EFFECTS OF LIGHT INTENSITY ON THE GROWTH AND PIGMENT PRODUCTION IN *Haematococcus pluvialis*

KARTIKA SARI DEWI BINTI MOHD TAHIR

THIS DISSERTATION IS SUBMITTED IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS FOR BACHOLER OF SCIENCE WITH HONOURS

AQUACULTURE PROGRAMME

FACULTY OF SCIENCE AND NATURAL RESOURCE

UNIVERSITI MALAYSIA SABAH

2015

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN STATUS TESIS

JUDUL: EFFECT OF LIGHT INTENSITY ON THE GROWTH AND PIGMENT PRODUCTION IN Haematococcus Pluvialis

IJAZAH: SARJANA MUDA SAINS DENGAN KEPUJIAN (AKUAKULTUR)

SAYA: KARTIKA SARI DEWI BINTI MOHD TAHIR (HURUF BESAR) SESI PENGAJIAN: 2014/2015

Mengaku membenarkan tesis *(LPSM/Sarjana/Doktor Falsafah) ini disimpan di Perpustakaan Universiti Malaysia Sabah dengan syarat-syarat kegunaan seperti berikut:-

1. Tesis adalah hakmilik Universiti Malaysia Sabah.
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. Sila tandakan (/)

☐ SULIT (Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di AKTA RAHSIA RASMI 1972)

☐ TERHAD (Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana Penyelidikan dijalankan)

☐ TIDAK TERHAD

PERPUSTAKAAN
UNIVERSITI MALAYSIA SABAH

Disahkan oleh: NURULAIN BINTI ISMAIL
LIBRARIAN
UNIVERSITI MALAYSIA SABAH

(TANDATANGAN PENULIS)

(TANDATANGAN PUSTAKAWAN)

Alamat tetap: NO. 1473 TAMAN TAWAU LAMA, LORONG 4, 91000 TAWAU, SABAH

PROF. MADYA DR. SITTI RAEHANAH
NAMA PENYELIA

Tarikh: 18 JUN 2015

Tarikh:

Catatan :- * Potong yang tidak berkenaan.
*Jika tesis ini SULIT atau TERHAD, sila lampirkan surat daripada pihak berkuasa/organisasi berkenaan dengan menyatakan sekali sebab dan tempoh tesis ini perlu dikelaskan sebagai SULIT dan TERHAD.
*Tesis dimaksudkan sebagai tesis bagi Ijazah Doktor Falsafah dan Sarjana Secara penyelidikan atau disertai bagi pengajian secara kerja kursus dan Laporan Projek Sarjana Muda (LPSM)

DECLARATION

I declare that this dissertation is the results of my own independent work, except where otherwise stated.

JUNE 2015

KARTIKA SARI DEWI BINTI MOHD TAHIR

(BS12110234)

19 JUNE 2015

VERIFIED BY

SIGNATURE

1. SUPERVISOR

(ASSOC. PROF. DR. SITTI RAEHANAH MUHAMAD SHALEH) _____

2. DEAN

(PROF. MADYA DR. BABA MUSTA) _____

ACKNOWLEDGEMENTS

This dissertation has accumulated debts of gratitude throughout its completion. I would like to acknowledge with deep and sincere gratitude to a great number of people who have contributed in completing this dissertation.

Firstly, I would like to thank my supervisor, Assoc. Prof. Dr. Sitti Raehanah binti Muhamad Shaleh for her guidance and advices for the study, for her patience comments and corrections on the preparation of the thesis.

Last but not least, I would like to thank the entire IPMB's staff and research assistants. My gratefulness also goes to my coursemates for who helped me during experiment.

KARTIKA SARI DEWI BINTI MOHD TAHIR

JUNE 2015

ABSTRAK

Mikroalga hijau, Haematococcus pluvialis telah dikultur di bawah aras keamatan cahaya yang berbeza untuk mengkaji kesan cahaya ke atas pertumbuhan dan penghasilan pigmen. Pertumbuhan H. pluvialis yang terbaik adalah apabila keamatan cahaya pada aras 250 µmol m^{-2} s^{-1} dengan kepadatan sel 18.95x10^{6} cells ml^{-1}. Pertumbuhan H. pluvialis pada 250 µmol m^{-2} s^{-1} adalah tinggi berbanding ($p<0.05$) dengan 25, 75, 150 dan 350 µmol m^{-2} s^{-1}. Analisis pigmen telah dibuat menggunakan dimethyl-sulphoxide (DMSO). Kandungan klorofil a paling tinggi adalah pada keamatan cahaya 350 µmol m^{-2} s^{-1} di hari ke-14 pengkulturan. Manakala, kandungan klorofil b paling tinggi pada hari ke-13 dengan keamatan cahaya 250 µmol m^{-2} s^{-1}. Tambahan lagi, jumlah kandungan karotenoid terkumpul kebanyakannya pada keamatan cahaya 250 µmol m^{-2} s^{-1} dan paling tinggi pada hari ke-14. Kesimpulannya, factor keamatan cahaya sangat mempengaruhi akumulasi klorofil a dan b dan juga jumlah karotenoid dalam H. pluvialis. Di samping itu ia juga turut mempengaruhi kepadatan sel.

ABSTRACT

The green microalga *Haematococcus pluvialis* was cultured with different light intensity to determine the effect on cell growth and pigment production. The best growth among the light intensity sources was achieved at 250 µmol m^{-2} s^{-1} with cell density of $18.95x10^6$ cells ml^{-1}. Cell growth was significantly higher ($p<0.05$) in 250 µmol m^{-2} s^{-1} compared to 25, 75, 150 and 350 µmol m^{-2} s^{-1}. The pigment analysis was performed using dimethyl-sulphoxide (DMSO). The highest Chlorophyll a content was found at light intensity 350 µmol m^{-2} s^{-1} on the 14^{th} day cultivation. Meanwhile, Chlorophyll b content of 26.295 µg/m was the highest on the 13^{th} day at 250 µmol m^{-2} s^{-1}. Moreover, the total carotenoid content accumulated mostly at 250 µmol m^{-2} s^{-1} and at the highest on 14^{th} day. It was concluded that light intensity were greatly influencing the accumulation of Chlorophyll a and b and total carotenoids of the *H. pluvialis*. Besides, it also affects the cell density.

List of content

Declaration		ii
Verified by		iii
Acknowledgement		iv
Abstrak		v
Abstract		vi
List of content		vii
List of table		ix
List of figure		x
Chapter 1	Introduction	1
1.1	Freshwater Microalgae	1
1.2	Microalgae in Aquaculture	1
1.3	Literature Review	2
1.3.1	General Characteristic of *Haematococcus pluvialis*	2
1.3.2	Composition of *Haematococcus* Algae	3
1.3.3	Astaxanthin of *Haematococcus pluvialis*	4
1.3.4	Astaxanthin in Aquaculture	5
1.3.5	Life Cycle and Growth of *Haematococcus sp.*	5
1.3.6	Effect of Light Intensity on Astaxanthin Production	7
1.4	Problem Statement	7
1.5	Significant of The Study	8
1.6	Objectives	8
1.7	Hypothesis	8
Chapter 2	Materials and Methods	9
2.1	Organism and Culture Condition	9
2.2	Experimental Design	9
2.3	Cell Counting	10
2.4	Absorbance (Optical Density)	11
2.5	Pigment Determination	11
2.6	Statistical Analysis	12
Chapter 3	Results	13
3.1	Growth of *H. pluvialis* in Various Light Intensity Culture	13
3.2	Pigment Formation of *H. pluvialis* in Various Light Intensity Culture	15
3.3	Microscopic Observation	18
Chapter 4	Discussion	20

Chapter 5 Conclusion 23
Reference 24
Appendix 29

List of table

No.of table		page
Table 1.2.1	High value products from microalgae	2
Table 1.3.2	Typical common components of *Haematococcus* algae	3
Table 3.1	Growth rate (μ) and doubling times (days) for *H. pluvialis* under different light intensity culture.	14

No. of figure		Page
Figure 1.3.1	Taxonomy of *H. pluvialis*	3
Figure 1.3.5.1	(a)Vegetative actively growing *Haematococcus* cells and (b)*Haematococcus* haematocysts that have accumulated astaxanthin as a result of nutrient starvation and sunlight	6
Figure 1.3.5.2	The growth phases of micro-algae cultures.	6
Figure 2.2	The experimental layout of the experiment to determine the effects of light intensity on the growth and pigment production in *Haematococcus pluvialis*	10
Figure 3.1	Growth curve of *H. pluvialis* cells grown in different light intensity.	13
Figure 3.2.1	The effect of light intensity on pigment content in *H. pluvialis*; (A) Chlorophyll$_a$ (B) Chlorophyll$_b$ and (C) Total Carotenioid.	16
Figure 3.2.2	(A) Chlorophyll a, (B) Chlorophyll b, (C) Total carotenoid content of *H. pluvialis* at different light intensity.	17
Figure 3.3	The size of *H. pluvialis* grown under different light intensity; (A) 25 µmol $m^{-2} s^{-1}$, (B) 75 µmol $m^{-2} s^{-1}$, (C) 150 µmol $m^{-2} s^{-1}$, (D) 250 µmol $m^{-2} s^{-1}$, (E) 350 µmol $m^{-2} s^{-1}$ at the (1) 7th day and (2) 14th day of culture.	19

CHAPTER 1

INTRODUCTION

1.1 Freshwater Microalgae

Algae are the single cell organisms which live in either fresh or saline water by producing its on food through photosynthesis using carbon dioxide. Freshwater microalgae can usually found in many freshwater habitats, especially in standing waters. Freshwater algae are important components of water systems as they form the base of aquatic food chain even though they are tiny in size. Therefore, microalgae are indispensable in the commercial rearing of various species of marine animals as a food source for all growth stages of bivalve molluscs, larval stages of some crustacean species, and very early growth stages of some fish species.

1.2 Microalgae in Aquaculture

Universally, more than 40 species of microalgae are used in aquaculture. Successful commercial utilization of microalgae has been established in the production of nutritional supplements, antioxidants, cosmetics, natural dyes and polyunsaturated fatty acids (PUFA) (Spolaore et al., 2006). In aquaculture, their applications are mainly to provide nutrition and to enhance the color of the flesh of salmonids. Besides, larvae of molluscs, echinoderms and crustaceans as well as some fish larvae feed on microalgae.

In the year 1999, the production of microalgae for aquaculture reached 1,000 tonnes (62% for molluscs, 21% for shrimps and 16% for fish) (Spolaore et al., 2006; Gagneux-Moreaux et al. 2007). Microalga have many inherent advantages, some of them are higher productivity (biomass) in few days, easy adaptability to new environments and high-lipid content. Due to that, microalgae culture has been done

CHAPTER 1

INTRODUCTION

1.1 Freshwater Microalgae

Algae are the single cell organisms which live in either fresh or saline water by producing its on food through photosynthesis using carbon dioxide. Freshwater microalgae can usually found in many freshwater habitats, especially in standing waters. Freshwater algae are important components of water systems as they form the base of aquatic food chain even though they are tiny in size. Therefore, microalgae are indispensable in the commercial rearing of various species of marine animals as a food source for all growth stages of bivalve molluscs, larval stages of some crustacean species, and very early growth stages of some fish species.

1.2 Microalgae in Aquaculture

Universally, more than 40 species of microalgae are used in aquaculture. Successful commercial utilization of microalgae has been established in the production of nutritional supplements, antioxidants, cosmetics, natural dyes and polyunsaturated fatty acids (PUFA) (Spolaore et al., 2006). In aquaculture, their applications are mainly to provide nutrition and to enhance the color of the flesh of salmonids. Besides, larvae of molluscs, echinoderms and crustaceans as well as some fish larvae feed on microalgae.

In the year 1999, the production of microalgae for aquaculture reached 1,000 tonnes (62% for molluscs, 21% for shrimps and 16% for fish) (Spolaore et al., 2006; Gagneux-Moreaux et al. 2007). Microalga have many inherent advantages, some of them are higher productivity (biomass) in few days, easy adaptability to new environments and high-lipid content. Due to that, microalgae culture has been done

commercial. Table below shows the microalgae product that has high value to aquaculture (Rathinam Raja et al., 2014):

Table 1.2.1 High value products from microalgae.

Microalgae	**Products**	**References**
Pavlova, Nannochloropsis, Phaeodactylum	Eicosapentaenoic acid (EPA)	Borowitzka and Borowitzka, 1988; Chisti, 2007; Raja *et al.*, 2008
Crypthecodiuimu and Schizochytrium	Docohexaenoic acid (DHA)	Borowitzka and Borowitzka, 1988; Chisti, 2007
Spirulina	γ-linolenic acid (GLA)	
Porphyridium	Arachidonic acid (AA)	
Spirulina platensis	Phycocyanin	
Porphyridium cruentum	Phycoerythrin Polysaccharides	
Dunaliella salina	β-carotene	Borowitzka and Borowitzka, 1988; Chisti, 2007; Raja *et al.*, 2008
Haematococcus pluvialis	Astaxanthin	
Aphanizomenon flosaquae	Mycosporine-like amino acids (MAA)	

1.3 Literature Review

1.3.1 General Characteristic of *Haematococcus pluvialis*

Algae can be categorized as phytoplankton derived from Greek "phyto" meaning plant while "plankton" means wanderer. *Haematococcus pluvialis* is a freshwater species of

Chlorophyta from the family Haematococcaceae. It is also referred to as *Haematococcus lacustris* or *Sphaerella lacustris*, is a ubiquitous green alga. *Haematococcus pluvialis* is well known for its high content of astaxanthin which is important in aquaculture and cosmetics.

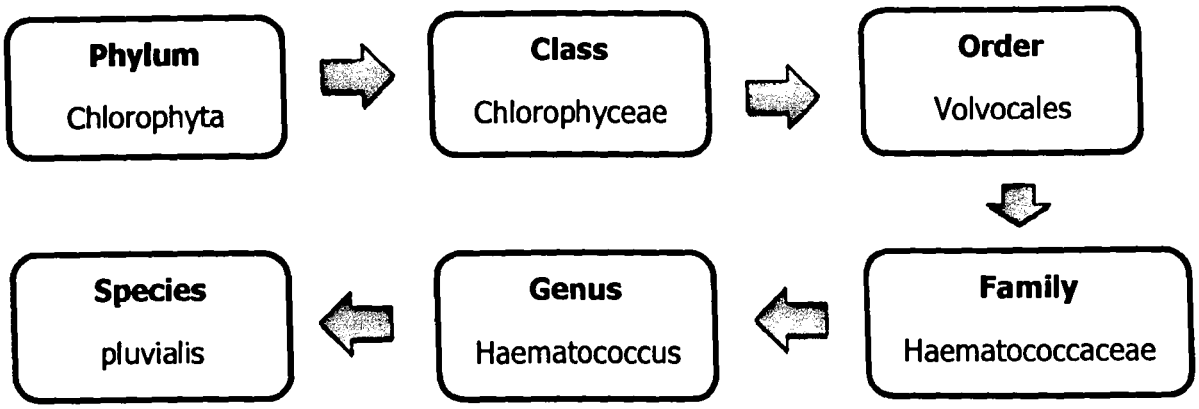

Figure 1.3.1 Taxonomy of *H. pluvialis*

1.3.2 Composition of *Haematococcus* Algae

Haematococcus algae are red to dark red in color. It has particle size of 5-25microns and moisture between 4-9%. *H. pluvialis* has high lipid concentration and produces the pigment astaxanthin. The general composition of *Haematococcus* algae consists of common carotenoids, fatty acids, proteins, carbohydrates, and minerals. The content is listed in Table 1.3.2:

Table 1.3.2 Typical common components of *Haematococcus* algae (NatuRoseÔ Technical Bulletin, 1999)

Components	Minimum	Maximum	Mean
Protein	17.30	27.16	23.62
Carbohydrates	36.9	40.0	38.0
Fat	7.14	21.22	13.80
Iron (%)	0.14	1.0	0.73
Moisture	3.0	9.00	6.0
Magnesium (%)	0.85	1.4	1.14

Calcium (%)	0.93	3.3	1.58
Biotin (mg/lb)	0.108	0.665	0.337
L-carnitine (ug/g)	7.0	12	7.5
Folic acid (mg/100g)	0.936	1.48	1.30
Niacin (mg/lb)	20.2	35.2	29.8
Panthothenic acid (mg/lb)	2.80	10.57	6.14
Vitamin B1 (mg/lb)	<0.050	4.81	2.17
Vitamin B2 (mg/lb)	5.17	9.36	7.67
Vitamin B6 (mg/lb)	0.659	4.5	1.63
Vitamin B12 (mg/lb)	0.381	0.912	0.549
Vitamin C (mg/lb)	6.42	82.7	38.86
Vitamin E (IU/lb)	58.4	333	186.1
Ash	11.07	24.47	17.71

1.3.3 Astaxanthin of *Haematococcus pluvialis*

The freshwater green unicellular alga *Haematococcus pluvialis* (Chlorophyceae) has been exhaustively studied due to its ability to accumulate the red carotenoid astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) and other related carotenoids and their esters (Grung et al., 1992; Yuan and Chen, 1998). The astaxanthin accumulates in *H. pluvialis* under unfavorable growth conditions such as nitrogen, phosphorus starvation (Boussiba and Vonshak 1991, Boussiba *et al.*, 1999), high temperature (Tjahono *et al.*, 1994), oxidative stress (Kobayashi *et al.*, 1993), salt stress (Cordero *et al.*, 1996, Sarada *et al.*, 2002), C/N ratio (Kakizono *et al.*, 1992, Boussiba and Vonshak 1991) and light condition (Kobayashi *et al.*, (1992).

Astaxanthin accumulation by this microalga is related to the formation of the palmella and aplanospore stages of the life cycle of the microalga (Elliot, 1934). It has attracted significant attention due to its wide application in pharmaceutical, cosmetic and food industries (Vidhyavathi *et al.*, 2008), especially as feed supplement for farmed trout, salmon and prawns (Holtin *et al.*, 2009).

Though many organisms are able to produce astaxanthin, only a few of them have commercially been cultivated. In this respect, the freshwater microalga *Haematococcus pluvialis* Flotow the most promising organism due to the higher cellular astaxanthin content compared to the others, i.e., more than 4% of dry weight (Lee and Soh, 1991; Torzillo *et al.*, 2003).

1.3.4 Astaxanthin in Aquaculture

Astaxanthin is one of the most important carotenoids in marine organisms, being responsible for the pigmentation of skin and flesh of fish, mainly salmonids (Lorenz and Cysewski, 2000). Other than that, it also influences survival and growth of fish larvae, even though the exact mechanism is not known (Christiansen *et al.*, 1995). Most crustaceans, including shrimp, crawfish, crabs and lobsters, are tinted red by as result of accumulation of astaxanthin from their diet. In commercial fish and crustacean farms, astaxanthin is commonly added to their diet in order to compensate for the lack of a natural food source of the pigment (Torrissen *et al.*, 1989).

1.3.5 Life Cycle and Growth of *Haematococcus sp.*

Life cycle of *Haematococcus pluvialis* was divided into four cell stages that are vegetative cell growth, encystment (vegetative to immature cyst), maturation (immature to mature cyst cells), and germination (mature cyst to vegetative cells). During the vegetative stage cells contained high levels of chlorophyll and proteins but had very low carotenoid contents, whereas encystment was accompanied by the degradation of chlorophyll and protein. The maturation of cyst cell was accompanied by enhanced carotenoid biosynthesis and accelerated protein degradation. Germination coincided with chlorophyll and protein synthesis, and carotenoid degradation. Carotenoid accumulated in cyst cells only on maturation.

Maturation stage is also known as resting hematocyst stage of *Haematococcus pluvialis* and responsible for the red coloration. These hematocysts had an oval shape, a thickened cell wall and no flagella. During this stage, the cell hematocysts contain large amount of astaxanthin.

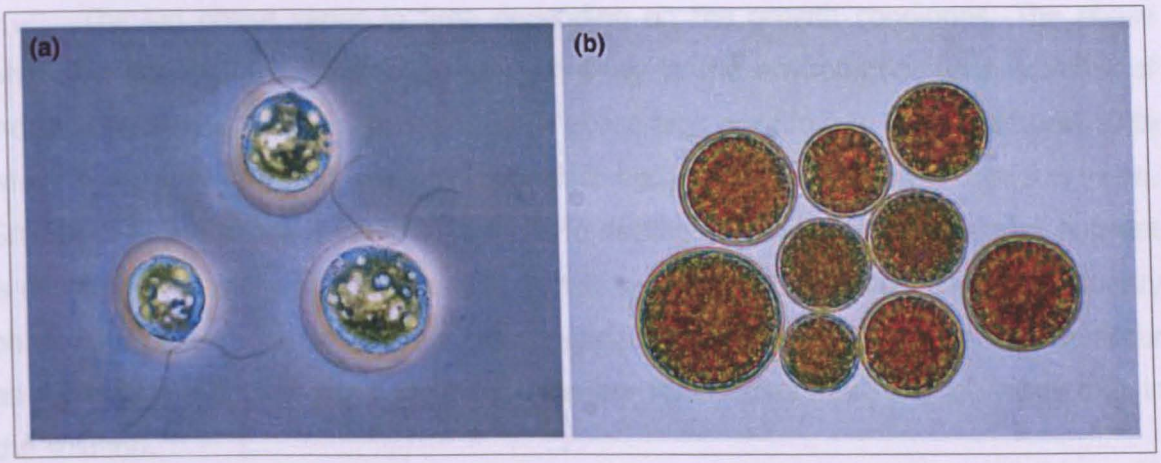

Figure 1.3.5.1 (a)Vegetative actively growing *Haematococcus* cells and (b)*Haematococcus* haematocysts that have accumulated astaxanthin as a result of nutrient starvation and sunlight. 400×magnification. Source : (Lorenz and Cysewski, 2000)

There are five phases of growth applicable to all organisms which consist of lag or induction phase, exponential phase, phase of declining relative growth, stationary phase, and death phase. Figure 1.7.2 shows the sigmoid growth curve of an algal population with indications of each phase:

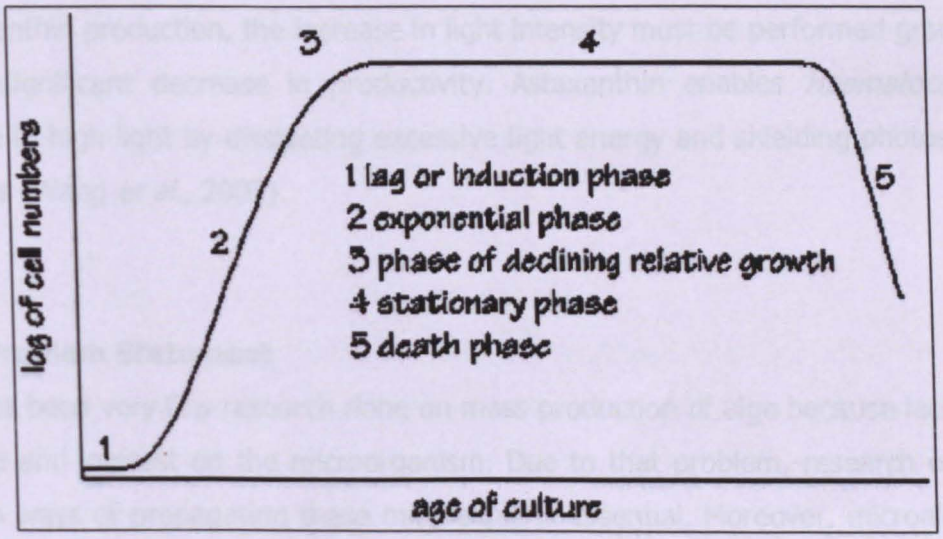

Figure 1.3.5.2 The growth phases of micro-algae cultures.

The lag phase varies in time depending on the growth conditions. The phase where an adaptation occurs, algae are adjusting to the environment upon transfer of inoculum into new medium. In exponential phase alga are often in their healthiest state where one cell divides into two new cells which each divide into two new cells and so on. Growth declining phase is when alga starts to decline due to either the essential nutrient is depleted or waste and toxic products accumulates and limit growth. During stationary phase, the population number remains constant for a certain period. Death phase occur as nutrients are depleted or toxic substances are released as there are too many cells in the solution.

1.3.6 Effect of Light Intensity on Astaxanthin Production.

The pattern of cell growth and astaxanthin accumulation has generated two productive strategies for growing *H. pluvialis*: one, in a single step using a suitable medium (sub-optimum) for both biomass and astaxanthin production. Two, vegetative growth followed by astaxanthin production in non-growing cells (Cifuentes *et al.,* 2003).

Increasing light intensity usually result in better growth and faster division of algal cells (Laing, 1991). In vegetative growth, the optimum intensity of light is about 2000 – 6000 lux for the cultivation of *Haematococcus pluvialis* microalgae. Meanwhile, for astaxanthin production, the increase in light intensity must be performed gradually to avoid a significant decrease in productivity. Astaxanthin enables *Haematococcus* to acclimate to high light by dissipating excessive light energy and shielding photosynthetic apparatus (Wang *et al.*, 2003).

1.4 Problem Statement

There has been very few research done on mass production of alga because lack of cost, expertise and interest on the microorganism. Due to that problem, research on finding optimum ways of propagating these microalgae is essential. Moreover, microalgae have potential in the economic sector.

Microalgae are the major part of the food chain. *Haematococcus pluvialis* for example, contain astaxanthin that is commonly added to the diet of commercial fish and

crustacean farms in order to compensate for the lack of a natural food source of the pigment. Besides, it can improve the survival rate in freshwater larvae. However, the mass production of *H. pluvialis* is hindered by the slow growth.

1.5 Significant of The Study

In algae culturing industry, the most challenging part is to produce it at large scale. Mass production will help supply sufficient volume of algae needed by the aquaculture industry especially. There is lack of study in Malaysia on microalgae propagating techniques. Other than that, astaxanthin have increasingly demand in aquaculture but their slow growths make it hard to produce in mass scale production. Through this study, light intensity parameter may provide an alternative to culturing *H. pluvialis* for mass scale production.

1.6 Objectives

This study was conducted to answer how to propagate *H. pluvialis* at the best light intensity for mass production at indoor culture system and determine the pigment production. Below are the specific objectives:

1.6.1 To determine the optimum light intensity for the growth of *Haematococcus pluvialis*.

1.6.2 To determine pigment production in *Haematococcus pluvialis*.

1.7 Hypothesis

In this study, light intensity will be used as the parameter for growth of *H. pluvialis* because it can induce astaxanthin production under condition of stress (Kobayashi *et al.*, 1997). Below are the hypotheses:

1.7.1 The growth rate of *Haematococcus pluvialis* is optimum at 250 µmol m^{-2} s^{-1}.

CHAPTER 2

MATERIALS AND METHODS

2.1 Organism and Culture Condition

Haematococcus pluvialis used for this project was obtained from UTEX Collection, University Texas at Austin, USA and was maintained at Borneo Marine Research Institute (BMRI), Universiti Malaysia Sabah since July 2012. Microalgae were cultured in liquid fertilizer NPK 10:8:6 . It is a less expensive medium compared to Bold Basald Medium (BBM) . The main requirement for any nutrient to be used in a mass production program is to be easily obtained in adequate quantities and have a low cost (Howell, 1973).

To determine the suitable light intensity to obtain the best growth and total carotenoid production a range of light intensity was tested: 25, 75, 150, 250 and 350 µmol m^{-2} s^{-1}.

Cultures were carried out in a temperature controlled room that was maintained at 26 ± 1°C, with a dark:light cycle of 12:12 hours. Cultures were aerated vigorously and were carried out in duplicate.

2.2 Experimental Design

H. pluvialis was grown in NPK medium at 3 ml NPK per 1000 ml distilled water. The cultures were carried out in 3 L round bottom flask with working volume of 2 L, arranged on the culture. Light intensity tested were 25, 75, 150, 250 and 350 µmol m^{-2} s^{-1}. The required light intensity was obtained by covering each of the partitions using white polystyrene at the back and each side of the shelf. There were two different ways to obtain the variation of light intensity; (i) by adjusting the height of the lamp from the

culture flask, (ii) by adding more lamps and covering the front part of the partition using polystyrene.

The experiment was conducted for a period of 14 days and the day of inoculation was considered as the 1st day. Cultures were inoculated at a cell density of 250,000 cell ml^{-1}. Cell density was monitored daily by cell counting using an improved Neubauer haematocytometer. On a daily basis, cell counting, optical density, pigment analysis and observation of the size of the cell were done.

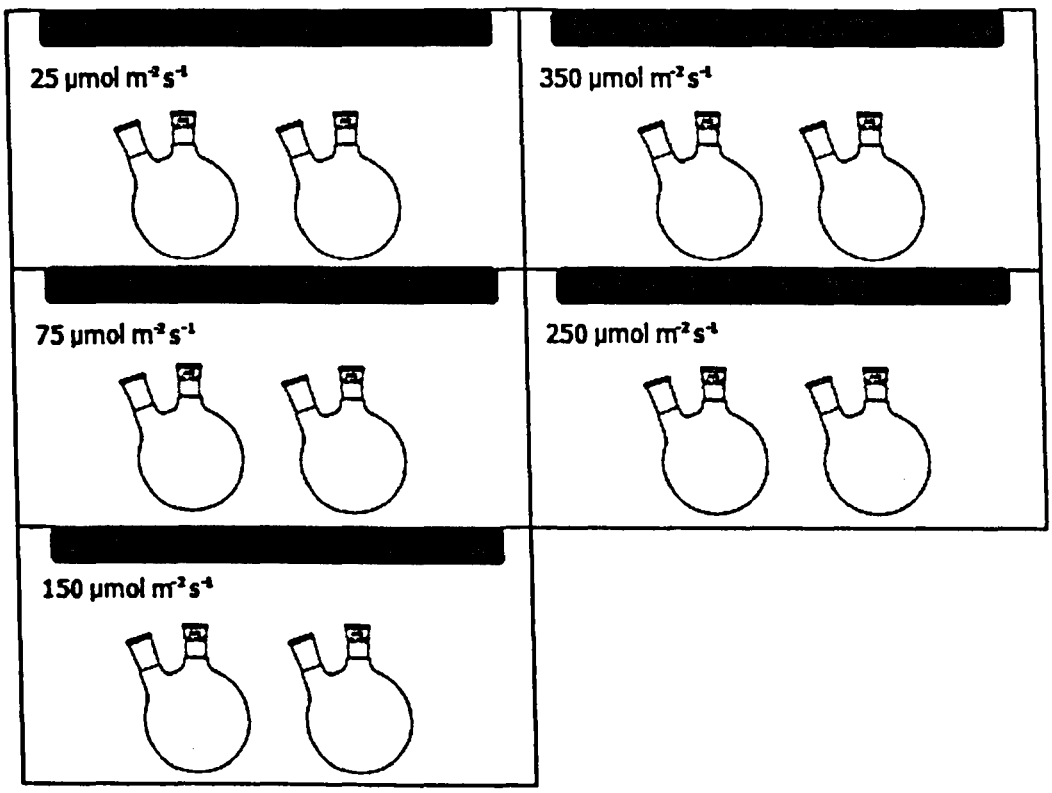

Figure 2.2 The experimental layout of the experiment to determine the effects of light intensity on the growth and pigment production in *Haematococcus pluvialis.*

2.3 Cell Counting

Cell counting was used to measure the growth of algae cultures. Cell numbers were determined by daily counting duplicate samples using an improved Neubauer

haemocytometer (Guillard and Sieracki 2005). It is recommended that more than one replicate samples are counted to reduce sampling errors. Neubauer haemocytometer is a special, thick, microscope slide originally developed for counting blood cells, but has proven excellent for counting single celled algae.

It is necessary to dilute the original cell suspension to be able to count the cells easily; however one must remember to include this dilution factor when doing calculations later. For the Neubauer Haemocytometer the volume over one of the 1 mm squares is 0.0001 mL. Therefore the cell number per mL can be calculated as follows, and expressed as 10^6 cell ml^{-1}:

$$\text{Cell number (cells ml}^{-1}) = \frac{\text{Total number of cells counted}}{\text{Number of squares counted}} \times 1000 \times \text{dilution factor}$$

2.4 Absorbance (Optical Density)

Absorbance is another method used widely for measuring growth of algae cultures. UV visualization spectrophotometer was used to read the absorbance. In order to avoid interference with absorbance by chlorophyll or other photosynthetic pigments, the usual wave lengths used are 550 or 750 nm. *H. pluvialis* was spectrophotometrically determined by recording the absorbance at 750 nm.

2.5 Pigment Determination

For pigment analysis, 2 ml samples with 3 replicate for each culture were centrifuged at 4,400 rpm for 10 min. Approximately 1.9 ml of the supernatant was separated and was mixed with 1.9 ml dimethyl sulfoxide (DMSO) and vortexed vigorously for 30 seconds (Sedmak *et al.*, 1990). The solution mixture in 15 ml falcon tube was covered with aluminum foil to prevent penetration of light. It is then kept in fridge with -5°C for overnight.

Next, the solution mixture was vortexed again before it was centrifuged. The supernatant was taken and measured in spectrophotometer. Chlorophyll a, Chlorophyll b and total carotenoids were spectrophotometrically determined by recording the absorbance at 665, 649 and 480 nm, respectively.

Dimethyl sulfoxide (DMSO) is the best solvent for use with green algae, and is as good a solvent as 90% acetone for diatoms and cyanobacteria (Shoaf and Lium, 1976, Oceanogr. & Limnol). In the study of Sumanta Nayek *et al.* (2014), DMSO is recognized for the highest extractions of carotenoids from the experimented fern species. Even though toxicity of DMSO is low, it penetrates skin and thus contact with this solvent should be limited.

2.6 Statistical Analysis

Data was analyzed by plotting the graph of number of cells against time of culture. One-way ANOVA was used to analyse and to compare the significant level in the growth performance among the five light intensity. The data was analyse using SPSS computer software. The statistical significant that used is ($P<0.05$). The specific growth rate (μ) was calculated during the exponential growth period by using formula as follows:

$$\mu = \frac{\ln N_2 - \ln N_1}{t_1 - t_2}$$

Where N_1 and N_2 = biomass at time1 (t_1) and time2 (t_2) respectively; Levasseur *et al* (1993).

The equations to determine concentrations (µg/ml) of Chlorophyll a (Ch-a), Chlorophyll b (Ch-b) and total carotenoids (C x+c) by using dimethyl sulphoxide (DMSO) (Sumanta Nayek *et al.*, 2014) in spectrophotometer are as follows

$$\text{Ch-a} = 12.47\, A_{665.1} - 3.62\, A_{649.1}$$

$$\text{Ch-b} = 25.06\, A_{649.1} - 6.5\, A_{665.1}$$

$$\text{C x+c} = (1000\, A_{480} - 1.29\, _{Ca} - 53.78\, _{Cb}) / 220$$

REFERENCES

Aflalo, C., Meshulam, Y., Zarka, A. and Boussiba, S. 2007. On the relative efficiency of two vs. one-stage production of astaxanthin by the green alga *Haematococcus pluvialis*. Biotechnol. Bioeng., 98: 300–305. DOI: 10.1002/bit.21391

Borowitzka, M.A., J.M. Huisman & A.Osborn. 1991. Culture of astaxanthin -producing green alga *Haematococcus pluvialis*. 1. Effects of nutrients on growth and cell type. Journal of Applied Phycology 3: 95-304

Borowitzka, M.A. 1999. Commercial production of microalgae: ponds, tanks, tubes and fermenters. Journal of Biotechnology 70: 313-321

Boussiba, S. and A. Vonshak. 1991. Astaxanthin accumulation in the green alga *Haematococcus pluvialis*. Plant Cell Physiol. 32(7): 1077-1082

Boussiba S., Bing W., Yuan J.P, Zarka A., Chen F. 1999. Changes in pigments profile in the green alga *Haematococcus pluvialis* exposed to environmental stresses. Biotech Lett., 21: 601-604

Boussiba, S. 2000. Carotenogenesis in the green alga *Haematococcus pluvialis*: cellular physiology and stress response. Physiol. Plant. 108: 111–117.

Britton G., Liaaen-Jensen S. & Pfander H. 1995. Carotenoids today and challenges for the future. In: G. Britton, S. Liaaen-Jensen & H. Pfander (eds.). Carotenoids vol 1A: 13-26. Isolation and Analysis. Basel Birkkhäuser

Brown, M. R., Jeffrey, S. W., Volkman, J. K., Dunstan, G. A., 1997. Nutritional properties of microalgae for mariculture. *Aquaculture,* 151: 315-331

Christiansen, R., Lie, Ø., Torrissen, O.J., 1995. Growth and survival of Atlantic salmon, *Salmo salar* L., fed different dietary levels of astaxanthin. First feeding fry. Aquac. Nutr. 1, 189–198

Choi, S. L., Suh, I. S. and Lee, C. G. 2003. Lumostatic operation of bubble column photobioreactors for *Haematococcus pluvialis* cultures using a specific light uptake rate as a control parameter. Enzyme Microbiol. Technol. 33: 403–409.

Cordero B., Otero A., Patino M., Arredondo B.O., Fabregas J. 1996. Astaxanthin production from the green alga *Haematococcus pluvialis* with different stress conditions. Biotech Lett., 18:213-218

Dominguez-Bocanegra, A.R., Guerrero Lagarreta, I., Martinez Jeronimo, F. and Tomasini Compocosio, F. 2004. Influence of environmental and nutiritional factors in the production of astaxanthin from *Haematococcus pluvialis*. Bioresource Technol., 92: 209–214. DOI: 10.1016/j.biortech.2003.04.001

Elliot, A.M., 1934. Morphology and life history of *Haematococcus pluvialis*. Arch. Protistenk. 82, 250–272

Enright, C. T., Newkirk, G. F., Craigie, J. S., Castell, J. D., 1986a. Growth of juvenile Ostrea edulis L. fed *Chaetoceros calcitrans* Schütt of varied chemical composition *Journal of Experimental Marine Biology and Ecology,* 96: 15–26

Fan L., A. Vonshak and S. Boussiba. 1994. Effect of temperature and irradiance on growth of *Haematococcus pluvialis*. J. Phycol. 30:829-833

Fábregas, J., Domínguez, A., García-Álvarez, D., Lamela, T., Otero, A., 1998. Induction of astaxanthin accumulation by nitrogen and magnesium deficiencies in *Haematococcus pluvialis*. Biotechnol. Lett. 20, 623–626

Fabregas, J., Otero, A., Maseda, A. and Dominguez, A. 2001. Two-stage cultures for the production of astaxanthin from *Haematococcus pluvialis*. J. Biotechnol., 89: 65 71. DOI: 10.1016/S0168-1656 (01)00289-9

Fujii K, Nakashima H, Hashidzume Y, Uchiyama T, Mishiro K, et al. (2010) Potential use of the astaxanthin-producing microalga, Monoraphidium sp. GK12, as a functional aquafeed for prawns. J Appl Phycol 22: 363-369

Gagneux-Moreaux S, Moreau C, Gonzalez JL, Cosson RP (2007) Diatom artificial medium (DAM): a new artificial medium for the diatom *Haslea ostrearia* and other marine microalgae. J Appl Phycol 19:549–556

Gong, X. and Chen, F. 1997. Optimization of culture medium for growth of *Haematococcus pluvialis*. J. Appl. Phycol. 9: 437-444.

Grung, M., D'Souza, F.M.L., Borowitzka, M., Liaaen-Jensen, S., 1992. Algal carotenoids 51, secondary carotenoids 2. *Haematococcus pluvialis* aplanospores as a source of (3S,30S)- astaxanthin esters. J. Appl. Phycol. 4, 165–171

Guillard RRL, Sieracki MS (2005) Counting cells in cultures with the light microscope. In: Andersen RA (ed) Algal culturing techniques. Elsevier Academic Press, London, pp 239–252

Guillard, R.R.L. 1973. Division rates. In, Stein (ed), Handbook of Phycological Methods, V. 1, Cambridge University Press, Cambridge, pp. 289-312

Harker, M., Tsavalos, A.J., Young, A.J., 1996. Factors responsible for astaxanthin formation in the chlorophyte *Haematococcus pluvialis*. Bioresource Technol., 207–214

Hejazi, M. A. and Wijffels, R. H. 2003. Effect of light intensity on β-carotene production and extraction by Dunaliella salina in two-phase bioreactors. Biomol. Eng. 20: 171-175

Howell, B.R. Marine fish culture in Britain VIII: A marine rotifer, Brachionus plicatilis Müller, and the larvae of mussel, Mitylus edulis L., as food for larvae flat - fish. J. Cons. Int. Explor. Mer., 35:1-6, 1973

Kakizono T., M. Kobayashi, and S. Nagai. 1992. Effect of carbon/nitrogen ratio on encystment accompanied with astaxanthin formation in a green alga, *Haematococcus pluvialis*. J. Ferm. Bioeng. 74: 403-405

Kobayashi. M. et al. 1992a. Effects of light intensity, light quality, and illumination cycle on astaxanthin formation in green alga, *Haematococcus pluvialis*. J. Ferm. Bioeng. 74(1): 61-63

Kobayashi M. et al. 1992b. Growth and astaxanthin formation *Haematococcus pluvialis* in heterotrophic and mixotrophic conditions. J. Ferm. Bioeng. 74(1): 17-20

Kobayashi M., Kakizono T., Nagai S. 1993. Enhanced carotenoid biosynthesis by oxidative stress induced cyst cells of a green alga *Haematococcus pluvial&.* Appl. Environ. Microbiol., 59: 867-873

Kobayashi, M., Kurimura, Y., Tsuji, Y., 1997. Light-independent, astaxanthin production by the green microalga *Haematococcus pluvialis* under salt stress. Biotechnol. Lett. 19, 507– 509.

Laing, I. 1991. Cultivation of marine unicellular algae. *Laboratory leaflet*. 67:7 Lincymol, K. P., Ittoop, G., Devi, P. L. & Joseph, A. 2012. Modulation in nutritional quality

of microalgae Chaetoceroscalcitrans in different culture media. *Fishery Technology*, 49:120-124

Lee, Y.-K. and Soh, C.-W. 1991. Accumulation of astaxanthin in *Haematococcus lacustris* (Chlorophyta). J. Phycol., 27: 575–577. DOI: 10.1111/j.0022-3646.1991.00575.x

Lee, Y.-K. and Zhang, D.-H. 1999. Production of astaxanthin by *Haematococcus*. In: Z. Cohen (Ed.), Chemicals from Microalgae, Taylor and Francis, London: 173–190

Lorentz, R. T., and G. R. Cysewski. 2000. Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. Trends Biotechnol. 18: 160-167.

Lubzens E, Gibson O, Zmora O, Sukenik A (1995) Potential advantages of frozen algae (*Nannochloropsis* sp.) for rotifer (*Brachionus plicatilis*) culture. Aquacul 133:295–309

Marusich, W.L., Bauerfeind, J.C., 1981. Oxycarotenoids in poultry feed. In: Bauerfeind, J.C. (Ed.), Carotenoids as Colorants and Retinal Precursors. Academic Press, New York, pp. 320–462

Muller-Feuga A (2000) The role of microalgae in aquaculture: situation and trends. J Appl Phycol 12:527–534

Muller-Feuga A (2004) Microalgae for aquaculture: the current global situation and future trends. In: Richmond A (ed) Handbook of microalgal culture. Blackwell Science, pp 352–364

Park, K. H. and Lee, C. G. 2000. Optimization of algal photobioreactors using flashing lights. Biotechnol Bioprocess Eng. 5:186–190.

Park, K. H. and Lee, C. G. 2001. Effectiveness of flashing light for increasing photosynthetic efficiency of microalgal cultures over a critical cell density. Biotechnol Bioprocess Eng. 6:189–193

Pedro C, Fernández-Díaz JC (2001) Pilot evaluation of freeze-dried microalgae in the mass rearing of gilthead seabream (*Sparus aurata*) larvae. Aquacul 193:257–269

Ponis E, Probert I, Véron B, Mathieu M, Robert R (2006) New microalgae for the Pacific oyster *Crassostrea gigas* larvae. Aquacul 253:618–627

Pringsheim, E.G. 1966. Nutritional requirements of *Haematococcus pluvialis* and related species. J. Phycol., 2: 1–7. DOI: 10.1111/j.1529-8817.1966. tb04584.x.

Sumanta Nayek, Choudhury I.H., Jaishee N, and Roy S. 2014. Spectrophotometric Analysis of Chlorophylls and Carotenoids from Commonly Grown Fern Species by

Using Various Extracting Solvents. Research Journal of Science. Vol. 4(9), 63-69, September 2014

Sarada R., Usha T., Ravishankar G.A. 2002. Influence of stress on astaxanthin production in *Haematococcus pluvial&* grown under different culture conditions. Proc. Biochem., 37: 623-627.

Shay, L. K, Hunt, H. R and Wegner, G. H. 1987. High-productivity fermentation process for cultivation industrial microorganisms. J. Ind. Microbiol. 2: 79-85.

Spolaore P, Joannis-Cassan C, Duran E, Isambert A (2006) Commercial applications of microalgae. J Biosci Bioeng 101:87–96

Steinbrenner, J. and Linden, H. 2003. Light induction of carotenoid biosynthesis genes in the green alga *Haematococcus pluvialis*: regulation by photosynthetic redox control. Plant Mol. Biol. 52:343–356

Tanaka, Y. et al. (1976) The carotenoids in marine red fish and the metabolism of the carotenoids in Sea bream, Chrysophrys major Temminch and Schegel. Bull. Jap. Soc Sci. Fish. 42, 1177–1182

Tjahjono A.E., Hayama Y., Kakizono T., Tereda Y., Nishio N., Nagai S. 1994. Hyper accumulation of astaxanthin in a green alga *Haematococcus pluvialis* at elevated temperatures. Biotech. Lett., 16:133-138

Torrissen O. J., Hardy R. W. & Shearer K. D. 1989. Pigmentation of salmonids-carotenoid deposition and metabolism. CRC Crit. Rev. Aquat. Sci. 1: 209-225

Torzillo, G., Goksan, T., Faraloni, C., Kopecky, J. and Masojidek, J. 2003. Interplay between photochemical activities and pigment composition in an outdoor culture of *Haematococcus pluvialis* during the shift from the green to red stage. J. Appl. Phycol., 15: 127– 136. DOI: 10.1023/A:1023854904163

Yamasaki S, Tanabe K, Hirata H (1989) Efficiency of chilled and frozen *Nannochloropsis* sp. (marine Chlorella) for culture of rotifer. Mem Fac Fish Kagoshima Univ 38:77–82

Yong, Y. Y. R. and Lee, Y. K. 1991. Do carotenoids play a photoprotective role in the cytoplasm of *Haematococcus lacustris* (Chlorophyta)? Phycologia 30:257–61.

Yuan, J.P., Chen, F., 1998. Chromatographic separation and purification of trans-astaxanthin from the extracts of *Haematococcus pluvialis*. J. Agric. Food Chem., 3371–3375