# การเพิ่มผลผลิตของแอสตาแซนธินในสาหร่ายขนาดเล็ก *Haematococcus pluvialis* NIES-144

นาย ณัฏฐวี ธรรมเจษฎา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีทางชีวภาพ หลักสูตรเทคโนโลยีทางชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2543
ISBN 974-347-301-7

# ENHANCED PRODUCTION OF ASTAXANTHIN IN A MICROALGA *Haematococcus pluvialis* NIES-144

Mr. Nutthawee Thamjedsada

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Biotechnology

Programme of Biotechnology

Faculty of Science

Chulalongkorn University

Academic Year 2000

ISBN 974-347-301-7

| | |
|---|---|
| Thesis Title | Enhanced Production of Astaxanthin in a Microalga *Haematococcus pluvialis* NIES-144 |
| By | Mr. Nutthawee Thamjedsada |
| Field of Study | Biotechnology |
| Thesis Advisor | Associate Professor Aran Incharoensakdi, Ph.D. |

---

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of Requirement for the Master's Degree

……Dean of Faculty of Science
(Associate Professor Wanchai Phothiphichitr, Ph.D.)

Thesis Committee

……Chairman
(Associate Professor Piamsook Pongsawasdi, Ph.D.)

……Thesis Advisor
( Associate Professor Aran Incharoensakdi, Ph.D.)

……Member
( Associate Professor Sirirat Rengpipat, Ph.D.)

ณัฏฐวี ธรรมเจษฎา : การเพิ่มผลผลิตของแอสตาแซนธินในสาหร่ายขนาดเล็ก *Haematococcus pluvialis* (ENHANCED PRODUCTION OF ASTAXANTHIN IN A MICROALGA *Haematococcus pluvialis* NIES-144) อ.ที่ปรึกษา : รศ.ดร.อรัญ อินเจริญศักดิ์, 119 หน้า ISBN 974-347-301-7

แอสตาแซนธินเป็นรงควัตถุประเภทคีโทคาโรธินนอยด์ ประโยชน์และความสำคัญคือสามารถผสมในอาหารสัตว์ปีกเพื่อเพิ่มสีสันให้แก่ไข่แดง รวมทั้งใช้ผสมในอาหารสัตว์น้ำที่มีคุณค่าทางเศรษฐกิจ ในธรรมชาติมีสิ่งมีชีวิตหลายชนิดสามารถผลิตแอสตาแซนธินได้ เช่น ครัสตาเซียน จุลินทรีย์พวกยีสต์ แบคทีเรีย แต่พบในปริมาณน้อย ในขณะที่สาหร่ายสีเขียว *Haematococcus pluvialis* มีปริมาณแอสตาแซนธินในระดับสูง คืออยู่ในช่วง 0.2-2 เปอร์เซ็นต์ ขึ้นอยู่กับสภาวะการเพาะเลี้ยง งานวิจัยนี้จึงสนใจเพาะเลี้ยงสาหร่ายสีเขียว *Haematococcus pluvialis* โดยใช้เทคนิคการเพาะเลี้ยงแบบ 2 ขั้นตอน คือ ขั้นแรกเลี้ยงเซลล์ให้ได้ปริมาณเซลล์สูงสุด จากนั้นเหนี่ยวนำให้เซลล์สร้างแอสตาแซนธิน พบว่า ภาวะที่เหมาะสมสำหรับการเจริญเติบโต คือ เลี้ยงเซลล์ในสูตรอาหาร Basal Medium โดยมีโซเดียมอะซีเตทและสารสกัดจากยีสต์ 1.5 และ 3.0 กรัมต่อลิตร ที่อุณหภูมิ 20 องศาเซลเซียส เซลล์ตั้งต้น $2x10^5$ เซลล์ต่อมิลลิลิตร ความเข้มแสง 30 $\mu Em^{-2}s^{-1}$ ให้แสง 12 ชั่วโมง ได้จำนวนเซลล์ $5.6x10^5$ เซลล์ต่อมิลลิลิตร ภาวะที่เหมาะสมเหนี่ยวนำให้เซลล์สร้างแอสตาแซนธินคือ เลี้ยงเซลล์ในสูตรอาหาร Basal Medium อุณหภูมิ 20 องศาเซลเซียส ความเข้มแสง 30 $\mu Em^{-2}s^{-1}$ ให้แสงต่อเนื่อง 24 ชั่วโมง และเติมโซเดียมอะซีเตท 45 มิลลิโมลาร์ ผลิตแอสตาแซนธินได้ 7.41 มิลลิกรัมต่อลิตร ผลของการวิเคราะห์แอสตาแซนธินโดยเทคนิค HPLC โดยใช้คอลัมน์คาร์บอน 18 และใช้ระบบตัวทำละลายซึ่งประกอบด้วยอะซิโตไนไตร เมทานอล ไดคลอโรมีเทน น้ำ พบว่าคาโรธินนอยด์ส่วนใหญ่อยู่ในรูปแอสตาแซนธิน โดยแอสตาแซนธินมีปริมาณเท่ากับ 75 เปอร์เซ็นต์



หลักสูตร.....เทคโนโลยีทางชีวภาพ ..... ลายมือชื่อนิสิต.....

สาขาวิชา....เทคโนโลยีทางชีวภาพ..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2543.....

## 4172279923 : MAJOR BIOTECHNOLOGY

KEY WORD : *Heamatococcus pluvialis* NIES-144 / ASTAXANTHIN

NUTTHAWEE THAMJEDSADA : ENHANCED PRODUCTION OF ASTAXANTHIN IN A MICROALGA *Haematococcus pluvialis* NIES-144. THESIS ADVISOR ASSO.PROF. ARAN INCHAROENSAKDI, Ph.D

119 pp. ISBN 974-347-301-7

Astaxanthin is a ketocarotenoid, which is used as a feed supplement in aquaculture for the production of salmon, trout, shrimp as well as a colorant for egg yolk in the poultry industry. In nature, astaxanthin occurs in aquatic crustaceans, yeast, bacteria but natural pigment sources have relatively low content of astaxanthin. The alga *Haematococcus pluvialis* NIES-144 has a high concentration of astaxanthin ranging from 0.2-2 % (w/v) depending on the method and control of culture. The aims of the present study are to culture *Haematococcus pluvialis* NIES-144 by two-stage batch culture technique. The first-stage , to attain a high cell concentration and for the second-stage, to enhance astaxanthin formation. *Haematococcus pluvialis* NIES-144 could grow best in the Basal Medium with 1.5 g/l sodium acetate and 3.0 g/l yeast extract , initial cell number of $2x10^5$ cell per ml under light intensity of 30 $\mu Em^{-2}s^{-1}$ , 12-h light at 20 $^0C$. The cell concentration of up to $5.6x10^5$ cell per ml was obtained. The optimal condition for astaxanthin accumulation in *Haematococcus pluvialis* NIES-144 was obtained by cultivation in Basal Medium at 20 $^0C$ under 30 $\mu Em^{-2}s^{-1}$ continuous illumination with the supplementation of 45 mM sodium acetate and was able to produce 7.41 mg/l of astaxanthin. The analysis of astaxanthin was performed by HPLC using $C_{18}$ column with dichloromethane : methanol : acetonitrile : water solvent systems. Astaxanthins content were found to account for 75 % of total carotenoids.

Programme…….. Biotechnology …… Student's signature……...

Field of study……Biotechnology……. Advisor's signature……

Academic year……2000……..

# ACKNOWLEDGEMENT

I wish to express my honest gratitude to Associate Professor Aran Incharoensakdi , for their helpful suggestions and supports throughout this research and also to Dr. Sirirat Rengpipat, Dr. Piamsook Pongsawasdi for service as thesis committee , for their valuable comments and also for useful suggestions.

Sincere thanks are also expressed to all friends and members of the Biotechnology and Biochemistry Department for their assistance and friendship.

Lastly , I would like to give a special thank to my parents, aunts , sister and Thitima for their understanding , support , patience and just being.

# Contents

Page

Thai Abstract ..... iv

English Abstract ..... v

Acknowledgement ..... vi

Contents ..... vii

List of tables ..... x

List of Figures ..... xi

Abbreviations ..... xvi

Chapters I Introduction ..... 17

Chapters II Materials and methods ..... 37

1 The first-stage (optimization for growth)

(Effect of environmental factors on growth of

*Haematococcus pluvialis* NIES-144)..... 40

1.1 Type of medium..... 40

1.2 Light intensity..... 40

1.3 Initial cell number..... 41

1.4 Content of Carbon and nitrogen ratio in media.... 41

2 The second-stage (optimization for astaxanthin synthesis)

.....43

2.1 Physical factor.....43

2.1.1 Effect of light intensity.....43

2.1.2 Effect of temperature.....43

2.2 Chemical factors.....43

2.2.1 Effect of sodium chloride.....43

2.2.2 Oxidative stress……44
2.3 Nutritional factors……44
2.3.1 Nutrient deficiency……44
2.3.2 Carbon and nitrogen ratio……44
3 Effect of combined treatments……45
3.1 Effect of sodium acetate and high salinity……..45
3.2 Effect of sodium acetate and high temperature….45
3.3 Effect of sodium acetate and N-deficiency…….45
4 Analysis of astaxanthin from *Haematococcus pluvialis* NIES-144 by High-Performance Liquid Chromatography Method ……..…….46
4.1 For Mature cyst cells……46
4.2 Saponification of astaxanthin esters……46
5 Growth and pigment analysis ……..48
Chapter III Results …….. 49
1 The first-stage (optimization for growth) (Effect of environmental factors on growth of *Haematococcus pluvialis* NIES-144)…….. 49
1.1 Type of medium…….. 49
1.2 Light intensity…….. 51
1.3 Initial cell number…… 54
1.4 Content of Carbon and nitrogen ratio in media…. 56
2 The second-stage (optimization for astaxanthin synthesis)
2.1 Physical factor……..65
2.1.1 Effect of light intensity…….. 65

2.1.2 Effect of temperature..... 67
2.2 Chemical factors..... 69
2.2.1 Effect of sodium chloride..... 69
2.2.2 Oxidative stress..... 71
2.3 Nutritional factors..... 73
2.3.1 Nutrient deficiency..... 73
2.3.2 Carbon and nitrogen ratio..... 75
3 Effect of combined treatments..... 79
3.1 Effect of sodium acetate and high salinity..... 79
3.2 Effect of sodium acetate and high temperature... 79
1.3 Effect of sodium acetate and N-deficiency..... 79
4 Analysis of astaxanthin from *Haematococcus pluvialis* NIES-144 by High-Performance Liquid Chromatography Method ..... 86
4.1 For Mature cyst cells..... 86
4.2 Saponification of astaxanthin esters..... 86
Chapter IV Discussions ..... 91
Chapters V Summary ..... 101
References .....102
Appendix .....112
Biography .....119

## List of Tables

page


**Table 1** Fraction of carotenoids produced by *Haematococcus pluvialis* NIES-144..... 24

**Table 2** Sources of astaxanthin..... 29

**Table 3** Carbon and nitrogen ratio in media ..... 41

**Table 4** Sodium acetate and yeast extract content in media ... 42

**Table 5** Effect of oxidative stress on pigment content of *Haematococcus pluvialis* NIES-144 after 4-day cultivation (mean value ± standard deviation )..... 72

**Table 6** Effect of nutrient deficiency on pigment content of *Haematococcus pluvialis*NIES-144 after 10-day cultivation (mean value ± standard deviation) ..... 74

**Table 7** Effect of carbon and nitrogen ratio in media on pigment content per cell of *Haematococcus pluvialis* NIES-144 after 10-day cultivation .....77

**Table 8** Optimum conditions for astaxanthin formation of *Haematococcus pluvialis* NIES-144..... 83

**Table 9** Percent astaxanthins and carotenoids of pigment extracts from *Haematococcus pluvialis* NIES-144..... 90

## List of Figures


page

**Figure 1** Structures of the biotechnologically most important Carotenoids..... 18

**Figure 2** *Haematococcus pluvialis*..... 20

**Figure 3** Characteristics of the reproduction of *Haematococcus pluvialis* ..... 22

**Figure 4** Suggested routes for astaxanthin biosynthesis of *Haematococcus pluvialis*..... 26

**Figure 5** Effect of types of media on growth of *Haematococcus pluvialis* NIES-144..... 50

**Figure 6** Specific growth rate of *Haematococcus pluvialis* NIES-144 in three types of media ..... 50

**Figure 7a** Effect of light intensity on growth of *Haematococcus pluvialis* NIES-144..... 52

**Figure 7b** Effect of light intensity on growth of *Haematococcus pluvialis* NIES-144..... 52

**Figure 8a** Specific growth rate of *Haematococcus pluvialis* NIES-144 with various light intensity..... 53

**Figure 8b** Specific growth rate of *Haematococcus pluvialis* NIES-144 with various light intensity..... 53

**Figure 9** Effect of initial cell number on growth of *Haematococcus pluvialis* NIES-144..... 55

**Figure 10** Specific growth rate of *Haematococcus pluvialis* NIES-144 with various initial cell numbers..... 55

**Figure 11** Effect of carbon and nitrogen ratio in media on growth of *Haematococcus pluvialis* NIES-144..... 57

**Figure 12** Specific growth rate of *Haematococcus pluvialis* NIES-144 with various carbon and nitrogen ratio in media..... 58

**Figure 13** Effect of sodium acetate and yeast extract content in media growth of *Haematococcus pluvialis* NIES-144... 59

**Figure 14** Specific growth rate of *Haematococcus pluvialis* NIES-144 with various sodium acetate and yeast extract content in media ..... 59

**Figure 15** Effect of sodium acetate and yeast extract content in media growth of *Haematococcus pluvialis* NIES-144.. 60

**Figure 16** Specific growth rate of *Haematococcus pluvialis* NIES-144 with various sodium acetate and yeast extract content in media ..... 60

**Figure 17** Effect of sodium acetate and yeast extract content in media growth of *Haematococcus pluvialis* NIES-144... 61

**Figure 18** Specific growth rate of *Haematococcus pluvialis* NIES-144 with various sodium acetate and yeast extract content in media ..... 61

**Figure 19** Effect of sodium acetate and yeast extract content in media growth of *Haematococcus pluvialis* NIES-144.. 62

**Figure 20** Specific growth rate of *Haematococcus pluvialis* NIES-144 with various sodium acetate and yeast extract content in media ..... 62

**Figure 21** Effect of combined conditions on growth of *Haematococcus pluvialis* NIES-144…….. 63

**Figure 22** Specific growth rate of *Haematococcus pluvialis* NIES-144 with combined condition……..…… 64

**Figure 23** Effect of light intensity on the cell numbers of *Haematococcus pluvialis* NIES-144 (mean value + standard deviation) ……. 66

**Figure 24** Effect of light intensity on pigment content of *Haematococcus pluvialis* NIES-144 after 10-day cultivation (mean value ± standard deviation) ……. 66

**Figure 25** Effect of temperature on the cell numbers of *Haematococcus pluvialis* NIES-144 (mean value + standard deviation)…… …….. 68

**Figure 26** Effect of temperature on pigment content of *Haematococcus pluvialis* NIES-144 after 8-day cultivation (mean value ± standard deviation)……. 68

**Figure 27** Effect of NaCl on the cell numbers of *Haematococcus pluvialis* NIES-144(mean value + standard deviation)…70

**Figure 28** Effect of NaCl on pigment content of *Haematococcus pluvialis* NIES-144 after 10-day cultivation (mean value ± standard deviation) …… 70

**Figure 29** Effect of oxidative stress on the cell numbers of *Haematococcus pluvialis* NIES-144 (mean value + standard deviation) …… …….. 72

**Figure 30** Effect of nutrient deficiency on the cell numbers of *Haematococcus pluvialis* NIES-144 (mean value + standard deviation) …….. 74

**Figure 31** Effect of carbon and nitrogen ratio in media on the cell numbers of *Haematococcus pluvialis* NIES-144 ….. 76

**Figure 32** Effect of combined treatments on the cell numbers of *Haematococcus pluvialis* NIES-144 (mean value + standard deviation) ……. 80

**Figure 33** Effect of combined treatments on astaxanthin content per cell of *Haematococcus pluvialis* NIES-144 (mean value+ standard deviation) ……. 81

**Figure 34** Effect of combined treatments on total astaxanthin content of *Haematococcus pluvialis* NIES-144 (mean value + standard deviation) …….. 81

**Figure 35** Effect of combined treatments on chlorophyll content per cell of *Haematococcus pluvialis* NIES-144 (mean value + standard deviation)…… …….. 82

**Figure 36** Effect of combined treatments on total chlorophyll content of *Haematococcus pluvialis* NIES-144 (mean value + standard deviation) ……82

**Figure 37** Morphological changes in the life cycle of a green alga *Haematococcus pluvialis* NIES-144 ..... 84

**Figure 38** Appearance of culture at different life cycle of *Haematococcus pluvialis* NIES-144 ..... 85

**Figure 39** HPLC chromatogram of standard astaxanthin (a) ; pigment extracts (b) ; pigment saponified extracts (c) from *Haematococcus pluvialis* NIES-144 .....88

# Abbreviations

| | |
|---|---|
| A | Absorbance |
| BM | The Basal medium |
| BBM | The Bold Basal Modified medium |
| CHU | CHU-10 modified medium |
| C/N ratio | carbon per nitrogen ratio |
| $^{0}C$ | Degree Celsius |
| $E^{1\%}_{1cm}$ | the specific extinction coefficient |
| g | gram |
| $in^2$ | square inch |
| lb | pound |
| ml | millilitre |
| mM | millimolar |
| m | metre |
| min | minute |
| nm | nanometre |
| pg | picogram |
| rpm | revolution per minute |
| $\mu$ | specific growth rate |
| $\mu$M | micromolar |
| $\mu Em^{-2}s^{-1}$ | photon density (light intensity) |
| w/v | weight by volume |
| % | percent |
| $\lambda$ | wavelength of the light |

# Chapter I

# Introduction

Carotenoids, over the years, have been extensively used commercially due to the several advantages they offer. Their commercial applications are indeed, very diverse, such as in cosmetic, pharmaceutical, and food industries. With the recent awareness towards the damages caused by several synthetic chemicals in our daily use items, there is a growing tendency for using natural, non-toxic substances; this has resulted in increased interest in carotenoids as substitutes for the chemicals already in use.

Carotenoids occur in animals, plants, and microorganisms giving them characteristic colour and are probably the most widespread and diverse of all the natural pigments. Traditionally, carotenoids have been given trivial names, usually derived from the biological material from which they were first isolated. Carotenoids have been defined as “a class of hydrocarbons and their oxygenated derivatives that consist of eight isoprenoid units joined in such a manner that the arrangement of these units is reversed at the centre of the molecule ; the two central methyl side chains are thus in a 1,6-positional relationship”. All carotenoids may formally be derived from acyclic $C_{40}H_{56}$ structure of lycopene by hydrogenation, dehydrogenation, cyclisation or oxidation, or a combination of any of these processes. Compounds that arise from certain rearrangements of this carbon skeleton or by the formal removal of part of this structure

are also considered carotenoids (Britton,1983). The characteristic colour of the carotenoids is due to the conjugated double bonds (π-bond) in the polyene chain which absorb strongly in the visible region of the solar spectrum. Thus, the carotenoids in the thylakoid membranes of photosynthetic organisms also participate in the energy transfer reactions of the primary photochemistry (Krishna and Mohanty ,1998). Carotenoids are the most important pigments from the biotechnology view point (Fig 1).

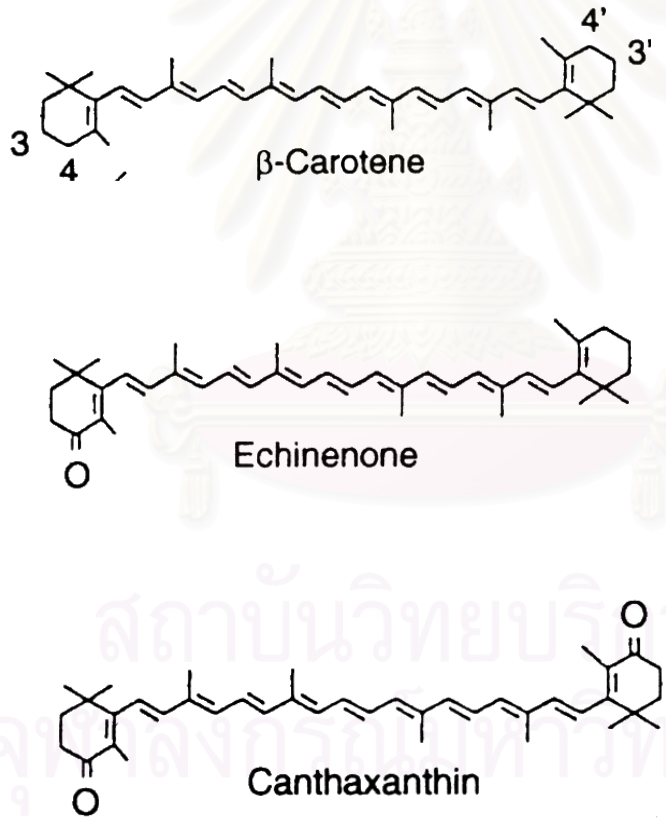
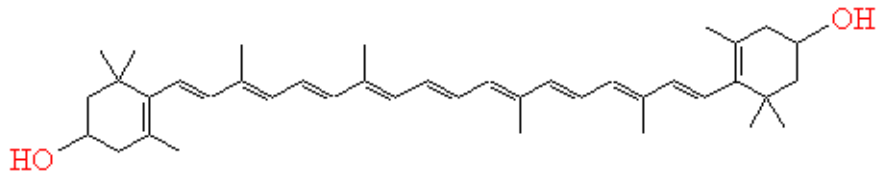
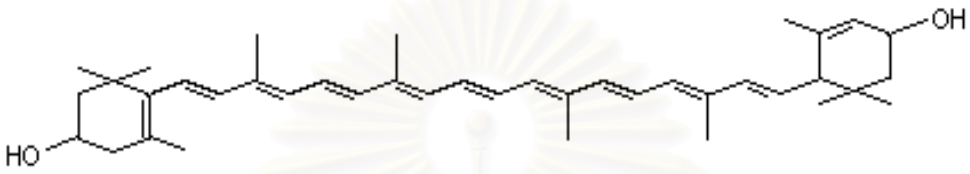


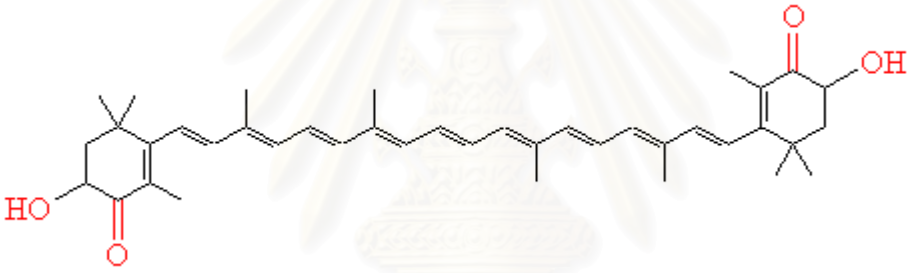

**Figure 1** Structures of the biotechnologically most important Carotenoids(Margalith,1999)




Zeaxanthin




Lutein




Astaxanthin

**Figure 1** Structures of the biotechnologically most important Carotenoids (continued)

*Haematococcus pluvialis*

The green microalga , *H. pluvialis* , Flotow , Volvocals , Chlorophyceae, is a unicellular fresh water biflagellate (Zoospore). Under optimal conditions the cells are spherical to ellipsoid and enclosed by a cell wall , which is separated from the protoplast by a region filled with a watery jelly and traversed by cytoplasmic threads (Santos and Mesquita ,1984). Figure 2 shows the morphological appearance of *H. pluvialis*.

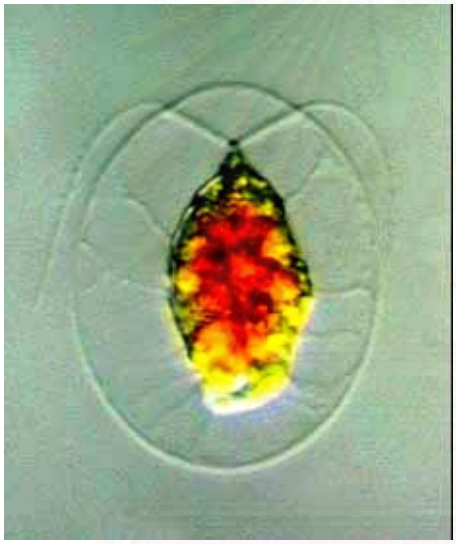


**Figure 2** *Haematococcus pluvialis* (http://www.microscopy-uk.org.uk)



**Taxonomy** (Smith,1950)

| | |
|---|---|
| Division | Chlorophyta |
| Class | Chlorophyceae |
| Order | Volvocales |
| Family | Chlamydomonadaceae |
| Genus | Haematococcus sp. |
| Species | *H. pluvialis* |

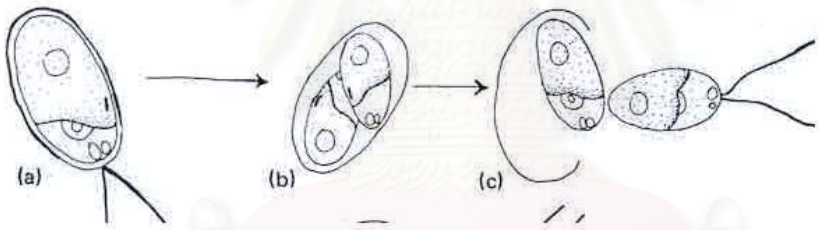
## Characteristics

The fresh-water unicellular alga *H. pluvialis* Flotow (Volvocales) occurs primarily in temporary, small fresh water pools (Droop,1954 and Czygan,1970). In its growth stages, it has both motile and non-motile forms. In the former, a pear-shaped cell ranges from 8 to 50 μm in diameter. The cellular structure of this stage is similar to most of its family members: a cup-shaped chloroplast with numerous and scattered pyrenoids, contractile vacuoles which are often numerous and apparently quite irregularly distributed near the surface of the protoplast, a nucleus and 2 flagella of equal length emerging from the anterior papilla which perforate the cellulose wall at a wide angle. The structure's uniqueness is marked by its cell wall which is strongly thickened, gelatinous, and is usually connected to its protoplast by simple or branched strands.

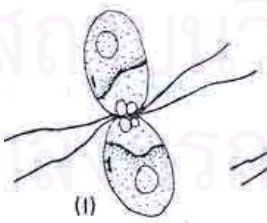
In its non-motile form, the so-called 'palmella' stage, the spherical protoplast is enveloped within a closely adherent palmella membrane, and, with the exception of the flagella, the cellular structure remains the same as its motile form. Once growing conditions become unfavorable, cell increase their volume drastically and enter a resting stage in which the cell is surrounded by a heavy resistant cellulose (Boussiba, unpublished). This overall process is termed 'encystment'. The protoplast is then a markedly red color, determined to be a secondary carotenoid, astaxanthin (Goodwin and Jamikorn, 1954).

## Reproduction

In asexual reproduction the zoospores may pass through a non-motile aplanospore stage before maturing (Fig 3) and this has developed to such an extent in *H. pluvialis* that it forms a major feature of the life history. In the addition, asexual reproduction is by division of the cells into two or four motile products (Droop,1955). Sexual reproduction, gamatic union is isogamous and the colonies are homotalic, but apparently with a fusion of gamete from different cells. Four or eight gametes are formed within a cell. A pair of fusion gametes become apposed at the anterior ends, and the flagella persist after fusion is completed (Donkin,1976).




(A) Asexual




(B) Sexual

**Figure 3** Characteristics of the reproduction of *Haematococcus pluvialis* (Bold and Wynne.1985)

## Astaxanthin

Astaxanthin (3,3'-dihydroxy-β,β'-carotene-4,4'-dione), a highly oxygenated carotenoid is synthesized by the unicellular green alga, *H. pluvialis* (Kobayashi et al. 1991) ; recently reviewed by Johnson and Schroeder(1985). Antioxidative activity of astaxanthin was shown superior to most of the hydrophobic antioxidants such as β-carotene and vitamin E (Miki,1991) . So that the ketocarotenoid has interest for pigmentation for aquaculture feeds (Choubert and Heinrich,1993).

Astaxanthin is an oxycarotenoid with the molecular formula $C_{40}H_{52}O_4$ and has a molecular weight of 596.86. Isolated crystalline astaxanthin has the appearance of a fine, dark violet-brown power. Its melting point is approximately 224 $^{o}$C. It is insoluble in aqueous solutions and most organic solvent but can be dissolved at room temperature in dichloromethane chloroform, acetone, dimethylsulfoxide(DMSO) and other nonpolar solvents. Because carotenoids contain a long conjugated double bond system, they are less stable than other isoprenoids and precautions must be taken to avoid artifacts and destruction of pigments. Light, heat, acids, and oxygen are particularly detrimental to carotenoid and enzymatic destruction also can occur during extraction from biological samples (Johnson and An ,1991). Astaxanthin may appear in various isomeric configurations. *Haematococcus* produces predominantly the 3S, 3S' isomer (Bjerkeng et al. 1997). Fraction of carotenoids produced by *Haematococcus pluvialis* are shown in Table 1.

**Table 1** Fraction of carotenoids produced by *Haematococcus pluvialis* (Kobayashi et al. 1991)

| Carotenoids (%) | *Haematococcus pluvialis* | | |
|---|---|---|---|
| | CCAP 34/7 | NIES 144 | NIVA CHL 9 |
| Astaxanthin | | | |
| monoester | 34 | 69 | 76 |
| diester | 46 | 20 | 10 |
| free form | 1 | nd | 1 |
| total | 81 | 89 | 87 |
| Lutein | 6 | 1 | 7 |
| Adonirubin ester | - | 9 | 3 |
| Echinenone | 4 | - | - |
| canthaxanthin | 4 | - | - |
| β-carotene | 5 | - | 1 |

## Biosynthesis

Although the biosynthesis of ketocarotenoids in *Haematococcus* has not been fully elucidated, it is widely assumed that the early part of carotenogenesis, i.e. the formation of phytone from mevalonate and the desaturation process up to β-carotene, follows a similar pathway to that found in other oxygenic carotenogenic organisms (Armstrong and Hearst ,1996). According to Donkin (1976) and Grung et al. (1992) the presence of small amounts of echinenone (one keto group) and canthaxanthin (two keto groups) indicates a pathway from β-carotene to astaxanthin involving the above ketocarotenoid prior to hydroxylation at the 3 and 3' carbons (Fig 4). This has been further corroborated by Orset et al. (1995) and Fan et al. (1995), who employed low concentration and astaxanthin formation with the accumulation of β-carotene. However, in a more recent paper it was claimed that zeaxanthin (β-carotene-3,3'diol) may also have served as an intermediate when a menbrane-bound enzyme fraction of *Haematococcus* was incubated with β-carotene in the presence of NADPH and $O_2$, yielding astaxanthin with high conversion yields (Chumpolkulwong et al. 1997).

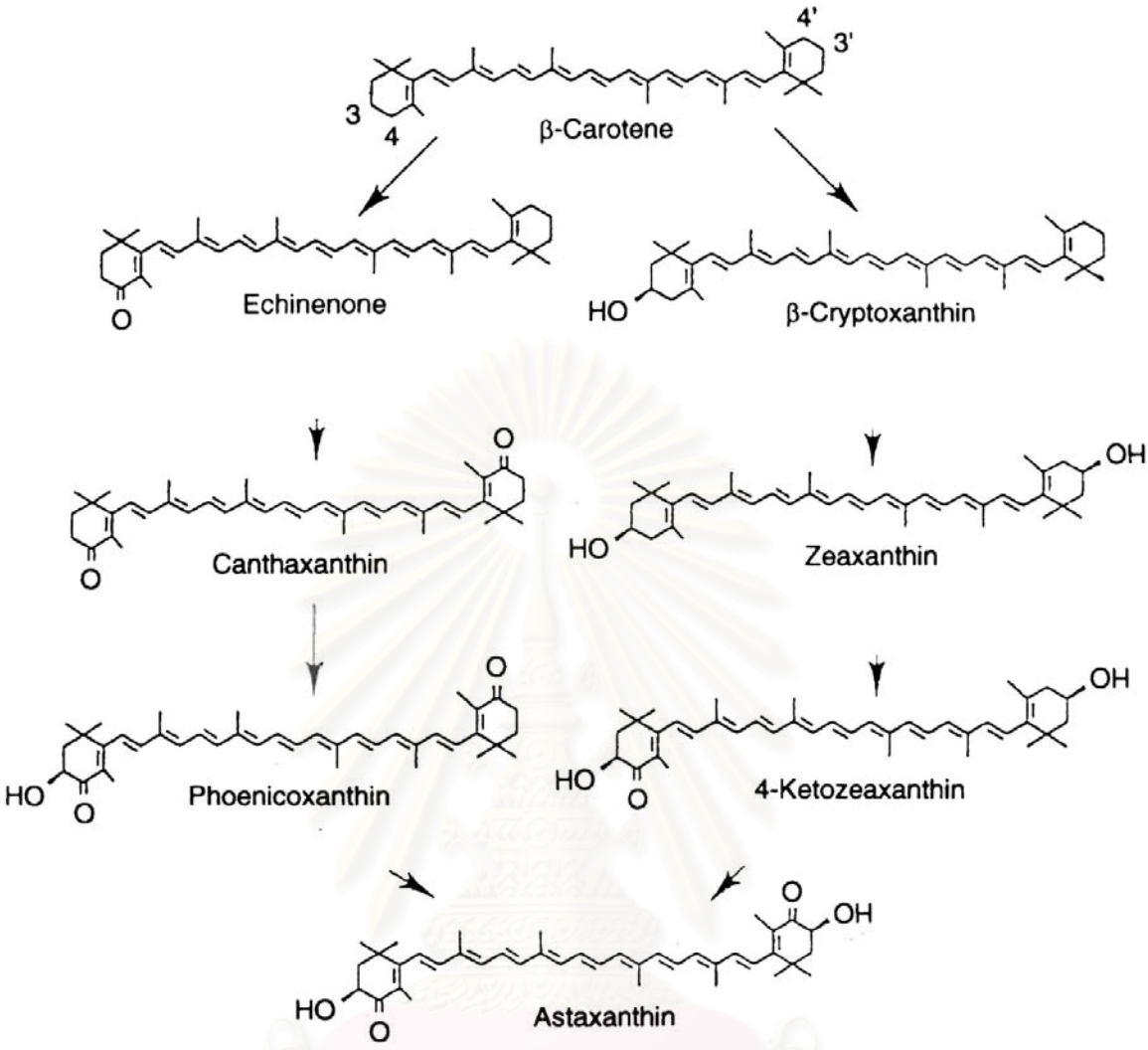



**Figure 4** Suggested routes for astaxanthin biosynthesis in *Haematococcus pluvialis* (Fraser et al.1997)

## Sources of astaxanthin

### 1 Synthetic astaxanthin

F.Hoffmann-La Roche, Basel, Switzerland have developed over many years advanced syntheses for various carotenoids. In 1964 they introduces canthaxanthin as a pigmenter for foods and feeds ("Roxanthin" or "Carophyll red"). Recently Roche accomplished the synthesis of trans-astaxanthin, which is marketed as "carophyll pink". Synthetic astaxanthin is presently the principal source used in feeds. Astaxanthin is preferred as a pigmenter over synthetic canthaxanthin for salmons since it is more efficiently absorbed and also imparts a more natural color to salmon. Chemically synthesized astaxanthin sells for $2000 to $2500/kg (dry weight basis) in beadlets containing 5 % astaxanthin and is stabilized by various ingredients, including gelatin, source, corn starch, modified food starch, ascorbyl palmitate, and ethoxyquin (Johnson and An,1991).

### 2 Crustaceans and crustacea by products

Several researchers have evaluated crustacea and derivatives as pigment sources (reviewed in Torrissen et al.1989). In Norway, shrimp (*Pandalus borealis*) wastes have been used traditionally as natural pigment sources for trout and salmon. Carotenoid levels in most crustacean preparations, however, are usually quit low (0 to 200 mg/kg) and satisfactory pigmentation requires the addition of 10 to 25 % by weight of the chitinous extract to the bulk diet. Crustacean wastes have high levels of ash, chitin, and moisture and low levels of

protein and other nutrients that limit their usefulness (Torrissen et al.1989).

### 3 Algae

Certain green algae in the subphylum *Chlorophyceae* possess astaxanthin as their primary carotenoid. Depending on the method and control of culture, very high levels accumulate in *Haematococcus pluvialis* (0.5 to 2 % astaxanthin on a dry weight basis). Most of the astaxanthin (87%) occurs esterified, which may affect its deposition and metabolism in some animals. Low deposition of astaxanthin by feeding algae to salmon was obtained by Kvalheim and Knutsen, and it was suggested that this was due to astaxanthin being present principally as esters; however, poor deposition caused solely by esterification was not confirmed by others. An other possible limiting factor is availability of carotenoids from the algal biomass. Highly pigmented algae occur in an encysted form surrounded by a thick cell wall, and this barrier could also impede the absorption of pigments(Johnson and An,1991).

### 4 Yeast

The genus *Phaffia* is characterized by the synthesis of carotenoid pigments, production of cell surface-associated amyloid compounds, a coenzyme Q-10 system, and the ability to ferment sugar. Other pertinent properties include its ability to assimilate

carbon compounds, including D-glucose, maltose, sucrose, cellobiose, trehalose, etc (Johnson and An,1991).

**5 Other microorganisms**

Some bacteria, including *Mycobacterium lacticola* and a *Brevibacterium* sp., and fungi in the genus *Peniophora* have been reported to contain astaxanthin. Carotenoid levels are low or growth is slow in these organisms and fermentation development has not been pursued. The industrially important xanthophylls, canthaxanthin and zeaxanthin, are produced by strains of *Brevibacterium* and *Flavobacterium*, but the productivity is too low for commercial fermentation (Johnson and An,1991). Sources of astaxanthin produced by microorganisms are shown in Table 1.

**Table 2 Sources of astaxanthin** (Modified from Simpson et al.1981 ; Schroeder et al.1996 ; Borowitzka,1989 ; Harker et al.1996)

| Sources | Astaxanthin (μg/g cell) | Astaxanthin (%) |
|---|---|---|
| Bacteria *Brevibacterium* | 30 | |
| *Mycobacterium* | 30 | |
| Yeast *Phaffia rhodozyma* | 200-300 | 0.02-0.03 |
| Algae *Chlamydomonas* | < 50 | |
| *Euglena rubida* | < 50 | |
| *Haematococcus* sp. | 7,000-55,000 | 0.2-2 |

## Physiological significance of secondary carotenoid

The secondary carotenoids are produced in large amount in the resting stage of the green algae. Why the algae need to produce them in such large amounts and that too in resting stages with the expense of energy ? What physiological role these secondary carotenoid play ?The possible functions of these carotenoids have been suggested and investigated.

### (A)Quenching of singlet oxygen

In *Haematococcus* , astaxanthin is present in cytoplasm and the active oxygen species which have very short life period are generated in the thylakoids, causing damage to them. Then, how can astaxanthin protect the thylakoid membrances from photoinhibition ? This physiological function in *Haematococcus*, thus appears unlikely.

### (B) Screening of excess light reaching the chloroplast

The secondary carotenoid both in *Dunaliella* and *Haematococcus* can act as a screen to absorb the excess radiation, thus protecting the photosynthetic membranes. This hypothesis is well accepted. It was observed that β-carotene rich *Dunaliella* cells show maximal photoprotection against high irradition with blue light and minimal protection with that of red light (Ben-Amotz and Shaish,1993). In *Haematococcus* ; (Johnson and An,1991) have observed that astaxanthin rich cells have relatively less photoprotection from high light intensity than the green cells. They suggested that astaxanthin

biosynthesis in *Haematococcus* is the outcome of protection process itself. Hagen et al.1993 have shown that the astaxanthin-rich *Haematococcus lacustris* cells in contrast to the green cells, show phototactic movement moving away from high light intensity.

### (C)Storage of carbon

Since accumulation of both β-carotene in *Dunaliella* and astaxanthin in *Haematococcus* takes place under adverse conditions when the cells have either limited growth (*Dunaliella*) or in resting stage (*Haematococcus*), these carbon-rich compounds could be considered as a storage material for later use.

## Uses of the secondary carotenoids

With the techniques of outdoor cultivation of algae becoming feasible, there is an immense interest in exploiting their products commercial value, some of which are given below.

### (A)As provitamin

Vitamin A plays an important role in vision. Vitamin A can be synthesized from β-carotene in two steps, catalyzed by dioxygenase and NAD(P)H-dependent reductase in animals (Johnson and An,1991). β-carotene is presently used as food additives as a source of provitamin A (Bauernfeind,1981).

**(B)As colourants in food and cosmetic industries**

There is perceptible change in the attitude of consumers world-wide to use natural products and desist from using synthetic chemicals, as far as possible, particularly in food and cosmetic industries. β-carotene is currently used as a food colouring agent and as an as additive in cosmetics(Johnson and An,1991). There has been growing interest to use astaxanthin too as colourant for egg yolk in poultry industry (Boussiba et al.1992).

**(C)As aquafeed**

Perhaps the most important commercial use of astaxanthin is an aquafeed. Salmoids owe their typical colour to astaxanthin. In commercial cultivation of these fish in captivity, carotenoid pigments are included in the feed to get desirable colouration, an important factor affecting consumer acceptance. Johnson and An (1991) have estimated that by the year 2000, more than 100,000 kg of carotenoid pigments might be required to be the fish feed.

**(D)As chemopreventive agent in cancer therapy**

Epidemiological and ontological studies suggest that normal to high level of β-carotene in the body may protect one from cancer. It has been suggested that this ability of β-carotene is based on its antioxidant function and the ability to scavenge various radical species. Miki (1994) has suggested that astaxanthin possesses higher anti-oxidant activity as lipophilic oxygen quencher than β-carotene

and α-tocopherol. A recent study indicates this differential ability of the carotenoids is mainly due to their stability in partial oxygen pressure and the antioxidative effect of the carotenoids has little to do with their structure (Jorgensen and Skibsted,1993).

## Factors governing secondary carotenoid green algae.

Several physiological parameters including nutritional and environmental factors affect the secondary carotenoid biosynthesis in these algae. The accumulation of these carotenoid is preceded by inhibition of growth as reflected in cell number. The factors reflected carotenoid accumulation have been studied by many word (Krishna and Mohanty,1998).

### 1 Physiological factors

#### (a) Light

Light intensity dependence of β-carotene and astaxanthin synthesis in *Dunaliella* ; Ben-Amotz et al.(1989) and *Haematococcus* ; Kobayashi et al. (1992a) ; Fan et al. (1994) , respectively , has been studied extensively. They have also observed similar results when the cells were grown under continuous illumination at different light intensities. They found that the astaxanthin content of the cells is directly related to the intensity of light to which the *Haematococcus* cells are exposed. This accumulation is more under continuous illumination than in 12 h and 12 h dark photoperiodic light.

(b) Temperature

The physiological growth temperature for both *Dunaliella* and *Haematococcus* is 20 to 25 $^{o}$C. When the temperature was increased to above 30 $^{o}$C to non-growth conditions, the vegetative growth of *Haematococcus* was found to retard and the cells rapidly turned into large dark red cysts with nearly 15 to 20-times increase in the astaxanthin content of the cells(Tjahjono et al.1994).

2 Chemical factor

(a) Salt stress

Besides these factors, many stress factors were also found to induce secondary carotenoid accumulation in green algae, the main being salinity. Effect of salinity on β-carotene production by *Dunaliella* has been extensively studied since the organism is able to survive under extremely high salt concentration , *Dunaliella* cells are able to accumulate large amounts of 0.8 percent NaCl was shown to induce astaxanthin accumulation (Boussiba and Vonshak,1991). Cordero et al.1996 have shown that under nitrogen deficiency conditions *Haematococcus* could accumulate high amounts of astaxanthin in the presence of 0.2 percent sodium chloride. However, it should be noted that while *Dunaliella* is tolerant towards a very high concentration of NaCl due to its unique structural and physiological properties, this limit is low for *Haematococcus*.

(b) Oxidative stress

Kobayashi et al.(1993) have observed that under high carbon to nitrogen ratio in the presence of $Fe^{2+}$, astaxanthin synthesis in *Haematococcus* was induced. Ferrous ion can generate hydroxyl radical by Fenton reaction (Halliwell and Gutteridge,1989). It is already known that high irradiance also induce oxidative stress in eukaryotic photosynthetic organisms by overexciting the photosynthetic system resulting in the formation of highly reactive oxygen radicals and singlet oxygen (Elstner,1987). These oxygen species are known to cause extensive damage to chlorophyll, lipids, and proteins in plant cells. They are also known to regulate the expression of extraplastidic and nuclear genes. Primary carotenoids have a role in quenching these reactive oxygen species protecting the thylakoid membranes. Whether these oxygen species induce the carotenoid biosynthesis by means of which the algal cells can overcome their damaging effect is not yet clearly understood. Beyer and Kleinig (1990) have suggested that the active oxygen species can directly participate in the enzymatic oxidation-reduction reactions of carotenogenesis.

3 Nutritional factor

The nutritional factors inducing secondary carotenoid biosynthesis in green algae have been extensively studied. Nitrogen deficiency was shown to induce β-carotene synthesis in *Dunaliella* (Tan ,1995) Increased astaxanthin production in *Haematococcus* on deficiency of nitrogen has also been reported earlier (Droop, 1954 ;

Goodwin and Jamikom,1954 ; Cyzgan ,1970). Phosphorous is one of the macronutrients essential for growth and metabolism of eukaryotic photosynthetic organisms. When *Haematococcus* cells were transferred from the normal growth medium to a medium lacking phosphate ; Boussiba and Vonshak (1991) have observed that the cellular content of astaxanthin increased.

## Objectives

To optimize the growth of *Haematococcus pluvialis* NIES-144 and to enhance high astaxanthin accumulation.

# Chapter II

# Materials and methods

## Materials

### 1.Equipment

| Equipment/model | Company/country |
|---|---|
| Autoclave HA-3D | Hirayama Manufactory Cooperation , Japan. |
| Balance | Sartorius |
| Centrifuge H-103 N series | Kokusan |
| Digital lux meter FT 710 | Taiwan |
| Haemacytometer | Boeco Germany |
| Hand tally counter | Bona |
| Illuminated / Refrigerated orbital incubator 101400 : XX2C | Sanyo , England |
| Laminar flow BVT-124 | International Scientific Supply , Co.Ltd Thailand. |
| pH meter PHM 83 | Radio , Copenhagen ; Denmark. |
| Microscope | Nikon , Japan |
| Refrigerated centrifuge J-21 C | Beckman Instrument Inc , USA |
| 6400 Spectrophotometer (UV/VIS) | Jenway |
| Water bath hearson | London , England |
| High Performance Liquid Chromatography | HP1050 Series HPLC Modules Germany |
| Centrifuge Model 1040 Series | Centurion Scientific LTD. UK |

## 2.Chemicals

| Chemicals | Company / Country |
|---|---|
| Acetone | BDH Laboratory chemical , England |
| Copper sulfate | “ |
| Cobalt chloride | “ |
| Dipotassium hydrogen phosphate | “ |
| Ferric chloride | “ |
| Magnesium sulfate | “ |
| Potassium hydrogen phosphate | “ |
| Sodium acetate | “ |
| Sodium chloride | “ |
| Sodium molydate | “ |
| Methanol HPLC grade | “ |
| Urea | “ |
| Zinc sulfate | “ |
| Yeast extract | DIFCO Laboratories , USA |
| Sodium hydroxide | EKA Wobel Industrial |
| Copper sulfate | Fluka Chemilka Biochemilk Switzerland |
| Ethylene diamine tetraacetic acid (EDTA) | “ |
| Ferrous sulfate | “ |

| Company / Country | Company / Country |
|---|---|
| Manganese chloride | Fluka Chemilka |
| Calcium chloride | Merck , Germany |
| Formaldehyde solution 40 % w/v | M & B Laboratory chemical |
| Dimethylsulfoxide | Labscan Laboratory |
| Acetonitrile HPLC grade | " |
| Dichloromethane HPLC grade | Sigma chemical company , USA |
| Thiamin | " |
| Hydrogen peroxide | " |

**3. Algal strain**

A green alga, *Haematococcus pluvialis* strain NIES-144 was a kind gift from Associate Professor Aran Inchroensakdi from Biochemistry Science Department, Chulalongkorn University. The organism was originally from the National Institue for Environmental Studies, Tsukuba, Japan.

**Methods**

The study consists of two stages. The first-stage , to attain a high cell concentration in the medium, and for the second-stage, to enhance astaxanthin formation by various treatments.

**Culture conditions**

A pure starter culture of *H. pluvialis* NIES-144 was prepared in the Basal medium (Kobayashi et al. 1991). For the Basal culture, a 10 ml portion of 4-d culture was transferred into 100 ml fresh Basal medium in a 200 ml Erlenmeyer flask. The flask was incubated at 20 $^{o}$C

under a 12-h light / 12-h dark illumination cycle (20 $\mu Em^{-2}s^{-1}$, fluorescent light). The flask was shaken manually once a day. The 4-d culture (vegetative growth phase, ca. $5.5x10^5$ cells per ml) was employed for the supplementation culture.

1. The first-stage (optimization for growth)

   (Effect of environmental factors on growth of *Haematococcus pluvialis* NIES-144)

   1.1 Type of medium

A pure starter culture of *H. pluvialis* NIES-144 was cultured in Basal Medium (Kobayashi et al. 1991) , Bold Basal Modified Medium (Spencer, 1989) and CHU-10 Medium (Bold and Wynne,1978) with initial cell numbers $2.5x10^4$ cells per ml . A 100 ml of culture in a 250 ml Erlenmeyer flask was incubated at 20 $^oC$ under a 12-h light / 12-h dark illumination cycle (20 $\mu Em^{-2}s^{-1}$ ,fluorescent light). The flask was shaken manually once a day. At 0, 3, 5, 7, 9, 11, 13, 15 days, the cell number was counted by a Haemacytometer under a microscope with magnification of 100x and a growth rate was analyzed in terms of specific growth rate ($\mu$) as shown in appendix 4.



   1.2 Light intensity

*H. pluvialis* NIES-144 was cultured in media which yielded highest growth obtained from section 1.1. Light intensities were adjusted to 20-200 $\mu Em^{-2}s^{-1}$ and dark. The cell number and cell growth were calculated as described in section 1.1.

### 1.3 Initial cell number

*H. pluvialis* NIES-144 was cultured in media which yielded highest growth obtained from section 1.1. Initial cell numbers were changed to 1, 2.5, 5, 10, 20, 30, 40, 50x$10^4$ cells per ml. The cell number and cell growth were calculated as described in section 1.1.

### 1.4 Content of carbon and nitrogen ratio in media

*H. pluvialis* NIES-144 was cultured in media which yielded highest growth obtained from section 1.1. The content of sodium acetate was fixed 15 mM (1.2g/l) and the contents of nitrogen were changed to 0, 5, 15, 30 mM $NaNO_3$ as shown in Table 3. In addition, sodium acetate and yeast extract were used as carbon and nitrogen source of media. The contents of sodium acetate were changed to 1.0-2.0 g/l and the contents of nitrogen were changed to 1.5-3.0 g/l as shown in Table 4 The cell number and cell growth were calculated as described in section 1.1.

**Table 3** Carbon and nitrogen ratio in media

| Type of medium | Carbon content | Nitrogen content | C/N ratio |
|---|---|---|---|
| Control | $CH_3COONa$ 15mM | Yeast extract | - |
| CN1 | " | $NaNO_3$ 0 mM | $\alpha$ |
| CN2 | " | " 5 mM | 3 |
| CN3 | " | " 15 mM | 1 |
| CN4 | " | " 30 mM | 0.5 |

**Table 4** Sodium acetate and yeast extract content in media

| Type of medium | Sodium acetate (g/l) | Yeast extract (g/l) |
|---|---|---|
| BM1 | 1.0 | 1.5 |
| BM2 | 1.2 | 1.5 |
| BM3 | 1.5 | 1.5 |
| BM4 | 1.8 | 1.5 |
| BM5 | 2.0 | 1.5 |
| BM6 | 1.0 | 2.0 |
| BM7 | 1.2 | 2.0 |
| BM8 | 1.5 | 2.0 |
| BM9 | 1.8 | 2.0 |
| BM10 | 2.0 | 2.0 |
| BM11 | 1.0 | 2.5 |
| BM12 | 1.2 | 2.5 |
| BM13 | 1.5 | 2.5 |
| BM14 | 1.8 | 2.5 |
| BM15 | 2.0 | 2.5 |
| BM16 | 1.0 | 3.0 |
| BM17 | 1.2 | 3.0 |
| BM18 | 1.5 | 3.0 |
| BM19 | 1.8 | 3.0 |
| BM20 | 2.0 | 3.0 |

**2. The second-stage (optimization for astaxanthin synthesis)**

The condition for promoting growth of *H. pluvialis* NIES-144 was obtained by culturing cells in the Basal medium. The initial cell number was $2.0 \times 10^5$ cells per ml and incubated at 20 $^{o}$C under a 12-h light 12-h dark illumination cycle at 30 $\mu Em^{-2}s^{-1}$, C/N ratio in media was 1.5:3.0 . The culture was shaken manually once a day.

**2.1 Physical factor**

**2.1.1 Effect of light intensity**

*H. pluvialis* NIES-144 was grown as described in section 2. After 5 days, the culture was incubated at 20 $^{o}$C under continuous illumination at 30, 80, 120, 160, 200, 240 $\mu Em^{-2}s^{-1}$. The cell numbers were counted by a Haemacytometer under a microscope with magnification of 100x and astaxanthin, chlorophyll were extracted with dimethylsulfoxide(DMSO).

**2.1.2 Effect of temperature**

*H. pluvialis* NIES-144 was grown as described in section 2.1.1. Temperatures were changed from 20 to 25, 30, 35 $^{o}$C. Pigments were extracted with DMSO.

**2.2 Chemical factors**

**2.2.1 Effect of sodium chloride**

*H. pluvialis* NIES-144 was grown as described in section 2.1.1. and NaCl contents were supplemented at 0, 0.2, 0.4, 0.6, 0.8, 1.0 % (w/v). Pigments were extracted with DMSO.

### 2.2.2 Oxidative stress

*H. pluvialis* NIES-144 was grown as described in section 2.2.1. and sodium acetate, ferrous sulfate, hydrogen peroxide were supplemented at 45 mM, 450 μM and 0.1 μM, respectively. Pigments were extracted with DMSO.

## 2.3 Nutritional factors

### 2.3.1 Nutrient deficiency

*H. pluvialis* NIES-144 was grown as described in section 2. After 5 days, the culture was harvested by centrifugation at 2,000xg for 10 min. Cells were grown in four culture media with a) control medium b)control medium without nitrogen source c) control medium without magnesium source d) control medium without phosphorus source. The condition of culture and pigment analysis were as described in section 2.1.1 ( control medium : $NaNO_3$ 250 mg/l, $K_2HPO_4$ 75 mg/l, $KHPO_4$ 175 mg/l, $MgSO_4$ 75 mg/l )

### 2.3.2 Carbon and nitrogen ratio

*Haematococcus pluvialis* NIES-144 was grown as described in section 2.1.1. The basal medium was supplemented with acetate and sodium nitrate so as to adjust the required C/N ratio of the medium. The acetate concentration supplemented to the culture was fixed at 45 mM, while sodium nitrate concentration were adjusted to 0, 15, 45, 90 mM. The cell numbers were counted and pigments were extracted with DMSO.

3. Effect of combined treatments

3.1 Effect of sodium acetate and high salinity

*H. pluvialis* NIES-144 was grown in Basal medium as described in section 2.2.1 After 5-days the culture was supplemented with 45 mM $CH_3COONa$ to stimulate astaxanthin synthesis and cultivation was continued for another 4 days. The culture was further treated with 0.6 % w/v NaCl for 2 days. Cell numbers were counted and pigments were extracted with DMSO.

3.2 Effect of sodium acetate and high temperature

*H. pluvialis* NIES-144 was grown in Basal medium as described in section 2.2.1 After 5-days the culture was supplemented with 45 mM $CH_3COONa$ to stimulate astaxanthin synthesis and cultivation was continued for another 4days. The culture was further treated with high temperature(35$^{o}$C) for 2 days. Cell numbers were counted and pigments were extracted with DMSO.

3.3 Effect of sodium acetate and N-deficiency

*H. pluvialis* NIES-144 was grown in Basal medium as described in section 2.2.1. After 5-days the culture was supplemented with 45 mM $CH_3COONa$ to stimulate astaxanthin synthesis and cultivation was continued for another 4days. The culture was further grown in N-deficient media for 2 days. Cell numbers were counted and pigments were extracted with DMSO.

**4. Analysis of astaxanthin from *Haematococcus pluvialis* NIES-144 by High-Performance Liquid Chromatography Method** (Yuan and Chen,1998)

**4.1 For Mature cyst cells**

*H. pluvialis* NIES-144 was cultured in basal medium as described in section 2.2.1. At 5-days cultivation, 45 mM sodium acetate was added. After 4-days cultivation, 100 ml of the cultures were centrifuged at 3000 g for 20 min, the supernatant was discarded, and the cell pellet was rinsed with distilled water twice and then freeze-dried using freeze-dryer. The cyst cells were lyophilized by Flexi-dry μp. The lyophilized cells were kept at –20 $^{o}$C in the dark.

The dry cells (25 mg) were homogenized using a 15-ml tissue homogenizer and the total pigments were extracted in the solvent mixture (2-mL aliquots) of dichloromethane and methanol (25:75,v/v). The mixture was then separated by centrifugation at 10,000 g for 5 min, and the supernatant containing pigments was collected. The extraction procedure was repeated at least three times until the cell debris was completely colorless, and 10 ml of pigment extract was obtained. The product could directly be used for HPLC determination and subsequent saponification. All of the above processes were conducted in darkness.

**4.2 Saponification of astaxanthin esters**

one milliliter of 0.018 M NaOH dissolved in methanol , which was freshly prepared, was added to 5 ml of the pigment extract solution

under a nitrogen atmosphere. The mixture (6 ml) was evaporated and concentrated to 5 ml under nitrogen and then kept overnight or for at least 8 h under nitrogen in darkness at room temperature for complete saponification of astaxanthin esters. The resulting product was directly separated and determined by HPLC.

**HPLC condition**

| | |
|---|---|
| Column | reversed-phase $C_{18}$ column ODS 150x4.6 mm |
| Mobile phase | solvent A ; dichloromethane : methanol : acetonitile : water (5 : 85 : 5.5 : 4.5) |
| | solvent B ; dichloromethane : methanol : acetonitile : water(22 : 28 :45.5 : 4.5) |
| Temperature | 25 $^{o}$C |
| Injection column | 20 μl |
| Flow rate | 1 ml/min |
| Detection | visible detector at 480 nm |
| Run time | 65 min |
| Step gradient | 100 % solvent A 8 min |
| | 0-100 % solvent B 6 min |
| | 100 % solvent B 51 min |
| Pressure | approx. 80 bar |

**5. Growth and pigment analysis** (Boussiba and Vonshak,1991)

The algal growth was determined by counting cell number with a haemacytometer. Growth rate ($\mu$) was calculated by the following equation.

$$\mu = (\ln x_2 - \ln x_1) / t_2 - t_1$$

where $x_2$ and $x_1$ are the number of cells at $t_2$ and $t_1$ , respectively.

For chlorophyll determination, cells were centrifuged at 3500 rpm for 10 min. The pellet was resuspended in 99.5 % DMSO and homogenized. The mixture was then heated for 10 min at 70 $^{o}$C. If necessary , the procedure was repeated until a white pellet was obtained. The absorbance of the combined DMSO extracts was determined at 673 nm, and chlorophyll content was calculated according to Seely et al.(1972) with $E^{1\%}_{1cm}$ of 898. For astaxanthin determination, harvested cells were first treated with a solution of 5 % KOH in 30 % methanol to destroy the chlorophyll. The supernatant was discarded, and the remaining pellet was extracted with DMSO to recover the astaxanthin. This last step was repeated if necessary until the cell debris was totally white. The absorbance of the combined extracts was determined at 492 nm, and the amount of the pigment was calculated according to Davies (1976) with $E^{1\%}_{1cm}$ of 2220.

# Chapter III

## Results

### 1. The first-stage (optimization for growth)

#### 1.1 Type of medium

Figure 5 shows the 15-day growth of *H. pluvialis* NIES-144 in three types of media with initial cell numbers of $2.5 \times 10^4$ cells per ml at 20 $^{o}$C under 12-h light and 12-h dark illumination cycle (20 $\mu Em^{-2}.s^{-1}$). It was found that *H. pluvialis* NIES-144 could grow best in Basal medium followed by Modified medium and CHU-10 medium respectively. Figure 6 shows that the specific growth rate ($d^{-1}$) of *H. pluvialis* NIES-144 grown in Basal medium was better than those in Bold Basal Modified medium and CHU-10 medium.

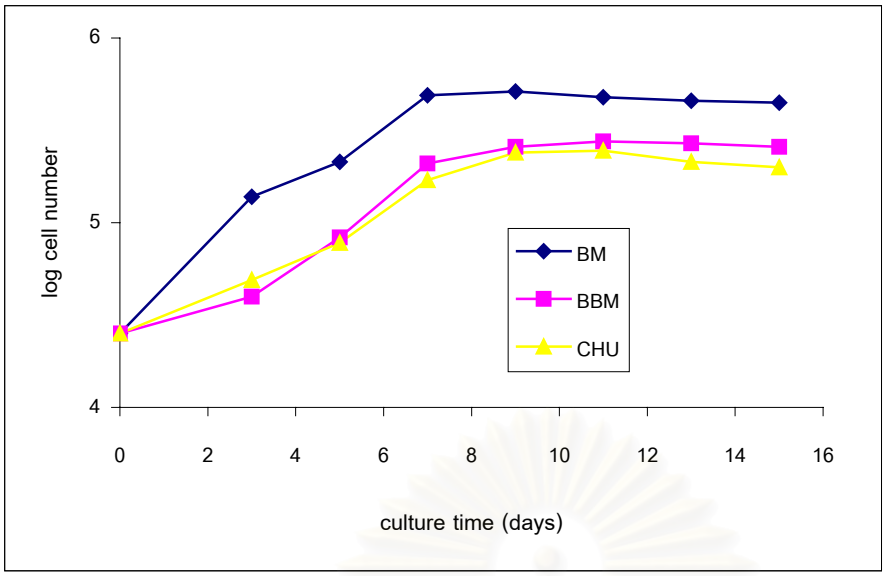
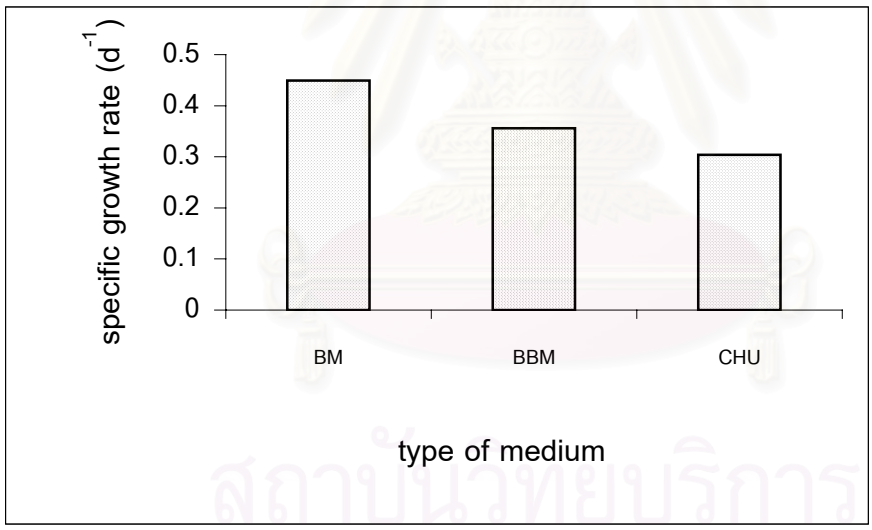



**Figure 5** Effect of types of media on growth of *Haematococcus pluvialis* NIES-144




BM = Basal Medium

BBM = Bold Basal Modified Medium

CHU = CHU-10 Medium

**Figure 6** Specific growth rate of *Haematococcus pluvialis* NIES-144 in three types of media

### 1.2 Light intensity

*H. pluvialis* NIES-144 was grown in Basal medium with initial cell number of $2.5x10^4$ cells per ml at 20 $^oC$ under 12-h light and 12-dark illumination condition. Light intensity was varied from 0 - 200 $\mu Em^{-2}.s^{-1}$. Fig.7a and 7b showed that the growth of *H. pluvialis* NIES-144 were slightly different when grow in the range of the light intensity from 0-200 $\mu Em^{-2}.s^{-1}$. Under the condition, the growth reached the stationary phase in day 5. The specific growth rates were slightly different when grown in different light intensity, whereas lower growth rate occurred in the dark. So, in this experiments the optimum light intensity for growth of *H. pluvialis* NIES-144 in the basal medium was 30 $\mu Em^{-2}.s^{-1}$. For this results, less light requirement at 30 $\mu Em^{-2}.s^{-1}$. Culture would provide advantageous operation for experiments to reduce expense of illumination(Fig.8a and 8b)

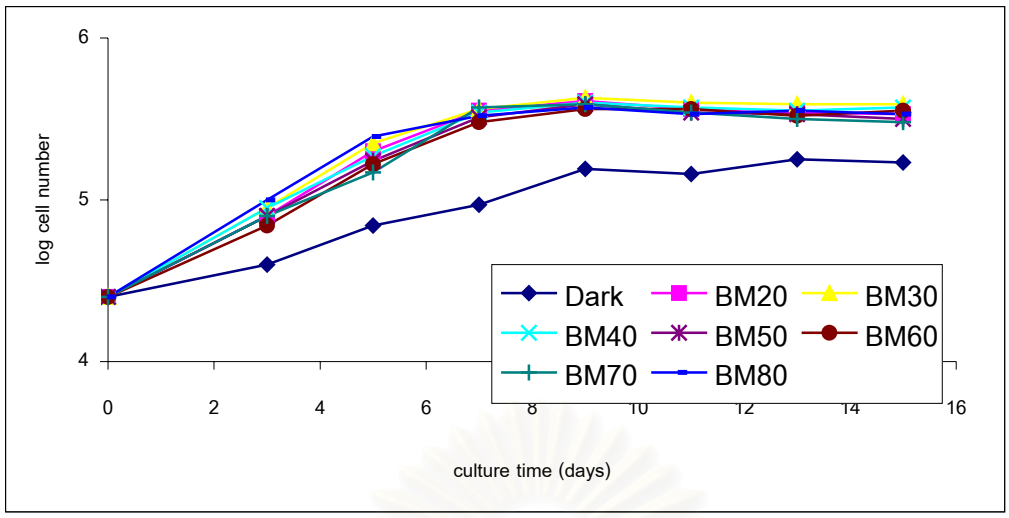
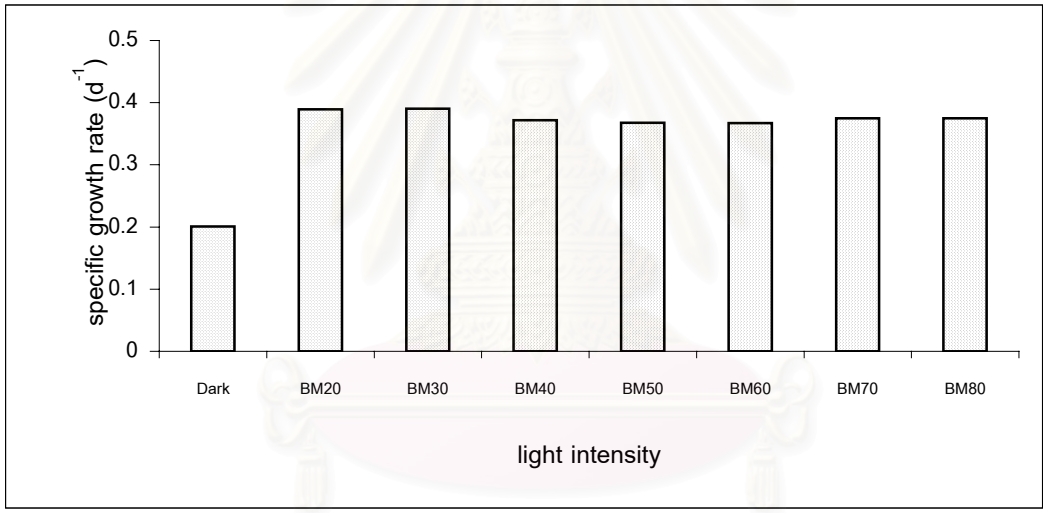



**Figure 7a** Effect of light intensity on growth of *Haematococcus pluvialis* NIES-144




Dark = 0 μEm$^{-2}$s$^{-1}$ BM 50 = 50 μEm$^{-2}$s$^{-1}$

BM20 = 20 μEm$^{-2}$s$^{-1}$ BM 60 = 60 μEm$^{-2}$s$^{-1}$

BM 30 = 30 μEm$^{-2}$s$^{-1}$ BM 70 = 70 μEm$^{-2}$s$^{-1}$

BM 40 = 40 μEm$^{-2}$s$^{-1}$ BM 80 = 80 μEm$^{-2}$s$^{-1}$

**Figure 8a** Specific growth rate of *Haematococcus pluvialis* NIES-144 with various light intensity

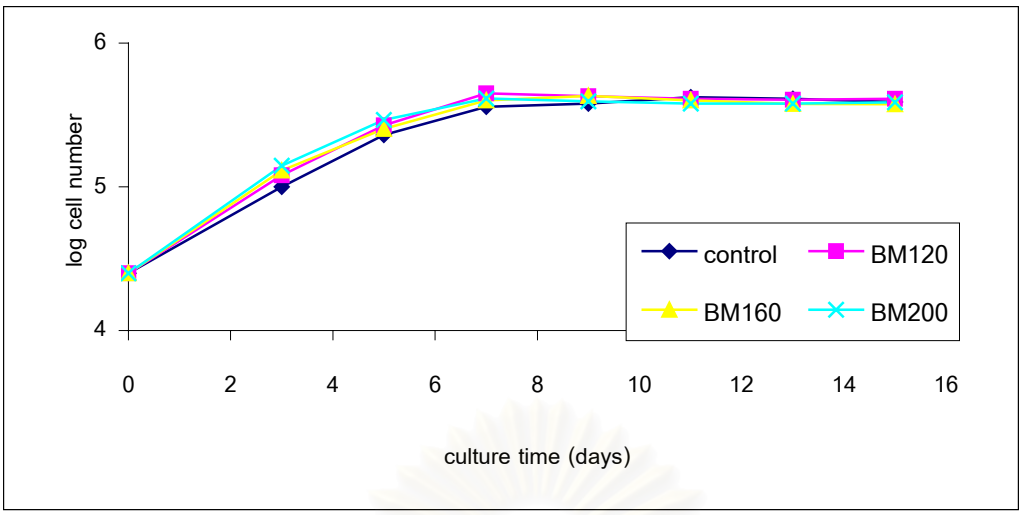
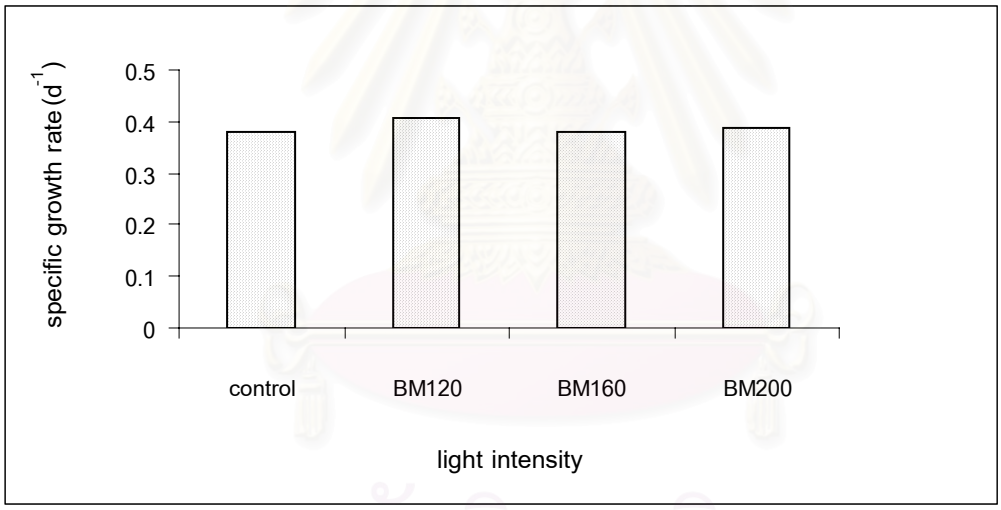



**Figure 7b** Effect of light intensity on growth of *Haematococcus pluvialis* NIES-144




Control = 20 μEm$^{-2}$s$^{-1}$ BM160 = 160 μEm$^{-2}$s$^{-1}$

BM 120 = 120 μEm$^{-2}$s$^{-1}$ BM 200 = 200 μEm$^{-2}$s$^{-1}$

**Figure 8b** Specific growth rate of *Haematococcus pluvialis* NIES-144 with various light intensity

### 1.3 Initial cell number

Under 12 hr light (30 $\mu Em^{-2}.s^{-1}$) and 12 hr dark cycle at 20 $^{o}C$. *H. pluvialis* NIES-144 was grown in Basal medium with initial cell number of $2.5x10^{4}$ cells per ml. The initial cell numbers were varied between 1 to $50x10^{4}$ cells per ml. As shown in Figure 9 and 10 the best initial cell number for growth and cell yield in *H. pluvialis* NIES-144 was $2x10^{5}$ cells per ml although specific growth rate decrease compared to other initial cell numbers. However, under the condition the growth reached the stationary phase in 5-day. It was found that *H. pluvialis* NIES-144 could grow best in basal medium with initial cell number of $2.0x10^{5}$ cells per ml. So , in the experiment , initial cell number for growth of *H. pluvialis* NIES-144 was fixed at $2x10^{5}$ cells per ml.

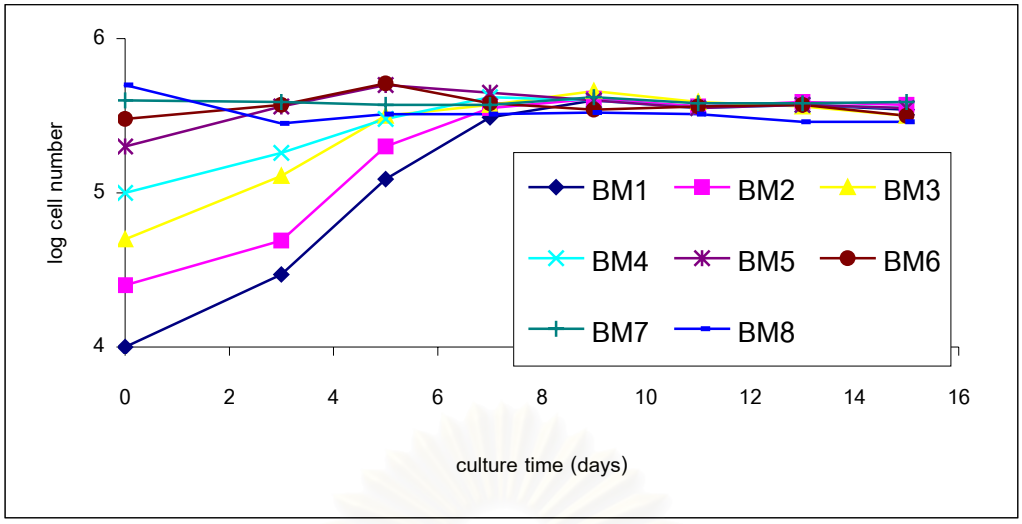
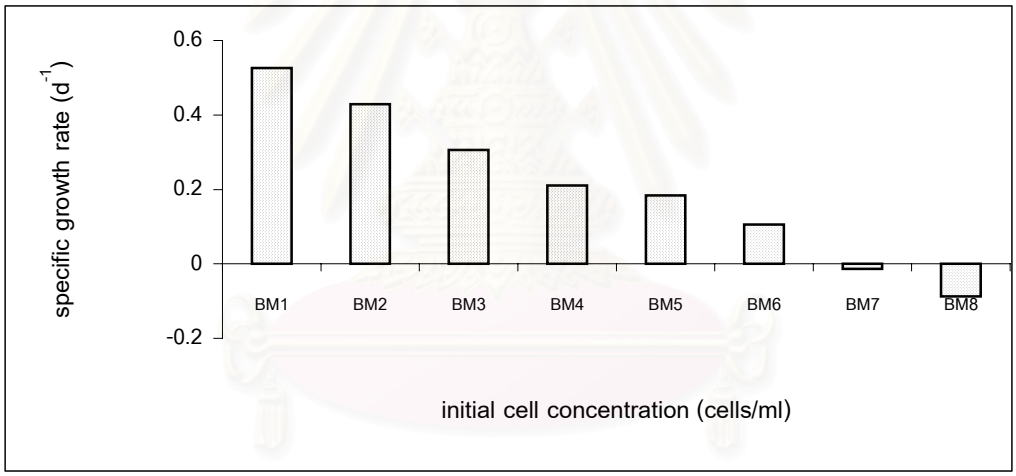



**Figure 9** Effect of initial cell number on growth of *Haematococcus pluvialis* NIES-144




BM1 = cell concentration : $1x10^4$ BM5 = cell concentration : $20x10^4$

BM2 = cell concentration : $2.5x10^4$ BM6 = cell concentration : $30x10^4$

BM3 = cell concentration : $5x10^4$ BM7 = cell concentration : $40x10^4$

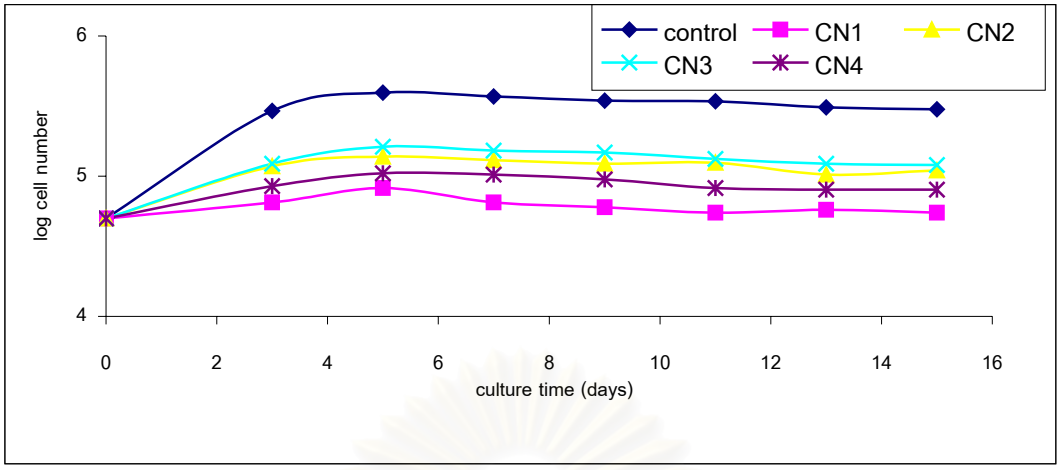
BM4 = cell concentration : $10x10^4$ BM8 = cell concentration : $50x10^4$

**Figure 10** Specific growth rate of *Haematococcus pluvialis* NIES-144 with various initial cell numbers

### 1.4 Carbon and nitrogen ratio in media

The culture of *H. pluvialis* NIES-144 was incubated at 20 $^{o}$C under 20 $\mu Em^{-2}.s^{-1}$ 12-h light and 12-h dark illumination condition. Figures 11 and 12 show that low growth occurred in types of media containing $NaNO_3$ as nitrogen source. It was found that *H. pluvialis* NIES-144 could grow best in the control(basal medium) containing $CH_3COONa$ as carbon source and yeast extract as nitrogen source. Over the range of $CH_3COONa$ concentrations (1.0-2.0 g/l) and yeast extract (1.5-3.0 g/l) tested growth was best at 1.5 and 3.0 g/l respectively. There were slight differences on growth. However under this best condition the maximum cell number was reached after 5-day of growth (Fig.13-20).

Figures 21 and 22 show the effect of combined conditions on growth of *H. pluvialis* NIES-144. It was found that specific growth rate was best in BM18. So , in the experiments , the optimum condition for growth of *H. pluvialis* NIES-144 was obtained culturing by cells in Basal medium with sodium acetate and yeast extract of 1.5:3.0 g/l, initial cell number $2x10^5$ cells per ml under light intensity of 30 $\mu Em^{-2}.s^{-1}$ 12-h light at 20 $^{o}$C.




Control = control (Basal medium)

CN1 = Basal medium ( yeast extract replaced with $NaNO_3$ )

($CH_3COONa$ : $NaNO_3$ = 15: 0 mM) , (C/N = $\alpha$)

CN2 = Basal medium (yeast extract replaced with $NaNO_3$ )

($CH_3COONa$ : $NaNO_3$ = 15 : 5 mM) ,(C/N = 3)

CN3 = Basal medium (yeast extract replaced with $NaNO_3$ )

($CH_3COONa$ : $NaNO_3$ = 15 : 15 mM) ,(C/N = 1)

CN4 = Basal medium (yeast extract replaced with $NaNO_3$ )

($CH_3COONa$ : $NaNO_3$ = 15 : 30 mM) , (C/N = 0.5)

**Figure 11** Effect of carbon and nitrogen ratio in media on growth of *Haematococcus pluvialis* NIES-144

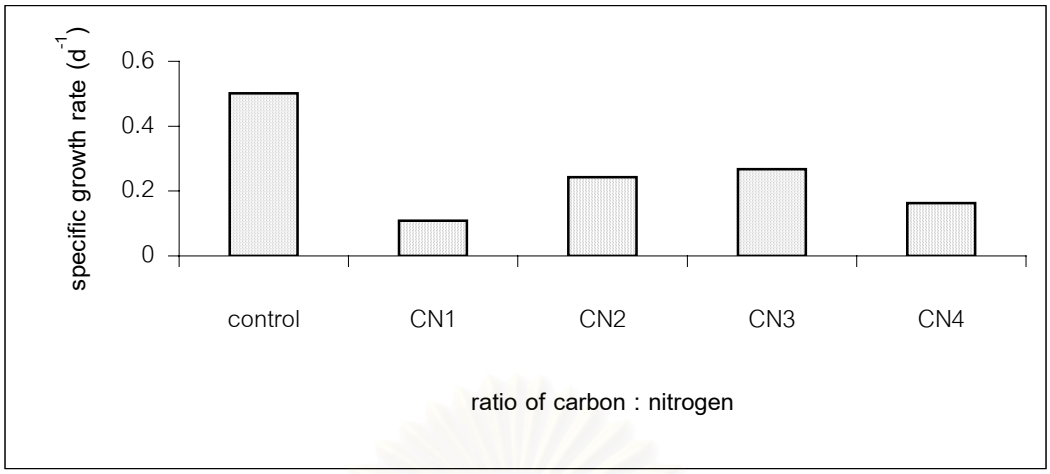



**Figure 12** Specific growth rate of *Haematococcus pluvialis* NIES-144 with various carbon and nitrogen ratio in media

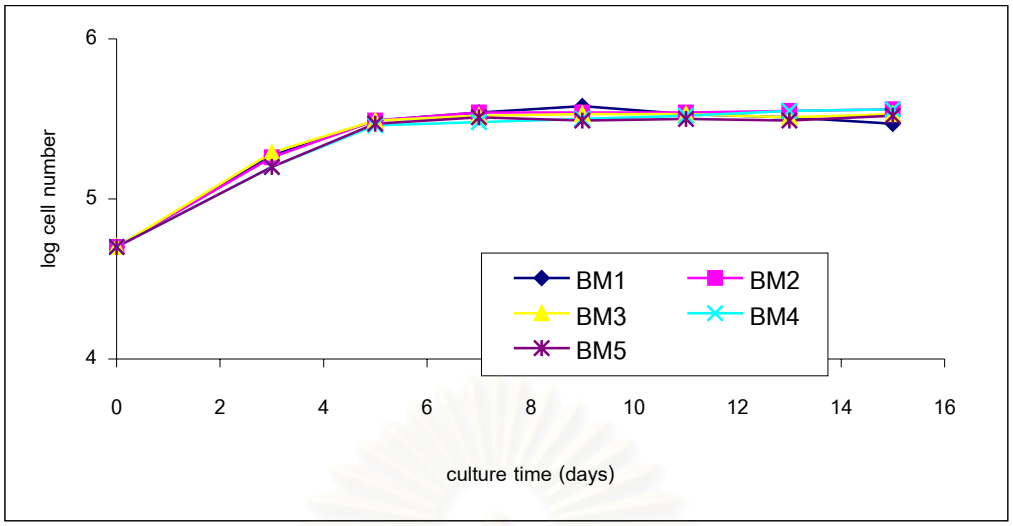
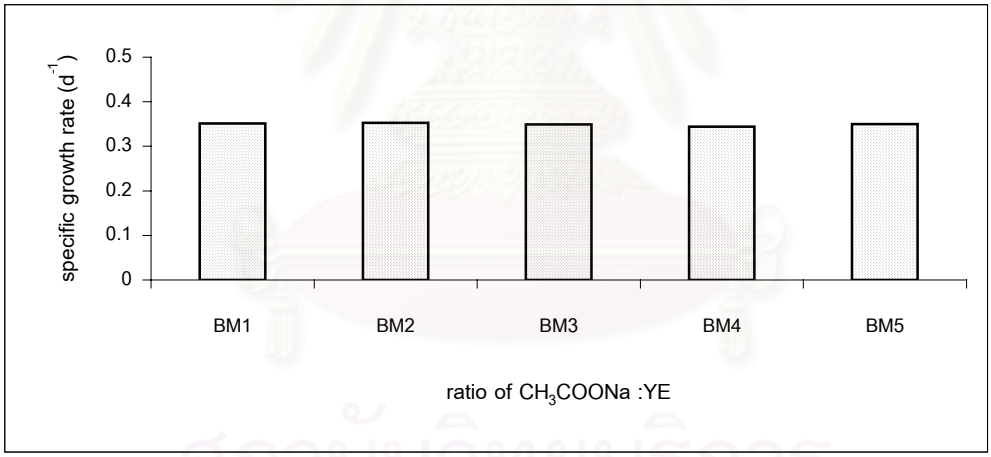



**Figure 13** Effect of sodium acetate and yeast extract content in media growth of *Haematococcus pluvialis* NIES-144




BM1 = Basal Medium ($CH_3COONa$ : Yeast extract = 1.0:1.5) g/l

BM2 = Basal Medium ($CH_3COONa$ : Yeast extract = 1.2:1.5) g/l

BM3 = Basal Medium ($CH_3COONa$ : Yeast extract = 1.5:1.5) g/l

BM4 = Basal Medium ($CH_3COONa$ : Yeast extract = 1.8:1.5) g/l

BM5 = Basal Medium ($CH_3COONa$ : Yeast extract = 2.0:1.5) g/l

**Figure 14** Specific growth rate of *Haematococcus pluvialis* NIES-144 with various sodium acetate and yeast extract content in media

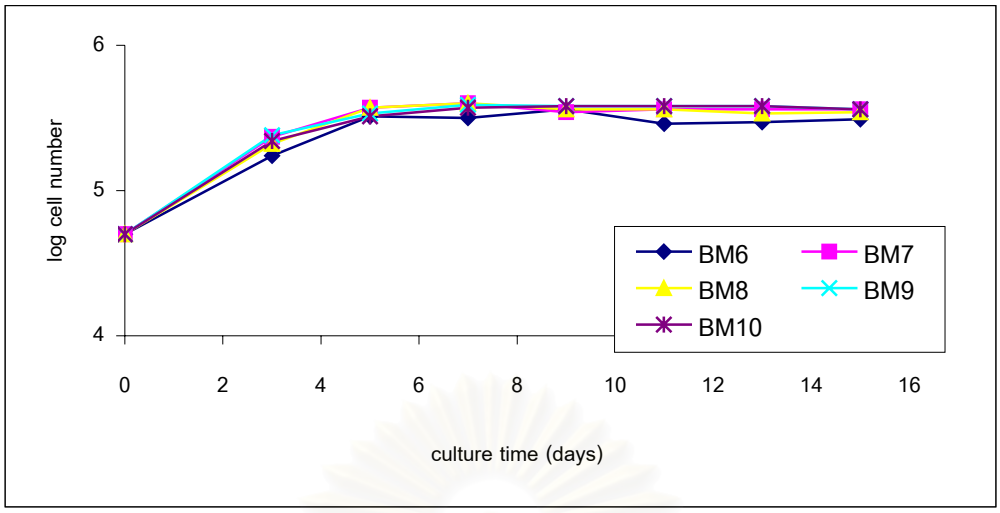
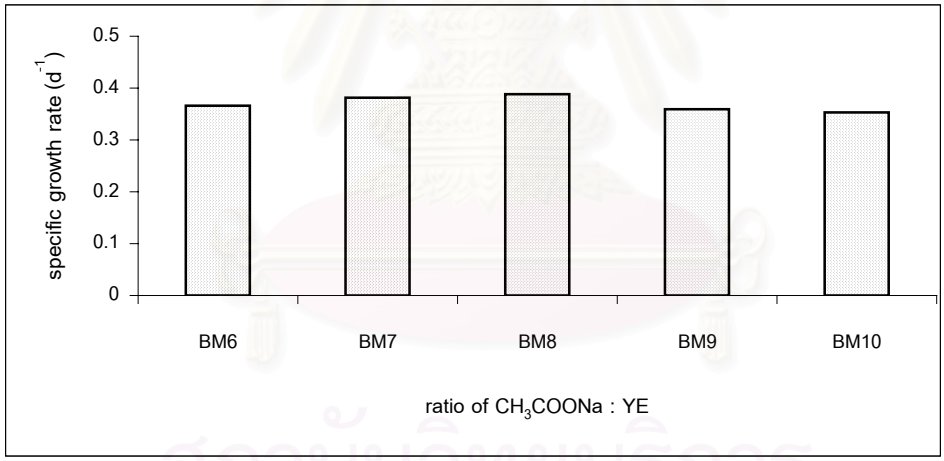



**Figure 15** Effect of sodium acetate and yeast extract content in media on growth of *Haematococcus pluvialis* NIES-144




BM6 = Basal Medium ($CH_3COONa$ : Yeast extract = 1.0 : 2.0) g/l

BM7 = Basal Medium ($CH_3COONa$ : Yeast extract = 1.2 : 2.0) g/l

BM8 = Basal Medium ($CH_3COONa$ : Yeast extract = 1.5 : 2.0) g/l

BM9 = Basal Medium ($CH_3COONa$ : Yeast extract = 1.8 : 2.0) g/l

BM10 = Basal Medium ($CH_3COONa$ : Yeast extract = 2.0 : 2.0) g/l

**Figure 16** Specific growth rate of *Haematococcus pluvialis* NIES-144 with various sodium acetate and yeast extract content in media

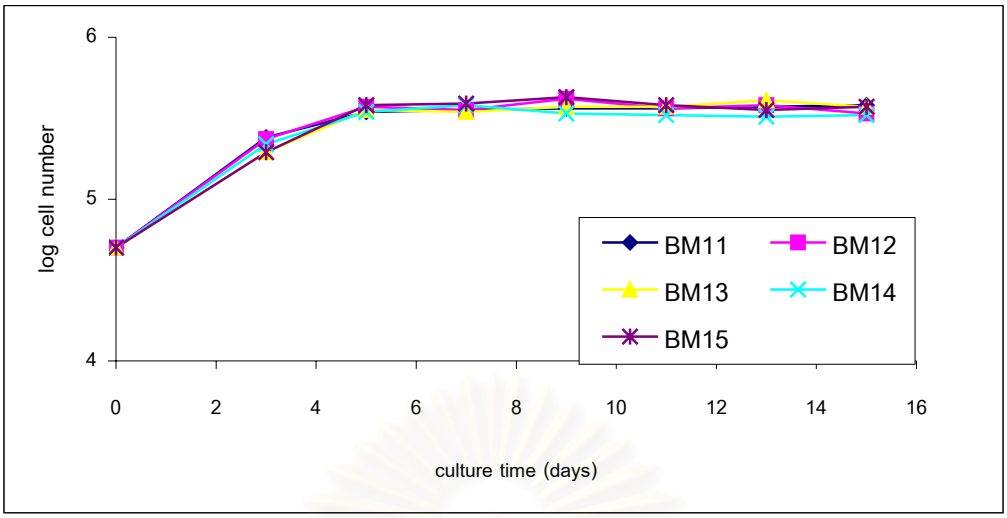
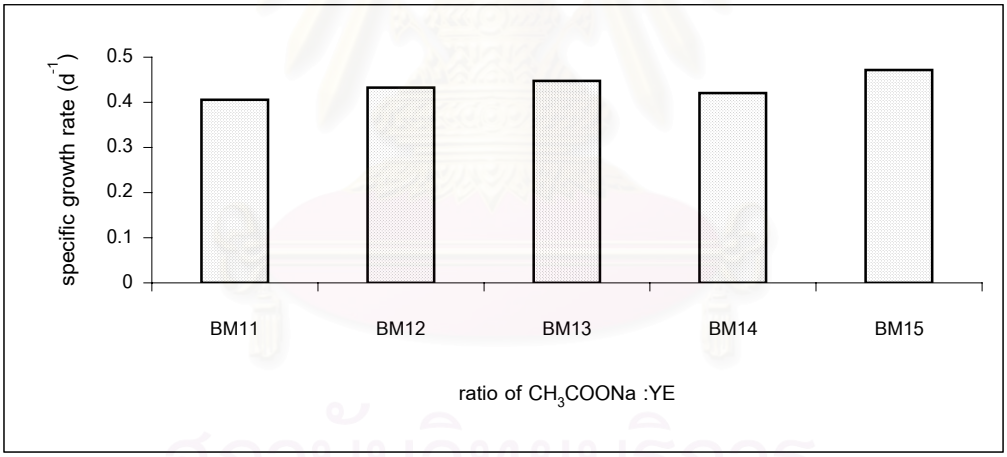



**Figure 17** Effect of sodium acetate and yeast extract content in media on growth of *Haematococcus pluvialis* NIES-144




BM11 = Basal Medium ($CH_3COONa$ : Yeast extract = 1.0 : 2.5) g/l

BM12 = Basal Medium ($CH_3COONa$ : Yeast extract = 1.2 : 2.5) g/l

BM13 = Basal Medium ($CH_3COONa$ : Yeast extract = 1.5 : 2.5) g/l

BM14 = Basal Medium ($CH_3COONa$ : Yeast extract = 1.8 : 2.5) g/l

BM15 = Basal Medium ($CH_3COONa$ : Yeast extract = 2.0 : 2.5) g/l

**Figure 18** Specific growth rate of *Haematococcus pluvialis* NIES-144 with various sodium acetate and yeast extract content in media

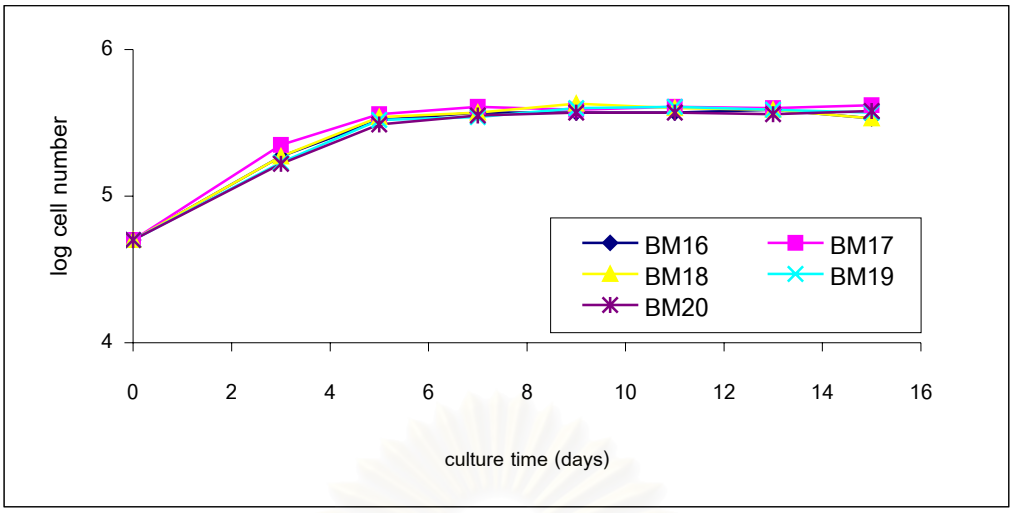
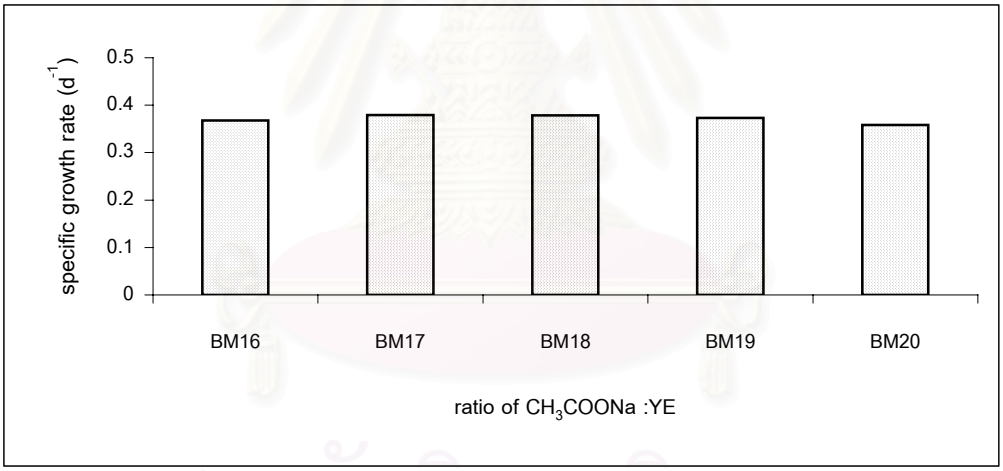



**Figure 19** Effect of sodium acetate and yeast extract content in media on growth of *Haematococcus pluvialis* NIES-144




BM16 = Basal Medium ($CH_3COONa$ : Yeast extract = 1.0 : 3.0) g/l

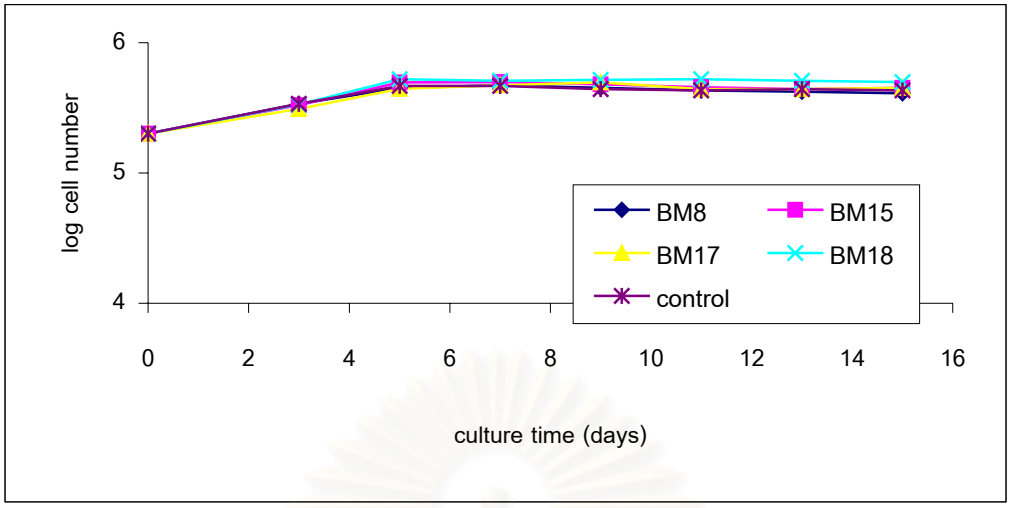
BM17 = Basal Medium ($CH_3COONa$ : Yeast extract = 1.2 : 3.0) g/l

BM18 = Basal Medium ($CH_3COONa$ : Yeast extract = 1.5 : 3.0) g/l

BM19 = Basal Medium ($CH_3COONa$ : Yeast extract = 1.8 : 3.0) g/l

BM20 = Basal Medium ($CH_3COONa$ : Yeast extract = 2.0 : 3.0) g/l

**Figure 20** Specific growth rate of *Haematococcus pluvialis* NIES-144 with various sodium acetate and yeast extract content in media




BM8 = Basal Medium ($CH_3COONa$ : Yeast extract = 1.5:2.0) g/l

Light = 30 μmol$m^2s^{-1}$ , cell = 20x$10^4$ cells/ml

BM15 = Basal Medium ($CH_3COONa$ : Yeast extract = 2.0:2.5) g/l

Light = 30 μmol$m^2s^{-1}$ , cell = 20x$10^4$ cells/ml

BM17 = Basal Medium ($CH_3COONa$ : Yeast extract = 1.2:3.0) g/l

Light = 30 μmol$m^2s^{-1}$ , cell = 20x$10^4$ cells/ml

BM18 = Basal Medium ($CH_3COONa$ : Yeast extract = 1.5:3.0) g/l

Light = 30 μmol$m^2s^{-1}$ , cell = 20x$10^4$ cells/ml

control = Basal Medium ($CH_3COONa$ : Yeast extract = 1.2:2.0) g/l

Light = 20 μmol$m^2s^{-1}$ , cell = 20x$10^4$ cells/ml

**Figure 21** Effect of combined conditions on growth of *Haematococcus pluvialis* NIES-144

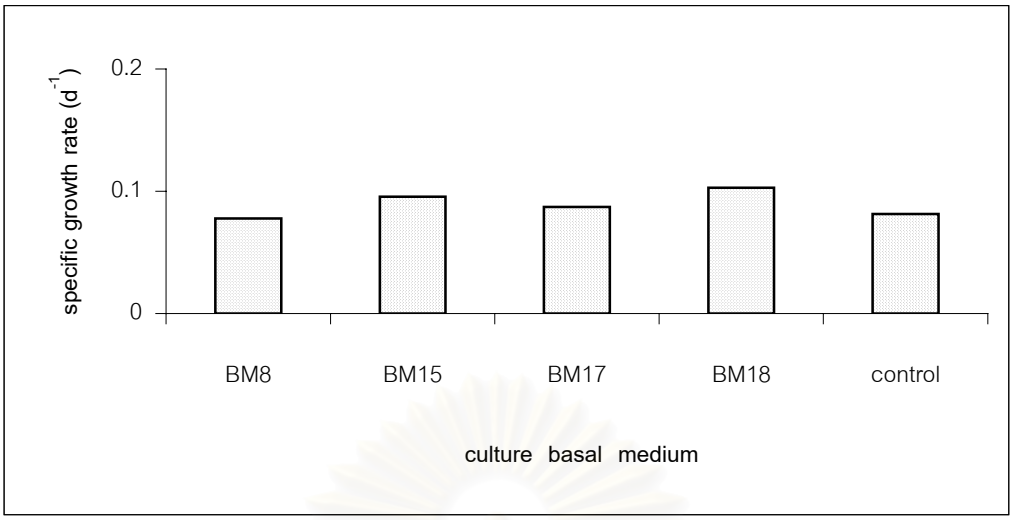



**Figure 22** Specific growth rate of *Haematococcus pluvialis* NIES-144 with combined conditions

2.The second-stage (optimization for astaxanthin synthesis)

2.1 Physical factor

2.1.1 Effect of light intensity

The 5-day culture (vegetative cells in the exponential growth phase) was used for second-stage culture to induce astaxanthin accumulation of *H. pluvialis* NIES-144. These cultures were exposed to various light intensities ; 30, 80, 120, 160, 200, 240 $\mu Em^{-2}.s^{-1}$ under 24 h continuous lighting at 20 $^{o}C$. Under different light intensity the cell numbers and pigment content did not show significant differences. Except, after 6 days the cell numbers was decreased when exposed at 240 $\mu Em^{-2}.s^{-1}$. Maximal astaxanthin was 18.19±1.20 pg/cell when exposed to 200$\mu Em^{-2}.s^{-1}$ after 10-day cultivation as shown in Fig. 23 and 24.

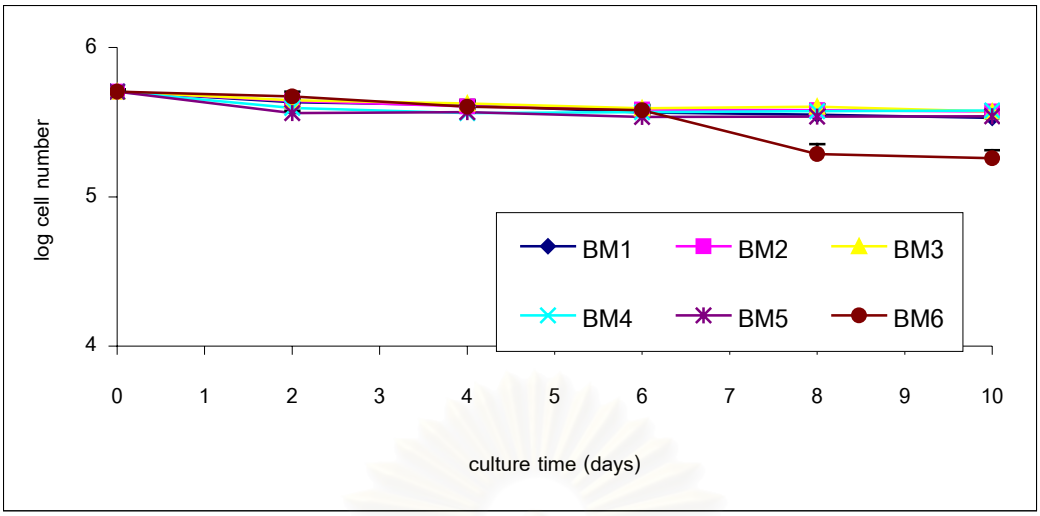
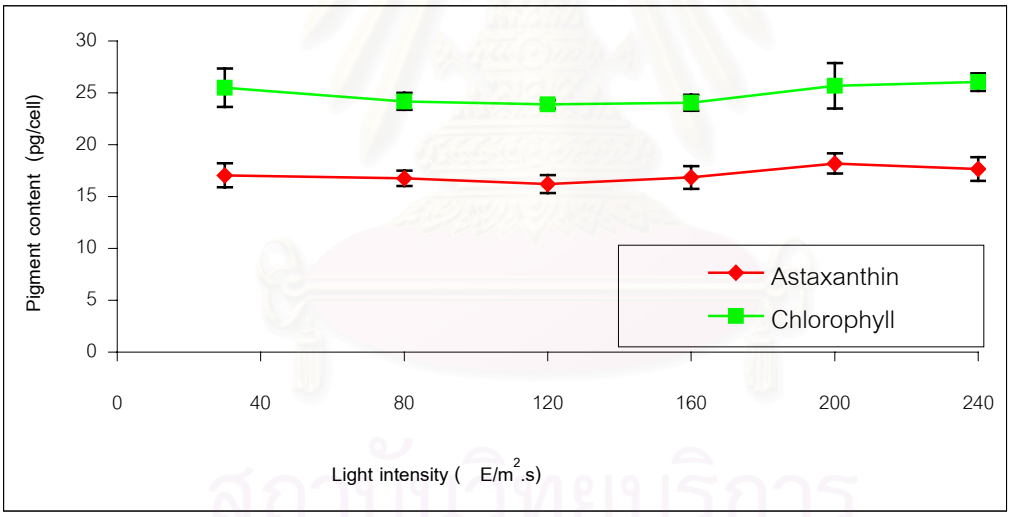



**Figure 23** Effect of light intensity on cell numbers of *Haematococcus pluvialis* NIES-144 ( mean value + standard deviation)




BM1 = 30 μEm$^{-2}$s$^{-1}$ BM 4 = 160 μEm$^{-2}$s$^{-1}$

BM 2 = 80 μEm$^{-2}$s$^{-1}$ BM 5 = 200 μEm$^{-2}$s$^{-1}$

BM 3 = 120 μEm$^{-2}$s$^{-1}$ BM 6 = 240 μEm$^{-2}$s$^{-1}$

**Figure 24** Effect of light intensity on pigment content of *Haematococcus pluvialis* NIES-144 after 10-day cultivation (mean value ± standard deviation)

### 2.1.2 Effect of temperatures

To investigate the effect of temperature on pigment formation in *H. pluvialis* NIES-144, the alga was cultivated in Basal Medium at various temperatures from 20 to 35 $^{o}$C under 30 $\mu Em^{-2}.s^{-1}$ continuous illumination. The algal cell numbers decreased when the cultures were cultivated at high temperature (Fig 25). Astaxanthin accumulation was induced by high temperature at 35 $^{o}$C. The astaxanthin content increased and reached 38.37$\pm$6.30 pg/cell after 8-day cultivation. Furthermore, the chlorophyll content increased when the cultures were exposed to high temperature as shown in Fig. 26.

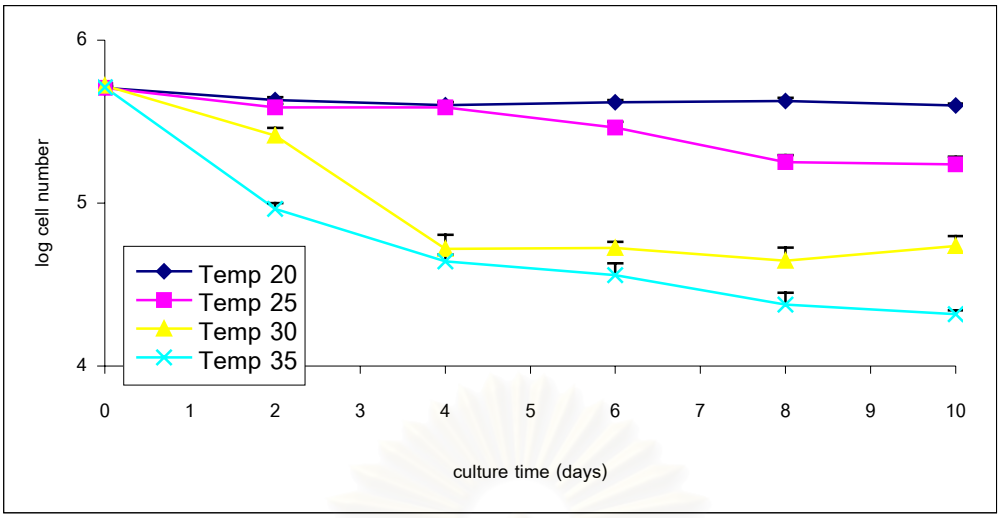
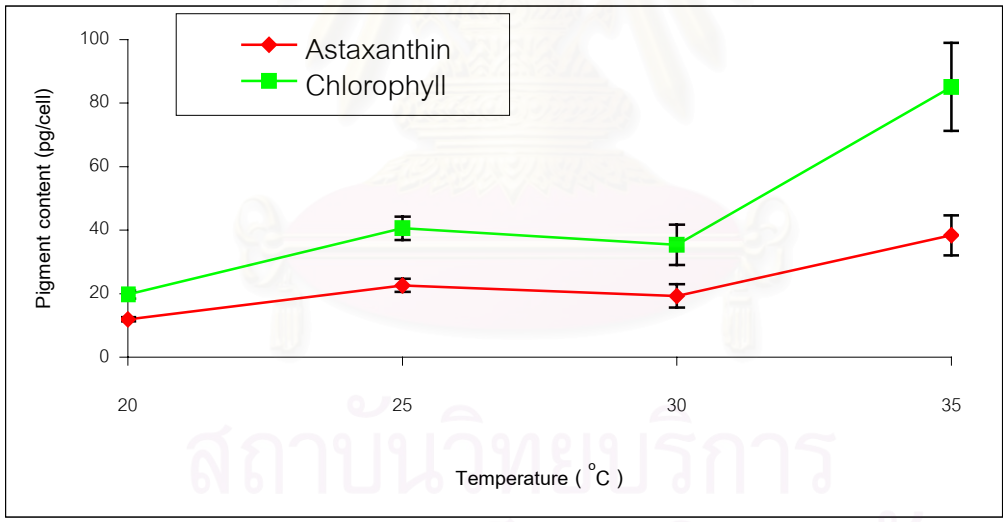



**Figure 25** Effect of temperature on the cell numbers of *Haematococcus pluvialis* NIES-144 (mean value + standard deviation)




Temp 20 = 20 °C Temp 30 = 30 °C

Temp 25 = 25 °C Temp 35 = 35 °C

**Figure 26** Effect of temperature on pigment content of *Haematococcus pluvialis* NIES-144 after 8-day cultivation (mean value ± standard deviation )

## 2.2 Chemical factor

### 2.2.1 Effect of sodium chloride

The 5-day culture in the Basal medium was used to test the effect of sodium chloride at 0, 0.2, 0.4, 0.6, 0.8, 1.0 %(w/v). The cultures were cultivated at 20 $^{o}$C under 30 $\mu Em^{-2}.s^{-1}$ continuous illumination for various times. The cell numbers were decreased when added with 0.4 to 1.0 %(w/v) NaCl(Fig 27). A high concentration of NaCl inhibited cell growth and caused the death of many cells. Maximal astaxanthin content was 36.21 $\pm$3.18 pg/cell when supplemented with 0.6 %(w/v) NaCl.(Fig.28)

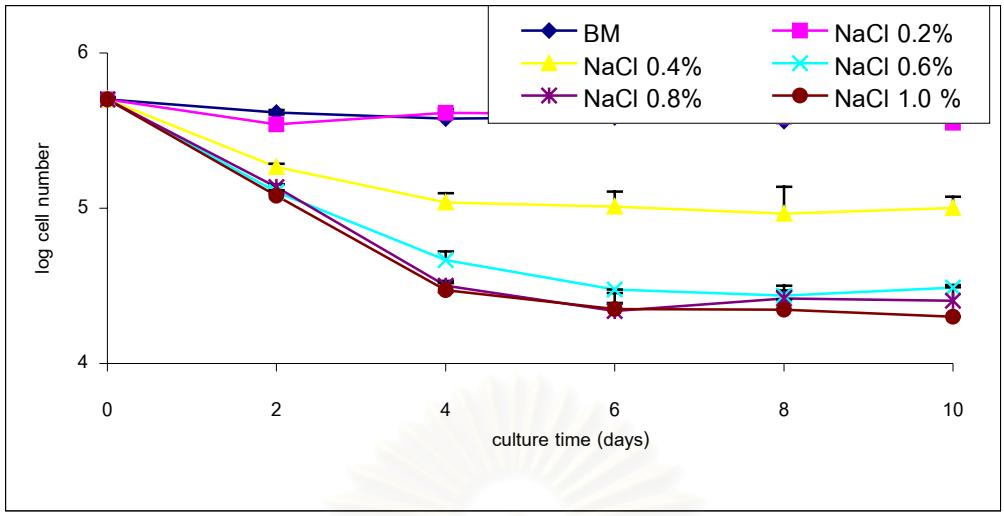



**Figure 27** Effect of NaCl on the cell numbers of *Haematococcus pluvialis* NIES-144 ( mean value + standard deviation)

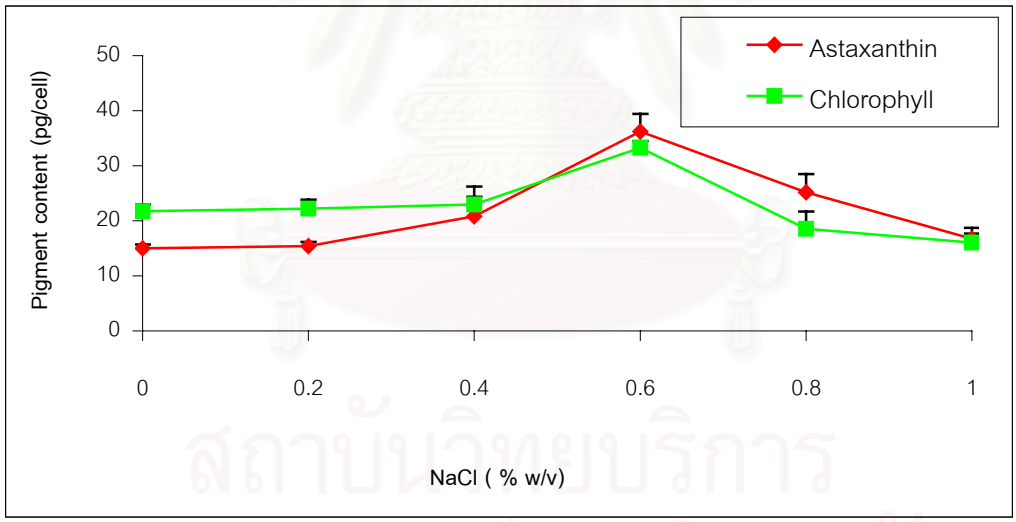



**Figure 28** Effect of NaCl on pigment content of *Haematococcus pluvialis* NIES-144 after 10-day cultivation (mean value ± standard deviation)

### 2.2.2 Oxidative stress

The 5-day culture (vegetative cell in the exponential growth phase) on Basal Medium was used to test the effect of oxidative stress (45 mM $CH_3COONa$ , 450 μM $FeSO_4$ , 0.1 μM $H_2O_2$ as follows. The cultures were cultivated at 20 $^{o}C$ under the same condition as section 2.2.1. Growth of *H. pluvialis* NIES-144 under different oxidative stress was not significantly different (Fig.29). The highest astaxanthin obtained was 18.65 ±0.37 pg/cell. Highest chlorophyll content was also obtained under this condition from the culture with 45 mM $CH_3COONa$ after 4-day cultivation (Table 5).

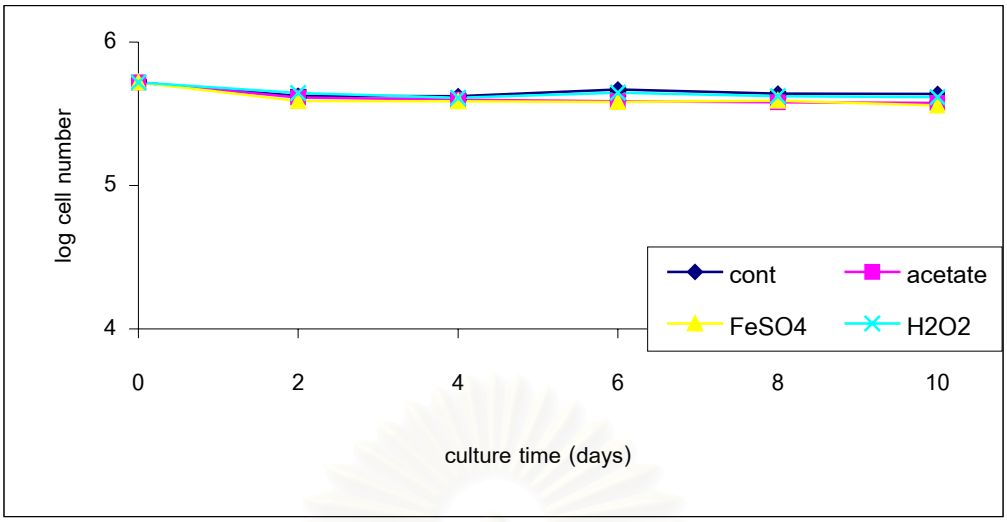



**Figure 29** Effect of oxidative stress on the cell numbers of *Haematococcus pluvialis* NIES-144 (mean value standard deviation)

**Table 5** Effect of oxidative stress on pigment content of *Haematococcus pluvialis* NIES-144 after 4-day cultivation (mean value ± standard deviation)

| Experiment | Astaxanthin (pg/cell) | Chlorophyll (pg/cell) |
|---|---|---|
| Control | 10.97 ± 0.80 | 18.51 ± 1.30 |
| With acetate 45 mM | 18.65 ± 0.37 | 18.58 ± 0.18 |
| With $Fe^{2+}$ 450 μM | 11.12 ± 0.43 | 15.79 ± 0.38 |
| With $H_2O_2$ 0.1 μM | 7.85 ± 1.51 | 16.85 ± 0.40 |

cont = basal medium $FeSO_4$ = + 450 μM ferrous sulfate

acetate = + 45 mM acetate $H_2O_2$ = + 0.1 μM hydrogen peroxide

## 2.3 Nutritional factor

### 2.3.1 Nutrient deficiency

The 5-day culture on Basal medium was harvested by centrifugation at 2000xg. The culture was then grown in four culture media, ie., (a) complete medium (b) complete medium without N-source (c) complete medium without Mg-source (d) complete medium without P-source at 20 $^{o}$C under 30 $\mu Em^{-2}.s^{-1}$ continuous illumination for various times. The cell number was decreased when grown in deficient media (Fig 30). After 10-day of cultivation , the highest concentration of astaxanthin (44.09 $\pm$ 5.75 pg/cell) was obtained with N-deficiency followed by that with Mg-deficiency (36.34 $\pm$ 7.68 pg/cell (Table 6). On the other hand, chlorophyll content was highest (45.61$\pm$1.45 pg/cell) when cultured in complete medium.

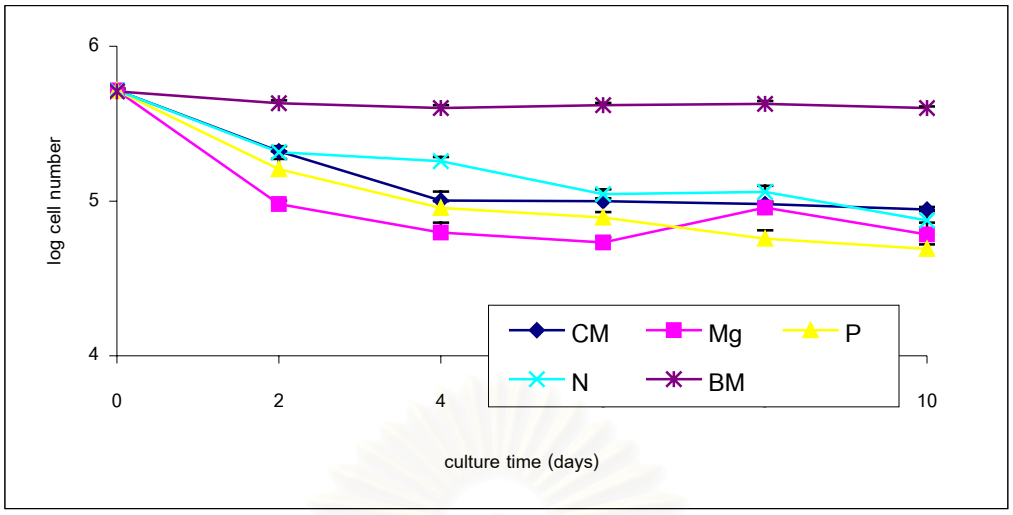



**Figure 30** Effect of nutrient deficiency on the cell numbers of *Haematococcus pluvialis* NIES-144 (mean value + standard deviation)

**Table 6** Effect of nutrient deficiency on pigment content of *Haematococcus pluvialis* NIES-144 after 10-day cultivation (mean value ± standard deviation)

| Experiment | Astaxanthin (pg/cell) | Chlorophyll (pg/cell) |
|---|---|---|
| Control | 16.04 ± 0.39 | 45.61 ± 1.45 |
| Without Mg | 36.34 ± 7.68 | 29.35 ± 6.07 |
| Without P | 26.64 ± 0.93 | 25.41 ± 1.60 |
| Without N | 44.09 ± 5.75 | 15.37 ± 1.96 |

BM = basal medium (medium for growth, no P, Mg)

CM = complete medium (with N, Mg , P)

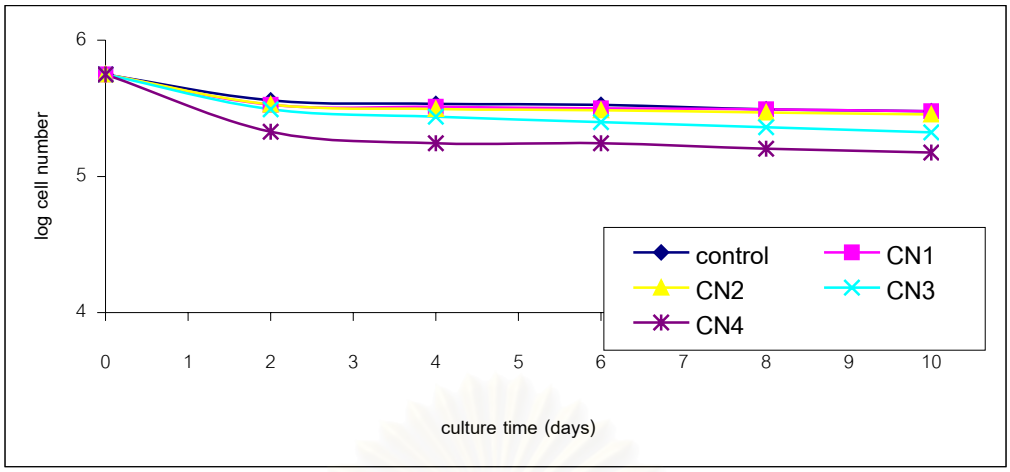
Mg = complete medium without Mg

P = complete medium without P

N = complete medium without N

### 2.3.2 Carbon and nitrogen ratio

The 5-day culture on Basal medium was supplemented with sodium acetate and sodium nitrate so as to adjust the C/N ratio of the medium. The acetate concentration supplemented to the culture was fixed at 45 mM , while sodium nitrate concentrations were adjusted to 0, 15, 45, 90 mM. The culture was cultivated at 20°C under the same condition as section 2.1.1. The alga cells slightly decreased after nitrate addition (Fig.31). As shown in Table 7 , under the high C/N ratio (C/N=$\alpha$) balance the astaxanthin content increased with the cultivation time after the supplementation. The astaxanthin content increased reaching 18 pg/cell after 10 day cultivation. Under high C/N ratio the chlorophyll content decreased.




Control = control (Basal medium)

CN1 = Basal medium ( yeast extract replaced with $NaNO_3$ )
($CH_3COONa$ : $NaNO_3$ = 45 : 0 mM) , (C/N = $\alpha$)

CN2 = Basal medium (yeast extract replaced with $NaNO_3$ )
($CH_3COONa$ : $NaNO_3$ = 45 : 15 mM) ,(C/N = 3)

CN3 = Basal medium (yeast extract replaced with $NaNO_3$
($CH_3COONa$ : $NaNO_3$ = 45 : 45 mM) ,(C/N = 1)

CN4 = Basal medium (yeast extract replaced with $NaNO_3$ )
($CH_3COONa$ : $NaNO_3$ = 45 : 90 mM) , (C/N = 0.5)

**Figure 31** Effect of carbon and nitrogen ratio in media on the cell number of *Haematococcus pluvialis* NIES-144

**Table 7** Effect of carbon and nitrogen ratio in media on pigment content per cell of *Haematococcus pluvialis* NIES-144 after 10–day cultivation

| ratio C:N | Astaxanthin(pg/cell) | Chlorophyll(pg/cell) |
|---|---|---|
| Control | 14.19 | **22.14** |
| CN1 | **17.45** | 18.50 |
| CN2 | 16.85 | 16.39 |
| CN3 | 16.54 | 21.05 |
| CN4 | 16.40 | 17.45 |

### 3. Effect of combined treatments

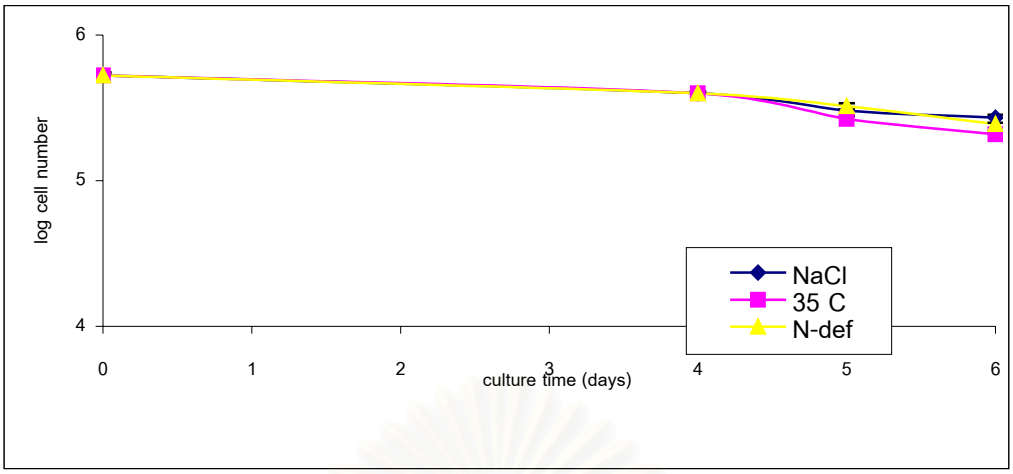
Green vegetative cells of *H. pluvialis* NIES-144 grew mixotrophically in the Basal Medium. When acetate was added to vegetative cells after 5-day, encystment and carotenogenesis occurred in cells. From section 2 , maximal astaxanthin content was obtained when cells were cultivated at 20 $^{o}$C under 30 $\mu Em^{-2}.s^{-1}$ continuous illumination with the supplementation 45 mM sodium acetate. The astaxanthin content was 18.65±0.37 pg/cell and 7.41 mg/l after 4 days cultivation(Table 8).

For the stimulation of astaxanthin accumulation in *H. pluvialis* NIES-144, the strategy of combined treatment was attempted, i.e. high temperature , high salinity, N-deficient medium after 9 days cultivation in Basal medium (with 45 mM sodium acetate). The cell number gradually decreased as shown in Fig 32. Astaxanthin accumulation was sharply increased by the combined treatment with, slightly lower astaxanthin was obtained by the combined treatment with salinity and with N-deficient medium. Highest astaxanthin was 31.51±1.84 pg/cell after 2 days cultivation. (Fig 33) In addition, total astaxanthin content(mg/l) was decreased when the culture was cultivated by combined treatments after 2 days cultivation (Fig 34). On the other hand, chlorophyll contents (pg/cell, mg/l) were not different, but sharply decreased with combined treaments after 2 days cultivation(Fig 35 and 36).

In this study, we applied a two-stage batch process for astaxanthin production from *H. pluvialis* NIES-144 which allowed mixotrophic

vegetative cell growth on Basal Medium followed by stress. The maximal astaxanthin accumulation per cell was obtained when culturing in N-deficient medium (44.09±5.75pg/cell) whereas the maximal total astaxanthin accumulation was obtained upon supplementation with 45 mM sodium acetate( 7.41 mg/l) as shown in Table 8.

The unicellular green alga *H. pluvialis* NIES-144 could accumulate large amounts of ketocarotenoid astaxanthin under environmental stress. Fig 37 shows morphological changes in the life cycle of green alga *H. pluvialis* NIES-144, (a) vegetative green cell with biflagella at 5 day, (b) immature cyst cell, (c) mature cyst cell with astaxanthin enlarging round cell. Fig. 40 shows the appearance of *Haematococcus pluvialis* NIES-144 culture at different life cycle.




35 C = 35 $^{0}$C

NaCl = + NaCl 0.6 %

N-def = + Medium without N

**Figure 32** Effect of combined treatments on the cell numbers of *Haematococcus pluvialis* NIES-144 (mean value + standard deviation)

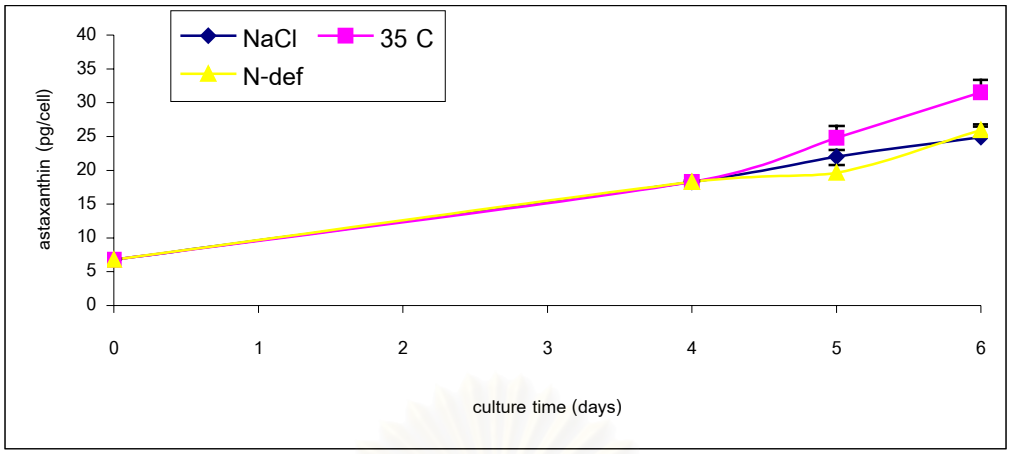



**Figure 33** Effect of combined treatments on astaxanthin content per cell of *Haematococcus pluvialis* NIES-144 (mean value + standard deviation)

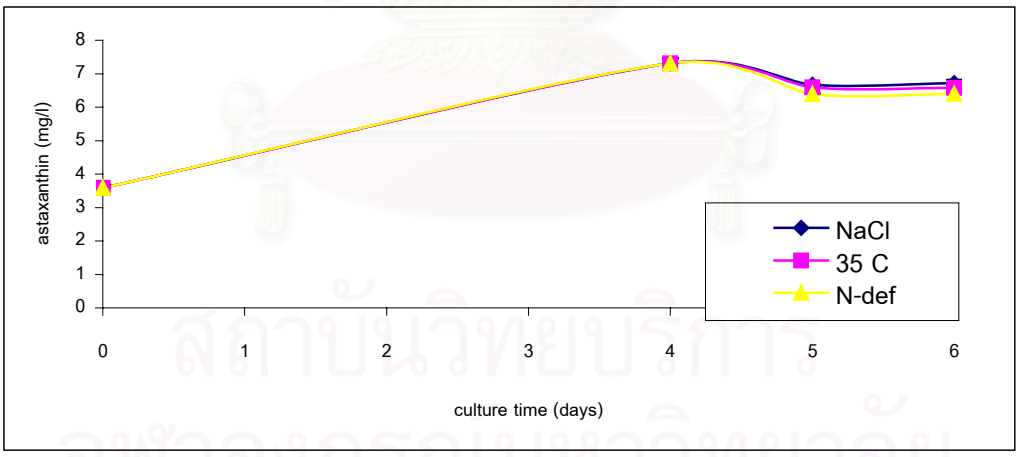



**Figure 34** Effect of combined treatments on total astaxanthin content of *Haematococcus pluvialis* NIES-144 (mean value + standard deviation)

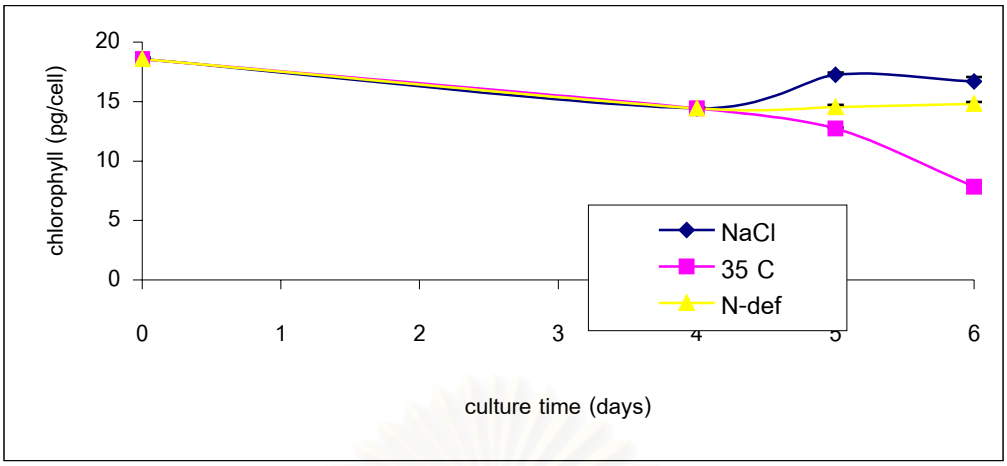



**Figure 35** Effect of combined treatments on chlorophyll content per cell of *Haematococcus pluvialis* NIES-144 (mean value + standard deviation)

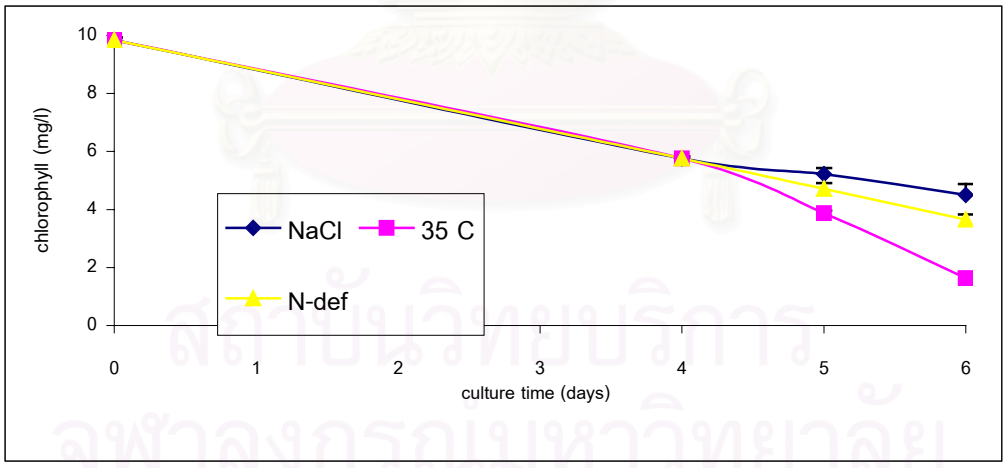



**Figure 36** Effect of combined treatments on total chlorophyll content of *Haematococcus pluvialis* NIES-144 (mean value + standard deviation)

**Table 8** Optimum conditions for astaxanthin formation of *Haematococcus pluvialis* NIES-144

| Factors | Condition for astaxanthin content per cell | | Condition for total astaxanthin produced | |
|---|---|---|---|---|
| | Condition | Max Astaxanthin content (pg/cell) | Condition | Max Total Astaxanthin (mg/l) |
| Light intensity | 200 μE/$m^2$.s for 10 days | 18.19 ± 1.20 | 160 μE/$m^2$s for 10 days | 6.38 |
| Temperature | 35 °C for 8 days | 38.37 ± 6.30 | 20 °C for 8 days | 5.07 |
| NaCl | 0.6 % NaCl for 10 days | 36.21 ± 3.18 | 0 % NaCl for 10 days | 5.09 |
| Nutrient | Without N For 10 days | **44.09 ± 5.75** | With N For 2 days | 4.33 |
| Oxidative stress | With Acetate 45 mM for 4 days | 18.65 ± 0.37 | With Acetate 45 mM for 4 days | **7.41** |
| C/N ratio | C/N = α (45:0) For 10 days | 17.45 | C/N = α(45:0) For 10 days | 5.23 |
| Combined treatment | Acetate+35°C For 6 days | 31.51±1.84 | Acetate+NaCl For 6 days | 6.71 |

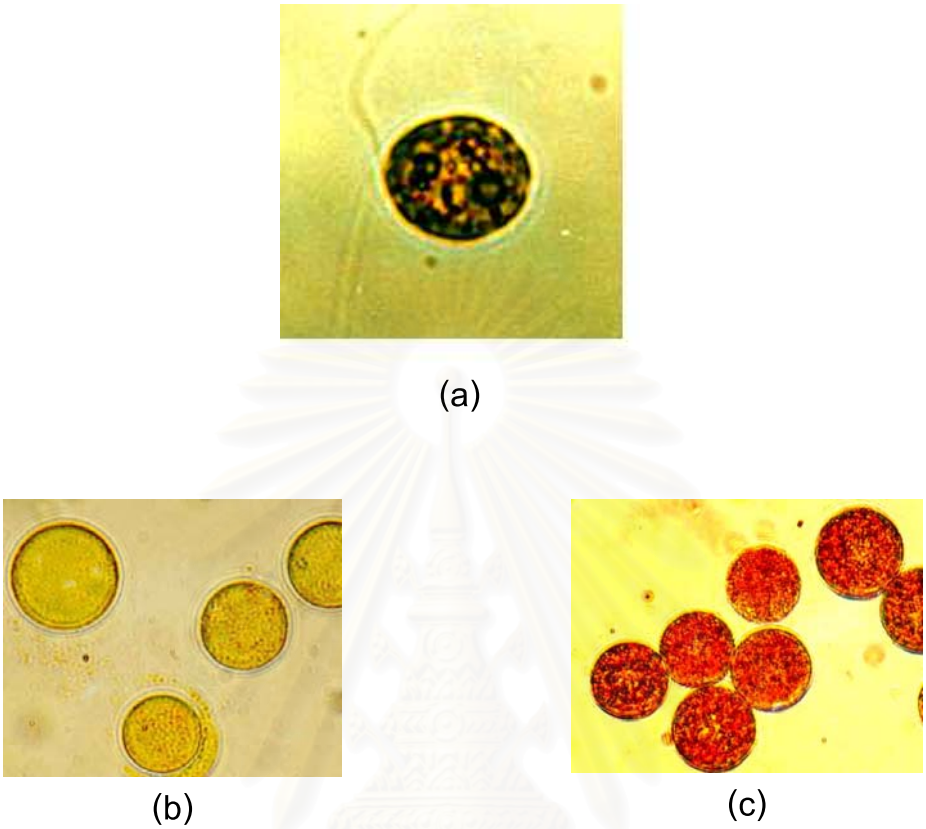



**Figure 37** Morphological changes in the life cycle of green alga *Haematococcus pluvialis* NIES-144 ; (a) vegetative green cell, (b) immature cyst cell, (c) mature cyst cell


a b c d

**Figure 38** Appearance of culture at different life cycle of *Haematococcus pluvialis* NIES-144

(a) Basal Medium, no culture

(b) Vegetative stage

(c) Immature cyst stage

(d) Cyst stage

**4. Analysis of astaxanthin from *Haematococcus pluvialis* NIES-144 by HPLC**

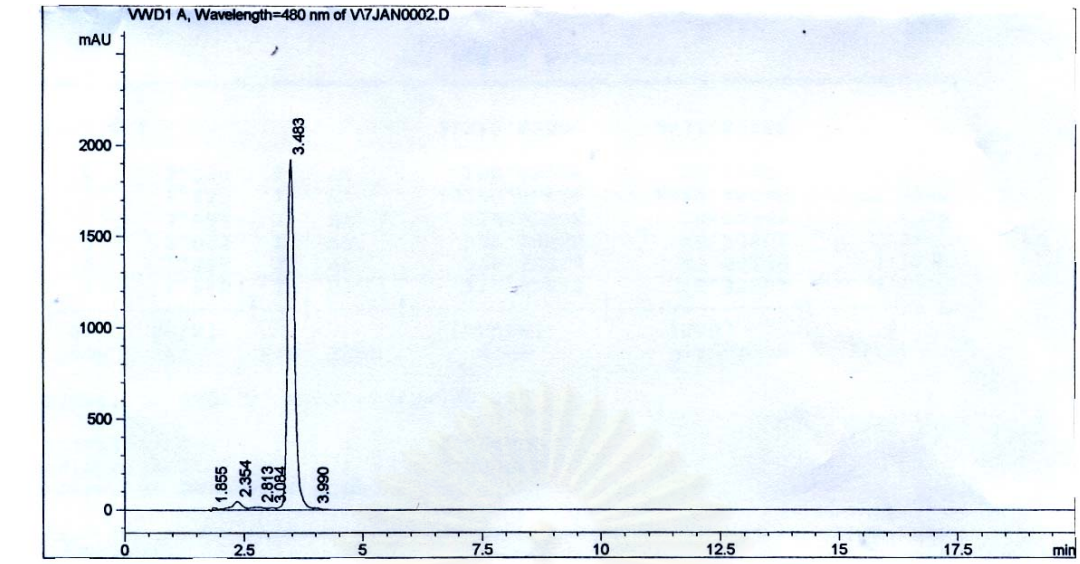
Pigments extracted from *Haematococcus pluvialis* NIES-144 were separated by HPLC reversed-phase $C_{18}$ ODS column. The solvent system consisted of solvent A (dichloromethane/methanol/acetonitrile/water, 5.0 : 85.0 : 5.5 : 4.5, v/v) and solvent B (dichloromethane/methanol/acetonitrile/water, 22.0 : 28.0 : 45.5 : 4.5 , v/v). The pigment extracts, pigment saponified extracts, standard astaxanthin solution were injected into a 20 μl loop. Step gradient from 100 % of solvent A for 8 min ; 0 to 100 % of solvent B for 6 min ; 100 % of solvent B for 51 min. The flow rate was 1.0 ml/min. Peaks were measured at a wavelength of 480 nm to facilitate the detection of astaxanthins.

As shown in Fig.39a, peaks were identified by typical retention time with standard astaxanthin at 3.4 min. The sharp peak of astaxanthin contained about 92% of total carotenoids. The chromatogram and chromatographic data obtained from the red pigment extract before saponification of *H. pluvialis* NIES-144 are shown in Fig.39b and Table 9a , respectively. Four main free astaxanthin fractions were separated and indicatified as free astaxanthins (RT 2.3, 2.5, 3.4, 3.7 min). Seven main astaxanthin ester fractions were separated and indicatified as astaxanthin esters (RT 3.1, 6.2, 11.6, 13.3, 15.1, 15.9, 22.0 min).

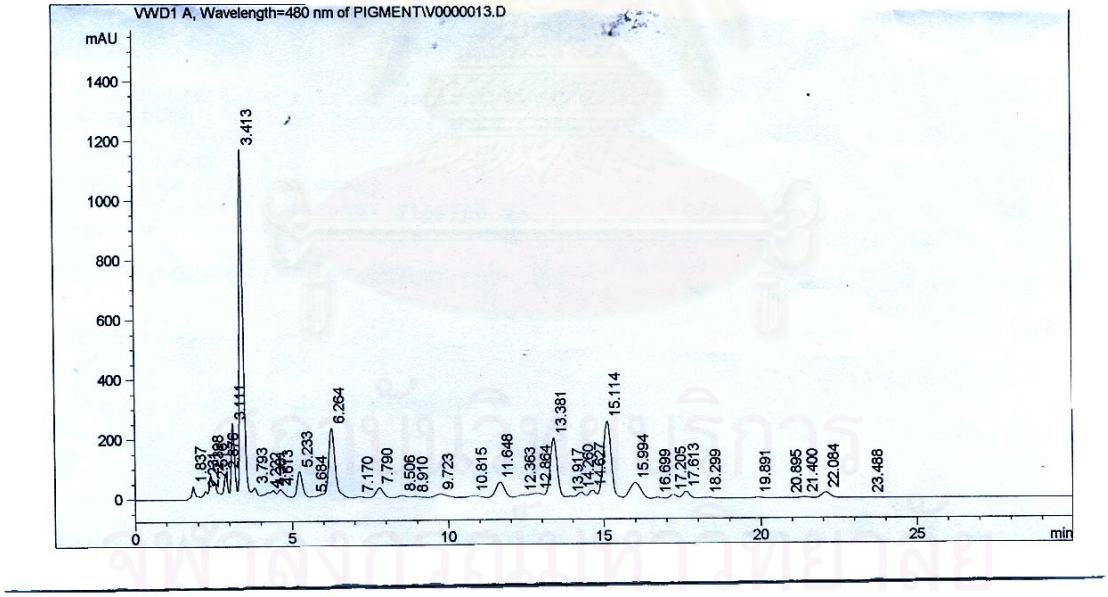
Fig.39c shows a typical chromatogram of the pigment saponified extract of *H. pluvialis* NIES-144. The identification of the

peak in Fig. 39c is the same as in Fig.39b. Five main free astaxanthin and three main astaxanthin ester fractions were separated and indicatified as free astaxanthins (RT 2.3, 2.5, 3.4, 3.7, 4.0min) and astaxanthin esters (RT 5.2, 13.3, 21.9 min).

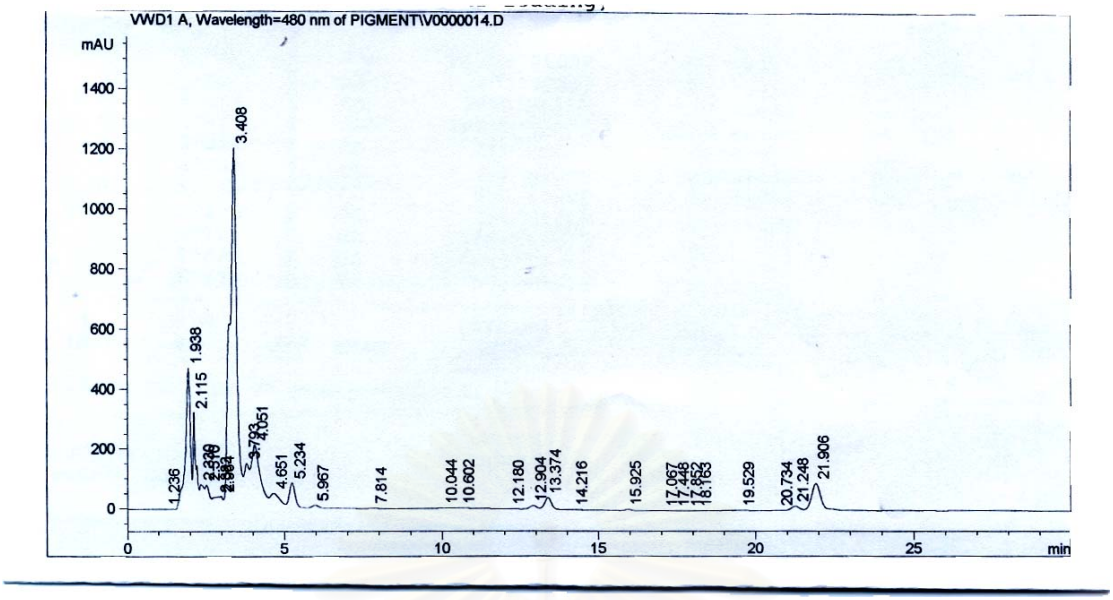
The results showed that pigment extracts from *H. pluvialis* NIES-144 contained about 75 % astaxanthin(free form and ester form) and 25 % other carotenoids(Table 9)




(a) standard astaxanthin




(b) pigment extracts




(c) pigment saponified extracts

**Figure 39** HPLC chromatogram of standard astaxanthin (a) ; pigment extracts (b) ; pigment saponified extracts (c) from *Haematococcus pluvialis* NIES-144.

**Table 9** Percent astaxanthins and carotenoids of pigment extracts from *Haematococcus.pluvialis* NIES-144.

(a) pigment extracts

| Free astaxanthin | | Astaxanthin esters | | Other carotenoid (free+esters) |
|---|---|---|---|---|
| RT (min) | % | RT (min) | % | % |
| 2.398 | 1.52 | 3.111 | 5.22 | |
| 2.516 | 1.45 | 6.264 | 10.62 | |
| 3.413 | 26.40 | 11.648 | 3.20 | |
| 3.793 | 1.22 | 13.381 | 8.90 | |
| | | 15.11 | 12.31 | |
| | | 15.949 | 3.47 | |
| | | 22.08 | 1.07 | |
| Total | 30.59 | Total | 44.79 | 24.62 |
| Total | 75.38 | | | 24.62 |

(b) pigment saponified extracts

| Free astaxanthin | | Astaxanthin esters | | Other carotenoid (free+esters) |
|---|---|---|---|---|
| RT (min) | % | RT (min) | % | % |
| 2.330 | 1.81 | 5.234 | 3.01 | |
| 2.510 | 1.99 | 13.37 | 1.52 | |
| 3.408 | 46.51 | 21.906 | 4.65 | |
| 3.793 | 4.09 | | | |
| 4.051 | 10.83 | | | |
| Total | 65.23 | Total | 9.18 | 25.59 |
| Total | 74.41 | | | 25.59 |

# Chapter IV

# Discussion

Two stage batch culture was used in this experiment. The first-stage was, to attain a high cell concentration in the growth medium and the second-stage was to enhance astaxanthin formation by various factors applied at the final period of the first-stage (Kobayashi et al. 1991). The best medium for biomass production was Basal Medium which contained yeast extract as the nitrogen source and sodium acetate as the carbon source. From the result in Fig. 5 when *H. pluvialis* NIES-144 was grown in Bold Basal Modified Medium and CHU-10 Medium, low specific growth rate and low cell numbers were obtained. The culture of *H. pluvialis* NIES-144 which was incubated at 20 $^{o}$C under 12-h light intensity and 12-d dark illumination in basal medium with 1.5 g/l of sodium acetate, 3.0 g/l yeast extract gave highest cell numbers and required only 5-day when cultured with $2x10^{5}$ cells per ml. The cell concentration of up to $5.6x10^{5}$ cells per ml as shown in Fig.21 and 22.



**Optimum conditions for astaxanthin synthesis in *H. pluvialis* NIES-144**

Fig.26 showed that the temperature to which the *H. pluvialis* NIES-144 culture were exposed had a significant effect on the level of astaxanthin to be accumulated in the cells. High temperature caused relatively large quantities of astaxanthin to be accumulated in the cells of *H. pluvialis* NIES-144. However exposure to high temperature

resulted in high rates of cell mortality. At lower temperatures the amount of astaxanthin accumulated was comparatively low but the survival rates of the alga were increased significantly. These results are in agreement with those of Tjahjono et al.(1994). Elevated culture temperatures may play two critical roles in carotenogenesis of *Haematococcus*. First, normal vegetative growth should be interrupted at a growth impermissible temperature, and second, higher temperature would stimulate formation and/or reactivity of active oxygen species that are endogenously generated from photosynthesis (Halliwell and Gutteridge,1989), which eventually contributed to hyper-accumulation of astaxanthin.

Light intensity dependence of both β-carotene and astaxanthin synthesis in *Dunaliella* and *H. pluvialis* NIES-144, respectively, has been studied extensively. (Krishna and Mohanty, 1998). However, as shown in Fig. 24 there were only slight differences for astaxanthin formation when *H. pluvialis* NIES-144 were exposed to 30, 80,120, 160, 200, 240 $\mu Em^{-2}.s^{-1}$. From these results, it seems that light energy was not so critical for generation of active oxygen species.(Tjahjono et al. 1994) Beside various stress factors were also found to induce astaxanthin accumulation in green alga, the main one being salinity. In *H. pluvialis* NIES-144, addition of 0.8 percent NaCl was shown to induce astaxanthin accumulation (Boussiba and Vonshak, 1991). From Fig.28 the addition of 0.2-1.0 % w/v NaCl to culture medium resulted in the initiation of astaxanthin formation in the alga, with highest level of astaxanthin being formed at 0.6 % w/v NaCl. However, such increase

in salinity was accompanied by high rates of cell mortality. It should be noted that algae living in an environment with high concentration of NaCl may have to adjust their physiological and structural properties to maintain the cells. (Krishna and Mohanty,1998)

Halliwell and Gutteridge, 1989 have observed that ferrous ion can generate hydroxyl radical by Fenton reaction as shown below

$$Fe^{2+} + H_2O_2 \longrightarrow OH^- + OH^- + Fe^{3+}$$

Sodium acetate can generate superoxide anion radical ($O^-_2$) and $H_2O_2$ respectively Kobayashi et al.1993. These oxygen species are known to cause extensive damage to chlorophyll , lipid and protein in plant cells.(Shaish et al.1993) Whether these oxygen species induce the carotenoid biosynthesis by means of which the algal cell can overcome their damaging effect is not yet clearly understood. Acetate , $Fe^{2+}$ , $H_2O_2$ were added to the 5-day culture to investigate their effect on astaxanthin formation (Table.5 and Fig.29). The results showed that thecell numbers were not changed.

It was found that with acetate addition astaxanthin was increased compared to that without addition whereas, hydrogen peroxide only slightly stimulated carotenogenesis. The result implied that intracellular active oxygen species, especially those generated in photosynthesis would be involved in the stimulation of carotenogenesis. (Tjahjono et al. 1994) In addition, inhibition of astaxanthin biosynthesis by $O^-_2$ and potassium iodide was explained to be due to the iron-catalyzed Haber-weiss reaction (Kobayashi et al. 1993), where $O^-_2$ is converted into OH by the following reaction :

$$O_2 + H_2O_2 \longrightarrow OH^- + OH^- + O_2$$

Similar results of triggering astaxanthin biosynthesis in *Haematococcus* were also obtained by Fan et al. (1996) with the help of singlet oxygen generators methyl blue and histidine. They suggested that it is a result of enzymatic antioxidant process.

The nutrition factors can affect astaxanthin biosynthesis in *H. pluvialis* NIES-144. In general, it was observed that deficiency of any nutrient leading to nutritional imbalance will induce secondary carotenoid production in this organism.(Krishna and Mohanty,1998) According to the result from Fig.30 where *H. pluvialis* NIES-144 were grown in four culture media with (a) complete medium (b) complete medium without N-source (c)complete medium without Mg-source (d) complete medium without P-source, astaxanthin cell content in N-deficient culture was the highest indicating that N-deficiency is most effective in blocking cell division and therefore in stimulating astaxanthin synthesis. Increased astaxanthin production in *H. pluvialis* NIES-144 deficient in nitrogen has also been reported earlier (Droop,1954 ; Goodwin, 1954 ; Czygan, 1970). Chodat (1938) and Borowitzka (1991) suggested that carbon to nitrogen ratio plays an important role in inducing astaxanthin biosynthesis in *H. pluvialis* NIES-144. These results were also confirmed by Kobayashi et al.(1992) who observed that high C/N ratio positively correlated with astaxanthin biosynthesis in *Haematococcus*. As acetate and sodium nitrate were the main carbon and nitrogen source respectively in the medium under the high C/N balance the astaxanthin content was

increased with the cultivation time after the supplementation. As shown in Table 7, astaxanthin content was higher when supplemented with the C/N ratio = 45: 0 than the C/N ratio = 45:15, 45:45, 45:90. From the results, it was suggested that cyst cells induced by a high C/N ratio possessed high astaxanthin content, whereas the chlorophyll content was drastically decreased. These results are in agreement with Spencer (1989) and Goodwin and Jamilcorn (1954) in that nitrogen deficient conditions induce astaxanthin formation in cell of *H. pluvialis* NIES-144. In addition, Coleman et al. 1988 reported that in unicellular green algae nitrogen starvation altered the partitioning of photosynthetically fixed carbon, resulting in an increased accumulation of carbohydrate and lipids.

The effect of oxidative stress was performed by combined treatments. Highest values of intracellular astaxanthin cellular content were obtained with high temperature whereas astaxanthin production (mg/l) was lower than that with 45 mM sodium acetate alone. It is possible that inclusive condition caused cell mortality, which would result in diminished astaxanthin accumulation.(Fig.33 and 34)

The results indicate clearly that physiological (light intensity, temperature) chemical (oxidative stress, salinity) and nutritional factors are potential inducers of astaxanthin formation in cells of *H. pluvialis* NIES-144. When cultivated in basal medium the alga can remain in a green, vegetative, state for a considerable period of time and it is only when the cultures age and nutrients such as nitrogen are depleted that growth becomes limited. The cell then encyst and accumulate

astaxanthin as shown in Fig 37. These results are in agreement with Boussiba (2000) in that astaxanthin accumulation in *Haematococcus* is induced by a variety of environmental stress which limits cell growth in the presence of light. This is accompanied by a remarkable morphological and biochemical 'transformation' from green motile cell into inert red cyst.

In conclusion, a number of factors relating to the cultivation of the microalga *H. pluvialis* NIES-144 can lead to the synthesis of the carotenoid astaxanthin and have been identified. Deficiency in nitrogen, high light intensity, high temperature, high C/N ratio and the addition of NaCl , to the culture medium are effective, especially when supplemented with 45 mM sodium acetate. Astaxanthin content reached 7.41 mg/l after 9 days cultivation. (Table 8 )

In order to increase astaxanthin content, the combined treatments were used. This strategy was based on preliminary experiments in our laboratory which showing that high temperature, salinity, nitrogen deficiency increased the astaxanthin concentration. The alga was grown in the basal medium as described in section 2.1.1. Firstly the culture was supplemented with 45 mM sodium acetate to stimulate astaxanthin synthesis and cultivation was continued for another 4 days. The final total astaxanthin content was 7.4 mg/l. Secondly, while the astaxanthin biosynthesis was actively operating, the culture was further treated with 0.6 % NaCl or high temperature (35$^{o}$C) or N-deficiency media for 2 days. Astaxanthin accumulation was sharply increased by the combined treatments.

However, total astaxanthin production in terms of mg/l was decreased. Our results seem to be in line with those reporting N-deficiency, high temperature (35°C), high salinity as major factors blocking cell division and stimulating the synthesis of astaxanthin (Borowitzka et al.1991 ; Kobayashi et al. 1997 ; Tjahjono et al.1994 ; Harker et al.1996). It can be concluded that combined treatments which interfere with certain cellular processes within the alga and hence limit growth or photosynthesis can act as a trigger for the process of encystment and astaxanthin formation.

The ketocarotenoid astaxanthin has been the focus of growing interest with respect to the use of this pigment as a food coloring agent, natural feed additive for the poultry industry and for aquaculture especially as a feed supplement in the culture of salmon, trout and shrimp (Johnson and Schroeder,1996). Recently, there have also been reports concerning its application in medicine due to its powerful antioxidant capacity. Though astaxanthin can be synthesized by plants, bacteria, a few fungi and green algae, the amounts accumulated by green alga *H. pluvialis* NIES-144 surpass any other reported sources (Boussiba, 2000).

The present experimental results showed the supplementation with 45 mM sodium acetate enhanced the astaxanthin accumulation. Highest concentration of astaxanthin (7.41 mg/l) was obtained after 9-day (Table 8). This gave similar astaxanthin yield obtained by various authors (Margalith, 1999). Current yield of astaxanthin 7-8 mg/l are still not sufficient to compete with chemical synthesis. However, the great

advantage of microbial astaxanthin as a nutriceutical will undoubtedly contribute to its commercial promotion. Furthermore, the synthetic formulations may contain unnatural configuration and cis-tran-isomers and carotenoid like compounds in the preparations that are not active form for animals. All these factors have contributed to interest in natural sources of carotenoid(Johnson and An, 1991)

In order to ascertain that astaxanthin is the major carotenoid, HPLC method was used for the separation and analysis of carotenoid in a green alga *H. pluvialis* NIES-144. Reversed-phase column, an octadecylsilane $C_{18}$ column, 150x4.6 mm was chosen for the separation of carotenoids. The solvent mixtures (as mobile phase) containing dichloromethane, acetonitrile, methanol and water.

According to the results from Fig. 39a and Table 9a retention time of standard astaxanthin at 3.4 min with a sharp peak was observed. Similar retention time of pigment extract from red-cyst cells was also observed at 3.4 min. Three main peaks (RT 2.3, 2.5, 3.7min) were separated , collected and indicated as isomers of astaxanthin. While seven main peaks(RT 3.1, 6.2, 11.6, 13.3, 15.1, 15.9, 22.0 min) were indicated as astaxanthin esters (Fan et al.1995 ; Yuan and Chen,1997). The total astaxanthins(free and its esters) content were estimated to be about 75 %.

In *H. pluvialis* NIES-144, astaxanthin is the major carotenoid and exists mainly as astaxanthin esters of various fatty acid(Johnson and An,1991). The complexity of the pigment composition in the algal extract makes it difficult to separate all of these pigments and

determine accurately the content of astaxanthin esters, because astaxanthin esters elute over a broad range of retention time(Sommer et al.1991 ; Juhl et al.1996). Therefore, the saponification of the algal extracts is recommended for reducing the number of chromatographic peaks. The addition of alkaline solutions(NaOH dissolved in methanol) which can break the pigment fatty acid bond (Ittah et al. 1993 ; Minquez-Mosquera and Perez-Galvez,1998), is necessary for the hydrolysis reaction of astaxanthin esters. The kinetic study of the hydrolysis of astaxanthin esters was carried out on the basis of the following reaction: (Yuan and Chen,1999)

Astaxanthin-FA + NaOH ⟶ Astaxanthin + Na-FA

Saponification was conducted to identify the individual astaxanthins. In this experiment, after saponification of the pigment extract by adding NaOH dissolved in methanol, the reaction mixture was separated by HPLC. Seven main peaks in Fig. 39b (RT 3.1, 6.2, 11.6, 13.3, 15.1, 15.9, 22.0 min) disappeared while five main peaks in Fig. 39c (RT 2.3, 2.5, 3.4, 3.7, 4.0min) appeared. The content of free astaxanthin and its isomer increased during saponification, indicating the inclusion of the product of saponification from the astaxanthin esters. The total free astaxanthin and its isomers content was determined by HPLC to be 65 % of total astaxanthins(74 %) and the individual content of the astaxanthin esters was 9 % of total astaxanthins, respectively.

Results of carotenoids in the unsaponified and saponified pigment extracts indicated no significant loss or isomerization of

carotenoids. As shown in Table 9, no differences in total carotenoids were observed after saponification. The contents of total carotenoids in the unsaponified and saponified pigment extracts were 24.62 and 25.59 %, respectively. The lack of carotenoid standards (canthaxanthin, lutein, echinenone, β-carotene) did not hinder the identification of astaxanthins from *H. pluvialis* NIES-144, because astaxanthins had been found to be the major carotenoids (over 80 % ) for *H. pluvialis* NIES-144 (Grung et al. 1992 ; Kobayashi et al. 1991).

The visible absorption spectra of astaxanthins and carotenoids were examined with a spectrophotometer and were found that the maximal absorption wavelengths of astaxanthin and astaxanthin esters were 475-493, respectively, which were in good agreement with those report by Yuan and Chen (1996,1998) ; Grung et al. (1992).

In this experiment, the detection wavelength was set at 492 nm to accept detection of astaxanthin and its esters. Thus, astaxanthins were detected in the extract of *H. pluvialis* NIES-144supporting the assumption that astaxanthin is the major carotenoids.

# Chapter V

## Summary

1.The optimal growth of *H. pluvialis* NIES-144 in the Basal medium with C/N ratio of 1.5:3.0 , initial cell number of $2x10^5$ cells per ml under light intensity of 30 $\mu Em^{-2}s^{-1}$, 12-h light 12-h dark at 20 $^oC$. The cell concentration of up to $5.6x10^5$ cells per ml was obtained.

2. Maximal astaxanthin content per cell was obtained in nitrogen deficient medium.

3. The highest total astaxanthin content (mg/l) was obtained by supplementation to the medium with 45mM sodium acetate. Astaxanthin content reached 7.41 mg/l after 9 days cultivation.

4. The optimal conditions for astaxanthin accumulation in *H. pluvialis* NIES-144 was obtained in Basal Medium at 20 $^oC$ under 30 $\mu Em^{-2}s^{-1}$ continuous illumination with the supplementation of 45 mM sodium acetate.

5. No effect of combined treatments on the total astaxanthin content (mg/l) was observed whereas the astaxanthin content per cell increased.

6. Astaxanthin analysis was accomplished by HPLC on a reversed-phase $C_{18}$ column with dichloromethane : methanol : acetonitrile : water solvent systems. Astaxanthins contents were found to account for 75 % of total carotenoids.

# References

Armstrong, GA and Hearst, JE.1996. Genetics and molecular biology of carotenoid pigment biosynthesis. **FASEB J.** 10: 228-237.

Barbera,E., Tomas,X., Moya,MJ., Ibanez,A., Molins,MB.1993. Significance test in the study of the specific growth rate of *Haematococcus lacustris* : Influence of carbon source and light intensity.**J Ferment.Bioeng.** 76(5) : 403-405.

Bauernfeind, J C.1981. **Carotenoids, Colorants and Vitamin A Precursors,Technological and Nutritional Applications.** New York: Academic Press, p.38.

Ben-Amotz, A ., Shaish,A., Avron, M.1989. Mode of action of the massively accumulated β–carotene of *Dunaliella badawil* in protecting the alga against damage by excess irradiation. **Plant physiol.91** : 1040-1043.

Ben-Amotz, A and Shaish, A.1993. **In *Dunaliella* Physiology, Biochemistry and Biotechnology.** edited by M Avron and A Ben-Amotz. London : CRC Press,p.205.

Beyer, P and Kleinig, H. 1990. **In Carotenoids chemistry and Biology** , edited by N I Krinsky,M M Mathews-Roth, and R F Taylor. New York : plenum Press, p.195.

Bjerkeng, B., Storebakken, T.,Jensen, S.L.1991. Response to carotenoids by rainbow trout in the sea : resorption and metabolism of dietary astaxanthin and canthaxanthin.**Aquaculture.** 91 : 153-162.

Bold, H.C. and Wynne, M.J. 1985. **Introduction to the Algae.** Englewood Cliffs, New Jersey :Prentice Hall, 573 pp.

Borowitzka, MA. 1989. **Fat, oils and hydrocarbons.Cambridge** : Cambridge University Press,p.27-58.

Borowitzka, MA., Huisman,JM., Osborn,A.1991. Culture of the astaxanthin producing green alga *Haematococcus pluvialis.* 1) Effect of nutrient on growth and cell type. **J Appl Phycol.** 3:295-304.

Boussiba, S., Fan, L., Vonshak, A.1992.Enhancement and determination of astaxanthin accumulation in green alga, *Haematococcus pluvialis*. **Methods Enzymol.** 213:386-391.

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

Boussiba, S.2000. Carotenogenesis in the green alga *Haematococcus pluvialis*: Cellular physiology and stress response.**Physiologia Plantarum.** 108 : 111-117.

Britton, G.1983. Carotenoid biosynsis in higher plants. **Physiol.Veg.**20, 735.

Chotdat, F.1938. Etudes sur la genes des carotenoid. **Arch Sci(Geneve).** 20 : 96.

Choubert, G and Heinrich, O.1993. Carotenoid pigments of the green alga *Haematococcus pluvialis* : assay on rainbow trout, *Oncorhynchus mykiss*, Pigmentation in comparison with synthetic astaxanthin and canthaxanthin. **Aquaculture.** 112 : 217-226.

Chumpolkulwong, N.,Kakizono, Y., Handa, T., Nishio, N. 1997.Enzymatic conversion of β-carotene to astaxanthin by cell-extracts of a green alga *Haematococcus pluvialis*. **Biotechnol Lett.** 19 : 443-446.

Coleman, L.W., Rosen, B.H., Schwartzbach, S.D.1988. Environmental control of carbohydrate and lipid synthesis in Euglena. **Plant Cell Physiol.**29 : 423-429.

Cordero, B ., Otero ,A., Patino,M., Arredondo, B.O., Fabregas, J.1996. Astaxanthin production from the green alga *Haematococcus pluvialis* with different stress condition. **Biotechnology letters.**18 (2): 213-218.

Czygan, F-C.1970.Blood-rain and Blood-snow : nitrogen deficient cells of *Haematococcus pluvialis* and *Chlamydomonas nivalis*. **Arch Mikrobiol.** 74 : 69-76.

Davies, B.H.1976. Carotenoids. **In Chemistry and Biochemistry of Plant Pigments**. 2nd Edition, Vol. 2. Edited by Goodwin, T.W.pp.38-165.

Donkin, P.1976. Ketocarotenoid biosynthesis by *Haematococcus lacustris*. **Phytochemistry.** 15 : 711.

Droop, M R.1954.Conditions governing *Haematococcus* formation and loss of alga *Haematococcus pluvialis* Flotow. **Arch Mikrobiol.** 20 : 391-397.

Droop, M.R.1955. Carotenogenesis in *Haematococcus pluvialis*, **Nature.**175,42.

Elstner, E F.1987. **in Biochemistry of Plants.** Vol.2., edited by Davies, p.253.

Fabregas, J. , Dominguez, A., Alvarez ,D.G ., Lamela ,T .1998. Induction of astaxanthin accumulation by nitrogen and magnesium deficiencies in *Haematococcus pluvialis.* **Biotechnology letters.** 20 (6) : 623-626.

Fan,L., Vonshak, A., Boussiba, S.1994. Effect of temperature and irradiance on growth of *Haematococcus pluvialis* (Chlorophyceae). **J.Phycol.** 30 (5) : 829-833.

Fan, L., Vonshak, A., Gabbay, R., Hirschberg, J., Cohen, Z., Boussiba, S.1995. The biosynthetic pathway of astaxanthin in green alga *Haematococcus pluvialis* as indicated by inhibition with diphenylamine.**Plant Cell Physiol.** 36 : 1519-1524.

Fraser, PD., Mivra, Y., Misawa, N.1997. In Vitro characterization of astaxanthin biosynthetic enzymes. **J.Biol Chem.** 272 : 6168-6135.

Goodwin, TW and Jamikom, M.1954.Studies in carotenogenesis : II Carotenoid synthesis in the algae *Haematococcus pluvialis*. **Biochem J.** 57 : 376-381.

Grung, C., Braune, W., Birckner, E.,Nuske, J.1993. Functional aspects of secondary carotenoids 2. *Haematococcus pluvialis* aplanospores as a source of (3S,3'S)-astaxanthin esters. **J Appl Phycol.** 4 :165-171.

Grung, M., Souza, D., Borowitzka, M., Lianaen-Jensen, S. 1992. Algal carotenoid 51.Secondary carotenoids 2. *Haematococcus pluvialis* aplanospores as a source of (3S,3'S) astaxanthin esters. **J.of Applied Phycology**. 4: 165-171.

Halliwell, B and Gutteridge, J.M.C.1989. Free radicals in biology and Medicine. **Oxford** : Oxford University Press.

Harker,M ., Tsavalos, AJ ., Young, AJ.1996. Factors responsible for astaxanthin formation in the chlorophyte *Haematococcus pluvialis*. **Bioresource Technology.** 55 : 207- 214.

http://www.microscopy-uk.org.uk.

Ittah,Y., Kanner, J., Granit, R.1993. Hydrolysis study of carotenoid pigments of paprika by HPLC/photodiode array detection. **J.Agric.Food Chem.**41 : 899-901.

Johnson, EA and AN, GH .1991. Astaxanthin from microbial sources.**Crit Rev Biotechnol.** 11:297-326.

Jorgensen, K and Skibsted, L H.1993.Carotenoid scarvenging of radicals. Effect of carotenoid structure and oxygen partial pressure on antioxidative activity. **Z Lebensm. Unters. Forsch.**196 : 197.

Juhl, A.R., Ohman, M.D., Goericke, R.1996. Astaxanthin in Calanus pacificus:Assessment of pigment-based measures of omnivory.Limnol. **Oceanogr.** 41 : 1198-1207.

Kobayashi, M ., Kakizono, T., Nishio , N ., Nagai, S. 1991. Astaxanthin production by a green alga, *Haematococcus pluvialis* accompanied with morphological changes in acetate media. **J.Ferment.Bioeng.** 71(5):335-339.

Kobayashi, M., Kakizono, T., Nishio, N., Nagai, S .1992 a. Effects of light intensity, light quality, and illumination cycle on astaxanthin formation in a green alga, *Haematococcus pluvialis*. **J.Ferment.Bioeng.** 74(1) : 61-63 .

Kobayashi, M., Kakizono, T., Nishio, N., Nagai, S .1992 b. Effect of carbon/nitrogen ratio on encystment accompanied with astaxanthin formation in a green alga, *Haematococcus pluvialis*. **J.Ferment.Bioeng.** 74(6):403-405.

Kobayashi, M., Kakizono,T ., Nishio, N., Nagai, S .1993. Enhanced carotenoid biosynthesis by oxidative stress in acetate-induce cyst cells of a green unicellular alga, *Haematococcus pluvialis*. **Applied and environmental microbiology.** 59(3) :867-873.

Kobayashi,M., Korimura, Y., Kakizono, T., Nishi, N., Tsuji, Y.1997. Light-independent, astaxanthin by the green microalga *Haematococcus pluvialis* under salt stress.**Biotechnology letters.** 19 (6) : 507-509.

Krishna, KB and Mohanty, P .1998. Secondary carotenoid production in green algae. **J of scientific and industrial research.** 57 : 51-63.

Margalith, P.Z.1999. Production of ketocarotenoids by microalgae. **Appl Microbiol Biotechnol.** 51 : 431-438.

Miki, W. 1991. Biological functions and activities of animal carotenoids. **Pure Appl Chem.** 63:141-146.

Orset, S., Harker, M., Tsavalos, AJ., Rijo, P., Mcgovern, K., Young, AJ.1995.Inhibition of astaxanthin synthesis in the green alga *Haematococcus pluvialis*. In: Mathis P.(ed) Photosynthesis : from light to bisphere. **Proceedings of the 10th International Photosynthesis Congress.** 4 : 59-62.

Santos, FM and Mesquita, JF.1984. Ultrastructural study of *Haematococcus lacustris* (Girod) Rostafinski (Volvocales). I. Some aspects of carotenogenesis.**Cytologia**. 49 : 215-228.

Schroeder, W.A., Calo, P., Declercq, M.L., Johnson, EA.1996.Selection For carotenogenesis in the yeast *Phaffia rhodozyma* by dark-generated singlet oxygen.**Microbiology**.142 : 2923-2929.

Seely, Gm., Duncan, M.J., Widaver, W.E.1972. Preparative and analyticalextraction of pigments from brown algae with dimethylsulfoxide. **Mar.Biol**.12:184-188.

Shaish, A., Avron, M., Pick, U., Ben-Amotz, A.1993. Are active oxygen species involved in induction of β-carotene in *Dunaliella bardawil*? **Planta.** 190 : 363-368.

Simpson, K.L., Katayama, T., Chichester, C.O.1981.**Carotenoids in fish feeds**.London : Academic Press, p.463-538.

Smith, G.M.1950.**Freshwater algae of the United States**.2nd ed. California : Mcgraw Hill Book Co, p109-111.

Sommer, T.R., Potts, W.T., Morrissy, N.M.1991.Utilization of microalgal astaxanthin by rainbow trout(Oncorhynchus mykiss). **Aquaculture**. 94 : 79-88.

Spencer, K.G .1989.Pigmentation supplements for animal feed composition.US. **Patent No.**4871551.

Tan, C K., Lee, Y K., Ho, K K. 1995. **J Appl Phycol.** 5 :547.

Tjahjono, AE., Hayama, Y., Kakizono, T., Terada, Y., Nishio, N ., Nagai,S .1994. Hyper-accumulation of astaxanthin in a green Alga *Haematococcus pluvialis* at elevated temperatures. **Biotechnology Letters.** 16 (2) :133-138.

Torrissen, O.J., Hardy, R.W., Shearer, K.D. 1989. Pigmentation of salmonids_carotenoid deposition and metabolism in salmonids. **Cri.Rev.Aquatic Sci.** 1, 209.

Yuan, J.P and Chen,F.1996. Separation and identification of astaxanthin ester and chlorophylls in *Haematococcus lacustris* by HPLC.**J.Agric.Food Chem.**10 : 655-660.

Yuan, J.P and Chen, F.1997. Identification of astaxanthin isomers in *Haematococcus lacustris* by HPLC-photodiode array detection. **J.Agric.Food Chem.**11 : 455-459.

Yuan, J.P and Chen.1998.Chromatographic Separation and Purification of trans-Astaxanthin from the Extracts of *Haematococcus pluvialis*. **J.Agric.Food Chem.**46:3371-3375.

Yuan, J.P and Chen, F.1999. Hydrolysis kinetics of astaxanthin esters and stability of astaxanthin of *Haematococcus pluvialis* during saponification. **J.Agric.Food Chem**.47:31-35.

# Appendix

# Appendix

## Appendix 1 The Basal medium (Kobayashi et al.1991)

| | | |
|---|---|---|
| $FeSO_4.7H_2O$ | 0.01 | g/l |
| $CaCl_2.2H_2O$ | 0.02 | g/l |
| $CH_3COONa.3H_2O$ | 1.2 | g/l |
| Yeast extract | 2.0 | g/l |
| L-asparagine | 0.4 | g/l |

pH 6.8

## Appendix 2 The Bold Basal modified Medium (Spencer , 1989)

| | | |
|---|---|---|
| $CH_3COONa.3H_2O$ | 1.4 | g/l |
| $(NH_2)_2CO$ | 0.12 | g/l |
| NaCl | 12.5 | mg/l |
| $CaCl_2.2H_2O$ | 12.5 | mg/l |
| $MgSO_4.7H_2O$ | 38 | mg/l |
| $K_2HPO_4$ | 93 | mg/l |
| $KH_2PO_4$ | 44 | mg/l |
| EDTA | 25 | mg/l |
| $FeCl_3$ | 2.5 | mg/l |
| $Na_2MoO_4.2H_2O$ | 0.3 | mg/l |
| $CoCl_3$ | 0.23 | mg/l |
| $CuSO_4$ | 0.77 | mg/l |
| $MnCl_2$ | 0.73 | mg/l |
| $ZnSO_4$ | 4.4 | mg/l |

pH 7.3

## Appendix 3 Modified CHU-10 Medium (H.C.Bold ,1978)

| | g/100 ml of distilled $H_2O$ | 982 ml of distilled $H_2O$ |
|---|---|---|
| $CaCl_2.2H_2O$ | 3.67 | 1 |
| $MgSO_4.7H_2O$ | 3.69 | 1 |
| $NaHCO_3$ | 1.26 | 1 |
| $K_2HPO_4$ | 0.87 | 1 |
| NaNO3 | 8.50 | 1 |
| $Na_2SiO_3.H_2O$ | 2.84 | 1 |
| *Citrate ferric solution | | 1 |
| **Micro nutrient solution | | 1 |

* Citrate ferric solution

| | 100 ml of distilled $H_2O$ |
|---|---|
| Citric acid | 3.35 g |
| Ferric citrate | 3.35 g |

* Keep the solution in amber recipient

* * Micronutrient solution

| | 1000 ml distilled $H_2O$ |
|---|---|
| $Na_2$-EDTA | 50 mg |
| $H_3BO_3$ | 618.8 mg |
| $CuSO_4.5H_2O$ | 19.6 mg |
| $ZnSO_4.7H_20$ | 44 mg |
| $CoCl_2$ $6H_20$ | 20 mg |
| $MnCl_2.4H_20$ | 12.6 mg |
| $Na_2MoO_4.2H_20$ | 12.6 mg |

Dissolve the cited reactions in 100 ml of distilled water. Autoclave at 15 lb/$in^2$/120$^oC$ for 15 minutes.

* The citrate ferric solution is sterilized under the same conditions . Kept refrigerated in amber recipient.

* * Micronutrient reactants are dissolved in 1000 ml of distilled water , which is sterilized using same conditions cited above. Keep refrigerated.

Final mixture of substances is done once the solution are at room temperature.

Appendix 4 The medium for *Haematococcus lacustris* ATCC 30453 (Barbera et al.1993) : (complete medium, CM)

| | | |
|---|---|---|
| $K_2HPO_4$ | 75 | mg/l |
| $KH_2PO_4$ | 175 | mg/l |
| $CaCl_2.2H_2O$ | 25 | mg/l |
| NaCl | 25 | mg/l |
| $MgSO_4.7H_2O$ | 75 | mg/l |
| $NaNO_3$ | 250 | mg/l |
| EDTA | 25 | mg/l |
| $FeCl_3$ | 2.5 | mg/l |
| $Na_2MoO_4.2H_2O$ | 0.35 | mg/l |
| $ZnSO_4$ | 4.4 | mg/l |
| $MnCl_2$ | 0.73 | mg/l |
| $CuSO_4$ | 0.77 | mg/l |
| $CoCl_3$ | 0.23 | mg/l |
| $CH_3COONa.3H_2O$ | 1.0 | g/l |

pH 7.0

## Appendix 5 Specific growth rate (Black , 1996)

$$\ln N - \ln N_o = \mu(t\text{-}to)$$

$$\log N - \log N_o = \frac{\mu(t\text{-}to)}{2.303}$$

$$\mu = \frac{2.303}{t\text{-}t_o} \log \frac{N}{No}$$

when $N_o$ = number of algal at zero time

$N$ = number of algal at the end time

$t_o$ = time at zero

$t$ = time at the end of a selected period

$\mu$ = specific growth rate

# Biography

Mr. Nutthawee Thamjedsada was born on June 30 , 1976 in Yala , Thailand. He graduated with a Bachelor Degree in Biochemistry from Faculty of Science, Chulalongkorn University in 1998 and studied for a Master Degree in Biotechnology programme in 1998.