase of temperature in the past few decades (Forster et al., 2007). The burning of fossil fuels is the main source of CO_2 in the atmosphere (Raj, 2016). According to the Intergovernmental Panel on Climate Change (IPCC), a global warming trend of 0.85 °C was documented from 1880 – 2012, with the average increase of 4–5 °C expected by the end of the 21st century (IPCC, 2013). In addition to increasing the mean annual temperatures, the period, incidence, and severity of periods with extraordinarily high temperatures are all also increasing (Easterling et al., 2000; Tripathi et al., 2016).

Global warming is one of the most deleterious factors affecting aquatic ecosystems, which sequentially leads to significant shifts in aquatic biogeochemical cycles, dynamics, biodiversity, and aquatic food web structure (Harley et al., 2006; Wrona et al., 2006). Temperatures increase caused by global climate change affect species' phenology and distributions (Parmesan, 2006). The impacts of global warming on species interactions are more challenging to elucidate, as warming might alter the outcome of competition and change the flow of energy via food webs (West and Post, 2016). In aquatic ecosystems, algae as primary producers play a vital role in carbon sequestration and forming the basis of both freshwater and marine food chains (Sayre, 2010; Tsai et al., 2015). Their roles and distribution are influenced by the shift in environmental factors due to global warming (Beardall and Raven, 2004; Bopp et al,

University of Malaya

2005; Paul, 2008; Olsenz, 2011). Acute changes in temperature, irradiance, salinity, or pH will influence their cellular integrity and biomolecules, thereby disrupting cellular homoeostasis (Jauzein and Erdner, 2013; Van et al., 2015). Environmental stress could lead to severe damage and initiate responses that result in either acclimation or programmed cell death, depending on the sensitivity of the algae (Zuppini et al., 2007; Sulmon et al., 2015). The effect of elevated temperature on algae can be investigated by focusing on several algae cell elements, such as lipids and membrane, proteins, carbohydrates, and photosynthetic components (Juneja et al., 2013; Minhas et al., 2016).

2.1.1 Global Warming and Antarctic

The Antarctic is Earth's southernmost continent. It contains the South Pole and is situated in the Antarctic region of the Southern Hemisphere, almost entirely south of the Antarctic Circle, surrounded by the Southern Ocean. It is the 5th largest continent, at 14,000,000 km^2. It is known that climate change in the polar regions could affect the mass balance of the polar ice sheets, and the resulting changes in sea-level would have global implications (King, 1991).

Since the 1950s, the surface temperatures have increased in the Antarctic. The highest ever temperature of 17.5 °C was reported in March 2015 by Esperanza Base (Argentina), at the northern tip of the Antarctic Peninsula (Howard, 2015). Model simulations on the Thwaites Glaciers Basin, West Antarctica by Joughin *et al*., (2014) reported the breaking up of a glacier that was previously assumed to be stable for at least a few millennia. Rignot *et al.* (2014) analyzed observational data from 1992 – 2011 from Earth Remote Sensing (ERS-1/2) satellite radar interferometry. The data confirmed that the glaciers have withdrawn from their original locations. The Pine Island Glacier shrunk to 31 km at its core, with the highest withdrawal reported

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occurred during 2005–2009. Thwaites Glacier withdrew 14 km to its core and 1–9 km on its side. Haynes Glacier withdrew 10 km on its sides. Smith/Kohler Glacier withdrew 35 km across its ice plain (Rignot *et al.*, 2014). Based on the aforementioned reports, this area of Antarctica is undergoing marine ice instability, which will increase the sea level in a few decades. Greenbaum *et al.* (2015) anticipated a minimum of 3.5 m sea level increase and identified ocean water infiltration underneath the glaciers. The instabilities from ice-ocean interaction in East Antarctica could have substantial global impacts.

Climate change in the Antarctic can adversely affect habitats at macro and micro levels. For example, Lynch and LaRue (2014) reported the first global survey of the Adélie Penguin, which was done using high resolution (~0.6m) satellite imageries and field counts. They showed that the decrease of Adélie Penguin on the Antarctic Peninsula is offset by increases in East Antarctica. Another research reported that the reduction of Adélie Penguin population is linked to the increase of sea ice in the Ross Sea region, possibly due to the elimination of Antarctic toothfish, which competes for the same prey as the penguins (Lyver *et al.*, 2014). Cimino *et al.* (2016) anticipated that a third of Adélie Penguin colonies in Antarctica could vanish by 2060 due to the effect of climate change on its food supply. Individual penguin might respond to global warming with an enhanced metabolic rate, but as long as the metabolic necessities do not surpass ingestion rates, krill productivity can be boosted with temperature increase in Antarctica (Constable *et al.*, 2014). However, this response is expected to be negative around South Georgia with increasing water temperatures. Further temperature increases could result in, metabolic costs, which could go beyond its physiological limit (Wiedenmann *et al.*, 2009; Hill *et al.*, 2013). A reduction in winter sea-ice coverage in the Antarctic Peninsula and Scotia Sea could result in a decreased number of krill (Trivelpiece *et al.*, 2011). Future Ocean warming may lead to a shift in krill habitat (Hill

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et al., 2013). Furthermore, the decline of the food supply of krill and the rapidly changing climate in the Southern Ocean are threatening fur seals (Hill *et al.*, 2013). Crabeater, leopard, and Ross seals, all of which inhabit the pack ice surrounding the Antarctic Treaty zone, are mostly influenced by sea ice decrease (Turner *et al.*, 2014). Constable *et al.* (2014) reviewed the impact of climate change on autecology of marine biota of Antarctic regions, such as zooplankton, microbes, Antarctic krill, salps, fish, cephalopods, seabirds, marine mammals, and benthos. The impacts of climate change may be associated with ecological adaptivity and tolerance potentials of the taxa, but it is more important that we examine how well species respond evolutionarily to the constant and rapid changes in ecosystems (Turner *et al.*, 2014). There were several studies predicting the effect of elevated temperatures on Antarctic algae which shown their ability to tolerate temperature above their ambient, however, their biochemical composition (nutritional content) were significantly affected (Hosono et al., 1994; Teoh et al., 2004; Chen et al., 2012;.Wong et al., 2015; Cao et al., 2016).

2.1.2 Global Warming and Temperate

Shanley *et al.* (2015) discussed the consequences of climate change in the northern coastal temperate rainforest of North America, focusing on the terrestrial ecological and hydro-ecological systems. These changes are expected to result in a cascade of ecosystem-level effects together with amplified rate of flooding and rain and snow, an higher snowline and reduced snowpack, changes in the timing and extent of stream flow, freshwater thermal regimes and riverine nutrient exports, and reduction in alpine habitats (Shanley *et al.*, 2015).

Peters *et al.* (2013) studied the long-term impacts of two possible future climate and atmospheric CO_2 scenarios on ecosystem function in jungles throughout the Great Lakes region of North America by ecosystem model PnET-CN. The result proposed that the

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productivity of Great Lake jungles will shift from being temperature-limited to water-limited by the end of the century. Campioli *et al.* (2011) reviewed the current status of production and biogeochemistry and their respective exposure and sensitivity to climate change in Belgium. The climate, environment, and forest conditions of Belgium are typical of the temperate oceanic region (Lindner *et al.*, 2010; Campioli *et al.*, 2011). In the long run, nutrient deficiencies at deprived sites may reduce forest production. Also, sensitive species, such as Norway spruce and beech, could experience limited growth due to drought. Drought conditions are anticipated to increase in the future, but opposing effects are more likely upon a rather limited number of tree species (Campioli *et al.*, 2011). The relationship between climate changes and disease risks from some pathogens is fast gaining attention. Hakalahti *et al.* (2006) pointed out that temperature significantly controls the transmission and reproduction of parasites in northern latitudes. A study on climate warming that changed the life history dynamics of the directly transmitted crustacean ectoparasite *Argulus coregoni* and complex life cycle trematode *Diplostomum spathaceum* could be risky to fish farming via amplified infection pressure (Hakalahti *et al.*, 2006).

Galicia and Gomez-Mendoz (2010) stated that the influence of variations in temperature and precipitation modelled under climate change scenarios will decrease the current ranges of geographic distribution of almost all species of oaks and pines. The most influenced species would be *Pinus rudis*, *P. chihuahuana, P. culminicola, P.oocarpa, Quercus acutifolia, Q. crispipilis* and *Q. peduncularis* under both conservative and severe climate change scenarios (Galicia and Gomez-Mendoz, 2010). The temperate region of Australia, also is known as a global centre for marine biodiversity and its waters have undergone a higher degree of warming than the global ocean. Projected rises in temperature are expected to cause further range shifts of algae and associated species and local extinctions for species that have their northern borders

University of Malaya

with the southern coastline (Wernberg et al., 2011). In a study of two temperate benthic diatoms species, (*Amphora cf. coffeaeformis* and *Cocconeis cf.sublittoralis*) exposed to temperatures ranging from 5 °C to 50 °C after a short-term exposure to a range of UV irradiances, significant differences were observed in their growth and photosynthetic performance and sign of photoinhibition observed suggesting high possibility of growth and photosynthesis inhibition due to warmer environment (Salah et al., 2011).

2.1.3 Global Warming and Tropics

The climate is also changing in the tropics (IPCC, 2013). Tropical jungles are the functional lung of the earth, and minor changes in the tropics can have an abundant impact on the entire planet's ecosystem. The tropics will experience the impacts of global warming earlier than the poles. Many regions of the subtropical and tropical continents, such as southern Amazonia, Australia, and southwestern and central United States have frequently experienced extreme droughts over the past few decades, accompanied by a growing wetness over the equatorial regions (Fu, 2015). The tropics have warmed to a mean ratio of 0.26 °C/decade (Malhi and Wright, 2004). A slight alteration in the environment in the tropics will be quickly felt. Due to the model's uncertainty, Corlett (2012) anticipated that the tropics are getting warmer by at least 2–3 °C by 2100, 4–6 °C, or even 7 °C.

Salazar *et al.* (2007) studied the impacts of anticipated climate change on the biome distribution in South America. Their results in the worst-case scenario suggested an increased coverage of savanna areas by the cost of tropical jungle areas. The climate variability, drought incidence, and seasonality of soil moisture, along with other aspects that may interact synergistically like fire, are critical factors for determining forest-savanna boundaries and vegetations' vulnerability in the Amazon (Hutyra *et al.*, 2005). In Central America, factors such as El Niño are projected to increase under future

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climate scenarios (Karmalkar *et al.*, 2011). Similar to the Amazon, Central America risk huge losses of jungle biomass under increased drying and warming scenarios in the 21st century (Lyra *et al.*, 2016; Lyra *et al.*, 2017).

Based on the analysis of nearly 50 years of temperature data sets in Malaysia, Wai *et al.* (2005) reported a substantial increase in the average yearly temperature, ranging from 0.99 – 3.44 °C/century. They also examined the average yearly temperature regression lines (for all the analysed stations), which confirmed that the global warming trend has increased in the past 30 years. This study agreed with IPCC's report. A similar study conducted by Tangang *et al.* (2007) reported notable warming trends of 2.7 – 4.0 °C/century for the past 42 years, from 1961 to 2002. Climate change may result in increased frequency and intensity of extreme weather events, such as droughts, storms, and floods in Malaysia (Rahman, 2009). Fourteen Global Climate Models (GCM's) stated a prediction, which illustrates that Malaysia could experience temperature increases from 0.7 °C to 2.6 °C (Rahman, 2009). The potential impacts of climate change in the Malaysian environment would consist of reduced crop yields, sea level increase, higher risk of diseases among forest species and biodiversity loss, tidal inundation of coastal areas, coral reef bleaching, decreased water availability, loss of biodiversity, added droughts, and others (Rahman, 2009). The impact of climate change on Malaysia's rich biodiversity is of foremost concern, where, with the complex interspecific relationship between animal and plant species, impact on one could well impact the other. In the years between 1997 and 1998, the most severe and widespread bleaching of corals happened and the reefs in 42 countries were affected, with extensive coral mortality being reported in South of Japan, Seri Lanka, the Maldives, India, Kenya, Tanzania, Seychelles and other sites in the Indopacific (Fitt et al., 2001). Rising sea surface temperature (SST) in tropical/subtropical waters have moved reef-building corals, 0.5 °C nearer to their upper thermal boundaries. Natural temperature fluctuations

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can drive corals into temperatures that result in bleaching faster than in the past. During summer when SST exceed the maximum by more than 1 °C for one month or more, corals bleach by expelling their symbiotic algae, showing either the pale colours of coral pigments or the brilliant white skeleton (Eakin et al., 2008). In the 2010/11 austral summer a severe marine heatwave which influenced 2000 km of the midwest coast of Australia occurred with SST anomalies of 2-5 °C above normal climatology. The heat wave was influenced by a strong Leeuwin Current during an extreme La Nina event at a global warming hot spot in the Indian Ocean. This contest had a vital effect on the oceanic ecosystem with changes to seagrass or algae and coral habitats, as well as fish kills and southern expansion of the range of several tropical species (Caputi et al., 2016).

2.2 Abiotic Stress and Algae

Algae, like other marine organisms living in intertidal or shallow subtidal habitats, are regularly exposed to strong water motion and subjected to extreme fluctuations in temperature, pH, irradiance, salinity or nutrient availability, and the amplitude of these fluctuations are far exceeded climate changes prediction in the coming decades (Olsenz, 2011). Principally, at the population level, physiological responses to climate change will display as changes in the timing of annually recurring events (phenology), abundance, and the spatial organization (dispersion and distribution) of organisms. Suboptimal states and weak individual performance can result in reduced population productivity as well as decreased resilience to disturbance. Well-documented biological impacts from climate change involve shifts in population range and distributions (Doney et al., 2001). While, at the species level, responses to environmental changes can be described to a small set of basic alternatives: (i) persistence without acclimatization or adaptation (tolerance), (ii) persistence with acclimatization or

University of Malaya

adaptation, (iii) persistence enabled by migration to remain within some particular climatic niche and (iv) extinction (Harley et al., 2012).

Algae respond to environmental changes by utilizing diverse strategies. For example, heat stress affects membrane fluidity, and algae, in response, can modify their membrane fatty acid composition to maintain homoeostasis (Morgan-Kiss et al., 2006). Moreover, algae protect their proteins and enzymes using molecular chaperones, such as heat shock proteins (HSPs) or degrade denatured proteins in processes involving ubiquitin (Craig et al., 1993). In addition, algae regulate their carbohydrate concentrations and structures to utilise the energy of endogenous carbon sources efficiently and protect other molecules via accumulation of compatible solutes (Welsh, 2000; Daroch et al., 2013). Algae regulate the photosynthetic machinery in order to acclimatise to stress conditions. Furthermore, these characteristics of algae can be exploited for the production of desired metabolites through the abiotic stress as a tool integrated with microalgal biorefinery for its sustainable development (Paliwal et al., 2017).

2.2.1 Abiotic Stress Effects on Lipids and Membrane of Algae

The capability of algae to survive against various ecological conditions is strongly reflected in the unusual pattern of their cellular lipids and their capacities for changing lipid metabolism (Thompson, 1996; Wang et al., 2016). The physical properties of the membrane rely on its fatty acid profile and the degree of saturation which regulates the fluidity of membranes (Singh et al., 2002). The fluidity of membranes is affected by fluctuations in temperature. Low temperature reduces membrane fluidity, which is ameliorated via desaturation of membrane lipids by fatty acid desaturases (FADs) (Sakamoto and Murata, 2002). Thus, cells maintain their membrane fluidity at low temperature by increasing the content of membrane unsaturated fatty acids (UFAs) to

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achieve a looser packing of lipids and decrease the solidification of membrane lipids (Lyon and Mock, 2014). Conversely, heat increases membrane fluidity, which is ameliorated by the incorporation of *de novo* synthesised saturated fatty acids (SFAs) into membrane lipids, and the presence of membrane-stabilizing proteins (Los et al., 2013). Moreover, shortening the length of fatty acids in membrane lipids is another acclimation strategy vis-à-vis elevated temperature (Dodson et al., 2014).

Basically, two main family groups of enzymes control fatty acid structures; desaturases and elongases (Khozin-Goldberg and Cohen, 2011). During stress conditions, cells also show an increase in fatty acid synthesis. The mechanisms involved in lipid homoeostasis can be monitored by analysing the expression of the central regulatory genes, such as genes encoding subunits of acetyl-CoA carboxylase (ACCase), ketoacyl-ACP synthase (KAS), desaturase, and elongases. ACCase, which consists of four subunits, namely *accA*, *accB*, *accC*, *accD*, regulates the rates of fatty acid synthesis (Podkowinski and Tworak, 2011; Singh et al., 2016). In certain stress conditions, such as metal stress (e.g. iron), up-regulation of ACCase subunits was observed along with elevated ACCase activity and fatty acid synthesis in *Chlorella sorokiniana* (Wan et al., 2014). ACCase activity yields malonyl-ACP, where the malonyl entity participates in a series of condensation reactions, leading to the lengthening of the precursor fatty acid in which KAS is the rate-limiting enzyme (Rismani-Yazdi et al., 2011; Lei et al., 2012). In *Dunaliella salina*, higher levels of KAS were observed when cells were exposed to higher salinity (Azachi et al., 2002). It is important to note that the SFAs formed might undergo desaturation and elongation steps to form longer chains of unsaturated fatty acids, or polyunsaturated fatty acids (PUFAs), such as arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) (Pereira et al., 2003).

University of Malaya

In eukaryotes, energy can be stored as triacylglycerols (TAGs), which act an essential carbon source (Merchant et al., 2012), contributing to a vital part of survival in stressful conditions (Singh et al., 2002; Fan et al., 2014). Most microalgae accumulate TAG under nitrogen or phosphorus limiting conditions (Fan et al., 2014), high salinity (Bartley et al., 2013) and also temperature stress (Fakhry and Maghraby, 2015; Elsayed et al., 2017). Two pathways to form TAG are emphasised; namely the acyl-CoA dependent (Kennedy pathway) and the acyl-CoA-independent (Lenka et al., 2016). In algae, the Kennedy pathway is the primary pathway where TAG is produced through the sequential transfer of acyl groups from acyl-CoA to the various positions of the glycerol-3-phosphate backbone, facilitated by acyltransferases such as glycerol-3-phosphate acyltransferase (GPAT), acyl-CoA: lysophosphatidic acid acyltransferase (LPAAT), and the acyl-CoA: diacylglycerol acyltransferase (DGAT). GPAT transfers the FA from the acyl-CoA pool (acyl-ACP in plastids) and its overexpression resulted in up to 50% increase of TAG in *Chlamydomonas reinhardtii* without affecting cell growth (Iskandarov et al., 2016). DGAT catalyses the last step of TAG assembly and is proposed as a rate-limiting step of TAG biosynthesis (Lenka et al., 2016). Transformation of the gene encoding DGAT was used as a strategy to obtain higher lipid content in *C. reinhardtii* (Ahmad et al., 2014). Alternatively, TAG can also be produced by three enzymatic reactions in the acyl-CoA-independent pathway, involving phospholipid diacylglycerol acyltransferase (PDAT), diacylglycerol acyltransferase (DGAT), and diacylglycerol transacylase (DGTA) (Chen and Smith, 2012). PDAT is reported as the main enzyme for TAG biosynthesis in *C. reinhardtii* (Yoon et al., 2012) and increased levels of PDAT were observed when the microalga was subjected to nutrient limitations, such as iron (Urzica et al., 2013) and nitrogen (Boyle et al., 2012).

PUFAs are fatty acids containing 18 carbons or more with the presence of two or more double bonds (Leonard et al., 2004). They affect membrane fluidity owing to their

University of Malaya

very low melting points. Therefore, the solidification of the membrane lipids can be reduced by increasing the ratio of PUFAs in the membrane (Brett and Müller-Navarra, 1997). Among the enzymes involved in lipid metabolism, Δ9 fatty acid desaturases (FADs) catalyse the first committed step in desaturation and initiate the conversion of SFAs to mono-unsaturated fatty acids (MUFAs), which are essential for the generation of PUFAs (Xue et al., 2016). These desaturases introduce the first double bond to palmitic acid (C16:0) and stearic acid (C18:0) and convert them to palmitoleic acid (C16:1) and oleic acid (C18:1), respectively (Xue et al., 2016). Up-regulation of several fatty acid desaturases (*Δ9ACPCiFAD*, *Δ12CiFAD*, *ω3CiFAD2* and *Δ6CiFAD*) was observed in the Antarctic ice *Chlamydomonas* sp. *ICE-L* when it was exposed to salinity stress (An et al., 2013). Antarctic *Chlorella vulgaris,* in response to cold and salt stress, up-regulated the Δ12 FAD, which catalyses the desaturation at the Δ12 position (Lu et al., 2010). Under low temperature (0 °C), *Δ9CiFAD*, *ω3CiFAD1* and *ω3CiFAD2* were up-regulated in the microalga while *Δ6CiFAD* increased with increasing temperature. However, the temperature did not cause deregulation of *Δ12CiFAD* except at a specific temperature (15 °C). These observations suggested that *ω3CiFADs* might have important roles in cold adaptation while *Δ6CiFAD* enhanced survival under high temperature (An et al., 2013). Stearoyl-ACP desaturase (SAD) is another enzyme involved in oleic acid (18:1) synthesis via the insertion of the first double bond into stearic acid (18:0) (John and Cahoon, 1998; Shanklin and Somerville, 1991). Stress conditions such as high light and nitrogen deficiency (N-) drastically up-regulated the transcripts of *SAD* in *Chlorella zofingiensi* and resulted in the accumulation of total fatty acids including oleic acid (Liu et al., 2012). Interestingly, it was noted that by increasing the levels of unsaturated fatty acids in the membrane, the photosynthetic machinery of algae could also be stabilised under chilling (Wada, et al., 1994) and salinity stresses (Allakhverdiev et al., 2001).

University of Malaya

2.2.2 Abiotic Stress Effects on Protein

Heat stress has been shown to induce the aggregation and denaturation of proteins, resulting in membrane injury and alterations to metabolic fluxes (Fu et al., 2008). Consequently, accumulation of unfolded proteins under stress conditions triggers the expression of molecular chaperones, such as heat shock proteins (HSPs), that act to re-establish protein homoeostasis (Fulda et al., 2010). Expression of HSPs induced by high-temperature is commonly observed in various living organisms (Parsell and Lindquist, 1993; Gupta, 2010). According to their molecular masses, HSPs are categorised into HSP100, HSP90, HSP70, HSP60, HSP40 and the small HSPs (Waters et al., 1996). HSP90s known to have a noticeable function in conserving protein homoeostasis by stabilising and maintaining the conformation of unstable proteins close to their native forms. In addition to its role in maintaining proper assembly of protein complexes, HSP90 is also involved in various signalling and cellular pathways (Richter and Buchner, 2001; Young et al., 2001). HSP90 varies from 82 to 96kD. Proteins that belong to this class function as ATP-dependent chaperones that bind to highly structured folding intermediates, preventing aggregation. HSP90s can act alone or in concert with other proteins (Reddy et al., 1998). It has been suggested that the *HSP90* complex performs this "buffering" activity by the activation/folding of signalling proteins that have variable domains such as R proteins (Sangster and Queitsch, 2005), which are part of the disease resistance response in plants (Krishna and Gloor, 2001). HSP90 is essential for maintaining the activity of numerous signalling proteins; it plays a key role in cellular signal transduction networks. At a molecular level, Hsp90 binds to substrate proteins, which are in a near-native state and thus at a late stage of folding (Jakob et., 1995) poised for activation by ligand binding or interaction with other factors. In fulfilling its role, HSP90 operates as part of multi-chaperone machinery in the cytosol, which includes HSP70 (Bose et al., 1996). On the other hand, HSP70

chaperones are involved in other aspects of protein processing, specifically protein translocation and protein folding (Bukau and Horwich, 1998). Some members are constitutively expressed (HSC) whereas others are expressed only under environmental stresses. They cannot act by themselves and work with two co-chaperones namely DnaJ homologs and HSP40 in eukaryotes in an ATP dependent reaction. The major function of this protein seems to be protein folding (Diefenbach and Kindl, 2000). Another function is folding and transport of proteins into the chloroplast and the mitochondrion (Zhang and Glaser, 2002). Chloroplast stromal HSP70B in *C. reinhardtii* and *Dunaliella* were reported to play a role in molecular protection and repair of PSII (Schroda et al., 1999; Yokthongwattana et al., 2001). HSP70B was also suggested as a stress marker for *Chlorella* sp. from different habitats (Chankova et al., 2013). For the Antarctic algae, an increase of 5–10 °C above normal temperature triggered the expression of *HSP70B* gene (Vayda and Yuan, 1994). Moreover, the expression of several small *HSPs* gene was up-regulated at species-specific threshold temperature indicating the association of temperature sensing systems in algae (Kobayashi et al., 2014). Also, significantly higher levels of *HSP* gene were detected in *C. acidophila*, suggesting that these chaperones might contribute to the survival of acidophilic algae under extreme conditions (Gerloff-Elias et al., 2006).

In addition to HSPs, several other proteins are involved in protecting cells against stress-induced damages. Late-embryogenesis abundant (LEA) proteins in the higher plant *Macrotyloma uniflorum* act as molecular shields to prevent interactions of aggregation-prone protein species by steric or electrostatic repulsion (Tunnacliffe and Wise, 2007; Veeranagamallaiah et al., 2011). These proteins can be classified into six groups, localised in both nuclear and cytoplasm regions (Hong-Bo et al., 2005). LEA proteins have also been identified in algae (Honjoh et al., 1995; Honjoh et al., 2000; Lan et al., 2013). They are highly hydrophilic and are thought to stabilise other proteins

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and membranes during dehydration via a mechanism involving hydrogen-bond stabilisation (Shih et al., 2008). Expression of LEA could be triggered by water stress such as cold shock or desiccation during cold acclimation and drought stress in Arabidopsis thaliana (Wang et al., 2011). Stable protein 1 (SP1) is another protein that plays a role in protein homoeostasis and is expressed during temperature stress (Wang et al., 2002). Its overexpression in plants noticeably decreases levels of reactive oxygen species (ROS) (Ling and Jarvis, 2015).

During stress, proteins that are irreversibly denatured or misfolded are labelled by ubiquitins for degradation (Hershko and Ciechanover, 1998). The ubiquitinated proteins can then be acted upon by proteolytic enzymes known as proteasomes. Ubiquitination can be induced by different stresses and it regulates various biological processes in eukaryotes, namely receptor endocytosis, cell-cycle control, intracellular signalling, transcription, gene silencing, DNA repair, and stress responses (Kwapisz et al., 2005; Kaliszewski and Zoladek, 2008). Enhanced ubiquitin levels are associated with increased levels of protein degradation and turnover (Hawkins, 1991; Shahsavarani et al., 2012). Up-regulation of ubiquitin has been observed in algae in response to heat (Pearson et al., 2010) and light stress (Heinrich et al., 2012). Furthermore, under unfavourable conditions such as heat stress, protein synthesis might be reduced as a strategy to limit the risk of generating misfolded proteins (Allakhverdiev and Murata, 2004; Chankova et al., 2013; Pancha et al., 2014). During stress conditions, the accumulation of several amino acids originating from either protein breakdown or *de novo* synthesis was reported (Lankadurai et al., 2013). These include several amino acids, mainly those derived from oxaloacetate and pyruvate (Ala, Asp, Ile, Leu, Pro, Thr, Val) (Kaplan et al., 2004; Guy et al., 2008). Eventually, degradation of denatured proteins results in a pool of amino acids that serve as building blocks for *de novo*

University of Malaya

protein (re)synthesis, although some of these amino acids, such as proline, might function as compatible solutes as well.

2.2.3 Abiotic Stress Effects on Carbohydrate

According to the energy allocation models, there is an increase of cell maintenance costs under harsh conditions due to the higher energy demand to maintain homoeostasis (Calow, 1991). Hence, carbohydrate metabolism is affected by abiotic stress resulting in a decrease of carbon storage and an increase in levels of dissolved sugars (Arbona et al., 2013). González and Ballesteros (2012) reviewed the effects of several environmental factors on the carbohydrate content of algae. Cultivation parameters such as limiting nutrients or starvation, inorganic carbon supply, sodium chloride concentration, irradiance and temperature can all affect the activity of enzymes associated with carbohydrate accumulation in algae (Vinocur and Altman, 2005). Interestingly, different algae have shown different patterns in carbohydrate homoeostasis when exposing to temperature changes; for example, in *Spirulina maxima* the elevated temperature significantly increased the carbohydrate content, (Oliveira et al., 1999) while in six *Caulerpa* spp. isolated from the Gulf of Mexico, the total carbohydrate content increased during cold seasons (Rathore et al., 2009). In contrast, in a study on tropical microalgae from Australia, no consistent pattern was observed in carbohydrate content in response to temperature treatments (Renaud et al., 2002). Among carbohydrates, sucrose is the major product of photosynthesis and plays crucial roles in growth, development, storage, signal transduction and acclimation to environmental stresses in plants and algae (Jiang et al., 2015). Sucrose formation is heavily temperature dependent and is enhanced by an increase in temperature, which may be due to the involvement of a thermophilic enzyme system in sucrose synthesis (Müller and Wegmann, 1978). However, algae respond differently to altering sucrose levels when

University of Malaya

subjected to temperature changes; for example in *Dunaliella*, sucrose was accumulated at elevated temperatures, while in *Chlorella* (Guy, 1990) and *Klebsormidium flaccidum* (Nagao and Uemura, 2012) sucrose accumulation was instead reported at low temperatures. In addition, an increased level of sucrose was observed in the cyanobacterium *Synechocystis* sp. during salt stress, suggesting a role for sucrose as an osmoprotectant (Miao et al., 2003). The enzyme sucrose phosphate phosphatase (SPP), known to be an essential component of the sucrose synthesis pathway, is involved in hydrolysing sucrose 6-phosphate (S6P) into sucrose and inorganic phosphate (Nagao and Uemura, 2012). The SPP family ubiquitously exists in algae (Jiang et al., 2015). In *C. reinhardtii*, the enhanced expression of the *SPP* gene in response to cold stress resulted in a corresponding increase in sucrose levels (Valledor, et al., 2013).

Algae also respond to environmental stress, especially nutrient deficiency, by accumulating starch as the primary carbon and energy storage (Geider and Roche, 2002; Wang et al., 2014). The biosynthesis of starch is regulated by the rate-limiting enzyme ADP-glucose pyrophosphorylase (AGPase) whose activity is affected by temperature and redox mechanisms (Ball and Morell, 2003; Thitisaksakul et al., 2012). For example, in *Chlorella zofingiensi* starch abundance increased significantly at an early stage of nitrogen starvation, with a significant reduction of starch synthesis rate at a later stage followed by an increase in starch degradation and lipid production (Huang, et al., 2014). Similarly, *AGPase* was highly expressed in *Chlorella vulgaris* in the first few hours of response to N-starved conditions, while its expression reduced after 120 hours of incubation (Ikaran et al., 2015). Vitova et al., (2015) conducted a review of the approaches to enhance the bioaccumulation of starch and lipids, namely the use of inhibitors to prevent DNA synthesis or cell division as well as by nutrient starvation involving the macro elements such as nitrogen, phosphorus and sulphur. Starch is known as the most common storage form of carbohydrate and as a dense and

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osmotically inert form of energy storage (Pfister and Zeeman, 2016). In phototrophically grown algae, starch accumulates during the cell cycle, prior to the period of nuclear and cellular division, and is utilised as a carbon and energy reserve to ensure completion of cell division processes is independent of external supplies of carbon and energy either during photosynthesis or in the dark (Vitova et al., 2015). Hence, starch degradation is an essential process in the cell, especially in photosynthetic organisms during the dark (Smith et al., 2005). Starch phosphorylase plays a vital role in starch degradation (Rathore et al., 2009) and its expression has been shown to be significantly induced by nitrogen starvation (Li et al., 2012).

2.2.4 Abiotic Stress Effects on Antioxidant Defence Mechanisms

Under optimal growth conditions, ROS such as superoxide ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), peroxyl radicals ($RO_2^{\bullet}$), hydroperoxyl radicals ($HO_2^{\bullet}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) are primarily produced as a part of cellular growth at low levels in various organelles, namely chloroplasts, mitochondria, and peroxisomes (Ahmad et al., 2014). However, the equilibrium between the production and scavenging of ROS could be affected by stress, resulting in increased levels of ROS and eventually oxidative stress (Malan et al., 1990; Apel and Hirt, 2004; Murik et al., 2014). In chloroplasts, the main reason for ROS production is the limitation of CO_2 fixation, linked to the over-reduction of the electron transport chain (Davidson and Schiestl, 2001). The accumulation of ROS in chloroplasts may occur via the Mehler reaction where the formation of superoxide occurs due to the transfer of electrons from photosystem I (PSI) to O_2 instead of to ferredoxin (Dietz et al., 2016). During oxidative stress, superoxide radicals act as oxidants and facilitate the production of short-lived hydroxyl radicals (Fridovich, 1995) which are highly reactive and can induce DNA damage via denaturation (Lesser, 2006). Primarily ROS act by inhibiting the repair of

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PSII and not by damaging PSII (Nishiyama et al., 2004). It is suggested that both the singlet oxygen and superoxide radical are strong oxidants and both oxidise elongation factor G (EF-G), which is involved in the synthesis of the D1 protein (Nishiyama and Murata 2014). At first, ROS were thought to be toxic by-products of aerobic metabolism, however more recent studies revealed that ROS play a key role as signal transduction molecules in many organisms (Mittler et al., 2004), so a basal level of ROS is vital for the functions of living cells (Mittler, 2017).

Principally, ROS elevation induces the synthesis of ROS scavenging enzymes, such as superoxide dismutases (SODs), peroxidases (PODs), catalases (CATs) and thioredoxins (TRXs) as well as enzymes involved in the biosynthesis of glutathione (Lemaire et al., 2007; Gill and Tuteja, 2010). As the first line of defence, superoxide dismutases (SODs) rapidly convert $O_2^{\bullet -}$ to O_2 and H_2O_2 and the generated H_2O_2 can be converted into H_2O by peroxidases, such as ascorbate peroxidase (APX) and glutathione peroxidase (GPX) found in vacuoles, cell walls and the cytosol (Alscher et al., 2002; Elbaz et al., 2010; Caverzan et al., 2012). It is suggested that high levels of SOD were associated with the acclimation of *Grateloupia turuturu* to stress (Liu and Pang, 2010). Induced activities of SODs, APX and GPX under diverse abiotic stress conditions have been reported in microalgae (Fischer et al., 2006; Park et al., 2006; Yildiztugay et al., 2014; Chen et al., 2015; Yanguez et al., 2015). The high APX activity seen in *Chlamydomonas* under stressed conditions indicated that APX potentially contributes to this alga's tolerance to oxidative stress (Tanaka et al., 2011). Similarly, in *Ulva limnetica*, APX was up-regulated more than 20 fold under salinity stress (Ichihara et al., 2011). In addition to APX, catalase (CAT) is recognised as a dominant enzyme that catalyses the conversion of H_2O_2 to H_2O or other non-toxic molecules (Mittler et al., 2004; Luis et al., 2006). In plants and algae, catalase (CAT) scavenges H_2O_2 from photorespiration and oxidation of fatty acids (Kato et al., 1997; Vega et al., 2005).

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Interestingly, CAT activity was correlated with the chlorophyll content during photoacclimation and exhibited protection against photodynamic damage induced by ROS (Dykens and Shick, 1984). Also, increased carotenoid content, along with increased catalyse activity, was observed in response to elevated ROS in *Porphyra umbilicalis* Kutzing (Sampath-Wiley et al., 2008). It is recognized that manganese superoxide dismutase (MnSOD) and CAT are the predominant enzymes responsible for mitochondrion protection. Up-regulation of CAT was observed in *C. reinhardtii* cells subjected to various stresses, including acidic conditions (Aksmann et al., 2014). In addition, CAT activity also contributed to the antioxidant defence and acclimation of the green alga *Scenedesmus vacuolatus* when exposed to copper (Sabatini et al., 2009). However, in the cyanobacterium *Anabaena doliolum,* the activity of CAT is salt sensitive and its inhibition resulted in cells' susceptibility to salt stress (Srivastava et al., 2008). The other reported antioxidant compounds in relation to photosynthesis, are carotenoids and α-tocopherols. Carotenoids are essential components found in photosynthetic algae. They are mainly involved in the light-harvesting process during photosynthesis while protecting the photosynthetic system against photo-induced oxidative stress (Vidhyavathi et al., 2008). As antioxidants, carotenoids are capable of quenching $^1O_2$ and excitation energy from chlorophyll, hence reducing the production and accumulation of $^1O_2$ (Demmig-Adams, 1990; Young, 1991). Carotenoids have been reported to increase antioxidant metabolism of *P. umbilicalis*, leading to its ability to withstand harsh environmental conditions such as high temperature, high irradiance and dehydration (Sampath-Wiley et al., 2008). It was also noted that ROS triggers the parallel accumulation of carotenoids in *Dunaliella* (Ye et al., 2008) and increased expression of carotenoid biosynthesis related genes was observed in *Haematococcus pluvialis* under several stress conditions (Vidhyavathi et al., 2008). On the other hand, tocopherols (Toc) also have vital roles in maintaining redox homoeostasis and their

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biosynthesis has been shown to increase under stress conditions. Toc functions by deactivating ROS (mainly $^1O_2$ and $OH^•$) generated in thylakoid membranes during photosynthesis and by inhibiting lipid peroxidation via removal of lipid peroxyl radicals ($LOO^•$) (Maeda et al., 2005). Although ROS are produced in both normal and stress conditions, cell defence mechanisms counter radical damage by limiting the formation of ROS as well as by facilitating its removal.

2.2.5 Photosynthesis

Photosynthesis is a reaction driven by light, whereby energy from the sun is used to fix carbon from carbon dioxide (CO_2) into organic carbohydrates via reducing agents from the splitting of water to release oxygen (Characklis and Marshall, 1990). Photosynthesis can be divided into two reactions: (1) the thylakoid membrane-bound light reactions (where H_2O is split) and (2) the carbon reduction reactions (formerly known as the dark reactions), which takes place in the stroma (Reinfelder et al., 2000; Riebesell, 2000). Pigment molecules have a native state, where all their electrons inhabit low stable electron orbitals (E^o). If the photon receives a specific amount of energy, it can boost electrons to the next orbital. A pigment molecule with an electron boosted to a higher orbital is regarded as excited. The excited state is transient, where the energy is altered to one of four de-excitation routes including; (1) to non-specific neighbouring molecules as molecular motion (heat), (2) via fluorescence by emitting a photon in the red band (lower energy, longer wavelength), (3) by transferring energy to an adjacent chlorophyll molecule and boosting another electron to a higher orbital (enabling this molecule to reach an excited state), and (4) through driving a chemical reaction by loss of the excited electron from the chlorophyll *a* molecule (used in photosynthesis) (Consalvey et al., 2005).

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2.2.5.1 Abiotic Stress Effects on Photosynthesis

Environmental changes can disrupt the balance between energy supply and consumption in photosynthetic algae leading to alterations to the photosynthetic apparatus. In algae and higher plants, the reaction centre of PSII is bounded by the core antenna (CP43 and CP47) and the outer antenna, which includes the less-abundant minor light-harvesting complex I (LHC) and the major light-harvesting complex II (LHCII) (Elrad et al., 2002). These LHCs comprise proteins that bind chlorophylls (Chl *a* and Chl *b*). The *LHCb* gene family encodes both the minor LHC proteins and the LHCII. In the thylakoid membrane, LHCII is the most abundant pigment-protein complex and about half of all chlorophylls are associated with it. The main role of LHCII is to capture and pass on light energy to the reaction centre of PSII and take part in regulating the distribution of excitation energy between PSII and PSI. Transcriptional regulation of genes encoding LHCII plays an essential role in antenna size adjustment (Teramoto et al., 2001; Elrad et al., 2002). Algae regulate the expression of nuclear chlorophyll *a–b* light-harvesting complex (*LHCa, LHCb*), thereby controlling the antenna size. For example, when *C. reinhardtii* was grown under light-limiting conditions, the up-regulation of the *LHC* gene expression resulted in a larger antenna size (Elrad et al., 2002). Moreover, environmental changes can affect the subunit composition of reaction centres (PSI and PSII). For example, the *psbC* gene encodes a PSII chlorophyll-binding protein, involved in splitting and acting as an oxygen-evolving enzyme of photosynthesis (Qian et al., 2009), and is affected by harsh conditions like temperature stress (Chong et al., 2011). The *psaB* gene encoding one of the reaction centre subunits of PSI (Qian et al., 2009) was down-regulated in *Chlorella vulgaris* when this alga was exposed to toxic chemicals (Qian et al., 2011). Although other stresses, such as salinity, might have an effect on the expression of these genes, high light illumination is more likely to be associated with their regulation (Shapira et al.,

1997; Nama, Madireddi et al., 2015). It is noteworthy to consider that gene expression is controlled at several steps starting with transcription of a gene, splicing, editing steps, and finally mature mRNA. The expression of chloroplast genes (*psbC, psaB* and *rbcL*) were initially thought to be controlled at the post-transcriptional level, associated with mRNA stability (Pfannschmidt, 2003). While it is likely that photosynthetic gene expression is controlled at all levels of expression (Qian et al., 2008), studying their expression in transcriptomic level can provide a suitable resolution of their synthesis.

Photosynthetic fixation of carbon dioxide is essential for algal growth and development, providing the carbohydrates required for metabolism, as structural components and supplying cellular building blocks. Also, photosynthesis acts as a global sensor of environmental stress that induces cellular energy imbalance leading to the distinct changes in redox chemistry linked to thylakoid membranes and adjustment of cellular sugar status (Biswal et al., 2011). Photosystems and key enzymes associated with carbon dioxide fixation, such as ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) play critical roles in photosynthesis. Studies have shown that the expression levels of genes involved in photosystem structure and RubisCO subunits were significantly influenced by various stresses (Qian et al., 2009; Qian et al., 2012; Luo et al., 2015). For example, *rbcL*, which encodes the large subunit of RubisCO (Spreitzer and Salvucci, 2002) was up-regulated upon temperature increase to 20 °C, then the further rise, down-regulated its expression (Deng, 2014). Also, expression of *rbcL* was inhibited under desiccation, high salinity, and low salinity conditions as well as at temperatures above and below the normal ambient temperature (Xu et al., 2013). The map of photosynthesis pathway with its components is shown in Figure 1.

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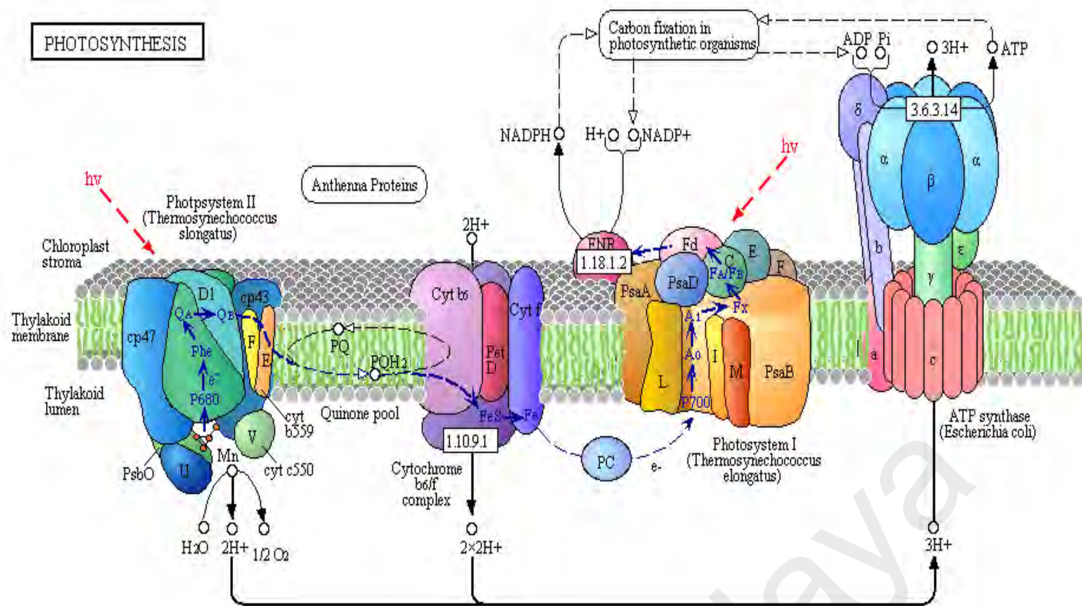

Figure 2.1: KEGG map of photosynthesis pathway (www.genome.jp/kegg).

Photoinhibition associated with damage to the D1 protein of PSII can occur whenever algal cells are receiving light, and the damage is continuously repaired by *de novo* synthesis of D1, followed by the activation of the reaction centre to achieve a balance between the photodamage of PSII and its repair (Takahashi and Murata, 2008). Three main steps in PSII repair are conspicuously sensitive to abiotic stress, namely, degradation of the D1 protein in photodamaged PSII, synthesis of pre-D1 and processing of pre-D1 to the mature D1 protein (Nishiyama and Murata, 2014). It was suggested that adverse stresses might not increase photodamage directly but rather affect the PSII repair process by inhibiting the (re)synthesis of D1 (Nishiyama and Murata, 2014). For example, in *Synechocystis sp. PCC6803* salt stress inhibited protein synthesis, degradation of the D1 protein, and processing of pre-D1 into the mature D1 protein, which is necessary for the assembly of the active PSII complex (Allakhverdiev et al., 2002). In cyanobacteria, synthesis of D1 protein is mainly regulated at the transcription of its coding gene, *psbA* with fine-tuning during the elongation phase of translation. However, in chloroplasts of *C. reinhardtii*, the expression of *psbA* is highly

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controlled by mRNA processing especially during the initiation of translation (Mulo et al., 2012). Besides the regulation of D1 protein synthesis during photoinhibition, amino acid changes in D1 protein were observed in response to temperature. Together with the physicochemical properties of the thylakoid membrane, these changes might contribute to the structural flexibility required for electron transfer in the PSII system (Giardi et al., 1997; Lukes et al., 2014).

2.2.5.2 Photosynthetic Parameters Measurement Principle

Fluorescence is the re-emission of a photon of light, with a lower energy than the photon absorbed. Chlorophyll *a* re-emits light energy in the red band (Govindjee, 1995) (Kautsky and Hirsch 1931, Govindjee 1995). At room temperature, most of the fluorescence that we measure comes from PSII, with PSI only having a minor influence (Krause and Weis, 1991; Pflindel, 1998; Hall and Rao, 1999). In microalgae, only a very minor ratio (typically 1-5%) of the light energy absorbed is used in this way (Kirk, 1983). When no photons are available to strike the antennae complexes (i.e. in the dark), the reaction centres are pronounced as "open". This shows that no electrons have been provided from the splitting of water to the reaction centres of the PSII. The rate-limiting step for energy transmission via PSII is the oxidation/reduction of Q_A. As a result of this, the reaction centre is known to be open once Q_A is oxidised and the yield of fluorescence is at its minimum, (F_o). If a short pulse of high light is introduced by the fluorimeter to the algal cells, this is adequate to close all of the reaction centres (i.e. reduce all Q_A). This is called the saturating pulse, which differs among photosynthetic organisms, with a higher intensity and longer exposure to vascular plants compared to algae. When the reaction centres are all closed, the yield of fluorescence, in the course of the length of the saturating pulse, reaches a maximum ($F_m{}^o$).

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In the light, photons strike the antenna complex and excite the pigment molecules. Some of the reaction centres at PSII will close (Q_A^-), and the fluorescence signal will increase to a yield between F_o and F_m (F_s). F_s is the fluorescence yield relative to the closure of the reaction centres induced by actinic light (drives photosynthesis). Basically, it takes several minutes for F_s to stabilise (an approximation after 30 secs to 2 mins are used) and back to normal, F' (Oxborough et al., 2000). The prime superscript represents a measurement of fluorescence yield achieved in the light, therefore, a maximum fluorescence yield in the light is meant to be F_m'. F' may increase with increasing light level. Mostly, F_m' is lower than F_m, due to the non-photochemical quenching (NPQ), which is a way of down-regulation that turn away some of the light energy from the PSII reaction centres. If light energy is diverted away from the reaction centres, then there is less energy to induce fluorescence, and the yield decreases.

By understanding the pathways involved in fluorescence and photosynthesis, the aforementioned parameters can be utilised to speculate about the electron transport rate (ETR) and photosynthetic efficiency, which means that fluorescence can be used as an ecological tool. The difference between the dark-adapted maximum (F_m) and minimum (F_o) fluorescence is the variable fluorescence (F_v). In the light-adapted state, the variance between the fluorescence yield at a certain light level (F') and the light-adapted maximum fluorescence (F_m') is defined as the fluorescence that has been quenched by photochemistry, F_q'. F_q' is relative to the number of open reaction centres and the amount of harvested light energy actively used to drive the photochemistry. Some researchers use the notation $\Delta F/F_m'$, where ΔF equals F_m'-F'. The light utilisation efficiency can then be calculated using these yields of fluorescence, either as a theoretical maximum efficiency (F_v/F_m) in the dark-adapted state or as the efficiency at a particular light level (F_q'/F_m') in the light-adapted state. As light level increases, the light utilisation efficiency decreases because there are more reaction centres closed, and

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therefore unable to take advantage of increases in light energy. A decrease in this parameter can also be indicative of stress (Underwood and Kromkamp 1999).

2.3 Algal Gene Regulatory Mechanisms and Gene Manipulation

Understanding the biological principles and regulatory networks by which algae live by can help explain their successful survival, proliferation, and distribution. Furthermore, knowledge on their responses toward stress conditions is required if successful outcomes are expected for the role of algae to help deal with increasing demand for feed, food, and biomass (Moreno-Risueno et al., 2010). A large body of literature has been devoted to studies of the effects of abiotic stress on algae at the molecular level in order to determine the roles and functions of stress-related genes and proteins (Hema et al., 2007; Jamers et al., 2009; Kebeish et al., 2014). Gene expression of *Chlorella* strains in response to different abiotic stress is presented in Table 2.1. This knowledge could be translated into practical industrial applications, for example, stress-induced production of lipids and antioxidants. Lipid content is extremely important to biofuel production, whereas antioxidants such as astaxanthin are known to be important biomolecules in the pharmaceutical industry (Hu et al., 2008; Ambati, et al., 2014). Although the desired product can be obtained by introducing the associated stress, if the growth is compromised by stress, its overall productivity is also reduced. Recently, advances in OMICS technologies, such as metabolomics, proteomics, transcriptomics, and genomics have provided deeper insights (Nouri et al., 2015), leading to the possibility of engineering strains with higher yields. However, for the mass cultivation of genetically engineered algae, biosafety guidelines, in addition to a cost-effective production system, must be in place.

University of Malaya

Table 2.1: The list of several studies reporting gene expression of *Chlorella* strains in response to abiotic stress.

Species	Class of Genes	Functions	Associated Stress	Ref.
Chlorella sp.	*HSP90*	Keep inherently unstable proteins in a close-to-native conformation	Temperature and salinity	Liu et al. (2014)
Chlorella. vulgaris	*HIC6, HIC12*	Encoding LEA, hydrogen-bond stabilising effects on enzymes	Low temperature	Machida et al. (2008)
Chlorella pyrenoidosa	*accA, accD*	Encoding subunit of ACCase regulate fatty acid synthesis rate	Nutrition limitation	Fan et al., (2014)
Chlorella vulgaris	*AGPase*	ADP-glucose pyrophosphorylase, Starch synthesis	Nitrogen starvation	Ikaran et al. (2015)
Chlorella vulgaris	*SP*	Starch phosphorylase, starch degradation	Nitrogen starvation	Ikaran et al. (2015)
Chlorella sorokiniana	*rbcL*	Large subunit of RubisCO	metal toxicity	Wan et al., (2014) 9
Chlorella vulgaris	*psbC*	Encodes a PSII chlorophyll-binding protein	salinity	Kebeish et al.,2014
Chlorella vulgaris	*psaB*	Reaction centre subunits of PSI	herbicide	Qian et al., (2009)
Chlorella vulgaris	*psbA*	D1 protein synthesis	UV radiation	Garcia-Gomez et al., (2016)

With advances in the technology of gene editing using approaches such as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 (CRISPR-associated gene 9), precise gene manipulation can be realised to obtain stress-tolerant algae. This technology has been adapted to the model microalga, *C. reinhardtii* (Jiang et al., 2014),

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and also employed in the marine diatom *P. tricornutum* to efficiently produce stable targeted mutations (Nymark et al., 2016). CRISPR can directly edit the genome precisely at the region of interest by either NHEJ or HDR mutations, which leads to a defined DNA replacement, deletion, and insertion (Xu et al., 2014). With this technology, further accomplishments can also be achieved in the functional study of algae proteins and genes associated with abiotic stress, characterization of their metabolic pathways, and the understanding of their biology (Lander et al., 2016). There are extensive interactions between components of signalling, regulatory and metabolic pathways, which lead to abiotic stress response/adaptation (Nakashima, 2009; Garg et al., 2014; Mickelbart et al., 2015). In many instances, knock-out of a single gene may not produce the desired phenotype, making it difficult to pinpoint its function. Multiple genes can be targeted simultaneously using the CRISPR-Cas9 system, which can overcome the problem posed by functional redundancy of genes (Jain, 2015). CRISPR technology is still in its infancy in algal studies, and it is expected that it can, in the future; it can contribute to the improvement of our understanding of algal metabolism and responses to environment, and be used to produce more stress-tolerant algae that can be used for industrial applications.

2.4 *Chlorella*

Chlorella is a genus of single-cell green algae belonging to the phylum Chlorophyta. It is sub-spherical, spherical, or ellipsoid in shape, ~2–10 µm in diameter, single or forming colonies, and lacks flagella. *Chlorella* has a single chloroplast, rigid cell wall, and lacks flagella (Eckardt, 2010). It is made up of green photosynthetic pigments chlorophyll *a* and b in its chloroplast. According to Guiry and Guiry (2017), the genus *Chlorella* can be classified as follows (Empire: Eukaryota, Kingdom: Plantae, Phylum: Chlorophyta, Class: Trebouxiophyceae, Order: *Chlorellales,* Family: *Chlorellaceae,*

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Genus: *Chlorella*). These taxa can be found in diverse habitats, such as freshwater bodies, ponds, soil, marine, brackish water, as well as hot springs (Phang, 2004). These small organisms are simply propagating plants, demonstrating the suitable experimental model representing biochemical and physiological properties of macro-and green microphytes (Krienitz et al., 2015).

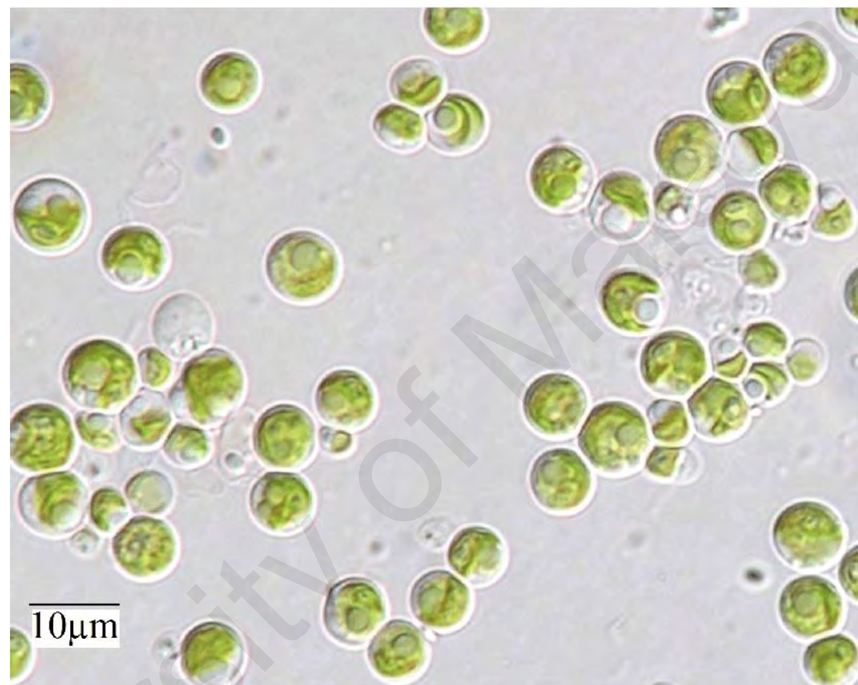

Figure 2.2: Image of *Chlorella* sp. using optical microscope (Salbitani and Carfagna 2016).

The microalgae demonstrate substantial roles: as primary producers in aquatic ecosystems, it induces photo-oxygenation and removes CO_2, which is used as a source of nutraceuticals, wastewater treatment, and feedstocks biofuel (Chu et al., 2009; Lim et al., 2010). *Chlorella* is used for biochemical and physiological studies since the cultures are easy to handle and it displays feasible growth performance (Krienitz et al., 2015). Ng et al. (2014) confirmed the potential of *Chlorella* sp. in producing electrical power due to its high photosynthetic rate and its ability to form a biofilm on indium tin oxide

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(ITO). The immune-modulating and anticancer properties from the *Chlorella* extracts are used for medical treatment in certain countries (Safi et al., 2014). Blanc et al. (2010) reported the first genome sequence of *Chlorella variabilis NC64A*. Its' genome size is 46.2 MB, with 12 % of the genome being repeated sequences. A total of 9791 protein-encoding genes are predicted in the genome. The mitochondrial genome accumulates as a circle of 78500bp, comprising of 62 genes, whereas the chloroplast genome forms a circle of 124793bp, consist of 114 genes (Orsini et al., 2016).

2.5 *Chlorella* Responses to Elevated Temperature

Several studies on the physiological responses of *Chlorella* strains towards elevated temperature have been carried out. Temperature is one of the important factors controlling the growth and biochemical composition of *Chlorella*. Teoh et al., (2005) investigated the influence of culture temperature on biochemical composition, growth and fatty acid profiles of Antarctic *Chlorella*. It was shown that temperature had the most significant effect on the protein content of *Chlorella* strains; by increasing 5 °C the protein content increased; however, the further rise of temperature inhibited protein synthesis. Also, the percentage of PUFA decreased as temperature increase (Teoh et al., 2004). In another study which compared the physiological responses of *Chlorella* strain from different latitudes in response to temperature stress reported that the three *Chlorella* strains from different regions are eurythermal, with a big overlap on tolerance ranging from 4 °C to 38 °C. It was concluded that the Antarctic strain was able to tolerate a broad range of temperature so that it is likely to survive if global warming continues (Teoh et al., 2013). Cao and co-workers reported that the Antarctic *Chlorella* strain was able to grow in the broad range of temperature. The reported Antarctic *Chlorella* strain showed the tendency to secrete soluble sugar into the culture medium with increasing temperature, whereas its intracellular soluble sugar content did not

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change with temperature variations. Accordingly, it was proposed that the algal cells might suffer from osmotic stress at high temperature, which could be regulated by excretion of soluble sugar (Cao et al., 2016). Photochemical responses of *Chlorella* strain studied in another research stated that elevated increasing temperature had significant effects on the photosynthetic properties and growth rates of *Chlorella pyrenoidosa* where by gradually increasing temperature, photosynthesis and the growth were inhibited (Zhang et al., 2012). In *Chlorella vulgaris*, heat stress resulted in retardation of cell division, inhibition of photosynthetic oxygen evolution and phase shifts of circadian patterns. However, cells appeared to possess the potential for adaptation to high temperature induced by growth temperature in the range 25–35 ºC which was demonstrated by the enhanced thermal stability of PSII as revealed by analysis of chlorophyll fluorescence induction kinetics (Sayed et al., 2000).

In the mass cultivation of *Chlorella,* it is economically important to screen strains adaptive to broad temperature fluctuation for outdoor cultivation where there is no temperature control being carried out. In a study conducted by Yang et al., (2016) three *Chlorella* strains were selected from different latitudes. All the three *Chlorella* strains showed the abilities to accumulate lipid under daytime temperature variations and their fatty acid profiles were suitable for biodiesel production, though the biochemical composition and growth were appeared to be region-specific. In the other study, the effect of elevated temperature on the composition of intracellular fatty acids and the release of free fatty acids (FFA) into a culture medium by *Chlorella vulgaris* was studied; it was reported that the relative content of intracellular more unsaturated fatty acids decreased with the elevation of temperature, while no change observed in extracellular unsaturated free (Sushchik et al., 2003). Results showed 30 ºC was optimal for achieving high lipid and biomass; by raising daytime temperature can lessen night biomass loss and stimulate lipid accumulation (Han et al., 2013). Besides, the effects of

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elevated temperature on protein synthesis were also being studied as protein has important use as a food and fertilizer. For example, in a study conducted by Valliammai and Gnanam (1987), on *Chlorella protothecoides* demonstrated that during heat stress the synthesis of soluble protein inhibited more than the synthesis of membrane-bound proteins. They suggested that in *Chlorella*, the membrane-bound ribosomes are less prone to damage by the heat shock than the free ribosomal components involved in the synthesis of soluble proteins. Also, the temperature has a substantial influence on the utilization efficiency of glucose and growth. In a separate study, *Chlorella pyrenoidosa* was heterotrophically grown at diverse temperatures (15, 20, 25, 30 and 35 °C) in order to investigate the effects of temperature on algae productivity, the consumption rate of glucose and conversion ratio of glucose to algae cells. The heterotrophic productivity and consumption rate of glucose increased with the increase of temperature. When the temperature was increased from 15 to 30 °C, the conversion ratio of glucose to algae cells enhanced, but with the additional temperature rise from 30 to 35 °C, the conversion ratio severely plummeted. The highest heterotrophic productivity (0.161 g/L.d) was found at 35 °C. Therefore, at 30 °C, *Chlorella pyrenoidosa* will achieve a high growth rate with a low cost of cultivation (Zhao et al., 2015).

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CHAPTER 3: MATERIALS AND METHODS

3.1 *Chlorella* Strains and Culture Conditions

Three *Chlorella* species from different latitudes were used in this study: tropical (UMACC 245) and Antarctic (UMACC 250) were obtained from the University of Malaya Algae Culture Collection (UMACC). The temperate *Chlorella* (UMACC 373) was purchased from the Culture Collection of Algae and Protozoa (CCAP) and was originally isolated from Loch Linnhe, Argyll, Scotland where it is deposited as CCAP 211/75. The stock cultures were grown in Prov (Provasoli) medium (Phang and Chu, 1999; Appendix. A) and maintained in a controlled environment incubator, illuminated with cool white fluorescent lamps (40 µmol photons m^{-2} s^{-1} on a 12:12 light-dark cycle) at the following ambient temperatures; 4 °C (Antarctic), 18 °C (temperate), and 28 °C (tropical). For the ease of reference, the selected strains were referred to as *Chlorella*-Ant: Antarctic *Chlorella* (UMACC 250), *Chlorella*-Trop: Tropical *Chlorella* (UMACC 245), *Chlorella*-Temp: Temperate *Chlorella* (UMACC 373).

3.2 Experiment Setups

3.2.1 Experiment 1: Growth and Biochemical Characterization of *Chlorella* strains

The algal cultures were first kept at their respective ambient temperatures. For the experiments, 10% (v/v) inocula from exponential phase cultures were prepared using 70 mL of inoculum (from an exponential phase culture), where the cell density was standardized to an optical density of 0.2 at 620 nm (OD_{620}), which was then inoculated into 630 mL fresh Prov medium in a 2 L conical flask. The triplicate batch cultures of each strain were then grown at pre-set temperatures, which exceeded the ambient

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temperature (Table 3.1) for ten days. The cultures were shaken continuously at 80 rpm in an orbital shaker incubator (Model 718, Hotech Instrument Corp., Taipei, Taiwan). The light was provided by cool-white fluorescent lamps (40 µmol photons m^{-2} s^{-1}) on a 12:12 light-dark cycle. Shaking and cotton plugs were used on the growth vessels, and no more than half of the vessel volume was used as a culture to ensure adequate gas exchange. In order to study growth and photosynthetic parameters, daily sampling was conducted. On day 10, when the cultures reached the stationary phase, it was harvested for a biochemical test. The flowchart of experiment 1 is shown in Figure 3.1.

Table 3.1: List of the selected temperature for each *Chlorella* strains

Latitudes	*Chlorella* Strains	Growth Medium	Ambient Temperature	Temperature Range
Tropical	UMACC 245	Provasoli	28 °C	28, 33, 35, 38 and 40 °C
Temperate	UMACC 373	Provasoli	18 °C	18, 25, 28, 33, 35 and 38 °C
Antarctic	UMACC 250	Provasoli	4 °C	4,13,18, 25, 28, 33, 35 and 38 °C

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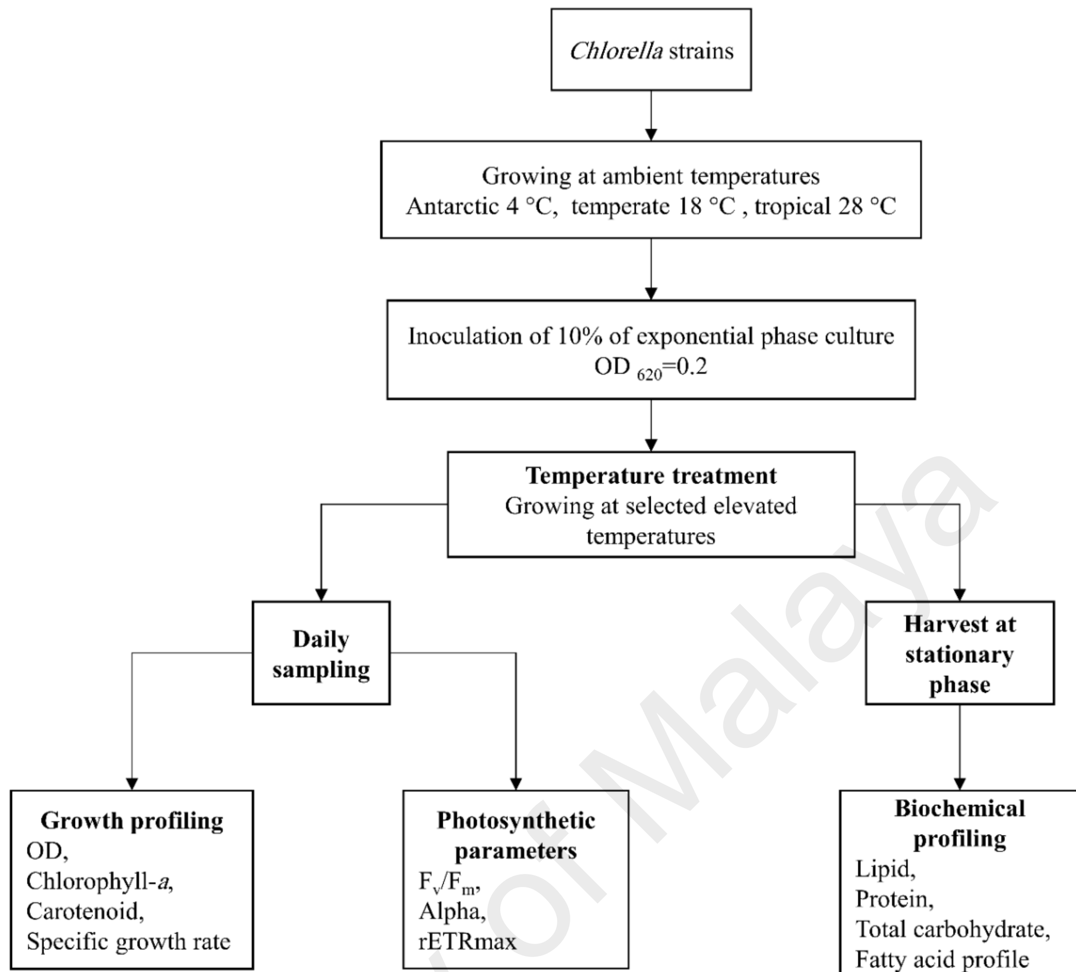

Figure 3.1: The flowchart of experiment 1.

3.2.2 Experiment 2: Stress and Recovery Treatment

After the first experiment (growth studies at various temperatures), for each strain, the temperatures inducing severe damage to photosynthesis (caused F_v/F_m to decrease to zero) and inhibited growth were selected for the temperature stress and recovery experiments. 38 °C was selected for both *Chlorella*-Ant and *Chlorella*-Temp, while 40 °C was selected for *Chlorella*-Trop. In this experiment, an inoculum size of 40 % (v/v) was prepared by inoculating 1200 mL of culture (from the exponential phase), where the cell density was standardised at an optical density of 0.2 at 620 nm (OD_{620}), into 1800 mL of fresh Prov media. During the stress treatment, the cultures were incubated

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at their stress-inducing temperatures, and the decrease in F_v/F_m was closely monitored to assess stress levels. When the F_v/F_m value decreased to ~0.4, 0.2, and 0.0, 500 mL of the cultures were transferred to the empty, sterilised, 1 L conical flask, and returned to the normal ambient temperature to determine their capacity for recovery. The cultures were considered recovered when the F_v/F_m regained the value above 0.4. The flowchart of experiment 2 is presented in Figure 3.2.

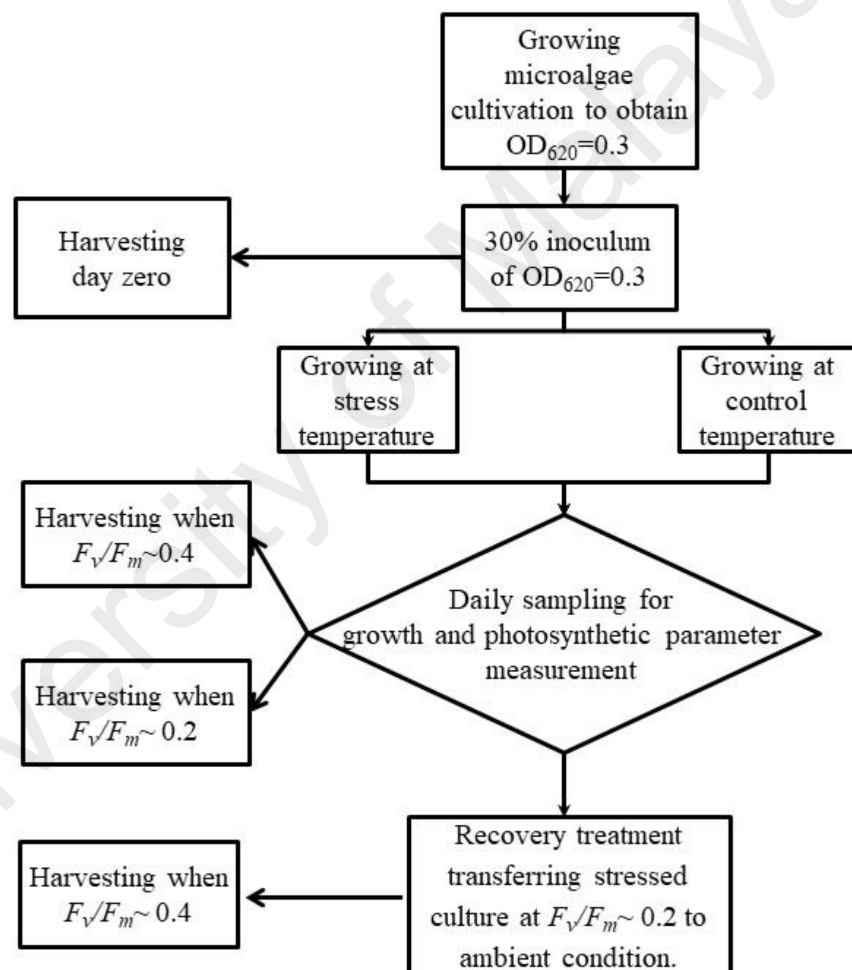

Figure 3.2: The flowchart of experiment 2.

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3.2.3 Experiment 3: Gene Expression Profiling and ROS Measurement upon Stress and Recovery

During stress and recovery treatments, the cultures were harvested at four-time points including; day zero, when F_v/F_m decrease to 0.4 and 0.2 and when it recovered to 0.4. At each time point, the ROS level was measured. This was followed by RNA isolation steps, as per 3.3.12. The RNA samples were purified using genomic DNA removal Kit based on manufacturer protocol, and then the synthesized cDNA was used as a template for RT-qPCR. The flowchart of experiment 3 is presented in Figure 3.3.

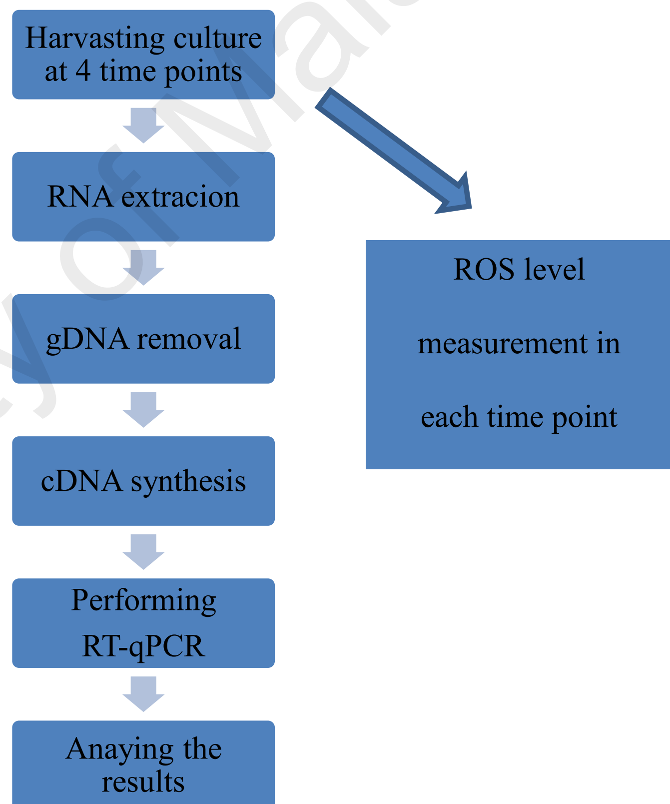

Figure 3.3: The flowchart of experiment 3.

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3.3 Analytical Methods

3.3.1 Growth study

3.3.1.1 Monitoring of Growth by Optical Density (OD_{620})

The optical density of the cultures was measured at an absorbance of 620 nm (OD_{620}) using UV-vis spectrophotometer (Shimadzu, Japan). First, three millilitres of distilled water was placed in the clean cuvette then the auto-zero function was selected to blank before reading the samples. A growth curve based on optical density was determined by plotting the OD versus time (day).

3.3.1.2 Specific Growth Rate

The specific growth rate was calculated according to the natural logarithm of optical density (OD_{620}) plotted versus time (day) within the exponential phase of cultures. The following formula was used to calculate the specific growth rate (μ, day^{-1}):

$$\mu\ (day^{-1}) = (Ln\ N_2 - Ln\ N_1) / (t_2 - t_1)$$

Where N_2 is OD_{620} at t_2, N_1 is OD_{620} at t_1, and t_2 and t_1 are times within the exponential phase (Claquin, 2008).

3.3.2 Photosynthetic Parameters Measurement

To measure the photosynthetic properties of the microalgae, chlorophyll *a* variable fluorescence parameters were measured by a Pulse-Amplitude-Modulation (PAM) chlorophyll fluorometer (Water PAM; Heinz Walz, Effeltrich, Germany). Rapid light curves (RLCs) were obtained under software control (Win control, Walz) (Ralph and Gademann, 2005). The samples were kept in dark for at least 15 min prior to measurement (Wong, 2015; Cao et al., 2016; Wang and Xu, 2016). RLCs were

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constructed by exposing the samples to 8 increasing red actinic irradiances (48, 105, 158, 233, 358, 530, 812 and 1216 μmol photons m^{-2} s^{-1}) for an interval of 10 s, each separated by a 0.8 s saturating flash (2000 μmol photons m^{-2} s^{-1}). The maximum quantum efficiency (F_v/F_m), was calculated as F_v/F_m = $(Fm-F_0)/F_m$, where F_m is the maximum fluorescence, F_0 is the minimum fluorescence and F_v is the variable fluorescence. The relative electron transport rate (rETR) was deliberate by multiplying the irradiance by quantum yield measured at the end of each light interval (Harbinson et al., 1989). Alpha (α), defined as the initial slope of the rETR vs irradiance curve, is used as a measure of photosynthetic efficiency. Values for α and the maximum relative electron transport rate (rETRmax) were calculated by fitting the data from RLCs to an exponential function using a multiple non-linear regression (Platt et al., 1980). The program setup is presented in Appendix B.

3.3.3 Biochemical studies

3.3.3.1 Chl-*a* Concentration and Carotenoid Content

Extraction of chlorophyll *a* and carotenoids was accompanied by filtering 20 mL of the sample on a glass-fibre filter (Whatman GF/C, 0.45 μm). The filtered samples were smashed with a glass hand-homogenizer in a 10 mL of acetone (100%). The samples were then covered with aluminium foil and incubated at 4 °C overnight. Prior to spectrophotometric measurement, the samples were centrifuged at 1409 g for 10 min to yield a clear supernatant. Chlorophyll *a* concentration was determined by measuring the absorbance of the cell extracts at 665, 645, and 630 nm using a UV–vis spectrophotometer (Shimadzu UV1700, Japan) using the formula of Strickland and Parsons (1972).

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Chlorophyll *a* (μg/mL) = (A× volume of acetone in mL (10 mL)/volume of sample in mL (20 mL)

Where A = 11.6 (A_{665nm}) - 1.31 (A_{645nm}) - 0.14 (A_{630nm}).

In addition, carotenoids were measured from the same supernatant by measuring the absorbance at 452 nm and the carotenoid concentration obtained using the formula of Strickland and Parsons (1972).

Carotenoid concentration (μg/mL) = A_{452nm} × 3.86 × Volume of acetone (10 mL)/Volume of sample (20 mL).

3.3.3.2 Dry Weight Determination

The glass-fibre filter (Whatman GF/C, 0.45 μm) were dried in the oven at 80 °C for 24 h, and then cooled down in a desiccator for 6 hours then the filter papers weighted. The known volume of samples (50 mL) was filtered. The filtered samples were flushed with ammonium formate, since they are all marine species to eliminate salt. The filtered samples were kept in the oven at 80 °C for 24 h and later transferred into desiccator for 6 hours. The samples were finally weighed and recorded, and they were returned back to the desiccator for 24 h. The samples were weighed again and recorded until a constant weight is achieved. The weight variance between the filter paper and the constant weight was recorded as the algal dry weight. Biomass concentration is expressed in g Dry Weight L^{-1} (g DW L^{-1}).

$$\text{Dry weight} = \frac{[\text{Filter with microalgae weight}] - [\text{Pre-weighed filter}]}{\text{Volume of culture}}$$

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3.3.3.3 Lipid Extraction

Lipids were extracted from the filtered samples and its concentration determined by gravimetric method (Bligh and Dyer, 1959). In 5 mL of methanol-chloroform (2:1, v/v) the samples were mashed with a hand homogenizer and transferred into 15 mL centrifuge tube and then centrifuged at 3,000 rpm for 10 min. The clear supernatant was transferred to a new centrifuge tube containing 2 mL of distilled water and 2 mL of chloroform. The mixture was vortex vigorously and centrifuged at 3,000 rpm for 10 min to separate it into two phases. The lower phase (green colour) pulled out with a specially drawn Pasteur pipette and transferred into a screw-capped test tube. Following that the extract was blown with a mild stream of Nitrogen gas, and the dried extract was kept in a desiccator for 24 h before measuring the weight. The difference in weights was taken as the weight of the lipid extract.

3.3.3.4 Fatty Acid Transesterification

Lipid transesterification was conducted by the adding 1 mL of sodium methoxide (1% H_2SO_4-MeOH) to dissolve the lipid extract and transferred into a screw-capped test tube. The tube was heated at 90 °C for 1 h using a test tube heater (Stuart SHT1, Netherland). The test tubes were shaken frequently. The tubes were cooled down in room temperature before the addition of 1 mL hexane to the heated extract, the mixture was carefully mixed using vortex and left to separate into two phases. The superior layer containing the fatty acid methyl ester (FAME) was aspirated into a new clean screw-capped test tube. Extraction of FAME was repeated with the addition of another 1 mL of hexane to the extract. The pooled hexane extracts were evaporated by a mild stream of nitrogen gas. The FAME was re-dissolved in 100 µL of hexane and

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transferred into a clean glass vial (capacity: 3.5 mL) and wrapped with Parafilm. Then the samples were kept in the freezer at −20 °C for gas chromatography analysis.

3.3.3.5 Gas Chromatography

The gas chromatography system (Shimadzu GC 14A, Japan) comprised of a flame ionization detector (FID) and an integrator (Shimadzu CR6A Chromatopac, Japan). The system was equipped with a polar capillary column (DB 23, J & W Scientific, USA) with dimensions of 30 m X 0.25 mm and 0.25 μm thickness. The split ratio of the injector was set at 1:60 and the carrier gas (nitrogen) was at a flow rate of 0.6 mL min^{-1}. Flame ionisation detector (FID) with temperatures of both the injector and detector fixed at 260 °C. Helium was used as the carrier gas at 2 mL min^{-1}. The flows of hydrogen gas and purified air for the FID were provided at rates of 40 and 400 mL min^{-1}, respectively. The injections were performed in duplicate for each extraction consisting 1 μL of sample. The quantification was according to the integrated peak areas of the chromatogram. The amount of each fatty acid (in % total fatty acid) was inferred from the standard curves based on the peak area.

3.3.3.6 Determination of Protein Content

Bradford (1976) method used to determine the protein content of algal cells. For the protein extraction, 50 mL of each replicate was filtered on Whatman GF/C, 0.45 μm. The filtered samples were mashed in a hand homogenizer in 6 mL of NaOH (0.5 M) and transferred to plastic centrifuge tubes. The mashed samples were incubated at 80 ℃ for the duration of 20 min in a water bath. The samples were then centrifuged for 10 min at 3,000 rpm. The supernatants were removed to a screw-capped test tube while the extraction was repeated by addition of another 6 mL of NaOH. The supernatants were

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pooled together and made up to 10 mL. Then protein assay was done using aliquots of 100 µL.

Table 3.2: Protein Standard Preparation

Concentration (µg)	**0**	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**100**
BSA (mL)	0	1	2	3	4	5	6	7	8	9	10
dH_2O (mL)	10	9	8	7	6	5	4	3	2	1	0

Here, 100 µL aliquots of each concentration were added to 3 mL of protein reagents. The samples were then incubated for 30 minutes before reading the absorbance at 595 nm (OD_{595}) using Shimadzu UV-vis spectrophotometer. Then, a standard curve of Absorbance against BSA concentration was plotted using prepared standard dilutions according to table 3.2.

3.3.3.7 Determination of Carbohydrate Content

Carbohydrate extraction was conducted according to Dubois's (1956) method. 50 mL of each replicate was filtered on Whatman GF/C (0.45 µm) filter paper. The filtered samples were mashed in a hand homogenizer in 6 mL of HCl (2 M) and transferred to plastic centrifuge tubes. The homogenized samples were incubated in a water bath at 80 °C for 1 h. Then, were centrifuged at 3,000 rpm for 10 min and the supernatants were removed to new clean screw-capped test tubes while the extraction was repeated by the adding another 6 mL of HCl. The supernatants were pooled together to make up a total volume of 10 mL. Aliquots of 0.5 mL were pipetted into glass test tubes and 1.5 mL of distilled water was added followed by the addition of 0.5 mL of newly prepared phenol

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solution via the phenol-sulphuric acid method as defined by Kochert (1978). Next, 5 mL of concentrated H_2SO_4 (95.5% v/v) was added and vortex using low speed. After incubation for 30 minutes, the optical density was measured at 485 nm (OD_{485}). The exact amounts of carbohydrates were extrapolated from the standard curve which was prepared according to table 3.3.

Table 3.3: Carbohydrate Standard Preparation

Concentration (mg/mL)	**0**	**5**	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**45**	**50**
Glucose (mL)	0	1	2	3	4	5	6	7	8	9	10
dH_2O (mL)	10	9	8	7	6	5	4	3	2	1	0

For carbohydrate standard, a sequence of glucose solution was prepared from the stock solution. 100 µL of aliquots from each concentration was transferred into glass test tubes. 1.9 mL of distilled water was added to the tubes followed by the addition of 100 µL of 100% aqueous phenol. Lastly, 5 mL of concentrated sulphuric acid was gently added to the tubes. The samples were mixed by vortex until they homogenized, and incubated for 30 minutes. The absorbance was taken at 485 nm and a standard graph of absorbance versus glucose concentration was extrapolated.

3.3.4 Detection of Reactive Oxygen Species (ROS)

To determine the ROS level, the oxidant-sensing probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) purchased from Sigma Aldrich and was used as per the method described by Rastogi et al., (2010). The 10 mM stock solution was prepared by dissolving the powder into Dimethyl Sulfoxide (DMSO).

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Then, a 2,000 µM working solution was used for the measurement. Samples were centrifuged at 10,000 rpm for 10 mins, washed thrice with Prov medium to remove extracellular ROS, then re-suspended in 1 mL Prov medium, and finally, 10 µL of 10 mM DCFH-DA was introduced. The cells were incubated at 37 ºC for 15 mins in the dark, then centrifuged and washed with the same method in order to remove extracellular DCFH-DA. Finally, the fluorescence intensity of the DCF compound was measured using a microplate reader at excitation/emission wavelength 488/525 nm and was directly correlated to the concentration of intracellular ROS by normalizing it to the OD.

3.3.5 Gene Expression Assay

3.3.5.1 RNA extraction

The RNA extraction protocol was obtained from a recently optimized protocol by Poong et al. (2017). The consumables used for RNA extraction were pre-treated with 0.1% diethyl pyrocarbonate and autoclaved for 45 minutes. All RNA extractions were performed in triplicate using ~300 mg fresh weight. The cells were centrifuged at 4500 g for 7 min at 4 °C. RNA was obtained by elution in 50 µL of RNase-free water (Invitrogen, USA), then after assessing their quality and quantity were stored at −80 °C. This method utilizes the standard TRIzol method plus, with an additional 1.2 M NaCl precipitation.

The cells were homogenized with a mini pestle mixer (Kimble Chase, Vineland, NJ, USA) after the addition of 1 mL TRIzol reagent. They were simultaneously flash frozen with liquid nitrogen in a pre-chilled mortar and grinded with a pestle mixer, followed by continual grinding after the addition of 1 mL TRIzol reagent to break the cells and extract RNA more efficiently. Caution was taken not to allow cells to thaw before

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TRIzol was added into the tube. As the frozen TRIzol-cell mixture started to melt, the homogenization was continued. Then, the homogenized mixture was centrifuged at 12,000 g for 15 min at 4 °C. Following that, the upper layer was removed to a new pre-chilled centrifuge tube and 200 µL of chloroform was added and left at room temperature for 5 minutes. It was then centrifuged at 12,000 g for 15 minutes at 4 °C. In the next step, which is the upper aqueous phase, one volume (500 µL) of ice-cold isopropanol and a half volume of 1.2 M NaCl (250 µL) were added, mixed well, and incubated for an hour at −20 °C. The RNA was collected by centrifugation at 12,000 g for 15 min at 4 °C. At this point, a white colour pellet should be apparent. In order to purify the RNA, the pellet was washed twice with 1 mL 70% ethanol, and then the remaining ethanol was evaporated at 55 °C for 10 mins in a heating block. At the final step, 50 µL of DNase free water was added to dissolve the pellet at 55 °C using a heating block.

3.3.5.2 RNA Purification and quantification

In order to purify the extracted RNA and remove any existing genomic DNA (gDNA), the TURBO DNA-free™ Kit (Ambion, Cat. No. AM1907, Lithuania) was used for each RNA extract. Briefly, 5 µL of TURBO DNase buffer was added to the 50 µL of RNA solution and incubated at 37 ºC for 30 mins to break down existing genomic DNA. Next, 5 µL of DNase inactivation reagent was added and kept at room temperature for 5 mins to inactivate reagents, subsequently, the added reagent was removed using centrifugation. The procedure is detailed in Figure 3.4.

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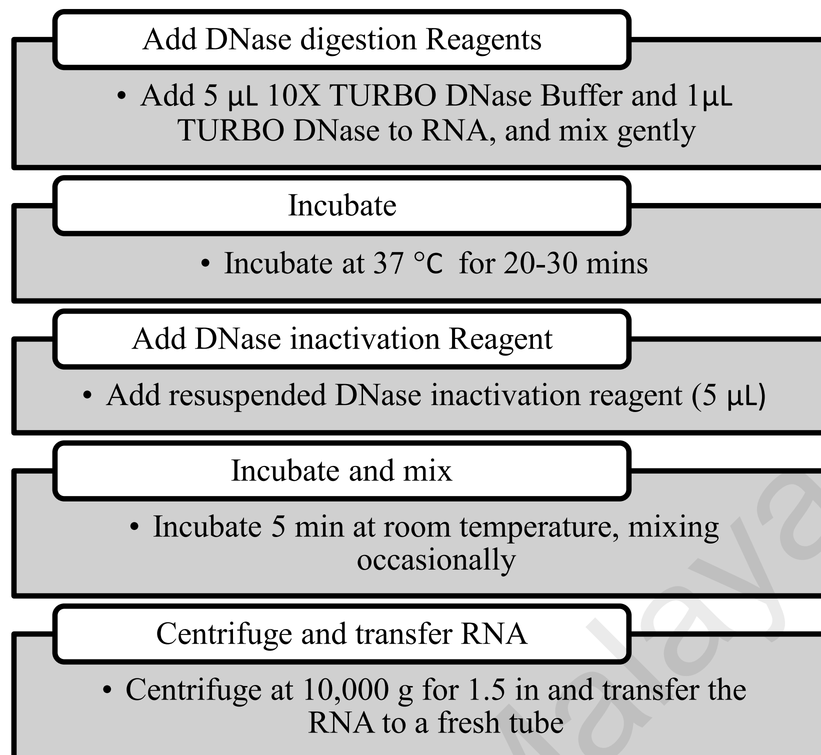

Figure 3.4: Summary of genomic DNA removal from RNA isolate.

NanoDrop Spectrophotometers (NDS) 2000c was used to assess the absorption of RNA sample at several wavelengths, such as 260/280 and 260/230 ratios, which are important indicators of RNA quality and concentration.

3.3.5.3 cDNA Synthesis

The cDNA synthesis was performed with 3200 ng of RNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Cat. No. 4387406, USA). The total volume of 35 µL reactions was made by adding 16 µL reverse transcriptase buffer and 17.25 µL of RNA sample, then 1.75 µL reverse transcriptase enzyme was added. The reaction tube was mixed, then spun down and placed in the thermal cycler set at parameters, as per the manufacturer protocol (Table 3.4).

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Table 3.4: Thermal cycler set up for cDNA synthesis.

Parameters	Step1	Step 2	Step 3
Temperature	37 °C	95 °C	4 °C
Time	60 min	5 min	As required

3.3.5.4 Primer Design and Validation

To obtain the primer sequence for the photosynthetic genes and gene related to fatty acid synthesis which are responding to heat stress, literature was searched, then accordingly the reported corresponding primers were tested (all tested primers are shown in Appendix C). Out of the reported primers, only the primers for *rbcL* and *SAD* were able to amplify the corresponding genes. For the rest of the genes (*H3, psbC, psaB, psbA, accD* and *FAD3*) primers were designed. The gene encoding the protein of interest obtained then the gene was searched among *Chlorella* strain sequences reported in National Center for Biotechnology Information (NCBI) database. The mRNA sequences of the genes of interest obtained from NCBI were used to do multiple sequence alignment (MSA) using ClustalW2 (www.ebi.ac.uk/). From MSA result the conserved regions were highlighted, subsequently from the highlighted region, the primers were designed using IDT online tool (http://sg.idtdna.com/calc/analyzer) based on the criteria such as; a melting temperature (Tm) in the range of 55 °C to 65 °C, absence of dimerization capability, the product size between 70 to 200bp, absence of significant hairpin formation, lack of secondary priming sites, low to moderate specific binding at the 3' end (avoid high GC content to prevent mispriming) the best primers were selected. The example of conserved regions of the selected genes where primers were chosen was highlighted (Appendix D). The housekeeping gene, histone protein subunit (*H3*) was used for data normalization. At least three technical repeats per

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biological repeat were analysed. The following genes were amplified: *psaB, psbC, rbcL, psbA, accD, FAD3* and *SAD*. Initially, primers were tested by performing conventional PCR, and then the PCR product was run in 3% agarose gel for 35 minutes (Appendix E). PCR amplification was performed using i-Taq Plus DNA polymerase (iNtRON Biotechnology). To run PCR an initial DNA denaturation step at 94 °C for 2 min was followed by 35 amplification cycles (0:20 melting at 94 °C, 0:20 annealing at 60 °C, 0:30 extension at 68 °C). All primers pairs were tested for dimer formation and the PCR products were sent for sequencing for the validation (the result of sequencing for each primer is presented in Appendix F) before using them with the actual samples. The final list of primers used in this study is presented in Table 3.5.

Table 3.5: List of genes and primers used for RT-qPCR amplification.

Gene name	Primer sequence	Function	Amplicon size	Ref.
H3	F: GAGATCCGCAAGTACCAGAAG R: GGTCTTGAAGTCCTGGGC	Endogenous control	93bp	This study
psaB	F: GCTGGTCAATCTTTGGCTTC R: AAAGTCTCCGGTCCGATGGT	encodes for the PSI	314bp	Xiong et al. (2014).
psbC	F: CTATGCGTTTCTGGGATTTCCGTG R: GCGTTAATTTCAGTTGCTACACCA	A component of PSII.	184bp	This study
psbA	F: GGTCCTACCAACTTATCGTTTG R: GGACGCATACCTAAACGGAAAGA	Encodes for the D1 protein	94bp	This study
rbcL	F: ATACCGTGTGGAGGACCTTG R: AGCCAGTTCCAGGTGAAGAA	Carbon fixation	235bp	Wan et al., 2014.
FAD3	F:TGTGGCTGGACGTGGTGACCTACCT R: TGAAGATGCCGTAGTCGCGGTC	Omega-3 biosynthesis	137bp	This study
SAD	F: AGTTCTTCAGGCTTGATCCTG R: TCGTTGAACAGGTTCCTGCC	First step of desaturation	136bp	Jusoh, et al., 2015
accD	F: TTAGTTTGTGCTTCTGGTGG R: AGCACAATTTTGATGAACATG	fatty acid synthesis regulation	102bp	This study

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3.3.5.5 Performing RT-qPCR

The PCR reaction was prepared according to PowerUP SYBR® Green Master Mix (Applied Biosystems Cat. No. A25776, USA) guideline. The total volume of 10 µl was prepared by adding 5 µl PowerUP SYBR® Green Master Mix (Applied Biosystems Cat. No. A25776, USA), with 4 µl forward and reverse primers (0.5 µM primer final concentration), and 1 µl cDNA sample. The primers stock was prepared based on the conventional protocol by standardising the concentration to 100 ng/ µl. No template control (NTC) was prepared using all of the reaction components, except samples. Real-time qPCR (RT-qPCR) was performed on an ABI 7500 Fast (Applied Biosystems, USA), and the following cycling steps were set: initial Uracil-DNA Glycosylase (UDG) activation at 50 °C for 2 mins and dual-lock DNA polymerase at 95 °C for 2 mins, followed by 40 cycles with 15 secs 95 °C, 60 secs 60 °C (Table 3.6). The data was collected at the extension step (60 °C). In order to obtain the melting curve, right after PCR amplification, the samples were heated from 60 to 95 °C in a 20 min gradient (Table 3.7).

Table 3.6: RT-qPCR cycling condition.

Steps	Temperature	Duration	Cycles
UDG activation	50 °C	2 minutes	Hold
Dual-Lock DNA polymerase	95 °C	2 minutes	Hold
Denature	95 °C	15 seconds	40
Anneal/extend	60 °C	1 minutes	

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Table 3.7: Dissociation curve condition (Melt curve stage).

Step	Temperature	Duration	Ramp rate
1	95 °C	15 sec	1.6 /Second
2	60 °C	1 minute	1.6 /Second
3	95 °C	15 seconds	0.15 /Second

3.3.6 Statistical Analyses

The effects of temperature on photosynthetic parameters, such as F_v/F_m, alpha and rETRmax were determined using repeated measures analysis of variance (ANOVA) followed by adjusted Bonferroni tests for pair-ways mean comparison. As cultures exhibited different initial values of rETRmax, its effects were excluded as a covariate using analysis of covariance (ANCOVA). In addition, independent sample t-tests were used to compare the ratio of chlorophyll to carotenoid between stress and recovery levels. Also, the effect of temperature on the fatty acid profile was analysed using one-way ANOVA, followed by a comparison of means using a Tukey test. In both analyses, the ANOVA assumptions (variance homogeneity, normal distribution) were examined using the Levene's and Kolmogorov-Smirnov tests. All the statistical analyses were carried out in SPSS 23.0 (SPSS Inc., Chicago, IL, U.S.A.), and the differences were considered significant when $P < 0.05$. For RT-qPCR analysis, all calculations were done using Microsoft Excel. The averages for cycle thresholds (CT) of the *H3* (normalizer) was calculated for each sample. The ΔCT for each of the time points was subtracted from the averaged CT of normalizers to get the ΔΔCT. Using this, the fold change was calculated using the equation: $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). Standard deviations were calculated using the fold changes for each replicate within the time points. Significant differences were determined using p-values from the Excel statistical tools.

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CHAPTER 4: RESULTS

4.1 Growth and Biochemical Characterization of Selected Marine *Chlorella* Strains.

4.1.1 Growth Study

4.1.1.1 Optical Density (OD)

The optical density of the three *Chlorella* strains were based at OD_{620} nm. For all of the strains, increasing the growth temperature increased the optical density as well, however, exceeding those temperature ranges would limit the growth. In *Chlorella*-Ant, growing the cultures at temperatures above the ambient (4 °C) to 28 °C improved growth, but further increasing the temperature hinders it. At 33 °C, slight growth was observed, but at 35 °C, growth was totally inhibited (Figure. 4.1a). In the temperate strain at 25°C, the growth performed is less than the ambient growth (18 °C), but at 28 °C, its performance was much better. However, at temperatures exceeding 28 °C, the growth decreased. Similar to the Antarctic strain, it showed no growth at 35 °C (Figure. 4.1b). In the tropical strain, the best growth performance was observed when cultures were grown at the ambient temperature (28 °C). In this strain, as the temperature increased, the growth decreased. It was able to grow at 38 °C, but cultivation at 40 °C caused serious damage to the cell, and the culture was gone after few days of cultivation (Figure. 4.1c).

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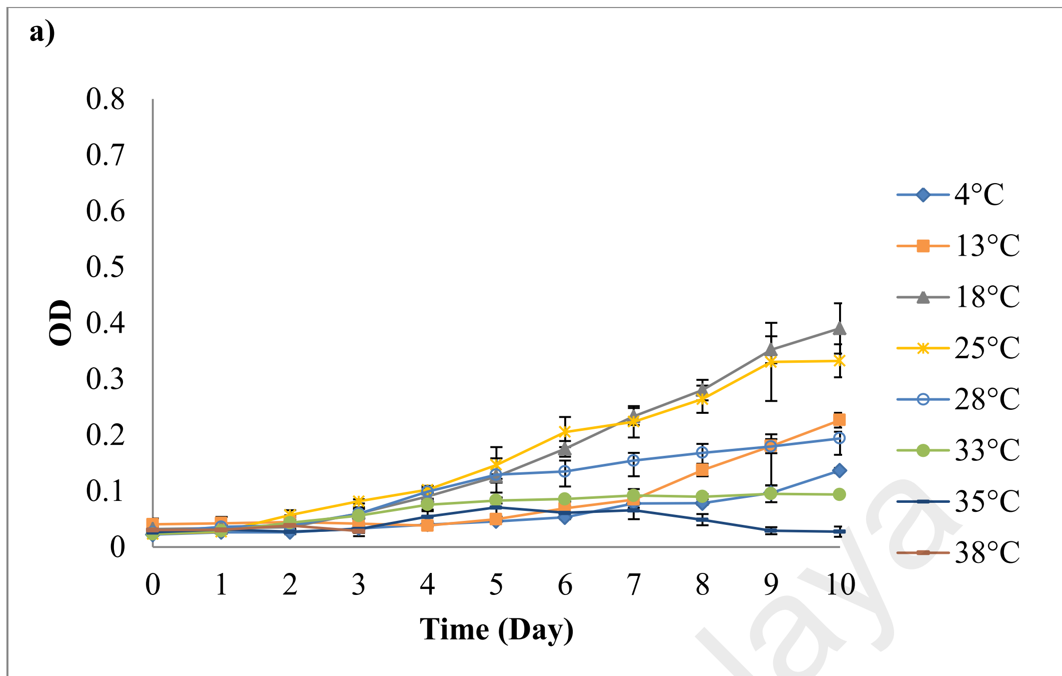

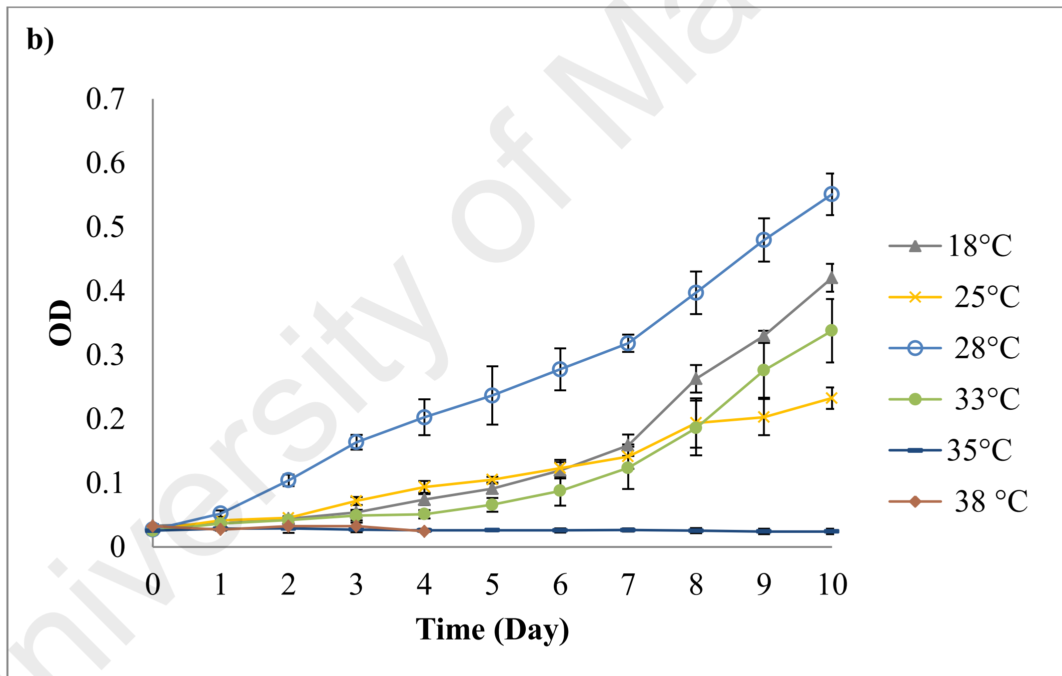

Figure 4.1: Optical density of *Chlorella* strains grown at different temperatures from day-0 to day-10 of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245). Data are presented as a mean ± standard division of the mean.

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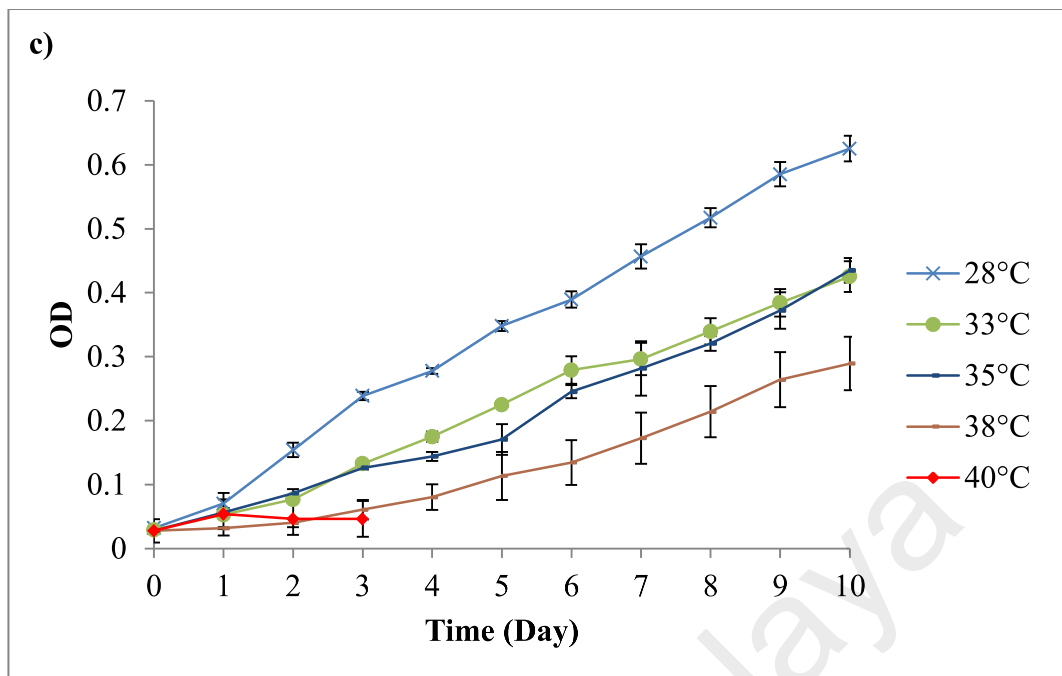

Figure 4.1, continued: Optical density of *Chlorella* strains grown at different temperatures from day-0 to day-10 of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245). Data are presented as a mean ± standard division of the mean.

4.1.1.2 Chlorophyll-*a* to Carotenoid Content

The ratio of chlorophyll to carotenoid of studied *Chlorella* strains are presented in Figure. 4.2. The response of strains toward changing the ratio of chlorophyll to carotenoid ratio was different between the strains. For example in *Chlorella*-Trop temperature did not impact on the ratio (Figure. 4.2c), while in *Chlorella*-Ant the ratio decreased at elevated temperature (Figure. 4.2a). In the Antarctic strain, in most of the temperatures, the ratio was fluctuating around 2. Although initially in treatment at 28 °C, the ratio was around 1.5, it increased during cultivation and reached 2.4 on day 10. In this strain, growing culture at 33 °C and 35 °C had a negative impact on the ratio and caused reduction as it continued to grow (Figure. 4.2a). In the temperate strain, the ratio was higher when cultures were grown at 25 °C and 28 °C but at higher temperatures,

the ratio slightly declined and remained constant during cultivation time (Figure. 4.2b). In the tropical strain, the ratio did not show any trend to temperature change but when it was grown at 38 °C the ratio declined as it continued to grow and it reached to 1.1 at day 10 (Figure. 4.2c).

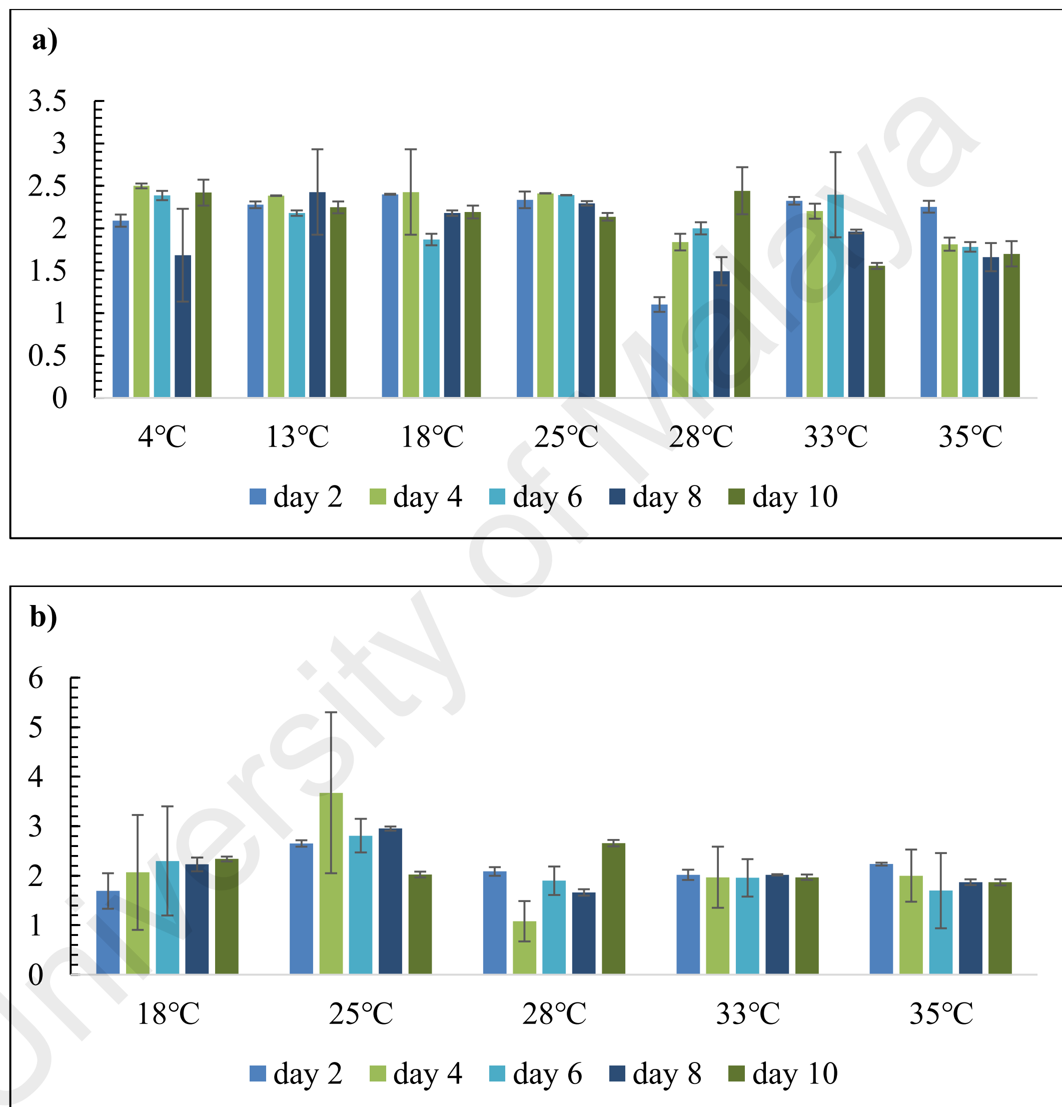

Figure 4.2: Ratio of chlorophyll *a* to carotenoid of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245). Data are presented as a mean ± standard division of the mean.

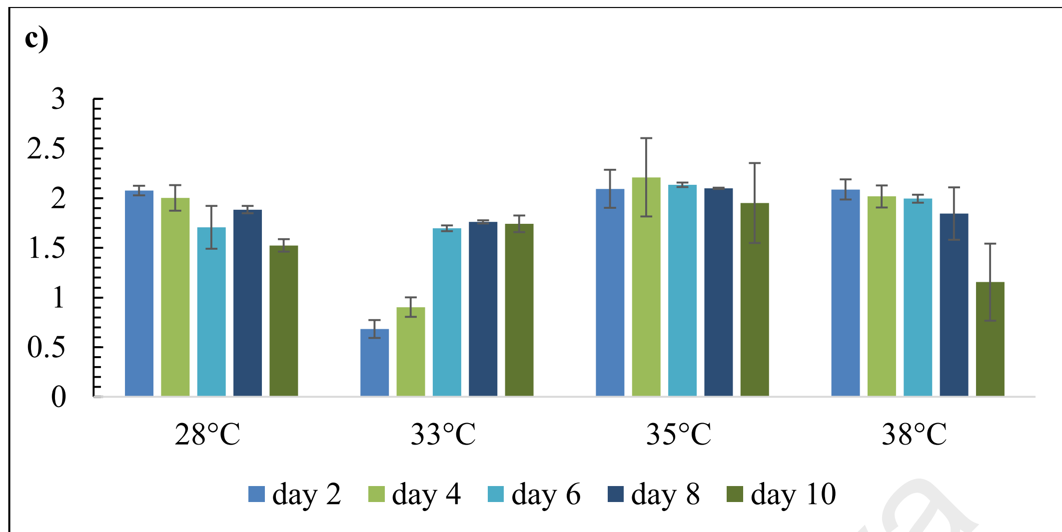

Figure 4.2, continued: Ratio of chlorophyll *a* to carotenoid of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245). Data are presented as a mean ± standard division of the mean.

4.1.1.3 Specific Growth Rate (μ)

The specific growth rates of three *Chlorella* strains are presented in Figure 4.3. Among the investigated strains, *Chlorella*-Ant and *Chlorella*-Trop showed the lowest and the highest specific growth rates at their ambient temperatures, respectively. The growth rate of *Chlorella*-Trop gradually declined with increasing temperature from 28 °C to 38 °C. *Chlorella*-Trop was grown at 28 °C exhibited the highest growth (μ= 0.670 Day^{-1}) compared to other temperatures while at 40 °C, the growth was completely halted. For *Chlorella*-Temp, the temperature increase from 18 °C to 28 °C resulted in a positive effect on the growth rate, while further increase beyond 28 °C decreased the specific growth rate. The highest growth rate was observed at 28 °C (μ= 0.569 Day^{-1}), while the lowest rate was at 35 °C (μ= 0.020 Day^{-1}). Similarly, in *Chlorella*-Ant, temperature increase up to 25 °C positively impacted the specific growth rate; however, further increase beyond 25 °C negatively affected the growth. For *Chlorella*-Ant, the

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maximum growth rate was recorded at 25 °C (μ= 0.553 Day $^{-1}$), while the minimum rate was recorded at 35 °C (μ= 0.111 Day $^{-1}$).

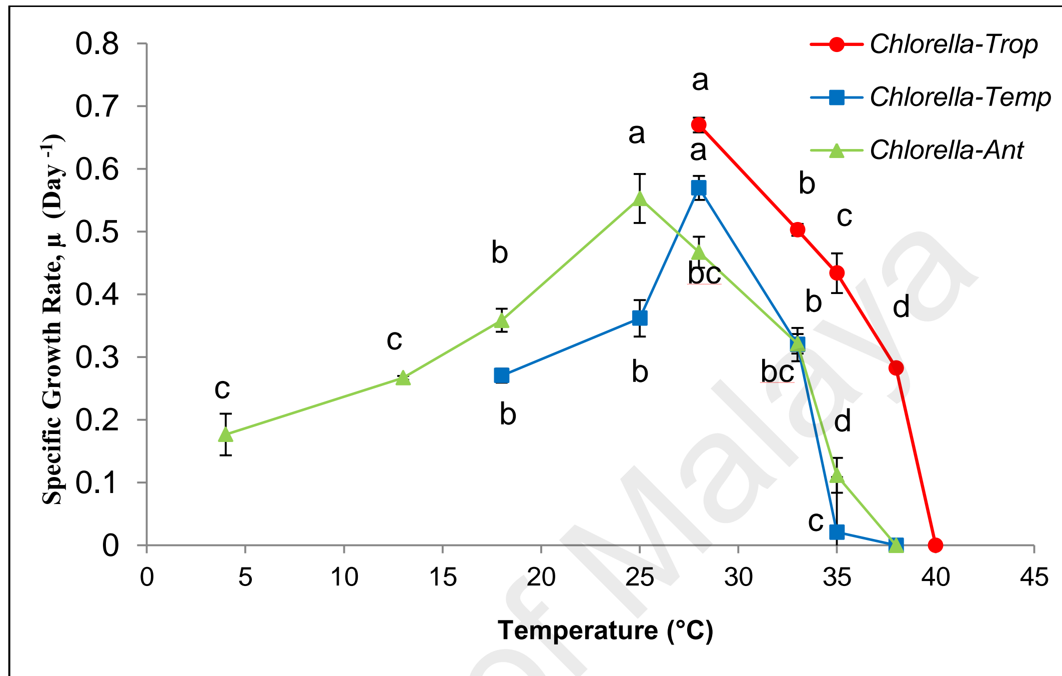

Figure 4.3: The specific growth rate of *Chlorella*-Ant, *Chlorella*-Temp, and *Chlorella*-Trop under different temperatures. Data represent the mean value of triplicates and error bars are standard deviations. Different letters in each strain indicate significant differences at P < 0.05.

4.1.2 Biochemical Composition

4.1.2.1 Protein Content

The response of different *Chlorella* strains differs based on its respective protein content. In *Chlorella*-Ant, the protein contents were not significantly changed by temperature increase, showing the slight fluctuation of ~46 % of its dry weight (Figure. 4.4a). In *Chlorella*-Temp, changes in protein content were insignificant, but a decreasing trend as per increasing temperature is evident. The protein content decreased

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from 45 % to 36 % when the cultures were grown at 28 °C and 35 °C, respectively (Figure. 4.4b). In *Chlorella*-Trop, the protein content significantly changed due to temperature fluctuations. The highest protein content measured at 28 °C by 36 %, while the lowest occurred when it was grown at the highest temperature (38 °C) by 23 % (Figure. 4.4c).

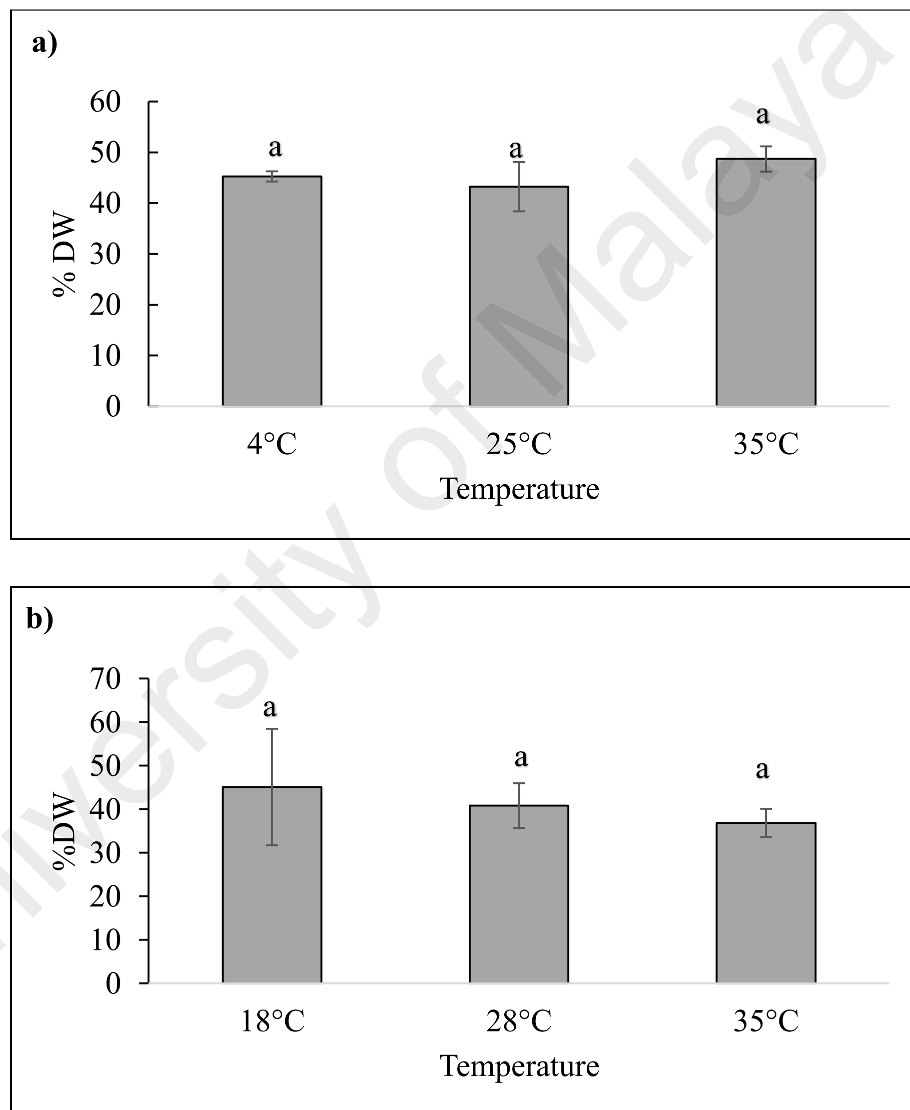

Figure 4.4: Protein content of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245). Data are presented as a mean ± standard division of the mean. Different letters in each strain indicate significant differences at P < 0.05.

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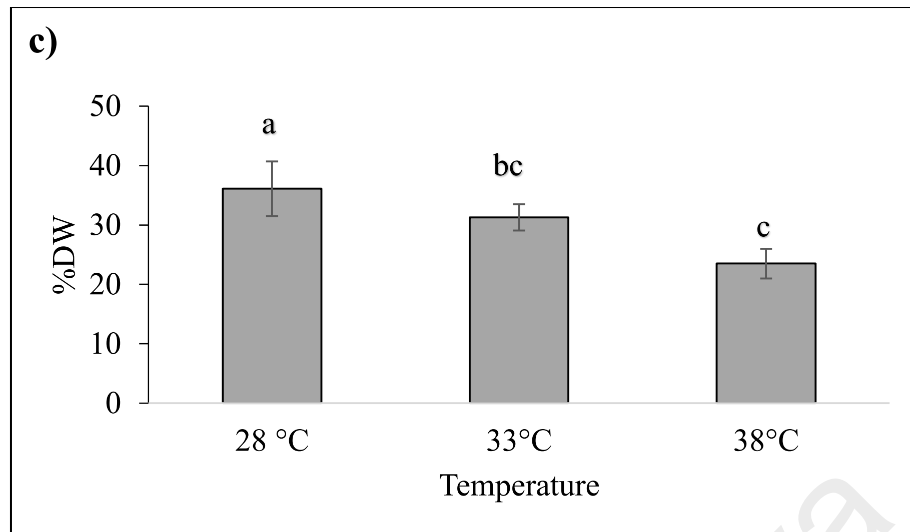

Figure 4.4, continued: Protein content of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245). Data are presented as a mean ± standard division of the mean. Different letters in each strain indicate significant differences at P < 0.05.

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4.1.2.2 Carbohydrate Content

The carbohydrate contents of the three *Chlorella* strains were generally lower than the protein content between 6 − 25 %DW. In Figure 4.5 the carbohydrate contents of the strains in different cultivation treatments are shown. The strains responded to temperature treatments significantly by changing its carbohydrate content, however, this response differed based on strains. In the Antarctic strain, changes in its carbohydrate content were significant among the studied temperatures. The carbohydrate content significantly decreased when it grew at 35 °C, while the highest proportion of carbohydrate was observed when it was grown at 25 °C (Figure. 4.5a). In the temperate strain, a decreasing trend in carbohydrate percentage was observed as per the increasing temperature. The carbohydrate percentage dropped from 23 to 13 % DW when the culture was grown at 18 °C and 35 °C, respectively (Figure. 4.5b). In the tropical strain, the trend was opposite to temperate strain, and it reported an increase in carbohydrate

content due to increasing temperature. The proportion of carbohydrate in the culture grown at ambient (28 °C) was reported to be 19 % DW, while when it was grown at 38 °C, its percentage increased to 24 % DW (Figure. 4.5c).

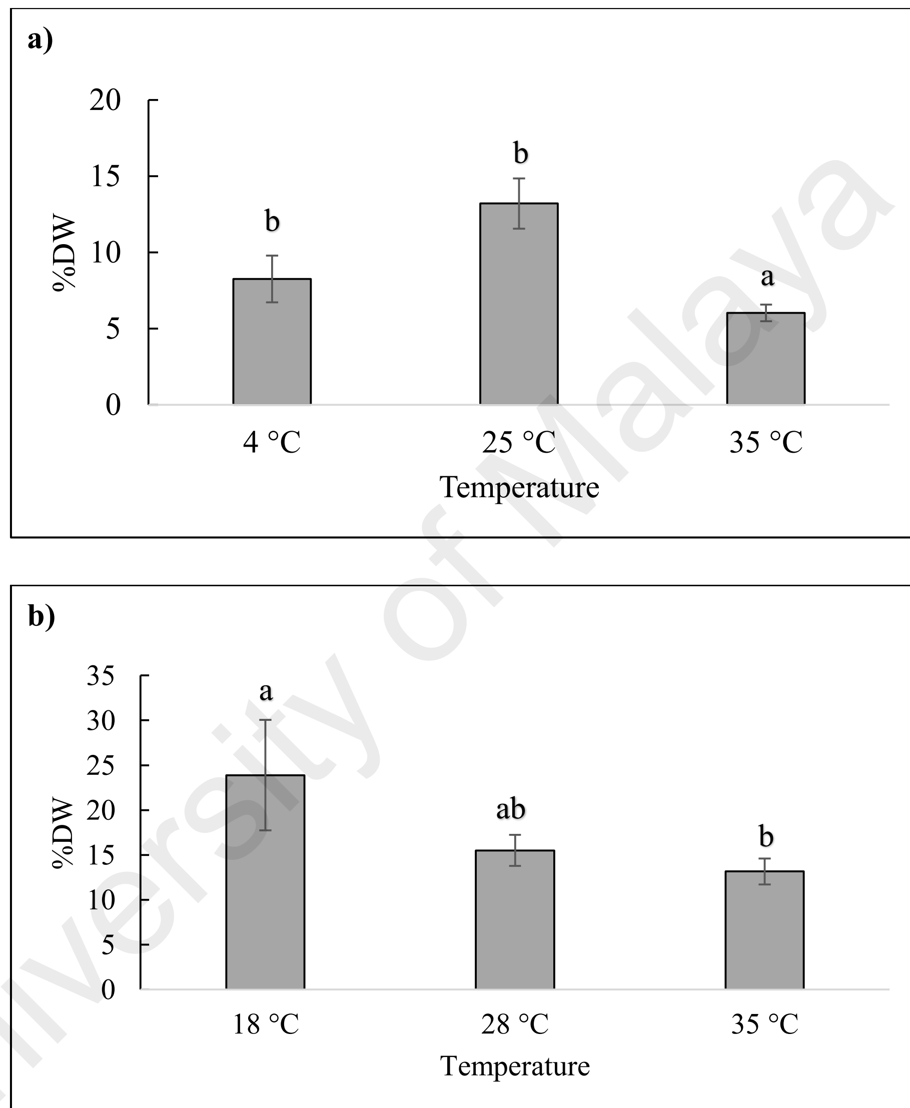

Figure 4.5: Carbohydrate content of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245). Data are presented as a mean ± standard division of the mean. Different letters in each strain indicate significant differences at P < 0.05.

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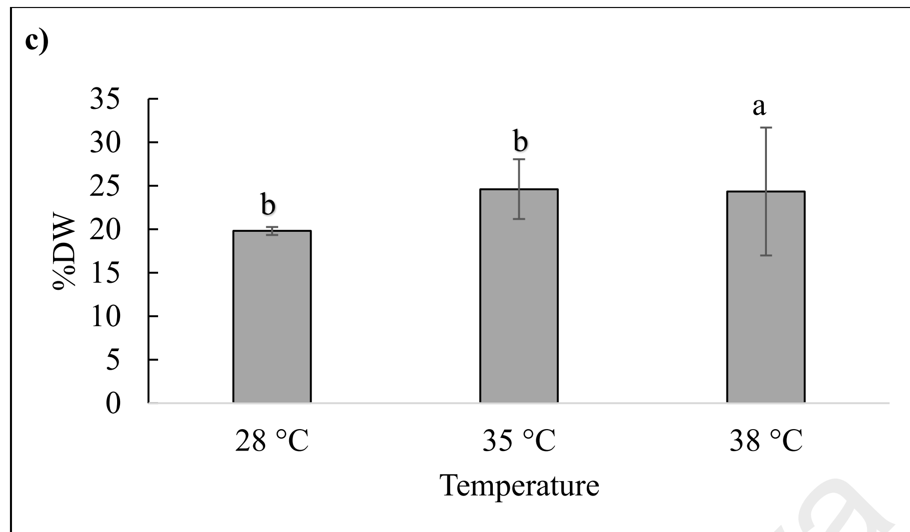

Figure 4.5, continued: Carbohydrate content of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245). Data are presented as a mean ± standard division of the mean. Different letters in each strain indicate significant differences at $P < 0.05$.

4.1.2.3 Lipid content

In Figure 4.6, the lipid contents of selected strains are presented for different temperatures. Generally, no significant changes in lipid content can be observed. The lowest lipid content was observed in *Chlorella*-Temp when it was cultivated at an ambient temperature (18 °C) by 28 % DW, while the highest lipid content observed in *Chlorella*-Trop when it was grown at a high temperature (38 °C). The lipid content in the Antarctic strain fluctuated ~34 - 37 % DW and did not show any significant changes due to temperature (Figure. 4.6a). In the temperate strain, the lipid content increased in tandem with the temperature. The lipid content increased from 28 % DW to 40 % DW when it was grown at 18 °C and 35 °C, respectively (Figure. 4.6b). In the tropical strain, similar to the temperate strain, it increased in tandem with increasing temperature. The lipid content increased from 34 % DW to 43% DW when the cultures were grown at 28 °C and 38 °C, respectively (Figure. 4.6c).

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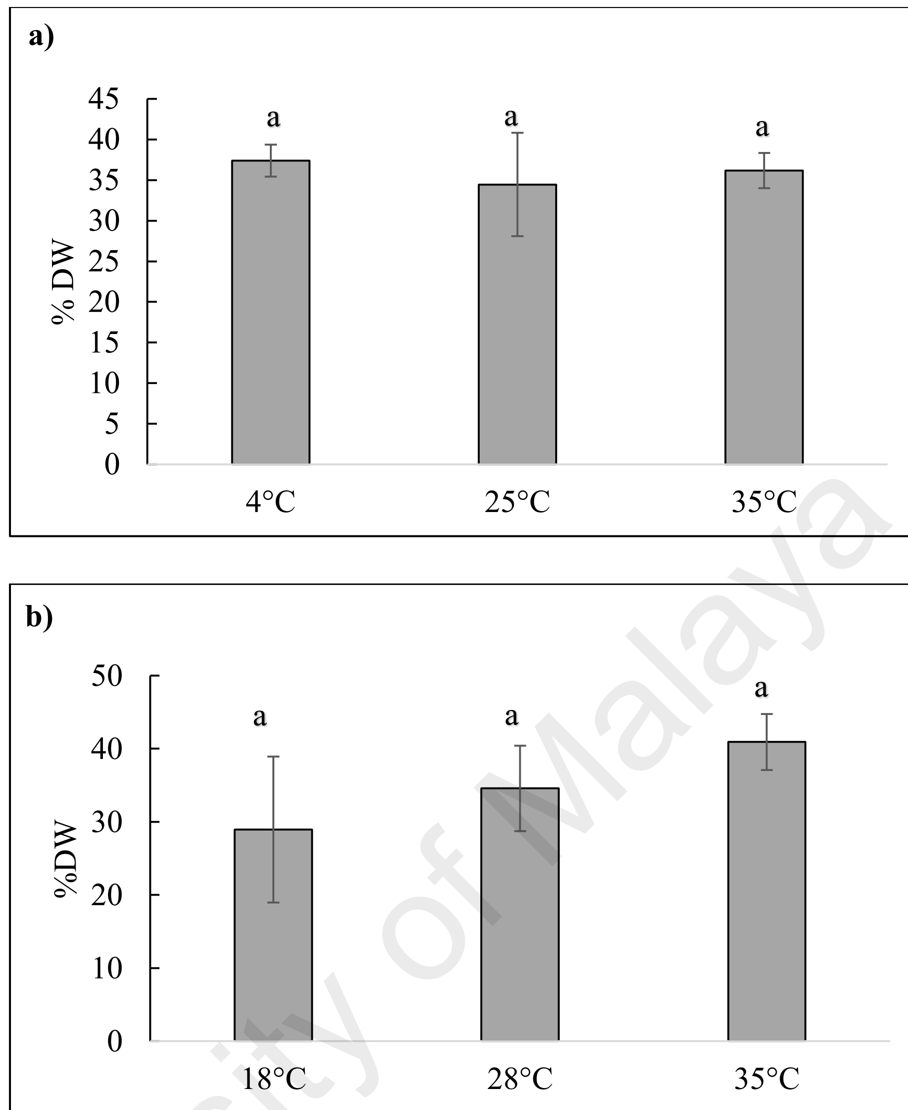

Figure 4.6: Lipid content of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245). Data are presented as a mean ± standard division of the mean. Different letters in each strain indicate significant differences at $P < 0.05$.

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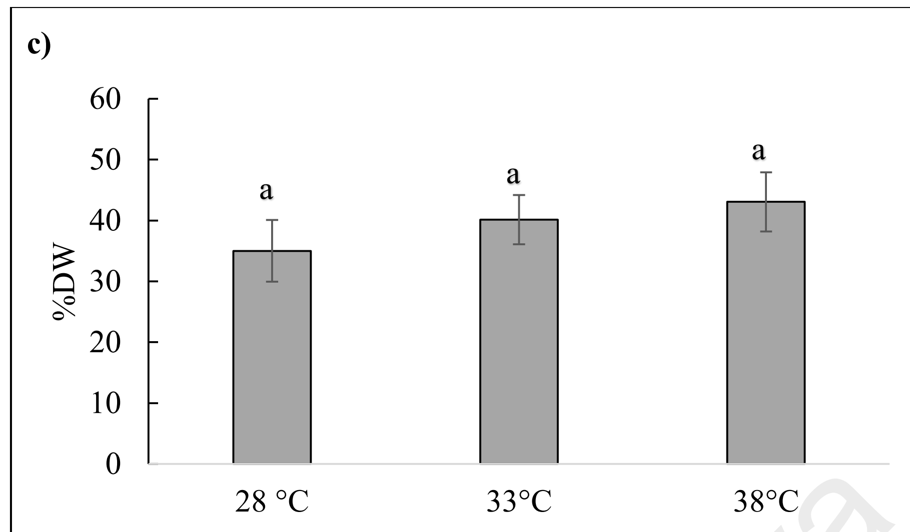

Figure 4.6, continued: Lipid content of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245). Data are presented as a mean ± standard division of the mean. Different letters in each strain indicate significant differences at $P < 0.05$.

4.1.2.4 Fatty Acid Composition

Generally, SFAs were the dominant type of fatty acids in all of the studied algae and constituted more than 50% of the total fatty acids in the cells. Overall, *Chlorella* tends to produce saturated fatty acids with carbon chain lengths of C16 and C18 (Vello et al., 2014). Similarly, in this study, the C16:0 was the dominant fatty acid in all of the treatments. However, the dominant monosaturated fatty acids (MUFA) varied between the strains. For example, in *Chlorella*-Trop and *Chlorella*-Temp, C16:1 and C18:1n9c were the dominant MUFA, respectively, while in *Chlorella*-Ant, a low amount of MUFA was detected. The main PUFA for all strains regardless of temperature was C18:2n6c. Fatty acid profiles in response to changes in temperature vary with strain. For example, in *Chlorella*-Trop and *Chlorella*-Temp, the percentage of MUFA was inversely correlated with temperature, although these changes were seen as being more significant in the temperate strain, while in *Chlorella*-Ant, the MUFA percentage

increased significantly (one-way ANOVA, $p<0.05$) at 25 °C, but decreased at 35 °C. Of the three strains, *Chlorella*-Trop accumulated the greatest amount of PUFA, while *Chlorella*-Ant accumulated a high percentage of SFA at the analyzed temperatures (above 73%). Furthermore, in *Chlorella*-Temp and *Chlorella*-Trop, no significant changes (one-way ANOVA, $p>0.05$) were observed in the proportions of PUFA when exposed to high temperatures (Table 4.1).

Table 4.1: Fatty acid profile of *Chlorella*-Trop, *Chlorella*-Temp and *Chlorella*-Ant incubated at three different temperatures. Data are presented as the mean percentage of total fatty acids; n=3. Different superscripts within a row indicate a significant difference between growth temperatures of each strain ($p<0.05$). (–) indicates that the fatty acid was not detected.

	Chlorella-Trop			*Chlorella*-Temp			*Chlorella*-Ant		
	28 °C	**33 °C**	**38 °C**	**18 °C**	**28 °C**	**35 °C**	**4 °C**	**28 °C**	**35 °C**
Saturated fatty acids									
C4:0	1.89	0.35	0.96	0.77	1.89	1.01	36.04	19.49	22.98
C6:0	-	-	-	-	-	-	-	-	-
C8:0	3.00	2.47	1.38	0.99	1.85	2.95	1.46	2.05	3.10
C10:0	6.23	4.32	1.97	2.90	4.02	5.28	2.06	4.50	4.90
C11:0	1.70	1.15	1.17	0.60	0.63	0.89	0.88	0.46	0.91
C12:0	1.73	2.50	1.21	1.10	1.16	0.69	0.15	0.96	0.23
C13:0	1.53	1.64	0.45	1.00	1.20	1.00	0.22	1.31	0.03
C14:0	0.99	0.76	0.85	0.61	0.70	0.93	0.68	0.47	0.85
C15:0	0.30	0.23	0.19	0.21	0.22	0.00	0.00	0.00	0.04
C16:0	35.87	33.60	38.96	35.29	47.97	50.57	35.85	36.23	45.84
C18:0	8.29	6.59	9.18	4.55	8.66	8.69	17.77	7.59	19.12
C23:0	0.09	0.13	-	0.02	0.02	-	-	-	0.05
SUM	**61.62±7.5** ^{a}	**53.75±0.8** ^{a}	**56.30±19.4** ^{a}	**48.04±5.9** ^{b}	**68.33±11.2** ^{a}	**72.01±0.8** ^{a}	**95.10±5.5** ^{ab}	**73.05±4.7** ^{b}	**98.05±1.9** ^{a}
Monounsaturated fatty acids									

C14:1	0.17	0.20	0.21	0.11	0.03	-	-	-	-
C15:1	1.63	0.39	0.03	1.91	0.55	-	-	0.18	-
C16:1	6.88	4.97	3.31	2.87	0.82	-	-	0.35	-
C18:1n9c	2.25	1.71	1.29	42.16	15.05	13.70	1.80	8.30	0.67
C20:1n9	3.56	2.74	2.27	-	-	-	-	-	-
C22:1n9	0.10	0.24	0.18	-	-	-	-	-	0.09
SUM	**14.60±2.7** [a]	**0.25±0.1** [b]	**7.28±1.3** [b]	**7.06±5.8** [a]	**16.45±3.6** [b]	**13.70±1.5** [b]	**1.80±1.7** [b]	**8.83±3.9** [a]	**0.76±1.1** [b]
Polyunsaturated fatty acids									
C18:2n6t			1.68	0.99	1.97	4.38	0.87	2.99	0.20
C18:2n6	23.78	36.00	24.39	3.92	13.26	9.91	2.23	15.14	0.99
C20:2			0.20	-	-	-	-	-	-
C20:3n3			10.15	-	-	-	-	-	-
SUM	**23.78±4.9** [a]	**6.00±0.9** [a]	**6.42±18.9** [a]	**4.90±0.6** [a]	**15.22±13.8** [a]	**14.29±0.7** [a]	**3.10±3.9** [ab]	**18.13±10.8** [a]	**1.19±0.8** [b]

4.1.3 Photosynthesis Properties

4.1.3.1 Maximum Quantum Yield (F_v/F_m)

The F_v/F_m values of *Chlorella*-Ant varied slightly between 0.49 and 0.67 at 4 – 28 °C, indicating a healthy state of the photosynthetic apparatus in the cells. Incubation at 33 °C and 35 °C negatively influenced the F_v/F_m, lowering it to under 0.4 by the end of the experiments. Further increase in temperature (at 38 °C) decreased the F_v/F_m from 0.680 to under 0.1 within four days of incubation (Figure. 4.7a). After seven days of incubation, F_v/F_m at 33 °C and 35 °C showed a significantly different trend compared with incubations under 28 °C (repeated-measure ANOVA, $P<0.05$). Despite the significant decline of F_v/F_m at 33 °C and 35 °C from day seven (repeated-measure ANOVA, $P<0.05$), they reached similar F_v/F_m values on day ten. The F_v/F_m values of *Chlorella*-Temp fluctuated between 0.510 – 0.670 at 18 °C to 33 °C, showing an active photosynthetic state, while incubation at 35 °C adversely affected the F_v/F_m value with

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a decrease to less than 0.2 within 8 days of incubation. Moreover, a further increase in temperature (at 38 °C) decreased the F_v/F_m to less than 0.2 after two days of incubation (Figure. 4.7b). The F_v/F_m of *Chlorella*-Temp showed a marked decline from day four at 33 °C and from day two at 35 °C, and the F_v/F_m was significantly different (repeated-measure ANOVA, P<0.05) at the end of treatment. The maximum quantum yield (F_v/F_m) of *Chlorella*-Trop incubated at 28 °C and 33 °C fluctuated around 0.5-0.7 displaying the natural variability of F_v/F_m in the *Chlorella* species, while at 35 °C and 38 °C, the F_v/F_m, dropped to 0.36 at the end of the treatment. As expected, a further increase in temperature (at 40 °C) reduced the F_v/F_m to less than 0.1 on day three (Figure. 4.7c). For *Chlorella*-Trop, the F_v/F_m at 33 °C to 40 °C were significantly different (repeated-measure ANOVA, P>0.05) compared to the control (28 °C) from day seven onwards, with 35 °C and 38 °C showing similar patterns of inhibition.

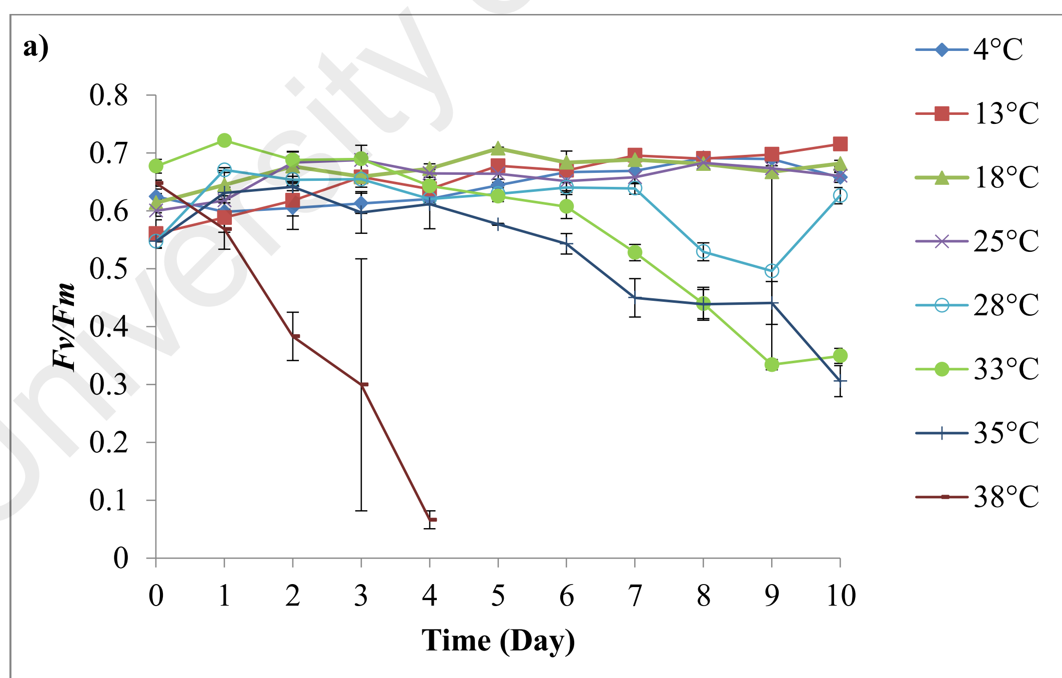

Figure 4.7: Maximum quantum yield (F_v/F_m) of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245). Data are presented as a mean ± standard division of the mean.

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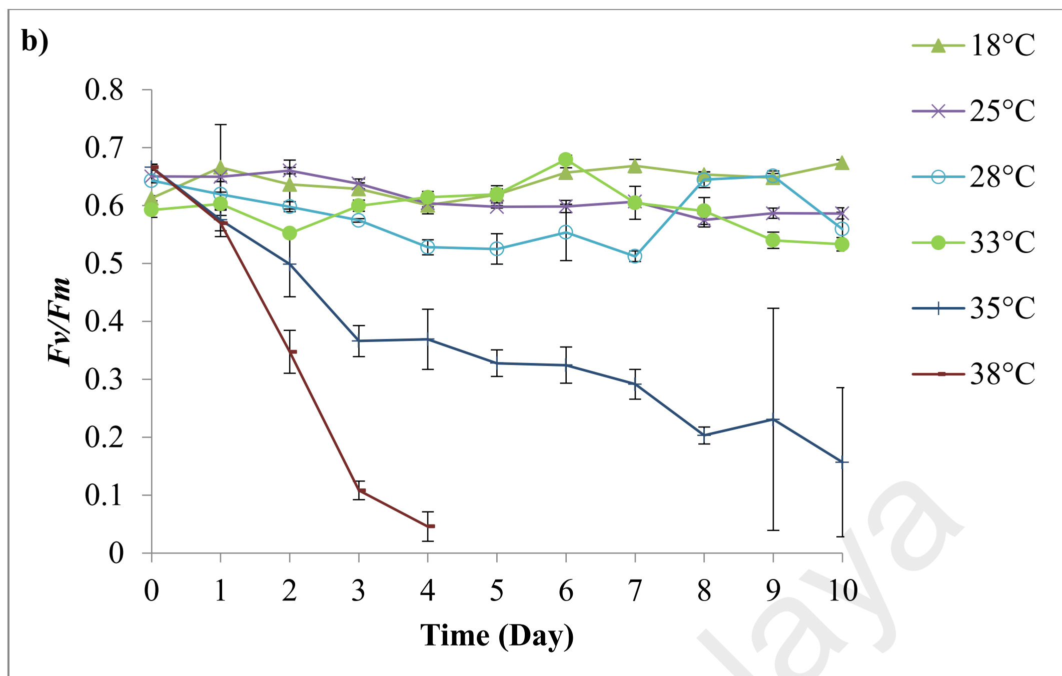

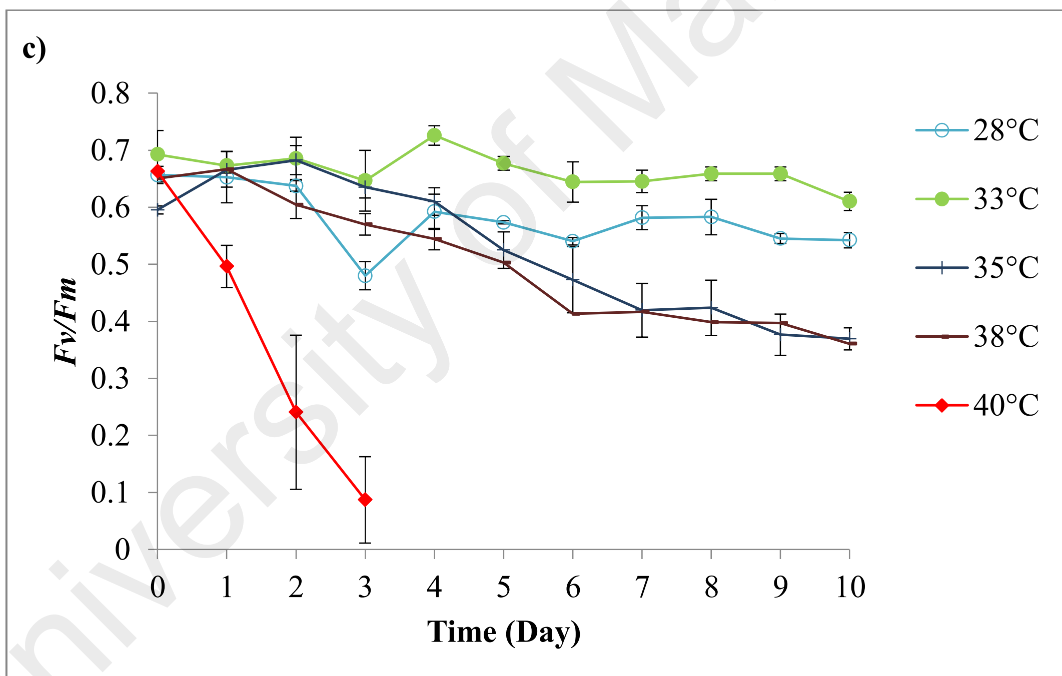

Figure 4.7, continued: Maximum quantum yield (F_v/F_m) of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245). Data are presented as a mean ± standard division of the mean.

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4.1.3.2 Photosynthetic Efficiency (α).

Incubation of *Chlorella*-Ant at 4 – 25 °C positively influenced the α value, while at 28 °C, the α value did not increase and only fluctuated between 0.341 and 0.485. In contrast, additional temperature increases (33 °C and 35 °C) adversely impacted the α value, and it declined significantly (repeated-measure ANOVA, $P<0.05$) after day seven. As can be expected at 38 °C, the α value decreased abruptly to under 0.1 in a short amount of time (Figure. 4.8a). Incubation of *Chlorella*-Temp at 18 °C to 33 °C positively influenced the α value, while at 25 °C, α remained quite similar. At 35 °C, the α value increased for the first two days, after which it dropped significantly (Figure. 4.8b). The α value of *Chlorella*-Trop at 28 °C was the highest (0.582) on day 1, thereafter it reduced and fluctuated between 0.409 – 0.440. *Chlorella*-Trop at 33 °C had a higher value of α, with a maximum of 0.759 at day four, and although α decreased slightly at day five, it remained higher compared to other temperatures. Incubation at 35 °C increased the α value in the first two days, but the effect was not constant, and it began to decrease gradually from day three. In contrast, incubation at 38 °C did not increase the α value, and from day two, it suffered a precipitous decline. As expected, incubation at 40 °C significantly reduced the α value in a short period of time (Figure. 4.8c).

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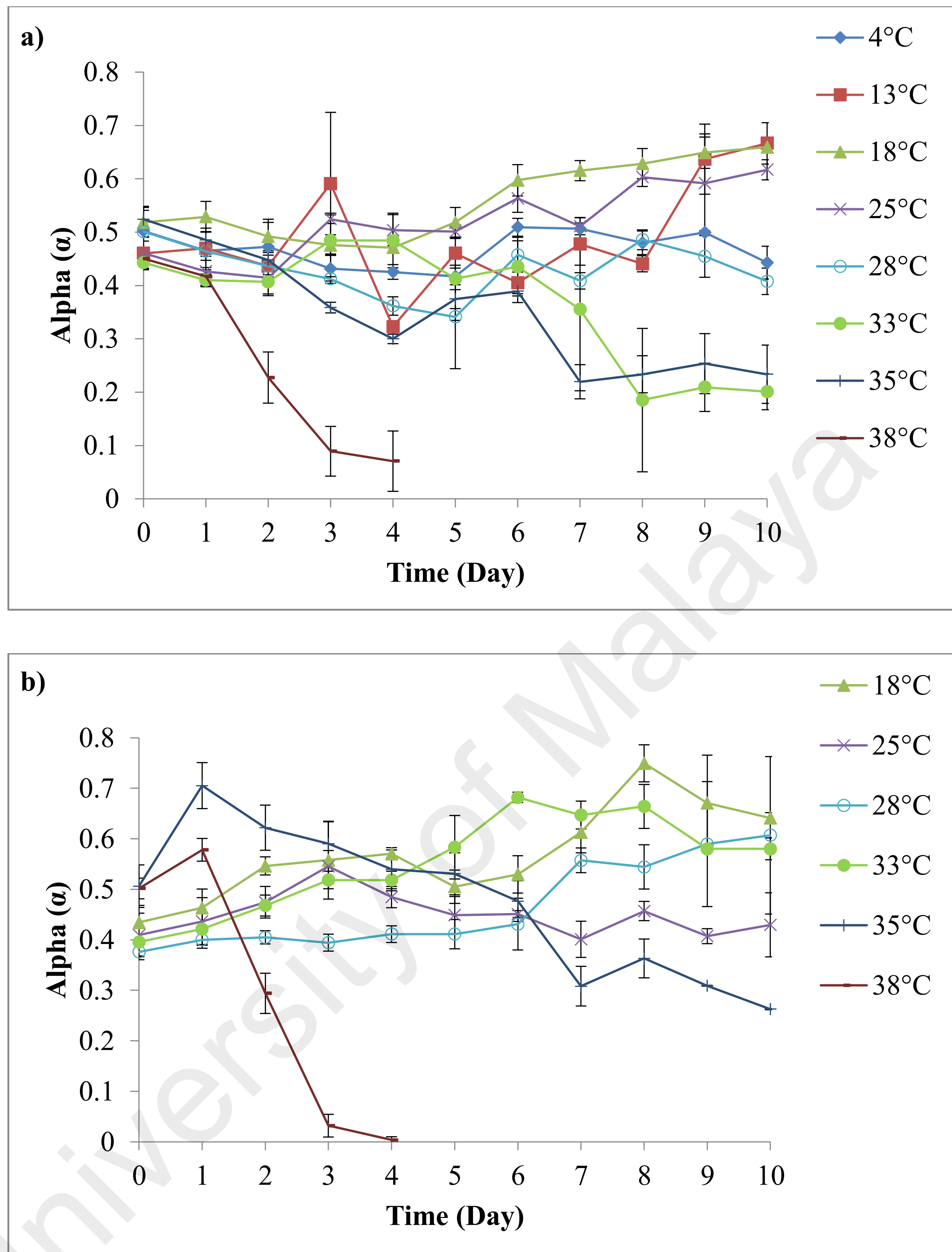

Figure 4.8: Photosynthetic efficiency (α) of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245). Data are presented as a mean ± standard division of the mean.

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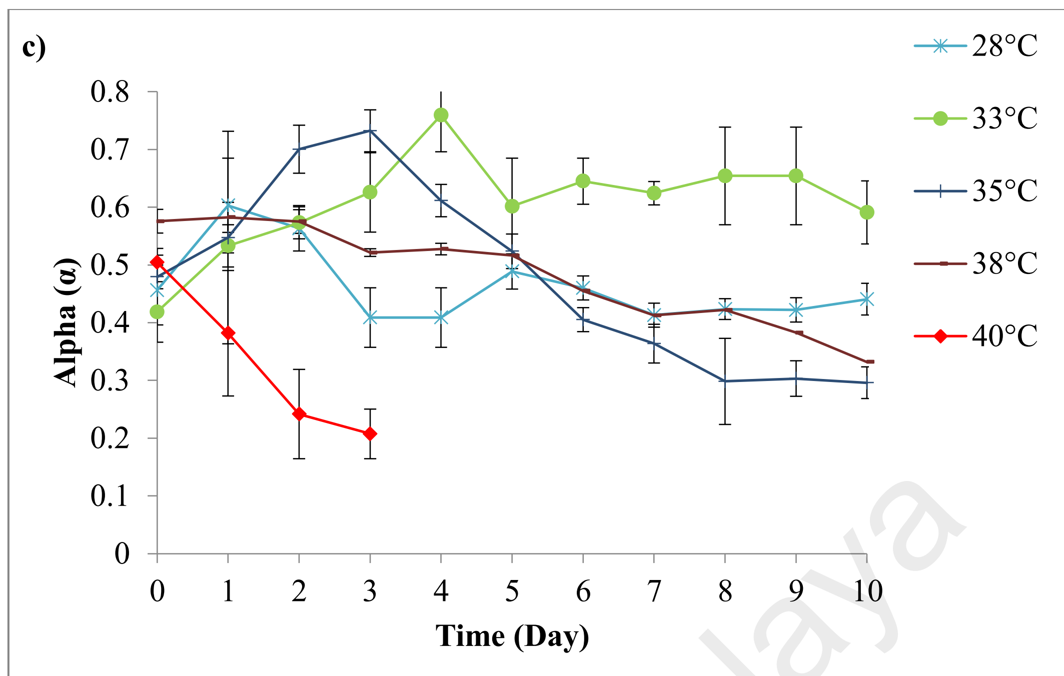

Figure 4.8, continued: Photosynthetic efficiency (α) of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245). Data are presented as a mean ± standard division of the mean.

4.1.3.3 rETRmax

Incubation of *Chlorella*-Ant at ambient temperature saw an increase of rETRmax throughout the experiment. However, incubations above the ambient temperature negatively impacted the rETRmax, whereby the magnitude of inhibition increased with incubation time (Figure. 4.9a). For *Chlorella*-Temp, rETRmax showed different trends in response to temperature treatments. For example, incubation at 18 °C and 25 °C did not result in a significant change in rETRmax, however, the values of rETRmax increased throughout the incubation at 28 °C and 33 °C. Meanwhile, incubation at 35 °C noticeably inhibited rETRmax (Figure. 4.9b). The rETRmax of *Chlorella*-Trop showed a decreasing trend during incubation at all temperatures. At 38 °C, rETRmax showed an extreme reduction after several days of incubation (Figure. 4.9c).

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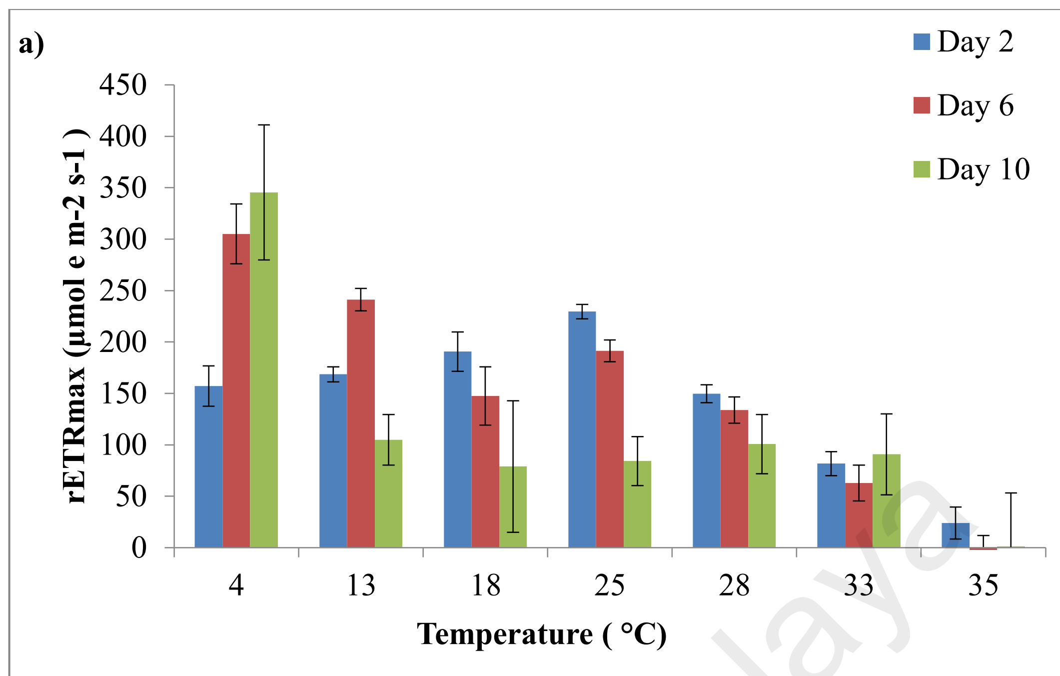

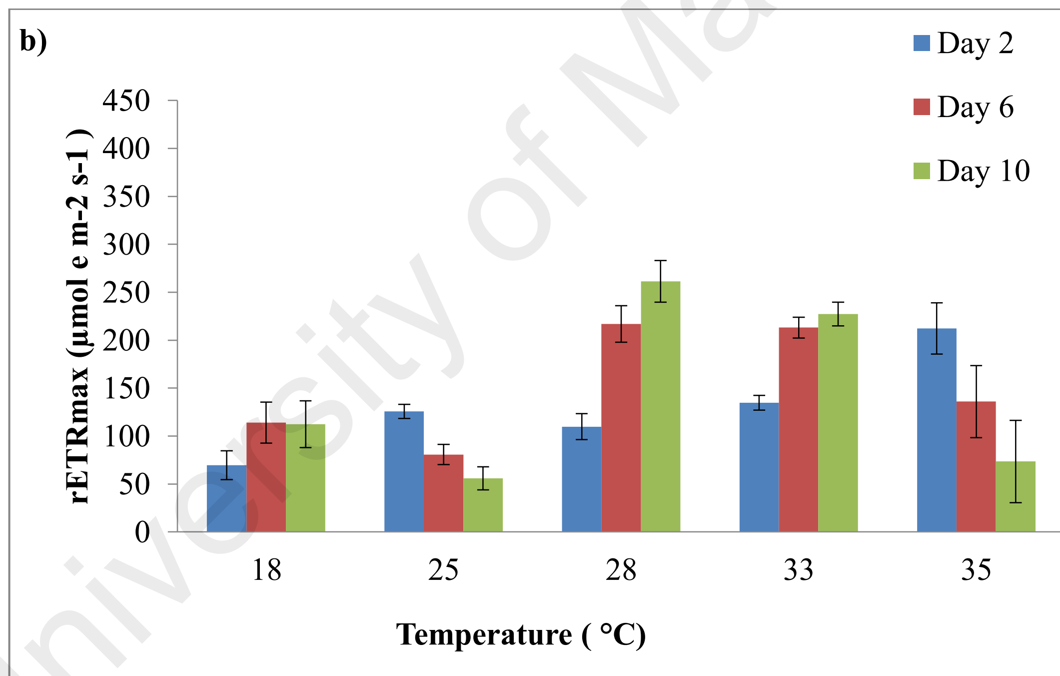

Figure 4.9: rETRmax of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245). Data are presented as mean ± standard division of the mean.

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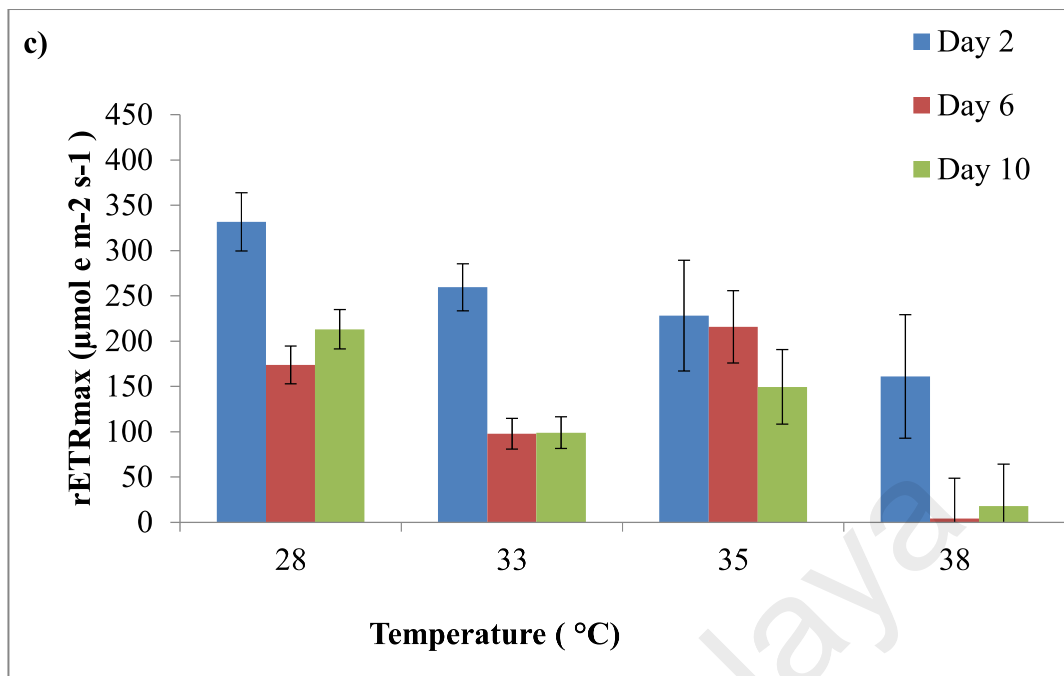

Figure 4.9, continued: rETRmax of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245). Data are presented as mean ± standard division of the mean.

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4.2 Experiment 2: Stress and Recovery Treatment

After the first experiment (growth studies at various temperatures), for each strain, the temperature that induced severe damage to photosynthesis (caused F_v/F_m to drop to zero) and inhibited growth was selected for the temperature stress experiment. 38 °C was selected for both *Chlorella*-Ant and *Chlorella*-Temp, while 40 °C was selected for *Chlorella*-Trop. In this experiment, an inoculum size of 40% (v/v) was prepared by inoculating 1,200 mL of culture (from exponential phase), where the cell density was standardised at an optical density of 0.2 at 620 nm (OD_{620}), into 1800 mL fresh Prov media. During stress treatment, the cultures were incubated at their stress-inducing temperatures, and the decrease in F_v/F_m was closely monitored to determine the stress levels. When the F_v/F_m value decreased to ~0.4, 0.2, and 0.0, 500 mL of the culture was transferred to an empty, sterilised, 1 L conical flask, and returned to the normal ambient

temperature to determine their capacity for recovery. The cultures were regarded as recovered when the F_v/F_m regained its value of above 0.5.

4.2.1 Growth and Photosynthesis

After incubation at a temperature that caused a severe reduction in F_v/F_m (stress-inducing temperatures), the cultures were returned to ambient temperatures for recovery. Generally, the stressed cells exhibited photosynthetic activity after being transferred to control temperatures, and the period of recovery varies with strain and condition. The *Chlorella*-Trop exhibited quick recovery in both growth and photosynthesis. In contrast, the F_v/F_m of *Chlorella*-Ant continuously decreased even after the cultures were returned to its respective ambient temperature. The incubation of *Chlorella*-Ant at the stress-inducing temperature of (38 °C) reduced the F_v/F_m from 0.649 to ~0.4, 0.2 and 0.0, in 48, 72, and 120 hours, respectively, while their photosynthetic recovery took 96, 144, and more than 196 hours, individually (Figure. 4.10a). In *Chlorella*-Ant, a slight increase in biomass was observed when it was transferred to ambient temperature after enduring a set lag time (Fig 4.11a). For *Chlorella*-Temp, its incubation at the stress-inducing temperature (38 °C) decreased the F_v/F_m value from 0.520 to ~0.4, 0.2, and 0.0, in 6, 24, and 72 hours respectively, with a photosynthetic recovery periods of 66, 72, and 168 hours, individually (Figure. 4.10b). However, the *Chlorella*-Temp that recovered from having its F_v/F_m value reduced to 0.0 was not able to grow even after 168 hours of incubation at its ambient temperature (Figure. 4.11b). In *Chlorella*-Trop, incubation at the stress-inducing temperature (40 °C) reduced the F_v/F_m from 0.637 to ~0.4, 0.2, and 0.0, in 54, 72, and 192 hours respectively, with a photosynthetic (F_v/F_m) recovery periods of 24, 48, and 120 hours, respectively (Figure. 4.10c). The recovered cultures from F_v/F_m 0.4 were able to grow, while the recovered cells from the other stress levels did not show any significant

growth (Figure. 4.11c). The *Chlorella*-Trop reported increasing growth upon recovery from all stress levels (Figure. 4.11c).

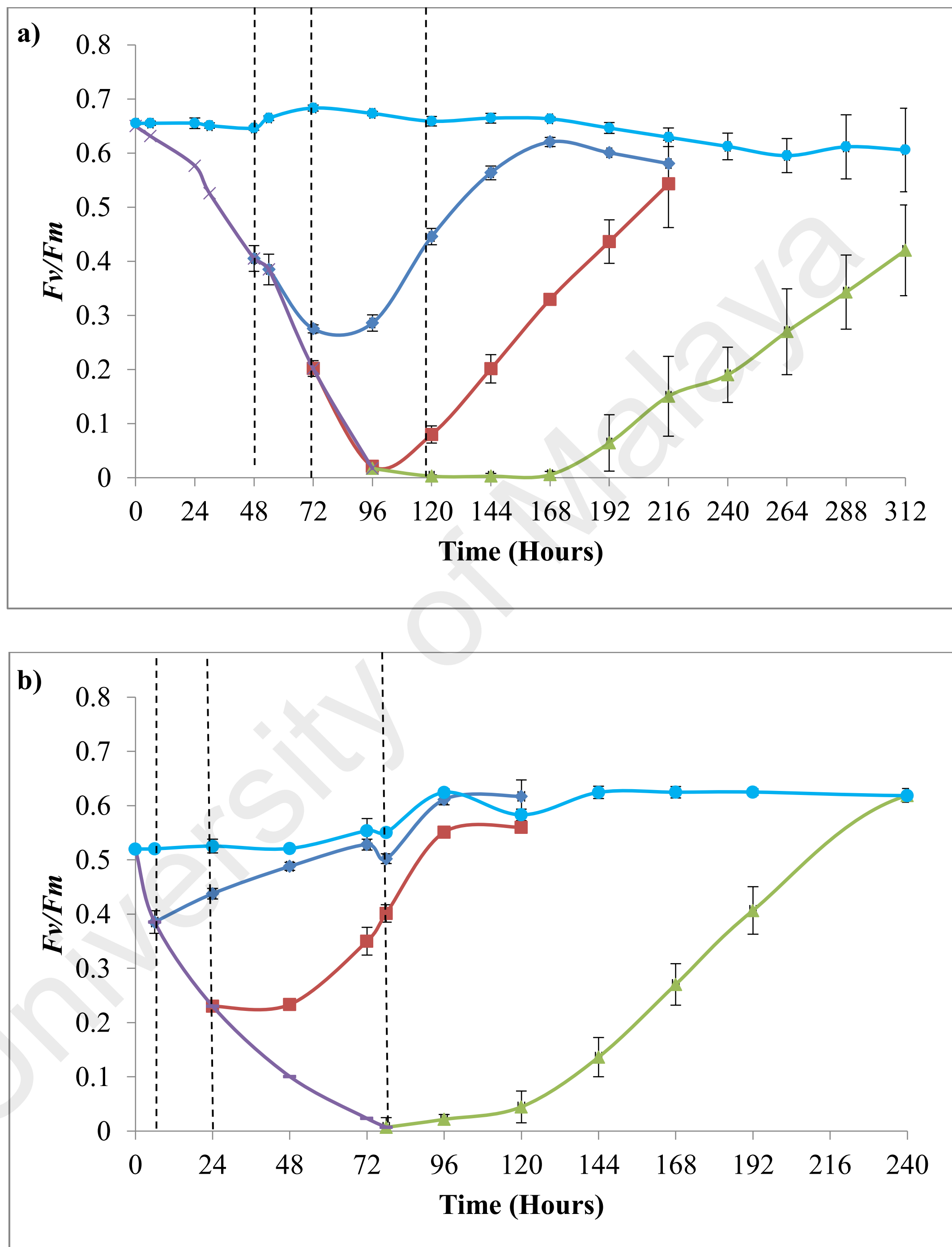

Figure 4.10: Maximum quantum yield (F_v/F_m) of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245) during stress and recovery. Data are presented as a mean ± standard division of the mean.

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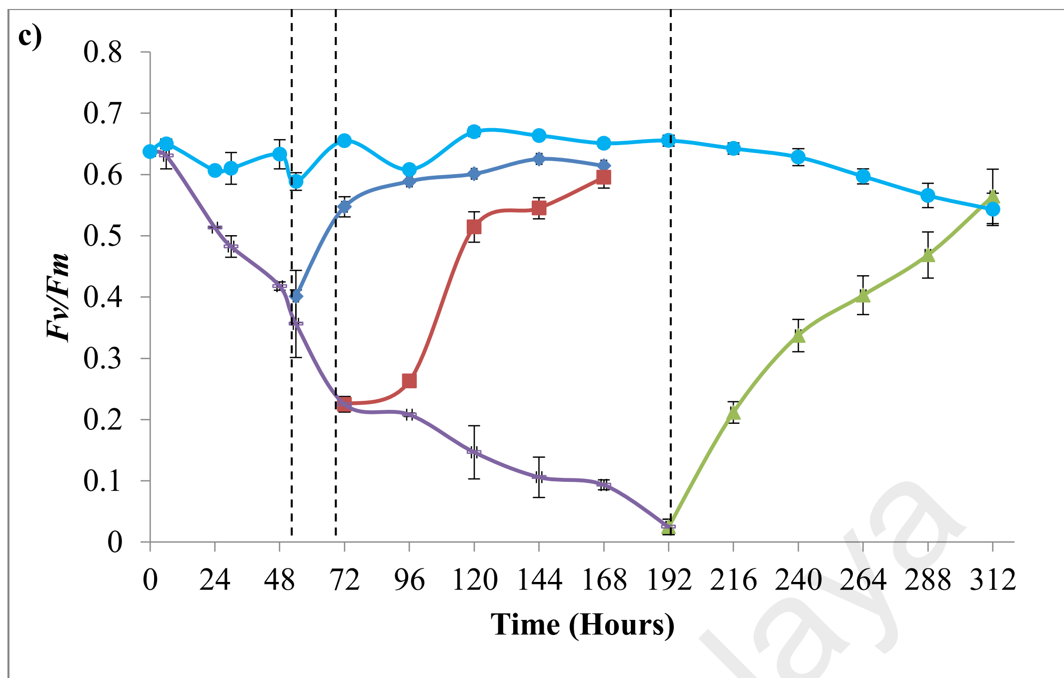

Figure 4.10, continued: Maximum quantum yield (F_v/F_m) of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245) during stress and recovery. Data are presented as a mean ± standard division of the mean.

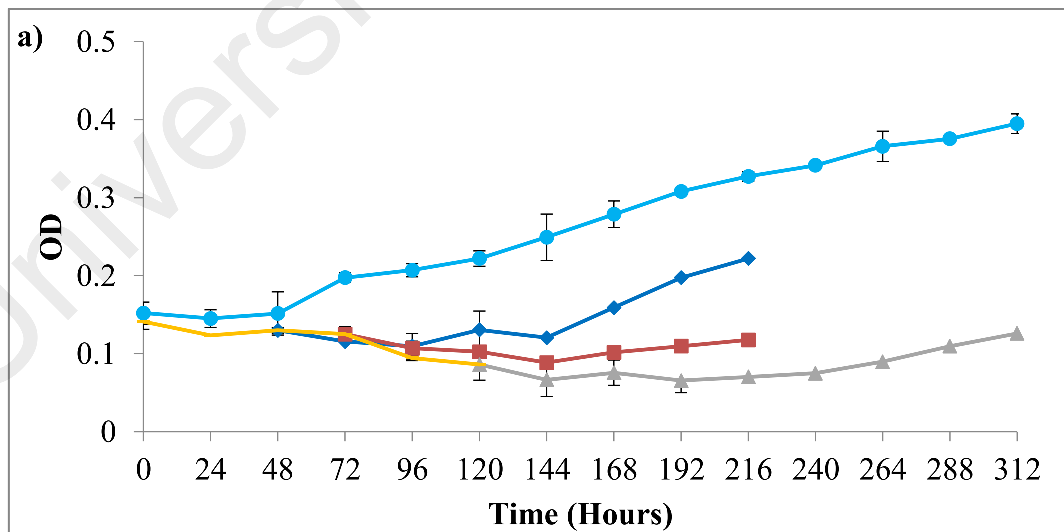

Figure 4.11: Optical density (OD) of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245) during stress and recovery. Data are presented as a mean ± standard division of the mean.

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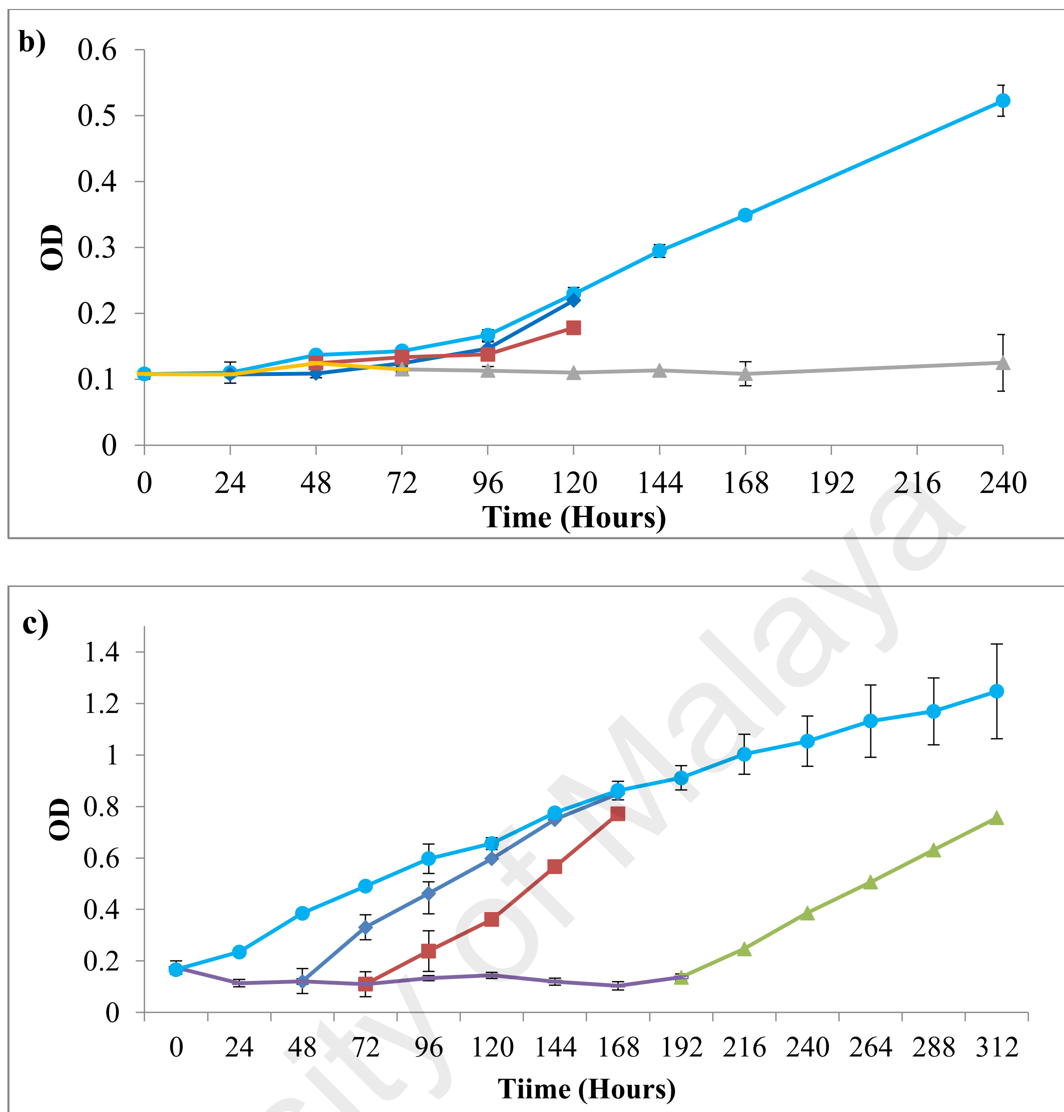

Figure 4.11, continued: Optical density (OD) of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245) during stress and recovery. Data are presented as a mean ± standard division of the mean.

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4.2.2 Carotenoid to Chlorophyll Content

The chlorophyll to carotenoid ratio was calculated at each stress level and upon their recovery (Figure. 4.12). In *Chlorella*-Ant, the ratio significantly decreased (t-test, P<0.05) during exposure to its stress-inducing temperature. The ratio was only partially restored upon recovery (Figure. 4.12a). In *Chlorella*-Temp, the ratio remained unchanged with increasing exposure time, and no substantial change was observed upon

recovery (Figure. 4.12b). In *Chlorella*-Trop, the ratio did not report any significant changes (t-test, P>0.05) with increasing exposure time, however, its ratio significantly increased (t-test, P<0.05) after recovery from the high-stress levels (F_v/F_m ~0.2 and 0.0) (Figure. 4.12c).

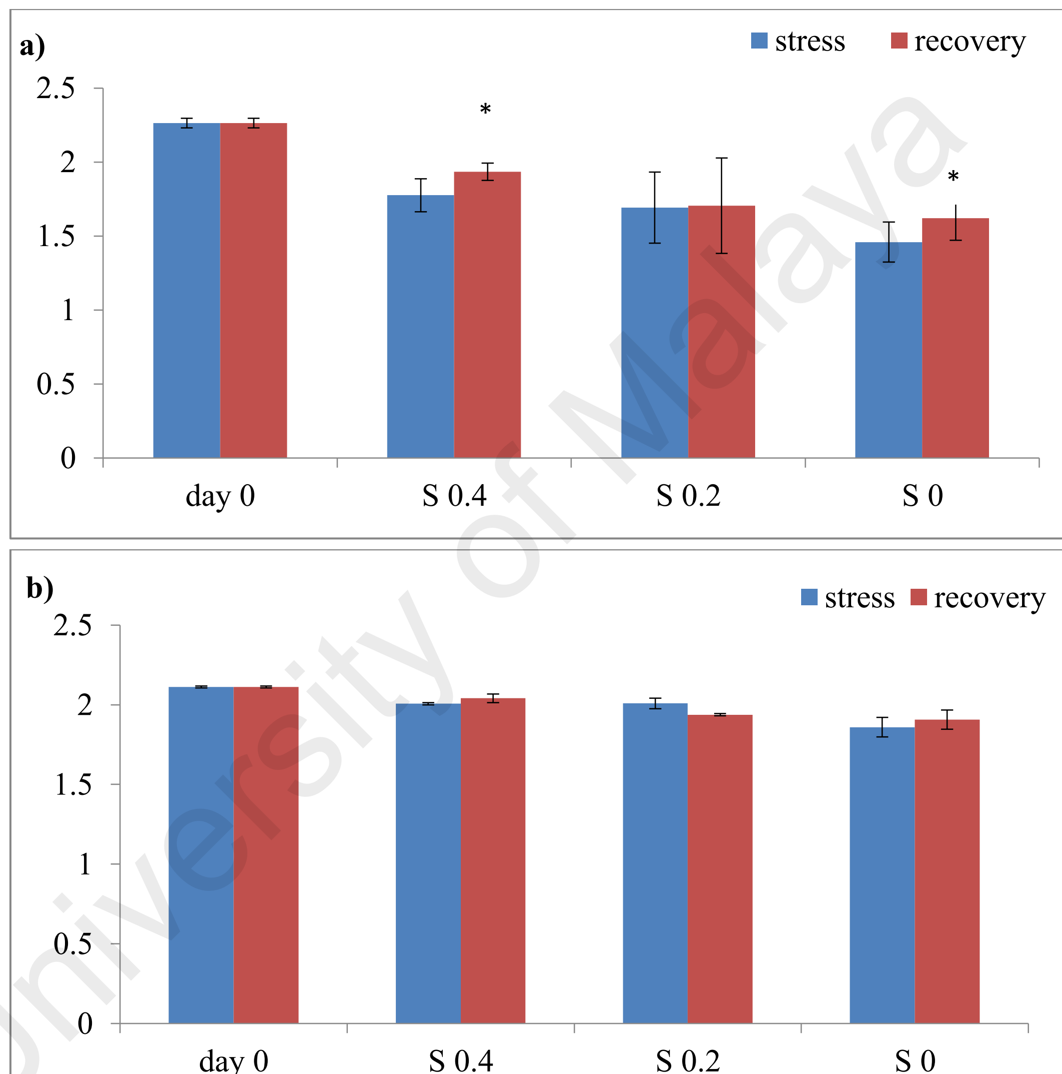

Figure 4.12: Comparison of Chlorophyll-*a* to the carotenoid ratio of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245) at different stress level and upon the corresponding recovery. Error bars denote standard deviations from triplicate samples. An asterisk (*) refers to the significant difference between chlorophyll to carotenoid ratio during stress and recovery. S0.4, F_v/F_m ~0.4; S0.2, F_v/F_m ~0.2; S0, F_v/F_m ~0.

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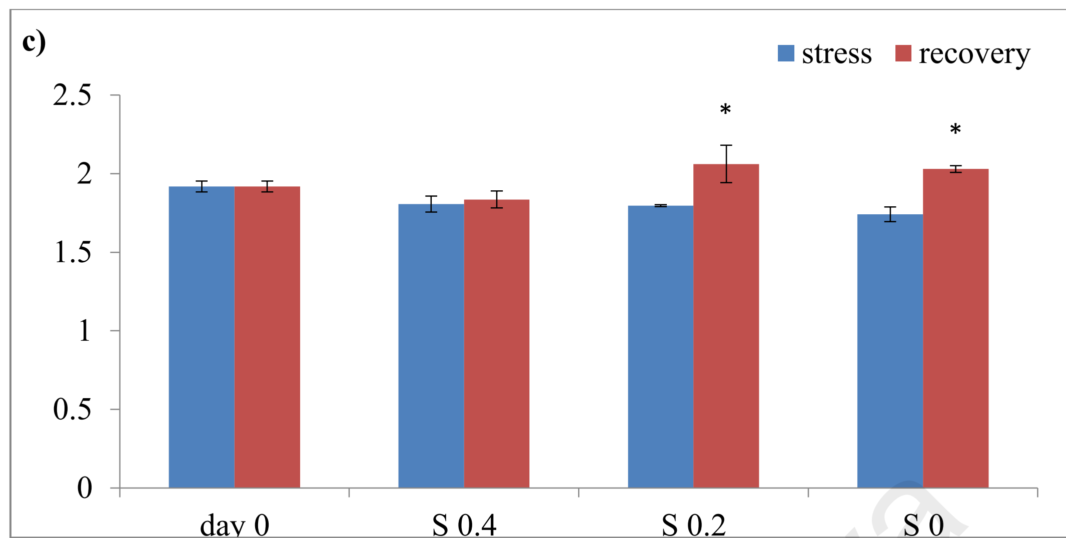

Figure 4.12, continued: Comparison of Chlorophyll-*a* to the carotenoid ratio of (a) *Chlorella*-Ant (UMACC 250), (b) *Chlorella*-Temp (UMACC 373) and (c) *Chlorella*-Trop (UMACC 245) at different stress level and upon the corresponding recovery. Error bars denote standard deviations from triplicate samples. An asterisk (*) refers to the significant difference between chlorophyll to carotenoid ratio during stress and recovery. S0.4, F_v/F_m ~0.4; S0.2, F_v/F_m ~0.2; S0, F_v/F_m ~0.

4.3 Experiment 3: Gene Expression Studies and ROS measurement

4.3.1 Gene Expression Result

The expression profiles of the selected genes in *Chlorella*-Ant during stress and recovery is shown in Figure 4.13. In *Chlorella*-Ant, the transcript abundance of *psbA* increased by 1.6 and 3.3-fold when the F_v/F_m decreased to ~0.4 and 0.2, individually. However, during recovery, its abundance decreased to the value of the control sample. In *Chlorella*-Ant, *psaB* transcript abundance increased to 2.1 and 4.6-fold when the cultures were under stress and F_v/F_m decreased to ~0.4 and 0.2, respectively. Although the transcript abundance declined after the culture was transferred to its ambient temperature, its abundance remained higher than that of the control sample (1.5-fold).

In *Chlorella*-Ant, the expression of *psbC* during stress increased to a maximum of 24.8-fold when F_v/F_m decreased to ~0.2, and after being transferred to ambient temperature, although the transcript abundance decreased, it remained up-regulated by 6.1-fold. In *Chlorella*-Ant during stress, *rbcL* transcript abundance decreased to 0.6-fold when F_v/F_m declined to ~0.4, but it increased to 1.9-fold due to additional stress (F_v/F_m ∽ 0.2). However, during recovery, it was unexpectedly down-regulated 0.2-fold (Fig 4.13). *FAD3* transcript abundance increased to ~3-fold during stress, and continued to increase (7.6-fold) during recovery. The expression of *accD* was up-regulated during stress and further increased stress, the expression continued to increase. When F_v/F_m dropped to ~0.2 it reached a maximum of 18.9 fold, however, it was slightly down-regulated by 14.1 fold during recovery. The SAD expression was highly affected during stress and up-regulated by 11.4 and 66.9-fold when F_v/F_m decreased to ~0.4 and 0.2, respectively (Figure. 4.13).

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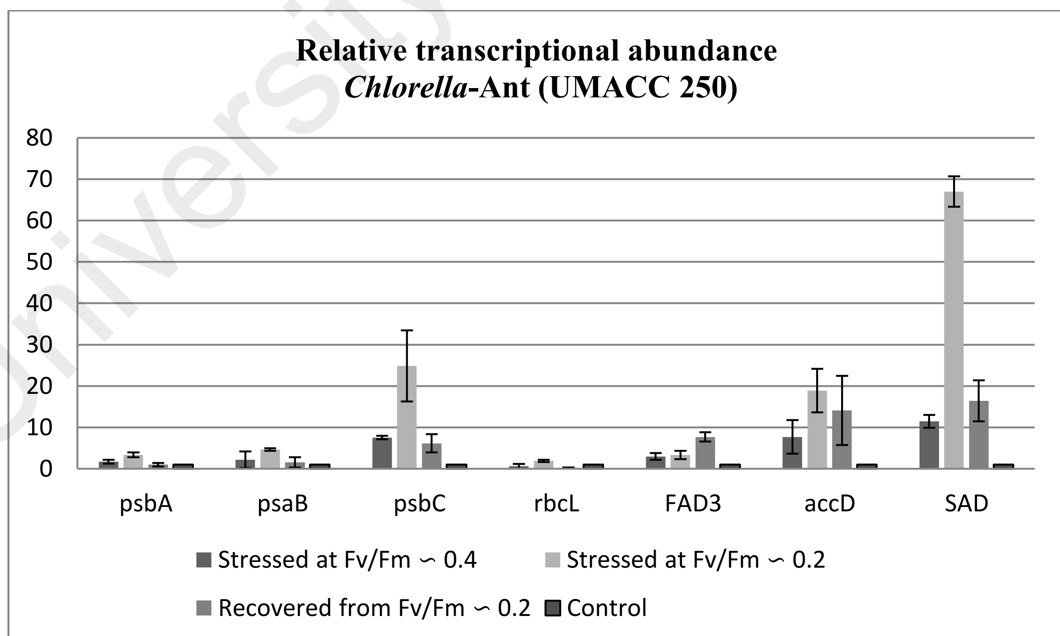

Figure 4.13: Transcript abundance of *psbA psaB, psbC, rbcL, FAD3, accD* and *SAD* of *Chlorella*-Ant during stress and recovery. Error bars denote standard error from triplicate samples.

The expression patterns of the selected genes in *Chlorella*-Temp are presented in Figure 4.14. In *Chlorella*-Temp, the expression of *psbA* was down-regulated by 0.12 and 0.77-fold of change when F_v/F_m decreased to ~0.4 and 0.2, respectively. After being transferred to its ambient temperature, the expression was considerably up-regulated and reached to a fold change of 7. In the *Chlorella*-Temp, the expression *psaB* was inhibited during stress, and reached ~0.15-fold, however, after being transferred to its ambient temperature, it was up-regulated to 3.4-fold. In *Chlorella*-Temp, the expression of *psbC* was inhibited during stress, however, during recovery, the expression of *psbC* increased slightly, but remained lower than its control counterpart. In *Chlorella*-Temp, during stress, *rbcL* expression was down-regulated by 0.2 and 0.6-fold when F_v/F_m declined to ~0.4 and 0.2, respectively; while during recovery, it was up-regulated by 15.6-fold. The expression of *FAD3* was inhibited during stress and was down-regulated by 0.6 and 0.4-fold when F_v/F_m declined to ~0.4 and 0.2, respectively. The transcript abundance of *accD* was initially inhibited when F_v/F_m decreased to 0.4, but it began to increase due to further stress (F_v/F_m ~0.2), and the expression continued to increase even when subjected to the ambient temperature and reached to a maximum of 19.6-fold. The expression of *SAD* showed a similar pattern to *accD*, but it reached its maximum of 2.7-fold during recovery (Figure. 4.14).

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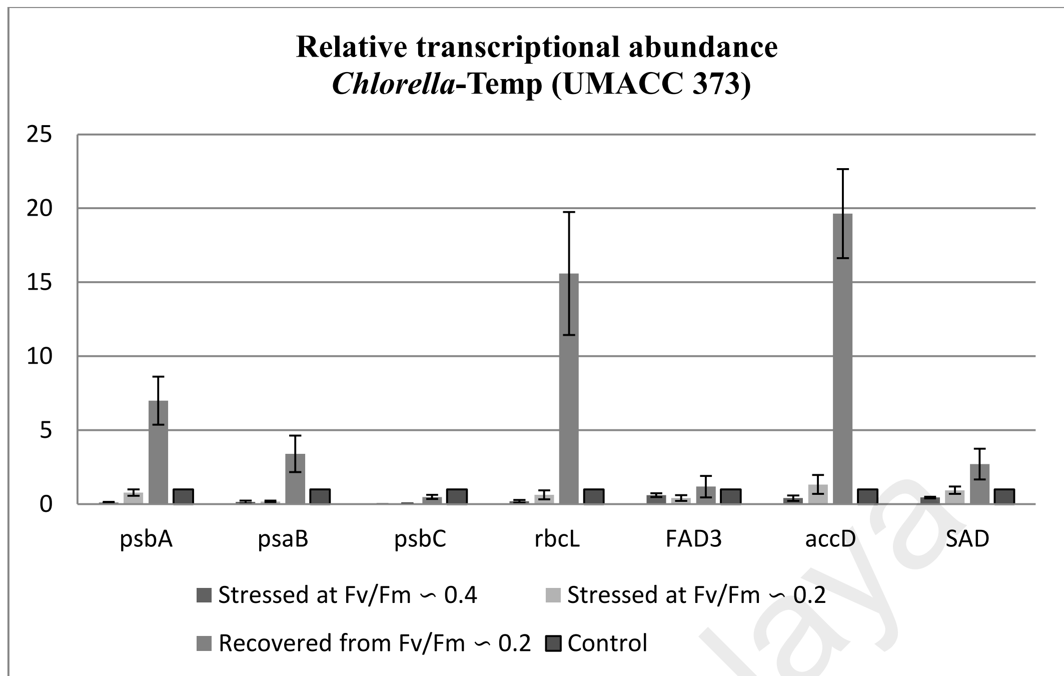

Figure 4.14: Transcript abundance of *psbA psaB, psbC, rbcL, FAD3, accD* and *SAD* of *Chlorella*-Temp during stress and recovery. Error bars denote standard error from triplicate samples.

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The expression profiles of selected genes in *Chlorella*-Trop are presented in Figure 4.15. The expression of *psbA* in *Chlorella*-Trop was not considerably affected by the temperature stress at 40 ^{o}C. The expression *psaB* in *Chlorella*-Trop did not show any remarkable changes when F_v/F_m was ~0.4, while further stress (F_v/F_m ∽0.2) up-regulated its expression by 1.68-fold, although during recovery it was down-regulated by 0.5-fold. The expression of *psbC* in *Chlorella*-Trop was down-regulated by 0.3-fold when F_v/F_m decreased to 0.4, but further stress (F_v/F_m ∽0.2) up-regulated it by 2.5-fold, and during recovery, it was up-regulated to 7.6-fold. In *Chlorella*-Trop, the expression of *rbcL* was initially inhibited, and it decreased 0.34-fold, but as stress increased (F_v/F_m ∽0.2), it was up-regulated by 1.5-fold. In *Chlorella*-Trop, *FAD3* transcript abundance decreased considerably during stress, and it did not exhibit any increase during recovery. The *accD* expression was up-regulated during stress, and it was intensified by

an increase in temperature. However, it recovered to its normal level during recovery. The expression of *SAD* was unaffected when F_v/F_m decreased to ~0.4, while additional stress caused a reduction in the *SAD* transcripts, where the expression did not increase during recovery (Figure. 4.15).

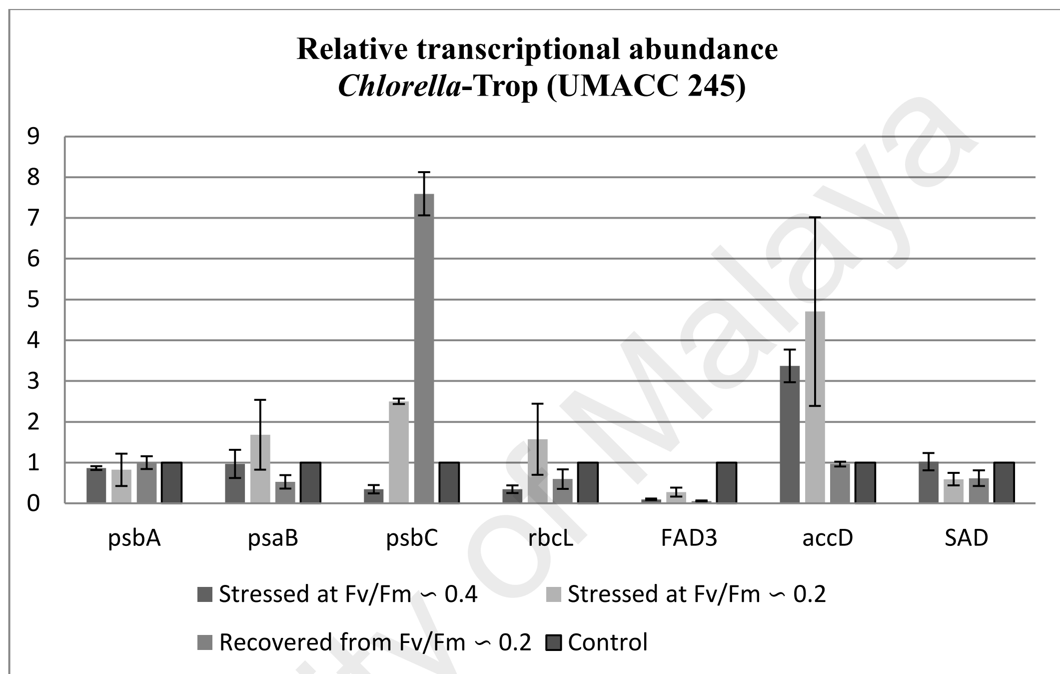

Figure 4.15: Transcript abundance of *psbA psaB, psbC, rbcL, FAD3, accD* and *SAD* of *Chlorella*-Trop during stress and recovery. Error bars denote standard error from triplicate samples.

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4.3.2 ROS Level Measurement

The level of reactive oxygen species showed different patterns among the studied *Chlorella* strains. For example, *Chlorella*-Trop exposed to heat stress showed a reduction in ROS level, while *Chlorella*-Ant displayed increased ROS level when exposed to heat. In *Chlorella*-Ant, due to its exposure to heat, the ROS level increased by 5.8-fold and reached to 8.4-fold when F_v/F_m ~0.2, continuing to increase to 10.60-

fold when the culture was recovered (Figure. 4.16a). In *Chlorella*-Temp, by exposing culture to heat, ROS level reduced to 0.30-fold in the first 2 hours of exposure. Further stress (F_v/F_m ~0.4) slightly increased it to 0.38-fold and increasing this to (F_v/F_m ~0.2) resulted in 0.31-fold. During recovery, it again decreased to 0.19-fold (Figure. 4.16b). In *Chlorella*-Trop, ROS level notably changed after exposure to heat stress and decreased to 0.25-fold in the first 2 hours, and remained roughly at the same level during stress. Also, during recovery, the ROS level decreased 0.16-fold (Figure. 4.16b).

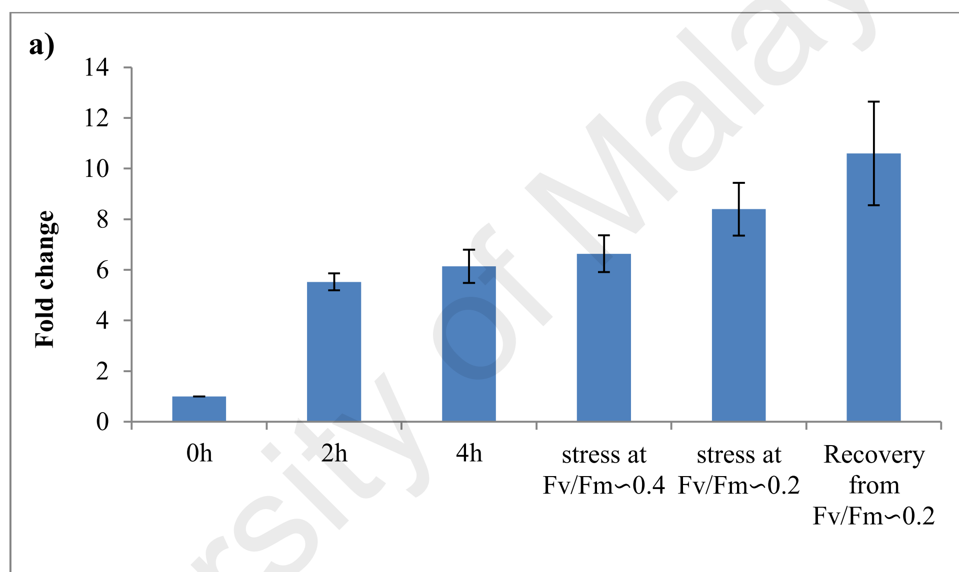

Figure 4.16: ROS level of (a) *Chlorella*-Trop, (b) *Chlorella*-Temp and (c) *Chlorella*-Ant at different stress level and upon recovery. Error bars denote standard deviations from triplicate samples.

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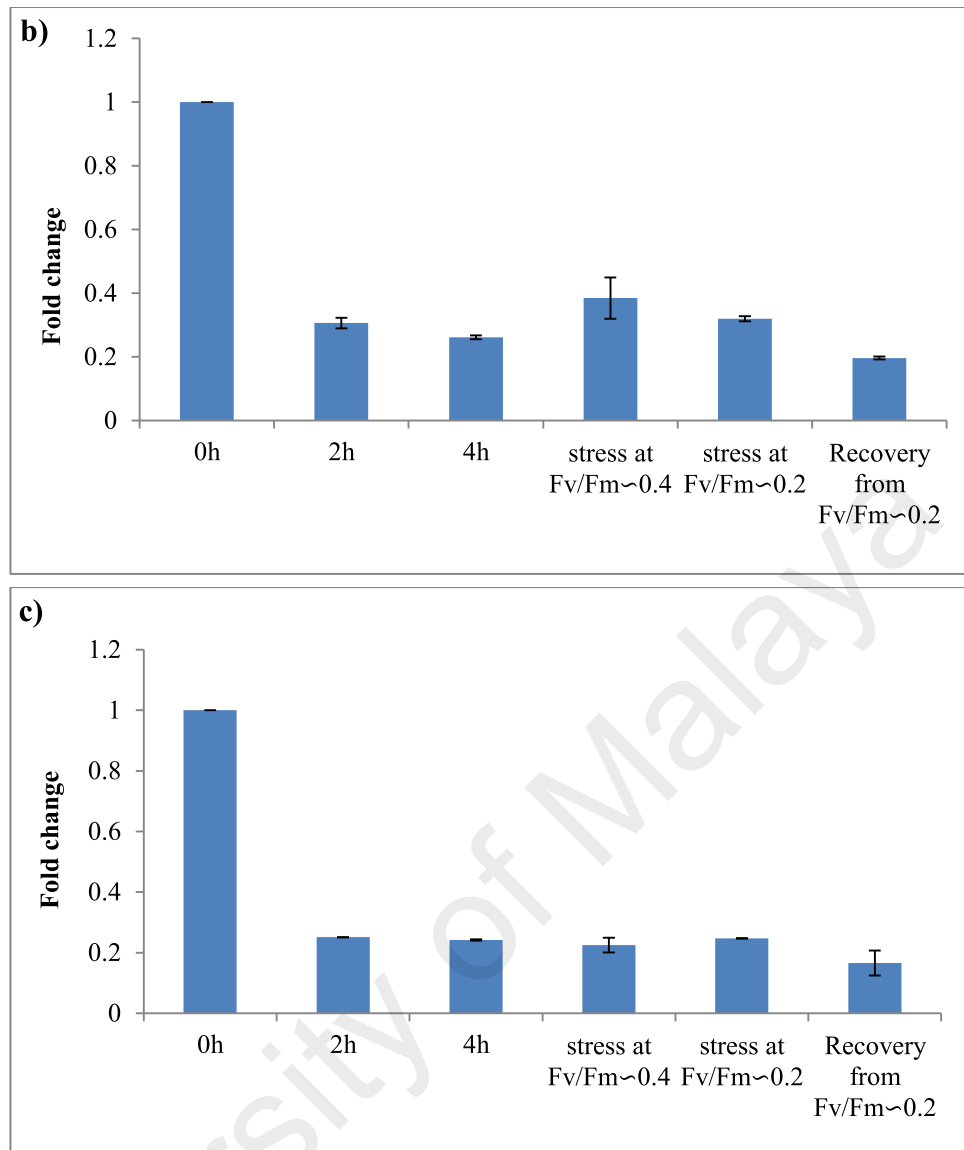

Figure 4.16, continued: ROS level of (a) *Chlorella*-Trop, (b) *Chlorella*-Temp and (c) *Chlorella*-Ant at different stress level and upon recovery. Error bars denote standard deviations from triplicate samples.

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CHAPTER 5: DISCUSSION

5.1 Experiment 1: Growth, Photosynthetic Performance and Biochemical Compositions of the *Chlorella* Strains

5.1.1 Growth study

Microalgae often show physiological acclimations and/or adaptations to cope with changes in their respective natural environments (Hodaifa, Martínez, & Sánchez, 2010). Among the approaches for modelling the relationship between the temperature and growth rate, the temperature coefficient Q10 has been the most commonly used (Ahlgren, 1987). The temperature coefficient Q10 (growth rate increase by a 10 °C rise in temperature) is often parameterized using the Arrhenius function and is expected to present a value near 2. In other words, for each 10 °C increase, the growth is expected to double until unfavourable temperatures are reached (Ras et al., 2013). Generally, beyond the optimum temperature, the growth rate begins to decrease, as enzymes are denatured, until it stops completely (Robert et al., 2002). Meanwhile, in low-temperature conditions, the growth rate decreases due to the reduced enzyme activity in metabolic processes such as glycolysis and the Krebs cycle (Jiang and Chen, 2000). Also, at low temperatures, photosynthetic activity is decreased (Öquist, 1983) while the energy requirement for protective metabolism increases (Mock and Valentin, 2004), these together leading to a lower growth rate. In this study, the specific growth rate of *Chlorella*-Ant increased from 0.266 to 0.553 day^{-1} with increasing temperature from 13 to 25 °C, showing a two-fold increase in growth rate by an approximately 10 °C increase. Likewise, the specific growth rate of *Chlorella*-Temp doubled (increased from 0.270 to 0.569 day^{-1}) by increasing the temperature from 18 to 28 °C. This indicates that both strains fitted the Arrhenius equation whereby their growth approximately doubled with a 10 °C rise in temperature. *Chlorella*-Ant and *Chlorella*-Temp showed

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similar trends in terms of their growth response to temperature (Figure. 4.3). Surprisingly, *Chlorella*-Ant was able to tolerate a broad range of temperature, from 4 to 38 °C, showing the highest growth rate at 25 °C. This suggests that this strain is more psychotrophic rather than psychrophilic. Generally, it is believed that Antarctic algae are mostly psychrophilic (being able to grow at 0 °C or less but cannot grow at temperatures above 15 °C) (Smith et al., 1994). However, in some studies, Antarctic algae showed growth over a wide range of temperature and were able to survive even at 30 °C (Seaburg et al., 1981; Tang et al., 1997; Teoh, 2004; Hu, 2012). These studies suggested that the Antarctic algae are psychrotrophs rather than psychrophilic. *Chlorella*-Ant exhibited growth patterns similar to *Chlorella*-Temp. For example, both showed an optimum temperature range from 25 to 28 °C, and also shared a similar upper limit temperature range (35 to 38 °C). It can be suggested that *Chlorella*-Ant might have originated from the temperate region possibly by natural dispersal or human activity (Broady and Smith, 1994) since it is expected that microalgae demonstrate physiological responses that are consistent with their respective climate zones (source location) (Montes-Hugo et al., 2009). Our studied *Chlorella* strains were unable to tolerate temperatures above 40 °C, which agreed with a previous study involving 72 *Chlorella* strains that suggested an upper limit of temperature for *Chlorella* species to be between 38 and 42 °C (Kessler, 1985). This implies that the temperature range of 38 to 40 °C is the upper limit for *Chlorella* strains.

5.1.2 Photosynthetic Performance

Stresses caused by environmental factors are often manifested in changes to physiological parameters such as F_v/F_m, α and ETRmax. Algae exhibit multiple photosynthetic responses when exposed to temperatures above and under their optimal

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temperatures. F_v/F_m is a measure of the efficiency with which photons absorbed by PSII are used in photochemistry instead of being quenched (Maxwell and Johnson, 2000). Thus, a decrease in F_v/F_m is often a reflection of reduced PSII activity (Ralph and Gademann, 2005). However, it must be recognized that a reduction in F_v/F_m is not necessarily associated with damage to PSII, considering that stressed cells might initiate defence mechanisms like non-photochemical fluorescence quenching (NPQ), causing F_v/F_m to decline (Baker, 2008). Many environmental factors, such as temperature, light, and nutrient status, will directly or indirectly affect PSII (Pereira et al., 2000; Murkowski, 2001; Cao et al., 2016). The reduction in F_v/F_m ratio does, however, suggest a loss in the efficiency of primary photochemistry of stressed cells. Generally, in this study, the F_v/F_m values at ambient temperature were lower for *Chlorella*-Temp and *Chlorella*-Trop than for *Chlorella*-Ant, indicating that *Chlorella*-Ant was more efficient at processing absorbed light for photosynthesis (containing more reaction centres to be open). The α values were roughly similar between the strains at ambient temperatures, suggesting similar efficiencies of light harvesting; although it has been observed that α may be higher in polar algae (Stamenković and Hanelt, 2013). In *Chlorella*-Ant, rETRmax was considerably higher at ambient temperatures compared to other strains, showing strong adaptation of electron transport machinery to the ambient temperature. For *Chlorella*-Trop, as temperature increased up to 33 °C, both *Fv/Fm* and α increased, while rETRmax decreased. This also suggests that rETRmax was the most sensitive parameter of the RLC. For *Chlorella*-Temp, temperature increase up to 28 °C had a positive effect on photosynthesis, but further increase caused inhibition. *Chlorella*-Temp showed higher photosynthetic performance at its ambient temperature (18 °C) compared to other temperatures, as shown by the higher and more stable α value and F_v/F_m. However, temperature increase up to 33 °C increased both the α and *Fv/Fm* to near ambient values. Surprisingly, rETRmax showed a higher value at 28 °C

University of Malaya

and 33°C than at the ambient temperature (18 °C), indicating enhanced electron flow in the electron transport chain of thylakoid to optimize photosynthesis (Nixon and Mullineaux, 2001). The effect of temperature on photosynthetic efficiency differed from one study to another. For example, in a study of eight species of temperate marine microalgae, it was reported that the α value varied between species, and the species isolated from the same growth environment showed different photosynthetic efficiencies when exposed to a series of temperatures (Salleh and McMinn, 2011). The reduction in the capacity of photosynthesis (rETRmax) at high temperatures was seen in all strains. The rETRmax is greatly inhibited at high temperatures, proving its sensitivity to high temperatures. More importantly, photosynthetic capacity is considered to be temperature dependent in marine microalgae (Meiners et al., 2009). A similar pattern is observed in a temperate microalgae community, with rETRmax increasing alongside temperature, up to an optimal value of (20 °C), then declining as per the enzymes that control the RUBISCO activity (i.e., RUBISCO activase), while others involved in carbon fixation were deactivated (Macintyre, 1997). *Chlorella* has been a target for mass cultivation due to its wide range biotechnological applications (Mata et al., 2010). However, a major issue in establishing a new flourishing, microalgae-based, agrobiotechnology lies in achieving large-scale biomass under outdoor conditions (Varshney et al., 2015). Nevertheless, mass cultivation poses several limitations including difficulty in controlling culture temperature (Ugwu et al., 2008). Thus, the ability of *Chlorella* strains to grow at a broad range of temperature is an important consideration in mass culture. In our study, *Chlorella*-Ant, *Chlorella*-Temp and *Chlorella*-Trop were able to grow in a wide range of temperature from 4 to 38 °C and showed photosynthesis efficiency. This suggests a potential for the studied strains to be cultivated in various climatic regions.

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5.1.3 Biochemical Compositions

Due to the fact that microalgae are the primary producers in the food chain, they were extensively studied, especially in the context of its biochemical composition. Changes to the optimal temperature were accompanied by the physiological modifications in the cell (Show *et al.*, 2017). There are no steady trends in the proximate biochemical composition as a function of temperature (Teoh *et al.*, 2010). It is known that the response of biochemical composition to temperature variations is more likely to be species-specific. For example, de Castro Araújo and Garcia (2005) reported carbohydrate content and lipid of diatom *Chaetoceros cf. wighamii* to be higher at lower temperatures (20 and 25 °C) compared to a higher temperature (30 °C), while protein content was not influenced. An increase in temperature from 20 °C to 25 °C practically doubled the lipid content of *Nannochloropsis oculata,* while an increase from 25 °C to 30 °C resulted in a reduction of the lipid content of *Chlorella vulgaris* from 14.71 to 5.90 % (Converti *et al.*, 2009). A similar trend was reported on the temperature effect on lipid accumulation in *Scenedesmus* sp. Temperature reductions from 25 °C to 20 °C increased the lipid content by 1.7-fold, with only 8 % decreases in the growth rate (Li *et al.*, 2011). *Chlorella* isolated from the Arctic had been shown to accumulate protein and lipids under lower temperatures (<15°C) (Cao *et al.*, 2016).

The response of different *Chlorella* strains is based on its respective protein contents. In *Chlorella*-Ant, the protein contents did not significantly change due to increased temperatures, but it reported a slight fluctuation of ~46 %. This suggests that this strain synthesised a new protein to acclimate to the high temperature. In *Chlorella*-Temp, a decreasing trend due to increasing temperature was observed, which means that temperature had a negative impact on protein synthesis. In *Chlorella*-Trop, the protein content changed in tandem with temperature, with an observed decreasing response to

University of Malaya

temperate strain. This suggests that both strains partially decreased their protein synthesis when the temperature increased to avoid protein aggregation.

The studied strains responded to temperature treatments significantly due to changing carbohydrate content. However, this response differs from the studied strains, suggesting the important role of high temperature in carbohydrate acclimation. In the Antarctic strain, changes in carbohydrate content were significant. In the temperate strain, a decreasing trend in carbohydrate percentage was observed due to increasing temperature. This suggests that the temperate strain tend to utilize carbohydrate as an energy source when the temperature increases. In the tropical strain, the trend was opposite of the temperate strain, where it reported an increase in carbohydrate content due to increasing temperature. This seems to suggest that tropical strain accumulates carbohydrate when experiencing high temperature, probably as a mechanism to lower energy consumption. Generally, no significant changes in lipid content were observed in all studied strains. In the Antarctic strain, no trend was observed due to temperature changes in the lipid content. Changes may be reflected in the composition of lipids, as the cells may accumulate higher levels of saturated/unsaturated fatty acids. In both temperate and tropical strains, the lipid content reported increasing trend due to increasing temperature, indicating that lipid might be the reserve energy source instead of carbohydrate when exposed to high temperature.

The ability of algae to acclimate/adapt to changing thermal environment is also reflected in the distinct changes to the lipids in their membrane (Thompson, 1996). As mentioned earlier, temperature affects membrane fluidity by altering the saturation level of membrane fatty acids. In our study, the Antarctic strain showed a high proportion of saturated fatty acids, which is consistent with suggestions that algae acclimate to higher temperatures by using SFA to modulate membrane fluidity (Hu et al., 2008; Yamori et al., 2014), which is also in agreement with several other studies (Horváth et al., 2012;

University of Malaya

Olofsson et al., 2012; Luo et al., 2015). However, SFAs were also found to increase under nitrogen limitation and UVB radiation, suggesting SFA accumulation as a common adaptation method during stressful conditions (Platt et al., 1980; Zheng et al., 2014). Increases in SFA have been observed in UV stressed cells and this has been suggested as an adaptation strategy under stress because these fatty acids can serve as an energy source under such conditions (Wong et al., 2004). SFAs are more efficient energy storage compounds comparing to PUFAs as they have a more compact structure and are more resistant to oxidative damage (Holman, 1954). Usually, microalgae under stress tend to divert carbon into storage, particularly into triacylglycerols (TAGs) (BenMoussa-Dahmen et al., 2016) due to a limited storage capacity for carbohydrate (Yang et al., 2016). PUFAs play several important roles in cellular and tissue metabolism, such as regulating membrane fluidity and electron transport as well as the thermal adaptation (Cardozo et al., 2007). For instance, lipids function in the stability of membrane-bound proteins (Pick et al., 1985) such as PSII (Murata, et al., 1990), and Los et al., (2013) suggest that unsaturation of membrane lipid fatty acids protects the PSII complex from photoinhibition at low temperatures by enhancing the recovery from the photoinhibitory damage. Our results indicated that the percentage of PUFA decreased in stressful temperatures, and increased at optimal temperatures, supporting *Chlorella's* potential capability for producing fatty acids (PUFAs) for the nutraceutical industry at optimum temperatures. A decrease in PUFAs has also been reported in cultures exposed to UV stress (Liang et al., 2006). In terms of ecological implications, a decrease in PUFAs could influence the nutritional quality of the microalgae for consumption by organisms at higher trophic levels (Wong et al., 2007).

University of Malaya

5.2 Experiment 2: Stress and Recovery Treatments

The selected strains were able to recover their respective photosynthetic activities when transferred to their respective normal ambient temperatures from different stress levels. However, the photosynthetic recovery period and ability to regain growth varied. *Chlorella*-Trop was able to regain and retain the ability to grow from various stress levels, which is indicative of the high recovery capacity of *Chlorella*-Trop. *Chlorella*-Temp and *Chlorella*-Ant were able to grow after being transferred from (F_v/F_m ~0.4 and 0.2), while growth was not observed when recovered from the highest stress level (F_v/F_m ~0). The latter outlined the magnitude of damage in the proliferation ability, despite the full recovery of the photosynthetic machinery. As discussed earlier, the tropical strain reported the fastest recovery from each stress levels, while the Antarctic strain reported the slowest recovery. Chlorophyll to the carotenoid ratio in the tropical strain did not change significantly during stress but increased during recovery. Contrarily, the ratio decreased in the Antarctic strain and was not fully restored during recovery. This can be due to the fact that metabolisms in the Antarctic strains are slower, which means that it needs more time to repair any damages (Bowden et al., 2006). There are several target sites for high temperature-induced damage, such as the CO_2 fixation system, photophosphorylation, and the electron transport chain (Yoshitaka et al., 2001). Damage can result from the inhibition of the Calvin cycle, and consequently, increases in the reactive oxygen species (ROS) level (Saibo et al., 2008). Recent studies in the cyanobacterium *Synechocystis* revealed that ROS inhibits the *de novo* synthesis of D1 protein (Shunichi Takahashi et al., 2004). In addition, an inverse correlation of ROS and D1 protein accumulation was found in *Dunaliella tertiolecta* (Chlorophyta) (Segovia et al., 2015). In their review, Nishiyama and Murata (2014) suggested that abiotic stresses act by inhibiting the repair of PSII, rather than accelerating the photodamage itself via inhibiting the synthesis of D1 protein. In a study

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on Antarctic microalgae, it was suggested that the values of F_v/F_m below 0.1 were considered a "recovery" threshold for Antarctic microalgae, below which the cells were unable to recover (Reeves et al., 2011). In our study, it was observed that all of the strains, including the Antarctic strain, were able to recover their photosynthetic activity from even the highest stress level (F_v/F_m ~0.0). Although some of the *Chlorella*-Ant cells could not survive stress, those that do were able to recover their photosynthetic activity upon recovery. There are many instances in nature whereby the stressed algal cells reach the maximum stress level that they can tolerate without triggering programmed cell death. In fact, their performance could decline to a certain extent, followed by a full recovery (Tzan-Chain and Ban-Dar, 2013). For example, desiccated desert green algae can recover high levels of photosynthetic quantum yield after rehydration (Gray et al., 2007). Photoinhibited cells are often able to recover to their prior photosynthetic capacity levels rapidly after its removal from extreme environments (Salleh and McMinn, 2011). The ability of algae to recover from high-temperature fluctuations is a vital characteristic enabling them to survive, particularly in the intertidal ecosystems, where organisms experience substantial variation in temperature (Campbell et al., 2006). Understanding species-specific thermal stress is important in understanding how temperature changes could lead to changes in the *Chlorella* species' composition. The ability of photoautotrophs such as *Chlorella* to survive high-temperature fluctuations is ecologically important for primary production and marine ecosystem dynamics. Overheating during the day could occur in closed system mass cultivation (Endres et al., 2016). Therefore, the selection of algae with high recovery capacity from temperature stress is important.

Apart from the role of carotenoids in the assembly of photosystems, they are also photoprotectors in photosynthesis (Fanciullino et al., 2014). In addition, it is known that carotenoids, such as those involved in energy dissipation by heat via xanthophyll cycle

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and quenching of singlet oxygen formed during photo-oxidation, increase stress conditions (Thompson, 1996; Demmig-Adams and Adams, 1996; Boussiba, 2000; Behera and Choudhury, 2003; Pintó-Marijuan and Munné-Bosch, 2014). In certain instances, the chlorophyll to carotenoid ratio has been proposed as a sensitive indicator of photooxidative damage (Hendry and Price, 1993), with its ratio decreasing during stressful conditions. The stress and recovery experiments reported here also showed changes in chlorophyll: carotenoid under heat stress, possibly due to increased levels of carotenoids and reduced synthesis of chlorophyll.

5.3 Experiment 3: Gene Expression Studies

F_v/F_m is a measure of the efficiency of photons absorbed by PSII being used in photochemistry instead of being quenched (Maxwell & Johnson, 2000). It is important to note that a decrease in F_v/F_m is not essentially related to the damage to PSII, as the stressed cells might recruit defence mechanisms such as non-photochemical fluorescence quenching (NPQ), causing F_v/F_m to decrease (Baker, 2008). Hence, in this study, the gene expression of *psbA*, *psaB, psbC* and *rbcL*, which are the key genes involved in PSI, PSII, and carbon assimilation (CO_2 fixation), were evaluated.

It was reported that the PSII of cyanobacteria, green algae, and higher plants is prone to light-induced inactivation, and the D1 protein is the primary target of such damage. As a consequence of this, the D1 protein, which is encoded by *psbA,* is degraded and re-synthesized in a multistep process called PSII repair cycle (Mulo et al., 2012). As shown in Figure. 4.15, the abundance of *psbA* in *Chlorella*-Trop was only marginally reduced during stress, but it could reach control levels during recovery. This confirms *Chlorella*-Trop's ability to maintain *psbA* expression level during stress, and its fast recovery suggests that the damage was rapidly repaired. In *Chlorella*-Temp, the initial

abundance of *psbA* during stress exhibited a significant decrease, but increased stress causes the cells to increase *psbA* abundance and activate their respective repair mechanisms. During recovery, the *psbA* remarkably increased to continue its repair. In the case of *Chlorella*-Ant, there was a substantial increase in the *psbA* abundance, which intensified as stress increased. This suggests the high competency of *Chlorella*-Ant in repairing its D1 protein, even during stress, and it might be one of the factors for its high-temperature photosynthetic tolerance.

PsaB is one of the main constitutes of PSI biogenesis and involves the formation of the chlorophyll *a* protein complex I (CPI) that binds most of the pigments and redox cofactors of PSI (Balczun et al., 2005). It also binds a total of ~100 Chl *a* molecules with *psaA* (Melis, 1991). The decrease in *psaB* transcript's abundance may result in the loss of PSI, and consequently, its reduced activity (Morgan-Kiss et al., 2006). In this study, *psaB* transcript abundance in both *Chlorella*-Ant and *Chlorella*-Trop increased during temperature stress and decreased during recovery, which indicates that both strains are capable of synthesising *psaB* subunit that could repair the PSI. Although the transcript abundance of *psaB* in *Chlorella*-Temp decreased significantly during stress, it increased considerably during recovery, which implies that *Chlorella*-Temp was not able to resynthesize *psaB* during stress, but was able to do so during recovery to build up PSI. The deregulation of *psaB* was also reported in *Chlorella vulgaris* when exposed to toxic chemicals (Qian et al., 2011).

Another photosynthetic gene, *psbC*, encodes a PSII chlorophyll-binding protein, involved in water-splitting and acting as an oxygen-evolving enzyme of photosynthesis (Qian et al., 2009). It is also affected by harsh conditions, such as temperature stress (Chong et al., 2011). During temperature stress, *Chlorella* strains from different latitudes responded differently to *psbC* transcript abundance. In *Chlorella*-Trop, the *psbC* abundance increased during stress and kept increasing even during recovery

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(reaching almost 8-fold). The damage occurred during stress might not be fully repaired, and the cell tried to compensate for this during recovery as well. In contrast, no deregulation of *psbC* was observed in *Chlorella*-Temp during stress/recovery. In *Chlorella*-Ant, *psbC* was up-regulated as stress intensified, and reached up to 25-fold relative to its control. This strain has a high capacity for maintaining the *psbC* level in harsh conditions to build up PSII for stress adaptation. Both *psaB* and *psbC* were involved in electron transport, and their down-regulation might hinder mitochondrial electron transport, which resulted in the accumulation of surplus electron, reduced transcription of PSI and PSII genes, as well as oxidative stress (Liu et al., 2015).

Photosynthetic fixation of carbon dioxide is essential for algal growth and development due to its provision of the carbohydrates required for metabolism, structural components, and cellular building blocks (Biswal et al., 2011). Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) play critical roles in photosynthesis and expression of genes encoding its subunits, and *rbcL* is significantly influenced by various stresses (Qian et al., 2009; Qian et al., 2012). The expression of *rbcL* was inhibited due to high salinity, desiccation, and low salinity conditions, as well as temperatures above and below its normal ambient temperature (Xu et al., 2013). In both *Chlorella*-Ant and *Chlorella*-Trop, the *rbcL* transcript abundance increased when F_v/F_m reached 0.2 during stress. The cells have the capacity to undergo the carbon fixation process even during stress by up-regulating the expression of *rbcL*. In *Chlorella*-Temp, *rbcL* transcript levels decreased during stress, but significantly increased 16-fold during recovery. This might be due to the susceptibility of *Chlorella*-Temp *rbcL* transcription machinery to temperature stress and its need to compensate post-recovery.

These expression analyses of key metabolic genes may provide insight into the actual mechanism of lipid accumulation in the microalgae in response to cultivation conditions (Jianhua et al., 2014). Recently, evolved molecular techniques, such as

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transcriptome analysis, microarray analysis, and full-length or EST (expressed sequence tag) transcript sequencing can generate knowledge of how lipid biosynthetic genes are expressed under different stress conditions and the key genes involved in triggering lipid accumulation (Shin et al., 2015). Therefore, gene expression analysis could help us improve existing stress strategies and develop novel strategies for better lipid yields in microalgae. Gene expression analysis also reveals the key functional genes involved in lipid biosynthesis, which could be exploited for improved applicability of molecular techniques, such as metabolic and genetic engineering (Poonam Singh et al., 2016).

Omega-3 is an important very long-chain polyunsaturated fatty acids. In all of the studied strains, the expression of gene encoding Omega-3 desaturase, *FAD3*, was inhibited during stress, indicating that the temperature stress can result in the inhibition of *Omega*-3 desaturation in *Chlorella* strains, which could result in the reduction of the nutritional value of *Chlorella* strains in the food web. However, *FAD3* in *Chlorella*-Temp and *Chlorella*-Ant were able to recover to levels of the control sample during recovery. This confirms its capability for producing high-value products after enduring temperature stress. The level of *FAD3* transcript increased by up to 5.4 times after only 3 h of cold exposure (Guschina and Harwood, 2006). Similarly, it was reported that the expression of *FAD3* in *Chlamydomonas sp. ICE-L* contributed to its adaptation to low temperature when it showed higher *FAD3* transcript abundance due to exposure to low temperature (Kumari et al., 2013). Recently, Zhang et al. (2011) demonstrated enhanced mRNA level of *CiFAD3* in *Chlamydomonas sp. ICE-L* that increased 3.8-fold at a salinity of 62 %.

Acetyl-CoA carboxylase (ACCase) catalyses the first rate-limiting step in the fatty acid biosynthetic pathway via the formation of malonyl-CoA from acetyl-CoA (Jianhua et al., 2014). On the other hand, the *accD* gene, which encodes the beta subunit of ACCase, was up-regulated in *Chlorella sorokiniana* when grown under N limited

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condition, while *accD* was up-regulated under higher Mg concentration (Singh, 2017). The effect of iron on lipid accumulation in *Chlorella sorokiniana* showed that the expression of genes, such as *accD*, was up-regulated at higher concentrations of iron resulting in high lipid productivity (Singh et al., 2016). In our study, the expression of *accD* varied between strains when exposed to heat stress. Both *Chlorella*-Trop and *Chlorella*-Ant enhanced their respective transcript's abundance, which may result in enhanced fatty acid biosynthesis. However, it was reduced during recovery, which could be due to the lack of available energy. In *Chlorella*-Temp, during stress, the *accD* was down-regulated to confirm reduced fatty acid synthesis, while during recovery, fatty acid synthesis was interestingly boosted, and cells tried to accumulate fatty acids.

In green algae, the soluble stromal enzyme stearoyl-ACP desaturase (SAD) determines the ratio of saturated-to-unsaturated fatty acids and catalyzes the first desaturation reaction, converting 18:0-ACP to 18:1 Δ9-ACP (Sangram et al., 2016). In *Chlorella zofingiensis,* the *SAD* gene was up-regulated in response to high light irradiation; as a result of this, the treated cells accumulated a higher amount of TFA including oleic acid (Jin, 2010). Changes in *SAD* were more apparent in *Chlorella*-Ant relative to its studied counterparts. In the Antarctic strain, *SAD* significantly increased during stress, and its expression intensified in tandem with stress.

ROS included free radicals (hydroxyl radical $OH^{\bullet}$, phenoxy radicals $RO^{\bullet}$, peroxy radicals $ROO^{\bullet}$) and other ROS (superoxide radical anion O2 $^{\bullet-}$, singlet oxygen $1O_2$, hydrogen peroxide H_2O_2) (Qian et al., 2009). Chloroplasts are the main source of ROS, which can cause cell damage in various ways when electron transport is blocked. These surplus electrons are transported to molecular oxygen, generating ROS (Kumar et al., 2008). The consequences of the formation of ROS include the gradual peroxidation of lipid structures (Baryla et al., 2000), oxidative DNA damage (Kasprzak, 2002), and photosynthetic apparatus damage (Dewez et al., 2005). In this research, it was found

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that the ROS content did not report any trend in responding to heat stress among the studied *Chlorella* strains, indicating different defence mechanisms being activated in each strain.

In *Chlorella*-Trop, the ROS level notably changed post-exposure to heat stress and declined in the first 2 hours. It remained at similar levels during stress and recovery. This suggests that this strain may activate the ROS scavenging enzyme to avoid oxidative damage. In *Chlorella*-Temp, similar to *Chlorella*-Trop exposing culture to heat, the ROS level was reduced to almost half in the first 2 hours of exposure and remained lower compared to the ROS level prior to treatment. By contrast, in *Chlorella*-Ant, by exposing the culture to heat, the ROS level increased during stress and recovery. This confirmed the failure of this strain in removing ROS effectively, while the damage was unfixed during recovery. Also, the role of ROS level as a signal transduction must be undertaken.

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CHAPTER 6: CONCLUSION

6.1 Conclusion

The following conclusions can be deduced based on the findings of this study:

Growing the selected *Chlorella* strains at temperatures above their ambient caused an increase in their growth rate, although further temperature increase decreased the specific growth rate. The studied *Chlorella* strains were able to grow at temperatures of up to 38 °C, although exceeding this limit inhibit both photosynthesis and growth. Surprisingly, *Chlorella*-Ant was able to grow well by 20 °C more than its ambient, and showed active photosynthesis properties even at 35 °C, although its growth was inhibited, which indicates that the Antarctic strain seems to be psychrotolerant rather than psychrophilic. The tropical strain appears to be living near their upper-temperature limit, as per its inhibition of growth and photosynthesis with further increase in temperature. The tropical and temperate strains showed a preference for specific niches, as presented by their in vitro growth and photosynthetic behaviour.

Changes in biochemical compositions, such as lipid, carbohydrates, and proteins were inconsistent with temperature changes. These tests were only able to detect total contents, while changes due to temperature were more apparent in the molecules. For example, cells might change its fatty acids saturation level, but maintain their total fatty acid percentage in the cell. *Chlorella* cells tend to accumulate more PUFA at ambient temperature instead of stress, indicating that high temperature significantly affects the quality of *Chlorella* as food in an aquatic system. Growing the studied strains at high temperature caused changes in its fatty acid profile. For instance, in *Chlorella*-Trop at 38 °C, the saturated fatty acid decreased by 10%, while, mono-saturated and poly saturated acids increased by 3%. In *Chlorella*-Temp, at 35 °C, the saturated fatty acid increased by 14%, while the mono-saturated and poly saturated decreased by 4% and

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increased by 10 %, respectively, and in *Chlorella*-Temp, at 35 °C saturated fatty acid increased by 3%, mono-saturated and poly saturated acids decreased by 1% and 2%, respectively.

The stressed *Chlorella* strains were able to recover even from a state in which photosynthesis was totally inhibited. This confirms its ability to recover from high-temperature stress even when their photosynthesis and growth were inhibited, although a portion of biomass was lost during stress. Among the studied strain, *Chlorella*-Trop reported the fastest recovery in both photosynthesis and growth ability. The Antarctic strain was able to recover; however, it was slowest in regaining its photosynthesis and growth ability. The ability of the *Chlorella* strain to recover from temperature fluctuation is important, particularly in the mass cultivation project. The main concern in setting a successful, microalgae-based, agrobiotechnology rests in producing large-scale biomass under outdoor conditions (Varshney et al., 2015). Considering mass cultivation poses some obstacles including difficulties in controlling culture temperature (Ugwu et al., 2008). Thus, the ability of *Chlorella* strains to grow at a broad range of temperature is an important factor in mass culture.

In the topical strain (*Chlorella*_Trop), the expression of photosynthetic genes amplified as stress increased but down-regulated during recovery except for *psbC* which continued to increase. This indicates the significant role of *psbC* in repairing PSII as the main subunit. Also, during stress the gene expression profile showed this strain tend to accumulate saturated fatty acid which is in line with numerous studies that *Chlorella* accumulates fatty acids during stress to store energy. Expression of the photosynthetic genes in *Chlorella*-Temp was inhibited during stress but increased substantially during recovery. This is in line with several reports in which different stressors including temperature (Chong et al. 2011), salinity (Kebeish et al. 2014b) and toxins (Qian et al. 2009a; Kebeish et al. 2014a; Liu et al. 2015). This may indicate a strategy to conserve

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existing resources and energy to cope with possible extended periods of stress (Poong et al. 2018). The subsequent up-regulated expression of these genes during recovery is perhaps a measure to resynthesize and restore the damaged components. Active transcription of the photosynthetic genes in *Chlorella*-Ant (even when it was under stress) proposes that this strain uses a strategy of increasing the expression of photosynthetic genes to compensate for the decline in photosynthetic activity caused by the heat-induced damage to the photosynthetic components. In this strain, the obvious changes in *accD* and *SAD* expression implying that this strain accumulates fatty acids during stress similar to tropical strain, however, increased *FAD3* during recovery suggests then they converted to PUFAs. While, in the *Chlorella*-Trop, *FAD3* down-regulated during recovery presenting decreased PUFA synthesis.

Based on the growth rate and photosynthetic parameters (F_v/F_m, Alpha and rETRmax), the temperature changes had a significant effect on growth and photosynthesis. Also, the changes in biochemical compositions, such as protein and carbohydrate content, were observable. However, the lipid percentage of the cell did not report substantial variations. The lipid composition itself was modulated during temperature change via changes in the saturation level of the fatty acid in order to adapt to temperature.

The expression pattern of the studied *Chlorella* strains was different. *Chlorella*-Trop and *Chlorella*-Ant showed a more similar pattern in response to temperature stress. Also, in both *Chlorella*-Trop and *Chlorella*-Ant, the *psbC* showed remarkable up-regulation during stress and recovery. In *Chlorella*-Temp, during stress, both photosynthetic genes and fatty acid synthesis related genes were down-regulated, while their expressions were up-regulated during recovery.

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6.2 Appraisal of Study

This study dealt with the actual topic of the impacts of climate change, especially global warming on the microalgae in marine ecosystems. In this research, the growth and photosynthetic performance of three *Chlorella* sp. from different habitats were analyzed. To a greater extent, the expression pattern of several selected genes related to photosynthesis and fatty acid synthesis during stress and recovery were studied. Some interesting findings in this research could well benefit future studies on global warming and ocean ecology. Studies on stress tolerance mechanisms are important towards understanding and predicting future impacts of climate change on microalgae. The understanding of the responses and adaptation of microalgae to changing climates could provide invaluable information towards mitigating climate change and bring our attention to the increasing vulnerability of species in the ecosystems. As pointed out earlier, microalgae are primary producers that form the basis of many aquatic food chains, therefore, any changes to the size and composition of algal communities can result in profound impacts to the ecosystems and fisheries productivity. Many species of microalgae, including *Chlorella,* are economically important as sources of health supplement, pigments, biofuel, and others. Thus, by understanding the response of microalgae to various stressors, such as thermal stress, they can be optimized for various purposes, such as lipid production, which can be used as biofuel in the future. Furthermore, by understanding the consequences of the abiotic stressors on these organisms, we can potentially predict possible changes to biogeochemical and nutrient cycles, and how they affect Earth.

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6.3 Future Research Direction

Understanding a single factor might not be able to bring an explicit conclusion to express the complexity of an actual environment. It is possible to combine additional factors, such as introducing UV radiation, enhanced CO_2 concentrations, and community study. It is important to understand how the interactive effects can be additive, synergistic, or antagonistic towards influencing the growth of microalgae. Work on additional strains from each latitudinal region and ecological niches are required for a stronger conclusion on the relationship between latitude and temperature response. In a natural environment, the interaction of biotic and abiotic factors displays a substantial effect on the population dynamic of microalgae. More advanced techniques can be used to identify genes involved in temperature adaptation and acclimated, which can be used to engineer more tolerant organisms/organisms with a particular feature for use in industrial/medical applications.

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List of Publications and Paper Presented

1) Bahram Barati, Phaik-Eem Lim, Sook-Yee Gan, Sze-Wan Poong, Siew-Moi Phang, John Beardall (2017) Effect of Elevated Temperature on The Physiological Responses of Marine *Chlorella* Strains From Different Latitudes. *Journal of Applied Phycology* DOI 10.1007/s10811-017-1198-z (ISI-Indexed).

2) Bahram Barati, Phaik-Eem Lim, Sook-Yee Gan, Sze-Wan Poong, Siew-Moi Phang (2018). Gene expression profile of marine *Chlorella* strains from different latitudes: Stress and recovery under elevated temperatures. *Journal of Applied Phycology* (ISI-Indexed).

3) Bahram Barati, Phaik-Eem Lim, Sook-Yee Gan, Sze-Wan Poong, Siew-Moi Phang, John Beardall (2018) Green Algae Molecular Responses to Temperature Stress. *Acta Physiologiae Plantarum* (under review).

4) Profiling Expression of Fatty Acids Key Regulatory Genes of Marine *Chlorella* Strains from Different Latitudes during Heat Stress and Upon Recovery (In Preparation).

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Conference Presentation

1) Barati, B., Lim, P.E., Gan, S.Y., Poong, S.W. & Phang, S.M. (2017) Gene expression profile of marine *Chlorella* strains from different latitudes: Stress and recovery under elevated temperatures. 8th Asian Pacific Phycological Forum 8-13 October 2017, Kuala Lumpur, Malaysia (Non-ISI/Non-SCOPUS), (oral presentation).

2) Barati, B., Lim, P.E., Gan, S.Y., Poong, S.W. & Phang, S.M. (2016) Physiological responses of marine *Chlorella* from different latitudes toward elevated temperature. Asia-Pacific Conference on Algal Biotechnology, 14 18 November 2016, Bangkok, Thailand (Non-ISI/Non-SCOPUS), (poster presentation).

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