UNIVERSITI PUTRA MALAYSIA

MORPHOLOGICAL, BIOCHEMICAL AND TRANSCRIPTOMIC CHARACTERISATION OF Chlorella sorokiniana AND Chlorella zofingiensis DURING NORMAL AND STRESS CONDITIONS

SITI NOR ANI BINTI AZAMAN

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MORPHOLOGICAL, BIOCHEMICAL AND TRANSCRIPTOMIC CHARACTERISATION OF *Chlorella sorokiniana* AND *Chlorella zofingiensis* DURING NORMAL AND STRESS CONDITIONS

By

SITI NOR ANI BINTI AZAMAN

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

July 2017

This thesis is lovingly dedicated to

My late father Azaman bin Dio
and my mother Aminah binti Jusoh

My beloved husband,
Wan Amirul Faiz bin Wan Muhammad

My dearest kids,
Wan Nurin Auni
and Wan Aisyah Inara

Who leads me with the light of their endless love, support and encourage me throughout my life.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

MORPHOLOGICAL, BIOCHEMICAL AND TRANSCRIPTOMIC CHARACTERISATION OF *Chlorella sorokiniana* AND *Chlorella zofingiensis* DURING NORMAL AND STRESS CONDITIONS

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SITI NOR ANI BINTI AZAMAN

July 2017

Chairman : Yeap Swee Keong, PhD
Faculty : Institute of Bioscience

Chlorella has been identified as one of the most interesting microalgae species, which has high nutritional values, high growth rate, and is able to produce a wide range of metabolites in response to environmental changes. The objectives of this study are to characterise the morphology and biochemical contents and to identify the genes and miRNAs involved in regulating the production of carotenoids and lipids in *Chlorella sorokiniana* and *Chlorella zofingiensis* when cultured under high light intensity combined with glucose supplementation. In this study, stress was introduced to the *Chlorella* cultures by adding 2% glucose and increasing the light intensity from 10 to 100 µmol photons m^{-1} s^{-1}. Then, the pigments, total phenolic contents, and antioxidant activities of both *Chlorella* species were evaluated. The results showed that both strains grew larger when cultured under stress condition. Although the total carotenoid content was increased under stress condition, reduction of the pigment and total phenolic contents associated with lower antioxidant activity were also recorded. Subsequently, the transcriptome of *C. sorokiniana* was sequenced using Illumina paired-end sequencing, and 198,844,110 raw reads with the length of 100 bp were produced. After pre-processing, ~95% of high quality reads were *de novo* assembled using Trinity software into 18,310 contigs. Analysis of differential gene expression by DESeq2 package showed that a total of 767 genes were upregulated and 948 genes were downregulated in stress conditions. Then, miRNAs that regulate the genes during normal and stress conditions of both *C. sorokiniana* and *C. zofingiensis* were profiled and analysed using CLC Genomic Workbench and OmiRas. From both analysis pipelines, the known and predicted novel miRNAs were identified. Although most of the identified miRNAs were not functionally determined, this study suggests that they were species-specific, which may have roles in regulating genes during stress condition. In conclusion, identifying the genes and the regulation of various metabolite productions under different growth conditions are useful for further strain enhancement of the microalgae.

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

PENCIRIAN MORFOLOGI, BIOKIMIA DAN TRANSKRIPTOMIK BAGI *Chlorella sorokiniana* DAN *Chlorella zofingiensis* SEMASA KEADAAN NORMAL DAN TEKANAN

Oleh

SITI NOR ANI BINTI AZAMAN

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Chlorella telah dikenalpasti sebagai salah satu spesies microalga yang paling menarik dan mempunyai nilai pemakanan dan kadar pertumbuhan yang tinggi, serta dapat menghasilkan pelbagai metabolit sebagai tindak balas kepada perubahan persekitaran. Objektif kajian ini adalah untuk mencirikan kandungan morfologi dan biokimia dan mengenal pasti gen dan miRNA yang terlibat dalam mengawal pengeluaran karotenoid dan lipid dalam *Chlorella sorokiniana* dan *Chlorella zofingiensis* apabila dibiakkan di bawah keamatan cahaya tinggi yang digabungkan dengan penambahan glukosa. Dalam kajian ini, tekanan telah diberikan kepada kultur *Chlorella* dengan menambahkan 2% glukosa dan meningkatkan keamatan cahaya dari 10 ke 100 µmol foton m^{-1} s^{-1}. Kemudian, pigmen, jumlah kandungan fenol, dan aktiviti bahan antioksida bagi kedua-dua spesies *Chlorella* telah dinilai. Keputusan menunjukkan bahawa kedua-dua strain tumbuh lebih besar apabila dikultur dalam keadaan tekanan. Walaupun jumlah kandungan karotenoid meningkat dibawah keadaan tekanan, penurunan pigmen dan jumlah kandungan fenol yang dikaitkan dengan aktiviti bahan antioksidan yang rendah juga direkodkan. Seterusnya, transkriptom *C. sorokiniana* telah dijujukkan menggunakan teknologi penjujukan hujung berpasangan Illumina, dan sebanyak 198,844,110 jujukan nukleotid mentah dengan panjang 100 bp telah dihasilkan. Setelah dipraproses, ~95% bacaan berkualiti tinggi dihimpunkan secara *de novo* menggunakan perisian Trinity menjadi 18,310 kontig. Analisis pembezaan pengekspresan gen oleh pakej DESeq2 menunjukkan sebanyak 767 gen dikawalatur menaik dan 948 gen dikawalatur menurun dalam keadaan tekanan. Kemudian, miRNA yang mengawal gen semasa keadaan normal dan tekanan kedua-dua *C. sorokiniana* dan *C. zofingiensis* diprofilkan dan dianalisis menggunakan CLC Genomic Workbench dan OmiRas. Berdasarkan kedua-dua saluran kaedah analisis ini, miRNA sedia diketahui serta miRNA ramalan novel telah dikenal pasti. Walaupun kebanyakan miRNA yang dikenal pasti ini tidak ditentukan fungsinya, kajian ini mencadangkan bahawa ia adalah khusus kepada spesies tersebut

yang mungkin mempunyai peranan tertentu dalam mengawalatur gen semasa keadaan tekanan. Kesimpulannya, mengenal pasti gen dan pengawalaturan pengeluaran pelbagai metabolit dalam keadaan pertumbuhan yang berbeza adalah sangat berguna bagi peningkatan strain mikroalga ini.

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Thank you so much.

I certify that a Thesis Examination Committee has met on 14 July 2017 to conduct the final examination of Siti Nor Ani binti Azaman on her thesis entitled "Morphological, Biochemical and Transcriptomic Characterisation of *Chlorella sorokiniana* and *Chlorella zofingiensis* during Normal and Stress Conditions" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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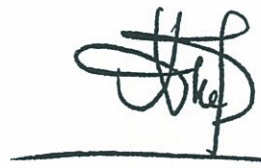

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

AAE	Ascorbic acid equivalent
acetyl-ACP	Acetyl-acyl carrier protein
ADP	Adenosine diphosphate
AGPAT	1-acylglycerol-3-phosphate O-acyltransferase
ath-miR	*Arabidopsis thaliana*-microRNA
ATP	Adenosine tryphosphate
BBM	Bold basal medium
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BLAST	Basic local alignment search tool
B-tub	Beta-tubulin
BWA	Burrows-wheeler aligner
CAL	Calmodulin
CAM	Crassulacean acid metabolism
CBF/DREBs	C-repeat-binding factor/dehydration-responsive element
CD	Chloroplast division
CDKA	Cyclin dependent kinase A
Cdna	Complementary DNA
CO2	Carbon dioxide
Cq	Quantification cycle
DAG	Diglycerides or diacylglycerol
DEGs	Differentially expressed genes
DGAT1	Diacylglycerol O-acyltransferase
DMAPP	Dimethylallyl pyrophosphate
DNA	Deoxyribonucleic acid
DOXP	Deoxyxylulose-5-phosphate
DPPH	2,2-diphenyl-1-picrylhydrazyl
DREs	Dehydration-responsive element
dsRNA	Double-stranded RNA
EFA	Essential fatty acids
EST	Expressed sequence tag
FADH	Flavin adenine dinucleotide
FDR	False discovery rate
FRAP	Ferric-reducing antioxidant power
GAE	Gallic acid equivalent
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC content	Guanine-cytosine content
Gdna	Genomic DNA
GO	Gene ontology
GPAT	Glycerol-3-phosphate acyltransferase
GtRNAdb	Genomic tRNA database
HA	Hatching autospore
HCl	Hydrochloric acid
HKG	Housekeeping gene
HSP90	Heat shock proteins 90
IPP	Isopentenyl pyrophosphate
ITS	Internal transcribed spacer

KAAS	KEGG Automatic Annotation Server
KEGG	Kyoto encyclopedia of genes and genomes
Kmer	Short DNA sequence consisting of a fixed number (K) of bases
KO	KEGG orthology
KOALA	KEGG Orthology And Links Annotation
log_2FC	Log 2 fold change
MA	Mature autospore
MAGs	Monoglycerides or monoacylglycerol
Mbp	Mega base pairs
MEP pathway	Non-mevalonate pathway or Methylerythritol 4-phosphate
miRBase	MicroRNA database
miRNA	MicroRNA
mRNA	Mesengger RNA
N content	If a sequencer is unable to make a base call with sufficient confidence then it will normally substitute an N rather than a conventional base call
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NCBI	National center for biotechnology information
NGS	Next generation sequencing
NTC	Non-template control
OD	Optical density
ORF	Open reading frame
PC	Phosphatidylcholine
PCA	Principal component analysis
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
PGM	Phosphoglucomutase
piRNA	Piwi-interacting RNA
PPAP2	Phosphatidate phosphatase
PSII	Photosystem II
PUFA	Polyunsaturated fatty acid
Repbase	Repetitive DNA database
rfam	Non-coding RNA (ncRNA) database
RNA	Ribonucleic acid
RNA-SEQ	RNA sequencing
ROS	Reactive oxygen species
RPL19	Ribosomal protein L19
rRNA	Ribosomal RNA
RT-QPCR	Quantitative reverse transcription PCR
SAGE	Serial analysis of gene expression
SEM	Scanning electron microscope
siRNA	Small interfering RNA
snoRNA	Small nucleolar RNAs
snoRNABase	Small nucleolar RNA database
SNP	Single nucleotide polymorphism
snRNA	Small nuclear RNA
spp	Several species
SSU	Small subunit

TAE	Tris base, acetic acid and EDTA
TAG	Triglycerides or triacylglycerol
TCA cycle	Tricarboxylic acid or citric acid cycle
TEM	Transmission electron microscope
TPC	Total phenolic content
tRNA	Transfer RNA
UBQ	Ubiquitin
UCP	Universal chlorophyte primer
UV	Ultraviolet
UV/VIS	Ultraviolet /visible
UV-B	Ultraviolet B
VST	Variance stabilization normalization

CHAPTER 1

GENERAL INTRODUCTION

Microalgae have been identified as good source of lipids for biofuels, protein and health metabolites including polyphenols, vitamins and antioxidants (Mostafa 2012). *Chlorella* sp. has received considerable attention, in view of its relatively high nutritional value, its capacity to modify its metabolites in response to changes in its growth medium, relatively rapid rates of reproduction and the possession of a thick cell wall that protect its nutrients (Iwamoto 2004). The microalgae *Chlorella* can produce an unusually wide range of metabolites during both normal growth and growth under stress (de Morais et al. 2015). It is well known that different microalgae produce different metabolites during stress (Skjanes et al. 2013).

In this study, two species of microalgae *Chlorella sorokiniana* and *Chlorella zofingiensis* were selected and evaluated based on their potential for producing high value metabolites under different culture conditions. The relative concentrations and profiles of various metabolites in microalgae are similar under optimal growth. However, the metabolite profile undergoes considerable changes under sub-optimal growth. Different algae species use different methods for managing these changes in the environment. Depending on their ability to handle various stresses, the microalgae produce different secondary metabolites to increase their chances of survival (Skjanes et al. 2013).

Numerous studies concerning the effects of stress upon the metabolome of microalgae have been documented in the literature (Ip and Chen 2005a; Lemoine and Schoefs 2010). However, our understanding of how microalgae respond to physiological stress at the molecular level is largely confined to model organisms, and the relevant pathways in microalgae have not been fully documented. Furthermore, the size and the appearance of microalgae can change profoundly depending on the environmental conditions and associated levels and types of stress. There is a clear need to improve the set of molecular methods and tools for working with microalgae. Previous studies suggested that, when the microalgae *Chlorella* was grown under different growth condition, the colour of the *Chlorella* changed from green to red or yellow, as a consequences of contributed by the pigment production (Ip et al. 2004; Del Campo et al. 2004; Ip and Chen 2005b; Cordero et al. 2011).

Phenotypic details surrounding the influence of stress on the production of pigments and metabolites that contribute to microalgae survival, can be understood by performing comparative transcriptome profiling (Fu et al. 2014; Sun et al. 2015). Transcriptomic study is an appropriate tool which provides an initial, broad view of the regulation of secondary metabolite biosynthetic pathways during a microalgal stress response. So far, the studies have focused on growth experiments and metabolite content screening, and have yielded

very limited information in respect of gene expression, in microalgae under normal and stress conditions (Doan et al. 2011; Perez-Garcia et al. 2011; Baba and Shiraiwa 2013; Goiris et al. 2015). With the discovery of specific classes of RNA molecules as gene regulatory in addition to the more widely accepted protein based transcriptional regulatory (Bartel 2004), transcriptome sequencing can provide a valuable approach for obtaining microalgae functional genomics information. However, transcriptomes relating to the biosynthetic pathways of lipids, polyphenols and vitamin D of *Chlorella* species remain to be profiled. Therefore, it is important to study the transcriptomic profile as well as the metabolite composition of *Chlorella.* It is important and timely to determine the true potential of these species and to support the potential for genetic engineering of these microalgae as they become an increasing focus for their development as alternative source of biofuel, food and health supplements.

In this study, *C. sorokiniana* and *C. zofingiensis*, two *Chlorella* species that exhibit relatively fast growth rates and have potential to produce high yields of secondary metabolites, including secondary pigments, during mixothropic growth were chosen. By performing systematic studies including both physiological (morphological and biochemical) and molecular response of both *Chlorellas* towards normal and stress conditions, this study would provide necessary information for understanding the molecular basis of some important metabolite biosynthesis pathways such as lipids and carotenoids.

1.1 Statement of the hypotheses

The hypotheses of this study are:

1. High light intensity combined with glucose supplementation that increase pigments and lipids production would alter antioxidant activity in *C. sorokiniana* and *C. zofingiensis*.

2. Transcriptomic analysis would reveal the upregulation of genes involve in carotenoid and lipid biosynthesis pathways of *C. sorokiniana* and *C. zofingiensis* when cultured under high light intensity combined with glucose supplementation.

3. Small RNA transcriptomic analysis would reveal the specific miRNA involve in regulating the production of carotenoid and lipid in *C. sorokiniana and C. zofingiensis* when cultured under high light intensity combined with glucose supplementation.

1.2 Research objectives

The objectives of this study are:

1. To characterise the morphology and biochemical contents (such as pigments, phenolic and antioxidant) of *C. sorokiniana* and *C. zofingiensis* under normal and high light intensity combined with glucose supplementation stress conditions.

2. To identify the genes involved in regulating the production of carotenoids and lipids in *C. sorokiniana and C. zofingiensis* when cultured under high light intensity combined with glucose supplementation through RNA-sequencing technique.

3. To identify the miRNAs involved in regulating the production of carotenoids and lipids in *C. sorokiniana and C. zofingiensis* when cultured under high light intensity combined with glucose supplementation through small-RNA sequencing technique.

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