CHAPTER II

MATERIALS AND METHODS

Materials

1. Equipments

1.1 Controlled Environment Incubator Shaker , Psycrotherm. New Brunswick Scientific Edison , N.J., U.S.A.

1.2 Spectrophotometer Shimadzu UV-20 Shimadzu , Japan.

1.3 Oven Temperature 60 °C Memmert , Germany.

1.4 pH Meter PHM 83 Radiometer Copenhagen , Denmark.

1.5 Autoclave Model HA-3D Hirayama Manufacturing Corporation , Japan

1.6 Laminar Flow Model BVT-124 International Scientific Supply Co. Ltd., Thailand.

1.7 Filter Whatman GF/C 4.7 cm. Whatman International Ltd. Maidstone , England.

1.8 Illumination Meter Topcorn MD-2

1.9 Rotary Shaker New Brunswick Scientific Edison , N.J. , U.S.A.

1.10 UV Lamp

1.11 Micropipette

1.12 Fraction collector

2. Chemicals

Lysozyme , DEAE cellulose (fine mesh), Glycerol, acrylamide , N',N'-methylene bis acrylamide(BIS), SDS-VII™ Dalton Mark,NTG (N-methyl-N'-nitro- N-nitrosoguanidine) were from Sigma Chemical Company , U.S.A.

N,N,N,N'tetramethylenediamine (TEMED), coomassie brillant blue were from BDH Laboratory Chemicals Ltd.

2-Mercaptoethanol was from Fluka , Switzerland.

3. Specimen

Aphanothece halophytica was isolated from marine lake in Isarael.

Methods

1. Growth of *Aphanothece halophytica* in Turk Island Salt Solution + modified BG_{11} medium

10% inoculum of *Aphanothece halophytica* was inoculated into a 250 ml flask containing 100 ml of Turk Island Salt Solution + modified BG_{11} medium (see Appendix 1) and grown on a rotary shaker with 160 rpm at 27-30 °C. Three 20-W fluorescent lamps placed overhead provided the flasks with 1,900 lux of continuous illumination (Figure 3).

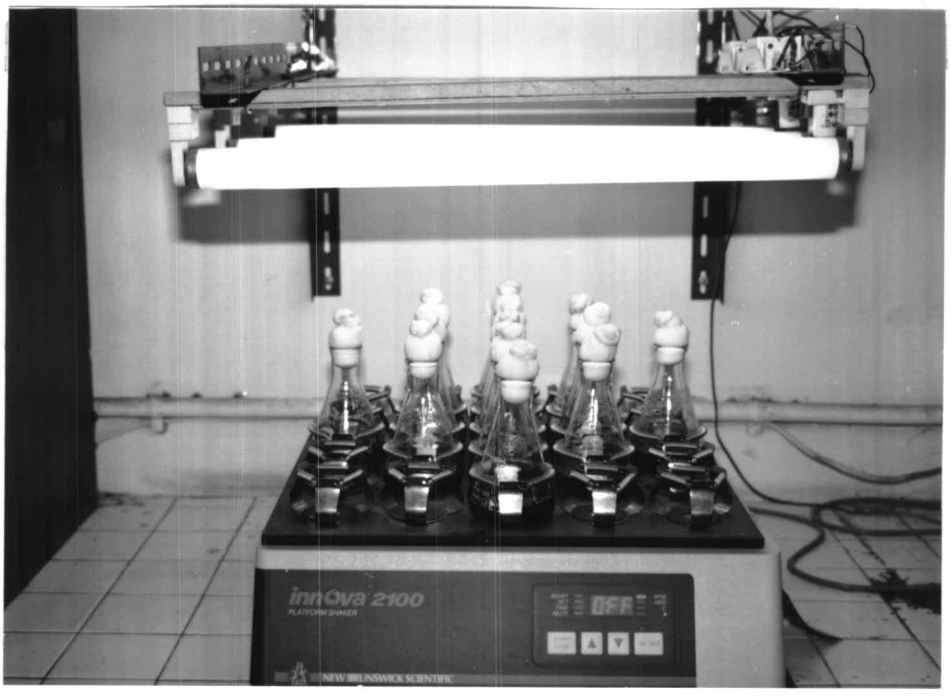

Figure 3 Cultivation of *Aphanothece halophytica* in a 250 ml flask

2. Methods for Phycocyanin Extraction

2.1 Extraction by Lysozyme Digestion

Two ml of *A. halophytica* culture was centrifuged at 3,500 rpm , 15 min. Phycocyanin was extracted from the pellet with 4 ml of 2 mg/ml lysozyme in 10 mM EDTA in 0.1 M sodium phosphate buffer, pH 7. The mixture was incubated at 37 °C for 1 hour, and centrifuged at 3500 rpm , 15 min. The supernatant was collected and the pellet was re-extracted with 4 ml of 2 mg/ml Lysozyme in 10 mM EDTA IN 0.1 m sodium phosphate buffer, pH 7. The blue supernatant was combined and kept in the dark before measuring the absorbance at 620 nm. The content of phycocyanin was calculated using $E^{1\%}_{1cm}$ =73 (Boussiba and Richmond ,1979 ; see Appendix 2).

2.2 Extraction with Freeze Thaw Method

Two ml of *A. halophytica* culture was centrifuged at 3,500 rpm , 15 min. The pellet was suspended in a small volume of 0.1 M sodium phosphate buffer , pH 7. The suspension was frozen at -70 °C , 30 min and thawed at 37 °C , 30 min before centrifuging at 3,500 rpm , 15 min. The supernatant was collected and the pellet was re-extracted with 0.1 M sodium phosphate buffer, pH 7 before centrifuging at 3,500 rpm , 15 min. The blue supernatant was combined and kept in the dark before measuring the absorbance at 620 nm. The content of phycocyanin was calculated using $E^{1\%}_{1cm}$ =73 (Boussiba and Richmond , 1979).

3. Determination of Dry Weight

A 20 ml culture was filtered through a Whatman GF/C by means of a suction pump. Prior to filtration, the paper was dried in an oven at 60 °C until the constant weight was obtained. The algal residue on the paper was dried in an oven at 60 °C until no further change in the weight occurred. The dry weight of *A. halophytica* was then determined by the difference between the weight of the paper before and after filtration.

4. Effect of Environmental Factors on *Aphanothece halophytica* Cultivation for High Phycocyanin Production

4.1 Effectt of NaCl Concentration

A. halophytica was grown in Turk Island Salt Solution + modified BG_{11} medium containing 0 , 0.125 , 0.25, 0.5 , 1.0 , 1.5 and 2.0 M NaCl , the pH of the medium was adjusted to 7.6. A 100 ml culture in 250 ml flask was shaken on a rotary shaker (160 rpm) at 27-30 °C with 1500 lux of continuous white light illumination. At 3 days intervals , the culture was collected for growth analysis. For phycocyanin determination , 2 ml of the day 9 culture was used.

4.2 Effect of Nitrogen Source

The experiment was performed as described in effect of NaCl concentration except concentration of NaCl in Turk Island Salt Solution + modified BG_{11} medium was 0.5 M and the nitrogen sources

were ammonium nitrate , ammonium acetate , sodium nitrate and urea (The concentrations of all the nitrogen-containing compounds were made equal on the N-atom basis, fixing the concentration of sodium nitrate at 1.5 g/l).

4.3 Effect of $NaNO_3$ Concentration

The experiment was performed as described in effect of NaCl concentration except concentration of NaCl in Turk Island Salt Solution + modified BG_{11} medium was 0.5 M , $NaNO_3$ was used as nitrogen source and the final $NaNO_3$ concentration were 0 , 0.1 , 0.25 , 0.5 , 1.0 , 1.5 , 2.0 and 2.5 g/l

4.4 Effect of Nitrate Starvation

A. halophytica was initially grown in Turk Island Salt Solution + modified BG_{11} medium containing $NaNO_3$ content of 1.5 g/l in the same condition as described in section 1. After 9 days the cells were harvested, washed and inoculated into Turk Island Salt Solution + modified BG_{11} medium containing 1.5 (as a control) and 0 g/l of $NaNO_3$. The culture was daily analyzed for growth and phycocyanin. At 68 hours $NaNO_3$ was added to $NaNO_3$-free culture flask until final concentration was 1.5 g/l $NaNO_3$. At 2 days intervals , the culture was collected for growth analysis and for phycocyanin determination.

4.5 Effect of Light Intensity

A. halophytica was grown in Turk Island Salt Solution + modified BG_{11} medium contained in a

250 ml flask. The intensities of light were 1,500 , 3,000 , 5,000 and 8,000 lux. At 2 days intervals , the culture was collected for growth analysis and for phycocyanin determination.

4.6 Effect of Light Quality

A. halophytica was grown in Turk Island Salt Solution + modified BG_{11} medium contained in a 250 ml flask under normal white light , red light and green light. The red and green light were provided by interposing the red and green plastic filter between the white light and culture flask , respectively.

4.7 Effect of Initial Chlorophyll Concentration

A. halophytica was grown in Turk Island Salt Solution + modified BG_{11} medium with different initial chlorophyll concentration , i.e., 50 , 100 , 250 or 500 $\mu g/ml$. A 100 ml of culture in 250 ml flask was grown in optimized condition. At 3 days intervals , the culture was collected for growth analysis. For phycocyanin determination , the culture at day 9 was used.

4.8 Effect of Cultivation Temperature on Growth and Phycocyanin Content

A. halophytica was grown in Turk Island Salt Solution + modified BG_{11} medium on a photosynthetic growth incubator (New Brunswick Psycrotherm). The temperature of the incubator was set at either 25 , 30 ,

35 or 40 °C with a shaking speed at 160 rpm.The illumination at about 1,900 lux was provided by cool white fluorescent lamps. The culture was daily analyzed for growth. For phycocyanin content determination , the culture at day 5 was used.

5. Effect of Mutagenesis on *Aphanothece halophytica* Cultivation for High Phycocyanin Production

5.1 Mutagenesis of *A. halophytica* by irradiation with Ultraviolet Light

A. halophytica was grown in 250 ml flask containing 100 ml of Turk Island Salt Solution + modified BG_{11} medium as described in section 1 for 10 days. Cells were centrifuged at 3,500 rpm , 15 min and resuspended in fresh medium. Two ml was removed and kept as a control sample. Ten ml of cells in a 100 mm petridish were irradiated at a distance of 30 cm from 30 W germicidal UV-light and 1 ml portions were removed at 5 second intervals.

5.1.1 Determination of Survival Curve of Mutagenesis of *A.halophytica* by Irradiation with UV-Light

The mutagenized cells and control sample were incubated under yellow lights at the same intensity used for normal growth for at least 36 hours. A 0.5 ml aliquot of each culture sample was spread on 100 mm plates containing 30 ml of Turk Island Salt Solution+ modified BG_{11} medium solidified with 1.5 % agar. The plates

were incubated under normal growth condition until colonies formed (10 days).

5.1.2 Selection for Mutants of *A. halophytica*

The experiment was performed as described in section 5.1.1 except the cells were irradiated with UV-light at 20 second which caused greater than 99 % killing. A 0.5 ml aliquot of mutated cells and normal sample were spread on agar plates and incubated under the normal growth condition. In another set of experiment, 0.5 ml aliquot of mutated cells and normal sample were treated with 150 μg/ml of ampicillin or cycloserine (selective agents) and incubated under normal growth condition for 24 hr. The selective agent was removed from the cells by two centrifugal washes and the cells were spread on agar plates and incubated under normal growth condition until colonies formed (10 days).

5.1.3 Growth and Phycocyanin Content from UV-mutated *A. halophytica*

UV-mutated cells were picked up from agar plates using a sterile loop and dispersed into a 125 ml flask containing 20 ml of Turk Island Salt Solution + modified BG_{11} medium and grown on rotary shaker with 160 rpm at 27-30 °C as described in section 1 for 10 days to scale up the cells. 10 % inoculum of *A.halophytica* mutant was inoculated into a 250 ml flask containing

100 ml of Turk Island Salt Solution + modified BG_{11} medium and grown on rotary shaker on condition as described in section 1. At 3-day intervals, the culture was collected for growth analysis. Dry weight and phycocyanin were also determined as described in section 3 and section 2.1.

5.2 Mutagenesis of *Aphanothece halophytica* by Chemical Method

Chemical mutagen used in this study was N-methyl-N´-nitro-N-nitrosoguanidine (NTG).

5.2.1 Determination of Survival Curve of Mutagenesis of *A.halophytica* by treated with NTG

A. halophytica was grown in a 250 ml flask containing 100 ml of Turk Island Salt Solution + modified BG_{11} medium as described in section 1 for 10 days. A 1.0 ml aliquot of each culture sample was added with various NTG concentrations ; NTG was dissolved in 0.2 M Tris-malate buffer, pH 7.6.The final concentrations of NTG in the culture samples were 0(control), 10, 20, 30, 40, 60, 80 and 100 μg/ml. Each culture sample with NTG treatment was incubated under normal growth condition for 5 min. NTG was removed from the cells by three centrifugal washes with normal culture medium. The pellets were resuspended in the culture medium and incubated under normal growth condition for 3 days. A 0.5 ml aliquot of each culture sample was spread on 100 mm plates containing 30 ml of Turk Island Salt Solution + modified BG_{11} medium

solidified with 1.5 %.The plates were incubated under normal growth condition until colonies formed.

5.2.2 Selection for Mutants of *A. halophytica*

The experiment was performed as described in section 5.2.1 except the cells were treated with 10 or 20 µg/ml NTG which caused greater than 99% killing. Aliquots (0.5 ml) of mutagenized cells and normal sample were spread on agar plates and incubated under the normal growth condition. In another set of experiment , 0.5 ml aliquot of mutated cells and normal sample were treated with 150 µg/ml of ampicillin or cycloserine (selective agents) and incubated under normal growth condition for 24 hr. The selective agent was removed from the cells by two centrifugal washes and the cells were spread on agar plate and incubated under normal growth condition until colonies formed (10 days).

5.2.3 Growth and Phycocyanin Content from NTG-mutated *A. halophytica*

NTG-mutated cells were picked up from agar plates using a sterile loop and dispersed into a 125 ml flask containing 20 ml of Turk Island Salt Solution + modified BG_{11} medium and grown on rotary shaker with 160 rpm at 27-30 °C as described in section 1 for 10 days to scale up the cells. 10 % inoculum of *A. halophytica* mutant was inoculated into a 250 ml flask

containing 100 ml Turk Island Salt Solution + modified BG_{11} medium and grown on rotary shaker on condition as described in section 1. At 3-day intervals , the culture was collected for growth analysis. Dry weight was determined as described in section 3 and Phycocyanin was determined as described in extraction by lysozyme digestion..

6. Partial Purification of Phycocyanin from *Aphanothece halophytica*

6.1 Growth of *Aphanothece haloophytica*

A. halophytica was grown in 100 ml of Turk Island Salt Solution + modified BG_{11} medium in a 250 ml flask as described in section 1. After 14 days, the cells were harvested by centrifugation and used for the purification of phycocyanin.

6.2 Ammonium Sulfate Precipitation of Crude Extract

About 5.0 g wet weight of *A. halophytica* was suspended in 200 ml of 2 mg/ml of lysozyme in 10 mM EDTA in 0.1 M sodium phosphate buffer,pH 7 and incubated at 37 °C for 1 hour then the suspension was centrifuged at 3500 rpm , 15 min as described in extraction by lysozyme digestion. The supernatant was precipitated with 0-50 %, 20-45 %, 20-65 % and 20-75 % ammonium sulfate. The final pellet was suspended in 0.02 M sodium phosphate buffer, pH 7.5 and the absorbance was measured at 620 nm and phycocyanin content was calculated by using $E^{1\%}_{1cm} = 73$ (Boussiba and Richmond, 1979).

6.3. DEAE-cellulose Column Chromatography

About 10 g of DEAE-cellulose was swelled in 1 litre of distilled water and then washed several times at room temperature to remove the fine particles. The resin was activated by washing sequentially with excess volume of 0.1 M HCl for 30 min followed by distilled water until the pH was 7. The resin was then treated with 0.1 M NaOH for 30 min followed by distilled water until the pH was 7. The activated resin was pre-equilibrated with 0.2 M sodium phosphate buffer, pH 7.5 for 30 min and then equilibrated with 0.02 M sodium phosphate buffer, pH 7.5 overnight. The prepared DEAE-cellulose was packed into a column (1.5X12 cm) at the height of 10 cm. The column was equilibrated with 0.02 M sodium phosphate buffer pH 7.5 at least 4-6 hr at the flow rate of 25-30 ml/hr.Seven ml of the protein solution containing about 50 mg was loaded and allowed to be absorbed. Elution was carried out by a continuous linear gradient of 0 to 0.5 M NaCl prepared in the buffer. A flow rate of 25-30 ml/hr was maintained and 5 ml fractions were collected. The concentration of NaCl in the fraction was estimated by using a conductivity meter. The absorbances of the fractions were read at 280 nm and 620 nm.

6.4 Determination of Protein

Protein content was determined by Lowry ' s method (Lowry and Rosenbrough , 1951). The sample

(0.5 ml) was mixed with 3 ml of Reagent A and left for at least 15 min. Reagent B (0.3 ml) was then added and the solution was left in the dark for 20 min. The absorbance was then read at 650 nm. The standard curve was constructed using bovine serum albumin as protein sample.

The compositions of Reagent A and B were

Reagent A : 2 % Na_2CO_3 in 0.1 M NaOH

1 % Na_2-Tartrate

1 % $CuSO_4$

These 3 solutions were mixed at 100 : 1 : 1 ratio

Reagent B : 1 M phenol reagent

6.5 Polyacrylamide Gel Electrophoresis

6.5.1 Preparation of the slab gel

The slab gel electrophoresis was done according to Laemmli (Laemmli,1970) either in the presence or absence of SDS. The gel was formed between 18 X 16 cm glasses assembled with 1 mm thick spacer. In SDS-PAGE all slab gels were prepared using a linear gradient of 10-18 % (w/v) of acrylamide for seperating gel and 3 % acrylamide for stacking gel. The concentration of acrylamide was prepared from a stock solution as shown below. In non-denaturing polyacrylamide gel electrophoresis (PAGE) 10 % acrylamide of seperating gel was formed.

A. Stock solution

a) 29.2 % (w/v) of acrylamide and 0.8 % (w/v) of N',N'-methylene bisacrylamide (BIS)

b) 1.5 M Tris-HCl pH 8.8 (Seperating buffer)

c) 0.5 M Tris-HCl pH 6.8 (Stacking buffer)

d) 10 % Ammonium persulphate

e) 0.025 M Tris,0.192 M glycine (electrode buffer)

f) 20 % SDS

B. Working solution prepared from stock solution

a. Running solution

for 10 % gel : 6 ml of stock a, 4.5 ml of stock b, 0.07 ml of d,7.5 ml of distilled water, 0.36 ml of stock f and 0.01 ml of TEMED

for 18 % gel : 10.5 ml of stock a, 4.5 ml of stock b, 0.07 ml of d,3.0 ml of distilled water , 0.36 ml of stock f and 0.01 ml of TEMED

b. Stacking gel solution

0.9 ml of stock a, 1.5 ml of stock c, 0.018 ml of d , 3.6 ml of distilled water , 0.03 ml of stock f and 0.006 ml of TEMED

C. Electrode buffer for normal gel: 0.025 M Tris,0.192 M glycine and distilled water to 1 litre.

D. Electrode buffer for denaturing gel: 0.025 M Tris, 0.192 M glycine , 0.1 % SDS and distilled water to 1 litre.

E. Staining solution : Coomassie brilliant blue R 0.2 % , methanol 50 % and acetic acid 10 %

F. Destaining solution : Acetic acid 7 % and methanol 5 %

6.5.2 Preparation of sample for electrophoresis

The phycocyanin was characterized by SDS-PAGE. The sample was dissolved in SDS-sample buffer containing (final concentration) 0.0625 M Tris-HCl pH 6.8 , 2 % SDS , 10 % glycerol , 5 % mercaptoethanol and 0.02 % bromphenol blue as the dye. Before loading the sample , the protein in the sample buffer was dissociated by boiling for 5 min.

6.5.3 Running condition

The non-SDS and SDS-PAGE were run from cathode to anode. A constant current of 30 mA was applied until the dye marker was 1 cm from the bottom of the gel (about 3-4 hr).

6.5.4 Staining and destaining

After electrophoresis , the proteins in the slabs were fixed for 2 hr with freshly prepared staining solution at room temperature and destaining was performed by repeated changes of destaining solution until the clear band was observed.