

QUANTIFICATION OF ASTAXANTHIN PRODUCTION BY *Haematococcus pluvialis*
INDUCED BY COPPPER NANOPARTICLES

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DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
BACHELOR OF BIOTECHNOLOGY (HONOURS)

FACULTY OF HEALTH AND LIFE SCIENCES
INTI INTERNATIONAL UNIVERSITY
PUTRA NILAI MALAYSIA

JAN 2018

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ACKNOWLEDGEMENT

First of all, I would like to bid my thank you to my supervisor, Assoc. Prof. Dr. Wong Ling Shing for his guidance and assistance in completing my research. I am very grateful for his aid and patience to look through my research every time and to correct my mistakes accordingly. Besides, I would like to bid my special gratitude to Dr. Cheng Wan Hee, who provided me assistance and suggestion whenever I was stumbled upon an obstacle in completing my thesis writing and my data analysis.

Next, I would also like to thank my lab assistant, Ms Quah for assisting me in the completion of my project. Not only that, I would also like to thank my friends and course-mates to be accompanying me to pull all-nighters in completing my thesis writing. A tonne of appreciations to their willingness in sharing their knowledge and ideas as well. Lastly, I would also like to bid my thank you to my family members, Mr and Mrs. Batumalai Devi and Ms. Chithra, who have given me their support throughout the last 8 months on providing financial support and lending their shoulders always.

ABSTRACT

Haematococcus pluvialis is green microalgae that is found in harsh-free regions like Africa, Europe and North America. It has the ability to produce high yield of astaxanthin under stress as such as when exposed to high temperature, pH, salinity, light and nitrogen deficiency. Astaxanthin is an unsaturated antioxidant that can also be found in various marine animals and microorganism. Astaxanthin were produced under stress condition. The aim of this study is to (i) develop *H. pluvialis* culture and (ii) investigate the effect of Cu nanoparticles to the production of astaxanthin in *H. pluvialis*. The *H. pluvialis* cells were grown in laboratory condition using circadian light, plus their growth patterns and cell count were measured using cell count chamber and light microscope and further analysed under 470 nm in the spectrophotometer in every 2 -3 days. The *H. pluvialis* cells were induced with different concentration of Cu nanoparticle at 100 mg/L, 200 mg/L and 300 mg/L. The astaxanthin were extracted and quantified at 470 nm using a spectrophotometer in every 4 - 5 days. The data were statistically analyzed using simple tools in Microsoft Excel 2016. Copper nanoparticles cannot enhance the production of astaxanthin in *H. pluvialis* due to high toxic level.

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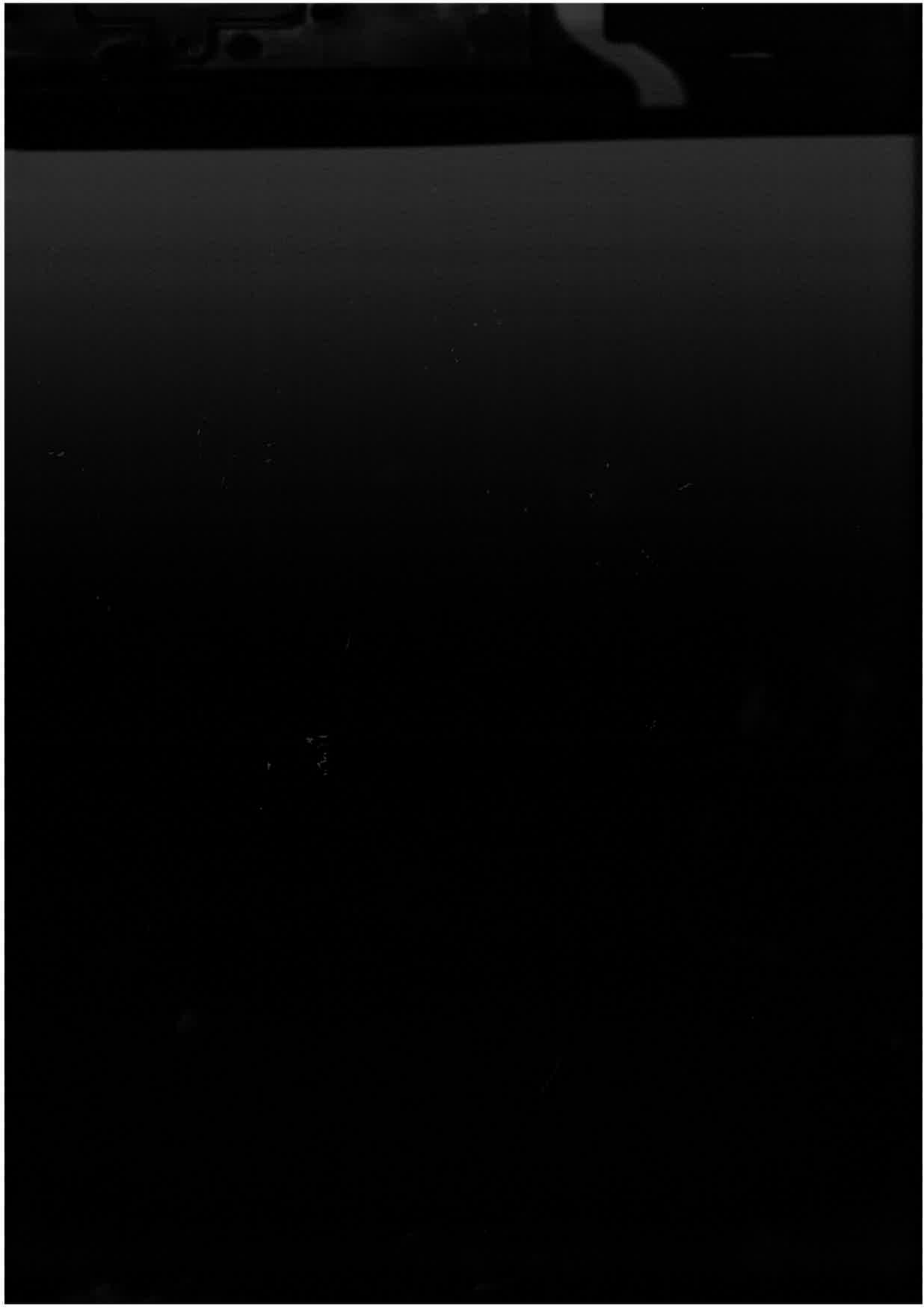
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LIST OF ABBREVIATIONS

°C	Degree Celsius: Unit of temperature
<i>H. pluvialis</i>	<i>Haematococcus pluvialis</i>
ANOVA	Analysis of Variance
UV	Ultra-violet
Fe ²⁺	ferrous
cells/mL	cells per millilitre: Unit of cell density
mg/L	milligram per Litre: Unit of concentration
mg/L	milligram per Litre: Unit of concentration
%	percentage
nm	nanometre
BBM	Bold Basal Medium
µm	micrometre
µL	microlitre
mg	milligram
ROS	Reactive Oxidative Species
klx	kilolux
min	minute
rpm	revolutions per minute
KOH	potassium hydroxide
NiO	nickel oxide



Pt	platinum
ZnO	zinc oxide
TiO ₂	titanium dioxide
Al ₂ O ₃	aluminium oxide
CeO ₂	ceric dioxide
BaTiO ₃	barium titanate
Ag	silver

CHAPTER 1

INTRODUCTION

Astaxanthin (3, 3'-dihydroxy- β -carotene-4, 4'-dione) is an unsaturated antioxidant that can be found in various marine animals and microorganisms such as shrimps, salmon, microalgae, yeast, red sea bream, crayfish and lobster (Chen, J-h., Liu, L. & Wei, D., 2017). As for its biochemical properties, astaxanthin is produced via carotenoid pathway from pyruvate and glyceraldehyde-3-phosphate which are products of glycolysis or/and photosynthesis relying on development conditions (Borowitzka, M. A., 2013). Astaxanthin contains ketone, hydroxyl groups and double bonds (Mahfuzur. M. R. S., Liang, Y., Cheng, J. J. & Daroch, M., 2016). It is both hydrophilic and lipophilic keto-carotenoid that exists as a bright red soluble pigment from the same family as β -carotene, lycopene, and lutein. Other than that, astaxanthin is further classified as xanthophyll carotenoid as it contains oxygen, hydrogen and carbon atoms that has been proven to trap high amounts of free radicals in cell membranes (Ranga, Sarada, A. R., V. Baskaran, V. & Ravishankar, G. A., 2009).

Astaxanthin is widely used in cosmetics, dietary supplements, nutraceutical industry, beverages, the aquaculture industry and functional foods as it has strong oxidation occurrence and makes the product more stable by donating electrons to the free radicals (Larissa, M. J. & Pedrosa, L. F. C., 2010). Plus, astaxanthin have been approved by The United States Food and Drug Administration (USFDA) to be used as animal feed supplements to transmit coloration to salmonids which is salmon, trout, graylings and chars (Ambati, R. R., Siew, M. P., Sarada Ravi & Gokare, R. A., 2013). Natural astaxanthin costs several thousand US dollars per kilogram compared to chemically-synthesized astaxanthin in the global market (Mahfuzur. M. R. S. et al, 2016). However, there are several doubts about the safety of consuming synthetic astaxanthin in human. Reason being, the intermediates and diverse stereochemistry in the chemically synthesized astaxanthin makes the normal astaxanthin to be a safer recommendation of consumption. For instance, they are 65 times more effective compared to vitamin C, providing a boost in energy, preventing atherosclerotic cardiovascular disease, nervous system and neurodegenerative diseases, reducing UV ray damage resulted by the exposure to the sun, improving vision and reducing the risk of Alzheimer's disease (Mahfuzur. M. R. S. et al, 2016).

Haematococcus pluvialis is a green microalga that is found to produce a large amount of astaxanthin (bright red pigment) under frequent cellular stress or unfamiliar conditions like high temperature, high salinity, light and nitrogen deficiency (Guerin, M., Huntley, M. E. & Olaizola, M., 2003). The accumulation of astaxanthin occurs during the conversion of the green vegetative cells to the aplanospore phase (Borowitzka, M. A., 2013). There are different types of cultivation and production of astaxanthin in mixotrophic, photoautotrophic and heterotrophic conditions that can be observed via indoor cultivation. The cultivation prioritizes the emergence of a single colony or closed photobioreactors by using fed-batch or batch culture with suitable nutritional requirements or medium in order to grow *H. pluvialis* (Eriksen, N. T., 2016).

One of the major challenge faced by the company by using *H. pluvialis* is the extraction process as the production yields are highly complicated. The complications of producing astaxanthin by the *H. pluvialis* happens because it gets trapped behind thick cells walls (Oilgae, 2017). Besides that, the *H. pluvialis* reverts bright red pigment to normal green microalgae after adapting to frequent stress conditions. Furthermore, *H. pluvialis* requires high amounts of radiation as it is hard to cultivate in dark heterotrophic conditions. However, the results from this procedure to be financially less sensible. Thus, the research and development team has conducted a number of experiments in order to overcome the challenges that are faced by the company. As stated, the experiments were to improve the production of astaxanthin by stress conditions like minimal light supply (Chen, J-h. et al, 2017). Plus, it has been proven that one of the experiments conducted on *H. pluvialis* produced more astaxanthin with the addition of acetate/ acetate plus Fe^{2+} (Makio, K.; Toshihide K. & Shiro, N., 1992). However, major improvements were needed to be made to produce a higher yield of astaxanthin such as improvement in changes of cell morphology under different environmental conditions, slow cell development rate and sensitivity of the cells towards the hydrodynamic stress (Mahfuzur. M. R. S. et al, 2016). There were no studies on how the Cu nanoparticles affect the *H. pluvialis* in production of astaxanthin. However, there were studies on other heavy metals such as cadmium used to stress the *H. pluvialis* to stimulates the production of astaxanthin (Hong, Y. Z., 2016).

This experiment is aimed to (i) develop *H. pluvialis* cultures in laboratory condition and (ii) the culture will be stressed with Cu nanoparticles to identify the effect of the nanoparticles on the astaxanthin production in the laboratory.

CHAPTER 2

LITERATURE REVIEW

2.1 *Haematococcus pluvialis*

2.1.1 Taxonomy and Living System of *Haematococcus pluvialis*

Haematococcus pluvialis is a unicellular freshwater green microalgae (Tocquin, P., Fratamico, A. & Franck, F., 2011). *Haematococcus pluvialis* can be classified as shown in Table 2.1. It is also commonly known as *Haematococcus lacustris* or *Sphaerella lacustris* (Eom, H., Lee, C. G., & Jin, E., 2006). *Haematococcus pluvialis* usually can be found in harsh-free regions around the world such as Himachal Pradesh, North America, Europe and Africa (Suseela, M. R., & Toppo, K., 2006).

In addition, *H. pluvialis* has been more likely found in temperate climates around the world, for instance, freshwater bowl of the stone loaded with liquefied snow on Blomstrandhalvøya Island in Norway (Klochkova, T. A., Kwak, M. S., Hân, J. W., Motomura, T., Nagasato, C., & Kim, G. H., 2013), salty water on the stones of seashores, freshwater fishpond in Bihor, Romania (Dragos, N., Bercea, V., Bica, A., Druga, B., Nicoara, A., & Coman, C., 2010); and dried wellspring close Rozhen and house top surface of a building of Korea Institute of Ocean Science and Technology (KIOST) in Seoul Korea. Not only that they can also survive under certain conditions like salt concentration, light and temperature area's that would be malicious to numerous other microalgae for a better capacity to encyst in a faster way (Shah, M. R., Yuanmei, L., Jay, J. C. & Maurycy D., 2016).


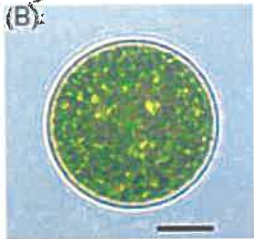
Table 2.1 Classification of *H. pluvialis*

Empire	Kingdom	Family	Class	Order
Eukaryota	Plantae	Haematococcaceae	<u>Chlorophyceae</u>	<u>Chlamydomonadales</u>

2.1.2 Life Cycle of *Haematococcus pluvialis*

The traits of *H. pluvialis* are observed light microscope. Life cycle of *H. pluvialis* comprise of four sorts of detectable cellular morphologies consisting hematocysts, macrozooids, palmella and microzooid (Shah, M. R., Yuanmei, L., Jay, J. C. & Maurycy D., 2016). Macrozooids, palmella and microzooid are typically referred to as green vegetative stage, while hematocysts encysted phase that would accumulate astaxanthin.

Table 2.2 Life cycle of *H. pluvialis*

Phase	Stage	Explanation
Vegetative phase	(A) 	<ul style="list-style-type: none"> • Motile cell • Spherical, ellipsoidal or pear-shaped cell with 2 flagella. • Thickness are between 8 - 20 μm • Can divide 2 – 32 daughter cells through mitosis. • Various contractile vacuoles are irregularly packed close to the protoplast surface of the cell.
	Microzooid stage of <i>H. pluvialis</i> (Shah et al, 2016)	
Resting phase	(B) 	<ul style="list-style-type: none"> • Non-motile cell • Round shaped cell • Form when unfamiliar culture or environmental condition (eg. high salinity, high temperature) • Macrozooids will lose their flagella and enlarge their size. • Form multilayer structure of the inward districts of the primary cell wall.
	Palmella stage of <i>H. pluvialis</i> (Shah et al, 2016)	