CHAPTER 2

MATERIALS AND METHODS

MATERIALS

1) Equipment

Equipment/Model	Company/Country
pH meter PHM 83	Radio, Copenhagen; Denmark.
Autoclave HA-3D	Hirayama Manufactory Cooperation, Japan.
Laminar Flow BVT-124	International Scientific Supply, Co. Ltd. Thailand.
Microscope	Olympus, USA.
Digital Lux Meter FT 710	Taiwan.
Spectrophotometer DU 650	Beckman, USA.
Illuminated/Refrigerated Orbital Incubator 101400: XX2C	Sanyo,England.
Rotavapor RE 111	Switzerland.
Water bath Buchi 461	Switzerland.
Flexi-Dryer µP	PST System, Stone Ridge, New York, USA.

Equipment/Model	Company/Country
Centrifuge H-103 N Series	Kokusan.
Refrigerated centrifuge J-21C	Beckman Instrument Inc., USA.
High Performance Liquid Chromatography Equipments	Shimadzu Cooperation, Kyoto, Japan.
Liquid Chromatograph Model LC- 3A	
Gradient Programmer Model GRE- 3A	
LDC analytical spectromonitor- Programmable Visible Wavelength detector	
C-R1A chromatopac recorder	

2) Chemicals

Chemicals	Company/Country
Calcium chloride Magnesium sulfate Dipotassium hydrogen - phosphate Dihydrogen potassium-phosphate Potassium nitrate Ethylenediaminetetra acetic acid	Merck Ag Darmstadt, Germany.
Sodium acetate Urea Sodium chloride	Carlo Erba Division Chemical, Italy.
Sodium molybdate Zinc sulfate Ethyl acetate Copper sulfate Cobolt chloride	BDH Laboratory Chemical Division, England.
Ferric sulfate Acetone	Mallinckrodt Chemical, USA.

Chemicals	Company/Country
Yeast extract	Oxoid Unipath Ltd., England.
Dimethyl sulfoxide	Sigma Chemical Company, USA.
N-Hexane	Genzyme Koch-Light Limited
Chloroform	England.

3) Specimen

A green alga, *Haematococcus pluvialis* strain NIES 144 was a kind gift from Assistant Professor Somkiat Piyatiratitivorakul from Marine Science Department, Chulalongkorn University. The organism was originally from the National Institute for Environment Studies, Tsukuba, Japan

METHODS

H. pluvialis NIES144 was cultured in The Basal medium (Kakizono, Kobayashi, and Nagai, 1992). For the basal culture, a 20-ml portion of a 4-day culture was inoculated into 200 ml of fresh medium in 500-ml Erlenmeyer flask. The flask was incubated at 21-23° C under a 12-h light -12-h dark illumination cycle at 20μ mol m^{-2} s^{-1} (fluoresence light). The flask was shaken manually once a day. The 4-day

culture(vegetative growth phase,ca. 5.5 x 10^5 cells per ml) was employed for the study.

1. Effect of Environmental Factors on Growth of *H. pluvialis* NIES 144

1.1. Type of Medium

H. pluvialis NIES 144 was cultured in Basal Medium ;BM(Kakizono, Kobayashi, and Nagai,1992), Bold Basal Medium; BBM(Spencer, 1989) and medium for *H. lacustris* ATCC 30453;MFH (Eduard, Xavier, Maria-Jose, Augusti, and Maria-Begona, 1993) with different initial cell numbers of 1, 5, 10, 15 and 20 x 10^4 cells per ml. A 50 ml of culture in a 250 ml Erlenmeyer flask was incubated at 21-23° C under a 12-h light - 12-h dark illumination cycle at 20 μ mol m^{-2} s^{-1}. The culture was shaken manually once a day. At 2 days intervals, the cell number was counted by a Haemacytometer under a microscope with a magnification of 100X and a growth rate was analyzed in terms of specific growth rate (μ)as shown in appendix 4.

1.2. Type and Concentration of Carbon Source

H. pluvialis NIES 144 was cultured in their types of media as described in section 1.1. But carbon source was changed from CH_3COONa to Na_2CO_3 and to CO_2 from air and carbon source concentration was changed to 1.2gram per litre as shown in table1. Initial cell number was kept at 5x10^4 cells per ml and the growth rate was performed as described in section 1.1.

Type of Medium	Source of Carbon (g/l)	Source of Nitrogen (g/l)
BM control	(1.2) CH_3COONa	(2.0) yeast extract
BM mod.1	no addition	(2.0) yeast extract
BM mod.2	(3.1) Na_2CO_3	(2.0) yeast extract
BBM control	(1.4) CH_3COONa	(0.12) urea
BBM mod.1	(1.2) CH_3COONa	(0.12) urea
BBM mod.2	no addition	(0.12) urea
BBM mod.3	(3.1) Na_2CO_3	(0.12) urea
MFH control	(1.0) CH_3COONa	(0.25) $NaNO_3$
MFH mod.1	(1.2) CH_3COONa	(0.25) $NaNO_3$
MFH mod.2	no addition	(0.25) $NaNO_3$
MFH mod.3	(3.1) Na_2CO_3	(0.25) $NaNO_3$

Table 1 Carbon and nitrogen content for carbon modified in three types of media

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1.3. Concentration of Nitrogen Source

H. pluvialis NIES 144 was cultured as described in section 1.2. For Bold Basal Modified Medium and medium for *H. lacustris* ATCC 30453, Urea and $NaNO_3$ concentrations were changed to 0.42 and 1.195 gram per litre respectively as shown in table 2. The cell number and cell growth were calculated as described in section 1.1.

2. Effect of Environmental Factors on Growth of *H. pluvialis* NIES 144 in Basal Medium

2.1. Light Intensity

A 50 ml. of culture in a 250 ml Erlenmeyer flask was inoculated with initial concentration at $5x10^4$ cells per ml. under 12-h light - 12-h dark illumination cycle at 21-23° C. The culture was shaken once a day. The light intensities were adjusted to 20, 40, and 60 μ mol m^{-2} s^{-1}. At 2 days intervals, the cell number was counted and cell growth was analyzed in terms of specific growth rate (μ) as described in section 1.1.

2.2. Content of Carbon and Nitrogen Source

CH_3COONa and yeast extract were used as carbon and nitrogen source of Basal medium. The cell number was kept at $5x10^4$ cells per ml. The contents of CH_3COONa were changed to 0.8, 1.2, 1.6 and 2.0 gram per litre and the contents of yeast extract were changed to 1.0, 2.0 and 3.0 gram per litre as shown in table3 . At 2 days intervals, the growth analysis was performed as described in section 2.1.

Type of Medium	Sodium Acetate Content (g/l)	Source of Nitrogen (g/l)
BM control	1.2	(2.0) yeast extract
BBM control	1.4	(0.12) urea
BBM mod.1	1.2	(0.12) urea
BBM mod.2	1.2	(0.42) urea
MFH control	1.0	(0.25)$NaNO_3$
MFH mod.1	1.2	(0.25)$NaNO_3$
MFH mod.2	1.2	(1.20)$NaNO_3$

Table 2 Carbon and nitrogen content for nitrogen modified in three types of media

The Basal Medium	Sodium Acetate Content (g/l)	Yeast Extract Content (g/l)
mod.1	0.8	1.0
mod.2	1.2	1.0
mod.3	1.6	1.0
mod.4	2.0	1.0
mod.5	0.8	2.0
mod.6	1.2	2.0
mod.7	1.6	2.0
mod.8	2.0	2.0
mod.9	0.8	3.0
mod.10	1.2	3.0
mod.11	1.6	3.0
mod.12	2.0	3.0

Table 3 Sodium acetate and yeast extract content for the Basal modified medium

2.3. Initial Cell Number

H. pluvialis NIES 144 was cultured in Basal Medium. The initial cell numbers were changed to 10, 15, 20, 40, 80, 100, 150, and 200 $x10^4$ cells per ml. The condition of culture and the growth analysis was performed as described in section 1.1.

3. Effect of Environmental Factors on Astaxanthin Content in *H. pluvialis* NIES 144

H. pluvialis NIES 144 was cultured in the Basal Medium. The initial cell number was 20 x 10^4 cells per ml. and incubated at 21-23° C under a 12-h light - 12-h dark illumination cycle at 20 μ mol m^{-2} s^{-1}. The cultured was shaken manually once a day. At 2 days intervals, the cell number was counted by a Haemacytometer under microscope with a magnificent of 100X. The pigment were extracted by either dimethylsulfoxide (DMSO) or acetone.

Extraction with Dimethyl Sulfoxide (DMSO)

1-2 ml. of *H. pluvialis* NIES 144 was centrifuged at 3500 rpm for 10 min. The supernatant was discarded, the cell pellet was resuspended in 1-3 ml of DMSO and 1 drop of acetic was added and homogenized for 5 min. to recover the pigment. The mixture was heated at 70° C for 5 min. This last step was repeated if necessary until the cell debris was totally white. The mixture was centrifuged again of 3500 rpm for 10 min. and the content of astaxanthin was determined by spectrophotometric measurement of the supernatant at 492 nm. The amount of astaxanthin was calculated according to Davies (1976) with $E^{1\%}_{1cm}$ of 2220.

Chlorophyll content was determined at 673 nm and calculated with $E^{1\%}_{1cm}$ of 898 according to Seely et al. (1972) and the wavelengths were scanned from 300 to 800nm.

Extraction with 90% Acetone

1-2 ml of *H. pluvialis* NIES 144 was centrifuged at 3500 rpm for 10 min. The supernatant was discarded and the cell pellet was resuspended in 1-3 ml of 90% acetone and homogenized for 1 hour. This last step was repeat if necessary until the cell debris was totally white. The mixture was centrifuged and astaxanthin was determined at 480 nm. The amount of astaxanthin was calculated according to Kobayashi et al. (1991) with $E^{1\%}_{1cm}$ of 2500. Chlorophyll content was determined at 664 and 647 nm with Jeffrey and Hamphrey's equation (Geider and Osborne, 1992) in which μg/ml chlorophyll a = $11.93A_{664} - 1.93\ A_{647}$. The extraction should be conducted in darkness.

3.1. Effect of Light Intensity

H. pluvialis NIES 144 was grown as described in section 3. After 4 days, the culture was incubated at 21-23° C under continuous illumination at 20, 50, 100, 150, and 200 μmol m^{-2} s^{-1} and shaken at 100 rpm. At 2-day intervals, the cell numbers were counted, and astaxanthin and chlorophyll were extracted with both of DMSO and 90% acetone.

3.2. Effect of Sodium Chloride

H. pluvialis NIES 144 was grown and extracted as described in section 3.1. and NaCl contents were supplemented at 0, 0.2, 0.4, 0.8, 1.2 and 1.6% (w/v)

3.3. Effect of Carbon and Nitrogen

3.3.1. Effect of Carbon / Nitrogen Content

H. pluvialis NIES 144 was grown and extracted as described in section 3.1. The supplementation of $CH_3COONa/NaNO_3$ concentrations were 0:0, 43.8:0, 43.8:43.8, 43.8:21.9 mM respectively .

3.3.2. Effect of CH_3COONa (pH 7)

H. pluvialis NIES 144 was grown in The Basal Medium and extracted as described in section 3.1. and CH_3COONa concentrations were supplemented at 0, 21.9, 43.8 and 87.6 mM.

3.4. Effect of Temperature

H. pluvialis NIES 144 was grown in The Basal medium and extracted as described in section 3.1. After 4 days cultivation, the temperatures were changed from 22 to 25, 30 and 35° C as follows.

3.4.1.Effect of Temperature and Light intensity, Supplemented with NaCl

H. pluvialis NIES 144 was performed as described in section 3.4. The NaCl contents were fixed at 0.2% (w/v) and the light intensities were adjusted to 50, 100 and 140 μ mol m^{-2} s^{-1}.

3.4.2 Effect of Temperature and Light intensity, Supplemented with CH_3COONa

H. pluvialis NIES 144 was cultured as described in section 3.4.1. and the CH_3COONa contents were fixed at 21.9 mM, respectively.

3.5. Effect of $FeSO_4.7H_2O$ (pH 1.5)

H. pluvialis NIES 144 was cultured in The Basal medium with light intensity 20, 40 and 60 μ mol m^{-2} s^{-1} at 20-23° C. At 4 days cultivation, 21.9 and 43.8 mM CH_3COONa were added together with $FeSO_4.7H_2O$ at 0, 225, 450 and 900 μM respectively. Cell numbers were counted, astaxanthin and chlorophyll were extracted and determined as described in section 3.

4. Partial Analysis of Astaxanthin from *H. pluvialis* NIES 144 by High Performance Liquid Chromatography Method.

After 8 days cultivation, 50 ml of the cultures were centrifuged at 5000 rpm, 20 min and washed twice with distilled water. The supernatant was discarded and the cyst cells were lyophilized by Flexi-Dry μP. The lyophilized cells were kept at -20 °C in the dark.

Standard Solution (Applied from Weber, 1990)

Astaxanthin(1.5 μg/ml): Approximately 3 mg pure astaxanthin crystal was dissolved in 100 ml chloroform in a 100-ml volumetric flask. 5-ml of this solution was mixed with 95 ml of acetonitrile. To calculate the exact content of this standard solution the absorbance was measured in a

spectrophotometer at 470 nm using $E^{1\%}_{1cm} = 2100$. This standard solution could be stored for 2 days if kept in a refrigerator in the dark.

Method for Astaxanthin Determination in *H. pluvialis* (Applied from Fan et al, 1995)

Approximately 10 mg of lyophilized algal cells were extracted several times with acetone until almost colourless. The extract was evaporated at 55° C. The residue was dissolved with 5 ml of acetone and centrifuged at 4000 rpm for 10 minutes. The sample could directly be used for HPLC determination.

Specification for High Performance Liquid Chromatography (Applied from Fan et al.,1995)

Column	reverse phase C_{18} column, Spherisorb OD 52 (5 mm particle size)	
Mobile phase	The solvent system included	
	acetonitrile : H_2O (9:1)	solvent A
	100% ethyl acetate	solvent B
Flow rate	1 ml/min	
Pressure approx.	80 bar	
Detection	LDC analytical spectromonitor programmable visible wavelength detector at 450 nm	
Injection volume	40 µl	
Run time	30 min	

The pigments were separated by a step gradient between solvent a and B for 30 minutes as follows:

0-10 min , 0% → 60% B (rate 6% / min)

11-20 min , 60% → 100% B (rate 4% / min)

20-30 min , 100% B (hold for 10 min)